ORIGINAL ARTICLE

Polymorphisms in *CYP11B2* and *CYP11B1* genes associated with primary hyperaldosteronism

Guo-xi Zhang^{1,2,6}, Bao-jun Wang^{1,6}, Jin-zhi Ouyang^{3,6}, Xi-yuan Deng⁴, Xin Ma¹, Hong-zhao Li¹, Zhun Wu⁵, Shuang-lin Liu⁵, Hua Xu⁵ and Xu Zhang¹

Several frequent polymorphisms in the *CYP11B2* gene are suggested to be associated with essential hypertension and aldosterone secretion. In this study, we investigated the association of polymorphisms in *CYP11B2* and *CYP11B1* genes with the risk of primary hyperaldosteronism (PH). Three polymorphisms in the *CYP11B2* gene (intron 2 conversion, rs1799998 and rs4539) and two polymorphisms in the *CYP11B1* gene (rs6410 and rs6387) were analyzed in patients with PH and in the normal population. The rs6410 allelic frequencies in patients with aldosterone-producing adenoma (APA) and idiopathic hyperaldosteronism (IHA) were significantly different from those in controls at $P=1.09 \times 10^{-5}$ and 0.015, respectively. There was a relative excess of AA homozygotes and AG heterozygotes of the rs6410 allele in the APA group as compared with the control group ($P=2.19 \times 10^{-4}$). There were significantly different genotypes, AA and AG, of the rs6410 allele between the patients with IHA and the controls only after adjustments for age, gender and body mass index (odds ratio (OR)=4.06, 95% confidence interval (CI) 1.31–12.66; OR=2.41, 95% CI 1.02–5.72). One susceptible haplotype, AAAWT, was identified to be significantly associated with APA (OR=1.44, 95% CI 1.19–1.76), and three susceptible haplotypes, AAAWT, AGGWT and AGAWC, were identified to be significantly associated with IHA (OR=1.55, 95% CI 1.23–1.96; OR=1.49, 95% CI 1.17–1.89; OR=1.40, 95% CI 1.04–1.88). In contrast, one protective haplotype, GGAWT, showed a significant difference between the patients with APA and controls (OR=0.73, 95% CI 0.55–0.97). Several haplotypes were associated with ARR in both the controls and cases. Our data demonstrated that there was a significant association between polymorphisms in the *CYP11B2* and *CYP11B1* genes and a genetic predisposition to PH. The association with IHA seemed closer compared with APA.

Hypertension Research (2010) 33, 478–484; doi:10.1038/hr.2010.21; published online 26 March 2010

Keywords: *CYP11B2*; *CYP11B1*; polymorphism; PH

INTRODUCTION

Primary hyperaldosteronism (PH) is now recognized as the most common form of secondary hypertension. Cross-sectional and prospective studies reported PH in >10% of hypertensive patients, in both general and specific settings.¹ The most common clinical subtypes of PH are aldosterone-producing adenoma (APA) and idiopathic hyperaldosteronism (IHA).

Aldosterone has a key role in APA, and the terminal stages in its synthesis, the synthesis of aldosterone from 11-deoxycorticosterone in the glomerulosa zone, is catalyzed by aldosterone synthase, which is encoded by the *CYP11B2* gene.^{2,3} Parallel 11 β -hydroxylation of the 17-hydroxysteroid, which is catalyzed by the enzyme 11 β -hydroxylase encoded by the gene *CYP11B1*, produces cortisol in the zona fasciculata.⁴ *CYP11B2* and *CYP11B1* are situated ~40 kb apart, on chromosome 8, band 8q24 in human.⁵

Several frequent polymorphisms in *CYP11B2* are suggested to have associations with essential hypertension and may influence aldosterone secretion.^{6–8} Recently, several investigators have demonstrated that the –344C/T and the intron 2 conversion variants in *CYP11B2* are in strong linkage disequilibrium (LD) with variants in *CYP11B1*. This can account for the altered 11 β -hydroxylase efficiency and mineralocorticoid synthesis. Such effects may be relevant to the development of low-renin essential hypertension.^{9,10}

Inglis *et al.*¹¹ found that the frequencies of the rs1799998T allele and the intron 2 conversion allele in the *CYP11B2* gene were markedly higher in Conn's syndrome group than in the normal control group, which demonstrated that the relative genotypes may predispose the tumor to aldosterone production. Tanahashi *et al.*¹² considered that a *CYP11B2* haplotype, including –344T and K173 in APA, was associated with a higher gene expression than the –344C/R173 haplotype.

¹Department of Urology, Clinical Division of Surgery, Chinese PLA General Hospital, Beijing, PR China; ²Department of Urology, First Affiliated Hospital of Gannan Medical University, Ganzhou, PR China; ³Department of Endocrinology, Clinical Division of Medicine, Chinese PLA General Hospital, Beijing, PR China; ⁴Department of Urology, Pingdingshan People's Hospital No.1, Pingdingshan, PR China and ⁵Department of Urology, Tongji Hospital/Medical College, Huazhong University of Science and Technology, Wuhan, PR China

⁶These authors contributed equally to the work.

Correspondence: Professor Xu Zhang, Department of Urology, Clinical Division of Surgery, Chinese PLA General Hospital, No.28, Fu Xing Road, Hai Dian District, Beijing 100853, PR China.

E-mail: xzhang@tjh.tjmu.edu.cn

Received 16 August 2009; revised 9 January 2010; accepted 22 January 2010; published online 26 March 2010

However, in a study of normal subjects and patients with PH and essential hypertension, rs1799998, rs4539 and intron 2 conversion polymorphisms in the *CYP11B2* gene may contribute to the dysregulation of aldosterone synthesis and may lead to susceptibility to IHA but not to APA.¹³

To date, the association between *CYP11B2* and *CYP11B1* polymorphisms and the development of PH are still unclear. There has been no report on the association between *CYP11B1* polymorphisms and PH. In this study, we aimed to identify polymorphisms in *CYP11B2* and *CYP11B1* genes that are associated with PH, with the hypothesis that genetic variants in them may contribute to PH.

