

Kininogen 1 gene polymorphisms are not associated with intracerebral hemorrhage in a Chinese Han population

[Çin Han popülasyonunda kininojen 1 genindeki polimorfizmler intraserebral hemoraji ile ilişkili değililer*]

Qi-Dong Yang¹,
Hong-Xing Wang¹,
Le Zhang¹,
Bao-Qiong Liu²,
Zhong-Yang Hu³,
Ming-Ming Ma⁴,
Jian Xia¹, Hong-Wei Xu¹,
Xiao-Ping Du¹

Central South University, Departments of
¹Neurology and ²Rehabilitation, Xiangya Hospital,
Changsha 410008, ³Departments of Neurology,
Third Xiangya Hospital, Changsha 410013,
⁴Department of Neurology, Henan Province
People's Hospital, Zhengzhou, 450000, China.

Yazışma Adresi
[Correspondence Address]

Le Zhang, MD

Department of Neurology, Xiangya Hospital, Central South University
No.87 Xiangya Road, Changsha, Hunan 410008, China.
Tel: +86-13548970664
Fax: +86-731-84327936
E-mail: 0205whx.student@sina.com

* Translated by [Çeviri] Ebru Karabal.

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ABSTRACT

Aim: Little is known about the potential role of genes in the pathogenesis of most intracerebral hemorrhagic stroke. Kininogens (KNG1) are the precursor of potent vasoactive kinin peptides and also function as cysteine proteinase inhibitors which are involved in hypertension and aneurysm. The purpose of this study is to investigate whether KNG1 gene polymorphisms are associated with intracerebral hemorrhage (ICH) in a Chinese Han population.

Materials and methods: A hospital based case-control study was conducted and we investigated the rs1656922 and rs2304456 polymorphisms of KNG1 gene from 351 ICH patients and 312 unrelated age- and gender-matched controls by using the multiplex SNaPshot reaction.

Results: The results showed that the T allele of rs1656922 was significantly over represented in the ICH patients. However, multiple logistic regression analysis both under recessive and dominant model were failed to confirm the two variants as risk factors for ICH. Furthermore, no gender or hematoma site specific associations were discovered between the two variants of KNG1 gene and ICH. However, the prevalence of the rs2304456 GG genotype ($p=0.014$) and the frequency of the G allele ($p=0.012$) were significantly increased among hypertensive patients when compared with normotensive patients.

Conclusion: In conclusion, these findings represent an important negative result indicating that rs1656922C/T and rs2304456G/T polymorphisms of KNG1 gene are not associated with ICH while rs2304456 GG genotype may be a risk factor for hypertension in a Chinese Han population.

Conflict of interest: There are no any actual or potential conflicts of interest including any financial, personal or other relationships with other people or organizations.

Key Words: Kininogen, polymorphism, genetics, intracerebral hemorrhage

ÖZET

Amaç: İntraserebral hemorajik inmenin patogeneğinde genlerin potansiyel rolü ile ilgili mevcut pek fazla veri bulunmamaktadır. Kininojenler (KNG1) etkili vazoaaktif kinin peptidlerinin prekürsörleridir ve aynı zamanda hipertansiyon ve anevrizmada rol oynayan sistein proteinaz inhibitörü olarak da fonksiyon gösterirler. Bu çalışmanın amacı, Çin Han popülasyonunda KNG1 genindeki polimorfizmlerin intraserebral hemoraji (İSH) ile ilişkili olup olmadığını araştırmaktır.

Gereç ve Yöntemler: Hastane bazlı vaka-kontrol çalışması olarak tasarlanan çalışmada, 351 İSH hastası ile 312 yaş ve cins açısından eşleştirilmiş kontrol deneklerinde KNG1 genindeki rs1656922 ile rs2304456 polimorfizmleri Multiplex SNaPshot reaksiyonu ile araştırılmıştır.

Bulgular: Elde edilen bulgular, rs1656922 T alelinin İSH hastalarında belirgin olarak daha fazla bulunduğunu göstermektedir. Fakat, hem resesif hem de dominant modelle yapılan çoklu lojistik regresyon analizi, iki varyantın İSH risk faktörü olduğunu teyit etmemektedir. Ek olarak, KNG1 geninin iki varyantı ve İSH arasında cins ve hematoma bölgesine özgü ilişki bulunamamıştır. Buna rağmen, rs2304456 GG genotipi prevalansı ($p=0.014$) ve G alel sıklığı ($p=0.012$) hipertansif hastalarda normotansif hastalara göre belirgin olarak yüksektir.

