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Short communication

Genetic polymorphisms of *CYP3A4: CYP3A4*18* allele is found in five healthy Malaysian subjects

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Abstract

Background: Cytochrome *P*450 3A4 (CYP3A4) is the major cytochrome involved in metabolizing of >60% of all drugs used in humans. A number of allelic variations in *CYP3A4* gene are known to affect catalytic activity including *CYP3A4*4*, *CYP3A4*5* and *CYP3A4*18*. We investigated the frequencies of *CYP3A4*4*, *CYP3A4*5* and *CYP3A4*18* alleles in a Malaysian population. This will impact treatment of patients receiving drugs metabolized by these alleles.
Methods: The study was conducted in 121 healthy Malaysian volunteers. DNA was extracted from leucocytes and the 3 alleles were determined by PCR–RFLP. The PCR product was later digested with restriction enzymes *Bst*MA I, *Bsh*V I and *Hpa* II.
Results: No mutations were detected for *CYP3A4*4* and *CYP3A4*5* alleles. The frequency of the *CYP3A4*18* allele in the Malaysian population is 2.1%. All 5 subjects with *CYP3A4*18* mutations were found to be heterozygous.
Conclusion: The present study describes polymorphisms of *CYP3A4* among Malaysian subjects. Clinical relevance of these genetic variants in these healthy volunteers is under investigation.

Keywords: *CYP3A4*; Genetic polymorphism; PCR–RFLP; Malaysian

1. Introduction

Interindividual variation in drug metabolism is caused by many factors including environmental factors, concurrent drug therapy as well as genetic factors. Much of this variation, however, has shown to be caused by genetic polymorphisms of the human cytochrome *P*450 enzymes (CYP) [1]. CYP is the most important enzymatic system involved in drug metabolism. It is also involved in the metabolisms of a large number of certain endogenous compounds such as steroids, prostaglandins, thromboxanes, fatty acid derivatives and retinoic acid derivatives [2]. Approximately 65% of current drugs used are metabolized by CYP enzymes and half of them are mediated by the CYP3A subfamily [3]. The CYP3A subfamily consists of 4 members: CYP3A4, CYP3A5, CYP3A7 and CYP3A47 [4]. The CYP3A subfamily represents about 30% of the total CYP in the human liver [5].

CYP3A4 is involved in the metabolism of >60% of all drugs used in humans [6,7]. It is found in the human livers and intestines [8]. It plays important roles in the metabolism of a wide variety of drugs such as antidiabetics, antiarrhythmics, antihistamines and synthetic estrogens [9]. Variant *CYP3A4* alleles in the population may contribute to interindividual variability in CYP3A4 activity. Detecting genetic polymorphisms may therefore help to predict an individual's ability to respond to certain drugs. Among Asian subjects, a number of allelic variations in *CYP3A4* gene are known to affect catalytic activity including *CYP3A4*4*, *CYP3A4*5* and *CYP3A4*18* [10–14].

The *CYP3A4*4* allele has A13989G change leading to Ile118Val substitution in exon 5 [10]. The *CYP3A4*5* variant allele has a C to G point mutation in exon 7 causing amino acid change where Pro is substituted with Arg at site 218 [10]. *CYP3A4*18* is a variant allele in exon 10 involving T to C transition that changes Leu to Pro at site 293 [11]. Table 1 summarizes the

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Table 1
Position, sequences and effects of mutations in *CYP3A4*4*, *CYP3A4*5* and *CYP3A4*18* variant alleles

Polymorphism	Nucleotide sequences		Effect
	Wild-type (WT)	Mutation (M)	
*CYP3A4*4*	**G**tctc	**A**tctc	Ile118Val
*CYP3A4*5*	ggatc**C**attct	ggatc**G**attct	Pro218Arg
*CYP3A4*18*	cgatc**T**ggagc	cgatc**C**ggagc	Leu293Pro

Capital letters indicate mutations sites.

sequences and effect of mutations in these variant alleles. These polymorphisms may have important clinical implications in individuals for carrying these variants. This will also impact treatment of patients receiving drugs metabolized by these alleles. Dai et al. [12] for example indicated that *CYP3A4*18* showed significantly a higher turnover number for both testosterone and insecticide chlorpyrifos in vitro.

