**Short Communication**

**Two Novel CYP2D6*10 Haplotypes As Possible Causes of a Poor Metabolic Phenotype in Japanese**

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**ABSTRACT:**

During the course of sequencing for the CYP2D6 gene, we found a novel single nucleotide polymorphism of g.3318G>A (E383K) associated with CYP2D6*10, termed as CYP2D6*72. We also found a g.1611T>A (F120I) in the CYP2D6*49, which was previously identified as a CYP2D6*10-associated allele in an independent Japanese population. To clarify the effects of these novel CYP2D6*10 haplotypes on the functions of CYP2D6, kinetic analysis for dextromethorphan O-demethylation was performed using the *Escherichia coli* expression system and human liver microsomes. The $V_{\text{max}}/K_m$ values for dextromethorphan O-demethylation catalyzed by recombinant CYP2D6 forms encoded by CYP2D6*10, CYP2D6*49, and CYP2D6*72 were 3.0, 0.5, and 1.3%, respectively, compared with that catalyzed by CYP2D6.1. Liver microsomes from a human subject genotyped as CYP2D6*10/*49 also showed a reduced dextromethorphan O-demethylation activity. CYP2D6.49 formed a 7-hydroxydextromethorphan, with a roughly similar $V_{\text{max}}/K_m$ value to that of O-demethylation. These results suggest that these two CYP2D6*10 haplotypes are possible causes of interindividual variation in the activities and the substrate specificity of CYP2D6.

**Materials and Methods**

**Chemicals.** Dextromethorphan and dextrorphan were purchased from Wako Pure Chemicals (Osaka, Japan). NADP⁺, glucose 6-phosphate, and glucose-6-phosphate dehydrogenase were obtained from Oriental Yeast (Tokyo, Japan). All other chemicals were of the highest quality commercially available.

**Subjects.** A total of 349 unrelated healthy Japanese subjects, who received medical checkups in the Japanese Red Cross Society Kumamoto Health Care Center (Kumamoto, Japan), were recruited in this study. Liver samples were obtained as described previously (Yamaori et al., 2004). Written informed consent was obtained from each subject. This study was approved by the Ethics Committees of Hokkaido University and Kumamoto University.

**Genotyping for CYP2D6.** Genomic DNA was prepared from peripheral leukocytes or liver samples according to the method of phenol-chloroform extraction followed by ethanol precipitation. Genotyping for the CYP2D6*5 and CYP2D6*1×2 alleles was carried out according to the method described by Johansson et al. (1996). Detection of the other mutations was performed by the direct sequencing method described previously (Yamaori et al., 2003).

**Enzyme Sources.** Expression plasmids carrying cDNA coding for the CYP2D6.10 (from CYP2D6*10), CYP2D6.49 (from CYP2D6*49), or CYP2D6.72 (from CYP2D6*72), together with the NADPH-cytochrome P450 reductase cDNA were constructed by site-directed mutagenesis using wild-type CYP2D6.1 (from CYP2D6*1) constructed by Iwata et al. (1998) as a template. Each CYP2D6 together with NADPH-cytochrome P450 reductase was expressed in *Escherichia coli* cells as described by Iwata et al. (1998).

**Microsomes from human livers were suspended in 10 mM Tris-HCl buffer (pH 7.4) containing 1.0 mM EDTA and 20% (v/v) glycerol. Micromosomal protein contents were determined using Pierce BCA Protein Assay Kit (Pierce Chemical, Rockford, IL) with bovine serum albumin as a standard.**

**Measurement of Dextromethorphan O-Demethylase Activity.** Dextromethorphan O-demethylase activity was determined according to the method described below. A typical incubation mixture consisted of the membrane...
fraction of E. coli expressing CYP2D6 (50 pmol/ml) or human liver microsomes (0.2 mg protein/ml), dextromethorphan (3.5–2000 μM), and a NADPH-generating system (0.5 mM NADPH, 5 mM glucose 6-phosphate, and 1 unit/ml glucose phosphate dehydrogenase) and 50 mM potassium phosphate buffer (pH 7.4). After 15-min incubation at 37°C, the reaction was terminated by addition of 10 μl of HClO4 (60%, w/v). Protein was removed from the reaction mixture by centrifugation. A supernatant (10 μl) was injected into a liquid chromatography (LC) system (L-7100 pump, L-7200 autosampler, and L-7485 FL detector; Hitachi, Tokyo, Japan) equipped with a Mightysil RP-18 column (250 μm × 4.6 mm, 5 μm; Kanto Chemical, Tokyo, Japan). When incubated with E. coli membranes, dextromethorphan and its metabolites were separated after gradient program with mobile phase of 15% (v/v) CH3CN containing 20 mM NaClO4 (pH 2.5) for solvent A and 60% (v/v) CH3CN containing 20 mM NaClO4 (pH 2.5) for solvent B at 2.0 ml/min. Gradient program consisted of A at 100% for 20 min, and decrease of A from 100% to 0% from 20 to 35 min, followed by an isocratic segment maintaining A at 0% to 45 min. Under this chromatographic condition, the analytes had the following retention times; dextrorphan, 3.0 min; and dextromethorphan, 4.13 min. When dextromethorphan O-demethylase activity in human liver microsomes was measured, elution was performed using a Mightysil RP-18 GP Aqua column (150 μm × 4.6 mm, 5 μm; Kanto Chemical), and mobile phase consisted of 25% (v/v) CH3CN containing 20 mM NaClO4 (pH 2.5) at a flow rate of 1.5 ml/min. Under this chromatographic condition, the analytes had the following retention times; dextrorphan, 3.0 min; new metabolite, 3.9 min; 3-methoxymorphinan, 4.7 min; and dextromethorphan, 11.2 min. The elution of metabolites was monitored with excitation at 270 nm and emission at 312 nm.