Sonuç: Sonuç olarak, çalışma bulguları Çin Han popülasyonunda rs2304456 GG genotipinin hipertansiyon açısından risk faktörü olabileceğini gösterirken, önemli bir negatif sonuç olarak da, KNG1 genindeki rs1656922C/T ve rs2304456G/T polimorfizmlerinin İSH ile ilişkili olmadığını göstermektedir.

Ahahtar Kelimeler: Kininojen, polimorfizm, genetik, intraserebral hemoraji

Introduction

Strokes are a major public health problem worldwide, particularly in industrialized countries [1,2]. Intracerebral hemorrhage (ICH) accounts for 10-15% of first-ever stroke with high mortality and morbidity rates [3,4]. Strokes are generally more severe in patients with ICH [5]. There are racial and ethnic differences in stroke incidence [6]. However, Changsha, a city in the south of China, has an extraordinarily high incidence of ICH. Our research has shown that the annual average ICH incidence in Changsha is 131.0/100,000 per year from 1986 to 2000, and ICH accounts for 55.4% of all stroke cases [7], higher than those reported in many other studies [8,9]. So, it's representative to explore the causes and pathogenesis of ICH in this area.

Undoubtedly, ICH is a multifactorial disease. Hypertension has been recognized as the most common and important contributing factor to ICH [10,11]. Other risk factors may include dyslipidemia, diabetes, cerebral amyloid angiopathy, cigarette smoking, heavy alcohol consumption and being overweight, etc [12]. However, in many cases, the pathogenesis of ICH remains unclear. Considerable evidence has been accumulated to indicate that genetic factors may influence the incidence of ICH, especially among the patients with a positive family history of stroke or patients without a history of cerebral vascular risk factors [13-15].

Kininogens (KNG1) have been recognized as the precursors of potent vasoactive kinin that function as cysteine proteinase inhibitors which have multifunctional domains. They involve in many physiological and pathological processes, including the development of hypertension and sodium homeostasis, inflammation and the cardioprotective effects of preconditioning [16]. Meta-analysis indicates that KNG1 gene may be a candidate gene for intracranial aneurysm [17]. Furthermore, a gender-specific association between the KNG1 gene polymorphisms and essential hypertension in a Chinese Han population has been demonstrated [18]. All these findings indicate that KNG1 gene variants are important candidate genes for ICH. However, no association studies had been reported to investigate the contribution of these gene variants to human ICH.

The aim of the present study is to investigate whether these two single nucleotide polymorphisms (SNPs), rs1656922 and rs2304456 of KNG1 gene are associated with ICH in a Chinese Han population.

Material and Methods

Subjects

The present study enrolled 358 cases selected from 589 unrelated ICH patients who were consecutively admitted to the neurology department of Xiangya Hospital, Central South University, between January 2007 and January 2010. Patients having cerebral venous throm-

bosis, transient ischemic attacks (TIA), and migrainous events were excluded. Patients were also excluded if they didn't agree to participate in the study and if ICH was caused by trauma, neoplasm, coagulation disorder, thrombolytic therapy, arteriovenous malformation and systemic disease. As the control group, 320 age- and gender-matched subjects without a prior history of cerebrovascular disease were enrolled. All subjects are Chinese Han in Changsha area and informed consents were taken from all of them. The study was approved by the human ethics committee of Xiangya Hospital in January 2007.

The following data of patients and controls were recorded by trained doctors: age, gender, body mass index (BMI), presence of hypertension and diabetes mellitus, smoking status, alcohol consumption, and lipid levels. BMI was calculated as weight divided by height square (kg/m^2). Hypertension was defined as an average systolic blood pressure (SBP) of at least 140 mmHg, diastolic blood pressure (DBP) of at least 90 mmHg on three consecutive blood pressure measurements, and/or current use of antihypertensive medication. Patients were classified as diabetic if they had any previous diagnosis, history of antidiabetic medication use or fasting plasma glucose levels of ≥ 7 mmol/L. Smoking definition included both former smokers and active smokers. Alcohol drinkers were defined as consuming more than 3 drinks per week. Serum total cholesterol (TC) and triglyceride (TG) was tested enzymatically (CHOD-PAP and GPO-POD methods, respectively), and high density lipoprotein (HDL) and low density lipoprotein (LDL) were measured directly (Dimension HDL method and N-Geneous LDL, respectively).