To date, allelic frequencies and genotypes of *CYP3A4*4*, *CYP3A4*5* and *CYP3A4*18* have been reported among the Chinese [10–12], Korean [13], Japanese [14] and Caucasian [12] populations but none has been reported for the Malaysian population, so far. Therefore, the aim of this study is to investigate the allelic distribution of these three alleles in the Malaysian population by using a polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) method.

2. Materials and methods

2.1. Subjects

One hundred and twenty-one healthy volunteers were recruited, consisting of 61 men and 60 women. These were students and staff of Universiti Sains Malaysia, Health Campus, Kelantan, Malaysia. Subjects were informed about the experimental procedures and study aim before giving written informed consents. They were ascertained to be healthy from the medical history, physical examination and routine laboratory tests. All subjects ageing between 18 and 50 years with a normal body mass index (BMI) were included (Table 2). The study protocol was approved by our local Research and Ethics Committee, School of Medical Sciences, Universiti Sains Malaysia.

2.2. DNA extractions

About 5 ml of venous blood was drawn into a tube containing EDTA and stored at −20 °C until the isolation of genomic DNA. Genomic DNA was isolated from 300 μl of blood using QIAamp DNA Blood Mini Kit (QIAGEN, Hilden). The purity of the DNA and their concentrations were measured by spectrophotometry, Biophotometer Uvette (Eppendorf, Germany) at 280 nm absorbance.

2.3. Genotyping of CYP3A4*4, *5, and *6 using PCR–RFLP

DNA samples were investigated for *CYP3A4** mutations according to Hsieh et al. [10] with slight modifications. The PCR reaction was carried out in a total volume of 25 μl consisting of 2.5 μl of 10× PCR buffer with KCl (MBI Fermentas Inc., Hanover, MD), 0.1 μmol/l of the primer as listed in Table 3, 0.4 μmol/l of dNTP (MBI Fermentas), 1.5 mmol/l $MgCl_2$ and 2 U *Taq* DNA polymerase (MBI Fermentas). PCR parameters consisted of an initial denaturation step running for 1 min at 94 °C, followed by 35 cycles of 45 s at 94 °C, 45 s at 66 °C and 30 s at 72 °C with a 10 min final extension at 72 °C. A 249-bp fragment was yielded by running on a 1% agarose gel (Fig. 1a). After PCR amplification, 5 μl of the diluted PCR product (1:10) was digested with *Bst*MA I (1 U) (Vivantis, Malaysia) for 1 h at 55 °C before electrophoresis using 3% agarose gel. Digestion of the PCR product with *Bst*MA I results in 89 bp and 155 bp fragments for wild-type sequence (Fig. 1b) and 47 bp, 85 bp and 117 bp for *CYP3A4*4* variant alleles. No samples were detected to have this mutation.

The method for *CYP3A4*5* genotyping was also based Hsieh et al. [10] with some slight modifications. The PCR mixture consisted of a 2.5 μl of 10× PCR buffer with KCl (MBI Fermentas), 0.4 μmol/l of the primer as listed in Table 3, 0.2 μmol/l of dNTP, 1.5 mmol/l $MgCl_2$ and 2.5 U *Taq* DNA polymerase (MBI Fermentas). PCR conditions were as follows: an initial denaturation step for 7 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 1 min at 64 °C and 1 min at 72 °C with a 7-min final extension at 72 °C. About 5 μl of the PCR product was run on a 1% agarose gel for 1 h resulting in a fragment with a 450-bp band size (Fig. 2a).

