RESEARCH ARTICLE

Association of protein Z and factor VII gene polymorphisms with risk of cerebral hemorrhage: a case–control and a family-based association study in a Chinese Han population

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Abstract

Protein Z (PZ) and factor (F) VII are two important factors in the clotting pathway which have similar structure, linked function and nearby gene sites. The aims of this study were to investigate whether the common variants of PZ and FVII genes are associated with the risk of cerebral hemorrhage (CH) and to explore the combined effects of PZ and FVII polymorphisms for CH risk. We performed genotyping analysis for two single-nucleotide polymorphisms (SNPs) of FVII (rs510317 and rs6046) and three SNPs of PZ (rs2273971, rs3024718 and rs3024731) both in a population-based case–control study and in a family-based association study. Case–control analysis found no evidence of significant association. But family-based association study revealed that the G allele of PZ rs2273971, and three haplotypes carrying the 'G' allele of PZ rs2273971: haplotype GA, CG and CGA of PZ and FVII genes, all had a significant effect on CH susceptibility ($Z = 1.882$, $P = 0.049$; $Z = 1.922$, $P = 0.044$; $Z = 1.826$, $P = 0.047$; $Z = 1.977$, $P = 0.048$, respectively). While, the A allele of PZ rs2273971, and four haplotypes carrying or crossing the 'A' allele of PZ rs2273971: haplotypes CA, ACAA, ACAT and ACAAT of PZ and FVII genes, may confer protection against CH ($Z = -1.882$, $P = 0.049$; $Z = -2.000$, $P = 0.045$; $Z = -2.319$, $P = 0.020$; $Z = -2.002$, $P = 0.045$; $Z = -2.015$, $P = 0.043$, respectively). This is a first family-based association study providing genetic evidences that PZ and FVII genes, especially PZ rs2273971 are involved in the development of CH in Han-Chinese families.

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Introduction

Stroke is currently viewed as a complex disease caused by a combination of multiple risk factors. Family, twin and genomewide association studies (GWAS) suggest that it has a strong genetic component (Fornage 2009; Munshi and Kaul 2010; Giralt-Steinhauer *et al.* 2014). Recent reports proposed that ischaemic and hemorrhagic stroke have a shared genetic basis. Our previous study suggested that the gene variants of the clotting system, prime biomarker candidates for ischaemic stroke (IS) are also involved in cerebral hemorrhage (CH) (Zeng *et al.* 2012).

Recently, *in vitro* and *in vivo* studies suggested that normal protein Z (PZ) level is necessary for appropriate activated factor X (FXa) inhibition, the key factor of the final common coagulation pathway. PZ is a vitamin K-dependent single-chain glycoprotein synthesized in the liver, with highest homology to other vitamin K-dependent factors (FVII, FIX, FX, protein C and protein S) in structure (Broze and Miletich 1984; Vasse 2008; Almawi *et al.* 2013). In contrast to these factors, PZ does not function as a serine protease because it lacks the active centre in its amino acid sequence. Instead, in the company of Ca^{2+} and phospholipids, PZ acts as a cofactor in the downregulation of coagulation, enhancing the rapid inhibition of FXa by protein Z-dependent protease inhibitor (PZI) more than 1000-fold (Sofi *et al.* 2004; Koren-Michowitz *et al.* 2006; Almawi *et al.* 2013). In the extrinsic coagulation pathway, it is the activated FVII (FVIIa) that converts FX to FXa (Nakagaki *et al.* 1991).