METHODS

Subjects

A total of 179 Chinese Han in-patients with PH, including 134 with APA and 45 with IHA (with at least one dominant adrenal nodule and drug-resistant hypertension), were recruited from Tongji Hospital (Wuhan, China) between February 2002 and December 2007. The diagnosis of PH was a three-step process as previously described.¹⁴ Briefly, (1) all patients had refractory hypertension, elevated plasma aldosterone concentration (PAC) (>17.4 ng per 100 ml) and an increased ARR (>50 ng per 100 ml per $\text{ng ml}^{-1} \text{h}^{-1}$); (2) all had positive results of subsequent confirmatory testing (intravenous saline loading);¹⁵ (3) all patients underwent a computed tomography scan with fine cuts (2.5–3 mm), and 65% of the patients accepted adrenal venous sampling (AVS) for differentiating PH subtypes. For AVS, we followed the same criteria as those in Torino, Italy.¹⁶ Adrenal vein cannulation was considered successful if the adrenal vein/inferior vena cava cortisol gradient was at least 2. Lateralization was considered when the aldosterone/cortisol ratio from one adrenal was at least four times the ratio from the other adrenal gland. In addition, glucocorticoid remediable aldosteronism was excluded in all patients using a long PCR technique as previously described.¹⁴ The medications thought not to affect aldosterone and plasma renin activity (PRA) levels, including spironolactone, were withdrawn for at least 4 weeks before the diagnostic tests. All patients underwent unilateral adrenalectomy, and the diagnoses were surgically and histologically strengthened.

A total of 118 ethnically and geographically matched controls were randomly selected from normal individuals of community-based residents. All controls were free of adrenal ailment. The clinical characteristics of the patients and controls are given in Table 1.

Table 1 Clinical characteristics of the study population^a

	Controls (n=118)	Cases (n=179)		P-value
		APA (n=134)	IHA (n=45)	
Age (years)	41.5 ± 1.43	43.2 ± 1.07	43.6 ± 1.15	0.327
Gender (M/F)	61/57	61/73	22/23	0.691
BMI (kg m^{-2})	22.4 ± 0.31	22.8 ± 0.39	23.7 ± 0.44	0.085
SBP (mm Hg)	110.0 ± 2.32	172.4 ± 2.71	168.7 ± 3.43	<0.001
DBP (mm Hg)	76.6 ± 1.35	106.6 ± 1.66	107.4 ± 1.79	<0.001
K (mmol l^{-1}) ^b	4.5 ± 0.05	3.2 ± 0.07	3.1 ± 0.05	<0.001
PRA ($\text{ng ml}^{-1} \text{h}^{-1}$)	0.56 ± 0.01	0.04 ± 0.01	0.05 ± 0.01	<0.001
(supine) ^b				
PAC (ng per 100 ml)	10.15 ± 3.67	22.40 ± 1.09	23.30 ± 1.28	<0.001
(supine) ^b				
ARR (ng per 100 ml per $\text{ng ml}^{-1} \text{h}^{-1}$) ^b	23.98 ± 1.56	532.01 ± 25.65	564.91 ± 57.60	<0.001

Abbreviations: APA, aldosterone-producing adenoma; BMI, body mass index; DBP, diastolic blood pressure; F, female; IHA, idiopathic hyperaldosteronism; K, potassium; M, male; PAC, plasma aldosterone concentration; PRA, plasma renin activity; SBP, systolic blood pressure.

^aData were expressed as mean ± s.e. or *n* for variables.

^bThe normal range of serum potassium (K^+) was 3.5–5.5 mmol l^{-1} , 5.9–17.4 ng per 100 ml for PAC (supine), 0.05–0.79 $\text{ng ml}^{-1} \text{h}^{-1}$ for PRA (supine) and ≤ 50 ng per 100 ml per $\text{ng ml}^{-1} \text{h}^{-1}$ for ARR.

The study was approved by the Committee of Ethics at Tongji Medical College, Huazhong University of Science and Technology, and all subjects gave informed consents.

Genotyping

Among the participants, 119 cases (88 with APA and 31 with IHA) provided frozen tissues, 60 cases (46 with APA and 14 with IHA) provided paraffin-embedded tissues and 118 controls provided peripheral blood samples for genotyping. DNA was extracted from the tissues and peripheral blood using DNeasy Blood and Tissue Kit (Cat. No. 69504; Qiagen, Hilden, Germany), as instructed. It was maintained at -20°C .

For the *CYP11B2* and *CYP11B1* genes, four single-nucleotide polymorphisms (SNPs) of minor allele frequency ≥ 0.05 were selected in public databases (<http://ncbi.nlm.nih.gov/SNP/> and <http://www.hapmap.org/>) for study. This is based on the evidence suggesting functional relevance or reports of association with hypertension.^{11–13,17} Two SNPs span the *CYP11B2* gene: rs1799998 in the promoter region (also known in previous publications as C-344T or SF-1) and rs4539 in the third exon (also known as A2718G or K173R). Two SNPs span the *CYP11B1* gene: rs 6410 (G225A) in the first exon and rs6387 (A2803G) in the third intron. We also typed the biallelic intron 2 conversion polymorphism in the *CYP11B2* gene. This variant exists in two alternate forms, either the wild type (W) or the conversion (C), in which part of intron 2 is replaced by the corresponding intron of the adjacent *CYP11B1* gene¹⁸ (Figure 1).

