A Rapid and Simple Detection of Genetic Defects Responsible for the Phenotypic Polymorphism of Cytochrome P450 2C19

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A rapid and simple cytochrome P450 (CYP) 2C19 genotyping system was established by making several modifications in previously reported procedures. PCR conditions were modified to be capable of simultaneous amplification of CYP2C19m1 and CYP2C19m2 regions. Intensive bands of 169 bp for the CYP2C19m1 region and 329 bp for the CYP2C19m2 region with low background were obtained by using PCR condition involving an initial denaturation of 5 min at 94°C, 35 cycles of 1 min at 94°C; 1 min at 53°C; 1 min at 72°C, and final extension of 5 min at 72°C. Next, the optimal restriction enzyme digestion conditions were determined by using PCR products from a subject of wild genotype. Both products were completely digested with 5 U of the corresponding enzyme (Sma I for CYP2C19m1 and Bam HI for CYP2C19m2) for 1 h incubation at an optimal temperature. The incidence (16%; 5/32) of subjects homozygous for mutant alleles determined by an established assay system agreed well with the incidence of the poor metabolizer (PM) phenotype in the Japanese population. The established genotyping system would, therefore, be applicable to the clinical laboratory testing of patients with a PM phenotype of CYP2C19 to select appropriate and effective medication.

Key words CYP2C19; genotyping; PCR-restriction fragment length polymorphism (RFLP)

A genetic polymorphism associated with the 4'-hydroxylation of (S)-mephentoin is one of the most widely studied polymorphisms of cytochrome P450 (CYP) enzymes in humans.1-3 Individuals can be characterized as either extensive (EM) or poor (PM) metabolizers. The latter phenotype is inherited in an autosomal recessive fashion4-5 with the EM phenotype comprising both homozygous dominant and heterozygote genotypes. There are marked inter racial differences in the frequency of this polymorphism. The PM phenotype occurs in Oriental populations (18-23%) at a higher frequency than in Caucasian populations (2-5%).6,7 The 4'-hydroxylation of (S)-mephentoin has been shown to be mediated by CYP2C19.7,8 de Morais et al. reported that the two principal genetic defects responsible for the PM phenotype of (S)-mephentoin 4'-hydroxylation in Japanese subjects are a single-base pair mutation (guanine to adenine mutation) in exon 5 (CYP2C19m1) and/or exon 4 (CYP2C19m2) of the CYP2C19. The genotyping of subjects would, therefore, be useful for predicting the PM phenotype. The allele-specific polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) procedure has been employed for determining the genetic polymorphism of CYP2C19 in subjects. Since the previously reported PCR conditions for amplification of CYP2C19m1 and CYP2C19m2 regions differ, at least two cycles of PCR need to be performed to determine the genotype of subjects. This is laborious and time-consuming. In this report, we describe the development of a rapid and simple PCR-RFLP-based CYP2C19 genotyping system produced by optimization of the PCR conditions for simultaneous amplification of the two regions and restriction enzyme digestion conditions.

MATERIALS AND METHODS

Venous Blood Specimen One ml venous blood was collected from healthy subjects following informed consent. Samples were then divided into 200 μl aliquots in Eppendorf tubes, and kept at −20°C until required.

Simultaneous PCR Amplification of CYP2C19m1 and CYP2C19m2 Regions leukocyte genomic DNA was extracted directly from blood using a QiAamp Blood Kit (Qiagen, Tokyo) according to the instruction manual. The concentration and purity of the genomic DNA was then determined by spectrophotometer. Genomic DNA (100 ng) was amplified in 1×PCR buffer [10 mm Tris–HCl (pH 8.3), 50 mm KCl] containing 200 μm concentrations of dATP, dCTP, dGTP, and deoxyribosylthymin 5'-triphosphate (dThT), 0.3 μm of PCR primers, 1.25 units AmpliTaq DNA polymerase (Perkin-Elmer, Norwalk, CN, U.S.A.), and 3.0 mm MgCl2. Previously reported PCR primer sets8,9 were used in this study. Amplification of the two regions was performed in a separate tube with the GeneAmp 2400 PCR system (Perkin-Elmer) using an initial denaturation step of 5 min at 94°C, 35 cycles of 1 min at 94°C; 1 min at 53°C; 1 min at 72°C, and a final extension step of 5 min at 72°C. The amplified PCR products (169 bp for the CYP2C19m1 region and 329 bp for the CYP2C19m2 region) were analyzed by 2% agarose gel with a 50 bp ladder (Gibco-BRL, Rockville, MD, U.S.A.) as a molecular weight marker.

Restriction Enzyme Digestion of PCR Products For detection of the CYP2C19m1 defect, 15 μl PCR product was digested with 5 U Sma I (New England Bio.Lab, Beverly, MA, U.S.A.) in a supplemented reaction buffer in a total volume of 20 μl for 1 h at 25°C. For detection of the CYP2C19m2 defect, 15 μl of PCR product was digested with 5 U Bam HI (New England Bio.Lab) in a supplemented reaction buffer in a total volume of 20 μl for 1 h at 37°C. Both digested products were analyzed on 3% agarose gel.