Selection of SNPs and Genotype Determinations

Selection of SNPs was based on the following considerations: (1) as a small number of tagging SNPs (tSNPs) are sufficient to capture most of the genetic variation in high LD regions, Haplotype-tagging SNPs of KNG1 gene were selected using the publicly available HapMap CHB databank [19, 20] with Haploview version 4.0 software; (2) we further identified SNPs through a literature review seeking an association of SNPs with vascular diseases, such as hypertension, atherosclerosis, aneurysms and myocardial infarction; (3) compared with intronic and noncoding SNPs, we selected missense mutations which lead to amino acid changes and might influence the transcriptional rate and activity of enzymes; (4) in order to improve genetic calculator power, only those SNPs with minor frequency more than 10% were selected. After weighing all these considerations, the final SNPs analyzed in the present study included: rs1656922 and rs2304456.

Venipuncture was performed one day after admission to avoid the bias of early mortality. Whole blood sample (5 ml) from patients and controls was collected with potassium EDTA. Genomic DNA was extracted from white

blood cells using a standard phenol–chloroform method. The gene region of interest was amplified by a multiplex polymerase chain reaction (PCR) with the primers designed using the online Primer3 software [21,22]. To test for possible repetitive sequences, primers were aligned with the NCBI sequence databases using BLAST [23]. The rs1656922 primer sequence is as follows: forward: 5'-ATATCAACGCAGAGCCAGACCT-3', reverse: 5'-ACCTGTCTTTGGGCCCGTTTTAC-3'. The rs2304456 primer sequence is as follows: forward: 5'-ATTGTTTCAGGTGGTGGCTGGAT-3', reverse: 5'-CCAAAGGGACTTGCAGTCTGGAG-3'. The product size of rs1656922 and rs2304456 were 121 bp and 112 bp, respectively. PCR was performed in a mixture containing 10 ng of genomic DNA, 0.1 $\mu\text{mol/L}$ of each primer, 0.4 mmol/L dNTP, 3.0 mmol/L MgCl_2 , 10 \times PCR buffer, 1 U Taq polymerase (Qiagen Inc.) in a final volume of 20 μl . For negative control, water was used instead of genomic DNA in PCR. The reaction was carried out under the following conditions: initial denaturation at 95°C for 15 minutes, followed by 14 cycles of 20 seconds at 94°C, 60 seconds at 66°C, and 30 seconds at 72°C, 25 cycles of 20 seconds at 94°C, 60 seconds at 59°C, and 90 seconds at 72°C, with a final extension at 72°C for 2 minutes and a 4°C holding step. All PCR reactions were performed in 96-well microplates. No PCR product was detected from any of the negative control reactions. After amplification, PCR products were purified to remove primers and un-incorporated dNTP as follows: 15 μl of PCR product was incubated with 5 U SAP and 2 U Exo I enzyme (Promega Corporation) for 15 minutes at 37°C followed by 15 minutes at 75°C for enzyme inactivation. The minisequencing SNaPshot reaction was performed in a 10 μl reaction volume containing 5 μl SNaPshot ready reaction mix (Applied Biosystems, Inc.), 2 μl of purified PCR products, and 0.4 μM of extension primer for each variants (rs1656922SF: 15 poly(dT)+A CTCAACATTCCTCCCTCTTCA; rs2304456SR: 32 poly(dT)+CGTTTGCACAATTGAGTAGGT). Extension used 28 cycles of denaturation at 96°C for 10 seconds, annealing at 50°C for 5 seconds and extension at 60°C for 30 seconds. After the minisequencing reaction, 10 μl final volume was treated with 1 U of SAP for 60 minutes at 37°C, followed by 15 minutes at 75°C for enzyme inactivation. The minisequencing products (0.5 μl) were mixed with 9 μl of Hi-Di formamide and 0.5 μl of GeneScan-120 LIZ size standard (Applied Biosystems), denaturation at 95°C for 5 minutes. The fluorescently labeled fragments were resolved by capillary electrophoresis on an ABI 3130 Genetic Analyzer (Applied Biosystems). The resulting data was analyzed with the GeneMapper 4.0 software (Applied Biosystems Co. Ltd., USA) [24]. Finally, 351 ICH patients and 312 controls were successfully genotyped.

