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Genotype of *CYP3AP1* associated with CYP3A activity in Chinese Han population[1]

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KEY WORDS cytochrome P-450 CYP3A; cytochrome P-450 CYP3AP1; genotype

ABSTRACT

AIM: To investigate the distribution of genotype of *CYP3AP1* in Chinese Han population and the correlation with CYP3A activity. **METHODS**: Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was employed in *CYP3AP1* genotype analysis; using midazolam as probe drug, CYP3A activity of 191 Chinese healthy subjects was measured by plasma 1′-hydroxymidazolam/midazolam (1′-OH-MDZ/MDZ) ratio at 1 h after oral administration of 7.5 mg midazolam. **RESULTS**: There was significant difference of CYP3A activity in different genotypes of *CYP3AP1 in vivo* ($P < 0.05$). The activity of CYP3A in homozygous A_{-44} (*CYP3AP1* * *3/CYP3AP1* * *3*) is lower than heterozygous $A_{-44}G$ (*CYP3AP1* * *1/CYP3AP1* * *3*), and the CYP3A activity in homozygous G_{-44} (*CYP3AP1* * *1/CYP3AP1* * *1*) is the highest. **CONCLUSION**: There was association between the genotype of *CYP3AP1* and increased activity of CYP3A *in vivo*.

INTRODUCTION

The enzymes P450 encoded by the CYP3A gene subfamily are the most abundant forms of the total cytochrome P450 presenting in adult human liver and small bowel. CYP3A enzymes play an important role in the metabolism of most xenobiotics, including a wide variety of drugs in many different therapeutic classes[1,2].

To date, four *CYP3A* genes have been identified in humans: *CYP3A4*, *CYP3A5*, *CYP3A7*, and *CYP3A43*. Two CYP3A pseudogenes, *CYP3AP1* and *CYP3AP2*, were found in intergenic regions of *3A7-3A5* and *3A4-3A7* respectively[3]. *CYP3A7* is the major fetal hepatic cytochrome[4]. Relatively high CYP3A4 levels, about 50 % hepatic levels and 70 % total CYP protein, are also present in small intestinal epithelium[5]. CYP3A5 expression was detected in only 10 % to 30 % of human livers in earlier studies[6-9]. But in the study of Hustert E *et al*, analysis of 183 human liver samples using Western blot found that the expression of CYP3A5 was detected in all samples and had a clear bimodal distribution[10].

Overlapping substrate specificities between CYP3A4 and CYP3A5 have previously made it difficult to separate metabolism by these isoforms[11]. The term CYP3A is usually understood to reflect the collective activity of all the isoforms[5]. The level of CYP3A5-mediated metabolism, when it was present, was less than that of CYP3A4, but in the 1′-hydroxylation of midazolam (MDZ), CYP3A5 exhibited greater catalytic activity[12]. The biotransformation of midazolam to its major metabolite, 1′-hydroxymidazolam (1′-OH-MDZ), has also been proposed as a probe for CYP3A activity *in vivo*[13].

Both studies of Paulussen *et al* and Kuehl *et al* demonstrated that the mutation A/G_{-44} of *CYP3AP1* was associated with increased expression and activity of CYP3A5 *in vitro*[11,14]. Chou *et al* found that 28 % *CYP3AP1* alleles were G_{-44} in 110 Chinese Han subjects[15].

In this study, using midazolam 1′-hydroxylation as a marker of CYP3A activity, we investigated the association between *CYP3AP1* genotype and CYP3A activity *in vivo* in Chinese Han population.

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MATERIALS AND METHODS

Clinical protocol This study was approved by the Ethics Committee of Central South University. One hundred and ninety-one healthy unrelated volunteers (age range: 17 – 22 years old; body weight range: 44 – 78 kg) from the Chinese (Han) population living in Changsha were recruited for the study after giving their written informed consents. All healthy individuals were in good health based on their medical history, physical examination, and laboratory evaluation. Subjects were abstained from medication, including alcohol, caffeine – and grapefruit juice, for at least 7 d before the study. All subjects were non-smokers and ate normal diet.

