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JNMU
Journal of Nanjing Medical University, 2007, 21(2): 77–81
www.elsevier.com/locate/jnmu
Research Paper

No association between thrombospondin-4 A387P polymorphism and acute coronary syndrome in Chinese Han population

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Received 26 October 2006

Abstract

Objective: The thrombospondin-4 (TSP-4) gene G29926C (A387P) polymorphism was recently reported to be associated with an increased risk of MI (myocardial infarction) in American population. However, several subsequent studies produced controversial findings. The aim of this study was to explore the possible association between TSP-4 A387P polymorphism and ACS (acute coronary syndrome) in Chinese Han population. **Methods**: A case-control study including 412 patients with ACS and 337 controls free from CAD (coronary artery disease) was conducted. TSP-4 A387P polymorphism was determined by PCR (polymerase chain reaction) and RFLP (restriction fragment length polymorphism) analysis. **Results**: Slightly decreased frequency of GC genotype was observed in patients with ACS, compared with controls (5.3% *vs.* 7.1%), but the difference did not reach statistical significance ($P = 0.31$). Similarly, the prevalence of C allele was 2.7% and 3.6% for ACS and control groups, respectively ($P = 0.32$). None of homozygote was detected for C allele. Further analyses in subjects subgrouped according to sex and age also showed no association of TSP-4 A387P polymorphism with ACS. Furthermore, after adjustment for conventional risk factors by multiple logistic regression analysis, the carrier prevalence of C allele did not differ significantly between ACS and control groups (OR = 0.85; 95% CI: 0.45–1.59; $P = 0.60$). **Conclusion**: The present study suggested that the TSP-4 A387P variant showed a low prevalence compared with western populations and failed to associate with an altered risk of ACS in Chinese Han population. The findings further supplement experimental data for TSP-4 gene study of coronary disease.

Keywords: thrombospondin; polymorphism; acute coronary syndrome

INTRODUCTION

The thrombospondins (TSPs) are multimeric, calcium-binding, extracellular matrix glycoproteins, which consist of five family members at present: TSP-1, –2, –3, –4 and TSP-5/COMP (cartilage coligomeric matrix protein) [1,2]. It has been theorized that TSPs are able to bind multiple matrix proteins and cell-surface receptors, and participate in a variety of biology responses, such as stimulating platelet activation and aggregation, promoting vascular smooth muscular cell proliferation and migration, apoptosis of endothelial cell and angiogenesis [2-6]. They may be involved in the formation and injury of atherosclerotic plaque and thrombosis [7,8]. TSP genes are important candidates for atherosclerosis and CAD (coronary artery disease) or MI (myocardial infarction). In the GeneQuest study, one large-scale genetic association study (showed that), three different SNPs (simple mucleotide polymorphisms) in different TSP genes—TSP-1 (A8831G, predicting Asn700Ser), TSP-2 (T-G substitution in the 3′ untranslated region) and TSP-4 (G29926C, predicting Ala387Pro)—were associated with an altered risk of premature MI [9]. Of note, the TSP-4 A387P polymorphism showed the strongest association, with an OR for MI of 1.89 ($P = 0.002$, adjusted for covariates) for individuals carrying 387P allele. However, the following

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studies performed in Dutch, Japanese and northern Han Chinese populations produced controversial findings[10-12]. In view of these contradictory findings, we took this study to further explore the possible association of the TSP-4 A387P polymorphism with ACS(acute coronary syndrome) in Chinese Han population.

MATERIALS AND METHODS

Study population

A total of 749 unrelated subjects were included in the study. The subjects consisted of two groups:the ACS group and control group. The ACS group composed of 412 patients with ACS (298 men and 114 women) recruited from hospitalized patients at four participating hospitals between November 2003 and May 2006. The diagnosis of ACS was based on the criteria of AHA/ACC for 2002. The control group consisted of 337 subjects(232 men and 105 women) who were free of significant CAD, which were randomly selected from inpatients in the same hospitals. Significant CAD was defined as angiographic evidence of at least one coronary artery with more than 50% stenosis. Subjects with congenital heart disease, cardiomyopathy, tumor, and renal or hepatic disease were excluded from the study. Conventional risk factors of CAD were recorded:plasma lipid concentration, the presence of hypertension (systolic blood pressure ⩾ 140 mmHg, diastolic blood pressure ⩾ 90 mmHg, or both) or diabetes mellitus and so on.

