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Genetic relationship between serum pregnancy-associated plasma protein-A gene polymorphism and ischemic cerebrovascular disease in a Northern Han Chinese population☆

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Abstract

The present study recruited 193 patients with ischemic cerebrovascular disease from Inpatient and Outpatient Departments at the Affiliated Hospital of Qingdao University Medical College, China from August 2008 to May 2010, as well as 120 healthy volunteers from the Medical Examination Center at the Affiliated Hospital of Qingdao University Medical College, China, who served as controls for this study. Patients and control subjects were from the Han population in northern China. Enzyme- linked immunosorbent assay analysis revealed increased levels of serum pregnancy-associated plasma protein-A (PAPP-A) in ischemic cerebrovascular disease patients compared with healthy controls. In addition, the patients exhibited greater frequency of genotype CC and C alleles in a missense A/C (Tyr/Ser) polymorphism (dbSNP: rs7020782) of exon 14 in the PAPP-A gene. Multiple-factor logistic regression analysis on correction of age, gender, history of smoking, hypertension, diabetes mellitus, hypercholesteremia, and ischemic stroke family history showed that the risk for ischemic cerebrovascular disease in the population without the A allele at the A/C genetic locus in exon 14 of the PAPP-A was 2-folds greater than the population expressing the A allele. These experimental findings suggested that ischemic cerebrovascular disease correlated with the C allele in exon 14 of PAPP-A. In addition, the A allele is likely a protective gene; individuals carrying the A allele were less prone to ischemic cerebrovascular disease compared with individuals without the A allele.

Key Words: atherosclerosis; ischemic cerebrovascular disease; metalloproteinase-9; polymorphisms; pregnancy-associated plasma protein A

Abbreviations: PAPP-A, pregnancy-associated protein A; ICVD, ischemic cerebrovascular disease; CRP, C-reactive protein; MMP-9, metalloproteinase-9

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INTRODUCTION

Ischemic cerebrovascular disease (ICVD) occurs as a result of several risk factors, and atherosclerosis has been determined to be a major cause of ICVD[1-4]. Atherosclerotic plaque rupture is etiology for acute cardiovascular events[5-6]. Plaque rupture depends on plaque stability, and many factors lead to plaque instability, including inflammation, plaque matrix degradation, and changes in blood clotting mechanisms, with the inflammatory response playing a major role[7-10].

Early detection of unstable plaques is crucial for the prevention of acute cardiovascular and cerebrovascular events. Previous results have shown that a variety of inflammatory factors, such as matrix metalloproteinases secreted from foam cells, macrophages, and inflammatory cells in atherosclerotic plaques, promote atherosclerotic plaque rupture[11]. Animal experiments and clinical studies have also demonstrated that pregnancy-associated protein A (PAPP-A) is involved in atherosclerotic plaque occurrence and rupture[12-13]. As previously reported[14], PAPP-A is widely expressed in unstable plaques and can trigger acute coronary syndromes. A previous study[15] showed that serum PAPP-A levels are increased in ischemic stroke patients with coronary heart disease compared with healthy controls. These results suggest that an increase in serum PAPP-A levels is a hallmark for atherosclerotic plaque instability and can serve as a novel serum indicator for predicting artery plaque stability[16-17]. Previous results[18] showed that a single nucleotide polymorphism (rs: 7020782, TCC AGA GCT GGC TGT GGA GAA TGC TT [A/C] TCT CAA TTG CTC CAG CAG CGA CCG C) exists within exon 14 A/C (Tyr/Ser) of the PAPP-A gene, and the A/C allele has multiple functions. Studies have suggested that this gene polymorphism is associated

with recurrent pregnancy miscarriage, premature coronary heart disease, and other diseases[19-20]. Because PAPP-A is closely associated with formation of instable atherosclerotic plaques and the occurrence of ICVD, it was hypothesized that A/C gene polymorphism in exon 14 of the PAPP-A also affects ICVD. Frequency distribution of genetic polymorphisms vary amongst different populations, races, and living environments[21-22]. In the present study, a Chinese Han population was analyzed as follows: (1) serum PAPP-A levels were measured in ICVD patients to determine a correlation between PAPP-A and C-reactive protein (CRP), metalloproteinase-9 (MMP-9), and ICVD, as well as to better understand the mechanisms underlying PAPP-A action in cardiovascular and cerebrovascular disease; (2) correlation between A/C gene polymorphism in exon 14 of the PAPP-A gene and ICVD was analyzed to determine the pathogenesis of cerebrovascular disease at the molecular levels.