After an overnight fast, each subject received 7.5 mg oral midazolam. All subjects were continued to fast until the blood sample had been collected. Blood samples (5 mL) were drawn in edetic acid tubes at 1 h after drug administration. Harvested plasma was stored at – 20 ℃ until analysis for concentration of midazolam and 1′-hydroxymidazolam. The peripheral lymphocytes were isolated immediately and stored at – 20 ℃ until analysis.

Chemicals All materials for genotyping analysis were bought from Sangon Co (Shanghai, China). Midazolam and 1′-hydroxymidazolam were purchased from Ultrafine Company (Manchester, UK). Nortriptyline was purchased from Sigma Chemical Co (St Louis, USA). Acetonitrile and methanol of HPLC grade and doubly distilled water were required for HPLC with UV detector. All other chemicals were of AR grade available from commercial sources.

Analysis of midazolam and 1′-hydroxymidazolam in plasma Midazolam and 1′-hydroxymidazolam were determined by a method based on Carrillo *et al*[16]. After added 100 μL of nortriptyline 100 nmol/L as internal standard and 1 mL buffer glycine (0.75 mol/L, pH 9), 1 mL plasma was extracted with 4 mL diethylether. The organic phase was evaporated to dryness. The residue was dissolved in 50 μL of mobile phase, and 20 μL were injected onto the HPLC column. Midazolam and 1′-hydroxymidazolam were separated on a C8 column (4.6 mm × 150 mm, 5 μm particle size, Hewlett). The composition of the mobile phase was 32 % acetonitrile; 3 % methanol; 65 % buffer acetate 0.1 mol/L (v/v/v) (pH 4.34). The flow rate through the column at 35 ℃ was 1.1 mL/min, midazolam and 1′-hydroxymidazolam were monitored by ultraviolet absorbance at 234 nm.

Genotyping analysis was measured by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) Genomic DNA was extracted from peripheral lymphocytes with phenol-chloroform followed by ethanol precipitation[17], and DNA samples were dissolved in Tris 10 mmol/L and edetic acid 1 mmol/L (pH 8.0) and stored at 4 ℃ until used. The PCR was performed as described previously[10] with slight modification. A 1343 bp PCR-amplified form genomic DNA using primers 3A51 (5′-GGAAGCAACCTACATGTCCATC-3′) and 3A52 (5′-ATCGCCACTTGCCTTCTTC-3′). PCR conditions were: one cycle of 95 ℃ for 3 min, 30 cycles of 95 ℃ for 40 s, 57 ℃ for 30 s, 72 ℃ for 2.5 min, and one final cycle of 72 ℃ for 10 min. All PCR assays for detecting the A/G_{-44} and T/G_{-369} mutations were performed utilizing a 1 in 200 dilution of the original 3A51/3A52 PCR product as template, and using primers 3A5F2 (5′-GGGGTCTGTCTGGCTGAGC-3′) and 3A5R1 (5′-TTTATGTGCTGGAGAAGGACG-3′) based on the method of Paulussen *et al*[11]. The PCR conditions were as follows: one cycle of 95 ℃ for 1 min, 30 cycles of 95 ℃ for 30 s, 55 ℃ for 30 s, 72 ℃ for 1 min, and 1 final cycle of 72 ℃ for 10 min. For the T/G_{-369} mutation, 12 μL of PCR product was digested with 10 U of AluI for a minimum of 3 h, and restriction fragments were separated by electophoresis on a 8 % polyacrylamide gels. The fragments were visualized by silver staining. For the A/G_{-44} mutation, 12 μL of PCR product was digested for a minimum of 3 h at 65 ℃ using 10 U of TaiI, and the restriction fragments visualized by silver staining after electrophoresis on a 8 % polyacrylamide gels.

