Chinese Medicine, **2009, 1, 36-41**
Published Online September 2009 in SciRes (www.SciRP.org/journal/cm)

Association of CALCA Genetic Polymorphism with Essential Hypertension

ABSTRACT

Calcitonin gene-related peptide (CGRP) is the predominant neurotransmitter in capsaicin-sensitive sensory nerves. Participation of CGRP in hypertension is one of the most extensively studied topics in the field. There is growing evidence to the effect that CGRP is associated with essential hypertension (EH). The aims of this study were to pinpoint whether single nucleotide polymorphisms (SNPs) in the genes coding for CALCA were associated with EH susceptibility in a Hunan Han population. A total of 293 subjects with EH and 208 controls were enrolled in the study. Genomic DNA was extracted from peripheral blood leucocytes by a phenol-chloroform method. The CALCA T-692C was genotyped using a restriction fragment length polymorphism method. CALCA genetic polymorphism is associated with EH susceptibility. Carriers of at least one C allele at the polymorphic site CALCA T-692C showed increased risk for EH.

Keywords: *hypertension, calcitonin gene-related peptide, single nucleotide polymorphism*

1. Introduction

Epidemiologic studies have reported that essential hypertension (EH) is prevalent, affecting approx 20%–25% of the adult population in the world, becoming a major worldwide public health issue.1,2 Hypertension can increase the risk of coronary heart disease, stroke and congestive heart failure. EH is considered to be a multifactorial, complex disease. Family and twin studies have shown that about 80% EH is determined by genetic factors interacting with environmental factors.3-6 Human calcitonin gene-related peptide (CGRP), a very potent vasodilating neuropeptide, includes alpha-CGRP (CALCA) and beta-CGRP (CALCB). The growing evidence from animal and human studies indicates that CGRP may play an important role in the initiation, progression and maintenance of hypertension via the alterations in its synthesis and release.

The human CALCA gene is located on chromosome 11p15.2-p15.1 and codes for both calcitonin and alpha-CGRP through alternative RNA splicing of gene transcripts, which spans approximately 3.8 kb, and contains five exons. Single nucleotide polymorphisms (SNPs) are the most common form of human genetic variation, and they may contribute to the individual susceptibility to EH. Many studies have demonstrated that SNPs in genes may not only affect the expression or activities of the enzymes or proteins but are associated with the risk of EH; such as the genes for

angiotensinogen,7 endothelial NO synthase8 and CYP3A5.9 The aims of this study were to discover a polymorphism in the 5' flanking region of the CALCA gene in Chinese subjects and to assess the association between this gene and EH. To test this hypothesis, we performed genotyping analyses for the CALCA T-692C polymorphism in a population-based case-control study of the Chinese Han population.

2. Methods

2.1. Clinical Materials

From December 2005 to August 2006, eligible patients for this population-based case-control study of EH were recruited at our community program of EH prevention and cure in Hunan province. They were genetically unrelated ethnic Han Chinese and were from Hunan

province in middle China. There were 293 EH patients (male/female ratio=1.53). EH was diagnosed based on the following criteria: seated systolic blood pressure (BP) ≥140 mmHg or diastolic BP ≥90 mmHg on three occa-

sions within 2 months after the first BP reading. Patients diagnosed as having secondary hypertension were excluded. A total of 208 normotensive age-matched healthy individuals (male/female ratio =1.70) served as control subjects. All control subjects had systolic BP <140 mmHg and diastolic BP <90 mmHg. The study was approved by the ethnic committee of Xiangya Hosptial.

