Gene polymorphisms and related risk factors in Mongolian hypertensive stroke patients

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ABSTRACT

الأهداف: التعرف على مخاطر الأشكال المتعددة لإنزيم النوكليونيد الأحادي الذي يسبب بشكل أساسي انتشار ارتفاع ضغط الدم والجلطة في سكان مونوغوليا.

الطريقة: أجريت الدراسة في شركة جنيوم هودا بكين، ومركز الاختبار السريري لوسط مونوغوليا، كلية مونوغوليا الطبية، هوهيهوت، الصين خلال الفترة من مارس إلى نوفمبر 2005م، واشتملت الدراسة على 96 مريض مصاب بارتفاع ضغط الدم، متوسط العمر 11±53 عام و68 مريض مصاب بجلطة ارتفاع ضغط الدم، متوسط العمر 10±60 عام. تم فحص تعدد أشكال الجين بتقنية تعدد أشكال طول الشدف لتفاعل سلسلة المبلمرة، ونظام سوكونيم وتحليلها بالتحليل اللوجستي المزدوج.

النتائج: أظهر التحليل اللوجستي المزدوج اختلافات مهمة بين المجموعات في العمر، والتدخين. لم يكن هنالك اختلافات إحصائية بين تكرار الأنماط الجينية، وجين الأليل بين المجموعات. كانت مخاطر الإصابة هي 3.182 قيمة إحصائية p=0.011 للنمط الجيني ACE DD، و 6.179 قيمة إحصائية p=0.038 للنمط الجيني CYP CT، و6.089 قيمة إحصائية p=0.042 للنمط الجيني CYP TT. لا ترتبط جميع الأنماط الجينية الأخرى بارتفاع ضغط الدم، والجلطة.

خاتمة: تعد الأنماط الجينية ACE DD، وCYP CT، و TT مؤشرات لارتفاع ضغط الدم مسببة الجلطة في سكان مونوغوليا. كما أن خطر الإصابة بالمرض منخفض في الأنماط الجينية ACE II، وCYP CC.

Objectives: To identify susceptible single nucleotide polymorphisms causing prevailing essential hypertension complicating stroke in the Mongolian population.

Methods: This study was carried out at the Beijing Huada Genome Company, Beijing, and the Clinical Testing Center of Inner Mongolia Medical College, Hohhot, P. R. China from March to November 2005, and included 96 patients with hypertension (control group) with an average age of 53±11 years, and 68 patients with hypertensive stroke with an average age of 60±10 years. The gene polymorphisms were examined by the polymerase chain reaction-restriction fragment length polymorphism technique and the Sequenom system, and analyzed by multiple regression analysis.

Results: Logistic multiple regression analysis revealed significant differences between the groups for age and smoking. Genotypes and allele gene frequencies were not significantly different between the groups. The significant incidence risks were p=0.011, odds ration (OR)=3.182 for the ACE DD genotype, p=0.038, OR=6.179 for the CYP CT genotype, and p=0.042, OR=6.089 for the CYP TT genotype. All the other genotypes did not significantly correlate to hypertension and hypertensive stroke.

Conclusion: The ACE DD, CYP CT and TT genotypes are candidates for hypertension complicating stroke in the Mongolian population. The risk of disease was lowest among the ACE II and CYP CC genotypes.

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Cerebrovascular accident (stroke) is a disease strongly correlated with the external environment and internal heredity, with internal hereditary playing the preeminent role. Currently, stroke is thought to be consistent with a multiple-gene disease genetic model. Research protocols mainly include candidate gene methods and linkage analysis. Previous studies[1]

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indicated that the rennin-angiotensin system plays an essential role in regulating blood vascular tone and water-electrolyte balance, and the angiotensin II blood plasma levels of patients with cerebrovascular diseases with hypertensive complications were significantly increased compared with patients without stroke.[2] To provide new insights in clinical diagnosis and therapy, the aim of present study was to identify susceptible single nucleotide polymorphisms (SNPs) that may specifically cause prevailing essential hypertension complicating strokes in a Mongolian population.

Methods. *Patient data.* All specimens were collected between October 2002 and November 2005 from a Mongolian population living in the Wulate Back county of Inner Mongolia, P. R. China. In total, 164 samples were gathered for analysis. There were 96 patients with hypertension with an average age of 53±11 years, and 68 patients with hypertensive stroke (specifically denotes hemorrhagic stroke) with an average age of 60±10 years. In our study, we strictly selected patients of Mongolian ethnicity, with no kin relationships with other ethnicities for 3 generations, and no interracial history. All participants gave their informed consent prior to the start of the study. The discrimination of hypertension is based on the 1999 WHO standard:[3] systolic pressure ≥140 mm Hg and/or diastolic pressure ≥90 mm Hg. The stroke clinical standard is consistent with the one reformulated by the 4th National Cerebrovascular Diseases Meeting.[4] All participants were asked to complete a survey form, the content of which included age, gender, height, weight, hypertension history, relevant illnesses, and family history. Each participant was examined 3 times, at a minimum of 10 minute intervals to obtain their average value.

