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Correlation of PCSK9 Gene Polymorphism with Cerebral Ischemic Stroke in Xinjiang Han and Uygur Populations

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Background: Cerebral ischemic stroke (CIS) is a major cause of morbidity and mortality. Its main pathological basis is atherosclerosis (AS); in turn, the main risk factor in AS is dyslipidemia. Human proprotein convertase subtilisin/kexin9 (PCSK9) plays a key role in regulating plasma low-density lipoprotein (LDL) cholesterol levels. We sought to assess the association between PCSK9 and CIS in Chinese Han and Uygur populations.

Material/Methods: We selected 408 CIS patients and 348 control subjects and used a single-base terminal extension (SNaPshot) method to detect the genotypes of the 20 single-nucleotide polymorphisms (SNPs) in PCSK9.

Results: Distribution of SNP8 (rs529787) genotypes showed a significant difference between CIS and control participants (P=0.049). However, when analyzing Han and Uygur populations separately, we found that only Han subjects showed distribution of SNP1 (rs1711503), SNP2 (rs2479408), and SNP8 (rs529787) alleles that was significantly different between CIS and control participants (P=0.028, P=0.013, P=0.006, respectively), and distribution of SNP2 (rs2479408) in the dominant model (CC *vs.* CG + GG) was significantly different between CIS and control participants (P=0.013), even after adjustment for covariates (OR: 75.262, 95% confidence interval [CI]: 7.232–783.278, P<0.001). Distribution of the 2 haplotypes (A-C and G-C) (rs1711503 and rs2479408) was significantly different between CIS and control participants (both, P=0.011).

Conclusions: Both rs1711503 and rs2479408 of PCSK9 genes were associated with CIS in the Han population of China. A-C haplotype may be a genetic marker of CIS risk in this population.

MeSH Keywords: Atherosclerosis • Ethnic Groups • Ischemic Attack, Transient • Proprotein Convertases

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Background

Cerebral ischemic stroke (CIS) is a major cause of morbidity and mortality, and is expected to remain so until at least 2030 [1]. CIS and coronary heart disease (CHD) are major manifestations of atherosclerotic processes. High plasma levels of low-density lipoprotein cholesterol (LDL-C) have consistently been shown to be a risk factor for the development of atherosclerosis [2]. Plasma concentrations of LDL-C are determined primarily by the activity of the LDL receptor (LDLR) in the liver. Proprotein convertase subtilisin-like kexin type 9 (PCSK9) was recently discovered to be a major factor in cholesterol homeostasis through enhanced degradation of LDLR [3–6] and possibly in neural development. However, both rare mutations and common variants in the coding regions of PCSK9 can affect LDL cholesterol levels and stroke risk. Recent studies identified several PCSK9 variants influencing circulating LDL-C levels [7,8]. Since the first identification mutation of PCSK9 was implicated in autosomal dominant hypercholesterolemia by Abifadel [9], more than 53 missense variants have been identified. A common SNP, E670G (rs505151) in exon 12 of PCSK9, results in the substitution of glutamate for a glycine residue at position 670 in the protein [10] Carriers of 670 Gln in the general population presented increased plasma TC, LDL-C, and ApoB levels. Another study suggested a key role played by the E670G polymorphism in determining plasma LDL-C levels and the severity of coronary atherosclerosis in the United States [11]. More recently, the presence of the 670G allele was significantly associated with an increased risk of large-vessel atherosclerosis (LVA) stroke [12] and intimal media thickness (IMT) [13]. However, these studies were inconsistent with previous studies [14–16], which were conducted in Caucasian and African populations and failed to find this association. Furthermore, the carriers of 670G showed significantly increased LDL in men but not in women in a European population [17]. In addition, the rs72555377 insertion polymorphism in exon 1 of PCSK9 is associated with lower LDL-C in Caucasian populations [18], while the L11 allele, with insertion of 2 Leucines, is associated with higher LDL-C [11], and rs562556 (Ile474Val) in exon9 of the PCSK9 gene is associated with approximately 7% lower LDL cholesterol levels in carriers in a Japanese population [19].

In our study, we used a single-base terminal extension (SNaPshot) method to detect the genotypes of the 20 single-nucleotide polymorphisms (SNP) in the PCSK9 gene to assess the association between the human PCSK9 gene polymorphism and CIS in members of the Han and Uygur populations of China.

Material and Methods

Ethics approval of the study protocol

Written informed consent was obtained from all participants. All participants explicitly provided permission for DNA analyses as well as collection of relevant clinical data. This study was approved by the Ethics Committee of the First Affiliated Hospital of Xinjiang Medical University, Urumqi, China (NO. 20120510). It was conducted according to the standards of the Declaration of Helsinki.

