Identification of a novel variant *CYP2C9* allele in Chinese

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Objectives **Cytochrome P450 (CYP) 2C9 metabolizes about 16% of drugs in current clinical use, including lornoxicam and tolbutamide. SNPs in the *CYP2C9* gene have increasingly been recognized as determinants of the metabolic phenotype that underlies interindividual and ethnic differences.**

Methods **The present study focused on a Chinese poor metabolizer (PM) whose apparent genotype (*CYP2C9*1/CYP2C9*3*) did not agree with his PM phenotype for both lornoxicam and tolbutamide. By sequencing his CYP2C9 gene, we identified a new variant *CYP2C9* allele involving a T269C transversion in exon 2 that leads to a Leu90Pro substitution in the encoded protein.**

Results **The *CYP2C9* genotype analysis in the family of the poor metabolizer showed the new exon 2 change and *CYP2C9*3* occurred on different alleles. Thus, the PM status of this subject could be attributed to his being heterozygous for the *CYP2C9* T269C allele together with the *CYP2C9*3*. Frequency analysis in 147 unrelated Chinese males indicated approximately 2% of the Chinese population carry the allele.**

Conclusion **This study suggests that this novel *CYP2C9* allele was correlated with reduced plasma clearance of drugs that are substrates for *CYP2C9*. *Pharmacogenetics* 14:465–469 © 2004 Lippincott Williams & Wilkins**

Pharmacogenetics 2004, **14**:465–469

Keywords: CYP2C9, polymorphism, pharmacokinetics, lornoxicam, tolbutamide

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Project supported by the National Natural Science Foundation of China, No. 39930180.

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Received 22 December 2003
Accepted 15 March 2004

Introduction

Cytochrome P450 2C9 (CYP2C9), a member of the CYP2C enzyme subfamily, ranks amongst the most important drug metabolizing enzymes in humans. It makes up about 20% of the total cytochrome P-450 protein in liver microsomes [1,2], and hydroxylates about 16% of drugs in current clinical use [3]. These include the anticoagulant warfarin, the antidiabetic agents tolbutamide and glipizide, the anticonvulsant phenytoin, the antihypertensive losartan, the antidepressant fluoxetine [4] and a number of nonsteroidal antiinflammatory drugs (NSAIDs) including ibuprofen [5], celecoxib [6], meloxicam [7] and lornoxicam [8].

Human CYP2C9 has been shown to exhibit genetic polymorphism. In addition to the wild-type protein CYP2C9*1, at least five single nucleotide polymorphisms (SNPs) have been reported within the coding region of the *CYP2C9* gene producing the variant allozymes, CYP2C9*2 (Arg144Cys), CYP2C9*3 (Ile359Leu), CYP2C9*4 (Ile359Thr), CYP2C9*5 (Asp360Glu) and CYP2C9*6 (null allele) [3]. The 5′-noncoding region of the *CYP2C9* gene is also polymorphic, having at least seven SNPs [9]. Some of these SNPs have been shown to affect the metabolism of CYP2C9 substrates *in vivo* and have increasingly been recognized as determinants of the metabolic phenotype that underlies interindividual and ethnic differences. For example, compared with wild-type *CYP2C9*1/*1*, clearance of tolbutamide in subjects heterozygous and homozygous for the most common variant *CYP2C9*3* is reduced by some 42% and 84%, respectively [10]. Moreover, the genotype *CYP2C9*3/*3* has been found in subjects with poor metabolizer (PM) phenotype for most CYP2C9 substrates [11]. The frequency of *CYP2C9* alleles is different among Caucasian, African and Asian populations. In Chinese populations, the most important variant allele is *CYP2C9*3* with a gene frequency of about 3.3%; while *CYP2C9*2*, *CYP2C9*4* and CYP2C9*5 are rare or absent [11,12].