Data analysis The differences in allelic frequencies between male and female in Chinese Han population were analyzed by a chi-square test. The influence of A/G_{-44} polymorphism on the metabolism of midazolam among three different genotypes was estimated by one-way ANOVA. The comparisons of CYP3A activity in each pair genotypes were estimated by least significant difference (LSD) test.

These statistical analyses were carried out with SPSS for Windows version 10.0. $P < 0.05$ were considered significant.

RESULTS

A/G_{-44} and T/G_{-369} polymorphism The point

mutations of A/G_{-44} and T/G_{-369} existed in both Chinese populations. For detecting the A/G_{-44} mutation, the primer introduces a TaiI recognition site only when the wild-type "A" nucleotide is present a position −44. Digestion of the 369 bp product with TaiI yields fragments of 349 and 20 bp for the wild-type sequence, whilst the product remains undigested if the mutant "G" nucleotide is present (Fig 1, 20 bp was not visible). For the detection of T/G_{-369}, the primer introduces a recognition site for the restriction enzyme AluI, digesting the wild-type PCR product to yield fragments of 318, 33, and 18 bp. The fragments from heterozygous type gave three bands of 336, 318, and 33 bp; the homozygous for variant sequence gave only two bands of 336 and 33 bp[11] (Fig 1, 33 and 18 bp were not visible).

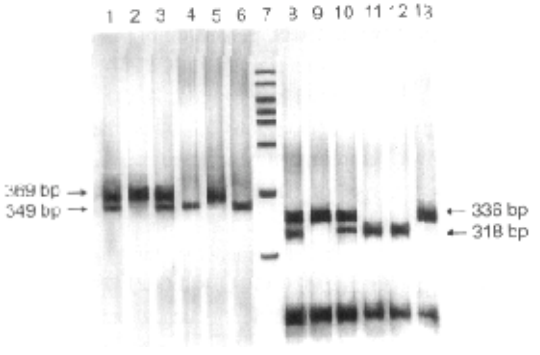

Fig 1. Restriction fragment length polymorphism (RFLP) analysis of *CYP3AP1* genotypes. Lane 4 and lane 6, genotype $A_{-44}A$; lane 1 and lane 3, genotype $A_{-44}G$; lane 2 and lane 5, genotype $G_{-44}G$; lane 7, DNA marker; lane 11 and lane 12, genotype $T_{-369}T$; lane 8 and lane 10, genotype $T_{-369}G$; lane 9 and lane 13, genotype $G_{-369}G$.

Genotypes and allelic variants One hundred and ninety-one healthy Chinese Han volunteers were screened for the presence of the A/G_{-44} and T/G_{-369} mutations. These two mutations were linked. The G_{-44} allele frequency was 0.2147 (82 out of 382) with the A_{-44} allele frequency being 0.7853 (300 out of 382). The frequency of homozygous for A_{-44} was 0.607 (116 out of 191), the heterozygous frequency was 0.356 (68 out of 191), and the frequency of the homozygous for G_{-44} was 0.037 (7 out of 191). The distribution of the genotypes was in Hardy-Weinberg equilibrium. There was no significant difference of frequencies of different genotypes between male and female in Chinese population ($P > 0.05$, Tab 1).

Tab 1. Frequencies of different genotypes for the A/G_{-44} genetic polymorphism of human *CYP3A5* in male and female Chinese Han population ($n = 191$).