Polymorphism analysis

Blood was collected from each subject into tubes containing 3.2% sodium citrate, and then, plasma and WBC were separated immediately and stored at −70℃. Genomic DNA was isolated from WBC by phenol-chloroform method. Genotyping of TSP-4 A387P polymorphism was determined with PCR-RFLP. The following primers were used:forward, 5′-AATTCCGCATCTTCACTTCAC-3′; and reverse, 5′-AACCGGTTCTGCTTTGATAAC-3′. The reaction mixture(50 μl) contained approximately 100 ng of DNA, 20 pmol of each primer, 2.0 mmol/l MgCl2, 0.2 mmol/l of each deoxynucleoside triphosphate and 1.25 units of Taq polymerase (TaKaRa) in the corresponding buffer. The conditions of amplification imcluded initial denaturation at 95℃ for 5 min; 33 cycles of denaturation at 95℃ for 40 s, annealing at 59℃ for 30 s, and extension at 72℃ for 40 s; and a final extension at 72℃ for 5 min. PCR products of A387P polymorphism were 221 bp, which generated 2 fragments of 143 and 78 bp in the presence of the C allele after digestion with *Ava* Ⅱ (37℃, 16 hours). The digested products were then electrophoresed in 2.5% agarose gel and visualized by ethidium bromide staining. Sequence analysis of some PCR products was performed to further confirm the accuracy of genotyping.

Statistical analysis

All statistical analyses were carried out using SPSS 10.0 software. Continuous variables were presented as mean ± SD and compared between patients with ACS and controls by the unpaired Student's *t* test. Categorical variables were expressed as counts or percentages and were compared using χ^2 test. Hardy-Weinberg equilibrium was assessed by χ^2 test. Univariate analysis, used to measure the association of the TSP-4 polymorphism with ACS, was tested by χ^2 test. Multiple logistic regression analysis, applied to adjust conventional risk factors, was performed to investigate the independent role of A387P polymorphism. The OR (odds ratio) and 95% CI(confidence interval) were calculated. A two-tailed *P* value of < 0.05 was considered statistically significant.

RESULTS

General characteristics

The main baseline characteristics of all study subjects were shown in ***Tab 1***. Compared with the control group, the ACS group had a greater proportion of smokers, more patients with hypertension and diabetes, and had a higher average systolic BP. The ACS group also had significantly higher serum triglyceride, fasting glucose levels and lower HDL-C (high density lipoprotein-cholesterol) than the control group.

Tab 1 **Clinical characteristics of the study subjects**

	ACS group (n = 412)	Control group (n = 337)	*P*
Male(%)	298.00(72.3)	232.00(68.80)	0.297
Age(years)	65.24(10.95)	63.66(11.76)	0.062
Systolic BP(mmHg)	135.01(18.62)	132.42(14.78)	0.041
Diastolic BP (mmHg)	80.63(12.04)	79.45(9.16)	0.143
Glucose(mmol/l)	5.71.00(2.35)	5.07(2.02)	< 0.001
TC(mmol/l)	4.66(1.30)	4.41(1.15)	0.274
TG(mmol/l)	1.81(0.98)	1.63(1.06)	0.016
HDL-C(mmol/l)	0.95(0.26)	1.16(0.32)	< 0.001
LDL-C(mmol/l)	2.72(0.78)	2.76(0.79)	0.498
Smoking(%)	208.00(50.50)	131.00(38.90)	0.001
Hypertension(%)	179.00(43.40)	116.00(34.40)	0.012
Diabetes(%)	44.00(10.70)	16.00(4.70)	0.003

Values were presented as mean ± SD for quantitative variables, and number (%) for qualitative variables. TC, total cholesterol; TG, triglyceride; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol.

TSP-4 A387P polymorphism

The genotype of A387P polymorphism was determined by amplification with PCR and restriction analysis with *Ava* Ⅱ enzyme. Two genotypes were distinguished: homozygous GG (221 bp) and heterozygous GC (221, 143 and 78 bp) (***Fig 1***). None of CC homozygote (143 and 78 bp) was detected. Sequence analysis for PCR products was shown in ***Fig 2***.

A387P polymorphism and ACS

The A387P polymorphism was in Hardy-Weinberg equilibrium ($P > 0.05$) in the ACS and control groups. The genotype distribution for the A387P polymorphism was shown in (***Tab 2***). Slightly decreased carrier frequency (genotypes of GC+CC) and allele frequency of 387P allele were observed in patients with ACS, compared with control subjects (5.3% *vs.* 7.1%, 2.7% *vs.* 3.6%, respectively), but the difference did not reach statistical significance. The distribution of the A387P variant was further analyzed by subgrouping patients and controls according to age and sex (***Tab 3***). Patients with ACS were divided into those with an early age of onset (≤ 55 years) and those with a late age of onset (> 55 years). The control population was also divided into two corresponding age groups. The prevalence of 387P allele carriers was 5.7% and 6.9% in male ACS patients and male controls, respectively (P = 0.57). Similarly, 387P allele showed a tendency toward lower prevalence in female patients with ACS, compared with the controls of same sex and given an OR of 0.56 (95% CI 0.18-1.76), the difference was also not statistically significant (P = 0.31). Further analysis in subjects subgrouped according to age showed no age-related difference in the distributions of A387P polymorphism. Furthermore, after adjustment for conventional risk factors by multiple logistic regression analysis, the carrier prevalence of C allele did not differ significantly between ACS and control groups (OR = 0.85; 95% CI: 0.45-1.59; P = 0.60) (***Tab 4***).