Subjects

Subjects were from a Han population and a Uygur population who lived in the Xinjiang Uygur Autonomous Region of China. We recruited the CIS group from the First Affiliated Hospital of Xinjiang Medical University Neurology Department between since October 2011 and May 2012, and the control group came from the same hospital in the same period.

In the CIS group, there were 408 CIS patients (158 Uygur, 250 Han), mean age 61.97±11.80 years. Inclusion criteria were: (1) diagnosed in accordance with the standards set at 10 international classifications of diseases (ICD10); (2) confirmed by MRI. Exclusion criteria were: (1) patients with CHD; (2) hemorrhagic cerebrovascular disease confirmed by CT or MRI; (3) refused to participate in trials.

In the Control group there were 348 of healthy controls (149 Uygur, 199 Han), mean age 61.84±11.65 years. Inclusion criteria were: (1) aged >40; (2) no known family history of cerebrovascular disease; (3) the cardiopulmonary physical examination and nervous system examination did not find abnormalities; (4) MRI negative except for cerebrovascular disease. Exclusion criteria: acute or chronic infection, malignant tumor, autoimmune diseases.

Clinical characteristics of the study participants

All patients completed the standard test registration form, and disclosed the following data: (1) General information: age, sex, race. (2) Personal history: smoking history (daily average smoking, smoking an average of ≥1 day or more, time >1 year, defined as smoking), (drinking alcohol an average of ≥3 times per week, more than 50 g each time >1 year, defined as drinking), hypertension, diabetes, hyperlipidemia, transient ischemic attack (TIA), atrial fibrillation (AF), heart valve disease, heart valve replacement, peripheral vascular disease. Hypertension: the Seventh World Health Organization /International Society of Hypertension League Conference defined the new standard for the diagnosis of hypertension; in our study, the diagnosis of hypertension was established if patients were treated with

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antihypertensive medication or if the mean of 3 measurements of systolic blood pressure (SBP) >140 mm Hg or diastolic blood pressure (DBP) >90 mm Hg, respectively. Diabetes mellitus was diagnosed according to the criteria of the American Diabetes Association [20]. Individuals with daytime random blood glucose ≥11.1 mmol/l or after fasting glucose ≥7.0mmol/l or glucose in line 2 h ≥11.1mmol/l or with a history of diabetes or treatment with insulin were considered diabetic. (3) Medical history prior to admission: treatment with antihypertensive drugs, antiplatelet drugs and anticoagulants, diabetes, lipid drug, anti-seizure medication, birth control pills, hormones. (4) Family history: whether grandparents, parents, siblings, and children had hypertension, diabetes, cerebral hemorrhage, cerebral infarction, myocardial infarction, coronary heart disease, or arrhythmia incidence. (5) Physical examination: height, weight, blood pressure, pulse, temperature. (6) Special tests: electrocardiogram, chest X-ray, heart neck ultrasound, blood routine, blood glucose, blood lipids.

Biochemical analysis

Serum concentrations of total cholesterol (TC), triglyceride (TG), glucose (Glu), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C), apolipo protein A1 (ApoA1), apolipo protein B (ApoB), and apolipo lipoprotein a (ApoLpa) were measured using standard methods in the Central Laboratory of First Affiliated Hospital of Xinjiang Medical University.

Blood collection and DNA extraction

Fasting blood samples (5 mL) drawn by venipuncture were taken from all participants early in the morning. The blood samples were drawn into a 5-mL ethylene diamine tetraacetic acid (EDTA) tube and centrifuged at 4000×g for 5 min to separate the plasma content. Genomic DNA was extracted from the peripheral leukocytes using standard phenol-chloroform method. The DNA samples were stored at –80°C until use, then diluted to 50 ng/μL concentration.

SNaPshot Reactions

We selected the genotypes of the 20 SNPs in the PCSK9 gene using the Haploview 4.2 software and the HapMap phrase II database by using minor allele frequency (MAF) ≤0.1 and linkage disequilibrium patterns with r2 ≥0.5 as a cut-off. The position of the 20 SNPs was by order of increasing distance from the gene PCSK9 5' end (Table 1). We used single-base terminal extension (SNaPshot) method to genotype. SNaPshot reactions were performed as described by the manufacturer (Applied Biosystems, Warrington, UK). Briefly, 4.0-μl of PCR product was incubated at 37°C for 60 min with 2-U shrimp alkaline phosphatase (SAP) and 2-U Exonuclease I (ExoI). Following a 15-min incubation to inactivate the enzymes, 1 ul of digested PCR product was mixed with 5 ul of ready reaction premix, 1 ul of 1.0- UM primer (Table 1), and 3 ul of dH2O. This mixture was placed in the thermal cycler and underwent 25 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 30 s. When completed, 0.5-U SAP was added and the reaction mixture was incubated for 60 min. Prior to loading onto the PRISM 310, 10 ul of formamide was added to 1 ul of reaction mixture and samples were heated to 95°C for 5 min.

