Differences in the Incidence of the CYP2C19 Polymorphism Affecting the S-Mephenytoin Phenotype in Chinese Han and Bai Populations and Identification of a New Rare CYP2C19 Mutant Allele

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ABSTRACT

The incidence of the S-mephenytoin polymorphism was compared in two Chinese ethnic groups, Han (n = 101) and Bai (n = 202) by phenotype and genotype analysis. The frequency of poor metabolizers (PMs) in Han vs. Bai subjects was 19.8% vs. 13.4%. Han subjects had a higher frequency of the mutant CYP2C19 m2 allele (0.366 vs. 0.257, P < .01) and a lower frequency of the wild-type allele (0.559 vs. 0.688, P < .01) than Bai subjects, which is consistent with the differences in the frequencies of PMs between the two ethnic groups. This results in a lower percentage of homozygous wild-type extensive metabolizers of mephenytoin (EMs) in Han subjects than in Bai subjects (40% vs. 59%, P = .005). Therefore, Han subjects may be more susceptible than Bai subjects to the drugs metabolized by the CYP2C19 enzyme. Ratios of urinary S/R-mephenytoin in homozygous EMs were lower than those of heterozygous EMs for both Han and Bai subjects, which shows a gene-dosage effect. Genotype analysis identified all but one PM as homozygous or heterozygous for the two known mutant CYP2C19 m1 and/or CYP2C19 m2 alleles. A single Bai PM outlier was shown to be heterozygous for CYP2C19 m1 and a new mutant CYP2C19 allele containing a single amino acid change of Arg433 → Trp433. A genotyping test demonstrated that only this one individual carried this rare allele (frequency of 0.0025 in Bai subjects).

S-mephenytoin hydroxylase is a polymorphically expressed cytochrome P450 that has been recently identified as CYP2C19 (Wrighton et al., 1993; Goldstein et al., 1994). This genetic polymorphism shows marked interracial differences. The incidence of the PM phenotype is much higher in Orientals (13%–23%) than in Caucasian populations (2%–5%) (Nakamura et al., 1985; Horai et al., 1989; Bertilsson et al., 1992). This polymorphism also affects the metabolism of several clinically used drugs, including omeprazole, barbiturates, chloroguanide, propranolol and diazepam (Wilkinson et al., 1989; Bertilsson, 1995).

Recent studies from our laboratory have demonstrated that two inactivating mutations in CYP2C19, CYP2C19 m1 (73%) and CYP2C19 m2 (13%), previously reported in Japanese populations (de Morais et al., 1994a; de Morais et al., 1994b), also occur in a Chinese Dong population (de Morais et al., 1995). CYP2C19 m1 is found in both Caucasians and Orientals, whereas CYP2C19 m2 is found principally in Orientals (de Morais et al., 1994a,b; Brøsen et al., 1995). Surprisingly, Jurima-Romet et al., (1995) found that the incidences of CYP2C19 m1 and CYP2C19 m2 in Canadian Inuits resemble those in Caucasians. China has 55 different minorities, accounting for approximately 100 million people, whose different genetic backgrounds and diverse environments distinguish them from the Han ethnic majority (Etler, 1992). It is of interest to determine whether the incidence of the polymorphisms in CYP2C19 varies among these different ethnic groups. This study was designed to compare S-mephenytoin hydroxylation and CYP2C19 genotypes in two Chinese ethnic groups, Han and Bai. The Han represents the largest ethnic group in China, accounting for ~95% of China’s total population (1.2 billion people) distributed throughout most of China. The Bai population represents one of the 15 largest

ABBREVIATIONS: PMs, poor metabolizers of mephenytoin; EMs, extensive metabolizers of mephenytoin; PCR, polymerase chain reaction; CYP2C19 m1, normal (wild-type allele); CYP2C19 m1 and CYP2C19 m2, mutant CYP2C19 alleles; 4′-OH-M, 4′-hydroxymephenytoin.
ethnic minorities in China. It consists of 1.6 million people living chiefly in Yunnan Province in the southwestern region of China.

Materials and Methods

Subjects. Unrelated Chinese subjects belonging to the Han nationality (n = 101, 53 men and 48 women) were recruited from students and staff of the Hunan Medical University. Chinese Bai subjects (n = 202, 120 men and 82 women) were recruited from students and staff of Dali Nationalities College (Yunnan, People’s Republic of China). All subjects were informed in writing about the purpose and experimental design of the study. None had taken any drug during the week before the study. All subjects were healthy and had no abnormalities, as shown by routine histories and physical examinations. The subjects were carefully interviewed and were considered to belong to either the Han or the Bai nationality, by lineage and birth. The criterion used for selection was that none of the three previous generations of the subjects had been married to members of other nationalities, as determined by questionnaire. This clinical protocol was approved by the Institutional Review Board of Hunan Medical University and was in compliance with DHHS regulations for protection of human research subjects.