2.2. Genetic Studies

Based on the information obtained from the NCBI SNP database, we chose a SNP CALCA T-692C that had a minor allele frequency of more than 10%. Blood samples were collected from all participants and genomic DNA was extracted from the peripheral blood leukocyte pellet by the standard method with proteinase K digestion and followed by phenol-chloroform extraction and ethanol precipitation. The CALCA genotypes were detected by the polymerase chain reaction (PCR) restriction fragment length polymorphism assay with primers of 5'CG CATCTGTACCTTGCAACT3' (forward) and 5'TCAA ATT CCCGCTCACTTTA3' (reverse). The 20 μl PCR mixture consisted of approximately 100 ng of genomic DNA, 40 pmol of each primer, 40 pmol dNTP, 2 μl 10×PCR buffer, and 2 U Taq DNA polymerase (Sangon, China). The PCR profile consisted of an initial melting step of 94℃ for 5 minutes, 38 cycles of 94°C for 50 seconds, 57℃ for 50 seconds and 72°C for 1 minute, and a final extension step of 72°C for 10 minutes. The 636 bp PCR product was then digested overnight by restriction enzyme of 1 U PshA I (TaKaRa, Japan). The -692TT genotype produced a single 636 bp fragment, the -692CC genotype produced two fragments of 235 bp and 401 bp, and the heterozygous -692CT genotype produced three fragments of 636 bp, 235 bp and 401 bp. Genotyping was performed without knowing the case/control status of the subjects. Twelve DNA samples of EH cases were randomly selected to have sequencing analysis and the results were 100% concordant with the restriction fragment length polymorphism (RFLP) results.

2.3. Statistical Analysis

All continuous variables were expressed as mean ± standard deviation. Differences in selected demographic characteristics, selected variables and frequencies of the genotypes and alleles of CALCA between the cases and controls were evaluated using the chi-square test and/or Student's *t* test. The association of CALCA genotypes with EH risk were estimated by computing the odds ratios (*OR*s) and their 95% *CI*s from multiple Logistic regression analyses with adjustment for gender, age, smoking history, dyslipoproteinemia, family history of premature cardiovascular disease and obesity. The Hardy-Weinberg equilibrium was tested by a chi-square test to compare the observed genotype frequencies with the expected frequency among the control subjects. Stratification analysis was performed according to different groups of selected variants. All the statistical analyses were performed with Statistical Package for the Social Science (SPSS) 15.0. All the *P* values were two sided and with the significant levels of $P < 0.05$.

3. Results

Human CGRP includes two subtypes, alpha- and beta-CGRP (CALCA and CALCB), and CALCB shows high sequence homology with CALCA. In the National Center for Biotechnology Information (NCBI) single nucleotide polymorphism (SNP) database (*http://www.ncbi.nlm.nih.gov/projects/SNP/*), 34 Chinese SNPs at CALCA (NT_009237, REGION: complement (137672 88.13791073)) and CALCB (NT_009237, REGION: complement (13872387.. 13897418)) have been recorded, as shown in Tables 1 and 2.

Table 1. Eleven SNPs loci of Chinese CALCA gene

Number	Polymorphism	Location	Minor allele	Minor allele frequency	rs*
1	T-9241C	5'Flank	C	0.1111	1496162
2	G-5357A	5'Flank	A	0.3444	903808
3	T-3905C	5'Flank	C	0.2222	1496163
4	T-3658C	5'Flank	C	0.1395	1496164
5	T-3580G	5'Flank	G	0.1111	1496166
6	T-692C	5'Flank	C	0.1111	3781719#
7	G-658C	5'Flank	C	0.1	1553005
8	C-3289A	Exon4	A	0.0227	5241
9	T-4711A	Intro4	A	0.1	2956
10	T-4852C	Intro4	C	0.1222	5242
11	G-5119A	Intro4	A	0.0111	5243

*The SNP rs number in dbSNP database. #The SNP locus of CALCA gene in this research

Table 2. Twenty-three SNPs loci of Chinese CALCB gene

Number	Polymorphism	Location	Minor allele	Minor allele frequency	rs
1	C-9634G	5'Flank	G	0.0238	7116679
2	A-7831G	5'Flank	G	0.1071	7110243
3	A-3945G	5'Flank	G	0.1351	10766205
4	C-2778T	5'Flank	T	0.1222	7945166
5	A-2208G	5'Flank	G	0.10227	10832355
6	G-1135T	5'Flank	T	0.11627	1540148
7	A-614G	5'Flank	G	0.12222	3812726
8	A-737G	Intro 3	G	0.44444	11603873
9	C-1225T	Intro 3	T	0.011111	3829220
10	A-1453T	Intro 3	T	0.12222	3829222
11	G-1802T	Intro 3	T	0.10227	1894128
12	G-1802T	Intro 3	T	0.11111	12274674
13	A-2453G	Intro 3	T	0.0909	1894128
14	C-2878T	Intro 4	G	0.1136	2282653
15	A-3082T	Intro 4	T	0.1222	2282654
16	C-3430T	Exon 5	T	0.03409	16930880
17	A-3941G	3'UTR	G	0.111111	1540140
18	C-5005T	3'UTR	T	0.12222	1945613
19	C-6500T	3'UTR	T	0.13333	1945614
20	C-6652G	3'UTR	G	0.01136	7121088
21	C-7824T	3'UTR	T	0.122222	10832356
22	A-8658T	3'UTR	T	0.10227	12791060
23	C-13436T	3'UTR	T	0.10227	6486224