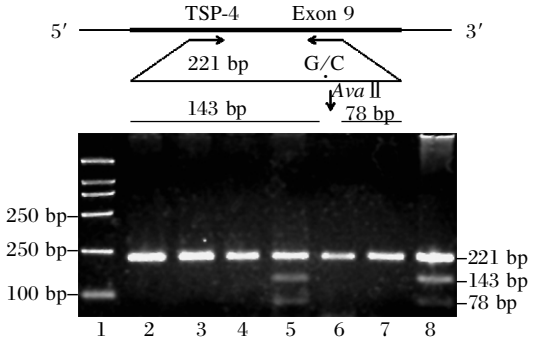

Fig 1 Determination of the A387P genotype by PCR amplification and restriction analysis. In the upper part, the G/C polymorphism position was indicated by an asterisk. When the nucleotide C was present, an *Ava* Ⅱ restriction site was created. In the lower part, 2.5% agarose gel electrophoresis of *Ava* Ⅱ digested PCR products was shown. The GG homozygote was not recognized by the *Ava* Ⅱ enzyme, and only showed one 221 bp band (lanes 2, 3, 4, 6 and 7). The GC heterozygote had three bands, with sizes of 221, 143 and 78 bp (lane 5 and 8). Lane 1: DNA Marker.

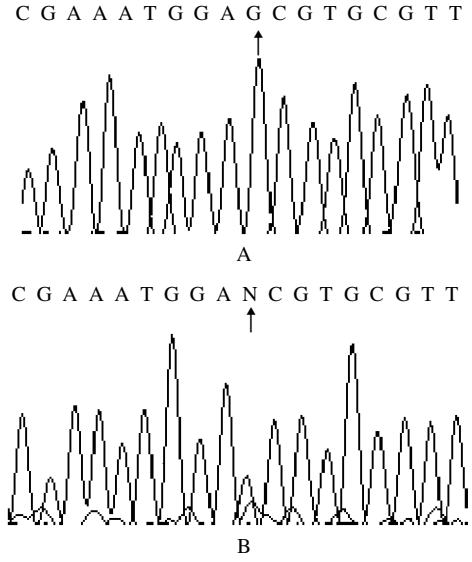

Fig 2 Sequencing chromatogram of TSP-4 gene polymorphism. A denoted GG genotype. B denoted the GC heterozygote. The arrow indicated different alleles at the polymorphic site.

DISCUSSION

We failed to find the association between A387P polymorphism and ACS in our population. Although slightly decreased variant allele and genotype frequency were observed in patients with ACS compared with control subjects (5.3% *vs.* 7.1%, P = 0.31; 2.7% *vs.* 3.6%, P = 0.32, respectively), the difference did not reach statistical significance. Further analysis in subgroup according to sex and age also showed no association of TSP-4 A387P polymor-

Tab 2 **Genotype distribution and allele prevalence of the TSP-4 polymorphism**

	ACS group	Control group	OR (95% CI)	P
Genotypes				
GG	390 (94.7)	313 (92.9)		
GC	22 (5.3)	24 (7.1)	0.74 (0.41-1.34)	0.31
CC	0	0		
Total	412	337		
Alleles				
G	802 (97.3)	650 (96.4)		
C	22 (2.7)	24 (3.6)		0.32

Values were number (%), OR (odd ratio) and 95% CI (95% confidence intervals).

Tab 3 **Distribution of TSP-4 gene polymorphism among ACS patients with different onsets of age, gender and comparison with age and gender of matched controls**

	ACS group	Control group	OR(95% CI)	*P*
Males				
GC+CC	17/298(5.7)	16/232(6.9)	0.82(0.40-1.65)	0.57
GG	281/298(94.3)	216/232(93.1)		
Females				
GC+CC	5/114(4.4)	8/105(7.6)	0.56(0.18-1.76)	0.31
GG	109/114(95.6)	97/105(92.4)		
Age ≤ 55Y				
GC+CC	3/75(4.0)	6/91(6.6)	0.59(0.14-2.44)	0.69
GG	72/75(96.0)	85/91(93.4)		
Age > 55Y				
GC+CC	19/337(5.6)	18/246(7.3)	0.76(0.39-1.47)	0.41
GG	318/337(94.4)	228/246(92.7)		

Values were number(%).

