The Association Between Gene Polymorphisms of Homocysteine Metabolism-Related Enzymes and Ischemic Cerebrovascular Diseases in Chinese Henan Han population

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Abstract: ***Background and Purpose***—During the last years, several studies suggested a role for genetic factors predisposing to thrombophilia and for moderate hyperhomocysteinemia.The mutations in homocysteine (Hcy) metabolism-related enzyme genes including methylenetetrahydrofolate reductase (MTHFR) C677T, cystathionine b-synthase (CBS) 844ins68, and methionine synthase (MS) A2756G have been identified as genetic risk factors for thromboembolic events. The evidence of a role for these gene variants in the risk of ischemic stroke is controversial and it has been noticed that these gene mutations have heterogeneous distributions among different ethnic groups or geographic areas. The data on the prevalence of the gene mutations in Chinese population is not yet available. The aim of the present study was to investigate the association between these gene polymorphisms and ischemic cerebrovascular diseases in Chinese Henan Han population in a large case-control study. ***Methods***—We investigated 512 cases (310 males, 202 females; mean_SD age, 60.58 years) as patient group with ischemic cerebrovascular disease in department of neurology in Henan province hospital were enrolled from December 2004 to July 2007 and 500 healthy subjects (274 males, 226 females; mean_SD age, 56.28 years) as control group in the study. All people are Henan Han Chinese and without cancer, epilepsy and hepatic or renal diseases. MTHFR C677T and MS A2756G polymorphism was genotyped by polymerase chain reaction and *Hin*fI digestion and HaeⅢ respectively, while the genotypes of CBS 844ins68 was detected by polymerase chain reaction. ***Results***—This investigation showed that MTHFR C677T TT-type 40%, CT-type 42.6% in patient group and TT-type 32.8%, CT-type 34.6%, in control group,and the T allele frequency was 61.3% versus 51.1% in the two groups. The frequencies of the three genetypes were significantly different between patient groups and controls ($\chi2=30.36$, $P<0.01$). The frequency of T allele was significantly higher in patient groups than that in controls ($\chi2=24.29$, $P<0.01$).But there were no significant differences in the frequencies of CBS 844ins 68 ($\chi2=0.093$, $P>0.05$), MS A2756G ($\chi2=4.101$, $P>0.05$) between the patient the and control groups. ***Conclusion***—The C677T polymorphism of the MTHFR gene was associated with increased risk of ischemic cerebrovascular diseases and MTHFR C677T may be independent risk factors for ischemic cardiovascular diseases. However, the mutations of CBS844ins68 and MS A2756G was no associated with the ischemic cerebrovascular diseases in Chinese Henan Han population. The prevalences of MTHFR C677T, CBS 844ins68 and MS A2756G may vary with different ethnic groups or geographic regions.
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1. Introduction

Ischemic cerebrovascular disease (ICVD) is a leading cause of death in the world.Over the last few years, several studies had been performed to elucidate the mechanisms of ischemic stroke, but this work was supported by the science foundation for prominent youth of Henan (No.06122001300) and Henan innovation project for university prominent research talents (N0.2005KYCX020) the etiology of ICVD is complicated and not understood completely until now. New researchs has discovered that ICVD is related with both hereditary and environmental factors. Recently, many researchers focus on the predisposing genes of the related risk factors of cerebral infarction. HHcy is a new important and independent risk factor to atherosclerosis and cerebral infarction. The level of plasma homocysteine are influenced by hereditary and environmental factors.The three key enzymes in the homocysteine metabolism are 5,10-methylenetrahydrofolate reductase (MTHFR), Methionine synthase (MS) and Cystathionine-βsynthase （CBS）. The gene mutations of MTHFR C677T 、MS

A2756G and CBS 844ins68 can result in loss or decrease of enzyme activity and consequently increase or desease of Hcy level.Therefore, some genes that code enzymes involved in Hcy metabolism are considered to be candidate gene. But the correlation between these gens and ICVD is still controversial[1,2,3]. In order to further investigate the correlation between the metabolism-relative gene polymorphism andICVD, the researcher detected the MTHFR, CBS and MS genes by PCR-RFLP in a large sample and observed the distribution of genotypic frequency and allel frequency among Han people in Henan province in China.