CYP2C9 has been shown as the primary enzyme responsible for the biotransformation of the NSAID lornoxicam to its major metabolites, 5′-hydroxylornoxicam in human liver microsomes [8,13]. Previously we studied the influence of genetic polymorphisms of *CYP2C9* on lornoxicam pharmacokinetics and found a subject with unusual pharmacokinetic parameters. The initial genotype results indicated that he was a *CYP2C9*1/*3* carrier, but his lornoxicam half-life of

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DOI: 10.1097/01.fpc.0000114749.08559.e4

about 105 h was markedly longer than that of other *CYP2C9*1/*3* and *CYP2C9*1/*1* carriers (half-lives of 5.8–8.1 and 3.2–6.3 h, respectively; unpublished data). The discrepancy between genotype and phenotype for lornoxicam in this individual suggested additional defects may contribute to phenotype. In the present study, after having examined the pharmacokinetics of the CYP2C9 probe tolbutamide, we performed genetic analysis of the *CYP2C9* gene to establish whether defective alleles were present in this Chinese poor metabolizer and subsequently in his parents and 147 unrelated Chinese males.

Materials and methods

Study subjects

The study subject was a healthy Chinese male identified as having PM phenotype for metabolism of lornoxicam in our earlier study. Prior to the pharmacokinetic study, he was required to refrain from ingesting medication or alcohol. Having identified a novel *CYP2C9* allele in this individual, its frequency in a Chinese population was determined by genotyping 147 unrelated Han Chinese male subjects. The study protocol was approved by the Ethics Committee of Liaoning Provincial People's Hospital and informed consent was obtained from each subject prior to the study.

Tolbutamide pharmacokinetics

Tolbutamide clearance of the poor metabolizer was determined to check the catalytic efficiency of CYP2C9. After a single oral dose of 500 mg tolbutamide, blood samples were collected at 6, 8, 12, 24, 36 and 48 h after drug administration. Plasma was separated and frozen at −20°C until assayed. Tolbutamide in plasma was determined using an high pressure liquid chromatography (HPLC) method. Tolbutamide and the internal standard gliclazide were extracted by liqud-liquid extraction with ether. The HPLC system consisted of a Perkin Elmer LC pump and a UV/Vis detector LC 295 (Norwalk, CT, USA) set at 237 nm. Chromatography was performed on a Hypersil C18 column (particle size 5 μm, 150 mm × 5.0 mm ID, Dalian, China), using a mobile phase of acetonitrile-$NH_4H_2PO_4$ (10 mmol/l, pH 4.0) (57:43) at a flow rate of 1.0 ml/min. The assay was shown to be linear from 1.0 to 100 μg/ml and the lower limit of quantification was 1.0 μg/ml. The intra-run precision was $> 10\%$. All samples were analyzed in a day with an intra-day precision of $> 10\%$.

Pharmacokinetic analysis

Noncompartmental analysis was used in the data processing of tolbutamide. k_e was determined by linear regression of the terminal linear portion of the ln (concentration)–time curve, and $t_{1/2}$ was calculated as $\ln(2)/k_e$.

Sequencing of *CYP2C9*

A 0.3-ml blood sample was drawn from the study subject into a tube containing ethylenediaminetetra acetic acid. Genomic DNA was then isolated from the whole blood using a commercially available kit (Sino-American, Luoyang, China). Each exon, intron–exon junction and −580 bp of the upstream region of the *CYP2C9* gene was amplified by PCR using intron-specific primers. To avoid amplification of sequences from homologous genes, highly specific primers were selected (Table 1). The amplification products were purified for sequencing using a Wizard PCR Preps DNA Purification System (Promega, Madison, WI, USA). They were then sequenced on an ABI Prism 310 Genetic Analyzer using a BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems Inc., Foster City, CA, USA).

Genotyping tests

To examine the *CYP2C9*3* alleles of the study subject's parents, PCR-based restriction fragment length polymorphism (RFLP) analysis was carried out using the method of Wang *et al.* with slight modification [18].

To determine the frequency of the newly discovered *CYP2C9* allele in the Chinese population, a PCR–RFLP genotyping test was developed. The primer pair designed for *CYP2C9*2* allele PCR–RFLP analysis by Dickmann *et al.* (shown in Table 1) was used to amplify *CYP2C9* [14]. The resulting 689-bp product were digested with 10 U of restriction enzyme *Eco*RII at 37°C for 3 h and electrophoresed on agarose gels. The T269C change in the sequence CTTGG creates a new recognition site of *Eco*RII (CCTGG) such that *Eco*RII digested the PCR products of the *CYP2C9* T269C allele into fragments of 192 bp, 195bp and 302 bp, whereas the PCR products of the *CYP2C9*1* allele were digested into fragments of 387 bp and 302 bp, as shown later in Fig. 1.