Allele	Genotype	Male		Female		χ^2, P
		n	%	n	%	
A/G_{-44}	$A_{-44}A$	63	64	53	57	$\chi^2 = 1.048$
	$A_{-44}G$	32	33	36	39	$P = 0.592$
	$G_{-44}G$	3	3	4	4	

Metabolic ratios of midazolam in different genotypes of *CYP3A5* Using the metabolism of midazolam to its 1′-hydroxylation metabolite as a marker of activity, CYP3A activities were detected in same population as above. The difference of metabolic ratio among different genotypes was statistically significant ($P < 0.05$). The activity of CYP3A in homozygous A_{-44} was the lowest, and the CYP3A activity in homozygous G_{-44} was the highest (Tab 2). The median metabolic ratio was significantly higher for heterozygous ($n = 68$) versus homozygous A_{-44} ($n = 116$) ($P < 0.05$). But there was no significant difference of metabolic ratio between homozygous A_{-44} ($n = 116$) and homozygous G_{-44} ($n = 7$), heterozygous ($n = 68$) and homozygous G_{-44} ($n = 7$) ($P > 0.05$).

Tab 2. The activities of CYP3A in different genotypes of *CYP3AP1* in Chinese Han population. $\bar{x} \pm s$.

Allele	Genotype	n	Activity [lg (1′-OH-MDZ/MDZ)]	Comparisons between each pair genotypes			Comparisons among three genotypes
				$A_{-44}A$ vs $A_{-44}G$	$A_{-44}A$ vs $G_{-44}G$	$A_{-44}G$ vs $G_{-44}G$	$A_{-44}A$ vs $A_{-44}G$ vs $G_{-44}G$
A/G_{-44}	$A_{-44}A$	116	−0.42 ± 0.21				
	$A_{-44}G$	68	−0.33 ± 0.18	$P = 0.004$	$P = 0.065$	$P = 0.485$	$P = 0.006$
	$G_{-44}G$	7	−0.28 ± 0.11				

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DISCUSSION

The human *CYP3A* genes are thought to be localized in a cluster on chromosome 7q21-q22.1. The genes lie in a head-to-tail orientation in the order of *CYP3A4*, *CYP3A7*, and *CYP3A5*. Both the cDNA sequences and the intron 5 of *CYP3A4* and *CYP3A5* have been characterized with 90 % similarity.

There is marked inter-individual heterogeneity in the expression of *CYP3A* genes. Patients with unusually high or low CYP3A activity should be at increased risk for subtherapeutic or toxic responses[1]. However, the molecular basis for the inter-individual variation in expression of the CYP3A subfamily members has so far remained unclear.

Paulussen *et al* described that increased CYP3A5 activity was resulted from two linked polymorphism including A/G_{-45} and T/G_{-369} in 5′ flanking region of the gene which caused increased levels of gene transcription *in vitro*[11]. But a CYP3A pseudogene, termed CYP3AP1, was identified afterwards[3]. *CYP3AP1* consists of three of the canonical 13 exons of CYP3As, exon 1, exon 2, and exon 13. Sequencing of the CYP3A locus revealed that the A/G_{-45} and T/G_{-369} are actually in the "CYP3A5 like" CYP3AP1 promoter region. Therefore, A/G_{-45} is referred to *CYP3AP1 * 1* (G at nt-44) now.

Pseudogene is non-functional copy of gene and plays important role in the evolution of gene family. A major goal of "Human Genome Project" has been completed with publication of the draft sequence of the human genome. Non-expression sequence which comprises of 97 % human genome has been becoming one of the "hotspots" in the deepening and widening of "Human Genome Project". Recently, people pay more attention to the structure characteristics of pseudogenes, the coding potentials, and the role in evolution. Pseudogenes is one of the problems which we encounter repeatedly in process of gene cloning and expression.

There was complete concordance between the *CYP3A5 * 1* and *CYP3AP1 * 1* genotypes and between *CYP3A5 * 3* and *CYP3AP1 * 3* (A at nt-44) genotypes in Caucasians. Kuehl *et al* found that CYP3A5 mRNA content was greater in people with a *CYP3AP1 * 1* allele than in those people homozygous for *CYP3AP1 * 3*[14]. In addition, analysis of human liver CYP3A5 cDNA revealed that only those people with a CYP3A5 * 1 allele produced high levels of full-length CYP3A5 mRNA and expressed CYP3A5[11].