A restriction enzyme, *Bsh*V I (1 U) (Vivantis, Malaysia) was used to digest 5 μl of the 1 in 10 diluted PCR products for 1 h at 37 °C before electrophoresis using 3% agarose gel. Samples with wild-type sequence gave a single 450-bp band as PCR product (Fig. 2b) while samples for *CYP3A4*5* variant allele were digested into 2 pieces of 200- and 250-bp band sizes, respectively. No samples were detected to have this mutation.

The method for PCR–RFLP for *CYP3A4*18* was adapted from Hu et al. [11]. A total of 50 ng of genomic DNA was used in a total PCR volume of 25 μl. The PCR mixture consisted of a 2.5 μl of 10× PCR buffer with KCl (MBI Fermentas), 0.4 μmol/l of the primer as listed in Table 3, 0.2 μmol/l of dNTP, 1.5 mmol/l $MgCl_2$ and 1 U *Taq* DNA polymerase (MBI Fermentas). The following PCR conditions were used: an initial denaturation step for 7 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 1 min at 58 °C and 1 min at 72 °C with a 7 min final extension at 72 °C. A 388-bp fragment was yielded by running on a 1% agarose gel (Fig. 3a). 5 μl of the 1 in 10 diluted PCR products was digested for 2 h at 37 °C with 1 U of *Hpa* II (Vivantis, Malaysia). The digested PCR products were analyzed by electrophoresis on a 3% agarose gel in the presence of ethidium bromide. Digestion of the PCR product with *Hpa* II resulted in 388-bp fragments for wild-type sequence and 2 fragments of 199 and 189 bp for the *CYP3A4*18* variant allele (Fig. 3b).

2.4. Direct DNA sequencing

DNA sequencing was performed to further confirm the genotyping results. Three samples from each different genotype were chosen at random and sent for

Table 2
Demographic data of subjects (n=121)

	Mean	S.D.	Range (min–max)
Age (year)	27.29	6.98	18–48
Body weight (kg)	58.17	8.43	40.0–77.8
BMI (kg/m^2)	21.94	1.99	18.5–25.0

Table 3
Primer sequences used in PCR–RFLP of *CYP3A4* alleles

Primers	Sequence	Size of PCR product (bp)	Endonuclease
Exon 5			
*CYP3A4*4* (F)	CAC ATT TTC TAC AAC CAT GGA GAC C	249	*Bst*MA I
*CYP3A4*4* (R)	TAC CTG TCC CCA CCA GAT TCA TTC T		
Exon 7			
*CYP3A4*5* (F)	TGT TGC ATG CAT AGA GGA AGG ATG G	450	*Bsh*V I
*CYP3A4*5* (R)	AGT GGT TGC ATA TGA TGA CAG GGT T		
Exon 10			
*CYP3A4*18* (F)	AAT GAT TTG CCT TAT TCT GGT TCT G	388	*Hpa* II
*CYP3A4*18* (R)	TTT CAC CTC CTC CCT CCT TCT C		

(F) – forward, (R) – reverse.