Tab 4 **Unadjusted and adjusted odds ratios for the association between the carrier frequency of 387P allele (GC+CC) and ACS**

	GC+CC		Univariate analysis			Multivariate analysis		
	n	%	OR	95% CI	*P*	OR*	95% CI	*P*
Cases(*n* = 412)	22	5.3	0.74	0.41-1.34	0.31	0.85	0.45-1.59	0.60
Controls(*n* = 337)	24	7.1						

*OR was adjusted for sex, smoking, hypertension, diabetes, TG and HDL-C.

phism with ACS. The frequency of the polymorphic allele was very different from the reported frequencies in western population:3.6% *vs.* 19.6%-23.2%, as well as the sum of heterozygous and homozygous phenotypes(A387/P387 + P387/P387) was 7.1% *vs.* 34.5%-40.5% in western populations[9,10]. These findings are consistent with the study by Zhou et al[12].

Numerous evidences have demonstrated that the TSP gene polymorphisms might have a potential significance in atherosclerotic lesions and acute vascular injury, but the results were often inconsistent and sometimes even contradictory. In the GeneQuest study, Topol and coworkers by using high-throughput microarray genotyping to shift through 62 genes from 352 patients with CAD and 418 controls, discovered three SNPs in the TSP family associated with altered risk of premature MI[9]. This discovery, therefore, was listed within "AHA top 10 advances for 2001". Of the three variants, the TSP-4 A387P showed the strongest association, with an OR for MI of 1.89(P = 0.002, adjusted for covariates) for individuals carrying 387P allele[9]. Two subsequent studies also in the American population further validated the results of GeneQuest study[13,14]. Furthermore, an in vitro functional study of TSP-4 A387P substitution provided additional evidence in support of a proatherogenic effect. This variant leaded to a substitution of proline for alanine in the third repeat type Ⅱ unit, which may affect the secondary structure of the protein and disrupt the Ca^{2+} binding site[9]. This "gain-of-function" mutation is correlated with extensive proinflammation including a striking reduction in endothelial proliferation or inability to repair from endothelial injury, and enhanced neu trophil function[15]. However, the studies performed in Dutch, Japanese and northern Han Chinese populations produced controversial findings. Yamada *et al*[11] assessed 112 candidate gene polymorphisms in Japanese individuals, including the TSP-4 A387P SNP, and confirmed the association between A387P polymorphism and MI only in men. Interestingly, Boekholdt *et al*[10] Showed that 387P allele was significantly associated with reduced risk, rather than increased risk, for MI in Netherland whites (OR = 0.43; 95% CI:0.22-0.85; P = 0.014). In addition, Zhou *et al*[12] failed to replicate the association between A387P polymorphism and CAD/MI.

Although the design of the present study made it difficult to compare our results with the data published so far, several points should be considered when interpreting the apparent discrepancy. The failure to repeat the association may have resulted from the following causes:heterogeneity across races and ethnicities, environmental and additional genetic factors, and differences in designs of the studies. Firstly, the best possible explanation was genetic heterogeneity across races and ethnicities. Secondly, failure to recognize gene-gene and gene-environment inter-

actions exerted a critical influence on the precise correlation between genes and CAD. It was possible that the TSP-4 variant conferred a modestly increased or reduced risk that might be added to, amplified or even overcome by other acquired environmental and/or additional genetic factors[16]. In addition, the controversial results were probably attributed to the selection bias of samples, mismatches between cases and controls and small sample sizes. No association between the TSP-4 polymorphism and ACS was likely due to the relatively small sample sizes in the present study. Furthermore, the average onset age of MI cases in our study was older than that in both American and Dutch studies[9,10].

Several limitations remaind in the present study. Firstly, the patients in the controls were not healthy volunteers representative of the general population and they had various atypical symptoms. However, the control participants were confirmed by coronary angiography to be free of significant coronary stenosis. Another limitation was that the study subjects were not recruited prospectively. Therefore, a survival bias could not be excluded. Lastly, the intermediate phenotypes of the polymorphism were not studied so that our report did not provide data on potential functionality of the TSP-4 polymorphism. Thus, this study went only as far as showing the association between TSP-4 polymorphisms and ACS.

In conclusion, the present findings showed that the TSP-4 A387P variant had a dramatically lower prevalence in the Chinese Han population compared with western populations and failed to associate with ACS. The precise association between TSP-4 A387P polymorphism and CAD/ACS awaits further investigations. More studies should be performed in populations at different risks of coronary events in order to further elucidate the exact contribution of this polymorphism to cardiovascular diseases.

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