Statistical analysis

All continuous variables (e.g., age, BMI, pulse, and cholesterol levels) are presented as means ± standard deviation (S.D.). The difference between the CIS and control groups was analyzed using an independent-sample T-test. The differences in the frequencies of sex, hypertension, diabetes mellitus, smoking, drinking, and genotypes were analyzed using chi-square test or Fisher's exact test, as appropriate. Hardy-Weinberg equilibrium was assessed by chi-square analysis. Logistic regression analyses with effect ratios (odds ratio [OR] and 95% CI) were used to assess the contribution of the major risk factors. All statistical analyses were performed using SPSS 17.0 for Windows (SPSS Institute, Chicago, USA). Haplotypes were estimated using the SHEsis platform [21,22]. P-values of less than 0.05 were considered to be statistically significant.

Results

Table 2 showed the clinical characteristics of the CIS patients (n=408) and control participants (n=348). For all Han and Uygur subjects, there were no significant differences in age and sex between CIS patients and control subjects, indicating the study was an age- and sex-matched case-control study. We observed several differences between the groups of patients. As expected, several common risk factors for CIS were significantly different between the 2 subgroups: Glu, low HDL-C, high LDL-C, EH, and DM. Other CIS risk factors, such as high TC, TG levels, and cigarette smoking and drinking, were not significantly different.

Table 1 shows the basic information and the distribution of genotypes and alleles of the 20 SNPs for the PCSK9 gene. The position of the 20 SNPs was by order of increasing distance from the gene PCSK9 5' end. We observed that the distribution of genotypes and alleles of 3 SNPs (SNP1, SNP2, and SNP8) were significantly different between CIS group and control participants. All SNPs were consistent with Hardy-Weinberg expectations (data not shown).

The 3 SNPs among the 3 groups (Total, Han, and Uyghur) were examined by Hardy-Weinberg equilibrium test and no significant differences were found in these 3 groups (data not shown).

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Table 1. Genotype and allele distributions of the twenty SNPs in patients with CIS and control subjects.

	SNP	Chr. 1 postion		Function	dbSNP allele	MAF			Total			Han			Uygur		
									CIS	Control	*P* value	CIS	Control	*P* value	CIS	Control	*P* value
1	rs17111503	55503448	5' near gene	Upstream variant 2KB	A/G	0.3375	Genotype	AA	81	59		42	22		39	37	
	SNP1							AG	197	158	0.223	115	86	0.094	82	72	0.757
								GG	130	131		93	91		37	40	
							Allele	A	359	276	0.088	199	130	0.028*	160	146	0.684
								G	457	420		301	268		156	152	
2	rs2479408	55504118	5' near gene	Upstream variant 2KB	C/G	0.1708	Genotype	CC	385	314		249	192		136	122	
	SNP2							CG	21	33	0.064	1	7	0.013*	20	36	0.446
								GG	2	1		0	0		2	1	
							Allele	C	791	661	0.050	499	391	0.013*	292	270	0.423
								G	25	35		1	7		24	28	
	rs2479409	55504650	5' near gene	Upstream variant 2KB	A/G	0.4362	Genotype	AA	75	58		34	24		41	34	
3	SNP3							AG	190	162	0.789	110	87	0.864	80	75	0.715
								GG	143	128		106	88		37	40	
							Allele	A	340	278	0.496	178	135	0.599	162	143	0.416
								G	476	418		322	263		154	155	
4	rs11583680	55505668	Exon 1	Missense (V-A)	T/C	0.0905	Genotype	CC	328	280		194	159		134	121	
	SNP4							CT	78	62	0.237	56	37	0.099	22	25	0.673
								TT	2	6		0	3		2	3	
							Allele	C	734	622	0.710	444	355	0.850	290	267	0.350
								T	82	74		56	43		26	31	
5	rs10888896	55550 9213	Intron 1	Intron variant	C/G	0.2374	Genotype	CC	326	280		203	169		123	111	
	SNP5							CG	76	61	0.797	45	27	0.360	31	34	0.782
								GG	6	7		2	3		4	4	
							Allele	C	728	621	0.995	451	365	0.436	277	256	0.521
								G	88	75		49	33		39	42	
6	rs4927193	55509872	Intron 2	Intron variant	C/T	0.1377	Genotype	CC	2	5		0	3		2	2	
	SNP6							CT	82	64	0.347	59	39	0.097	23	25	0.863
								TT	324	279		191	157		133	122	
							Allele	C	86	74	0.953	59	45	0.818	27	29	0.609
								T	730	622		441	353		289	269	
7	rs499718	55512549	Intron 3	Intron variant	C/T	0.247	Genotype	CC	276	240		154	130		122	110	
	SNP7							CT	116	97	0.829	86	64	0.564	30	33	0.778
								TT	16	11		10	5		6	6	
							Allele	C	668	577	0.570	394	324	0.332	274	253	0.520
								T	148	119		106	74		42	45	
8	rs529787	55513521	Intron 3	Intron variant	C/G	0.1166	Genotype	CC	384	312		249	191		135	121	
	SNP8							CG	22	35	0.049*	1	8	0.006*	21	27	0.452
								GG	2	1		0	0		2	1	
							Allele	C	790	659	0.039*	499	390	0.006*	291	269	0.426
								G	26	37		1	8		25	29	
9	rs11206514	55516004	Intron 3	Intron variant	A/C	0.4096	Genotype	AA	248	212		152	124		96	88	
	SNP9							AC	141	115	0.670	89	67	0.899	52	48	0.727
								CC	19	21		9	8		10	13	
							Allele	A	637	539	0.772	393	315	0.842	244	224	0.551
								C	179	157		107	83		72	74	
10	rs572512	55517344	Intron 3	Intron variant	C/T	0.4596	Genotype	CC	54	40		26	14		28	26	
	SNP10							CT	171	144	0.711	102	78	0.365	69	66	0.993
								TT	183	164		122	107		61	57	
							Allele	C	279	224	0.469	154	106	0.171	125	118	0.991
								T	537	472		346	292		191	180	