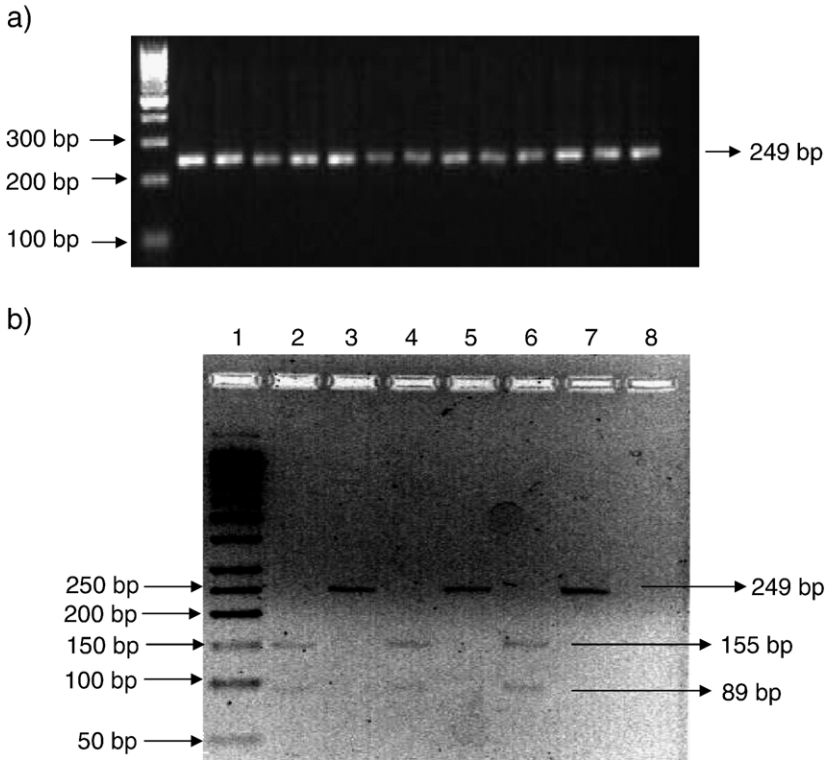

Fig. 1. (a) PCR products for amplification using *CYP3A4*4* primers. (b) RFLP results for wild-type *CYP3A4*1/*1* after digestion with *BstM*A I (Lanes 2, 4, 6). Lanes 3, 5, 7 contained undigested PCR product. Lane 8 contained a negative control (gel picture is printed in a reverse mode).

sequencing. The PCR products were purified using QIAquick PCR purification kit (Qiagen, Hilden, Germany) before being sent for DNA sequencing by standard Kit of ABI PRISM Big Dye Terminator. The sequencing results were then verified against the published sequence for *CYP3A4* (GenBank accession no.: AF20209389).

2.5. Statistics

Data were compiled according to the genotype and allele frequencies. 95% confidence intervals were calculated for all observed allele frequencies. Expected genotype frequencies were calculated using a Hardy–Weinberg equation from the allele frequencies ($p^2+2pq+q^2=1$). A $p<0.05$ was considered statistically significant. All statistics were performed using the SPSS package (ver. 11, SPSS, Chicago, IL).

3. Results and discussions

In all reactions, correct lengths of expected PCR products were obtained. In the PCR–RFLP analyses, the restriction enzymes used in the present study was found to have worked successfully

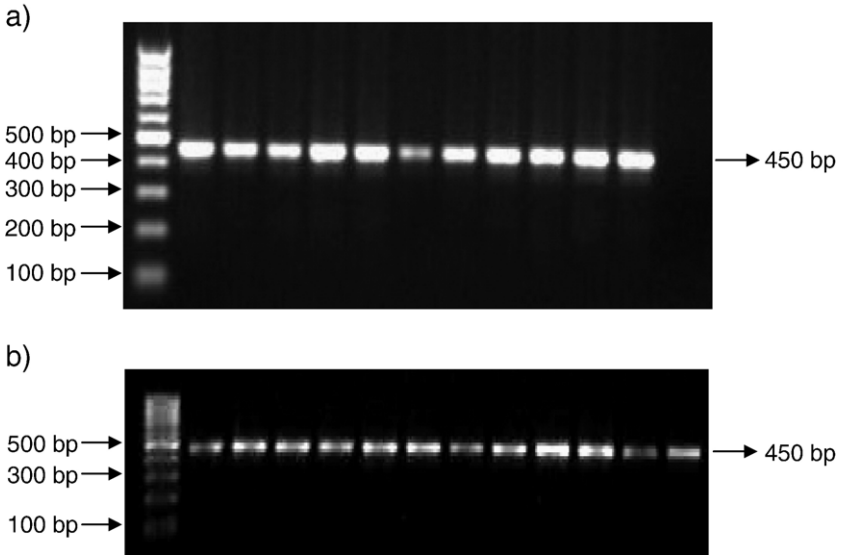

Fig. 2. (a) PCR products for amplification using *CYP3A4*5* primers. (b) RFLP results for wild-type *CYP3A4*1/*1* after digestion with *Bsh*V I.