Statistical Analysis

To describe the characteristics of the study population,

categorical data were reported as frequencies and percentages, and continuous data were reported as mean \pm SD. Hardy-Weinberg equilibrium was tested by the χ^2 test in the cases and controls separately. Differences in demographic characteristics and vascular risk factors between patients and controls were initially compared by univariate analysis with the use of Student's t test for continuous variables and the χ^2 test for all categorical variables. Logistic regression model were used to test the independent association of KNG1 gene and intracerebral hemorrhage adjusted for confounders. $P < 0.05$ was assumed as statistically significant. All statistical analyses were performed with SPSS software version 16.0.

Results

Baseline characteristics

The demographic and clinical data of the study subjects are summarized in Table 1. The two groups were well matched for age and gender. ICH patients had a significantly higher prevalence of hypertension, smoking and alcohol intake. Besides, systolic blood pressure (SBP) and diastolic blood pressure (DBP), serum TG and BMI were significantly higher, while HDL and TC were lower in cases than in controls. There were no significant differences in diabetes and LDL level between cases and controls.

Association study of single polymorphism with ICH

Table 2 shows the genotype distribution and allele frequencies of the KNG1 polymorphisms in ICH patients and controls. All SNPs were in Hardy-Weinberg equilibrium for both case and control groups ($P > 0.05$). The results showed that the T allele of rs1656922 was significantly over represented in the ICH patients ($P = 0.043$), while both allele and genotype frequencies of rs2304456 did not differ significantly between the two groups. Furthermore, when the case and control groups were divided into different subgroups according to gender, their blood pressure trait and site of hemorrhage (Table 3), there were no significant differences both in genotype and allele frequencies of rs1656922 and rs2304456 between male patients and male controls, and female cases and female controls. There were also no significant differences between lobar and deep hemorrhage (basal ganglia, thalamus, internal capsule, brain stem and cerebellum). However, it was found that G allele of rs2304456 was over-represented in hypertensive ICH patients, compared with normotensive cases ($\chi^2 = 6.338$, $P = 0.012$).

Multiple logistic regression analysis was conducted to identify possible independent risk factors for ICH, incorporating the KNG1 rs1656922 and rs2304456 polymorphisms, hypertension, cigarette smoking, alcohol consumption, diabetes mellitus, lipid indices. The results of the analyses are shown in Table 4. Hypertension showed the strongest association with ICH both under

Table 1. Demographic and clinical characteristics of ICH patients and controls

Variables	Patients (n=351)	Controls (n=312)	P Value
Age, y	58.35±12.65	57.24±8.38	0.177
Male, %	195 (55.56)	165 (52.88)	0.491
BMI, kg/m ²	23.76±2.92	23.04±2.98	0.002
Hypertension, %	221 (62.96)	69 (22.12)	<0.001
SBP (mmHg)	164.31±25.12	119.10±17.39	<0.001
DBP (mmHg)	95.29±16.09	75.39±10.25	<0.001
Diabetes, %	22 (6.27)	11 (3.53)	0.105
Smoking, %	115 (32.76)	67 (21.47)	0.001
Alcohol consumption, %	140 (39.88)	62 (19.87)	<0.001
TG (mmol/L)	1.81±1.09	1.55±1.08	0.002
TC (mmol/L)	4.33±1.10	4.70±1.18	<0.001
LDL (mmol/L)	2.52±0.69	2.45±0.80	0.276
HDL (mmol/L)	1.13±0.32	1.52±0.39	<0.001

Table 2. Genotype distribution and allele frequency of the two KNG1 gene SNPs in the participants.