Analysis of the intron 2 conversion polymorphism was performed using two separate PCRs. One amplifies the wide-type gene, and the other amplifies the conversion gene. The forward and reverse primers were respectively as follows: 5'-TGGAGAAAAGCCCTACCTGT-3' and 5'-AGGAACCTCTGCACGGCC-3' for the wide-type gene and 5'-CAGAAATCCCTCCCCCTA-3' and 5'-AGG AACCTCTGCACGGCC-3' for the conversion gene. Each 25 μl assay mix contained 12.5 μl 2 $\times$ Taq PCR MasterMix (Tiangen, Beijing, China), 2 μl mixture of forward and reverse primers and 1 μg DNA. The reaction was subjected to 31 cycles at 95°C for 30 s, at 64°C for 25 s and at 72°C for 30 s, followed by a final extension at 72°C for 10 min. The size of the amplicon in each reaction was ~418 bp. The SNPs in the *CYP11B2* and *CYP11B1* genes were analyzed using TaqMan probes. Primers and probes were designed and synthesized by Applied Biosystems (Foster City, CA, USA). All samples were genotyped using the Stratagene Mx3000P instrument (Applied Biosystems) as instructed. Each assay was carried out using 20 ng DNA in a 20 μl reaction containing a TaqMan universal PCR master mix (Applied Biosystems), forward and reverse primers, and FAM- and VIC-labeled probes designed and synthesized by Applied Biosystems. Allelic discrimination was measured automatically by Mxpro 6.0, and the genotype was determined. A random sample of 10% of the cases and controls was typed twice by different methods (independent PCRs or DNA sequencing) to confirm that the genotyping and reproducibility were 100%.

Statistical analysis

Demographic data were expressed as mean ± s.e. or *n* for variables and were performed with SPSS 13.0 (SPSS Inc, Chicago, IL, USA) for Windows. The differences between groups were assessed using one-way analysis of variance and the χ^2 -test. $P < 0.05$ was considered statistically significant.

The Hardy–Weinberg equilibrium and the difference in genotype frequencies between cases and controls of each polymorphism were determined by SNPAssoc 1.5–3 with Bonferroni correction for R 2.7.0 statistics program (freely obtained by <http://www.r-project.org/>). In addition, multivariable unconditional logistic regression was used to estimate odds ratio (OR) and 95% confidence interval (CI) after adjustments for gender, age and body mass index (BMI) by SNPAssoc. With the help of Haploview 4.0 for Windows, pairwise LD was estimated by D' , the difference in allelic frequencies between the case and control groups of each polymorphism was determined and allelic permutation test with 10 000 times was performed. The haplotype frequencies for various polymorphism combinations were estimated by Haplo.stats 1.3.8 for the R 2.7.0 statistics program. The differences in haplotype frequency profiles between the case and control groups were determined, and haplotype-based hypothesis tests of generalized linear models were conducted using the software haplo.stats with Bonferroni correction. The association between CYP11B2–CYP11B1 genotypes/haplotypes and ARR was

Figure 1 Schematic representation illustrating the five typed polymorphisms in the *CYP11B2* and *CYP11B1* genes (locations in the chromosome are shown). Exons are numbered and names for the polymorphisms are given below the arrow. IC represented *CYP11B2* intron 2 conversion.

Table 2 Allele frequencies in APA, IHA and the control group

Variation position	Allele frequencies						P-value	
	Controls (n=118)		APA (n=134)		IHA (n=45)		APA ^a	IHA ^a
rs6387	A	G	A	G	A	G	0.462	0.935
	0.73	0.27	0.76	0.24	0.74	0.26		
rs6410	A	G	A	G	A	G	1.09×10 ⁻⁵	0.015
	0.24	0.76	0.42	0.58	0.37	0.63		
rs4539	A	G	A	G	A	G	0.499	0.430
	0.71	0.29	0.74	0.26	0.76	0.24		
IC	W	C	W	C	W	C	0.546	0.168
	0.79	0.21	0.81	0.19	0.85	0.15		
rs1799998	C	T	C	T	C	T	0.335	0.160
	0.29	0.71	0.25	0.75	0.21	0.79		

Abbreviations: APA, aldosterone-producing adenoma; C, conversion; IHA, idiopathic hyperaldosteronism; IC, intron 2 conversion; W, wide type.
^aAPA and IHA vs. controls.

analyzed by SNPAssoc and haplo.stats. $P < 0.05$ or 95% CI of OR not including 1.0 implied statistical significance, and all statistical tests were two sided.

RESULTS

All selected diallelic polymorphisms in the case-control populations were successfully genotyped. The allele and genotype frequencies for cases and controls are shown in Tables 2 and 3. The distribution of genotypes was in accordance with the Hardy-Weinberg equilibrium in each group ($P > 0.05$). The degrees of pairwise LD among all selected variants were estimated by the standardized disequilibrium coefficient D' (Figure 2). It was shown that these five variants were in different strengths of LD, with the rs6410 and rs6387 polymorphisms in the tightest LD ($D' 0.66$), and rs4539 and intron 2 conversion having the weakest LD ($D' 0.06$) in the control subjects.

CYP11B2-CYP11B1 genotypes in relation to PH

Univariate allelic frequency analyses revealed that only rs6410 was significantly associated with APA and IHA at $P = 1.09 \times 10^{-5}$ and 0.015, respectively (Table 2). Further, this SNP was still significantly associated with APA after 10 000 times permutation ($P = 1 \times 10^{-4}$). There was a statistically significant difference in APA even after applying Bonferroni correction to this study ($P = 5 \times 10^{-4}$). There was a relative excess of AA homozygotes and AG heterozygotes of the rs6410 allele in the APA group as compared with the control group (Table 3). Multivariate unconditional logistic regression analyses demonstrated that

this significant association between rs6410 and APA was consistent even when there were adjustments for age, gender and BMI (Table 3). Simultaneously, a marginal difference was found in the rs6410 genotype frequencies between the control group and the IHA group, which became statistically significant after adjustments for age, gender and BMI (Table 3). In contrast, there was no significant difference in rs6387, rs4539, intron 2 conversion and rs1799998 between the control group and the APA or IHA group.