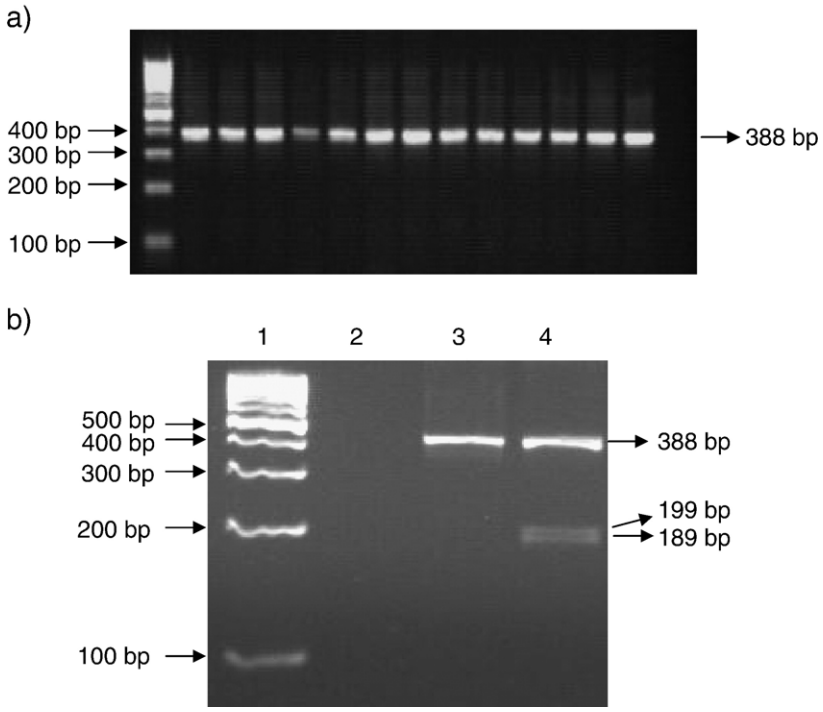

Fig. 3. (a) PCR products for amplification using *CYP3A4*18* primers. (b) RFLP results for *CYP3A4*18* allele after digestion with *Hpa* II. Lane 2 contained a negative control. Lane 3 is an individual wild-type *CYP3A4*1/*1*. Lane 4 is a heterozygous individual.

as depicted in Figs. 1b and 3b. The restriction enzyme used for *CYP3A4*5* genotyping (*Bsh*V I) was further confirmed to be correct by digestion of λ DNA for 1 h at 37 °C (result not shown). PCR–RFLP is one of the most reliable methods used for recognition of genetic mutations. The method is based on a specific PCR product cut by restriction endonucleases, which is capable of cutting the double-stranded DNA [15]. This method offers the advantage of cutting DNA by the appropriate restriction enzyme at the correct mutation site hence enhancing its reliability. Electrophoretic separation demonstrates the presence (or absence) of restriction sites.

In our study, no mutations were detected for *CYP3A4*4* and *CYP3A4*5* alleles and all 121 subjects were found to be of the wild-type. For the *CYP3A4*18* alleles, 116 of these volunteers were found to be of wild-type, 5 were heterozygous and none was homozygous. The direct sequencing results for the selected samples were found to be aligned and matched the published sequence for *CYP3A4* (GenBank accession no.: AF209389).

Table 4 summarizes the allele frequencies and genotypes for *CYP3A4*. Table 5 presents allelic frequencies for each *CYP3A4* mutation in Malaysians comparing it with other ethnic populations. Table 5 indicates that the *CYP3A4*4* and *CYP3A4*5* alleles are only seen among the Chinese population with a reported allele frequency of 1.47% and 0.98%, respectively [10,12]. On the other hand, *CYP3A4*18* allele is found among the Chinese [10,12] and Korean [13] populations but not in the Caucasians [12] and the Japanese [14]. The allele frequency of *CYP3A4*18* in the Malaysian populations is higher than that of the Korean and Chinese populations, i.e., at 2.1%. The allele frequency of *CYP3A4*18* in the Chinese population reported by