Experiments. All the experiments were performed in the Beijing Huada Genome Company, Beijing, and the Clinical Testing Center of the Inner Mongolia Medical College, Hohhot, P.R. China between March and November 2005. The gene loci MTHFR C677T, eNOS G894T, ADRB1 G1165C, GNB3 C825T, ACE I/D, AGT M235T, and CYP11B2 C-344T were examined. The selection of the above genes analyzed in our study is based on previous works,[5,6] and they are thought to be related to the incidence of hypertensive stroke. Five ml of peripheral venous blood was collected and we used heparin for anticoagulation. The leucocytes were isolated and the whole DNA was extracted using phenol/chloroform.

1) Using polymerase chain reaction (PCR) the sixteenth intron segment of the ACE gene was amplified. The PCR primers are shown in **Table 1**. The PCR products were observed under an ultraviolet lamp after being processed by 2% agarose gel electrophoresis and ethidium bromide (EB) pigmentation. We used the following methods to discriminate genotypes: If there is an I/D Alu inserting sequence in the ACE allele and the size of PCR product is 490 bp, it is the insertion genotype (type II); if there is no Alu sequence, and the length of PRC product is 190 bp, we named it the deletion genotype (Type DD). Finally, if the PCR products include 2 segments (490 bp and 190 bp), the

Table 1 - Polymerase chain reaction primers for the hypertensive stroke and general hypertension groups.

Sites	Primers (5'~ 3')	Endonuclease
ACE I/D	CTGGAGACCCCCATC-CTTTCT GTAGTGGCATCACATTCGTCAGAT	
AGT M235T	GGTTTGTGCAGGGCCTGGCTCTC AGGGTGCTGTCCACACCTGCTCC	TthIII
CYP11B2	CAG GAG GAG ACC CCA TGT GAC CCT CCA CCC TGT TCA GCC Crs1801133	HaeIII
MTHFR C677T	P1 ACGTTGGATGCTTGAAGGAGAAGGTGTCTG P2 ACGTTGGATGCTTCACAAAGCGGAAGAATG P3 GCGTGATGATGAAATCG	
eNOS G894T	P1 ACGTTGGATGACCTCAAGGACCAGCTCGG P2 ACGTTGGATGAAACGGTCGCTTCGACGTGC P3 CTGCAGGCCCCAGATGA	
ADRB1 G1165C	P1 ACGTTGGATGAGCCCTGCGCGCGCAGCAGA P2 ACGTTGGATGCCTTCAACCCCATCATCTAC P3 TCCGCAAGGCCTTCCAG	
GNB3 C825T	P1 ACGTTGGATGTCTCCCACGAGAGCATCATC P2 ACGTTGGATGTCGTAGCCAGCGAATAGTAG P3 CTGAGGGAGAAGGCCAC	

genotype type is ID, totalling 3 ACE genotypes: II, DD, and ID.

2) Using the PCR technique to amplify the sequence segment containing the target gene AGT. We used restriction endonuclease TthIII-1 to digest the PCR amplified product with the AGT gene, and then performed 2% agarose gel electrophoresis, and observed the result under an ultraviolet lamp after EB pigmentation. As the AGT gene contains the homozygous type of the M allele (235MM type), there are no restriction enzyme cutting sites. Therefore, only a 163 bp amplified segment was observed in the lamp. The homozygous type of the T allele (235TT type) for the AGT gene has shifted from T to C at dinucleotide site 704 of the exon, so it can be digested by We used restriction endonuclease TthIII-1 enzyme, and a new 140 bp segment was generated. However, the AGT gene M235T heterozygote (235MT type) contains both 163 bp and 140 bp segments.

3) Using the PCR technique to amplify the sequence segment containing the CYP11B2 target gene, and after processing the HaeIII endonuclease digestion, 2% agarose gel electrophoresis and EB pigmentation, the result was observed under ultraviolet lamp. If the 202 bp segment is found, the genotype is CC, while if the 273 bp segment is found, the genotype is TT. If both segments are found, then the genotype is the heterozygote (TC). We did not directly amplify the sequences for other genes, including C677T loci of the MTHFR gene, G894T loci of the eNOS, G1165C loci of the ADRB1 gene, C825T loci of the GNB3 gene. Instead, all these above gene polymorphisms were sequenced and identified by the Huangda Gene Company, Beijing, China.