RESULTS

Quantitative analysis of included subjects

A total of 193 patients with ICVD and 120 healthy controls were included in the final analysis. The case group matched the control group in age, number of males, and smoking status ($P > 0.05$). The number of cases with a disease history of hypertension, diabetes mellitus, or hyperlipidemia was significantly greater in the case group compared with the control group ($P < 0.01$ or $P < 0.05$; Table 1).

Table 1 Clinical data of subjects

Item	Case group	Control group	*P*
n	193	120	–
Age (mean ± SD, years)	66.06±7.94	64.52±6.57	0.08
Male [*n*(%)]	105(54.4)	64(53.3)	0.73
Smoking [*n*(%)]	64(33)	34(28)	0.37
Hypertension [*n*(%)]	93(48)	26(22)	< 0.01
Diabetes [*n*(%)]	51(26)	14(12)	< 0.01
Hyperlipidemia [*n*(%)]	52(27)	18(15)	< 0.05
≤ 65 years [*n*(%)]	96(59.6)	65(54.2)	< 0.05

Serum PAPP-A levels positively correlated with CRP and MMP-9 levels in ICVD patients

Serum PAPP-A levels were significantly greater in ICVD patients than in controls ($t_{PAPP-A} = 6.65$, $P < 0.01$), and serum CRP and MMP-9 levels were significantly greater than in controls ($t_{CRP} = 9.91$, $t_{MMP-9} = 8.14$, $P < 0.01$; Table 2).

Correlation analysis revealed that serum PAPP-A levels positively correlated with CRP and MMP-9 levels in ICVD patients ($r = 0.430$, $P < 0.01$; $r = 0.467$, $P < 0.01$; Figure 1).

A/C (Tyr/Ser) gene polymorphism in exon 14 of serum PAPP-A in ICVD patients

Chi-square goodness-of-fit test showed that genotype distribution in exon 14 of the PAPP-A met the Hardy-Weinberg equilibrium in all ICVD patients, demonstrating that all patients were from the same population. The target fragment length following PCR was 361 bp (Figure 2).

Table 2 Serum PAPP-A, CRP, and MMP-9 levels in ischemic cerebrovascular disease patients and healthy controls

Group	*n*	PAPP-A (mIU/L)	CRP (mg/L)	MMP-9 (μg/L)
Case	193	11.84±5.55[a]	5.26±2.78[a]	58.35±26.54[a]
Control	120	8.12±3.26	2.99±1.65	33.37± 9.71

[a]$P < 0.01$, *vs*. control group. Data are expressed as mean ± SD, and group comparisons were conducted using independent samples *t*-test. PAPP-A: Pregnancy-associated protein A; CRP: C-reactive protein; MMP-9: metalloproteinase-9.

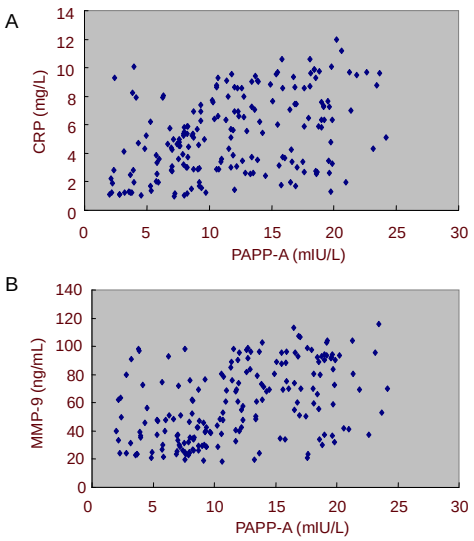

Figure 1 Pearson's correlation analysis of serum PAPP-A levels with CRP (A) and MMP-9 (B) levels in ischemic cerebrovascular disease patients. Correlation coefficients of serum PAPP-A levels with CRP and MMP-9 levels are 0.430 ($P < 0.01$) and 0.467 ($P < 0.01$), respectively, indicating a significant positive correlation.