2. Material and Methods

2.1. patients and controls

This study included 512 cases(310 males, 202 females; mean_SD age,60.58 years) as patient group with ischemic cerebrovascular disease in department of neurology in Henan province hospital were enrolled from December 2004 to July 2007 and 500 healthy subjects(274 males, 226 females; mean_SD age, 56.28 years) as control group. All people are Henan Han Chinese and without cancer, epilepsy and hepatic or renal diseases.

2.2.Genotype detection of the MTHFR C677T 、MS A2756G and CBS 844ins68

Genomic DNA were extracted from peripheral-blood lymphocytes by the standard phenol-chloroform method.Genotyping was performed according to previously described methods for MTHFR677 of the MTHFR gene, CBS ins 68 of the CBS gene and MS2756 of the MS gene polymorphism in details[4,5,6]. For the 677 C→T polymorphism, extracted DNA was amplified with the forward primer 5'-TGA AGG AGA AGG TGT CTG CGG GA-3'and the reverse primer 5'-AGG ACG GTG CGG TGA GAG TG-3'. Polymerase chain reaction (PCR) thermal cycling conditions were 2-minutes denaturation at 94℃,then 40 cycles of 94℃ for 30 seconds, 62℃ for 30 seconds, and 72℃ for 30 seconds. This was followed by 5-minutes extention at 72℃.Amplified 198-bp PCR products were digested with *Hin*fI and visualized under electrophoresis on 2％agarose gel with ethidium bromide. The C allele produced a 198-bp band, and the T allele produced 175-and 23-bp frements. For the MS 2756A→G polymorphism, DNA was amplified with the forward primer5'-CAT GGA AGA ATA TGA AGA TAT TAG AC-3' and the reverse primer5'-GAA CTA GAA GAC AGA AAT TCTCTA-3'. PCR thermal cycling conditions were 2-minutes denaturation at 92℃,then 35 cycles of 92℃ for 60 seconds, 56℃ for 60 seconds, and 72℃ for 90 seconds, with a 7-minutes extention at 72℃. PCR products were digested with HaeⅢ and visualized under electrophoresis on 2％agarose gel with ethidium bromide, resulting in a 189bp band for the A allele and 159- and 30-bp fragments for the G allele, after 2％ agarose gel with ethidium bromide. For the CBS 844ins68 polymorphism, DNA was amplified with the forward primer 5'-CTG GCC TTG AGC CCT GAA-3' and the reverse primer 5'-GGC CGG GCT CTG GAC TC -3'. PCR conditions were 3-minutes denaturation at 95℃, then 35 cycles of 95℃ for 60 seconds, 58℃ for 60 seconds, and 72℃ for 60 seconds, with a 3-minutes extention at 72℃. Mutated CBS 844ins68 fragment was 252bp

2.3.Statistical analysis

Allele frequencies in the ICVD patients and controls were determined by counting alleles and calculating proportions.The Hardy-weinberg equilibrium analysis was calculated using the chi-square statistics for goodness of fit (1 degree of freedom). The OR with an associated 95％confidence interval(CI)was calculated to estimate the relative risk of the different genotype combinations.MTHFR,MS and CBS alleles frequencies were determined for the study and control groups,and were compared by χ^2 analysis. P values ≤ 0.05 were considered statistically significant, and all P values were based on two-tailed tests.The SPSS 11.0 statistical software program was used for all analysis.

3.Results

3.1. Mutation Identification

The genotype of heterozygous MTHFR C677T mutation (C/T) showed three bands of 198, 175 and 23bp. The mutant homozygote (T/T) showed two bands of 175 and 23 bp; the wild type (C/C) showed only one band of 198 bp (Fig.1; the 23-bp band was out of gel). The heterozygote for MS A2756G (A/G) showed three bands of 189, 159 and 30 bp; the mutant homozygote (G/G) showed two bands of 159 and 30 bp; the wild type (A/A) showed only one band of 189 bp (Fig. 2; 30 bp band was not demonstrated here). On the gel containing PCR products of the CBS gene, the wild type (D/D) showed a band of 184 bp; the heterozygote for CBS 844ins68 (I/D) showed a 252-bp band in addition to the 184-bp band. No mutant homozygote (I/I) was found in this series (Fig. 3).