The characteristics of the 293 EH cases and 208 controls included in the study were summarized in Table 3. The mean age was 50 years (±12 years) for cases and 49 years (±11 years) for controls, which was adequately matched on age as suggested by the Student's *t* test (*P*=0.560). Compared with the control subjects, the EH cases had a significantly higher family history of EH (*P* <0.001), and had a significantly higher serum TBIL (*P* <0.001) and BS (*P*=0. 215). Moreover, as shown in Table 3, serum HDL was significantly lower in EH cases (*P* <0.001).

The observed genotypes for this polymorphism among the control subjects were both in Hardy-Weinberg equilibrium. The genotype distributions of CALCA T-692C in the cases and controls and their association with EH are shown in Table 4. For the CALCA T-692C polymorphism the genotype frequencies of TT, TC and CC were 85.1%, 14.9% and 0 in the controls, respectively, which were significantly different from the EH cases (71.7% TT , 26.9% TC and 1.4% CC) ($\chi 2$=13.734, *P*=0.001). Logistic regression analyses revealed that compared with the TT genotype, the adjusted *OR* of EH for subjects carrying TC and combined genotypes TC +CC were 1.942 (95% *CI*: 1.216–3.103) and 2.093 (95% *CI*: 1.317–3.326), respectively. The frequencies of the C allele was 14.85% in EH group, and 7.45% in control group, the prevalence of C alleles in the EH subjects and controls were significantly incomparable (*P* <0.001). In addition, the risk of EH associated with premature cardiovascular disease (adjusted *OR*=9.114, 95% *CI*: 2.105–39.455, *P*=0.003).

4. Discussion

It is generally believed that the neuropeptide CGRP plays an integral role in the pathophysiology of hypertension. In experimental animal models, strong evidence supports the hypothesis that reduction both in alpha- and beta-CGRP could contribute to blood pressure elevation.10 Up to now, differences between normal and hypertensive patients in the concentration of plasma CGRP

Table 3. Distributions of select variables in EH cases and controls

Variable	EH (*n*=293)	Control (*n*=208)	*P* value
Gender			0.560
Male (*n*, %)	177(60.4)	131(63.0)	
Female (*n*, %)	116(39.6)	77 (37.0)	
Age at first diagnosis(years)	50 ± 12	49 ± 11	0.608
Smoking history (*n*, %)	57(19.5)	40(19.2)	0.959
Drinking history (*n*, %)	54(18.4)	27(12.9)	0.164
Family history of EH (*n*, %)	23(7.8)	2(1.0)	<0.001
BMI(kg/m^2)	23.62±1.87	23.73±1.64	0.522
TBIL (mmol/L)	10.17±4.25	8.29±4.26	<0.001
BUN (mmol/L)	5.38±1.04	5.51±1.14	0.231
Cr (μmol/L)	85.68±15.12	84.65± 15.79	0.462
BS (mmol/L)	5.22±1.66	4.97±0.66	0.037
TC (mmol/L)	3.77±0.59	3.67±0.51	0.065
TG (mmol/L)	1.38±0.40	1.34±0.30	0.215
HDL (mmol/L)	1.40±0.43	1.61±0.50	<0.001
LDL-C (mmol/L)	2.71±2.18	2.23±1.12	0.211

BMI: body mass index, TBIL: total bilirubin, BUN: blood urea nitrogen, Cr: creatinine, BS: blood glucose, TC: total cholesterol, TG: triglyceride, HDL: high-density lipoprotein, LDL-C: low-density lipoprotein cholesterol

Table 4. Frequency distributions of the CALCA T-692C polymorphism among the EH cases and controls and their association with EH