No matter in which way, low or high, PZ plasma levels were believed to be associated with IS (Kobelt *et al.* 2001; Vasse *et al.* 2001; Heeb *et al.* 2002; McQuillan *et al.* 2003; Ayoub *et al.* 2004). Several PZ single-nucleotide polymorphisms (SNPs) were found to be correlated with PZ plasma

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levels and thus effect PZ coagulation function (Lichy *et al.* 2004; Santacroce *et al.* 2004; van Goor *et al.* 2008; Topalidou *et al.* 2009). This was same with FVII (Grant 1997; Heywood *et al.* 1997; Kang *et al.* 2004; Rubattu *et al.* 2005). The associations of PZ rs2273971, rs3024718, rs3024731; and FVII rs510317 and rs6046 with IS were the most commonly studied, but yielded conflicting results (Grant 1997; Heywood *et al.* 1997; Nishiuma *et al.* 1997; Kang *et al.* 2004; Lichy *et al.* 2004; Yeh *et al.* 2004; Altarescu *et al.* 2005; Rubattu *et al.* 2005; Staton *et al.* 2005; Funk *et al.* 2006; Roldan *et al.* 2008; van Goor *et al.* 2008; Nowak-Gottl *et al.* 2009). To our knowledge, there are only three case–control studies on association of PZ or FVII gene SNPs with CH (Obach *et al.* 2006; Greisenegger *et al.* 2007; Navarro-Nunez *et al.* 2007). All these three studies were performed in Caucasian populations.

PZ and FVII genes both located at chromosome 13q34 (de Grouchy *et al.* 1984; Fujimaki *et al.* 1998). The aims of this study were to investigate whether the common variants of PZ and FVII genes are associated with the risk of CH and to explore the combined effects of PZ and FVII SNPs for CH risk in a southern Han Chinese population.

Materials and methods

Study population

The study population consisted of 195 sporadic CH patients, 116 healthy controls and 281 individuals from 65 unrelated CH pedigrees. The CH patients had been admitted to Xiangya Hospital, Central South University. The healthy controls were randomly recruited from a population who underwent health examination in the Health Care Centre of Xiangya Hospital. All subjects enrolled were of southern Han Chinese descent. Brain CT and/or MRI were performed in all patients. The inclusion and exclusion criteria for patients and controls were the same as previously stated (Zeng *et al.* 2012). Baseline characteristics and classical vascular risk factors were recorded (Zeng *et al.* 2012). The study protocol was approved by the Ethics Committee of Xiangya Hospital, Central South University. Informed written consent was obtained from all participants.

Genotyping

From each subject, 5 mL of peripheral blood (heparin anticoagulant) was collected for genomic DNA extraction by the phenol/chloroform method. We used Multiplex SNaPshot method to genotype. SNaPshot reactions were performed as described by the manufacturer (Applied Biosystems, Foster City, USA). Briefly, 15 μL of touchdown PCR product was incubated at 37°C for 1 h with 1 U shrimp alkaline phosphatase (SAP) and 1 U exonuclease I (*Exo*I). Following a 15 min incubation at 75°C to inactivate the enzymes, 2 μL of digested PCR product was mixed with 5 μL of ready reaction premix, 1 μL of primers mixture and 2 μL of ultrapure water. PCR cycling conditions were as follows: predegeneration at 96°C for 1 min, then 28 cycles of 96°C for 10 s, 50°C for 5 s and 60°C for 30 s, followed by a reextension at 60°C for 1 min. When completed, 1 U SAP was added and the reaction mixture was incubated at 37°C for 1 h and then inactivated at 75°C for 15 min. Prior to loading onto the ABI3130XL (Applied Biosystems), 0.5 μL of Liz 120 Size Standard and 9 μL of Hi-Di was added to 0.5 μL of reaction mixture and samples were heated to 95°C for 5 min. Primers used for touchdown PCR amplification are listed in table 1 and extension primers used for SNaPshot are listed in table 2. Five randomly-selected duplicate samples of each genotype were included for the purpose of quality control. The genotyping rates for the five SNPs were 96.9–98.3% and the consistency rate for duplicate samples was 100%.

