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α-ADDUCIN GENE AND ESSENTIAL HYPERTENSION IN CHINA

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ABSTRACT

Adducin is a membrane skeletal protein that is involved in the regulation of membrane ion transport and cellular signal transduction. Essential hypertension has been linked to α-adducin gene locus, and association of a polymorphism of the gene has been found in some studies, but results of linkage or association studies on α-adducin gene are controversial among different populations. This study was designed to examine the linkage between α-adducin gene locus and essential hypertension and to reveal the relationship between an α-adducin gene polymorphism (Gly460Trp) and essential hypertension in a Chinese population. For the linkage study, one hundred and six Chinese nuclear families were recruited, including 417 hypertensive patients in all 474 individuals. Those samples were genotyped at D4S412 and D4S3038. The distances between the two microsatellite markers and the α-adducin gene locus are less than 3cM. Parametric, non-parametric linkage (NPL) analyses using the GENEHUNTER software were carried out. Sib transmission-dise-

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quilibrium test (S-TDT), as well as transmission-disequilibrium test (TDT), was also implemented with TDT/S-TDT Program 1.1. Serum levels of uric acid, creatinine, blood urea nitrogen (BUN), fasting glucose and lipids were determined as phenotypes. In an association study, 138 hypertensive and 121 normotensive subjects were genotyped at Gly460Trp of the α-adducin gene to examine a possible association between this polymorphism and blood pressure or other phenotypes. We fail to find the linkage between the two markers and essential hypertension by parametric, NPL analysis or TDT/S-TDT study. With the use of the simple association and the multivariate logistic regression analyses, we also fail to reveal a significant association between the Gly460Trp polymorphism in α-adducin gene and the blood pressure variation, or blood biochemical indices studied. The frequency of the 460Trp allele in Chinese (46–48%) is similar to that found in Japanese (54–60%) while the allele frequency is less common in Caucasian (13%–23%). These findings suggest that in our Chinese population, α-adducin 460Trp variant may not play an important role in the etiology of EH. And the negative results of linkage and TDT/S-TDT further supports this conclusion.

Key Words: Hypertension; Genetics; α-adducin

INTRODUCTION

Essential hypertension (EH) is a complex quantitative trait, which means that, multiple genes are involved in its pathogenesis and progress, which makes it difficult to reveal the essence of this disorder (1–3). Many researchers focused on the candidate gene strategy and so far, more than 150 candidate genes for EH were listed and more than forty of them were studied in human beings or in animal models, most of them act in the field of sodium balance or neuro-endocrine modulation (4). Among them, α-adducin gene had been paid much attention to in recent years for its particular and attractive role in EH.

Adducin is a membrane skeletal protein that is involved in cell-to-cell contact, cellular signal transduction and cell membrane ion transport. The determination of the adducin cDNA of Milan hypertensive strain (MHS) and its normotensive control—Milan normotensive strain (MNS) revealed a point mutation in each of the two genes coding for the α- and β- subunits of adducin. Genetic analysis in rats suggested that only the mutation of the α-subunit accounts for the most significant blood pressure (BP) variation (5).

Recent findings strongly supported the hypothesis that α-adducin gene variants may affect kidney function by modulating the overall capacity of the tubular epithelial cells to transport ions through both a modification in the assembly of actin cytoskeleton and a modulation of sodium pump activity (6). Evidence has shown that, the polymorphic locus Gly460Trp was associated with and linked to EH especially in relation to salt sensitivity (7). An independent association study between hypertension and some other markers close to the α-adducin gene locus was also found (8), although other studies got negative results (9,10). Among these

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studies, few were carried out in Chinese population, though replications in different races were quite indispensable to the reliability of the results in candidate gene strategy.

Arterial blood pressure is critically dependent on sodium balance and the kidney is the key player in maintaining sodium homeostasis. Salt-sensitivity hypertension is a common subtype of EH which showed some special clinical characteristics. Genetic predisposition may play a substantial role because salt sensitivity is more obvious in black individuals and in persons with non-insulin-dependent diabetes mellitus. The disease genes of some rare salt-sensitive genetic syndromes, such as glucocorticoid-remediable aldosteronism and Liddle's syndrome, have been identified. Molecular genetic techniques for identifying individuals with salt-sensitive and salt-resistant essential hypertension are not yet available (11). If the relationship between the α-adducin genotype and salt-sensitive hypertension were illuminated, it would shed light on the essence of EH and guide the diagnosis and therapy. So, in this study, we have implemented the combined sib-TDT and TDT, in addition to parametric and non-parametric linkage methods and association study to try to analyse the association and linkage between the α-adducin gene and hypertension in a Chinese population.