Statistical analysis. Using the Statistical Package for Social Sciences (SPSS Inc., Chicago, IL, USA) version 10.0 to perform the analysis, the measured data were expressed as the means ± standard errors. The mean comparison for multiple group data was analyzed by single-factor analysis of variance, and the comparison on genotype frequencies was analyzed by Chi-square test.

Results. On clinical comparison between the groups, the results of logistic multiple regression analysis revealed significant differences for age and smoking (**Tables 2 & 3**). On comparison of genotypes and allele frequencies, there were no significant differences between the groups (**Table 4**). The logistic stepwise-regression fitting model was used to analyze the genotype interaction effect. The results as summarized in **Table 5**, show significance for the ACE DD genotype, the CYP CT genotype, the CYP TT genotype.

Discussion. Brain stroke is a very complex disease caused by the co-effects from multiple genetic and environmental factors. The genetic model for brain stroke has not reached a clear consensus. So far, works from epidemiology and molecular genetics have shown that the genetic model for stroke is beyond current knowledge. Revealing the susceptible genes for stroke will be beneficial in controlling and monitoring the disease.[7]

As hypertension is a high-risk factor causing brain stroke, investigations into the genetic susceptibility of hypertension complicating brain stroke is meaningful. The ACE plays a vital role in the renin-angiotensin system (RASS) and kallikrein-kinin system (KKS), functioning in moderating blood tension and maintaining blood pressure. For individuals of a population, the ACE activity is not influenced by environmental factors, which are strongly correlated to the I/D genotype of the ACE gene.[8] In blood serum and blood tissue, the D allele is related to high ACE levels, indicating that it can affect the morbidity of brain stroke by changing the ACE concentration.[9] There are many inconsistencies in previous works on the correlation between ACE I/D polymorphism and hypertension complicating stroke.[10,11]

Table 2 - Comparison of clinical characteristics between hypertensive stroke and general hypertension groups.

Attributes	Hypertension with stroke (n=68)	Hypertension (n=96)	*P*-value
Age (years)	60±10	53±11	0.001
Male (%)	64	45	
Smoking (%)	59	37	0.027
Drinking (%)	43	52	
Pulse pressure index	0.38±0.11	0.38±0.08	
Waistline (cm)	90.72±12.74	92.22±12.29	
BMI (kg/m^2)	25.64±4.61	27.49±4.41	
Family history of hypertension (%)	37	41	
Family history of stroke (%)	27	20	

Table 3 - Logistic regression analysis of clinical characteristics between the hypertensive stroke and general hypertension groups.

Attributes	β	χ^2	*P*-value	OR	95% CI
Age (years)	0.062	12.015	0.001	1.032	1.007-1.057
Drinking	0.678	2.875	0.09	2.095	0.900-4.313
Family history of stroke	0.811	3.402	0.065	2.374	0.950-5.332
Gender	-0.428	1.226	0.268	0.652	0.306-1.391
Pulse pressure index	-1.691	0.625	0.429	0.184	0.003-12.175
BMI (kg/m^2)	0.002	0.171	0.679	1.002	0.994-1.009
Smoking	0.795	4.875	0.027	2.251	1.093-4.486
Waistline (cm)	-0.019	1.598	0.26	0.981	0.953-1.011

OR - odds ration, CI - confidence interval

Table 4 - Comparison of allele frequency between the genotypes of hypertensive stroke and general hypotension groups.

Site	Allele	Hypertension (n=96)	Hypertension with stroke (n=68)	*P*-value
MTHFR C677T	CC	32	26	
	CT	51	27	
	TT	13	15	0.177
	C/T	0.60:0.40	0.58:0.42	0.796
eNOS G894T	GG	73	51	
	GT	17	14	
	TT	6	3	0.808
	G/T	0.84:0.16	0.85:0.16	0.872
ADRB1 G1165C	GG	4	3	
	GC	30	19	
	CC	62	46	0.901
	G/C	0.20:0.80	0.17:0.83	0.473
GNB3 C825T	CC	43	35	
	CT	40	26	
	TT	13	7	0.659
	C/T	0.65:0.34	0.71:0.29	0.503
AGT M235T	MM	20	24	
	MT	71	42	
	TT	5	2	0.112
	M/T	0.58:42	0.66:0.34	0.292
ACE I/D	DD	18	24	
	ID	36	21	
	II	42	23	0.073
	D/I	0.37:0.62	0.51:0.49	0.093
CYP C-344T	CC	9	3	
	CT	42	33	
	TT	45	32	0.463
	C/T	0.31:0.69	0.29:0.71	0.801

Table 5 - Logistic regression analysis of genotypes between hypertension stroke and general hypertension groups.

