Evaluation of CYP2D6 Protein Expression and Activity in the Small Intestine to Determine Its Metabolic Capability in the Japanese Population

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CYP2D6 plays an important role in the metabolism of many drugs such as opioids and antidepressants. Polymorphisms of the CYP2D6 gene are widely observed in the Japanese population, and can affect the first-pass metabolism of orally administered drugs. Several CYP enzymes have been identified in the small intestine, but intestinal CYP enzymes have not been reported in the Japanese population, except for CYP3A4 and CYP2C19. In this study, we evaluated the CYP2D6 metabolic capacity by measurement of CYP2D6 mRNA and protein levels and activity in the small intestine of Japanese individuals. Normal jejunal tissues were obtained from 31 patients who had undergone pancreatectoduodenectomy, and the CYP2D6*10 variant was identified in these tissues. CYP2D6 mRNA and CYP2D6 protein levels were analyzed using real-time RT-PCR and Western blotting, respectively. Bufuralol 1'-hydroxylation, a marker of CYP2D6 activity, was analyzed using HPLC. Frequencies of the CYP2D6*1/*1, *1/*10, and *10/*10 genotypes in the jejunal tissue were 29.0% (n=9), 35.5% (n=11), and 35.5% (n=11), respectively. CYP2D6 protein and activity levels did not differ significantly between the genotypes. A positive correlation was found between CYP2D6 protein and activity levels. Furthermore, CYP2D6 protein levels and activity in the small intestine were significantly lower than those in the liver. These findings suggest that the metabolic capacity of CYP2D6 in the small intestine of the Japanese population has a relatively small effect on drug metabolism.

Key words CYP 2D6; small intestine; Japanese; polymorphism; bufuralol

CYP enzymes are present in most tissues in the body, but are most abundant in the liver. There, they play a pivotal role in the metabolism of xenobiotics and of endogenous compounds such as glucose,8–10 their presence in extrahepatic tissues, such as the esophagus, stomach, and small intestine,2–8 also contributes to drug metabolism.3–7 Paine et al. reported a significant contribution of CYP enzymes in the small intestine to the first-pass metabolism of midazolam, specifically by jejunal CYP3A.3 Expressions of CYP3A, CYP2C9, CYP2C19, CYP2D6, and CYP4F proteins have been demonstrated in the small intestine of Caucasians.8,9 Of these, CYP2D6 plays a particularly important role in drug metabolism. Although CYP2D6 accounts for only 2% of the total CYP protein in the liver, it is a very important isoform because it metabolizes approximately 20–25% of commonly used drugs, including codeine, tramadol, and tamoxifen.9 However, CYP2D6 expression and activity have not yet been characterized in the small intestine of the Japanese population.

CYP2D6 gene polymorphisms are produced from over 109 allelic variants.10 The phenotypes resulting from these polymorphisms are associated with abolished, decreased, and increased enzyme activity, and the resulting phenotypes can be classified as a poor metabolizer (PM), intermediate metabolizer (IM), extensive metabolizer (EM), or ultra-rapid metabolizer (UM), based on the level of enzyme activity.11 Thus, CYP2D6 polymorphisms greatly affect the pharmacokinetics of CYP2D6 substrates,12,13 though it is unknown whether CYP2D6 present in the small intestine also has an influence on the blood concentrations of its substrates.

CYP2D6*1 is the wild-type, or the so-called normal, allele and results in normal enzyme activity. Nishida et al. have reported allele frequencies for *10, *5, *4, and *3 of 38.1, 4.5, 0.2, and 0%, respectively, in healthy Japanese individuals.14 The reported frequencies of CYP2D6 *5, *4, and *3, which confer the PM phenotype and deficient CYP2D6 activity, are approximately 10% in Caucasians and less than 1% in the Japanese population. However, the frequency of CYP2D6*10, which confers the IM phenotype, is relatively high in Asians, and most Asian individuals have been categorized as IMs. CYP2D6*10 exhibits relatively low catalytic activity toward dextromethorphan, metoprolol, and nortriptyline.15–17

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Recently, we reported that the protein level and activity of CYP must be determined to estimate its metabolic capacity because we could not predict the metabolic capacity based on the CYP mRNA level and polymorphism in the human small intestine.\(^1\)\(^8\) A major purpose of this study was to evaluate the CYP2D6 metabolic capacity in the small intestine of Japanese individuals. We then examined CYP2D6 mRNA and protein levels and activity in small intestine tissue of Japanese surgical patients. Additionally, we compared the protein and enzyme activity levels in the intestine with those observed in the liver tissue. Finally, we determined whether CYP2D6*10 affects the metabolic capacity of CYP2D6 in the small intestine.