Table 4
Allele frequency with observed and predicted genotype frequencies of *CYP3A4* according to Hardy–Weinberg equation among Malaysians ($n=121$)

Allele	Allele frequency (%)±95% CI	Genotype	Samples (n)	Observed genotype frequency (%)±95% CI	Predicted genotype frequency (%) by Hardy–Weinberg Law±95% CI
*CYP3A4*1*	97.93±0.0179	*CYP3A4*1/*1*	116	95.87±0.0181	95.91±0.0353
*CYP3A4*4*	0	*CYP3A4*1/*4*	0	0	0
*CYP3A4*5*	0	*CYP3A4*1/*5*	0	0	0
*CYP3A4*18*	2.07±0.0179	*CYP3A4*1/*18*	5	4.13±0.0179	4.05±0.0351
		*CYP3A4*4/*4*	0	0	0
		*CYP3A4*4/*5*	0	0	0
		*CYP3A4*4/*18*	0	0	0
		*CYP3A4*5/*5*	0	0	0
		*CYP3A4*5/*18*	0	0	0
		*CYP3A4*18/*18*	0	0	0.04%±0.0037

Table 5
Allelic frequencies of *CYP3A4* polymorphisms in Malaysians and comparison with other ethnic populations

Population	No. (*n*)	*CYP3A4*4*	*CYP3A4*5*	*CYP3A4*18*	References
Malaysians	121	0%	0%	2.07%	Present study
Koreans	200	0%	0%	2%	Jung et al. [13]
Japanese	20	0%	0%	ND	Lamba et al. [14]
Chinese	102	1.47%	0.98%	2%	Hsieh et al. [10],
	24	1.47%	0.98%	2%	Dai et al. [12]
	302	ND	ND	0.01%	Hu et al. [11]
Caucasians	24	0%	0%	0%	Dai et al. [12]

ND – not determined.

Hu et al. [11], however, is the lowest. This inconsistency might be due to the differences in the sample size where Hu et al. [11] have used a larger number of subjects.

Polymorphisms in *CYP3A4* may have important physiological and pathological consequences in diseases such as in diabetes mellitus. In the case of repaglinide, a novel short-acting antidiabetic drug, CYP2C8 and CYP3A4 are the principal enzymes involved in its metabolism [16]. A study done by Niemi et al. [17] showed that even low doses of clarithromycin, a CYP3A4 inhibitor, may increase plasma concentrations and repaglinide's therapeutic effects. Therefore, concomitant use of clarithromycin or other potent inhibitors of CYP3A4 with repaglinide may enhance its blood glucose-lowering effect and increase the risk of hypoglycemic attack among patients. Variation in the *CYP3A4* gene may thus lead to variation in CYP3A4 activity and differences in drug metabolism which can lead to toxicity or therapeutic failure due to the differences in available concentration of the active constituents in the blood.

It can also be hypothesized that CYP3A4 is well conserved among the Malaysian populations because it is an important enzyme metabolizing >60% of current drugs used but yet no mutations were detected for *CYP3A4*4* and *CYP3A4*5*. It also plays important roles in the metabolism of some endogenous steroids as well as harmful environmental contaminants [1]. As the most abundant enzyme in the liver, CYP3A4 is also involved in the metabolism of endogenous cortisol to 6β-hydroxycortisol (6βOHF) [18]. The urinary excretion of 6βOHF and its ratio to free cortisol (6βOHF/F) reflected its activities [18]. Hsieh et al. [10] reported that measuring the urinary morning spot of 6βOHF/F is a good indication of CYP3A4 activity. They concluded that from the 6βOHF to 6βOHF/F ratio, those with *CYP3A4*4*, *CYP3A4*5* and *CYP3A4*6* alleles have shown decreased CYP3A4 activity compared to those with no mutations shown.