CYP11B2-CYP11B1 haplotypes in relation to APA

The interactions of multiple variants within a haplotype may have an impact on the biological phenotype.¹⁹ We therefore proceeded to analyze the effect of these five polymorphisms in *CYP11B2* and *CYP11B1* genes on the risk of APA in the context of haplotypes. Haplotype analysis for the five polymorphisms was implemented using haplo.stats software. Haplotypes with an estimated frequency of $> 3\%$ in patients with APA or the controls are shown in Table 4. A highly significant difference was observed in the haplotype frequencies between the control subjects and the patients with APA (Global-stat=31.95, degrees of freedom=9, $P = 0.0002$). Individually, a susceptible haplotype, AAAWT, was identified to be significantly associated with APA with empirical P -values of 5.0×10^{-5} (Table 4). In contrast, one protective haplotype, GGAWT, showed a significant difference between patients with APA and the controls (empirical $P = 1.0 \times 10^{-4}$). Importantly, all the above results of each haplotype remained significant even after Bonferroni correction ($P = 4.5 \times 10^{-4}$ and 9.0×10^{-4} , respectively). Comparisons between one haplotype vs. all others in both the cases and controls were made to determine the possible specific disease-predisposing haplotypes in patients with APA. The haplotype chosen for the reference in the model was AGAWT, which has the highest frequency. One susceptible haplotype, AAAWT, displayed a significantly increased risk for APA (OR 15.89, 95% CI 2.06–122.89), which was consistent even when there were adjustments for age, gender and BMI (OR 1.44, 95% CI 1.19–1.76). In contrast, a protective haplotype, GGAWT, exhibited significantly decreased effect on APA (OR 0.08, 95% CI 0.01–0.69), and there was a significant difference even after adjustments for age, gender and BMI (OR 0.73, 95% CI 0.55–0.97). In addition, two susceptible haplotypes, GAACT and AGGWT, were found with a marginal significance in the difference between the control group and the APA group after adjustments for age, gender and BMI (OR 1.28, 95% CI 0.97–1.71 and OR 1.23, 95% CI 0.99–1.54, respectively).

CYP11B2-CYP11B1 haplotypes in relation to IHA

Haplotypes with an estimated frequency of $> 3\%$ in patients with IHA or the controls are shown in Table 5. A significant difference was found in the haplotype frequencies between the control subjects and

Table 3 The genotype and variation frequencies of DNA polymorphisms in *CYP11B2* and *CYP11B1* genes

Variation position	Genotype	Genotype frequencies			P-value		Crude ORs (95% CI)		Adjusted ORs (95% CI) ^a	
		Controls (n=118)	APA (n=134)	IHA (n=45)	APA ^b	IHA ^b	APA ^b	IHA ^b	APA ^b	IHA ^b
rs6387	AA	59 (0.50)	73 (0.55)	22 (0.49)	0.776	0.661	1 (ref.)	1 (ref.)	1 (ref.)	1 (ref.)
	AG	53 (0.45)	55 (0.41)	22 (0.49)			0.84 (0.50–1.40)	1.11 (0.55–2.24)	1.04 (0.60–1.78)	2.01 (0.92–4.38)
	GG	6 (0.05)	6 (0.04)	1 (0.02)			0.81 (0.25–2.64)	0.45 (0.05–3.93)	0.79 (0.21–2.91)	0.00
	GG+AG	59 (0.50)	61 (0.45)	23 (0.51)			0.84 (0.51–1.37)	1.05 (0.53–2.08)	1.01 (0.60–1.71)	1.82 (0.84–3.97)
rs6410	GG	72 (0.61)	51 (0.38)	21 (0.47)	2.19×10 ⁻⁴	0.070	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)
	AG	37 (0.31)	54 (0.40)	15 (0.33)			2.06 (1.19–3.57)	1.39 (0.64–3.01)	2.58 (1.41–4.69)	2.41 (1.02–5.72)
	AA	9 (0.08)	29 (0.22)	9 (0.20)			4.55 (1.98–10.43)	3.43 (1.21–9.74)	5.02 (2.11–11.95)	4.06 (1.31–12.66)
	AA+AG	46 (0.39)	83 (0.62)	24 (0.53)			2.55 (1.53–4.24)	1.79 (0.89–3.58)	3.10 (1.78–5.40)	2.80 (1.27–6.18)
rs4539	AA	63 (0.53)	72 (0.54)	26 (0.58)	0.459	0.593	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)
	AG	41 (0.35)	52 (0.39)	16 (0.36)			1.11 (0.65–1.89)	0.95 (0.45–1.98)	1.02 (0.58–1.80)	1.09 (0.49–2.43)
	GG	14 (0.12)	10 (0.07)	3 (0.06)			0.63 (0.26–1.51)	0.52 (0.14–1.96)	0.50 (0.20–1.25)	0.57 (0.13–2.27)
	GG+AG	55 (0.47)	62 (0.46)	19 (0.42)			0.99 (0.60–1.62)	0.84 (0.42–1.67)	0.88 (0.52–1.49)	0.95 (0.44–2.05)
IC	WW	72 (0.61)	84 (0.63)	31 (0.69)	0.256	0.344	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)
	WC	40 (0.34)	48 (0.36)	14 (0.31)			1.03 (0.61–1.74)	0.81 (0.39–1.70)	1.33 (0.75–2.35)	1.05 (0.46–2.39)
	CC	6 (0.05)	2 (0.01)	0 (0.00)			0.29 (0.06–1.46)	0.00	0.11 (0.01–0.94)	0.00
	CC+WC	46 (0.39)	50 (0.37)	14 (0.31)			0.93 (0.56–1.55)	0.71 (0.34–1.47)	1.09 (0.63–1.88)	0.83 (0.37–1.85)
rs1799998	TT	58 (0.49)	77 (0.58)	29 (0.64)	0.226	0.184	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)	1.00 (ref.)
	CT	52 (0.44)	45 (0.33)	13 (0.29)			0.65 (0.39–1.10)	0.50 (0.24–1.06)	0.62 (0.35–1.09)	0.57 (0.25–1.32)
	CC	8 (0.07)	12 (0.09)	3 (0.07)			1.13 (0.43–2.94)	0.75 (0.18–3.04)	1.01 (0.38–2.71)	0.87 (0.20–3.77)
	CC+CT	60 (0.51)	57 (0.42)	16 (0.36)			0.72 (0.44–1.18)	0.53 (0.26–1.08)	0.68 (0.40–1.16)	0.61 (0.28–1.36)