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Table 1 continued. Genotype and allele distributions of the twenty SNPs in patients with CIS and control subjects.

	SNP	Chr. 1 postion		Function	dbSNP allele	MAF			Total			Han			Uygur		
									CIS	Control	*P* value	CIS	Control	*P* value	CIS	Control	*P* value
11	rs2479413	55518682	Intron 5	Intron variant	C/T	0.3191	Genotype	CC	225	193		141	124		84	69	
	SNP11							CT	150	140	0.090	95	70	0.183	55	70	0.054
								TT	33	15		14	5		19	10	
							Allele	C	600	526		377	318		223	208	
								T	216	170	0.363	123	80	0.109	93	90	0.834
12	rs7552841	55518752	Intron 5	Intron variant	C/T	0.284	Genotype	CC	264	235		176	143		88	92	
	SNP12							CT	126	96	0.602	65	48	0.886	61	48	0.502
								TT	18	17		9	8		9	9	
							Allele	C	654	566		417	334		237	232	
								T	162	130	0.564	83	64	0.834	79	66	0.406
13	rs557435	55520864	Intron 5	Intron variant	A/G	0.1662	Genotype	AA	5	1		1	0		4	1	
	SNP13							AG	56	56	0.242	32	25	0.668	24	31	0.260
								GG	347	291		217	174		130	117	
							Allele	A	66	58		34	25		32	33	
								G	750	638	0.862	466	373	0.755	284	265	0.703
14	rs693668	55521109	Intron 5	Intron variant	A/G	0.3912	Genotype	AA	212	189		131	116		81	73	
	SNP14							AG	164	135	0.772	100	71	0.441	64	64	0.908
								GG	32	24		19	12		13	12	
							Allele	A	588	513		362	303		226	210	
								G	228	183	0.472	138	95	0.205	90	88	0.774
15	R434W	5552339?	Exon 8	Missense (R-W)	C/T	/	Genotype	CC	408	348		250	199		158	149	
	SNP15							CT									
								TT									
							Allele	C	816	696		500	199		316	298	
								T									
16	rs540796	55524197	Exon 9	Synonymous codon (V-V)	G/A	0.1354	Genotype	AA	1	2		0	0		1	2	
	SNP16							AG	29	20	0.585	5	5	0.716	24	25	0.340
								GG	378	326		245	194		133	132	
							Allele	A	31	24		5	5		26	19	
								G	785	672	0.716	495	393	0.714	290	279	0.378
17	rs14931192655525315		Exon 10	Missense (E-Q)	G/C	0.0005	Genotype	CC	408	348		250	199		158	149	
	SNP17							CG									
								GG									
							Allele	C	816	696		500	398		316	298	
								G									
18	rs483462	55525400	Intron 10	Intron variant	A/G	0.3223	Genotype	AA	279	234		170	135		109	99	
	SNP18							AG	116	102	0.939	73	57	0.907	43	45	0.837
								GG	13	12		7	7		6	5	
							Allele	A	674	570		413	327		261	243	
								G	142	126	0.721	87	71	0.863	55	55	0.734
19	rs10465832	55528807	Intron 11	Intron variant	C/G	0.1483	Genotype	CC	2	3		1	2		1	1	
	SNP19							CG	75	67	0.778	54	39	0.654	21	28	0.419
								GG	331	278		195	158		136	120	
							Allele	C	79	73		56	43		23	30	
								G	737	623	0.603	444	355	0.850	293	268	0.218
20	rs505151	55529187	Exon 12	Missense (E-G)	A/G	0.0983	Genotype	AA	365	310		219	179		146	131	
	SNP20							AG	41	37	0.878	30	20	0.537	11	17	0.399
								GG	2	1		1	0		1	1	
							Allele	A	771	657		468	378		303	279	
								G	45	39	0.940	32	20	0.380	13	19	0.207