RESULTS AND DISCUSSION

Previously reported PCR conditions for the amplification of CYP2C19m1 (initial denaturation step of 5 min at 94°C, 30 cycles of 1 min at 94°C, 0.5 min at 52°C, 1 min at 72°C, and final extension step of 10 min at 72°C) and CYP2C19m2© 1999 Pharmaceutical Society of Japan
Fig. 1. Amplification of the CYP2C19m1 and CYP2C19m2 Regions of Five Subjects Using a Single Set of PCR Conditions
The upper arrow (329 bp) indicates the PCR product of the CYP2C19m2 region and the lower arrow (169 bp) indicates that of the CYP2C19m1 region.

Fig. 2. Genotyping of Five Subjects by Digestion of PCR Products with Restriction Enzymes
PCR products of the CYP2C19m1 (left) and CYP2C19m2 (right) regions as shown in Fig. 1 were digested with Sma I and Bam HI, respectively, under the optimized conditions. Digestion products (size of the product is shown on both sides) were then analyzed using 3% agarose gel.

(initial denaturation step of 5 min at 94 °C, 35 cycles of 1 min at 94 °C, 0.5 min at 53 °C, 0.5 min at 72 °C, and final extension step of 5 min at 72 °C) regions did not differ significantly from each other, so we first tried to amplify the two regions simultaneously using each set of PCR conditions. Both regions were amplified by either set of conditions, and the CYP2C19m2 amplification conditions were found to be better than those for CYP2C19m1 amplification with respect to the quality and quantity of the amplicons (data not shown). We, therefore, determined the optimal PCR conditions by modifying the CYP2C19m2 amplification conditions. As a result, intensive bands of both 169 bp (CYP2C19m1 region) and 329 bp (CYP2C19m2 region) with low background were obtained by PCR conditions using an initial denaturation of 5 min at 94 °C, 35 cycles of 1 min at 94 °C, 1 min at 53 °C, and final extension of 5 min at 72 °C (Fig. 1). Determination of the template amount for PCR amplification showed that 100 ng genomic DNA, i.e. half the amount of previous procedures, was optimal for obtaining sufficient PCR products for downstream analysis (data not shown).

We next determined the optimal restriction enzyme digestion conditions using the PCR products from a subject of wt/wt genotype. The PCR products were completely digested with 5 U of the corresponding enzymes (Sma I for CYP2C19m1 and Bam HI for CYP2C19m2) for 1 h incubation at an optimal temperature. Genotyping was then performed by digestion of the PCR products from the subjects as shown in Fig. 1 under the established conditions. The wild type is assigned by the appearance of two bands of digestion products (120 bp + 49 bp for CYP2C19m1, and 233 bp + 96 bp for CYP2C19m2). On the other hand, the type homozygous for mutation is assigned by the appearance of a single band of undigested product (169 bp for CYP2C19m1, and 329 bp for CYP2C19m2). When all the products (undigested and digested) appeared on the gel, the subject was assigned as a heterozygote. As shown in Fig. 2, since the migration pattern

Table 1. Genotype Analysis of Healthy Subjects with Respect to the Two Mutations in the CYP2C19

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. of subjects</th>
<th>Incidence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt/wt</td>
<td>10</td>
<td>31.3</td>
</tr>
<tr>
<td>wt/m1</td>
<td>11</td>
<td>34.4</td>
</tr>
<tr>
<td>wt/m2</td>
<td>6</td>
<td>18.6</td>
</tr>
<tr>
<td>m1/m1</td>
<td>1</td>
<td>3.2</td>
</tr>
<tr>
<td>m1/m2</td>
<td>3</td>
<td>9.3</td>
</tr>
<tr>
<td>m2/m2</td>
<td>1</td>
<td>3.2</td>
</tr>
<tr>
<td>Total</td>
<td>32</td>
<td>100.0</td>
</tr>
</tbody>
</table>

of the products can be classified into the above categories, the new assay system is applicable to the genotyping of subjects. To evaluate the reliability of the assay system, the genotyping of 32 subjects was performed by the new procedure. Approximately 16% (5/32) of subjects were found to be homozygous for the mutant alleles responsible for the PM phenotype (Table 1). This result agreed well with the previously reported incidence of the PM phenotype in the Japanese population.

CYP2C19 is known as a key enzyme in the in vivo metabolism of a number of related hydantoins and barbiturates, as well as in the metabolism of structurally unrelated drugs such as omeprazole, lansoprazole, proguanil, and citalopram. As a result, large interphenotypic differences occur in the disposition of these drugs, which may affect their efficacy and toxicity. Moreover, approximately 20% of the Japanese population is estimated to be deficient in this enzyme. These observations strongly support the necessity of identifying the CYP2C19 genotype of patients before selecting medication. This new assay system makes it possible to perform rapid and simple genotyping that will be useful for preventing the risk of drug toxicity and for planning effective medication in patients with the PM phenotype.
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REFERENCES