MATERIALS AND METHODS

Study Population

All subjects in this study are of Han Chinese currently residing in Greater Shanghai area. One hundred and six nuclear families including 417 EH patients and 474 individuals overall were studied in the linkage analyses. In these families, 7(7%) had two, 38(36%) had three, 31(29%) had four, 15(14%) had five, 6(5%) had six, 7(7%) had seven, 1(1%) had eight and 1(1%) had eleven affected siblings. The total number of affected sib-pairs used in this study was 777. All siblings were full siblings. For the case-control study, 138 hypertensive patients and 121 normotensive controls were recruited. The hypertensive probands were identified in the Outpatient Department of Hypertension of Ruijin Hospital, Shanghai or selected from a screening survey in Shanghai area on the basis of the following criteria: 1) Systolic BP $\geq$150mmHg and/or diastolic BP $\geq$95mmHg, or currently receiving antihypertensive medication for at least one year. 2) Having an onset of hypertension after 20 and before 60 years of age. 3) Free of secondary hypertension through extensive clinical examination. 4) With at least one affected brother or sister. If a hypertensive proband met these criteria, all his/her siblings and parents together with him/herself were examined using a standardized protocol in the hospital by trained physicians and epidemiologists from the Shanghai Institute of Hypertension. The BP of all normotensives was under 140/90mmHg.

Blood pressure was measured in the seated position after 10 minutes of rest using a mercury sphygmomanometer by experienced and certified examiners. Afterwards, a detailed questionnaire was filled out, a physical examination was carried-out and 10ml blood sample was drawn from all subjects for DNA extrac-

tion and biochemical examination. Informed consent was obtained from all participants.

Clinical and Biochemical Measurements

All participants were inquired after their medical histories, physical activities, personal habits and family histories. Body mass index (BMI) was computed as weight (kilogram) divided by height (meters squared).

10ml blood was taken and serum total cholesterol, triglycerides, creatinine, BUN, uric acid and fasting glucose were measured.

Genotyping

Genomic DNA was extracted from peripheral blood cells using a standard phenol-chloroform method and stored at −20°C.

The Gly460Trp polymorphism was interrogated by the technique of mutagenically separated polymerase chain reaction (MS-PCR) (12), which was used in other studies (8,13). In brief, both normal and mutant alleles were amplified in the same reaction tube using different length allele-specific primers and their nonselective complimentary primer. Deliberate differences were introduced into the allele-specific primers in addition to the base substitution to reduce possible cross-reactions. The primer sets were: 1) the wildtype primer (FP-614G), 5′-GGGGCGACGAAGCTTCCGAGGTAG-3; 2) the mutant primer (FP-614T), 5′-CTGAACTCTGGCCCAGGCGACGAAGCTTCCGAGGATT-3; 3) the complimentary primer (RP-614), 5-CCTCCGAAGCCCCAGCTACCCA-3′, in which deliberated differences and base substitutions are underlined.

Genomic DNA (150ng) was amplified in DNA Thermal Cycler 480 (Perkin Elmer) in a 30 μl reaction volume containing:15 pmol FP614G, 15 pmol FP614T, 20 pmol RP614, 10mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 80 μmol/L each of dNTPs, 2.0U Taq polymerase (Promega) and 2.5 mmol/L $MgCl_2$. The initial denaturation for 3min at 94°C was followed by 35 cycles of denaturation for 45s at 94°C, annealing for 1min at 65°C, and extension for 1min at 72°C. Then a final extension had been held on 72°C for 10min. PCR products were run on 8% polyacrylamide gels. By ethidium bromide staining and UV transillumination, the products were clearly visualized for 220bp and 234 bp for the 460Gly and 460Trp alleles, respectively.

