Single nucleotide polymorphisms in promoter of angiotensin II type 1 receptor gene associated with essential hypertension and coronary heart disease in Chinese population[1]

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ABSTRACT

AIM: To discover single nucleotide polymorphisms (SNPs) in the promoter region of angiotensin II type 1 receptor (AT1) gene and evaluate their associations with the occurrence of essential hypertension (EH) and coronary heart disease (CHD) in Chinese Han population. **METHODS:** SNPs detection was performed by PCR-sequencing. The genotype was determined by the same method in a total number of 473 unrelated patients including 160 EH cases, 128 CHD cases, and 185 EH combined with CHD cases as well as 160 healthy controls. **RESULTS:** Six SNPs were discovered in the promoter region of AT1 gene. -810A/T was almost in completely linkage disequilibrium with -713G/T, -214A/C, -213G/C, and -153A/G polymorphisms. No statistically association was found in our population between -810A/T polymorphism and EH, the association of -810A allele and CHD was of borderline significant (χ^2=3.649, *P*=0.056). However, significant differences of genotype distributions were observed in the EH combined with CHD group (TT=126, TA=51, AA=8) compared with the EH patients (TT=127,TA=26, AA=7, χ^2=6.410, *P*=0.041) and the healthy controls (TT=130, TA=24, AA=6, χ^2 =7.742, *P*=0.021). The EH combined with CHD patients had a significantly increased A allele frequency than the normal references (0.181 *vs* 0.106, χ^2=7.690, *P*=0.006) and the EH subjects (0.181 *vs* 0.125, χ^2=4.119, *P*=0.042). Hypertensive patients carrying TA genotype (OR=1.977, 95 % CI 1.160-3.354, *P*=0.011) or A allele (OR=1.548, 95 % CI 1.015-2.361, *P*=0.043) had an increased risk for CHD morbidity. **CONCLUSION:** we firstly report that -810A/T polymorphism in the promoter region of AT1 gene might be a genetic risk factor for the pathogenesis of CHD complicated with EH in Chinese Han population.

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INTRODUCTION

Human essential hypertension (EH) and coronary heart disease (CHD) are both common complex diseases believed to result from the interaction of environmental and genetic factors. In search of the disease-susceptibility genes has become the research frontier.

The renin-angiotensin system (RAS) plays a central role in salt and homeostasis, the maintenance of vascular tone and cardiovascular remodeling[1]. Consequently, each component of this system repre-

sents a potential candidate in the etiology of EH and CHD. Angiotensin II (Ang II) is the active component of the RAS. Ang II receptors, which mediate the vasoconstrictive and salt-conserving actions of the RAS, also represent interesting candidate genes for cardiovascular diseases. Two subtypes of cell surface receptors, angiotensin II type 1 receptor (AT1) and angiotensin II type 2 receptor (AT2), have been identified. Most of the known actions of Ang II are mediated through AT1, which is particularly prominent in vascular smooth muscle cells and myocardium[2,3].

A single nucleotide polymorphism (SNP) located in the 3' untranslated region of AT1 gene, +1166 A/C, has been characterized and investigated in relation to arterial hypertension[4,5], hypertension-induced hypertrophy[6], aortic stiffness[7], myocardial infarction[8,9], and carotid intimal-medial thickening[10]. Since the +1166 A/C polymorphism does not appear to be functional, it is postulated to be a genetic marker or in linkage disequilibrium with an unidentified functional loci which would affect the regulation of the gene in response to Ang II. Recently, some scientists have transferred their attention to other locus of AT1 gene[11-15].

In the present study, we screened the promoter region of AT1 gene in Chinese Han population using PCR-sequencing. We attempted to assess whether the possible SNPs might be involved in the pathology of EH and CHD through conducting case-control studies.

MATERIALS AND METHODS

Study population A total number of 633 unrelated participants were recruited from Ruijin Hospital, Shanghai Second Medical University. EH was diagnosed by either blood pressure >140 mmHg systolic or >90 mmHg diastolic, without clinical and biochemical signs of secondary hypertension. Diagnosis of CHD was established if the coronary artery presented a reduced lumen greater than 50 % on angiography. The research subjects were then divided into 4 groups: (1) The healthy controls: 160 citizens came from Eastern China who had received routine health examinations in the hospital. They had normal ECG and blood pressure, without family history of EH and CHD in first-degree relatives. (2) The EH group: 160 subjects accorded with the above criteria of EH. They had no clinical signs of CHD. (3) The CHD group: 128 normotensives in line with the above criteria of CHD. (4) The EH combined with CHD group (EH+CHD): 185 subjects both satisfied the above criteria of EH and CHD.