Abbreviations: APA, aldosterone-producing adenoma; C, conversion; CI, confidence interval; IHA, idiopathic hyperaldosteronism; IC, intron 2 conversion; OR, odds ratio; ref., reference; W, wide type.

^aAdjusted OR (95% CI) were computed with multivariate unconditional logistic regression analysis after adjustment in age, gender and body mass index.

^bAPA and IHA vs. controls.

Figure 2 LD structure of the locus in *CYP11B2* and *CYP11B1* genes. Estimated values of D' , generated by Haploview 4.0 from the genotypes of 118 controls, are in the grids. IC represented *CYP11B2* intron 2 conversion.

the patients with IHA (Global-stat=19.06, degrees of freedom=9, $P=0.025$). Interestingly, a haplotype, AAAWT, was also identified to be significantly associated with IHA ($P=2\times 10^{-3}$). There was a significant

difference even after Bonferroni correction ($P=0.018$). The most frequent haplotype, AGAWT, was chosen as the reference when comparisons between one haplotype vs. all others in patients with IHA and the controls were made. The subjects carrying a susceptible haplotype, AAAWT, showed a significant increased risk for IHA (OR 4.99, 95% CI 1.29–19.35), which was consistent even when there were adjustments for age, gender and BMI (OR 1.55, 95% CI 1.23–1.96). Moreover, the risk conferred by the haplotype, AAAWT, on IHA was higher as compared with APA (OR 1.55 vs. OR 1.44). A susceptible haplotype, AGGWT, was found with a marginal significance in the difference between the control group and the IHA group (OR 2.92, 95% CI 0.82–10.45), and had a significantly increased risk after adjustments for age, gender and BMI (OR 1.49, 95% CI 1.17–1.89). Another susceptible haplotype, AGAWC, was found to have a significantly increased risk only after adjustments for age, gender and BMI (OR 1.40, 95% CI 1.04–1.88).

CYP11B2-CYP11B1 genotypes/haplotypes in relation to ARR

None of the CYP11B2-CYP11B1 genotypes was found to be associated with ARR. However, two haplotypes, AAAWT and GAAWT, were associated with ARR when there were adjustments for age, gender and BMI in the control group (coefficient=65.09, $P<0.001$ and coefficient=18.86, $P<0.001$, respectively). In the APA group, three haplotypes, AAAWT, AGGWC and GAACT, were also found associated with ARR when there were adjustments for age, gender and BMI (coefficient=40.17, $P<0.001$; coefficient=56.81, $P<0.001$ and coefficient=-24.36, $P<0.001$, respectively). Likewise, two haplotypes,

Table 4 Assessment of association between CYP11B2-CYP11B1 haplotypes and APA

Haplotypes ^a	Frequencies of haplotype		Empirical P-value ^b	Crude ORs (95% CI) ^c	Crude P-value ^c	Adjusted ORs (95% CI) ^d	Adjusted P-value ^d
	Control (n=118)	APA (n=134)					
AGAWT	36 (0.31)	35 (0.26)	0.402	Reference		Reference	
AGGWC	18 (0.15)	16 (0.12)	0.184	0.80 (0.41–1.57)	0.436	0.93 (0.80–1.09)	0.375
GAAWT	14 (0.12)	15 (0.11)	0.281	0.94 (0.45–1.96)	0.971	1.07 (0.91–1.26)	0.414
AGACT	9 (0.08)	6 (0.04)	0.067	0.41 (0.13–1.30)	0.097	0.86 (0.68–1.09)	0.225
GGAWT	7 (0.06)	1 (0.01)	1.0×10 ⁻⁴	0.08 (0.01–0.69)	0.003	0.73 (0.55–0.97)	0.029
GAACT	3 (0.03)	9 (0.07)	0.426	2.29 (0.66–7.94)	0.246	1.28 (0.97–1.71)	0.088
AGGWT	3 (0.03)	9 (0.07)	0.125	2.56 (0.78–8.34)	0.089	1.23 (0.99–1.54)	0.067
AAAWT	1 (0.01)	15 (0.11)	5.0×10 ⁻⁵	15.89 (2.06–122.89)	0.0004	1.44 (1.19–1.76)	0.0003
AGAWC	5 (0.04)	6 (0.04)	0.776	1.11 (0.43–2.87)	0.860	1.05 (0.84–1.31)	0.686
Global-stat=31.95, d.f.=9, P=0.0002							

Abbreviations: APA, aldosterone-producing adenoma; CI, confidence interval; d.f., degrees of freedom; OR, odds ratio.