In conclusion, the PCR–RFLP method described in our study can be used to screen for and detect *CYP3A4*4*, *CYP3A4*5* and *CYP3A4*18* mutations. Out of these 3, only *CYP3A4*18* is detected among Malaysian subjects with a frequency of 2.1%. The clinical relevance of the genetic variants in these healthy volunteers receiving repaglinide is still under our investigation.

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References

[1] Nebert DW, Gonzalez FJ. *P450* genes: structure, evolution and regulation. Annu Rev Biochem 1987;56:945–93.
[2] Anzenbacher P, Anzenbacherova E. Cytochrome *P*450 and metabolism of xenobiotics. Cell Mol Life Sci 2001;58:737–47.
[3] Evans WE, Relling MV. Pharmacogenomics: translating functional genomics into rational therapeutics. Science 1999;286:487–91.
[4] Gellner K, Eiselt R, Hustert E, et al. Genomic organization of the human *CYP3A* locus: identification of a new, inducible *CYP3A* gene. Pharmacogenetics 2001;11:111–21.
[5] Nelson DR, Koymans L, Kamataki T, et al. *P450* superfamily: update on new sequences, gene mapping, accession numbers and nomenclature. Pharmacogenetics 1996;6:1–42.
[6] Thummel KE, Wilkinson GR. In vitro and in vivo drug interactions involving human *CYP3A4*. Annu Rev Pharmacol Toxicol 1998;38:389–430.
[7] Rendic S, Di Carlo FJ. Human cytochrome *P*450 enzymes: a status report summarizing their reactions, substrates, inducers and inhibitors. Drug Metab Rev 1997;29:413–580.
[8] Shimada T, Yamazaki H, Mimura M, Inui Y, Guengerich FP. Interindividual variations in human liver cytochrome *P*450 enzymes involved in the oxidation of drugs, carcinogens and toxic chemicals. J Pharmacol Exp Ther 1994;270:414–23.
[9] Watkins PB. Non-invasive tests of *CYP3A4* enzymes. Pharmacogenetics 1994;4:171–84.
[10] Hsieh KP, Lin YY, Cheng CC, et al. Novel mutations of *CYP3A4* in Chinese. Drug Metab Dispos 2001;29:268–73.
[11] Hu YF, He J, Chen GL, et al. *CYP3A5*3* and *CYP3A4*18* single nucleotide polymorphisms in a Chinese population. Clin Chim Acta 2005;253:187–92.
[12] Dai D, Tang J, Rose R, Hodgson E, Bienstock RL, Mohrenweiser HW. Identification of variants of *CYP3A4* and characterization of their abilities to metabolize testosterone and chlorpyrifos. J Pharmacol Exp Ther 2001;299:825–31.
[13] Jung H, Lee S, Jung H, Shon J, Cha I, Shin J. Allelic distribution of *CYP3A4* genetic polymorphisms in a Korean population. Clin Pharmacol Ther 2002;73(2):P41.
[14] Lamba JK, Lin YS, Thummel K, et al. Common allelic variants of cytochrome *P*4503A4 and their prevalence in different populations. Pharmacogenetics 2002;12:121–32.
[15] Topic E. Pharmacogenetics and clinical laboratory. Biochem Med 1999;9:97–106.
[16] Bidstrup TB, Bjornsdottir I, Sidelmann UG, Thamsen MS, Hansen KT. *CYP2C8* and *CYP3A4* are the principal enzymes involved in the human in vitro biotransformation of the insulin secretagogue repaglinide. Br J Clin Pharmacol 2003;56(3):301–14.
[17] Niemi M, Neuvonen PJ, Kivisto KT. The cytochrome *P*4503A4 inhibitor clarithromycin increases the plasma concentrations and effects of repaglinide. Clin Pharmacol Ther 2001;70:58–65.
[18] Ged C, Rouillon JM, Pichard L, et al. The increase in urinary excretion of 6 beta-hydroxycortisol as a marker of human hepatic cytochrome *P*450IIIA induction. Br J Clin Pharmacol 1989;28:373–87.