Kinetic parameters for the dextromethorphan O-demethylation were estimated with a computer program (Micral Origin; OriginLab Corp., Northampton, MA) designed for a nonlinear regression analysis. Statistical comparisons were made with Dunnett’s post hoc test using StatView version 5.0 (SAS Institute, Cary, NC), and differences were considered to be statistically significant when the p value was <0.05.

Results and Discussion

Novel SNP of g.3318G>A, causing amino acid substitution (E383K), was discovered by sequencing of the CYP2D6 gene locus containing in the 5′-flanking region, all exons and introns, and 3′-untranslated region of 349 unrelated healthy Japanese. Haplotype analysis clarified that g.3318G>A was completely linked with CYP2D6*10 allele. This CYP2D6 haplotype was assigned as CYP2D6*10 by Dunnett’s test. To investigate whether or not the novel genetic polymorphisms influenced the catalytic activities of CYP2D6 in hepatic microsomes, dextromethorphan O-demethylase activities of human liver microsomes from subjects carrying different CYP2D6 genotypes were measured (Table 2). The Km values of dextromethorphan O-demethylation seen in liver microsomal samples genotyped as CYP2D6*1/*1, CYP2D6*1/*10, CYP2D6*10/*10, and CYP2D6*10/*49 were 10.2, 15.3, 23.9, and 39.7 μM, respectively. The Vmax values for these liver microsomal samples were comparable, resulting in Vmax/Km values for microsomes from livers genotyped as CYP2D6*1/*10, CYP2D6*10/*10, and CYP2D6*10/*49 that were 51, 40, and 23% of CYP2D6*1/*1 (15.4 μmol/min/mg protein), respectively. The impaired dextromethorphan O-demethylase activity of CYP2D6*49 was also seen in human liver microsomes from a subject heterozygous for the variant allele.

It is interesting to note that a new peak, suggesting the formation of a new metabolite eluted at approximately 18 min, was seen when dextromethorphan was incubated with E. coli membrane expressing CYP2D6*49 (data not shown). In LC mass spectrometry analysis, an [M+H+] ion at 288 was observed for the new metabolite, demonstrating an addition of 16 units (a hydroxy group) to dextromethorphan.

### Table 1

| CYP2D6 Allele | CYP2D6 Protein | Amino Acid Changes | O-Demethylation | 7-Hydroxylation
<table>
<thead>
<tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Km (μM)</td>
<td>Vmax (mmol/min/mmol CYP2D6)</td>
</tr>
<tr>
<td>*1</td>
<td>CYP2D6.1</td>
<td>None</td>
<td>10 ± 1</td>
<td>6.3 ± 0.9</td>
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<td>*10</td>
<td>CYP2D6.10</td>
<td>P345, S486T</td>
<td>63 ± 7</td>
<td>1.2 ± 0.2*</td>
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<tr>
<td>*49</td>
<td>CYP2D6.49</td>
<td>P345, F120L, S486T</td>
<td>323 ± 44*</td>
<td>0.9 ± 0.1*</td>
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<tr>
<td>*72</td>
<td>CYP2D6.72</td>
<td>P345, E383K, S486T</td>
<td>87 ± 21*</td>
<td>0.7 ± 0.3**</td>
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N.D., not detectable.

### Table 2

<table>
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<tr>
<th>CYP2D6 Genotype</th>
<th>Km (μM)</th>
<th>Vmax (mmol/min/mg protein)</th>
<th>Vmax/Km</th>
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<td>*1/*1</td>
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<tr>
<td>(n = 3)</td>
<td>10.2</td>
<td>157 ± 77</td>
<td>15.4</td>
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<td>*1/*10</td>
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<td>120 ± 14</td>
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<td>*10/*10</td>
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<tr>
<td>(n = 3)</td>
<td>23.9</td>
<td>148 ± 103</td>
<td>6.2</td>
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<tr>
<td>*10/*49</td>
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<tr>
<td>(n = 3)</td>
<td>39.7</td>
<td>141</td>
<td>3.6</td>
</tr>
</tbody>
</table>

* Data are represented as mean ± S.D. of liver microsomes from different individuals.
variants might cause unexpected side effects. Further studies are needed to confirm this hypothesis.

In conclusion, two novel CYP2D6*10 haplotypes (CYP2D6*49 and CYP2D6*72) can be expected to demonstrate a reduced metabolic capacity toward drugs metabolized primarily by CYP2D6. We were able to explain one of the causes of a large interindividual variation in CYP2D6 enzyme activity within CYP2D6*10 genotype group.

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Fig. 1. Representative LC chromatograms for metabolite formation from dextromethorphan catalyzed by human liver microsomes. Dextromethorphan (400 μM) was incubated in the presence of human liver microsomes genotyped as CYP2D6*10/*10 (a) and CYP2D6*10/*49 (b). The peak of dextromethorphan was noted at a retention time of 11 min. DOR, dextrorphan; 3MM, 3-methoxymorphinan; NM, new metabolite.