^aThe sequence of the variants in the haplotypes rs6387, rs6410, rs4539, intron 2 conversion and rs1799998.^bEmpirical P-value based on 10 000 permutations.^cComparisons were made between one haplotype (reference) vs. all others in the cases and controls.^dAdjusted for age, gender and body mass index.**Table 5 Assessment of association between CYP11B2-CYP11B1 haplotypes and IHA**

Haplotypes ^a	Frequencies of haplotype		Empirical P-value ^b	Crude ORs (95% CI) ^c	Crude P-value ^c	Adjusted ORs (95% CI) ^d	Adjusted P-value ^d
	Control (n=118)	IHA (n=45)					
AGAWT	36 (0.31)	16 (0.35)	0.944	Reference		Reference	
AGGWC	18 (0.15)	3 (0.07)	0.115	0.58 (0.23–1.46)	0.270	0.96 (0.82–1.12)	0.580
GAAWT	14 (0.12)	5 (0.11)	0.381	1.33 (0.54–3.30)	0.477	1.12 (0.93–1.35)	0.218
AGACT	9 (0.08)	1 (0.02)	0.161	0.33 (0.06–1.76)	0.241	0.87 (0.69–1.08)	0.213
GGAWT	7 (0.06)	1 (0.02)	0.233	0.34 (0.04–3.08)	0.393	1.07 (0.81–1.41)	0.615
AGGWT	3 (0.03)	5 (0.11)	0.053	2.92 (0.82–10.45)	0.054	1.49 (1.17–1.89)	0.001
AAAWT	1 (0.01)	5 (0.11)	0.002	4.99 (1.29–19.35)	0.004	1.55 (1.23–1.96)	0.0003
AGAWC	5 (0.04)	1 (0.02)	0.908	1.39 (0.26–7.43)	0.664	1.40 (1.04–1.88)	0.027
GAACT	3 (0.03)	2 (0.03)	0.831	0.84 (0.13–5.29)	0.892	0.87 (0.69–1.08)	0.414
Global-stat=19.06, d.f.=9, P=0.025							

Abbreviations: CI, confidence interval; d.f., degrees of freedom; IHA, idiopathic hyperaldosteronism; OR, odds ratio.

^aThe sequence of the variants in the haplotypes rs6387, rs6410, rs4539, intron 2 conversion and rs1799998.^bEmpirical P-value based on 10 000 permutations.^cComparisons were made between one haplotype (reference) vs. all others in the cases and controls.^dAdjusted for age, gender and body mass index.

AAAWT and GAAWT, were associated with ARR when there were adjustments for age, gender and BMI in IHA group (coefficient=272.16, $P<0.001$ and coefficient=237.1, $P<0.001$, respectively).

DISCUSSION

No satisfactory explanation is yet available for the initiation of growth of PH. In this study, five polymorphisms spanning the CYP11B1 and CYP11B2 locus were genotyped. Among them, the rs6410 polymorphism showed a significant association with APA and IHA in the Chinese Han population, which was similar to Keavney's findings on the association between the rs6410 polymorphism and 11-deoxycortisol urinary metabolite excretion in the Caucasian population.²⁰ To our knowledge, this is the first report wherein a significant association of the polymorphisms of the CYP11B1 gene with PH was found. The present results further provided some useful information for the investigation of the association between the polymorphisms of the CYP11B2 and CYP11B1 genes and PH in the Chinese Han population.

To our knowledge, the CYP11B1 gene is responsible for the final step in cortisol synthesis, not for aldosterone synthesis. Furthermore, it is unlikely that the rs6410 polymorphism in CYP11B1 gene directly influences the phenotype because it involves a change from G to A at codon 75, which has no consequences for the amino-acid sequence of the final protein due to the same leucine encoded in both cases. There may be two possible mechanisms by which this polymorphism can influence the trait. One exists in the LD between the rs6410 variant in CYP11B1 and the undiscovered causative variant(s) in CYP11B2. Another may be that the rs6410 variant is in LD with causal variant(s) in CYP11B1 itself. This leads to the reduced 11 β -hydroxylase efficiency, then mildly activates the pituitary–adrenal axis and finally influences the aldosterone production. As a result, hypertension or even PH may occur. Our hypothesis is consistent with some previous findings. Connell and coworkers^{9,10} found that reduced 11 β -hydroxylase efficiency and increased aldosterone production are associated with genetic variations (for example, rs1799998, intron 2 conversion, rs6410, –1859G/T and –1889A/G) of CYP11B2 and CYP11B1 genes

in hypertension patients and normal objects. They also observed that variants in *CYP11B2* and *CYP11B1* are in strong LD and associated with the chronic upregulation of hypothalamic–pituitary–adrenal axis activity.^{20,21} Taken together, these findings suggest that reduced 11 β -hydroxylase efficiency in genetically susceptible individuals mildly activates the pituitary–adrenal axis by a slight and lifelong increase in adrenocorticotrophic hormone and influences the aldosterone production, which may lead to the development of hypertension within the general population.²¹ Moreover, these results were confirmed by others.^{17,22}

In this study, no correlation of APA and IHA with rs6387, rs4539, intron 2 conversion and rs1799998 polymorphisms in the *CYP11B2* and *CYP11B1* genes was found, which does not agree with previous reports.^{11,13} The allele and genotype frequencies as well as the *CYP11B2*–*CYP11B1* haplotypes identified also differed from the ones previously found by Ganapathipillai *et al*.¹⁷ In addition, in contrast to those previous studies, we only observed mild or weak, but not strong LD among rs6410, rs4539, intron 2 conversion and rs1799998 alleles. In some previous hypertension studies, the rs1799998 polymorphism is not associated with increased aldosterone and increased blood pressure.^{23–25} Maybe, the different groups with different ethnic backgrounds could account for the differences, but further research is needed to verify this.