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Table 2. Characteristics of study participants.

	Total			Han			Uygur		
	Stroke patients	Control subjects	p Value	Stroke patients	Control subjects	p Value	Stroke patients	Control subjects	p Value
Number (n)	408	348		250	199		158	149	
Sex(M/W)	242/166	183/165	0.063	144/106	102/97	0.183	98/60	81/68	0.203
Age (years)	61.97±11.80	61.84±11.65	0.885	63.56±11.37	62.35±11.79	0.269	59.44±12.01	61.17±11.45	0.198
BMI (kg/m^2)	24.67±3.36	24.51±2.93	0.508	24.30±3.30	24.20±3.13	0.728	25.23±3.37	24.93±2.60	0.386
Glu (mmol/L)	6.90±3.30	5.45±2.68	<0.001*	6.86±3.12	5.24±1.44	<0.001*	6.98±3.55	5.74±3.73	<0.003*
TG (mmol/L)	1.90±1.12	2.04±1.30	0.122	1.81±1.08	1.90±1.21	0.406	2.03±1.17	2.22±1.40	0.221
TC (mmol/L)	4.38±0.96	4.27±1.24	0.182	4.35±0.95	4.43±1.24	0.444	4.42±0.98	4.06±1.22	0.004*
HDL (mmol/L)	1.05±0.35	1.36±0.90	<0.001*	1.07±0.26	1.35±0.84	<0.001*	1.02±0.44	1.37±0.98	<0.001*
LDL (mmol/L)	2.76±0.88	2.52±0.78	<0.001*	2.68±0.86	2.51±0.78	0.038*	2.87±0.89	2.52±0.79	<0.001*
ApoA1 (mmol/L)	1.25±0.27	1.22±0.35	0.216	1.27±0.22	1.24±0.30	0.310	1.21±0.32	1.18±0.40	0.538
ApoB (mmol/L)	0.89±0.76	0.89±0.61	0.909	0.90±0.75	0.90±0.79	0.986	0.87±0.24	0.88±0.23	0.612
ApL(a) (mmol/L)	195.27± 146.14	172.94± 113.84	0.019*	199.72± 146.08	192.68± 136.62	0.602	188.20± 146.42	146.57± 64.73	0.001*
EH (Y/N)	284/118	98/246	<0.001*	175/72	54/143	<0.001*	129/46	44/103	<0.001*
DM (Y/N)	125/269	65/272	<0.001*	78/164	31/168	<0.001*	47/105	34/104	0.242
Smoke (Y/N)	117/279	86/250	0.234	79/169	51/144	0.208	38/110	35/106	0.893
Drinking(Y/N)	0.204	44/290	0.161	43/204	26/167	0.292	23/120	18/123	0.500

BMI – body mass index; BUN – blood urea nitrogen; Glu – glucose; TG – triglyceride; TC – total cholesterol; HDL – high density lipoprotein; LDL – low density lipoprotein; EH – essential hypertension; DM – diabetes mellitus. Continuous variable were expressed as mean ± standard deviation. P value of continuous variables was calculated by independent T-T test. The P value of categorical variable was calculated by Fisher's exact test. * P<0.05.

In the study, we confirmed the distribution of genotypes and alleles of the 3 SNPs (SNP1, SNP2, and SNP8) for the PCSK9 gene. For SNP1 (rs17111503), the distribution of alleles showed a significant difference between CIS and control participants (P=0.028) in the Han group, but not in the total group and Uygur group. For SNP2 (rs2479408), the distribution of alleles, the dominant model (CC *vs.* CG + GG), and the additive model (CG *vs.* CC + GG) showed a significant difference between CIS and control participants in total and Han groups, but not in the Uygur group. C allele of rs2479408 was significantly higher in CIS patients than in control participants (total: 96.94% *vs.* 94.97%; Han: 99.80% *vs.* 98.24%). For SNP3 (rs529787), the distribution of alleles, the dominant model (CC *vs.* CG + GG) and the additive model (CG *vs.* CC + GG) showed a significant difference between CIS and control participants in the total and Han groups, but not in the Uygur group. C allele of rs529787 was significantly higher in CIS patients than in control participants (Total: 96.81% *vs.* 94.68%; Han: 99.80% *vs.* 97.99%) (data no shown).