Phenotyping procedures. After emptying the bladder, all subjects took an oral dose of 100 mg racemic mephenytoin (Mesantoin). Urine was collected from 0 to 8 h after dosing. The total volume was recorded, and a 20-ml aliquot was stored at –20°C. Urinary concentration of 4′-OH-M was analyzed by means of HPLC with a UV detector as recently described by Xie et al. (1995). The mephenytoin oxidation capacity was assessed as the percent of both the dose of mephenytoin excreted as 4′-OH-M (% dose) and the mephenytoin S/R ratio in urine as determined by chiral gas chromatography according to Wedlund et al. (1984), as modified by de Morais et al. (1995). Samples from the subjects whose phenotypes were questionable or were not in agreement with their genotypes were verified by acid treatment according to Zhang et al. (1992). An acid-labile metabolite is present in the urine of some EMs, and this metabolite can decompose in the urine, regenerating S-mephenytoin and leading to an elevated S/R ratio and improper designation of phenotype. An increase in the S/R ratio after acidification indicates that the individual is an EM.

Genotyping procedures. Ten milliliters of venous blood were obtained from 101 Han and 202 Bai subjects, and DNA was isolated from peripheral leukocytes by standard methods (Sambrook et al., 1989). Genotyping procedures for the detection of the CYP2C19m1 defect were modified slightly by use of more specific primers, as described by Goldstein and Blaisdell (1996): the forward primer (5′-CAGGCGTGGCATATTTGAT-3′) annealing in intron 4, 71 bp upstream from the intron 4/exon 5 junction, and the reverse primer (5′-GTAACAAGACAGATGTCATG-3′) annealing in intron 5, 73 bp downstream from the exon 5/intron 5 junction. After PCR amplification, the DNA fragments of exon 5 were digested with Sma I before electrophoresis with a 4% agarose gel. Genotyping procedures for the identification of the CYP2C19m2 defect were performed by PCR amplification with intron 3- and intron 4-specific primers. The PCR products were digested with BamH I and analyzed by gel electrophoresis as described previously (de Morais et al., 1995). PCR amplification for both defects was performed with a Perkin-Elmer 9600 thermocycler (Perkin-Elmer Corp., Norwalk, CT). An initial denaturation step at 94°C for 5 min was followed by 37 cycles consisting of denaturation at 94°C for 20 s, annealing at 53°C for 10 s and extension at 72°C for 10 s. The normal (wild-type) allele is defined as CYP2C19_wt. The final assignment of genotype requires the comparison of digestion patterns of both sets of PCR products.

To identify a possible new genetic defect in one unexplained Bai phenotypic PM outlier that appeared to have the EM genotype CYP2C19_wt/CYP2C19_wt, all exons and exon-intron junctions of CYP2C19 were amplified using intron-specific primers for CYP2C19 and sequenced on an automated sequencer (Applied Biosystems, Inc., Foster City, CA) using the cycle-sequence reactions with fluorescence-tagged dye terminators from a PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems Inc., Foster City, CA). This individual was found to be heterozygous for a new C → T mutation at bp 1297 of exon 9 of the cDNA. This would result in an Arg433 → Trp433 amino acid change in the homeotic region.

A PCR-based genotyping test was subsequently developed for the detection of the new Trp433 variant. This test used a forward PCR primer with a 1-bp mismatch (underlined) (5′-TCCCTATGTTGT-TATTCCAGGG-3′) from intron 8 and a CYP2C19-specific reverse primer (5′-GAGCCAGCAGCACCATCTTG-3′) from the 3′ noncoding region of exon 9, in a method similar to that described by Wang et al. (1995) for CYP2C9 alleles. The mismatched primer introduced a BstXI site in the Trp433 allele but not in the normal allele. The amplification procedure was similar to that described for detection of CYP2C19m1 and CYP2C19m2 (Goldstein and Blaisdell, 1996) except that the annealing temperature was 55°C and the number of cycles was 38. The resulting 229-bp products were digested with 10 units of BstX I at 55°C for 6 h or overnight, and the fragments were separated on 4% agarose gels. Samples containing the Trp433 allele produced 203-bp and 26-bp fragments after BstX I digestion, whereas the 229-bp product generated from the Arg433 allele remained uncleaved (fig. 1).

Data analysis. Phenotypic data were analyzed by frequency distribution plots of mephenytoin S/R ratios. Statistical analysis of the data was performed with the SAS software for Vax computers (SAS Institute, Cary, NC).