**MATERIALS AND METHODS**

**Human Intestine and Liver Samples** We obtained tissue specimens from the jejunal portion of the small intestine from 31 Japanese patients (21 men, 10 women) undergoing pancreatecoduodenectomy for various cancers (Table 1). All specimens were taken from normal (unaffected) tissue. The mucosa of the tissues was immediately scraped off with a knife, frozen in liquid nitrogen, and preserved at \(-80^\circ\text{C}\) until use. We also obtained normal liver tissue from 13 Japanese patients (9 men and 4 women; age range 44 to 81 years, mean = 66.7 years) undergoing partial hepatectomy for hepatocellular carcinoma (\(n = 3\)), cholangiocarcinoma (\(n = 5\)), metastatic liver cancer (\(n = 4\)), and hepatoma (\(n = 1\)). The tissue was taken at least 10 cm from the tumorous part of the liver, examined pathologically, and confirmed to be free of tumor cells. Our study was approved by the St. Marianna University School of Medicine Ethics Committee (approval number #1082).

**Immunohistochemistry** Immunohistochemistry was used to detect CYP2D6 protein. Tissue samples were fixed in 10% formalin, embedded in paraffin, and cut into 3-µm-thick sections. The paraffin-embedded sections were deparaffinized in xylene and rehydrated in a graded series of ethanol washes. Endogenous peroxidase activity in the tissue sections was inactivated with 0.1% hydrogen peroxide. Immunoreactivity in sections was visualized with a Dako EnVision system (Carpinteria, CA, U.S.A.) according to the manufacturer’s instructions. Tissues were then counterstained with hematoxylin.

**Extraction of Genomic DNA and CYP2D6 Genotyping** Genomic DNA was extracted from the small intestine tissue using the illustra Tissue and Cells genomicPrep Mini Spin Kit (GE Healthcare, Buckinghamshire, U.K.). Genotyping for the CYP2D6*10 allele was performed by PCR.\(^1\)\(^9\) The CYP2D6*10 allele was identified as the 188C \(\rightarrow\) T mutation using a 2-step PCR protocol, as described by Johansson \(et\) \(al\).\(^2\)\(^0\) The thermal cycling process was performed in a PTC-1152 thermal cycler (Bio-Rad, Tokyo, Japan) under the following conditions: 40 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 2 min, extension at 72°C for 1 min, and annealing at 58°C for 2 min, and extension at 72°C for 3 min for a double-

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cDNA was synthesized from cDNA was synthesized from a cDNA kit (RNeasy Mini, Qiagen, Germantown, MD, U.S.A.). Total RNA was extracted from the small intestine tissue using a commercial kit (SNP typing kit Cytochrome p450 CYP2D6*5, Toyo-bo, Osaka, Japan). Mutant alleles that cause defective enzyme activity and are identified in very low frequencies in Japanese individuals (*2, *4, *14, and *36) were not examined.

**RNA Extraction and Real-Time RT-PCR** Total RNA was extracted from the small intestine tissue using a commercial kit (RNeasy Mini, Qiagen, Germantown, MD, U.S.A.). cDNA was synthesized from 1 μg of total RNA using a RETROscript kit (Ambion Inc., Austin, TX, U.S.A.). Quantitative mRNA analysis was performed with a 3502S Light Cycler (Roche Diagnostics, Basel, Switzerland). Briefly, the PCR was performed in 20 μL of total reaction volume containing 2 μL cDNA, 10 pmol primers, 3 mM MgCl2, and 2 μL SYBR Green with a Light Cycler Fast Start DNA Master SYBER Green Kit (Roche Diagnostics). The primers used for CYP2D6 and CYP3A4 were obtained from the Light Cycler–Primer Set for human CYP 2D6 and 3A4 (Search LC, Heidelberg, Germany). The primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were 5′-GACAACTTTGATCCGTGGA-3′ (sense) and 5′-TACCGAGAATGAGCTTGAC-3′ (antisense). The threshold cycles (Ct) were calculated using the Light Cycler software (Version. 5.32). Standard curves were plotted as Ct-versus-log cDNA quantities, and the sample quantities were determined from the standard curves. Expression was quantified by the multiplex comparative threshold method, in which the gene expression data were normalized to the expression level of the housekeeping gene for GAPDH in each sample.