Genotype	Controls (*n*=208)		EH (*n*=293)		*OR* (95%*CI*) *	*P* value
	n	%	*n*	%		
TT	177	85.1	210	71.7	1.00	
TC	31	14.9	79	26.9	1.942 (1.216–3.103)	0.005
CC	0	0	4	1.4	2.093 (1.317–3.326)	0.002
C allele	31	7.45	87	14.85		

*Adjusted by gender, age, smoking history, dyslipoproteinemia, family history of premature cardiovascular disease and obesity. Frequency distributions of TT, TC, CC and C alleles between control and EH groups were found significantly different ($\chi 2$=23.434, *P* <0.001)

are uncertain. Previous studies have shown that plasma CGRP concentrations were significantly lower in hypertensive patients and preeclamptic pregnancies than in normotensive controls.11,12 The present study demonstrates that calcium intake and anti-hypertension drugs enhance the release or response of endogenous CGRP, resulting in a decrease in blood pressure.13 In addition, intra-arterial CGRP infusions have been reported to induce vasodilation in a dose-dependent manner in the human forearm, increase forearm blood flow ratio and lower blood pressure.14,15 These results are consistent with the hypothesis that the decrease in CGRP synthesis and release contributes to elevated blood pressure. In contrast, other studies have shown increased plasma CGRP levels in patients with hypertension and shown a significant positive correlation between systolic and diastolic blood pressure.16 The increase in CGRP levels, or the enhancement of vascular sensitivity response to CGRP, plays a beneficial compensatory depressor role in the development of hypertension. Some functional studies reported that CALCA gene knockout accelerated hypertension-induced heart and kidney damage.17 The

systolic blood pressure of CALCA gene knockout mice has been reported to be significantly elevated.18 Recently, CALCA genetic polymorphisms show a possible association with Parkinson's disease, ovarian cancer and bone mineral density, suggesting that polymorphisms in the CALCA gene have functional significance.19-21 Therefore, we reasoned that polymorphisms of CALCA genes may contribute to EH susceptibility in the general population. However, there has been only one report dealing with the relationship between the CALCA gene variants and EH. In a study in Japanese a novel 2-bp microdeletion polymorphism was discovered in intron 1 of the CALCA and showed that the CALCA gene could be the susceptibility gene of EH.22 In our population-based case-control study of EH, we investigated, for the first time, the association of SNP (CALCA T-692C) with the risk of EH in a Han population in Hunan province of middle China. We found that CALCA T-692C was associated with EH risk, suggesting that this variant may play a role in the etiology of EH. However, these results are preliminary because of the limited sample size in the subgroup populations and need for validation in a further larger studies. Although the main findings of current study were positive and encouraging, several limitations of this study need to be addressed. First, we can not rule out the possibility that other, as yet unidentified, alterations in genes involved in cell cycle and DNA replication influence the risk of developing EH. In addition, because of the populationbased design of the study, these results may not be generalizable to the general population. Genetic polymorphisms often vary between ethnic groups. Furthermore, the sample size of the EH cases was not large enough to detect a small effect from very low penetrance genes or SNPs. In conclusion, our study provides evidence for association of the CALCA T-692C variant genotypes with risk of EH in this study population. A large prospective study is needed to verify our findings, particularly in subjects with a family history of EH.

References

[1] Zhao XL, Chen J, Cui YL, Wu F, Hu DY. Current status of primary hypertension in China: an epidemiological study of 12 provinces,1 autonomous region, and 1 municipality. Natl Med J China (Chin) 2006; 86: 1148-1152.

[2] Zdrojewski T, Wyrzykowski B, Szczech R, Wierucki L, Naruszewicz M, Narkiewicz K, et al. Epidemiology and prevention of arterial hypertension in Poland. Blood Press Suppl 2005; 14:10-16.

[3] Luft FC. Hypertension as a complex genetic trait. Semin Nephrol 2002; 22: 115-126.

[4] Kurland L, Liljedahl U, Lind L. Hypertension and SNP genotyping in antihypertensive treatment. Cardiovasc Toxicol 2005; 5: 133-142.

[5] Gong M, Hubner N. Molecular genetics of human hypertension. Clin Sci (Lond) 2006; 110: 315-326.

[6] Doris PA. Hypertension genetics, single nucleotide polymorphisms, and the common disease: common variant hypothesis. Hypertension 2002; 39: 323-331.