PAPP-A: Pregnancy-associated protein A; CRP: C-reactive protein; MMP-9: metalloproteinase-9.

Figure 2 Electrophoretogram of the pregnancy-associated protein A gene fragment in serum of ischemic cerebrovascular disease patients.

1–11: Electrophoresis of serum samples; band length: 361 bp; M: marker.

The PAPP-A gene target fragment was sequenced as follows: TGG TTT GTC TGT TGC CAT AAC TTG TGC TCT TAA TGA GAA ACT TTA ATC GCT GCC TCT AGA GTC ATC TTC ACC CTA GCC AGC ACC CCT TTC TGT CCT CAG TCT CAA GCC CCC TGA AAT CTT GGA GAA AGA GGC TCT TAG CCT TTA TCT TCC AAC TGA GCA TAG CTG TCC TGT GAG GAG ACT CCT TCT TCC CCT CGT TTC TTT CCT CCC CAG CTG CGT GCA CTT CGC ATG TGA GAA AAC TGA CTG TCC AGA GCT GGC TGT GGA GAA TGC TTC TCT CAA TTG CTC CAG CAG CGA CCG CTA CCA CGG TGC CCA GTG TAC TGT GAG CTG CCG GAC AGG CTA CGT GCT CCA GAT ACG GCG GGA TGA T (the underlying C is A/C gene locus in exon 14 of the PAPP-A). Exon 14 (A/C) can be characterized by three genotypes: homozygous CC, heterozygous CA, and homozygous AA (Figure 3).

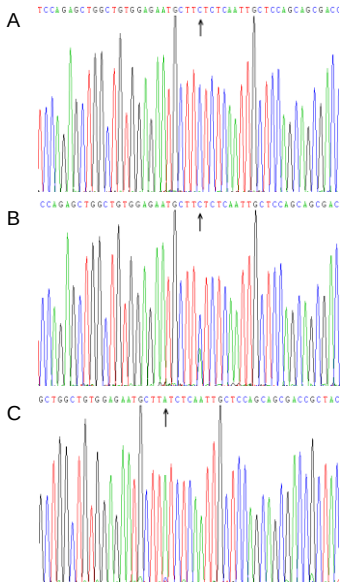

Figure 3 A/C (Tyr/Ser) gene polymorphism in exon 14 of pregnancy-associated protein A in serum of ischemic cerebrovascular disease patients. Arrow indicates gene polymorphism.

(A) Homozygous CC; (B) Heterozygous CA; (C) Homozygous AA.

Comparisons of PAPP-A genotype and allele distribution in ICVD patients and controls

The CC genotype frequency of PAPP-A was significantly greater in the case group than in the control group ($P < 0.01$). C-allele frequency exhibited a similar trend to the control group (Odds ratio (*OR*) = 1.742, 95% confidence interval (*CI*) = 1.26–2.42, $P = 0.001$; Table 3). According to grouping management for age and sex, the CC genotype frequency was greater in the ≤ 65-year-old case group and male case group than in the control group ($P < 0.01$), and C-allele frequency was also greater than in controls (*OR* = 2.17, 95% *CI* = 1.38–3.42, $P = 0.001$; *OR* = 2.13, 95% *CI* = 1.36–3.34, $P = 0.001$). Multivariate logistic regression adjusting for age, sex, smoking, hypertension, diabetes, and hyperlipidemia demonstrated that the risk for ICVD was 2-folds greater in individuals without the A allele compared with those carrying the A allele (*OR* = 2.03, 95% *CI* = 1.11–3.73, $P = 0.02$).