Table 3 and Figure1 show patterns of linkage disequilibrium in the PCSK9 gene, with their |D'| and r^2 values. |D'| values from 0.7 to 1 indicate strong LD between a pair of SNPs. |D'| values from 0.25 to 0.7 indicate moderate LD and |D'| values of 0–0.25 indicate low LD. In the study, 3 strong LD patterns were observed between SNP1 and SNP2 (|D'|=0.999), SNP2 and SNP8 (|D'|=0.983), and SNP1 and SNP8 (|D'|=0.999). We consider that all 3 SNPs were located in 1 haplotype block. The r^2 value of SNP2–SNP8 >0.5 means the SNP2 and SNP8 can replace each other [11] and they cannot construct haplotypes simultaneously. Therefore, given that the position of SNP1 and SNP2 are both in 2KB upstream of PCSK9 gene and the position of SNP8 is in intron3, we constructed the haplotypes using SNP1 and SNP2.

Table 4 shows the distribution of haplotypes in CIS patient and control participants. There were 4 haplotypes established in all subjects. The overall distribution of the haplotypes were significantly different between the CIS patients

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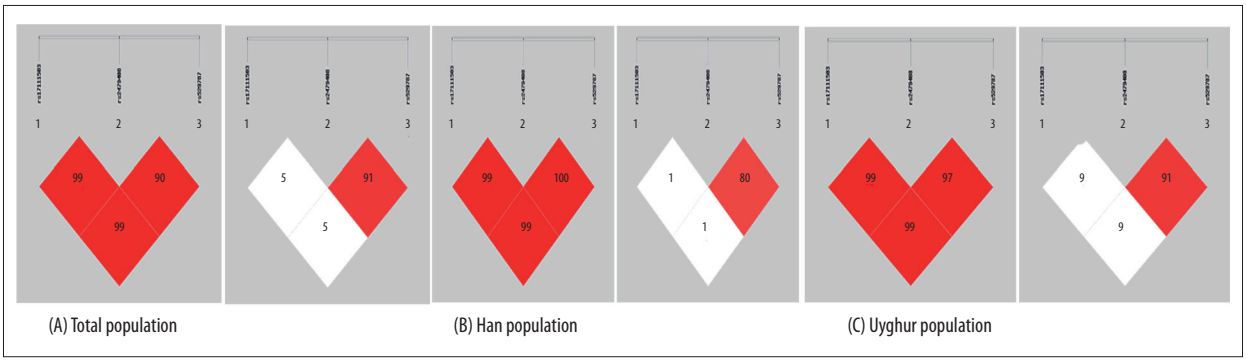
Table 3. Pairwise linkage disequilibrium (| D'| above diagonal and r^2 below diagonal) for the three SNPs.

	Total				Han				Uygur			
	|D'|				|D'|				|D'|			
	SNP	SNP1	SNP2	SNP8	SNP	SNP1	SNP2	SNP8	SNP	SNP1	SNP2	SNP8
r^2	SNP1		0.999	0.999	SNP1		1.000	0.988	SNP1		0.999	0.999
	SNP2	0.057		0.983	SNP2	0.016		1.000	SNP2	0.093		0.979
	SNP8	0.060	0.918		SNP3	0.017	0.888		SNP8	0.097	0.919	



(A) Total population (B) Han population (C) Uyghur population

Figure 1. Pairwise estimates of linkage disequilibrium (LD) between each PCSK9 polymorphism were plotted using SHEsis platform. Each polymorphism is numbered according to its position in the PCSK9 gene as presented (left shows |D'| and right shows r^2).

Table 4. Haplotype analysis of the two SNPs (rs17111503 and rs2479408).

