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Polymorphisms of Renin-angiotensin System in Essential Hypertension in Chinese Tibetans[1]

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Objective To evaluate the potential implications of the genetic variability of angiotensin converting enzyme, angiotensinogen and angiotensin Ⅱ type 1 receptor gene for essential hypertension in Tibetan. **Methods** A case-control study was conducted in 173 hypertensive individuals and 193 individuals with normal blood pressure. Multiple logistic regression analyses were used to estimate the risks of developing hypertension for different genotypes, and haplotype analyses of the angiotensinogen gene were used to determine the association between two-locus angiotensinogen gene polymorphisms and hypertension. **Results** As to the risk to high blood pressure and high systolic pressure, women with MM genotype were 7.7 (95% CI: 1.3-20.5) and 8.7 (95% CI: 1.8-20.1) times higher than those with TT genotype after adjustment for age and body mass index. Haplotype frequencies for M235T and G-6A were significantly different between hypertensive individuals and controls, which indicated an association of angiotensinogen gene haplotypes with hypertension, and a significant association of 235T/-6A haplotype with hypotensive effect. **Conclusion** Our results suggest that angiotensinogen gene 235MM is a predictor for hypertension development in Tibetan women but not in men, and may exert its hypertensive effect on linkage disequilibrum with a possible function locus of G-6A.

Key words: Angiotensinogen; Essential hypertension; Polymorphism; Tibetan

INTRODUCTION

Human essential hypertension is a multi-factorial trait and its genetic basis is a complex one. The renin-angiotensin system (RAS) genes have been extensively studied as hypertension candidate genes. Although variants in these genes have shown on association with hypertension in some studies, the association is often not reproducible in studies of other

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populations. Several recent meta-analyses concluded that the coding polymorphism of angiotensinogen (AGT) gene (M235T) was associated with an increased risk of hypertension[1,2], but the result has not been confirmed by several candidate gene linkage studies as reviewed by Corvol *et al*.[3]. As far as the angiotensin-converting enzyme (ACE) locus is concerned, the significant association and linkage were seen in two large size studies[4,5], whereas a number of other studies failed to detect a major genetic contribution of ACE[6]. Finally, the angiotensin Ⅱ type 1 receptor (AT_1R) gene has also shown an association and linkage with hypertension in a single Finnish population[7], but not in another study in a different white population[8].

These inconsistent results from previous studies concerning the association of various candidate genes with hypertension may have been derived from differences in the polymorphism frequencies that construct the specific genetic architecture of different ethnic populations. As a consequence, the relative contributions of any given variant vary among the different studied populations. Based on this concept, reproduction of the finding in a different ethnic population is considered to be important.

While there are plenty of confusing results between these three genes and risk to hypertension in other ethnic groups, no information is available for Tibetan populations. In this context, we studied a relative homogeneous Tibetan population from Lhasa, who had a relatively primitive socioeconomic status, limited mobility and the highest prevalence of hypertension in China[9]. The genotyped variants were in genes comprising the renin-angiotensin pathway, including ACE I/D, AGT M235T which have been associated with hypertension in some populations; AT_1R -2228G/A, -1424C/G and -521C/T, which were not in linkage disequilibrum and could provide information for all possible haplotypes in the promoter of AT_1R. Therefore, the present study was undertaken to investigate the distribution of these five polymorphisms in Tibetan population in China and to examine their association with and their susceptibility to essential hypertension.

METHODS

Subjects

This case-control study was approved by the institutional review committee, and all the subjects recruited gave informed consent. Participants in this case-control study comprised 173 hypertensive patients and 193 controls with normal blood pressure. All subjects were ethnic Tibetans without a history of intermarriage with other ethnic groups at least within four generations. Hypertensive subjects were selected in the People's Hospital of the Tibet Autonomous Region. Hypertension was defined according to the following criteria: (1) onset of hypertension at the age under 60 years, (2) systolic blood pressure (SBP)$\geqslant$140 mmHg and/or diastolic blood pressure (DBP)$\geqslant$90 mmHg on three consecutive measurements for those untreated, (3) absence of secondary hypertension and no history of diabetes mellitus or renal failure, (4) family history of hypertension with at least one parent being affected. In this study, we also defined systolic hypertension (SBP$\geqslant$140 mmHg) and diastolic hypertension (DBP$\geqslant$90 mmHg) to study the relationship between the polymorphisms and elevated SBP/DBP.

Another group of 193 individuals with no serious health problems and history of hypertension in their first-degree relatives were independently recruited from the same community, and used as a random population control. Their lifestyles were also similar to those of the hypertensive individuals, including salt intake. Subjects with a SBP<130 mmHg

and a DBP<90 mmHg were assigned to the control group.