Table 3 Genotype and allele frequency distribution in exon 14 (C/A) of pregnancy-associated protein A gene

Group	*n*	Genotype [*n*(%)]			
		CC	CA	AA	*P*
ICVD	193	58(29.7)	83(42.6)	52(26.7)	0.009
Control	120	21(17.5)	49(40.8)	50(41.7)	
≤65 years old					
ICVD	96	35(36.5)	39(40.6)	22(22.9)	0.006
Control	65	10(15.4)	29(44.6)	26(40.0)	
> 65 years old					
ICVD	97	23(23.7)	44(45.4)	30(30.9)	0.288
Control	55	11(20.0)	2036.4)	24(43.6)	
Male					
ICVD	105	37(35.2)	42(40.0)	26(24.8)	0.009
Control	64	10(15.6)	27(42.2)	27(42.2)	
Female					
ICVD	88	21(23.9)	41(46.6)	26(29.5)	0.363
Control	56	11(19.6)	22(39.3)	23(41.1)	

Group	*n*	Allele			
		C + A (*n*)	C [*n*(%)]	A [*n*(%)]	*P*
ICVD	193	386	199(51.6)	187(48.4)	0.001
Control	120	240	91(37.9)	149(62.1)	
≤65 years old					
ICVD	96	192	109(56.8)	83(43.2)	0.001
Control	65	130	49(37.7)	81(62.3)	
> 65 years old					
ICVD	97	194	90(46.4)	104(53.6)	0.165
Control	55	110	42(38.2)	68(61.8)	
Male					
ICVD	105	210	116(55.2)	94(44.8)	0.001
Control	64	128	47(36.7)	81(63.3)	
Female					
ICVD	88	176	83(47.2)	93(52.8)	0.190
Control	56	112	44(39.3)	68(60.7)	

ICVD: Ischemic cerebrovascular disease.

DISCUSSION

PAPP-A is a member of the matrix metalloproteinase superfamily, and matrix metalloproteinase degradation of the extracellular matrix is an important factor in atherosclerotic plaque instability[23]. PAPP-A is widely expressed in cells with unstable plaques and in the extracellular matrix, but expression is little or absent in stable plaques. PAPP-A levels are not elevated in patients without coronary artery disease[24], but are significantly increased in acute myocardial infarction patients[25]. In patients with stable coronary artery disease, increased serum PAPP-A levels help to determine prognosis and risk of death[26]. PAPP-A levels also serve as an independent predictor of restenosis following coronary angioplasty[27]. In the present study, serum PAPP-A levels in the case group were significantly greater than in the control group, and

PAPP-A levels positively correlated with CRP and MMP-9 levels.

CRP is a sensitive, non-specific, serological indicator for inflammatory processes[28], and an increase in serum CRP levels is an independent risk factor for atherosclerotic plaque rupture[29]. A previous study[14] showed that PAPP-A levels are related to CRP levels in patients with acute coronary syndrome. This study also demonstrated that PAPP-A is significantly associated with CRP in patients with ischemic stroke. These results suggest that CRP promotes inflammation and produces inflammatory cells that generate MMPs, including PAPP-A. In addition, CRP reverses development of inflammation, thereby leading to plaque instability and rupture.

MMPs are inflammatory markers that are involved in the process of atherosclerosis; MMPs promote plaque rupture through degradation of extracellular matrix and digestion of the fibrous cap[30-31]. MMP-9 protein and enzyme activity are significantly increased in the shoulder area of an unstable atherosclerotic plaque, which is 3–5 times greater than in stable plaques[32]. The Pearson correlation analysis reveals that PAPP-A significantly positively correlates with MMP-9 levels in ICVD patients. PAPP-A is a member of the matrix metalloproteinase zinc finger peptides super-family[12] and has similar structure and mechanisms of action to MMP-9. PAPP-A promotes atherosclerotic plaque formation and development, as well as triggers formation of unstable plaques and leads to cardiovascular and cerebrovascular diseases.

The PAPP-A gene is located on the ninth human somatic chromosome (9q33.1)[33] in the Sushi domain, which is the complement regulatory protein region that contains many complements and adhesion proteins[34]. These domains are characteristic of approximately 60 residue sequences that involve protein-protein and protein-ligand interactions[35]. PAPP-A IVS6 + 95 C allele has been shown to be associated with unstable angina[36] and is recognized an independent risk factor for acute myocardial infarction[37]. The present study showed that the CC genotype and C allele frequencies were greater in the case group than the control group, which was consistent with previous results[19-20].