	Haplotype	Case (freq)		Control (freq)		Odds Ratio [95% CI]	P
Total	AC	334.02	(0.409)	241.01	(0.346)	1.308 [1.061–1.613]	0.012*
	AG	24.98	(0.031)	34.99	(0.050)	0.597 [0.353–1.007]	0.051
	GC	456.98	(0.560)	419.99	(0.603)	0.837 [0.681–1.027]	0.088
	GG	0.02	(0.000)	0.01	(0.000)		
Han	AC	198.05	(0.396)	123.00	(0.309)	1.434 [1.085–1.895]	0.011*
	AG	0.95	(0.002)	7.00	(0.018)		
	GC	300.95	(0.602)	268.00	(0.673)	0.697 [0.528–0.922]	0.011*
	GG	0.05	(0.000)	0.00	(0.000)		
Uygur	AC	136.01	(0.430)	118.01	(0.396)	1.153 [0.836–1.590]	0.387
	AG	23.99	(0.076)	27.99	(0.094)	0.792 [0.448–1.401]	0.423
	GC	155.99	(0.494)	151.99	(0.510)	0.936 [0.682–1.285]	0.685
	GG	0.01	(0.000)	0.01	(0.000)		

All those frequency<0.03 will be ignored in analysis.

and the control subjects (all P<0.05). The most frequent haplotype in this study was A-C haplotype. For Han, the frequency of A-C was significantly higher in the CIS patients than in the control subjects (P=0.0011). In addition, the frequency of the G-C haplotype was lower in the CIS patients than in the control subjects (P=0.0011).

Table 5 showed that multiple logistic regression analyses were performed with age, sex, BMI, HDL-C, LDL-C, TC, TG, ApoA1 ApoB, ApoLpa, EH, DM, and smoking and drinking, because these variables were the major confounding factors for CIS. The significant difference of the dominant model (CC *vs.* CG + GG) of rs2479408 was retained after adjustment for covariates in the Han, but not in the Uygur group (OR: 75.262, 95% confidence interval [CI]: 7.232–783.278, P<0.001).

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Table 5. Multiple logistic regression analysis for stoke patients and control subjects.

	Total				Han				Uygur			
	OR	95% CI		P	OR	95% CI		P	OR	95% CI		P
		Lower	Upper			Lower	Upper			Lower	Upper	
rs2479408 (CC/CG+GG)	10.544	3.336	33.328	0.000*	75.262	7.232	783.278	0.000*	2.229	0.449	11.060	0.327
sex	10.544	3.336	33.328	0.613	1.147	0.651	2.019	0.635	1.045	0.558	1.956	0.891
age	1.001	0.986	1.016	0.901	0.997	0.976	1.018	0.762	1.017	0.991	1.043	0.196
BMI	0.981	0.924	1.041	0.522	0.983	0.905	1.068	0.686	0.987	0.897	1.086	0.789
TG	1.109	0.953	1.291	0.181	1.228	0.981	1.537	0.073	1.118	0.882	1.418	0.356
TC	1.031	0.851	1.250	0.756	1.239	0.933	1.646	0.139	0.715	0.508	1.008	0.055
HDL-C	1.783	1.288	2.468	0.000*	2.568	1.413	4.666	0.002*	1.297	0.854	1.970	0.223
LDL-C	0.685	0.528	0.889	0.004*	0.660	0.453	0.961	0.030*	0.752	0.483	1.169	0.205
APOA1	0.990	0.556	1.762	0.974	0.744	0.269	2.061	0.570	1.348	0.617	2.945	0.453
APOB	1.103	0.889	1.370	0.373	1.114	0.873	1.421	0.388	1.292	0.380	4.392	0.681
APL (a)	0.999	0.997	1.000	0.061	0.999	0.997	1.001	0.225	0.996	0.993	1.000	0.031
EH	5.308	3.700	7.615	0.000*	6.366	3.877	10.453	0.000*	5.112	2.836	9.215	0.000
DM	2.407	1.546	3.746	0.000*	4.746	2.403	9.376	0.000*	1.379	0.717	2.655	0.336
Smoking	1.137	0.656	1.972	0.647	1.133	0.542	2.370	0.739	0.956	0.376	2.433	0.925
Drinking	8.645	3.174	23.549	0.000*	52.408	5.808	472.912	0.000*	1.883	0.495	7.165	0.353

Discussion

PCSK9, also known as neural apoptosis-regulated convertase 1 (NARC1), is the ninth member of the proprotein convertase (PC) family [23]. The human PCSK9 gene is located on chromosome 1p32.3; it encompasses 12 exons and encodes a 692 amino acid glycoprotein. PCSK9 is synthesized as an inactive zymogen, pro-PCSK9 (73 kDa) and contains a signal peptide, a prodomain (residues 31–152) and a catalytic domain (residues 153–451) followed by a C-terminal domain (residues 452–692) [24]. PCSK9 acts as a serine protease and molecular chaperone that reduces both hepatic and extrahepatic low-density lipoprotein receptor levels through an endosomal/lysosomal pathway and increases plasma LDL cholesterol [4,25]. PCSK9 may also regulate apolipoprotein B-containing lipoprotein production and apoB secretion [26,27].