SNP	Genotype frequency (percentage)				Allele frequency (percentage)			
	Major homozygote	Heterozygote	Minor homozygote	P	Major allele	Minor allele	P	
rs1656922	TT	CT	CC		T	C		
	Case	148(42.2)	156(44.4)	47(13.4)	0.126	452(64.4)	249(35.6)	0.043
	Control	114(36.5)	140(44.9)	58(18.6)		368(59.0)	256(41.0)	
rs2304456	TT	GT	GG		T	G		
	Case	247(70.4)	92(26.2)	12(3.4)	0.791	586(83.5)	116(16.5)	0.572
	Control	224(71.8)	80(25.6)	8(2.6)		528(84.6)	96(15.4)	

recessive and dominant model, increasing the susceptibility more than 5-fold (OR=5.852, P<0.001). Gender, BMI, alcohol intake and LDL also had elevated relative risks to ICH, while HDL and TC were protective factors. However, the other 6 considered variables were not independent risk factors for ICH, including T allele of rs1656922 and G allele of rs2304456.

Discussion

In the present study, the data represented an important negative finding that rs1656922 and rs2304456 polymorphisms of KNG1 gene, previously implicated as genetic risk factors for essential hypertension in a Chinese Han population [18], were not associated with ICH in our

study population. However, this might be the first study focusing on the SNPs of KNG1 gene and ICH in Chinese Han population.

In humans, there are two different forms of plasma kininogens, high molecular weight kininogen (HMWK) and low molecular weight kininogen (LMWK) because of alternative splicing [25]. Kallikrein acts on HMWK and LMWK to release bradykinin. All of these are important components of kallikrein-kinin system (KKS). So far, there is plenty of evidence documenting the role of KKS in the pathogenesis of hypertension [26-28] and its cardiac protective effects [29-31]. Dysfunctional KKS may contribute to high blood pressure, heart failure and left ventricular hypertrophy. KNG1 gene consists of 11 exons encompassing approximately 27kb and was mapped

Table 3. KNG1 rs1656922 and rs2304456 allele and genotype distributions in the ICH patients and controls.

	n	rs1656922				rs2304456			
		TT, %	CT, %	CC, %	T, %	TT, %	GT, %	GG, %	G, %
Controls									
All	312	114(36.5)	140(44.9)	58(18.6)	368(59.0)	224(71.8)	80(25.7)	8(2.5)	96(15.4)
Hypertensive	69	25(36.2)	33(47.8)	11(16.0)	83(60.1)	49(71.0)	19(27.5)	1(1.4)	21(15.2)
Normotensive	243	89(36.6)	107(44.0)	47(17.4)	285(58.6)	175(72.0)	61(25.1)	7(2.9)	75(15.4)
Male	165	60(36.4)	74(44.8)	31(18.8)	194(58.8)	115(69.7)	46(27.9)	4(2.4)	54(16.4)
Female	147	54(36.7)	66(44.9)	27(18.4)	174(59.2)	109(74.2)	34(23.1)	4(2.7)	23(14.3)
PICH patients									
All	351	148 (42.2)	156 (44.4)	47 (9.4)	452 (64.4)	247 (70.4)	92 (26.2)	12 (3.4)	116 (16.5)
Hypertensive	221	98 (44.3)	91 (41.2)	32 (14.5)	287 (64.9)	148 (67.0)	61 (27.6)	12 (5.4)	85 (19.2)*
Normotensive	130	50 (38.5)	65 (50.0)	15(11.5)	165(63.5)	99 (76.2)	31 (23.8)	0 (0.0)	31(11.9)
Male	195	83 (42.6)	85 (43.6)	27 (13.8)	251 (64.4)	135 (69.2)	54 (27.7)	6(3.1)	66 (16.9)
Female	156	65 (41.7)	71 (45.5)	20 (12.8)	201 (64.4)	112 (71.8)	38 (24.4)	6 (3.8)	50 (16.0)
Lobar location	49	20(40.8)	21(42.9)	8(16.3)	61(62.2)	35(71.4)	13(26.5)	1(2.1)	15(15.3)
Deep location	302	128(42.4)	135(44.7)	39(12.9)	391(64.7)	212(70.2)	79(26.2)	11(3.6)	101(16.7)

*G allele of rs2304456 in Hypertensive vs normotensive patients: $\chi^2=6.338$, $P=0.012$.

Table 4. Multiple Logistic Regression Model Incorporating KNG1 gene rs1656922 and rs2304456 polymorphisms and Vascular Risk factors.