All subjects responded to a questionnaire on medical history, physical activities, medication and personal habits. Body height and weight were recorded in light clothing and body mass index (BMI) was calculated as weight divided by height in square meters (kg/m^2). Triplicate blood pressure was measured by trained technicians using mercuric sphygmomanometer with appropriate size cuffs, after 10 to 15 minutes of rest in a sitting position, and the average of these three readings was taken.

DNA Analyses

Blood samples (5 mL) were collected from each subject with consent and put into tubes containing EDTA. Genomic DNA was obtained from peripheral blood leukocytes by phenol/chloroform extraction. Aliquots of genomic DNA were genotyped for each of the above-mentioned polymorphisms with standard polymerase chain reaction (PCR) or PCR/ restriction fragment length polymorphism (RFLP) methods[10-12]. The M235T polymorphism of AGT gene was investigated by PCR, followed by digestion with the restriction enzyme Tth111I and agarose gel electrophoresis[10]. ACE gene genotypes were determined by PCR amplification[11]. Variants at -2228 and -521 in the promoter AT_1R were genotyped using the PCR based restriction fragment length polymorphism as previously described[12]. Digestion by enzyme Hinf I and Ssp I allowed determination of genotypes in positions -2228G/A and -521C/T, respectively.

Statistical Analyses

All statistical analyses were performed with the Statistical Analysis System (SAS) software (Version 8.0; SAS Institute Inc, Cary, North Carolina, USA). Between-group continuous variables were compared by the general linear model (GLM) procedure and Mantel-Haenszel χ^2 analysis was used for categorical variables. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated by unconditional logistic regression to provide an estimate of the relative risk of developing hypertension for the different genotypes excluding the effect of confounding. Hardy-Weinberg equilibrium was assessed by the χ^2 test. Haplotype frequencies were estimated by maximum likelihood analysis as implemented in the EH program[13].

RESULTS

The BMI, SBP and DBP of the hypertensive group were significantly higher than those in the group with normal blood pressure (Table 1). The genotype distributions and allele frequencies in each group are shown in Table 2. None of the genotype showed in each group was deviations from Hardy-Weinberg equilibrium. Allele frequency of the AGT 235T, ACE I, AT_1R -2228A and -521T in the Tibetans with normal blood pressure was 0.71, 0.64, 0.15 and 0.22, respectively. Being different from previous reports[1,2], the frequency of T235 variant was higher in normotensive subjects than in hypertensive subjects , but the difference was not significant either in men or women. Furthermore, ACE I/D, AT_1R -2228G/A and -521C/T polymorphisms did not differ greatly in frequency between the two groups. All subjects were homozygous for the C allele at the position -1424C/G of AT_1R gene.

TABLE 1

Characteristics of Study Population

Demographics	Hypertensives (n=173)	Normotensives (n=193)	P
Male/Female	88/85	102/91	0.705
Age[a], y	54.25±12.89	44.97±11.31	0.077
BMI, kg/m^2	23.63±0.18	21.35±0.17	<0.001
SBP[b], mmHg	163.3±1.64	118.1±0.82	<0.001
DBP[b], mmHg	106.4±0.82	78.69±0.77	<0.001

Note.[a] In $\bar{x} \pm s$. [b] In $\bar{x} \pm s$, adjusted for age, sex and BMI.

TABLE 2

Single-Locus Allele Frequencies and Genotype Distributions

Locus	Genotype (%)			Allele Frequency
ACE I/D	II	ID	DD	Frequency I
Hypertensives	83 (48)	75 (43)	15 (9)	0.70
Normotensives	76 (39)	96 (50)	21 (11)	0.64
AGT M235T	TT	MT	MM	Frequency T
Hypertensives	73 (42)	82 (47)	18 (11)	0.66
Normotensives	93 (48)	88 (46)	12 (6)	0.71
AT$_1$R G-2228A	GG	GA	AA	Frequency A
Hypertensives	131 (76)	41 (24)	1 (0.5)	0.12
Normotensives	138 (72)	53 (27)	2 (1)	0.15
AT$_1$R C-521T	CC	CT	TT	Frequency T
Hypertensives	126 (73)	45 (26)	2 (1)	0.14
Normotensives	128 (66)	60 (31)	5 (3)	0.22

Note. Figures in blankets are average percentage. ACE indicates angiotensin-converting enzyme; AGT, angiotensinogen; and AT$_1$R, angiotensin Ⅱ type 1 receptor. No significant difference in allele frequency or genotype distribution between hypertensives and normotensives was observed by χ^2 test.