Site	Genotype	β	χ^2	*P*-value	OR	95% CI
eNOS G894T	GT	0.359	0.624	0.429	1.431	0.588-3.484
	TT	-0.869	1.066	0.302	0.419	0.080-2.184
MTHFR C677T	CT	-0.730	3.354	0.067	0.482	0.221-1.053
	TT	0.381	0.573	0.449	1.463	0.546-3.923
ADRB1 G1165C	CG	-0.085	0.044	0.834	0.919	0.415-2.033
	GG	-0.411	0.200	0.655	0.663	0.109-4.017
GNB3 C825T	CT	-0.473	1.459	0.227	0.623	0.289-1.342
	TT	-0.501	0.750	0.387	0.606	0.195-1.884
AGT M235T	AGT(1)	0.051	0.003	0.955	1.052	0.180-6.145
	AGT (2)	0.947	1.036	0.309	2.579	0.416-15.984
ACE I/D	ID	-0.154	0.141	0.708	0.857	0.382-1.921
	DD	1.157	6.449	0.011	3.182	1.302-7.775
CYP C-344T	CT	1.821	4.287	0.038	6.179	1.102-34.639
	TT	1.807	4.154	0.042	6.089	1.072-34.599

Angiotensinogen is mainly synthesized in the liver, and AGT in the blood plasma is from the liver. The AGT can generate Ang II and induce the constriction of blood vessels of the kidney, which will increase the release of aldosterone and noradrenalin, promote the growth of smooth blood muscle cells, activate macrophages, resulting in the appearance of hypertension and atherosclerosis. The AGT level has a high association with AGT gene polymorphism.[12] A previous study showed that individuals from different genotypes of M235T have discrepancies in their blood plasma concentrations.[13] Another study found that the occurrence of TT and MT genotypes is the key independent forecasting factor of disease malignancy development, which is the genetic marker of pathologic changes of brain small blood vessels.[14] The AGT gene 235T increases the stroke risk for patients using an AGT transferase inhibitor.[15] The CYP11B2 C-344T is a single nucleotide polymorphism located in the promoter region, the specific site of which is 334. At this position exists the steriodogenic transcription factor-1 (SF-1), including 2 T and C alleles.[16] The gene polymorphism influences the combination of CYP11B2 gene promoter and SF-1, further influencing the transcription of aldosterone synthase mRNA and the synthesis of aldosterone. The ACE gene and CYP11B2 gene may have synergistic effects on ischemic stroke,[17] Verpillat et al[18] found that the CYP11B2 C-344T polymorphism is closely associated with the MR1 T2 white matter hyperintensities based on the comparison among the polymorphisms of CYP11B2, ACE, AGT, endothelin-1 and NO synthetase. The work showed that TT genotype is an essential risking factor of hypertension-related illnesses.

Our study investigates the complicated interaction between population genetics and environment in a Mongolian population with hypertension complicating stroke. The genotypes and allele frequency of the 7 studied genes did not significantly differ from those of the control group. The effect of environmental factors showed that age and smoking can significantly influence the presence of the disease. The ACE DD, CYP CT and CYP TT genotypes may be the hypertensive stroke susceptible sites for the Mongolian populations. Furthermore, these sites should have crossing effects with other nucleotide sites. For ACE II and CYP CC genotypes, the illness risks are conclusively the lowest among all the compared genotypes.

The Mongolian population was selected from the Wulate Back County of Inner Mongolia, a typical pure Mongolian population in China. They live on grazing land with cold weather, inconvenient transportation, and seldom intermarry with other ethnicities. From the geographic and historical perspective, this population is very isolated from other Mongolians and has a great degree of genetic homogeneity within the population, and the environmental factors influencing the risk of hypertension are consistent. Therefore, if our study can be verified in other Mongolian populations, it will have broad implications in monitoring hypertensive stroke.

In conclusion, hypertensive stroke has a complicated genetic formation mechanism. The micro-phenotype effect caused by a single gene is unlikely to cause the disease, but the sum effect caused by the accumulation of multiple genes increases the possibility of the disease arising. In our study, we identified the I/D polymorphism of the ACE gene and the C-344T of the CYP gene as candidates to synthetically induce the outbreak of hypertension complicating stroke. Our study has some pitfalls that need improvement, for example, we cannot exclude discrepancies caused by sample number and sampling bias. The limitation of our work is based on small sample size. We acknowledge that this problem can weaken the capability to reveal the crossing effects led by different factors. In the future, we plan to gather more samples to provide more extensive and reliable results, and to also perform comparative studies on the different subtypes of hypertensive stroke (namely, hemorrhagic stroke and ischemic stroke) so as to better understand their genetic differences. Also, a functional analysis for specific gene polymorphisms is necessary in successive studies.

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