Multivariate logistic regression analysis for adjusting age, sex, smoking, hypertension, diabetes, high cholesterol, and family history of ischemic stroke demonstrated that the ICVD risk was increased in individuals with the CC genotype, in particular in the ≤ 65-year-old age group and male group. Populations with an absence of the A allele had a 2.0 times greater risk for ICVD than the population with the A allele. It was speculated that the C allele in exon 14 of PAPP-A was associated with ICVD and increased susceptibility to atherosclerosis-related disease. Results suggested that the A allele served as a protective gene and individuals carrying the A allele were less prone to ICVD than individuals without the A allele. In summary, the A/C gene polymorphism in exon 14 of the PAPP-A was associated with ICVD susceptibility. Patients carrying the homozygous CC genotype were at high risk for ICVD. A better understanding of the correlation between PAPP-A gene polymorphism and ICVD will help to determine the molecular mechanisms underlying the pathogenesis of cerebrovascular disease, as well as screening for high-risk populations and developing novel methods of ICVD treatment.

SUBJECTS AND METHODS

Design

A non-randomized, concurrent, controlled analysis of gene polymorphism.

Time and setting

Experiments were performed from August 2008 to December 2010 at the Central Laboratory of the Affiliated Hospital at Qingdao University Medical College, China.

Subjects

A total of 193 ICVD patients were recruited from Inpatient and Outpatient Departments at the Affiliated Hospital of Qingdao University Medical College, China from August 2008 to May 2010.

Inclusion criteria

(1) All patients met diagnostic criteria of the Fourth National Cerebrovascular Disease Conference of China[38]. (2) All patients corresponded with large-artery atherosclerosis of Trial of Org 10172 in Acute Stroke Treatment classification. (3) Digital subtraction angiography or CT angiography confirmed large-artery atherosclerotic stenosis-associated ischemic stroke. (4) All patients provided informed consent.

Exclusion criteria

(1) Patients with acute coronary syndrome and other heart diseases, such as dilated cardiomyopathy or rheumatic heart disease, were excluded. (2) Patients with liver or kidney dysfunctions or severe heart failure were excluded. (3) Patients with infections or immune system diseases were excluded. (4) Patients with peripheral vascular disease were excluded. (5) Patients with cerebral infarction induced by arteritis, blood diseases, cancer, drugs, aneurysms, or vascular malformations were excluded.

In addition, 120 healthy volunteers were simultaneously selected from the Medical Examination Center at the Affiliated Hospital of Qingdao University Medical College, China, and served as the control group. The subjects matched the case group for age and sex, and included 64 males and 56 females with a mean age of 64.52 ± 6.57 years.

All patients and healthy controls were from a Chinese Han population. Clinical data included age, gender, smoking status, hypertension, diabetes mellitus, and hyperlipidemia. Hypertension was defined in accordance with guidelines from the World Health Organization in 1999 and International League of Hypertension, systolic blood pressure ≥ 140 mm Hg (18.7 kPa) and/or diastolic blood pressure ≥ 90 mm Hg (12.0 kPa), with the

exception of secondary hypertension[39]. Hyperlipidemia met diagnostic criteria from Recommendations of Dyslipidemia Prevention issued by the Chinese Society of Cardiovascular Disease[40]. Diabetes mellitus was diagnosed according to criteria from the World Health Organization in 1999[41].

Methods

Blood sample collection

A total of 5 mL fasting venous blood was collected within 24 hours after admission. The samples were anti-coagulated with ethylene diamine tetraacetic acid and were centrifuged at 2 500 r/min for 10 minutes within 30 minutes after sample collection. The samples were then transferred to 1.5-mL tubes after the supernatant was discarded and stored at −70°C for future measurements of serum CRP, PAPP-A, and MMP-9 levels. Blood cells were also stored in tubes at −70°C for DNA extraction.