Results

Tolbutamide pharmacokinetics

The tolbutamide plasma concentration–time curve in the study subject is shown in Fig. 2. The half-life of tolbutamide was calculated to be 103 h. Compared with the pharmacokinetic parameters reported by Kirchheiner *et al.*, Shon *et al.* and Lee *et al.* in which the subjects with the *CYP2C9*1/*1*, *CYP2C9*1/*3* and *CYP2C9*3/*3* genotypes have average tolbutamide plasma half-life of about 6.6–7.1, 11.5–13.2 and 42.8 h, respectively [10,19,20], the PM study subject's plasma half-life is about 9–15 times that of the *CYP2C9*1/*1* or *CYP2C9*1/*3* carriers and is even slower than the *CYP2C9*3/*3* genotype subjects.

Sequencing of *CYP2C9*

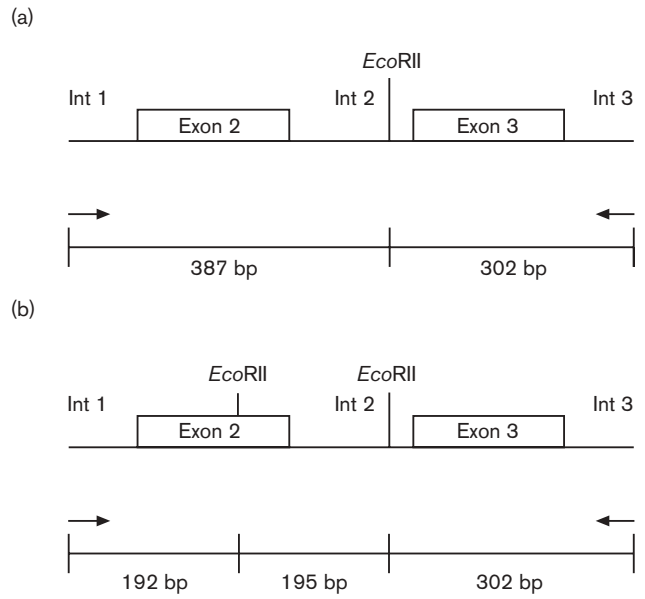
Gene sequencing of CYP2C9 revealed the study subject is heterozygous for *CYP2C9*3* and a previously

Table 1 Sequence and location of *CYP2C9* specific primers used in polymerase chain reaction

Sequence[a] (5′→3′) and direction	Location	Amplified region	Fragment size (bp)
GCCTTCAGGAATTTTTTTTA (F)	Upstream region	−580 to Exon 1	929
TTTTACTTTACCATTACCTCTTG (R)	Intron 1		
TACAAATACAATGAAAATATCATG (F) [14]	Intron 1	Exon 2, intron 2 & exon 3[b]	689
CTAACAACCAGACTCATAATG (R) [14]	Intron 3		
TGTTAAGGGAATTTGTAGG (F) [15]	Intron 3	Exon 4	340
AATTTTGGATTTGTCAGAA (R) [15]	Intron 4		
CAGAGCTTGGTATATGGTATG (F) [16]	Intron 4	Exon 5	321
GTAAACACAGAACTAGTCAAC (R) [16]	Intron 5		
GTTTGGGCAAGTTGGTCTA (F)	Intron 5	Exon 6	395
AGAAACAGGAAGGAGGACAC (R)	Intron 6		
CTCCTTTTCCATCAGTTTTTACT (F) [17]	Intron 6	Exon 7	284
GATACTATGAATTTGGGACTTC (R) [17]	Intron 7		
TTCATGGCTTCTTTACAGCT (F)	Intron 7	Exon 8	382
TCCCCAAAGTCCACTAATCT (R)	Intron 8		
TATTGCATATTCTGTTTGTGC (F)	Intron 8	Exon 9	803
CAAGTAACTCTAACACTCACCC (R)	Past the stop codon		