Statistical analysis

Hardy–Weinberg equilibrium (HWE) was assessed by chi-square test. Genotype frequencies were compared by chi-square test. Haploview ver. 2.02 was used to define haplotype blocks in case–control data (Zeng *et al.* 2012). Phase ver. 2.1.1 was used to infer the haplotype frequencies in case–control data (Zeng *et al.* 2012). The odds ratio (OR) and 95% confidence interval (CI) for the effect on sporadic CH risk were estimated by logistic regression analysis before and after adjusting for the recorded common risk factors. Chi-square analysis and logistic regression analysis were performed using SPSS 11.5 software (Chicago, USA). Software package family-based association test (FBAT) ver. 2.0.2 was

Table 1. Primer sequences used for touchdown PCR of the SNPs.

SNP	Primer sequence ($5' \rightarrow 3'$)	Fragment size (bp)
rs2273971	F: GGAAGCACACAGCTGCACAGG R: GACGCAGCCTGCCATTCC	244
rs3024718	F: CATGCTTTGGGACCCTCAGGT R: ACTGGGGACCAAGAGCCATCA	188
rs3024731	F: GGGAAACACTGACACAGAGGACAA R: TCAGTCCGCTCTGGGTGACAT	215
rs510317	F: GGCCTGGTCTGGAGGCTCTCT R: GTTGCCAGCGTGCAGGTGTTA	200
rs6046	F: CAAAGTGGCCCACGGTTGC R: ACTCGGATGGCAGCAAGGACT	237

Table 2. Extension primer sequences used for Multiplex SNaPshot of the SNPs.

SNP	Extension primer	Primer sequence (5′ → 3′)
rs2273971	rs2273971SR	TTTTTTTTTCTGCCATTCCCACCCGGAG
rs3024718	rs3024718SF	TTTTTCACTGCCGCGGGTCAACTC
rs3024731	rs3024731SF	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCACATGGACCAACACACCAGA
rs510317	rs510317SF	TTTTTTTTTTTTTTTTTCAAATATTTACATCCACACCCAAGATAC
rs6046	rs6046SF	CGTCAGGTACCACGTGCCC

used for SNP-based and haplotype-based association analyses in family-based data. Analysis was carried out under the additive model in the biallelic mode (with respect to the minor allele). For all statistical tests, a $P < 0.05$ was considered significant. In FBAT, $Z > 0$ and $P < 0.05$ indicated that a specific single allele or haplotype was overtransmitted to CH patients in informative families than expected under the null hypothesis of no linkage and no association, and thus was significantly associated with susceptibility to CH; while $Z < 0$ and $P < 0.05$ indicated a allele or haplotype was undertransmitted from informative parents to the affected children and thus conferred protection against CH.

Results

All PZ and FVII genotype distributions in controls were consistent with those expected based on HWE. In the case–control analysis, no statistically significant differences in genotype distributions between CH patients and controls were found (table 3). In addition, we compared the allele distributions of all the five SNPs involved in this study with that in other ethnics (data from the National Center for Biotechnology Information (NCBI) SNP database, http://www.ncbi.nlm.nih.gov/SNP, chosen from the largest population). The allele frequencies of all the five SNPs involved in this study were similar to those in Han Chinese background (HCB) or Chinese Han in Beijing, China (CHB) population, but were significantly different to those in Japanese from Tokyo, Japan (JPT), European, African-American and sub-Saharan African populations (table 4).

Figure 1 show plots of the pairwise linkage disequilibrium (LD, D′) values for the five selected SNPs in case–control data. One block of LD was defined (the D′ value between PZ rs3024718 and rs3024731 was 0.88, which was very close to 0.9, also suggested a strong recombination event, thus we presumed PZ rs2273971, rs3024718 and rs3024731were all in one block). We observed no statistically significant differences in haplotype frequency distribution. No haplotype was found to be associated with CH by further logistic regression analysis, even after adjusting for the different confounding variables.