D4S412 and D4S3038 are two polymorphic microsatellite markers whose genetic distances to α-adducin gene locus are within 3cM. D4S412 is located near the centromeric end of α-adducin gene whereas D4S3038 was located near the telomeric end. Genotyping of the markers was based on PCR amplification of the subjects' genomic DNA. The primer sequences were inquired from the Genome Database (http://gdbwww.gdb.org/) and both primers were labeled with fluorescence at the 5′ end. After DNA extraction, PCR reactions were carried out

in GeneAmp PCR System 9700 (Perkin-Elmer) (in 96-well trays). Each 5.0 μl reaction contained 20ng genomic DNA, 0.2U HotStart TaqTM DNA polymerase (Qiagen), 3.0 mmol/l $MgCl_2$, 200 mmol/l dNTPs, 0.2 pmol/L of both forward and reverse primers and 0.5 μl PCR buffer containing the antibody used for "hot-start". After an initial 15min of denaturation at 95°C, 38 cycles were carried out. Each cycle contained 30s at 94°C, 1min at 63°C and 1.5min at 72°C, except that in the first 15 cycles, the annealing temperatures decreased from 63°C to 56°C by 0.5°C a cycle. The products were run on 5% acrylamide sequencing gels in ABI Prism 377 sequencer (Perkin-Elmer). The gel analysis was operated with GeneScanTM (version 3.0) while the allelic status for each sample was determined using GenotyperTM (version 2.1). Genotypes were scored according to the size in base pairs.

Statistical Analyses

All phenotypes are expressed as means±SD (standard deviation). Differences in genotypes or allele distributions were tested with the $\chi 2$ test, and Hardy-Weinberg equilibrium was also checked by a $\chi 2$ test. Multivariate logistic regression analysis was used to assess the relationship between the G460T genotypes and the hypertension susceptibility while simultaneously considering the effects of other predictor variables. A probability value of 0.05 was taken to assess the statistical significance.

Because the mode of inheritance of hypertension is not well established, non-parametric linkage (NPL) analyses using the GENHUNTER computer program were carried out to assess evidence for linkage. Furthermore, parametric linkage analyses using GENEHUNTER software were applied to verify the results of NPL estimates (14). Briefly, GENEHUNTER performs multipoint analysis, which computes a *Z* score statistic comparing the observed identical-by-descent (IBD) sharing among all affected members to that expected under the null hypothesis of no linkage. Under the null hypothesis, the Z score is normally distributed with mean zero and variance one. We considered two alleles D and d at the disease locus with frequencies 0.075 and 0.925, respectively. The penetrances of the genotype DD, Dd and dd were assumed to be 0.725, 0.1 and 0.1, respectively. The calculated prevalence of hypertension from these values is consistent with that observed in the Shanghai area (11–15%).

The transmission-disequilibrium test, or TDT, has proved to be a powerful tool for linkage-disequilibrium analysis (15). However, the TDT relies on data from parental genotypes. Since EH is a late onset disease, data from parents were difficult to obtain. The sib transmission-disequilibrium test (S-TDT) was regarded as being more powerful to detecting the evidence of association in this circumstance. This method compares the marker genotypes in affected and unaffected offspring so it needn't reconstruct parental genotypes and does not depend on estimates of allele frequencies. Therefore, the limitations on the TDT imposed by lack of data from parents are circumvented by the procedures for the S-TDT program

(16). The TDT was also carried out to compare the power of these two methods in the adult onset disease. Both TDT and S-TDT were performed with the TDT/S-TDT Program 1.1.

RESULTS

Association Study

When the subjects were divided by their blood pressure status, the hypertensive patients had significantly higher systolic and diastolic blood pressure, BMI, fasting glucose, serum uric acid and triglycerides than the normal controls. But the frequencies of the α-adducin genotypes and alleles were not significantly different between the two groups (Table 1).

When all the subjects were grouped according to α-adducin genotype based on Gly460Trp polymorphism, no significant differences in their phenotypes were found between the groups (Table 2). Even when the effects of BMI, fasting glucose, triglycerides and uric acid were excluded, we still failed to find a significant association between α-adducin Gly460Trp polymorphism and hypertension status or other variables in a multiple logistic regression analysis (data not

Table 1. Baseline Characteristics of Hypertensive and Normotensive Subjects: Association Study

	Hypertensives (n = 138)	Normotensives (n = 121)
Age(years)	50.7 ± 7.6	49.4 ± 4.7
Gender, M/F	82/56	73/48
SBP(mmHg)	145.82 ± 16.69*	111.00 ± 10.26
DBP(mmHg)	97.96 ± 11.14*	74.00 ± 7.06
BMI (Kg/m^2)	24.81 ± 2.73*	22.55 ± 2.57
Cr (μmol/L)	79.11 ± 40.75	77.81 ± 16.42
BUN(mmol/L)	5.01 ± 1.84	5.30 ± 1.15
Uric acid(μmol/L)	330.83 ± 81.36*	280.13 ± 81.62
FG (mmol/L)	5.18 ± 1.11*	5.02 ± 0.55
TC (mmol/L)	4.67 ± 0.88	4.50 ± 080
TG (mmol/L)	1.74 ± 0.74*	1.40 ± 0.62
Genotype(Frequency):		
Gly/Gly	35(25.4%)	39(32.2%)
Gly/Trp	73(52.9%)	53(43.8%)
Trp/Trp	30(21.7%)	29(24.0%)
Allele Frequency:		
Gly	51.8%	54.1%
Trp	48.2%	45.9%

Values are means±SD. SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index; Cr, Creatinine; BUN, blood urea nitrogen; FG, Fasting glucose; TC, total cholesterol and TG, triglycerides. *$P < 0.01$, hypertensive versus normotensive subjects.