All subjects in this study are ethnically Eastern Chinese Han. Physical examinations, including height, weight, systolic blood pressure (SBP), and diastolic blood pressure (DBP) were recorded. Body mass index (BMI) was calculated as weight divided by height in metres-squared (kg/m^2). A full biochemical profile, including fasting lipids [total cholesterol (TC), triglycerides (TG), low-density lipoproteins (LDL), high- density lipoproteins (HDL)] and glucose was performed after 16-h fast. Informed consent from each subject was obtained regarding participation in this study. The participants' characteristics are shown in Tab 1.

SNPs identification and genotyping Genomic DNA was prepared from white blood cell using a standard phenol/chloroform protocol. Twenty-four individuals were selected for the initial SNPs identification. We chose to screen about 2000 bp of the promoter region for sequence variations. The entire promoter

Tab 1. Characterization of research subjects. Mean±SEM. $^bP<0.05$ *vs* control.

Parameter	Control (*n*=160)	EH (*n*=160)	CHD (*n*=128)	EH+CHD (*n*=185)
Gender, male/female	98/62	86/74	96/32	112/73
Age, year	62±9	63±11	63±12	62±9
BMI, kg/m^2	23.5±2.8	24±3^b	23.9±2.8^b	25±3^b
SBP, mmHg	113±12	168±22^b	122±13^b	160±19^b
DBP, mmHg	74±8	104±11^b	75±9	99±13^b
TG, mmol/L	1.3±0.6	1.3±1.0^b	1.27±0.13^b	1.35±0.09^b
TC, mmol/L	4.5±0.8	4.9±1.0^b	4.7±0.9	4.9±1.1^b
HDL, mmol/L	1.4±0.3	1.3±0.4^b	1.2±0.27^b	1.20±0.28^b
LDL, mmol/L	2.8±0.9	3.2±1.0^b	2.9±0.8	3.0±1.0
Glucose, mmol/L	4.7±0.6	5.4±1.8^b	5.8±1.0^b	5.8±2.1^b

sequence was divided into 5 overlapping fragments of 450-550 bp. The primer sequences for PCR are listed in Tab 2. Each fragment was amplified in PE-9700 (Perkin-Elmer, Fostor City,Calif) by touch-down PCR[16]. Following PCR, products were purified by the Wizard Purification kit (Promega, Madison, Wis), Sequencing reactions were run on an ABI 377 semi-automatic sequencer with BigDye and Dye Terminator chemistry (Perkin-Elmer), and were scored using Sequence Analysis and Sequence Navigator software (Perkin-Elmer). Identified SNPs were genotyped by the same method in the entire research population.

Statistical analysis Data analyses were performed with the Statistical Package for the Social Science (SPSS, version 10.0). Data were expressed as mean±SEM. For each SNP, Hardy-Weinberg equilibrium was tested by χ^2 analysis. Differences in allele frequencies and genotype distributions between groups were analyzed by χ^2 test or the Fisher's exact test when necessary. Odds ratio (OR) and 95 % confidence intervals (CI) were calculated as well. A 2-tailed $P<0.05$ was considered as significant. Arlequin software was used to estimate linkage disequilibrium.

RESULTS

SNPs discovery Twenty-four subjects were selected in the initial search for molecular variants within the promoter region of AT1 gene. Six SNPs at position -810, -713, -521, -214, -213, and -153 were identified and were further analyzed by PCR-sequencing in all study population.

Genotype analyses For each polymorphism, allele frequencies were satisfied the Hardy-Weinberg equilibrium. Among the six identified SNPs, the genotypes of -810A/T, -713G/T, -214A/C, -213G/C, and -153A/G polymorphisms were almost in completely linkage disequilibrium with one another. The Genotype distributions and allele frequencies for -810A/T and -521C/T were then determined in four research groups (Tab 3).

For -521C/T polymorphism, the allele frequencies and genotype distributions in three case groups did not differ significantly from those in the healthy control group.