In the family-based association analysis, FBAT revealed a significant positive association in distribution of allele G of PZ rs2273971 ($Z = 1.882$, $P = 0.049$) and a negative association of allele A of PZ rs2273971 ($Z = -1.882$, $P = 0.049$) with CH susceptibility (table 5). Moreover, we found that haplotypes GA, CG and CGA displayed significant associations with CH ($Z = 1.922$, $P = 0.044$; $Z = 1.826$, $P = 0.047$; $Z = 1.977$, $P = 0.048$, respectively), while haplotypes CA,

Table 3. Genotype distributions in this Han Chinese population and results of case–control genotype association analysis for the SNPs.

					Logistic regression			
SNP	Genotype	Patient *n* (%)	Control *n* (%)	*P* value (2df)	Crude OR (95% CI)	Crude *P* value	Adjusted OR (95% CI)	Adjusted *P* value
rs2273971	GG	63 (33.3)	42 (36.8)	0.535	1.00 (reference)		1.00 (reference)	
	GA	93 (49.2)	53 (46.5)		0.864 (0.435–1.716)	0.675	0.866 (0.439–1.718)	0.678
	AA	33 (17.5)	19 (16.7)		1.010 (0.523–1.950)	0.976	1.014 (0.526–1.952)	0.979
rs3024718	AA	78 (41.3)	43 (37.7)	0.542	1.00 (reference)		1.00 (reference)	
	AG	95 (50.3)	54 (47.4)		1.927 (0.886–4.194)	0.098	1.922 (0.884–4.192)	0.096
	GG	16 (8.5)	17 (14.9)		1.869 (0.874–3.997)	0.107	1.864 (0.871–3.999)	0.110
rs3024731	AA	57 (29.4)	39 (34.2)	0.378	1.00 (reference)		1.00 (reference)	
	AT	102 (52.6)	57 (50.0)		0.752 (0.374–1.513)	0.424	0.761 (0.382–1.517)	0.431
	TT	35 (18.0)	18 (15.8)		0.920 (0.478–1.771)	0.804	0.928 (0.484–1.780)	0.812
rs510317	GG	66 (34.0)	31 (27.2)	0.214	1.00 (reference)		1.00 (reference)	
	GA	91 (46.9)	64 (56.1)		1.093 (0.544–2.199)	0.802	1.102 (0.588–2.239)	0.821
	AA	37 (19.1)	19 (16.7)		0.730 (0.385–1.383)	0.335	0.741 (0.389–1.387)	0.338
rs6046	CC	173 (91.5)	104 (92.0)	0.879	1.00 (reference)		1.00 (reference)	
	CT	16 (8.5)	9 (8.0)		0.936 (0.399–2.194)	0.878	0.927 (0.393–2.190)	0.875

$P < 0.05$, considered to be statistically significant. Logistic regression adjusted for age, sex, body mass index, hypertension, ischaemic heart disease, diabetes, alcohol intake, smoking, serum levels of total cholesterol, triglycerides and low density lipoprotein.

Table 4. Comparison of allele frequencies of the SNPs between this study and other ethnics.

SNP	Allele	Population[b] This study	HCB	JPT	European	African-American	Sub-Saharan African
rs2273971	G	60.1	64.8	48.9	8.5	36.3	35.0
Chi-squared			0.533	2.440	58.177	11.538	12.531
P value			0.465	0.118	0.000[a]	0.001[a]	0.000[a]
rs3024718	A	61.4	58.1	80.2	85.0	88.7	90.7
Chi-squared			0.187	8.679	14.612	20.907	24.671
P value			0.666	0.003[a]	0.000[a]	0.000[a]	0.000[a]
rs3024731	A	59.2	54.5	39.8	24.2	42.1	53.6
Chi-squared			0.423	7.221	25.229	5.781	0.509
P value			0.516	0.007[a]	0.000[a]	0.016[a]	0.476
rs510317	G	57.5	48.0	64.0	76.1	87.5	–
Chi-squared			1.791	0.910	7.801	22.236	–
P value			0.181	0.340	0.005[a]	0.000[a]	–
rs6046	C	96.0	91.5	93.4	88.3	88.7	86.9
Chi-squared			2.003	0.866	4.348	3.532	5.207
P value			0.157	0.352	0.037[a]	0.060	0.022[a]

[a]$P < 0.05$ considered to be statistically significant. [b]Data of allele frequencies in other ethnics were found in NCBI (http://www.ncbi.nlm.nih.gov/projects/SNP), chosen from the largest population number.