Haplotype analysis can overcome the loss of information attributable to biallelic variants, which could make an existing association clearer and more credible.²⁶ To further supplement our understanding of the contributions of these genetic variants to APA and IHA, five-locus haplotypes were constructed, and their distribution was studied in the patients and control populations. AAAWT was identified as the susceptible haplotype, whereas GGAWT was identified as the protective haplotype in patients with APA. In the IHA group, the risk haplotype, AAAWT, was consistent with that in the APA group, but the risk was higher than that of APA. This demonstrated that there are both differences and similarities in the association between polymorphisms in *CYP11B2* and *CYP11B1* genes and APA and IHA. In the function study, ARR was found to increase in the carriers of the susceptible haplotype, AAAWT, not only in the controls but also in the cases, suggesting a functional significance. Ganapathipillai *et al*.¹⁷ also considered that the haplotypes of *CYP11B2* and *CYP11B1* genes tended to associate with the ARR, as well as predicted the urinary excretion ratio of THS/(THE+THF+5 α THF), a marker for 11 β -hydroxylase activity. Furthermore, the coefficient of association of haplotype, AAAWT, with ARR in the IHA group was more than that in the APA group (272.16 vs. 40.17). This demonstrated that the effect of the polymorphisms in *CYP11B2* and *CYP11B1* genes on IHA might be more than that on APA. In other words, the haplotypes analysis suggested that the polymorphisms in *CYP11B2* and *CYP11B1* genes seemed more closely associated with IHA than APA.

However, there are also some limitations in our study. First, the patients with APA and IHA were recruited from in-patients subjected to unilateral adrenalectomy, which was not completely free from some bias in the selection of study subjects. Further study in a typical population is useful to show the precise association between the polymorphisms in *CYP11B2* and *CYP11B1* genes and PH. Second, we could not obtain enough information on some environmental exposure factors, such as smoking and drinking status and occupational history in both case and control groups, which may make our results biased. Third, larger samples from a normal population and patients with APA and IHA will be needed in further studies.

In conclusion, our study results reveal a highly significant association between genetic variations in *CYP11B2* and *CYP11B1* genes and a

genetic predisposition to PH. Although we cannot conclude with confidence that the rs6410 variant is a causal polymorphism for PH, significant global and haplotype specific *P*-values support the potential role of *CYP11B2* and *CYP11B1* genes in the genetic etiology of PH. Moreover, the polymorphisms in *CYP11B2* and *CYP11B1* genes seemed more closely associated with IHA than APA. Additional research, especially involving more polymorphisms, typical objects, more environmental exposure factors and other study designs, is warranted to further explore the etiological relevance of polymorphisms in *CYP11B2* and *CYP11B1* genes for APA and IHA.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This work was supported by the National Natural Science Funds for Distinguished Young Scholar, People's Republic of China (No. 30725040).

- 1 Funder JW, Carey RM, Fardella C, Gomez-Sanchez CE, Mantero F, Stowasser M, Young Jr WF, Montori VM. Case detection, diagnosis, and treatment of patients with primary aldosteronism: An endocrine society clinical practice guideline. *J Clin Endocrinol Metab* 2008; **93**: 3266–3281.
- 2 Kawamoto T, Mitsuuchi Y, Toda K, Yokoyama Y, Miyahara K, Miura S, Ohnishi T, Ichikawa Y, Nakao K, Imura H, Ulick S, Shizuta Y. Role of steroid 11 beta-hydroxylase and steroid 18-hydroxylase in the biosynthesis of glucocorticoids and mineralocorticoids in humans. *Proc Natl Acad Sci USA* 1992; **89**: 1458–1462.
- 3 Curnow KM, Tusie-Luna MT, Pascoe L, Natarajan R, Gu JL, Nadler JL, White PC. The product of the *cyp11b2* gene is required for aldosterone biosynthesis in the human adrenal cortex. *Mol Endocrinol* 1991; **5**: 1513–1522.
- 4 Ferrari P, Bianchetti M, Frey FJ. Juvenile hypertension, the role of genetically altered steroid metabolism. *Horm Res* 2001; **55**: 213–223.
- 5 Mornet E, Dupont J, Vitek A, White PC. Characterization of two genes encoding human steroid 11 beta-hydroxylase (p-450(11) beta). *J Biol Chem* 1989; **264**: 20961–20967.
- 6 Davies E, Holloway CD, Ingram MC, Inglis GC, Friel EC, Morrison C, Anderson NH, Fraser R, Connell JM. Aldosterone excretion rate and blood pressure in essential hypertension are related to polymorphic differences in the aldosterone synthase gene *cyp11b2*. *Hypertension* 1999; **33**: 703–707.
- 7 Lim PO, Macdonald TM, Holloway C, Friel E, Anderson NH, Dow E, Jung RT, Davies E, Fraser R, Connell JM. Variation at the aldosterone synthase (*cyp11b2*) locus contributes to hypertension in subjects with a raised aldosterone-to-renin ratio. *J Clin Endocrinol Metab* 2002; **87**: 4398–4402.
- 8 Nicod J, Bruhin D, Auer L, Vogt B, Frey FJ, Ferrari P. A biallelic gene polymorphism of *cyp11b2* predicts increased aldosterone to renin ratio in selected hypertensive patients. *J Clin Endocrinol Metab* 2003; **88**: 2495–2500.
- 9 Barr M, MacKenzie SM, Friel EC, Holloway CD, Wilkinson DM, Brain NJ, Ingram MC, Fraser R, Brown M, Samani NJ, Caulfield M, Munroe PB, Farrall M, Webster J, Clayton D, Dominiczak AF, Connell JM, Davies E. Polymorphic variation in the 11beta-hydroxylase gene associates with reduced 11-hydroxylase efficiency. *Hypertension* 2007; **49**: 113–119.
- 10 Imrie H, Freil M, Mayosi BM, Davies E, Fraser R, Ingram M, Cordell HJ, Farrall M, Avery PJ, Watkins H, Keavney B, Connell JM. Association between aldosterone production and variation in the 11beta-hydroxylase (*cyp11b1*) gene. *J Clin Endocrinol Metab* 2006; **91**: 5051–5056.
- 11 Inglis GC, Plouin PF, Friel EC, Davies E, Fraser R, Connell JM. Polymorphic differences from normal in the aldosterone synthase gene (*cyp11b2*) in patients with primary hyperaldosteronism and adrenal tumour (Conn's syndrome). *Clin Endocrinol (Oxf)* 2001; **54**: 725–730.
- 12 Tanahashi H, Mune T, Takahashi Y, Isaji M, Suwa T, Morita H, Yamakita N, Yasuda K, Deguchi T, White PC, Takeda J. Association of *lys173arg* polymorphism with *cyp11b2* expression in normal adrenal glands and aldosterone-producing adenomas. *J Clin Endocrinol Metab* 2005; **90**: 6226–6231.
- 13 Mulatero P, Schiavone D, Fallo F, Rabbia F, Pilon C, Chianducci L, Pascoe L, Veglio F. *Cyp11b2* gene polymorphisms in idiopathic hyperaldosteronism. *Hypertension* 2000; **35**: 694–698.
- 14 Mulatero P, Veglio F, Pilon C, Rabbia F, Zocchi C, Limone P, Boscaro M, Sonino N, Fallo F. Diagnosis of glucocorticoid-remediable aldosteronism in primary aldosteronism: aldosterone response to dexamethasone and long polymerase chain reaction for chimeric gene. *J Clin Endocrinol Metab* 1998; **83**: 2573–2575.
- 15 Holland OB, Brown H, Kuhnert L, Fairchild C, Risk M, Gomez-Sanchez CE. Further evaluation of saline infusion for the diagnosis of primary aldosteronism. *Hypertension* 1984; **6**: 717–723.
- 16 Mulatero P, Stowasser M, Loh KC, Fardella CE, Gordon RD, Mosso L, Gomez-Sanchez CE, Veglio F, Young Jr WF. Increased diagnosis of primary aldosteronism, including surgically correctable forms, in centers from five continents. *J Clin Endocrinol Metab* 2004; **89**: 1045–1050.