Results

The frequency distribution of the S/R ratio of mephenytoin excreted in the urine was distributed bimodally in both Han and Bai populations (fig. 2). The frequency of the PM phenotype in Han (20%, 95% confidence interval: 12%–28%) was marginally higher than in Bai subjects (13%, 95% confidence interval: 10%–17%), although this difference was not statistically significant (P = .12). There was no significant difference in the distribution profile of the S/R ratios in EMs.

Fig. 1. A PCR mismatch test for CYP2C19_Trp433. BstX I digested exon 9 PCR products were electrophoresed on a 4% gel for the detection of CYP2C19_Trp433. The forward mismatched primer (5′-TCCCTATGTTGTATTCCAGGG-3′) introduces a BstXI site in the CYP2C19_Trp433 allele but not in the normal allele. Sizes of molecular weight markers (M) are shown on the left. PCR products in lane 1 from a wt/wt individual are not digested, whereas those in lane 2 (the heterozygous CYP2C19_wt/CYP2C19_Trp433 individual) show both bands. PCR products from individuals with CYP2C19_m1 or CYP2C19_m2 alleles are identical to the wild-type allele.
between the two populations, the frequency distribution mode for Chinese Bai EMs being similar to that for Chinese Han EMs (Kolmogorov-Smirnov test; \( P > .05 \)).

In our previous study of Chinese Dong subjects (de Morais et al., 1995), we found that the S/R ratios of the EMs and PMs overlapped slightly but that an antimode of 0.78 for the urinary S/R ratio was the primary determinant of the phenotype. However, we also took into consideration the percentage of the dose excreted as 4'-OH-M and the genotyping results when determining this antimode. When the S/R ratios were compared with the genotypes in the present study, three Han subjects had borderline S/R ratios that did not clearly define the phenotype. One subject (H38) with a S/R ratio of 0.76 was considered to be a probable PM, because the genotype (\( CYP2C19_{m1}/CYP2C19_{m1} \)) agreed with the low excretion of 4'-OH-M (only 2.2% of dose), and after acidification, the S/R ratio changed only from 0.76 to 1.76. In contrast, subject H39 had a similar S/R ratio (0.79), but his genotype (\( CYP2C19_{m1}/CYP2C19_{wt} \)) was consistent with the high excretion of 4'-OH-M (25% of the dose) and with an increase in the S/R ratio after acidification from 0.79 to 2.74, which indicates that he is a probable EM subject. H54 had an intermediate S/R ratio of 0.86 and an excretion of 4.3% of the dose as 4'-OH-M. However, after acidification the urinary S/R ratio increased dramatically (from 0.86 to 16.29); this suggests that H54 is also an EM, which is consistent with the genotype (\( CYP2C19_{wt}/CYP2C19_{wt} \)).

Three Bai subjects were possible outliers. The PM genotype of subject B139 (\( CYP2C19_{m1}/CYP2C19_{m1} \)) was not consistent with the EM phenotype, indicated by a relatively low S/R ratio (0.45) and the high excretion of 4'-OH-M (18% of the dose). Similarly, the EM genotype of B140 (\( CYP2C19_{m1}/CYP2C19_{wt} \)) did not agree with the PM phenotype, indicated by a urinary S/R ratio of 0.91 or the excretion of only 0.2% of the dose as 4'-OH-M. After acidification, the S/R ratio for B139 increased dramatically from 0.43 to 9.62, which further
confirms that this individual was an EM, whereas the S/R ratio for B140 changed only from 0.84 to 0.96, a result indicative of a PM. The proximity of the numbers and the discrepancy of the two genotypes with their phenotypes strongly suggests the possibility that these two samples were inadvertently exchanged, and their data were excluded from further analyses. In contrast, subject B100 appeared to be a PM phenotypically with a high S/R ratio (0.91) that changed marginally after acidification from 1.0 to 1.45 and a low excretion of 4'-OH-M (2.4% of the dose), but genotyping predicted this subject was an EM (CYP2C19m1/CYP2C19wt). This individual thus appeared to be an outlier, and perhaps it carries an allele for some unknown inactivating mutation.

All exons of CYP2C19 were subsequently amplified and sequenced in B100, who was found to be heterozygous for a new mutation consisting of a single C → T mutation at bp 1297 in exon 9. This mutation would result in the substitution of Arg433 → Trp433 in the heme-binding region. The fact that this individual phenotypically resembles a PM suggests that the CYP2C19TRP433 allele may represent a deleterious mutation. A mismatch PCR test was designed for the CYP2C19TRP433 allele. PCR amplification with a primer containing one mismatch introduces a BstXI I site in the CYP2C19TRP433 allele but not in the normal allele (fig. 1). BstXI I digestion of sample B100 verified that this individual was heterozygous for the CYP2C19TRP433 allele. All Han and Bai samples were screened using this genotyping test, but no additional individuals carrying the CYP2C19TRP433 allele were detected.