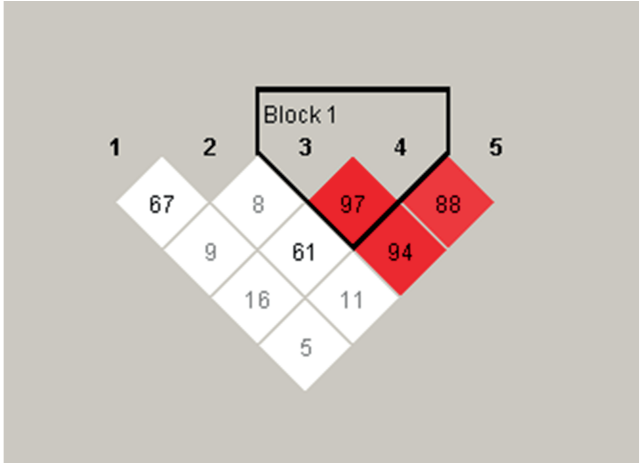

Figure 1. Intermarker linkage disequilibrium and haplotype blocks in FVII and PZ genes in case–control study. The block generated under confidence interval algorithm of HAPLOVIEW is marked. SNP 1 = rs510317, 2 = rs6046, 3 = rs2273971, 4 = rs3024718 and 5 = rs3024731, respectively, in the graph. The LD between two SNPs is D′ value. An indication of D′ value is shown graphically, with darker red representing higher D′. Since the D′ value is 0.88 between SNP 4 and 5 was very close to 0.9, which also suggested a strong recombination event, we presumed SNPs 3, 4 and 5 were all in one block.

ACAA, ACAT and ACAAT (Z = −2.000, $P = 0.045$; Z = −2.319, $P = 0.020$; Z = −2.002, $P = 0.045$; $Z = -2.015$, $P = 0.043$, respectively) was negatively associated with the disease (table 6).

Table 5. Results of family-based allele association analysis for the SNPs with LD.

Marker	Allele	Afreq	Fam#	S-*E*(S)	Var(S)	Z	*P*
rs2273971	**A**	0.487	20	−5.000	7.061	**−1.882**	**0.049**
	G	0.513	20	5.000	7.061	**1.882**	**0.049**
rs3024718	A	0.730	13	−1.993	3.689	−1.038	0.299
	G	0.270	13	1.993	3.689	1.038	0.299
rs3024731	A	0.526	20	3.133	6.221	1.256	0.209
	T	0.474	20	−3.133	6.221	−1.256	0.209
rs510317	A	0.437	19	−1.500	6.308	−0.597	0.550
	G	0.563	19	1.500	6.308	0.597	0.550

Afreq, estimated allelic frequencies; Fam#, number of informative families; S, test statistics for the observed number of transmitted alleles; *E*(S), expected value of S under the null hypothesis (i.e. no linkage or association). Significant *P* values ($P < 0.05$) are in bold.

Discussion

In this study, we found that the G allele of PZ rs2273971 and three haplotypes carrying the 'G' allele of PZ rs2273971: haplotype GA, CG and CGA of PZ and FVII genes were associated with higher risk of CH. While, the 'A' allele of PZ rs2273971, and four haplotypes carrying or crossing the 'A' allele of PZ rs2273971: haplotypes CA, ACAA, ACAT and ACAAT of PZ and FVII genes may decrease the susceptibility of CH. This is the first family-based association study providing genetic evidences that PZ and FVII genes are involved in the development of CH. PZ rs2273971 might

Table 6. Haplotypes associated with CH in the family-based association test.