To study the association of various genotypes with hypertension, we calculated an adjusted odds ratio (95% CI) for each genotype by multivariate logistic regression analysis (Table 3). When stratifying the subjects by gender, we found that women with MM genotype had a higher risk to hypertension and a high SBP pressure by 7.7 (95% CI: 1.3-20.5) and 8.7 (95% CI: 1.8-20.1) times than those with TT genotype after adjustment for age and BMI, respectively. No significant association was found between genotypes and risk of elevated DBP. In men, no significant associations were observed between these four variants and high blood pressure, neither SBP nor DBP. Results also showed that age and BMI were the significantly independent risk factors for hypertension. There was no significant association between blood pressure and genotype among the control subjects. Adjustment for the covariates did not change the results (data not shown).

TABLE 3

Odds Ratio (95% CI) of Genotypes for Developing Hypertension and Systolic/Diastolic Hypertension.

Genotypes	Women			Men		
	HP	SHP	DHP	HP	SHP	DHP
ACE I/D						
DD vs II	0.54 (0.15,1.95)	0.97 (0.26, 3.41)	0.62 (0.17, 2.15)	0.70 (0.23, 1.99)	1.14 (0.27, 4.11)	0.81 (0.27, 2.31)
ID vs II	0.69 (0.31,1.53)	0.73 (0.32,1.66)	0.69 (0.31,1.51)	0.81 (0.43,1.51)	0.84 (0.37,1.90)	0.90 (0.48,1.68)
AGT 235T/M						
MM vs TT	7.70* (1.27, 0.46)	8.66* (1.80, 0.10)	3.55 (0.72, 1.15)	1.18 (0.39, 3.54)	1.13 (0.26, 4.33)	1.25 (0.41, 3.72)
MT vs TT	0.91 (0.41,1.99)	1.97 (0.86, 4.69)	0.98 (0.46, 2.09)	1.81 (0.96, 3.45)	1.87 (0.82, 4.40)	1.63 (0.87, 3.10)
AT_1R -2228G/A						
GA+AA vs GG	0.81 (0.34,1.91)	0.78 31,1.86)	0.75 (0.32,1.73)	0.93 (0.47, 1.82)	1.47 (0.60, 3.54)	0.93 (0.47, 1.83)
AT_1R -512C/T						
CT+TT vs CC	0.80 (0.34,1.85)	0.51 (0.20,1.20)	0.76 (0.33,1.70)	0.67 (0.35, 1.28)	1.11 (0.46, 2.61)	0.68 (0.35, 1.31)

Note. Odds Ratios were adjusted for age and BMI and figures in parentheses were their 95% CI. SHP indicates systolic hypertension (Systolic blood pressure≥140 mmHg). DHP indicates diastolic hypertension (Diastolic blood pressure≥90 mmHg). HP indicates hypertension (Systolic blood pressure≥160 mmHg and/or Diastolic blood pressure≥95 mmHg). *P<0.05

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A two-locus haplotype frequency analysis between M235T and G-6A was conducted in both the hypertensive and normotensive subjects (Table 4). The data of G-6A were from the study reported by Liu *et al.*[14]. The omnibus haplotype profile test was significant (χ^2=17.3, P<0.01), indicating that the overall haplotype frequency profiles were different between the hypertensive cases and the controls. The frequency of haplotype containing -6A and 235T (4072C) allele was higher in the controls than in the hypertensive cases.

TABLE 4

Haplotype Frequency Estimates for M235T and A-6G of Angiotensinogen in Hypertensive Individuals and Normotensive Individuals

Haplotype		Hypertensives	Nomotensives	Overall	$\chi^{2,b}$	P
-6	4072[a]	(n=157)	(n=149)	(n=306)		
A	C	0.572	0.719	0.645	6.97	0.01
G	C	0.081	0.039	0.059	2.37	0.12
A	T	0.116	0.060	0.088	2.79	0.09
G	T	0.231	0.181	0.206	1.08	0.30
Log (ln) Likelihood		-271.31	-198.57	-478.52	17.28	<0.01

Note. [a]T4072C dinucleotide polymorphism corresponds to M235T amino acid polymorphism. [b]Likelihood ratio test statistic for omnibus haplotype profile.

DISCUSSION

This study has, for the first time, investigated these five variants in Chinese Tibetans. A significantly higher frequency of the AGT 235MM genotype was observed in the female hypertensive patients as compared in the controls, and this association was restricted to the aspect of elevated systolic blood pressure. Furthermore, 2-locus haplotype analysis composed of M235T and G-6A demonstrated that only 235T/-6A haplotype was significantly associated with hypotensive effect.