The results of the urinary S/R ratios are summarized in table 1. S/R ratios were lower in the homozygous EMs compared with heterozygous EMs (P < .05–.001), which indicates that gene dosage affects the 4'-hydroxylation of S-mephenytoin, as suggested in our earlier study of Dong subjects (de Morais et al., 1995). Han EMs had marginally higher S/R ratios than the Bai EMs phenotypes (0.23 ± 0.02 vs. 0.21 ± 0.01, P < .05). However, there were no statistically significant differences in S/R ratios between the Han and the Bai subjects within genotypes. There appeared to be a significant sex difference in the S/R ratios among Han EMs (males vs. females, P < .05), but this was not observed in Bai EMs.

Table 2 compares the CYP2C19 allele frequencies in Chinese Han and Bai subjects. The combined frequency of CYP2C19m1 and CYP2C19m2 alleles was significantly higher in Han subjects (44%, 95% confidence interval: 0.37–0.51) than in Bai subjects (31%, 95% confidence interval: 0.26–0.36) (P < .01). This difference in the frequency of mutant alleles was due to the higher frequency of the CYP2C19m1 mutation (0.37, 95% confidence interval 0.30–0.43) in Han vs. Bai (0.26, 95% confidence interval 0.21–0.30) (P < .01) and is consistent with the somewhat higher incidence of PMs in Han. As shown in table 1 and figure 3, a higher percentage of Han EMs (60.5%) than of Bai subjects (41.1%) also exhibited heterozygous genotypes. The new mutation, CYP2C19TRP433, was found only in one PM Bai individual with an estimated allele frequency of 0.0025.

**Discussion**

CYP2C19 genotype analysis was largely consistent with the mephenytoin phenotype in the present study by a variety of criteria, including urinary S/R ratios before and after acidification and assessment of the % dose excreted as 4'-OH-M. Of 101 Han subjects, 20 (19.8%) were classified as PMs phenotypically, and 100% of these phenotypes could be explained by the two known defective CYP2C19 alleles (CYP2C19m1 and/or CYP2C19m2). Of 202 Bai subjects, 27 (13.4%) were classified as PMs phenotypically, and only one

### Table 1

**Urinary mephenytoin S/R ratios in Han and Bai subjects separated by genotype**

<table>
<thead>
<tr>
<th>Ethnic Origin</th>
<th>CYP2C19 Genotype</th>
<th>n</th>
<th>S/R Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Han</td>
<td>wt/wt</td>
<td>32</td>
<td>0.17 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>m1/wt</td>
<td>42</td>
<td>0.27 ± 0.02b</td>
</tr>
<tr>
<td></td>
<td>m2/wt</td>
<td>7</td>
<td>0.26 ± 0.03c</td>
</tr>
<tr>
<td></td>
<td>m1/wt + m2/wt</td>
<td>49</td>
<td>0.27 ± 0.02c</td>
</tr>
<tr>
<td></td>
<td>m1/m1 + m1/m2 + m2/m2 (PMs)</td>
<td>20</td>
<td>1.00 ± 0.02b</td>
</tr>
<tr>
<td></td>
<td>wt/wt + m1/wt + m2/wt (EMs)</td>
<td>81</td>
<td>0.23 ± 0.02c</td>
</tr>
<tr>
<td>Bai</td>
<td>wt/wt</td>
<td>102</td>
<td>0.16 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>m1/wt</td>
<td>64</td>
<td>0.27 ± 0.02a</td>
</tr>
<tr>
<td></td>
<td>m2/wt</td>
<td>9</td>
<td>0.24 ± 0.03b</td>
</tr>
<tr>
<td></td>
<td>m1/wt + m2/wt</td>
<td>73</td>
<td>0.26 ± 0.02a</td>
</tr>
<tr>
<td></td>
<td>m1/m1 + m1/m2 + m2/m2 + m1/Trp433 (PMs)</td>
<td>27</td>
<td>0.99 ± 0.02b</td>
</tr>
<tr>
<td></td>
<td>wt/wt + m1/wt + m2/wt (EMs)</td>
<td>175</td>
<td>0.21 ± 0.01c</td>
</tr>
</tbody>
</table>

a Different from wt/wt (P < .001).
b Different from wt/wt (P < .01).
c Different from wt/wt (P < .05).d Different from Han ethnic group (P < .05).
PMs and a higher proportion of heterozygous EMs than do Bai. These data suggest that different Chinese ethnic groups may exhibit somewhat different sensitivities to drugs metabolized by CYP2C19. CYP2C19<sub>m1</sub> and CYP2C19<sub>m2</sub> accounted for 99% of the defective PM alleles in the study. A single new mutation consisting of the substitution of Arg<sub>433</sub>→Trp<sub>433</sub> in one PM outlier suggests that this represents a new rare PM allele that is termed CYP2C19<sub>m3</sub>.

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