Recent advances revealed a large number of genetic variants of PCSK9 that may modulate plasma cholesterol levels either positively or negatively. "Gain of function" missense mutations in PCSK9 were associated with autosomal-dominant hypercholesterolemia (ADH), a rare form of familial hypercholesterolemia (FH) in which neither the LDLR nor the ligand binding domain of apolipoprotein (apo) B100 are mutated [28,29]. "Loss of function" nonsense mutations in PCSK9 were associated with low plasma LDL-C levels and a reduced incidence of cardiovascular disease [30,31]. Later, many *in vitro* and *in vivo* overexpression and knockout/knockdown studies confirmed that PCSK9 targets the LDLR for degradation [32–34]. Studies have confirmed that both rare mutations and common variants in the coding regions of PCSK9 affect LDL cholesterol levels and stroke risk. In this study, we selected 20 SNPs of PCSK9 and used case-control analyses to assess the association between the human PCSK9 gene polymorphism and CIS in the Han and Uygur populations.

Our findings showed the distribution of SNP8 (rs529787) genotypes were significantly different between CIS and control participants (P=0.049). However, when analyzing Han and Uygur groups separately, we found that only in the Han population was the distribution of SNP1 (rs1711503), SNP2 (rs2479408), and SNP8 (rs529787) alleles significantly different between CIS and control participants (P=0.028, P=0.013, P=0.006, respectively). For SNP1 (rs17111503), the frequency of A allele was higher in CIS than in control participants (P=0.028, 39.80% *vs.* 32.66%) in the Han group, indicating that the risk of CIS was increased with the A allele of rs17111503. For SNP2 (rs2479408), the distribution of alleles, the dominant model (CC *vs.* CG + GG), and the additive model (CG *vs.* CC + GG) showed a significant difference between CIS and control participants

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in total and Han groups, but not in the Uygur group. C allele of rs2479408 was significantly higher in CIS patients than in control participants (total: 96.94% *vs.* 94.97%; Han: 99.80% *vs.* 98.24%). Moreover, the significant difference of the dominant model (CC *vs.* CG + GG) of rs2479408 was retained after adjustment for covariates: age, sex, BMI, HDL-C, LDL-C, TC, TG, ApoA1 ApoB, ApoLpa, EH, DM, and smoking and drinking in the Han group (OR: 75.262, 95% confidence interval [CI]: 7.232–783.278, P<0.001), indicating that the risk of CIS was increased with the C allele of rs2479408. For SNP3 (rs529787), the distribution of alleles, the dominant model (CC *vs.* CG + GG), and the additive model (CG *vs.* CC + GG) showed a significant difference between CIS and control participants in total and Han groups, but not in Uygurs. C allele of rs529787 was significantly higher in CIS patients than in control participants (total: 96.81% *vs.* 94.68%; Han: 99.80% *vs.* 97.99%). When we constructed the haplotypes using SNP1 and SNP2, we found that the most frequent haplotype in this study was A-C haplotype. For Han, the frequency of A-C was significantly higher in the CIS patients than in the control subjects (P=0.0011), but the frequency of the G-C haplotype was lower in the CIS patients than in the control subjects (P=0.0011). This fully showed that A allele of rs17111503 and C allele of rs2479408 may be the risk factor of CIS, and G allele of rs17111503 and G allele of rs2479408 may be the protective factor of CIS.

SNP20 (rs505151) was observed in the exon12 of the PCSK9 gene and the polymorphisms caused the substitution of glutamate for a glycine residue at position 670 in the protein. The studies about the association between rs505151 of PCSK9 gene polymorphisms (E670G) and the cardiovascular risk have provided inconsistent results, as the introduction of description. Our study was consistent with previous studies [14–16] showing no significant association between the polymorphism of PCSK9 (rs505151) and CIS. By comparison, we found the age of our control subjects was higher than the other studies [11,12] and the study by Afef Slimani [35]. In our study, there were no significant difference in age between CIS patients (age: 63.56±11.37) and control subjects (age: 62.35±11.79) (P=0.269), but in the study by Afef Slimani, there were significant difference in age between CIS patients (age: 66/54.5–76.50) and control subjects (age: 49/45–55) (P<0.0001). Age is a risk factor for stroke, and this may be why our conclusions were not consistent with their conclusions. In addition, there may be differences in populations and geographical factors that explain some differences.

Conclusions

We found that both rs1711503 and 2479408 of PCSK9 were associated with CIS in the Han population of China. A-C haplotype may be a risk genetic marker of CIS in Han in China. A allele of rs17111503 and C allele of rs2479408 may be the risk marker of CIS. Studies with statistically significant numbers of clinical samples are needed for further research in China.

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