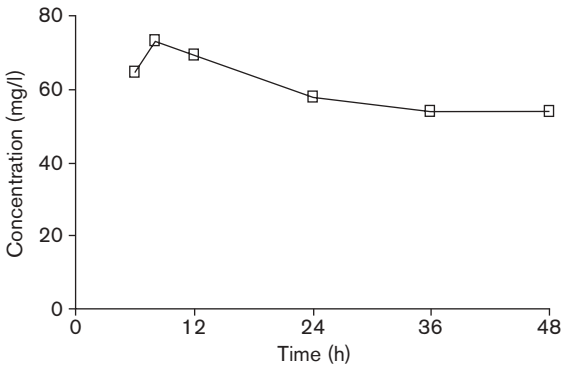
[a]The sequences are derived from the GenBank (accession nos L16877 through L16883 and NM_000771) and Human genome sequence data at NCBI. F, forward primer; R, reverse primer.

Fig. 1



Schematic depiction of the *CYP2C9 T269C* transversion allele genotyping test showing specific PCR amplification of exon 2 & 3 and restriction map of *Eco*RII sites. Arrows indicate the primers. (a) CYP2C9*1; (b) *CYP2C9 T269C*; the T269C transversion of exon 2 creates a new *Eco*RII site generating fragments of 192 and 195 bp.

Fig. 2



Tolbutamide plasma concentration–time curve in the study subject.

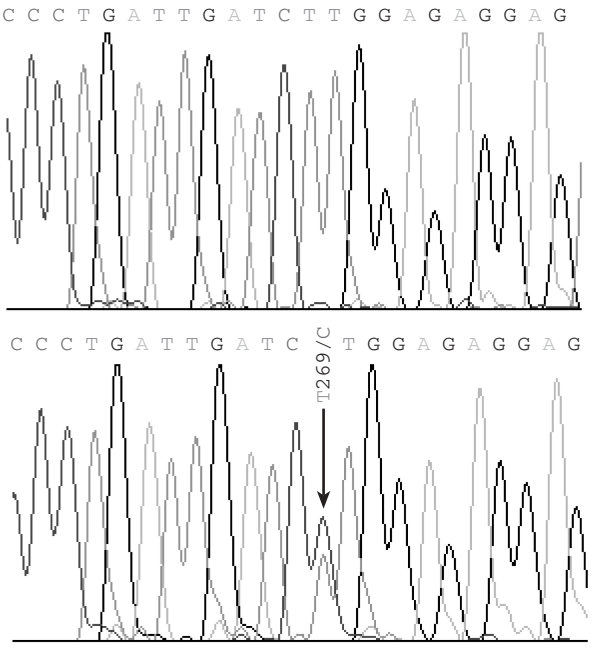
unreported mutation in exon 2. This mutation associates with a T269C transversion of the *CYP2C9* gene that leads to a Leu90Pro substitution in the encoded protein (Fig. 3).

To determine if the two mutations occurred on the same or separate alleles, genotyping for *CYP2C9 T269C* allele and *CYP2C9*3* was performed on DNA from the parents of the poor metabolizer. The results showed that the mother of the study subject is heterozygous for *CYP2C9*3*, but does not carry the new *CYP2C9* allele and the father is heterozygous for the new *CYP2C9 T269C* allele, but does not carry the *CYP2C9*3* allele. Thus, the *CYP2C9 T269C* and *CYP2C9*3* alleles of the poor metabolizer are derived separately from the father and mother, and the novel mutation and the *CYP2C9*3* mutation occur on separate alleles.

Frequency analysis

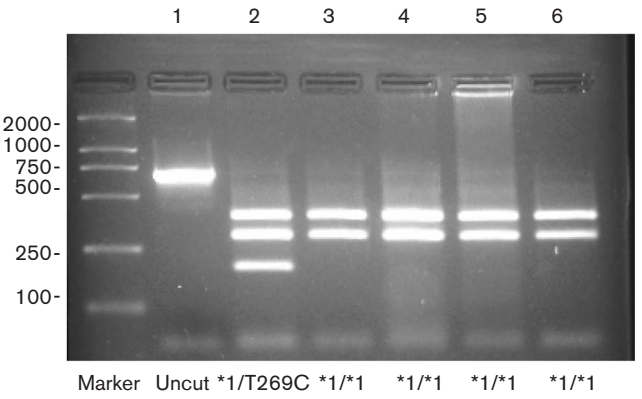
In PCR–RFLP analysis of the 147 Chinese males, three subjects were identified to be carriers of the CYP2C9 T269C allele. None of the subjects were homozygous for this newly identified variant. These data correspond to an allele frequency of 1.02% (95% confidence limits of 0.2 to 3.0%) in the Chinese population (Fig. 4).