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Table 2. Characteristics of Participants Grouped According to α-Adducin Genotypes

	Hypertensives			Normotensives		
	Gly/Gly (n = 35)	Gly/Trp (n = 73)	Trp/Trp (n = 30)	Gly/Gly (n = 39)	Gly/Trp (n = 53)	Trp/Trp (n = 29)
Age(years)	50.3 ± 7.8	49.8 ± 7.5	52.2 ± 7.4	50.1 ± 4.6	48.6 ± 4.5	51.5 ± 10.5
SBP(mmHg)	148.1 ± 13.9	145.8 ± 17.8	143.3 ± 17.1	110.6 ± 9.7	112.9 ± 11.9	112.2 ± 10.3
DBP(mmHg)	100.0 ± 8.9	97.8 ± 12.2	96.0 ± 11.0	13.3 ± 8.1	74.7 ± 7.5	74.0 ± 6.8
BMI (Kg/m^2)	25.1 ± 3.3	24.5 ± 2.5	25.3 ± 2.6	22.2 ± 2.1	23.3 ± 2.9	21.9 ± 2.5
Cr (μmol/L)	75.0 ± 18.7	76.9 ± 22.7	74.2 ± 16.4	75.7 ± 16.0	78.1 ± 15.9	80.7 ± 17.4
BUN(mmol/L)	5.0 ± 1.5	4.9 ± 1.8	5.2 ± 2.3	5.6 ± 1.1	5.3 ± 1.2	5.3 ± 1.2
UA(μmol/L)	317.5 ± 63.4	337.7 ± 81.4	329.8 ± 98.0	271.4 ± 68.3	281.6 ± 88.7	302.9 ± 89.4
FG(mmol/L)	5.1 ± 1.0	5.2 ± 1.0	5.1 ± 1.1	5.0 ± 0.6	4.8 ± 0.6	4.8 ± 0.5
TC(mmol/L)	4.4 ± 0.8	4.8 ± 0.9	4.7 ± 1.0	4.4 ± 0.9	4.5 ± 0.8	4.6 ± 0.8
TG(mmol/L)	1.7 ± 0.5	1.7 ± 0.7	1.8 ± 1.0	1.3 ± 0.5	1.3 ± 0.5	1.6 ± 0.9

Values are means±SD, SBP indicates systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index; Cr, Creatinine; BUN, blood urea nitrogen; FG, Fasting glucose; TC, total cholesterol and TG, triglycerides. Gly/Gly, homozygous wild type; Gly/Trp, heterozygous type; Trp/Trp, homozygous mutant type. No significant differences were found.

shown). No significant deviation from Hardy-Weinberg equilibrium was observed at this locus.

Linkage Study

In the linkage study, two microsatellite markers, D4S3038 and D4S412 were chosen for their highly polymorphism. The heterozygosity rate for D4S3038 was 0.78 and its allele distribution 0.01 for 211(in bp), 0.38 for 213, 0.01 for 215 and 221 respectively, 0.01 for 223, 0.21 for 205, 0.06 for 227,0.12 for 229, 0.05 for 231,0.13 for 233 and 0.01 for 235. For D4S412, the heterozygosity rate was 0.76, and the allele frequencies 0.01 for 235(in bp), 0.19 for 239, 0.01 for 241, 0.01 for 243, 0.17 for 245, 0.52 for 247, 0.05 for 249, 0.01 for 251, 0.02 for 253 and 0.01 for 257. Neither parametric nor NPL analysis supported such a linkage between essential hypertension and these two loci. The maximum LOD scores were 0.16 for the locus D4S412 and −0.51 for D4S3038, and the NPL *Z*-scores were −0.16 for D4S412 and 0.24 for D4S3038, respectively.