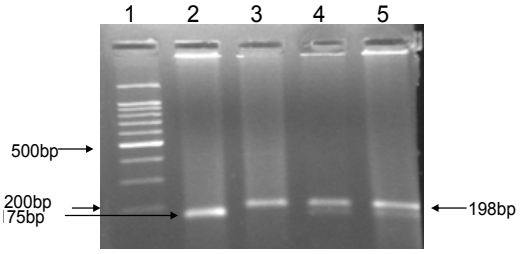

Fig.1 Genotyping of the MTHFR C677T gene

Lane 1 :100bp DNA ladder

Lane 2:TT homozygous

Lane 3:CC heterozygous

Lane 4,5:CT heterozygous

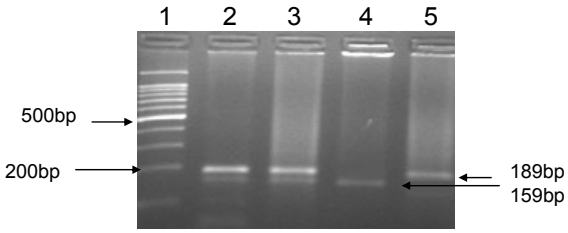

Fig.2 Genotyping of the MS A2756G gene

Lane 1:100bp DNA ladder

Lane 2,3:AG heterozygous

Lane 4:GG honozygous

Lane 5:AA honozygous

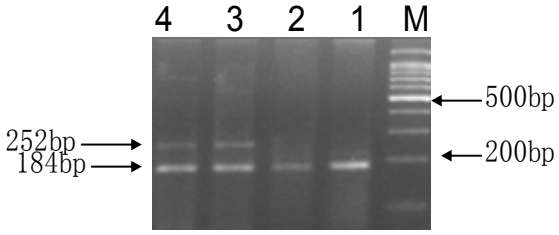

Fig.3 Genotyping of the CBS 844ins68 gene

Lane 1,2:DD homozygous

Lane 3,4:I/D heterozygous

3.2. Mutation Frequencies and Distribution in Patient and Control Groups

MTHFR,MS and CBS genotype distributions and allele frequencies for Chinese Henan Han population ICVD patients and controls were presented in Table 1,2,3.We found a genotype frequency distribution for the MTHFR,MS and CBS mutation that was as expected according to the Hardy-weiberg equilibrium. Between the patient groups and the control group, there was statistically significant difference in the distribution of MTHFR C677T genotype of the mutations ($P < 0.001$). However, The difference in frequency in the CBS allele and in the MS G allele between the patient groups and the control group there was not statistically significant. The T allele of MTHFR C677T yielded an OR of 2.583 for ICVD (95% confidence interval (CI) 1.92-3.12). The G allele of MS A2756G yielded an OR of 0.92 for ICVD (95% CI 0.47–1.81). The heterozygous state of CBS 844ins68 yielded an OR of 0.19 for ICVD(95% CI 0.02–1.43).All of the OR values were not significant in statistical sense.

The distributive pattern for combined mutation in single individual for both MS A2756G and MTHFR C677T was summarized in Table 4. The difference in the frequency of this combined mutations between the patient groups and the control group was not significant (P=.748). The OR was 0.97 for ICVD (95% CI 0.46–2.48), statistical significance was not obvious. The frequency of mutation of CBS 844ins68 was relatively low (2.96%) in the overall study series.