Fig. 3



Results of DNA Sequencing analysis in exon 2 of the *CYP2C9* gene showing spectrum of a wild-type (upper) and the heterozygote for a mutant allele (lower). Mutated points are indicated by an arrow. Sequencing was carried out using the primer shown in Table 1.

Fig. 4



CYP2C9 T269C transversion genotyping test showing an agarose gel of exon 2 PCR products digested with *Eco*RII. Lane 1 contains uncut sample and lanes 2 through 6 contain digested PCR products from individuals with the genotypes indicated below each lane. Lanes 3 through 6 show that *Eco*RII digests the PCR amplicon of the *CYP2C9*1* allele into fragments of 387 bp and 320 bp. Lane 2 shows that the PCR products of the *CYP2C9*1* and *CYP2C9 T269C* alleles are digested into fragments of 387 bp, 302 bp and 192 and 195 bp.

Discussion

In the present study, a new *CYP2C9* allele has been identified in a Chinese poor metabolizer of lornoxicam. It involves a T-to-C transversion at nucleotide position 269 causing a mutation at codon 90, which results in a Leu→Pro substitution.

The pharmacokinetics of tolbutamide in the PM subjects was investigated, and he was found to be a phenotypically poor metabolizer of tolbutamide as well. Since tolbutamide is widely accepted as a probe substrate for the assessment of hepatic CYP2C9 activity *in vivo* [21,22], this study confirms that the study subject has severely impaired CYP2C9 catalytic efficiency. The result provides further evidence that lornoxicam pharmacokinetics is a reliable indicator of the genetic polymorphism in the CYP2C9 gene.

Genetic analysis showed the poor metabolizer was heterozygous for both *CYP2C9*3* and the novel *CYP2C9 T269C* allele. If the two mutations occurred on the same allele, the other allele would be wild-type and part of the translated CYP2C9 would be normal and give rise to an intermediate catalytic efficiency. Our study shows the poor metabolizer has two mutations on the separate alleles, i.e. he has two copies of *CYP2C9* gene which are both abnormal resulting in a substantially reduced catalytic efficiency of the translated product. In fact, the pharmacokinetics are significantly different from *CYP2C9*1/*3* but similar to those of *CYP2C9*3/*3*. This strongly suggests that the new variant allele is important in determining *CYP2C9* metabolic capability.

As stated previously, previous studies have shown the *CYP2C9*3* allele is the major variant allele in Chinese and about 6.6% of the Chinese population are carriers of *CYP2C9*1/*3* allele [11]. This study reveals another allelic variant and genotyping tests indicate about 2.0% of Chinese are heterozygous carriers of this new allele. The frequency of the new allele is probably different in other ethnic groups, as is the case for other *CYP2C9* alleles.

As described above, the novel *CYP2C9 T269C* allele appears to contribute to interindividual variability in drug metabolism activity. So, the individuals with the same heterozygous *CYP2C9*3/T269C* genotype as the poor metabolizer will express CYP2C9 with low metabolic capability, and will be at potential risk of toxicity from a number of drugs metabolized by CYP2C9, as reported for homozygous *CYP2C9*3*. Subjects homozygous for *CYP2C9 T269C* may face a similar situation. To confirm the predicted clinical consequences, we have undertaken a lornoxicam pharmacokinetic study in several subjects heterozygous for the *CYP2C9 T269C* allele, and preliminary results support this hypothesis. In addition, the significance of these mutations can be revealed by site-directed mutagenesis work, which is ongoing at present in our laboratory.

Acknowledgements

The authors wish to thank Dr J. Paul Fawcett (School of Pharmacy, University of Otago, New Zealand) for his modification on the language of this manuscript.

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