- 17 Ganapathipillai S, Laval G, Hoffmann IS, Castejon AM, Nicod J, Dick B, Frey FJ, Frey BM, Cubeddu LX, Ferrari P. Cyp11b2-cyp11b1 haplotypes associated with decreased 11 beta-hydroxylase activity. *J Clin Endocrinol Metab* 2005; **90**: 1220–1225.
- 18 White P, Slutsker L. Haplotype analysis of cyp11b2. *Endocr Res* 1995; **21**: 437–442.
- 19 Drysdale CM, McGraw DW, Stack CB, Stephens JC, Judson RS, Nandabalan K, Arnold K, Ruano G, Liggett SB. Complex promoter and coding region beta 2-adrenergic receptor haplotypes alter receptor expression and predict *in vivo* responsiveness. *Proc Natl Acad Sci USA* 2000; **97**: 10483–10488.
- 20 Keavney B, Mayosi B, Gaukrodger N, Imrie H, Baker M, Fraser R, Ingram M, Watkins H, Farrall M, Davies E, Connell J. Genetic variation at the locus encompassing 11-beta hydroxylase and aldosterone synthase accounts for heritability in cortisol precursor (11-deoxycortisol) urinary metabolite excretion. *J Clin Endocrinol Metab* 2005; **90**: 1072–1077.
- 21 Freel EM, Ingram M, Wallace AM, White A, Fraser R, Davies E, Connell JM. Effect of variation in cyp11b1 and cyp11b2 on corticosteroid phenotype and hypothalamic-pituitary-adrenal axis activity in hypertensive and normotensive subjects. *Clin Endocrinol (Oxf)* 2008; **68**: 700–706.
- 22 Kennon B, Ingram MC, Friel EC, Anderson NH, MacKenzie SM, Davies E, Shakerdi L, Wallace AM, Fraser R, Connell JM. Aldosterone synthase gene variation and adrenocortical response to sodium status, angiotensin II and ACTH in normal male subjects. *Clin Endocrinol (Oxf)* 2004; **61**: 174–181.
- 23 Tsujita Y, Iwai N, Katsuya T, Higaki J, Ogihara T, Tamaki S, Kinoshita M, Mannami T, Ogata J, Baba S. Lack of association between genetic polymorphism of cyp11b2 and hypertension in japanese: the Suita Study. *Hypertens Res* 2001; **24**: 105–109.
- 24 Pojoga L, Gautier S, Blanc H, Guyene TT, Poirier O, Cambien F, Benetos A. Genetic determination of plasma aldosterone levels in essential hypertension. *Am J Hypertens* 1998; **11**: 856–860.
- 25 Yamagishi K, Tanigawa T, Cui R, Tabata M, Ikeda A, Yao M, Shimamoto T, Iso H. Aldosterone synthase gene T-344C polymorphism, sodium and blood pressure in a free-living population: a community-based study. *Hypertens Res* 2007; **30**: 497–502.
- 26 Kumar NN, Benjafield AV, Lin RC, Wang WY, Stowasser M, Morris BJ. Haplotype analysis of aldosterone synthase gene (cyp11b2) polymorphisms shows association with essential hypertension. *J Hypertens* 2003; **21**: 1331–1337.