The S-TDT determines whether the marker allele frequencies among affected offspring differ significantly from the frequencies among their unaffected sibs. And it is carried out entirely with the "*Z* score" approach in the TDT/S-TDT Program 1.1. The null hypothesis that disease and marker are unlinked is rejected if *Z* departs significantly from zero, and *P* values were determined by use of the normal distribution approximation. While S-TDT could yield lower *P* values than TDT in all alleles tested, neither TDT nor S-TDT could provide a significant linkage of hypertension in each allele of the two loci (Table 3. Only the five most common alleles of each microsatellite marker were listed).

Table 3. TDT and S-TDT Analyses in D4S3038 and D4S412

		TDT				S-TDT				
Marker	Allele	Trans-	Non-Trans	χ^2	*P* Value	Y	A	V	Z′	*P* Value
D4S3038	13	9	12	0.43	0.51	6	6.40	1.01	−0.10	0.46
	14	24	16	1.60	0.20	42	44.60	3.47	1.12	0.13
	6	14	19	0.76	0.38	69	66.46	6.05	0.83	0.20
	16	10	6	1.00	0.32	24	22.42	2.57	0.68	0.25
	12	20	22	0.10	0.76	45	43.56	4.16	0.46	0.32
D4S412	4	13	15	0.14	0.71	71	71.99	4.92	0.22	0.41
	8	16	15	0.03	0.86	73	72.96	6.88	−0.17	0.57
	9	5	4	0.11	0.74	10	8.39	1.29	0.98	0.16
	7	7	10	0.53	0.47	20	18.00	2.26	1.00	0.16
	13	1	2	0.33	0.56	8	8.56	1.09	0.06	0.48

Y, total observed number of the allele among affected sibs; A, mean of the number of the allele among affected sibs; V, variance of the allele among affected sibs; And Z′, the correction of the Z score calculated from $Z' = (|Y\text{-}A|\text{-}1/2)/V^{1/2}$. Only the five most common alleles of each microsatellite marker were listed.

DISCUSSION

As an important participant in the regulation of ion transportation, the α-adducin gene was regarded as a candidate for a "salt sensitivity gene" as envisioned by Cusi (7). However, studies in different populations always yielded different results. Despite the significant association reported by Cusi, negative results had already been reported in Chinese, Scottish, Anglo-Austrialians and African Americans (10,17–19). But the contradictions in results just remain among different populations and may reflect the different demographic histories of the populations.

However, several association studies in Japanese population have shown that, under the same ethnic background, some groups revealed a significant association between the G460T polymorphism and hypertension status (20,21), while others, failed to find the association in the same locus (9,22). These inconsistent results suggest the complex genetic background and the etiologic diversity of polygenic diseases, which are the results of complex interactions between environmental factors and susceptibility alleles of multiple genes. The gene-gene or gene-environment interactions, or even the different criteria of the subjects selected in the design (such as age, gender, blood pressure level, etc.) could affect the results. Therefore, well-designed experiments, more powerful statistical methods, as well as functional genomic research work, may be needed to confirm the precise role of the α-adducin.

To maximize the statistical power of our investigation, we adopted a combination of approaches, including two kinds of linkage studies (parametric and nonparametric linkage analyses), two kinds of transmission disequilibrium tests (TDT

and S-TDT) and an association study, to try to identify the role of α-adducin gene in hypertension susceptibility. However, in this study, we failed to observe a significant linkage between the hypertension and the chromosomal region where α-adducin is located. Nor did we find a significant association between the Gly460Trp, a polymorphism in α-adducin gene, and the hypertension status. In the Chinese population studied, the frequency of the 460Trp variant (45.9% to 48.2%) was similar to that in Japanese (54% to 60%) but much higher than that in Caucasians (13% to 23%) (2,4).

Recently, a new method, sib TDT (S-TDT), has been proposed to investigate linkage disequilibrium in late-onset diseases. When diseases with onset in adulthood or in old age are studied, it may be impossible to obtain genotypes for markers in the parents of the affected offspring. This difficulty has limited the applicability of TDT. Instead of using marker data from affected offspring and their parents, the S-TDT compares the marker genotypes in affected and unaffected offspring. So, it can obtain more information in late onset diseases. In the present study, both S-TDT and TDT were applied, while no allele could yield a significant evidence for linkage disequilibrium in these two loci. For the same allele, the *P* value in S-TDT is smaller than that in TDT, which confirmed the power of the S-TDT in the late onset disease such as hypertension.

These different approaches but consistent results show that, in the Chinese population studied here, α-adducin 460Trp variant may not play an important role in the etiology of EH. And the negative results of linkage and TDT/S-TDT further supports this conclusion.

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