Determination of serum CRP, PAPP-A, and MMP-9 levels

Human serum CRP levels were measured using the immunoresonance scattering assay (BN-II protein analyzer; Dade Behring Company, Marburg, Germany). Human serum PAPP-A and MMP- 9 levels were detected using the enzyme-linked immunosorbent assay[42] in accordance with kit instructions (Wuhan EIAab Science, Wuhan, Hubei Province, China). PAPP-A antibody was aliquoted into 96-well microplates to prepare a solid-phase carrier. Standards and samples were added to each well, and PAPP-A interacted with antibodies bound to the solid-phase carrier. Biotinylated PAPP-A antibody (enzyme-linked immunosorbent assay kit; Uscn Life Science, Wuhan, Wuhan city, Hubei Province, China) was then added to the plate and incubated, and non-adherent antibody was subsequently washed away. HRP-labeled avidin and a color substrate were then added to visualize the presence of antibody after thorough washing. Color intensity positively correlated with PAPP-A levels. The absorbance value at 450 nm was measured using a microplate reader (Microplate Reader 550; Bio-Rad, Hercules, CA, USA), and sample concentrations were calculated. Using the standard concentration for the vertical axis and the absorbance value for the horizontal axis. The standard curve was plotted on a logarithmic scale (best formula was based on R^2 value of regression equation; a R^2 value closer to 1 leads to better results). The obtained curve was analyzed using Professional Curve Expert 1.3 software. Sample concentrations were inferred according to absorbance values from the standard curve.

DNA extraction

DNA was extracted using the Promega whole blood DNA extraction kit (Promega Biotech, Beijing, China; Cat. # A1125). DNA (5 μL) was mixed with 195 μL ultrapure water. Absorbance at 260 and 280 nm was read using an UV spectrophotometer (Beckman, DUR640; Los Angeles, CA, USA), and the ratio of A_{260}/A_{280} was calculated. DNA concentration (μg/μL) = A_{260} × 50 × dilution factor/1 000. The A_{260}/A_{280} ratio was the hallmark for purity of extracted DNA, and measured levels were between 1.7 and 2.0, indicating that DNA samples were of high purity.

Determination of PAPP-A genotype

PAPP-A primers were designed using Primer Premier 5.0 software (Premier, Canada) and were synthesized by Takara (Dalian, China). The PAPP-A upstream primer was 5'-TGG TTT GTC TGT TGC CAT AA-3'; the downstream primer was 5'-ATC ATC CCG CCG TAT CTG-3'. PCR amplified products were obtained (Eppendorf 22331 PCR reaction device; Eppendorf, Hamburg, Germany). PCR reaction conditions were as follows: 12.5 μL Taq PCR MasterMix, 1 μL each of upstream and downstream primers, 1 μL DNA template (0.04–0.08 μg/μL), and ultra-pure water. The total volume was 25 μL. A total of 35 cycles of 94°C predenaturation for 10 minutes, 94°C denaturation for 30 seconds, 60°C annealing for 30 seconds and 72°C extension for 45 seconds, followed by a 72°C extension for 5 minutes. Samples were electrophoresed on a 2% agarose gel in 1 × Tris-acetate-EDTA electrophoresis buffer at 110–120 V for 40 minutes (PowerSupply PAC300 horizontal electrophoresis apparatus; Bio-Rad, Hercules, CA, USA) to obtain PCR amplification bands. The PCR amplified gene fragments were sequenced by Shanghai Sangon Biotechnology (Shanghai, China) to determine the A/C (Tyr/Ser) genotype and allele frequencies in exon 14 of PAPP-A.

Statistical analysis

Data were analyzed using SPSS 13.0 software (SPSS, Chicago, IL, USA). Measurement data were expressed as mean ± SD and compared with independent sample *t*-test between groups. Quantification data were compared using the chi-square test. Pearson's correlation analysis was utilized to analyze the correlation between serum PAPP-A levels and MMP-9 and CRP in the case group. PAPP-A genotype and allele frequencies were calculated using the frequency counting method. Genotype and allele frequencies between groups were compared using the chi-square test. The chi-square goodness-of-fit test was applied to determine Hardy-Weinberg genetic equilibrium of gene frequencies. The OR (*A* value) and 95% *CI* represented relative risk. Two-tailed $P < 0.05$ was considered statistically significant. Confounding factors, including age, sex, smoking, hypertension, diabetes, and hyperlipidemia, were adjusted using the multivariate logistic regression to determine the correlation between the A allele and risk of ICVD.

Author contributions: Haiping Wang had full access to the study concept and design. Zhang Chen supervised the study. Haiji Wang was responsible for data acquisition and integration. Yan Song and Jingjing Zhan were responsible for data analysis. Rui Zhang was responsible for statistical analysis.

Conflicts of interest: None declared.

Ethical approval: The pilot was approved by the Medical Ethics Committee, Affiliated Hospital of Qingdao University Medical College in China.

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