	Recessive model		Dominant model	
	OR	P	OR	P
KNG1 rs1656922TT genotype	1.006	0.978	0.732	0.259
KNG1 rs2304456GG genotype	1.691	0.425	0.895	0.970
Age	1.006	0.546	1.005	0.631
Gender	1.691	0.019	1.691	0.019
BMI	1.083	0.021	1.083	0.021
Hypertension	5.852	<0.001	5.852	<0.001
Cigarette smoking	1.351	0.285	1.330	0.312
Alcohol consumption	2.092	0.002	2.092	0.002
Diabetes mellitus	1.625	0.326	1.565	0.368
TG	1.001	0.992	1.002	0.985
TC	0.804	0.040	0.804	0.040
LDL	1.440	0.025	1.440	0.025
HDL	0.042	<0.001	0.042	<0.001

to 3q27, the location of the homologous α 2HS-glycoprotein and histidine-rich glycoprotein [32,33]. However, several SNPs of the KNG1 gene were demonstrated to be associated with intracranial aneurysm [17] and essential hypertension in a Chinese Han population [18], making these genes important candidates for cerebrovascular disease.

Many studies have reported that the pathogenesis of lobar ICH may differ from deep ICH [34,35]. ICH in the deep locations is proved to occur more frequently in individuals with hypertension, whereas those in lobar sites often occur in normotensives [12,36]. Researchers have also shown that disease susceptibility may vary widely

according to gender in many highly prevalent diseases that affect both women and men [37]. Yang *et al.* [38] observed that sex hormones can affect protein amount, leading to different gene expression in men and women, which may well explain the potential effects of sex on the different predisposition to the disease susceptibility between genders. So, we stratified the case and control groups into different subgroups according to their gender and site of hemorrhage. However, no gender or hematoma site specific associations were discovered between the two variants of KNG1 gene and ICH.

Nevertheless, despite negative results of the current study, we still cannot completely exclude the possible role of KNG1 gene in ICH. The present study may yield false-negative results. First, racial and ethnic difference in KNG1 gene genotype distribution, varying greatly in different populations [19], should be considered. Further studies in other populations are warranted. Second, it has been well recognized that the KNG1 gene region was in strong linkage disequilibrium [18]. Data released from the HapMap database show that there were only slight change in linkage disequilibrium pattern when the number of genotyped common SNP (minor allele frequency >0.1) increased from 16 to 29. So, the interactions among the different KNG1 genes need to be further investigated.

When compared the allele and genotypes distribution between hypertensive and normotensive ICH patients, a significant difference was observed (OR=1.98; 95%CI, 1.16 to 3.42; P=0.008). G allele of rs2304456 polymorphism was over-represented in hypertensive patients, similar to the results of a previous study by Zhao *et al* [18], which have demonstrated that rs2304456 GG genotype was significantly associated with hypertension and blood pressure trait in males, but not females in a Chinese Han population. It's supposed that rs2304456 may lead to an alteration of amino sequence of isoleucine (Ile) to methionine (Met) and play a physiological effect directly or has an interaction with rs5030024 polymorphism, which was found to be responsible for kininogen deficiency in Williams' family [39].

Some limitations of our study should be noted. First, although blood withdrawal was conducted on the first day after the ICH patients were admitted to the hospital, we still cannot exclude selection bias. In addition, the loss of patients who refused to take part in the study or died before admission to the hospital may influence the final results. Finally, the sample size of our study is relatively small, especially when classified into different subgroups, and may limit the efficiency of genetic discovery.

Conclusion

In conclusion, to the best of our knowledge, this is the first study to investigate the association of the KNG1 gene SNPs with the ICH in a Chinese Han population. Our results represented an important negative result that

KNG1 gene rs1656922 and rs2304456 polymorphisms were not associated with ICH and confirmed the role of rs2304456 G allele in the development of hypertension. Further studies involving a large number of subjects and patient population are required to assess the role of KNG1 gene polymorphisms in the development of ICH, especially based on different racial and geographical populations.

Ethical Considerations

The study was approved by the human ethics committee of Xiangya Hospital in January 2007 and informed consents were taken from all of the subjects.

Conflict of Interest

There are no any actual or potential conflicts of interest including any financial, personal or other relationships with other people or organizations.

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