Obviously, it is a good way to find appropriately homogenous hypertensive cases and control subjects, which is known to be of common origin, with similar environmental exposures and genetic or ethnic background with a view to cutting down the problematic heterogeneity hampering hypertension genetics research. Both living in the Tibetan plateau, original Tibetans exhibited hypertension twice more as frequently as Hans in Lhasa. In 1991, the third national blood pressure survey reported that the prevalence of hypertension was 19.5% in Tibetans, which was the highest among different populations[9]. In this context, we studied a Tibetan population from Lhasa, a remote mountainous region at an elevation of 3658 m. Because of their specificity, a relatively primitive socioeconomic status, limited population mobility and the highest prevalence in China, the Tibetans were thought to be a well appropriate population for genetic study.

The distribution of M235T genotypes in normal controls was similar to that found in Japanese and Chinese Hans, and differed markedly from Caucasians[2,15,16]. Among Chinese and Japanese, the T allele is more common than the M allele, whereas the reverse is true for Caucasians. The large difference in allele frequencies among ethnic groups may imply the different etiological link between this variant and hypertension. In this sample of Tibetan adults, it is surprising that individuals who were homozygous for the 235M allele had a significantly increased risk of hypertension as those with TT homozygotes, which differed from results obtained previously in other ethnic groups, and might indicate the importance of ethnic origin in the assessment of genetic risk identifier. The Qinghai-Tibet high altitude where Tibetans live is the highest and largest plateau in the world towering over southwestern China at an average elevation of 4000 m above sea level[17]. The Tibetan population is thought to be the original inhabitants of that region. According to the pervious study by mtDNA sequence diversity, Tibetans showed no conspicuous population expansion in the past and also exhibited a reduced genetic diversity compared with the other ethnic populations in China[18]. A great difference in genetic background may have existed between Tibetans and other populations, which may be implicated in the nature of the candidate locus predisposing to hypertension. In addition, the variants in AGT also bore on a question of natural selection[19]. This pattern led to a hypothesis that the T235 was selective and adaptive in the hypoxia and high-sodium environment, and the predisposing M235 variants were likely to have risen during the Tibetan evolution.

Haplotype analysis is a powerful tool for identifying candidate genes for complex trait disease. Our results showed that hypertensive subjects with an AGT haplotype profile, were significantly different from the normotensive ones, which implied that the AGT gene might be a susceptible locus for essential hypertension in the Tibetan population. Furthermore, the association was more evident for 235T/-6A haplotype, its frequency was higher in normotensive individuals than in hypertensive ones, and hypotensive effect of 235T or -6A was observed in the single-locus analysis. Recently, Wu *et al*. found G-6 variant was associated with a higher transcriptional activity than A-6 variant[20], and a significant linkage disequilibrum between M235T and G-6A was found in the present study. These recent findings led to the hypothesis that G-6A polymorphism, which is linked to nonfunctioning

M235T, increased the plasma AGT level via regulation of AGT gene transcription, and was involved in the pathogenesis of the predisposition to hypertension. Meanwhile, potential hormones such as estrogen might work with this core promoter region, and enhance the transcription of AGT gene, thus affecting the blood pressure.

Although we failed to detect an association between a single diallelic polymorphisms, such as ACE I/D, AT_1R -2228G/A, and -521C/T polymorphisms and hypertension, it should not exclude the possibility that these loci might be involved in the pathogenesis of essential hypertension. Essential hypertension is a multi-factorial trait, and it is assumed that multiple genes contribute to the etiology of hypertension independently or synergistically with each gene exerting a small effect. Thus, these three polymorphisms may play a synergistic role in the development of hypertension. There may be another possibility that in Tibetan population only AGT gene contributes to the etiology of hypertension because different genes may predispose it to the phenotype of hypertension in different populations.

Nevertheless, there were some limitations in our studies. Firstly, the normotensive subjects were relatively young as compared with the hypertensive ones. Although the difference in age between these two groups was not significant (P=0.07), and the estimated OR was adjusted for age. This age difference might reduce the power of our study because the penetrance of hypertension is age-dependent and some younger normotensive individuals may develop hypertension in their later life. Secondly, considering the relatively low allele frequency of 235M, a lager sample size was needed to evaluate haplotypes between M235T and G-6A more credible. Furthermore, many studies have proved that it is more powerful to focus on the transmission of multilocus haplotypes and combination of alleles at a variety of candidate loci than on the allele at single loci for genetic study of hypertension[21,22]. A large sample size is a safeguard due to the increased number of genotypes to be compared with each other. Therefore, further investigation needs to be performed in a larger population using not only hypertensive status but also intermediate phenotypes to confirm the role of AGT as a prognostic and genetic marker for hypertension.

In conclusion, the M235T polymorphism of AGT gene may be a predisposing factor in the development of essential hypertension in Tibetan women, but not in men. Women carrying the 235MM genotype have a high risk to develop elevated systolic blood pressure. The possible mechanism involved is that M235 allele is linked to G-6 allele and exerts its hypertension effect via upregulation of the level of transcription.

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