All the previous studies about the association between *CYP3AP1* genotype and CYP3A activity were *in vitro* studies[11,14]. Based on these studies, we studied the distribution of two linked polymorphism, A/G_{-44} and T/G_{-369} in Chinese Han population, and the association with CYP3A activity in population *in vivo* study.

We have confirmed that there was a significant correlation between plasma MDZ clearance and the 1′-OH-MDZ/MDZ plasma ratio, assessed at 1 h after 7.5 mg MDZ intake in the volunteers. The finding provides a simpler estimate for measuring liver and intestinal CYP3A activity, with a single blood measurement[18], in population study. Therefore, in this study, we use the plasma 1′-OH-MDZ/MDZ ratio at 1 h after oral administration of midazolam as CYP3A activity index.

We found that there was significant difference of CYP3A activity in different genotypes of *CYP3AP1 in vivo* ($P < 0.05$). The activity of CYP3A in homozygous A_{-44} is lower than heterozygous A/G_{-44}, and the CYP3A activity in homozygous G_{-44} is the highest. Therefore, we demonstrated that there was association between the genotype of *CYP3AP1* and increased activity of CYP3A *in vivo*.

In this study, we determined that the variant allele frequency of A/G_{-44} and T/G_{-369} was 0.2147 (82 out of 382) with the wild-type allele frequency being 0.7853 (300 out of 382) in Chinese. Paulussen *et al* found that the variant allele frequency was 0.092 and wild-type allele frequency was 0.908 in Caucasian volunteers[11]. Therefore, the incidence of homozygous for G_{-44} is higher in Chinese population than that in Caucasian. But the variant allele frequency in this study was lower than in the study of Chou *et al*[3]. Since CYP3A involved in the metabolism of many medications and environmental contaminants, there might be ethnic difference for toxic responses to some medications and risks of some environmental diseases.

The allele frequency is approximately equal between genders in the same population (Tab 1). This finding indicates that the sex-linked differences of CYP3A activity might not result from differences in genotypes of *CYP3AP1*[19]. For such a difference, the possible that sex-related hormonal status modifies gene expression can not be excluded.

In conclusion, in this study, we demonstrated that the mutant A/G_{-44}, referred to *CYP3AP1 * 1*, was related to increased CYP3A activity *in vivo*. Pseudogenes provide abundant pro-material for keeping

fluidity of eukaryote genome and some pseudogenes have been shown to relate to disease such as Gaucher disease[20]. The knowledge of pseudogene will allow identification of pseudogene-functional gene complex alleles that may aid in understanding the intricate phenotype-genotype relationship in disease.

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中国汉族人群中 *CYP3AP1* 基因型与 CYP3A 活性的相关性研究[1]

R96 A

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关键词 细胞色素 P-450 CYP3A；细胞色素 P-450 CYP3AP1；基因型

目的：研究中国汉族人群中 *CYP3AP1* 基因型的分布特征及其与 CYP3A 活性的相关性。**方法**：以口服 7.5 mg 咪哒唑仑后 1 小时血浆中 1′-羟化咪哒唑仑与咪哒唑仑的比值作为 CYP3A 活性的衡量指标，测定 191 名中国汉族健康受试者的 CYP3A 活性，并利用多聚酶链式反应-限制性片段长度多态性（PCR-RFLP）方法对已知 CYP3A 活性受试者的 DNA 进行 *CYP3AP1* 基因分型。**结果**：*CYP3AP1* 不同基因型个体的 CYP3A 活性存在显著差异（$P<0.05$）。A_{-44} 等位基因纯合子个体的 CYP3A 活性低于 $A_{-44}G$ 杂合子，而 G_{-44} 等位基因纯合子个体的 CYP3A 活性最高。**结论**：*CYP3AP1* 基因型与体内 CYP3A 活性的增加存在相关性。

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