For -810A/T polymorphism, the genotypes and allele frequencies did not show significant differences between the EH group and the healthy controls. Comparing CHD group with controls, the difference of A allele frequency was of borderline significant (0.165 *vs* 0.125, χ^2 =3.649, P=0.056). However, significant differences in genotypes were observed in the EH combined with CHD group (TT=126, TA=51, AA=8) compared with the EH patients (TT=127, TA=26, AA=7, χ^2=6.410, P=0.041) and the healthy controls (TT=130, TA=24, AA=6, χ^2 =7.742, P=0.021). The EH combined with CHD patients had a significantly increased A allele frequency than the controls (0.181 *vs* 0.106, χ^2=7.690, P=0.006) and the EH subjects (0.181 *vs* 0.125, χ^2 =4.119, P=0.042).

The odds ratio (OR) for EH combined with CHD associated with -810A/T polymorphism was measured (Tab 4). With the genotype TT as the reference, the OR for EH combined with CHD was 2.024 (95 % CI 1.194-3.43, P=0.009) for genotype TA. With the allele T as the reference, the odds ratio associated with the -810A were assessed as 1.860 (95 % CI 1.199-2.885, P=0.006). Similarly, Hypertensive patients carrying TA genotype (OR=1.977, 95 % CI 1.160-3.354, P=0.011) or A allele (OR=1.548, 95 % CI 1.015-2.361, P=0.043) had an increased risk for CHD morbidity.

DISCUSSION

Human AT1 gene is assigned to 3q21-q25, which has a length of more than 55 kb and is composed of five exons. Best evaluated with respect to the association with cardiovascular phenotypes is the +1166 A/C

Tab 2. Primer sequences of 5 overlapping fragments for PCR.

Fragment	Primer sequence, 5'-3'	Amplification fragment size/bp
1	F: AGTGGTGAGAAGCCAACAGTG R: TGGACAAAGCCTACATGATTGT	509
2	F: TTCCTATTCCTAGTTTGTTGAGTGT R: TTGATGCCAGAAAGCAAGATT	495
3	F: TGTCTTTTCTTCATGCTAGAAACATT R: TGGATTAATTCCAGGGTTATGA	496
4	F: ATGCAACTTGGGTAGCATGTC R: ATTTATAGTGAGGGGCGTTGC	459
5	F: AGGAGGAGGGAATGCAAAAC R: GAACTACGGTCGCTCTTTCCT	452

Tab 3. Allele frequencies and genotype distributions of -810A/T and -521C/T polymorphisms in patients with EH, CHD, EH combined with CHD, and healthy controls. [b]*P*<0.05 *vs* control.

	Subjects	Genotype distribution			χ^2	*P*	Allele frequency		χ^2	*P*
-810(A/T)		**TT**	**TA**	**AA**			**T**	**A**		
Control	160	130	26	4			286 (89.37 %)	34 (10.63 %)		
EH	160	127	26	7	0.853	0.653[b]	280 (87.50 %)	40 (12.50 %)	0.550	0.458[b]
CHD	128	90	35	3		0.070[b]	215 (83.98 %)	41 (16.02 %)	3.649	0.056[b]
EH+CHD	185	126	51	8	7.742	0.021[b]	303 (81.89 %)	67 (18.11 %)	7.690	0.006[b]
EH *vs* EH+CHD					6.410	0.041			4.119	0.042
CHD *vs* EH+CHD					0.899	0.638			0.464	0.496
-521(C/T)		**CC**	**CT**	**TT**			**C**	**T**		
Control	160	110	41	9			261 (81.56 %)	59 (18.44 %)		
EH	160	105	40	15	1.629	0.443[b]	250 (78.12 %)	70 (21.88 %)	1.175	0.278[b]
CHD	128	77	44	7	2.657	0.265[b]	198 (77.34 %)	58 (22.66 %)	1.564	0.211[b]
EH+CAD	185	108	65	12	4.091	0.129[b]	281 (75.95 %)	89 (24.05 %)	3.213	0.073[b]
EH *vs* EH+CHD					4.540	0.103			0.459	0.498
CHD *vs* EH+CHD					0.182	0.913			0.165	0.685

Tab 4. Odds ratio (OR) for EH combined with CHD associated with -810A/T polymorphism. [b]*P*<0.05, [c]*P*<0.01.