Table1. Prevalence of the MTHFR C677T genotypes and allele frequency in patients and controls

groups	subjects(n)	Genotype frequency n (%)			allele frequency (%)	
		CC	CT	TT	C	T
Patient	512	89(17.4)	218(42.6)	205(40)	38.7	61.3
control	500	163(32.6)	173(34.6)	164(32.8)	49.9	51.1
		χ^2=30.36，P<0.001			χ^2=24.29，P<0.001	

Table2. Prevalence of the MS A2756G genotypes and allele frequency in patients and controls

groups	subjects(n)	Genotype frequency n (%)			allele frequency (%)	
		AA	AG	GG	A	G
Patient	512	455(88.9)	55(10.7)	2(0.39)	965(94.2)	59(5.8)
control	500	423(84.6)	75(15)	2(0.4)	921(92.1)	79(7.9)
		χ^2=4.101 P＞0.05			χ^2=3.64 P＞0.05	

Table3. Prevalence of the CBS 844ins68 genotypes and allele frequency in patients and controls

groups	subjects(n)	Genotype frequency n (%)			allele frequency (%)	
		D/D	D/I	I/I	D	I
Patient	512	496(96.7)	16(3.3)	0(0)	1008(98.4)	16(1.6)
control	500	486(97.2)	14(2.8)	0(0)	986(98.6)	14(1.4)
		χ^2=0.093 P＞0.05			χ^2=0.186 P＞0.05	

Table 4. Prevalence of combined mutations of MS A2756G (A/G) and MTHFR C677T (C/T or T/T) between control and patient

combined mutations	Patients	Controls	P values
AG+CT	22	27	0.748
AG+TT	20	26	
Sum	42	53	

* The P value was obtained by χ^2test

4. Discussion

In this study we investigated whether the gene mutations of MTHFR C677T 、MS A2756G and CBS 844ins68 could have an impact on ischemic cerebravascular disease in the Henan Han population. We found the frequency of the T-containing allele was slightly higher in this study than in a pervious study in Japan [7]but was similar to that for Chinese other population[8], demonstraing that this polymorphism is common in the Chinese Henan Han population it is likely that discrepancies in alleles frequency result from ethnic or regional differences.The results of the present study indicate the T-containing allele have an increased occurrence of the ischemic cerebravascular disease (OR=2.583),it may be expected that the MTHFR C677T mutation is a risk factor for ICVD in the Chinese Henan Han population.

Our finding of no association between the A2756G polymorphism in the MS gene and the occurrence of the ICVD, which is in agreement with the findings of other groups who did not report a direct or significant role for this polymorphism in th etiology of ICVD[8,9,10].

MS plays an important role in the Hcy metabolism. The gene coding MS has been cloned, sequenced and located, and, with several mutations, have also been identified. The most prevalent mutation of the MS gene is the A2756G transition, which results in the substitution of aspartic acid by glycine. Among Western Caucasian populations, the prevalence of heterozygous and homozygous MS A2756G carriers has been reported to be around 32% and 4%, respectively, and does not exhibit a significant difference between patients with coronary artery disease and healthy controls [11,12]. Our data show that the prevalence of MS A2756G in the Chinese Henan Han population is lower than that in the Western Caucasian population, and the frequency of MS A2756G mutation in the patient groups are not different from that observed in the control group. The information indicates that the MS A2756G gene may not be an independent risk factor for ICVD in the Chinese Henan Han population.

Cystathionine beta-synthase (CBS) mediates conversion of homocysteine to cystathionine and deficiency in enzyme activity may lead to hyperhomocysteinemia/homocystinuria, which are often associated with vascular disease. A large number of polymorphisms have been reported in the CBS gene, some of which impair its activity and among these, a

T833C polymorphism in cis with a 68 bp insertion at 844 in the exon 8 is found to be associated with mild hyperhomocysteinemia in different ethnic groups[13,14]. From our observation, the prevalence of CBS 844ins68 in the Chinese Henan Han population is evidently lower than that in Western Caucasian population. The CBS 844ins68 mutation tends to be no significant difference in the healthy individuals (2.8%) and that in the patients (ICVD, 3.3%) and yields an OR of 0.19 for IVCD, which may suggest that CBS 844ins68 is not a risk factor for ischemic cerebravascular diseases.

The carriers of all the three mutations are few in the investigated population. Thus, combined mutations of MTHFR C677T and MS A2756G can be confirmed not to be an independent risk factors for ICVD in the Chinese Henan Han population.

In conclusion, the mutation of MTHFR C677T, may be a genetic risk factor for population with ICVD, while the gene polymorphisms MS A2756G or CBS 844ins68 cannot be a genetic risk factor for ICVD in the Henan Han population of Chinese.

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