[7] Markovic D, Tang X, Guruju M, Levenstien MA, Hoh J, Kumar A, et al. Association of angiotensinogen gene polymorphisms with essential hypertension in African-Americans and Caucasians. Hum Hered 2005; 60: 89-96.

[8] Sandrim VC, Coelho EB, Nobre F, Arado GM, Lanchote VL, Tanus-Santos JE. Susceptible and protective eNOS haplotypes in hypertensive black and white subjects. Atherosclerosis 2006; 186: 428-432.

[9] Kivisto KT, Niemi M, Schaeffeler E, Pitkala K, Tilvis R, Fromm MF, et al. CYP3A5 genotype is associated with diagnosis of hypertension in elderly patients: data from the DEBATE Study. Am J Pharmacogenomics 2005; 5: 191-195.

[10] Deng PY, Ye F, Cai WJ, Deng HW, Li YJ. Role of calcitonin gene-related peptide in the phenol-induced neurogenic hypertension in rats. Regul Pept 2004; 119: 155-161.

[11] Portaluppi F, Trasforini G, Margutti A, Vergnani L, Ambrosio MR, Rossi R, et al. Circadian rhythm of calcitonin gene-related peptide in uncomplicated essential hypertension. J Hypertens 1992; 10: 1227-1234.

[12] Dong YL, Green KE, Vegiragu S, Hankins GD, Martin E, Chauhan M, et al. Evidence for decreased calcitonin gene-related peptide (CGRP) receptors and compromised responsiveness to CGRP of fetoplacental vessels in preeclamptic pregnancies. J Clin Endocrinol Metab 2005; 90: 2336-2343.

[13] Wimalawansa SJ, Supowit SC, DiPette DJ. Mechanisms of the antihypertensive effects of dietary calcium and role of calcitonin gene related peptide in hypertension. Can J Physiol Pharmacol 1995; 73: 981-985.

[14] Vanmolkot FH, Van der Schueren BJ, de Hoon JN. Calcitonin gene-related peptide-induced vasodilation in the human forearm is antagonized by CGRP8-37: evaluation of a human *in vivo* pharmacodynamic model. Clin Pharmacol Ther 2006; 79: 263-273.

[15] de Hoon JN, Smits P, Troost J, Struijker-Boudier HA, Van Bortel LM. Forearm vascular response to nitric oxide and calcitonin gene-related peptide: comparison be-

tween migraine patients and control subjects. Cephalalgia 2006; 26: 56-63.

[16] Edvinsson L, Erlinge D, Ekman R, Thulin T. Sensory nerve terminal activity in severe hypertension as reflected by circulating calcitonin gene-related peptide (CGRP) and substance P. Blood Press 1992; 1: 223-229.

[17] Supowit SC, Rao A, Bowers MC, Zhao H, Fink G, Steficek B, et al. Calcitonin gene-related peptide protects against hypertension-induced heart and kidney damage. Hypertension 2005; 45: 109-114.

[18] Gangula PR, Zhao H, Supowit SC, Wimalawansa SJ, Dipette DJ, Westlund KN, et al. Increased blood pressure in alpha-calcitonin gene-related peptide/calcitonin gene knockout mice. Hypertension 2000; 35: 470-475.

[19] Goodman MT, Ferrell R, McDuffie K,Thompson PJ, Wilkens LR, Bushley AW, et al. Calcitonin gene polymorphism CALCA -624(T/C) and ovarian cancer. Environ Mol Mutagen 2005; 46: 53-58.

[20] Magana JJ, Gomez R, Cisneros B, Casas L, Castorena F, Miranda A, et al. Association of the CT gene (CA) polymorphism with BMD in osteoporotic mexican women. Clin Genet 2006; 70: 402-408.

[21] Buervenich S, Xiang F, Sydow O, Jonsson EG, Sedvall GC, Anvret M, et al. Identification of four novel polymorphisms in the calcitonin/alpha-CGRP (CALCA) gene and an investigation of their possible associations with Parkinson disease, schizophrenia, and manic depression. Hum Mutat 2001; 17: 435-436.

[22] Morita A, Nakayama T, Soma M, Mizutani T. Association between the calcitonin-related peptide alpha (CALCA) gene and essential hypertension in Japanese subjects. Am J Hypertens 2007; 20: 527-532.