	EH+CAD *vs* controls	EH+CHD *vs* EH
Genotype AA *vs* TT		
OR	2.063	1.152
95 % CI	(0.619-6.878)	0.405-3.276
Genotype TA *vs* TT		
OR	2.024	1.977
95 % CI	(1.194-3.430)[c]	(1.16- 3.354)[b]
Allele A *vs* T		
OR	1.860	1.548
95 % CI	(1.199-2.885)[c]	1.015-2.361[b]

polymorphism located in the 3' untranslated region, but this SNP's function is unclear. It has been speculated that +1166 A/C polymorphism probably might be a neutral marker in tight linkage to a functional variant yet to be identified. In this study, we focused our research on the promoter region of AT1 gene and identified 6 SNPs in Chinese Han population. 5 of these variations were almost in completely linkage disequilibrium with each other and none of the polymorphisms was in linkage disequilibrium with the previously reported +1166A/C polymorphism. We did not identify -1424C/G polymorphism which had been reported to have a low allele frequency of 0.963/0.037 in Caucasian population[11]. This might somewhat reflect the racial difference.

We used the candidate gene approach to determine the associations of these identified SNPs with the occurrence of EH and CHD. The allele frequencies and genotype distributions of -810T/A polymorphism did not show statistical differences compared with the EH patients and controls. This finding was in line with the former research conducted in Caucasian population[12] which found no association of -810T/A polymorphism with blood pressure either. The ECTIM study[11] indicated that -810T/A polymorphism might be associated with the risk of myocardial infarction in male, but in our study, the correlation of -810T/A polymorphism with CHD was of borderline significant. However, significant differences in genotypes and allele frequencies were observed in EH combined with CHD group compared with EH patients and healthy controls. Hypertensive subjects carrying TA genotype or A allele had an increased risk for CHD morbidity. Several explanations may account for the discrepancy between our result and that of the ECTIM study. First, the ECTIM study was conducted in Caucasian population, while

Tab 5. The predicted regulatory and transcription factor binding sites by Matinspector software (version 2.2).

Binding sites	Allele 1	Allele 2
	-713 G allele	**-713 T allele**
V$CHOP_01	ggaT**G**CAatttgc(+)	No such binding site
V$GFI1_01	No such binding site	gcaaattg**A**ATCcaataacatacg(-)
	-214A-213G allele	**-214C-213C allele**
V$E47_02	gggacC**AG**Gtgaacgc(+)	No such binding site
V$LMO2COM_01	gacC**AG**Gtgaac(+)	No such binding site
V$DELTAEF1_01	gttcAC**CT**ggt(-)	No such binding site
V$MYOD_Q6	ttCAC**C**tggt (-)	No such binding site
V$NMYC_01	No such binding site	gaccc**C**GTGaac(+)

DNA strands are denoted as (+) and (-). The position of the SNP allele is underlined**.**

our study was carried out in Eastern Chinese Han population. The SNP distributions might be divergent between different races. Second, the research population of the ECTIM study satisfied the definition of acute myocardial infarction (AMI), and only male subjects were recruited; In our study, patients who had a major arteries with >50 % stenosis on angiography were recruited, including both male and female subjects. Finally, the ECTIM study did not figure out whether the AMI patients were complicated by hypertension. In the present study, the CHD patients were subdivided into the CHD without hypertension group and the CHD combined with hypertension group. A statistically association had been found in the EH combined with CHD patients.

Till now, the function of AT1 gene promoter is unclear. The identified SNPs were interrogated for the presence of regulatory and transcription factor binding sites by Matinspector software, version 2.2 [17]. Some SNPs were predicted to both create and destroy transcription factor binding sites (Tab 5). We therefore speculate that although single loci's effect may be relatively modest, the SNPs that were almost in completely linkage disequilibrium with each other in the promoter region would produce synergistic effect to regulate the express of AT1 gene and then have an influence on the pathogenesis of cardiovascular diseases. However, these binding sites need to be experimentally verified.

From the perspective of epidemiology, hypertension is an important risk factor of CHD[18]. Predispositions to hypertension and CHD are possibly associated with gene polymorphisms. Our present research is the first study to implicate genetic variations in the promoter region of AT1 gene with susceptibility to EH complicated with CHD.

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