Block	Haplotype	Afreq	Fam#	S-*E*(S)	Var(S)	Z	*P*
rs2273971/rs3024731	G–A	0.511	19	4.633	5.814	1.922	0.044
rs6046/rs2273971	C–G	0.516	20	4.785	6.867	1.826	0.047
rs6046/rs2273971/rs3024731	C–G–A	0.494	20	4.786	5.863	1.977	0.048
rs6046/rs2273971	C–A	0.447	22	−5.452	7.431	−2.000	0.045
rs510317/rs6046/rs2273971/rs3024718	A–C–A–A	0.210	22	−4.795	4.274	−2.319	0.020
rs510317/rs6046/rs3024718/rs3024731	A–C–A–T	0.192	21	−3.873	3.744	−2.002	0.045
rs510317/rs6046/rs2273971/rs3024718/rs3024731	A–C–A–A–T	0.191	20	−3.961	3.862	−2.015	0.043

Afreq, estimated allelic frequencies; Fam#, number of informative families; S, test statistics for the observed number of transmitted alleles; *E*(S), expected value of S under the null hypothesis (i.e. no linkage or association).

play an important role in the aetiology of CH in Han Chinese families.

In the case–control study part, we found that all the five SNPs detected in this study (PZ rs2273971, rs3024718, rs3024731 and FVII rs510317, rs6046) have no association with CH in the Han Chinese population. Similarly, Greisenegger *et al.* (2007) also found that there was no relationship beween FVII rs510317 and rs6046 with CH in an Austrian population. However, Obach *et al.* (2006) found that PZ rs3024718 was associated with CH in Spain. Since the study of Obach *et al.* (2006) and the present study had similar case–control sample sizes, the most likely explanation for the inconsistence would be the genetic heterogeneity in different ethnicities. The 'A' allele frequency of PZ rs3024718 in this study was 0.614, while was only 0.220 in the study by Obach *et al.* (2006). Moreover, factors like differences in clinical features and gene–environment interaction may contribute to the inconsistent result. Further association studies in larger samples and different genetic background populations are warranted.

We found that the allele frequencies of all the five SNPs involved in this study were significantly different to those in JPT, European, African-American and sub-Saharan African populations. This adds some evidence to the racial differences of PZ and FVII gene polymorphisms and highlights the complex nature of polygenic diseases like CH.

Population stratification is one of the major causes of confounding in case–control association studies. But family-based studies are thought to be robust against the population stratification bias (Lin and Chen 2010). We proposed a traditional population-based case–control study and a family-based association study in this present study. Interestingly, all these positive results only came from the family-based association study of this report. This demonstrates that the extended family-based association study can provide important insights into the relationships between SNPs and CH that were revealed by the population-based case–control study.

The present study has several limitations. First, we only chose five common SNPs of PZ and FVII genes that were mostly reported in association studies with IS. There were other tagged SNPs of PZ and FVII genes, such as those related to diseases like thrombosis, coronary heart disease, atherosclerosis, etc. More possible tagged SNPs of PZ and FVII should be performed in further studies. Second, the power of this population-based case–control study was limited by sample size. Large epidemiological studies in different ethnics should be done to prove our preliminary result. Third, there was no TDT result for the alleles of FVII rs6046 in the family-based association study. This may be because the nuclear families carrying FVII rs6046 in family-based data of this study were less than the minimum required number (10 nuclear families) by the FBAT software when performing the command '.fbat'. Further family-based association study in a larger sample should be performed to validate our finding.

Conclusions

In conclusion, we observed a greater risk of CH in individuals with the 'G' allele of PZ rs2273971 and three PZ and FVII haplotypes carrying the 'G' allele of PZ rs2273971. While, the A allele of PZ rs2273971, and four haplotypes carrying or crossing the 'A' allele of PZ rs2273971 were found to be associated with decreased susceptibility of CH. This is the first family-based association study providing genetic evidences that PZ and FVII genes, especially PZ rs2273971, are involved in the development of CH in Han Chinese families. The roles of PZ and FVII genes in CH merit further investigation.

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