**Preparation of Microsomes** Microsomes were prepared as described previously. Briefly, the jejunal mucosae and liver were homogenized with 3 volumes of 1.15% KCl, and microsomes were prepared by sequential centrifugation of the homogenate at 9000×g for 20 min and of the resultant supernatant at 105000×g for 60 min at 4°C. The protein concentration was determined with a protein assay kit (Bio-Rad, Hercules, CA, U.S.A.).

**Western Blotting** The microsomes (100 μg protein) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% acrylamide), and then transferred to Hybond-ECL (Roche Diagnostics) membranes for 60 min. The membrane was blocked overnight at 4°C with 5% skimmed milk in Tris-buffered saline containing Tween 20 (NaCl 150 mM, Tris–HCl 100 mM, pH 7.5, Tween 0.5%). Anti-CYP2D6 mouse monoclonal antibody (BD Biosciences, Franklin Lakes, NJ, U.S.A.) was applied to the blots for 2 h at room temperature. Anti-polyclonal antibody (Wako, Osaka, Japan) was used as the loading control. Reactive proteins were viewed using enhanced chemiluminescence (Immunostar, Wako). Signal intensities of the detected bands were analyzed using a C-DiGit Chemiluminescent Western blot Scanner (LI-COR Biosciences, Lincoln, NE, U.S.A.). Protein expression was quantified by densitometric analysis. The expression level of α-tubulin was confirmed as a control. The inter-individual and inter-tissue differences in α-tubulin levels in the small intestine and liver samples were less than three-fold. The data of the CYP2D6 protein level was shown as the density in 1 mg protein.

**Determination of CYP2D6 Activity by HPLC** To investigate CYP2D6 activity, 1′-hydroxybufuralol was assayed as described previously, with minor modifications. The reaction mixtures contained human jejunal microsomes (HJMs) (50 μg protein) or liver microsomes (HLMs) (15 μg protein), 10 μM bufuralol, and a reduced nicotinamide adenine dinucleotide phosphate (NADPH)-generating system in 100 mM potassium phosphate buffer (pH 7.4), and were incubated at 37°C.
for 20 min (HJMs) or 15 min (HLMs). A Capcell Pak C18 SG120 HPLC column (4.6×250 mm², Shiseido, Tokyo, Japan) was used for detecting 1'-hydroxybufuralol. A portion of the reaction mixture was injected into the column and separated with a mobile phase of 20 mM NaClO₄ (pH 2.5) and acetonitrile (70:30) at a rate of 1 mL/min. The HPLC instrumentation included an SCL-10A VP system controller, a VC-10AD VP pump, a DGU-14-A degasser, an SIL-10AD VP auto-sampler (Shimadzu, Kyoto, Japan), a Hitachi L-7485 fluorescence detector (excitation 252 nm, emission 302 nm), and a Fujitsu computer running Shimadzu CLASS-VP software.

**Statistical Analyses** All values are expressed as mean±standard deviation (S.D.). The Steel–Dwass test was used for multiple comparisons, and the non-parametric Mann–Whitney test was used to analyze between-group differences in protein levels and in protein activity. Correlation between CYP2D6 activity and protein expression levels was tested by Pearson’s product–moment correlation coefficient. All statistical analyses were performed with KyPlot 5.0 (Kyes Lab, Tokyo, Japan), and p<0.05 was considered significant.

**RESULTS**

**Localization of CYP2D6 in the Small Intestine** To determine whether CYP2D6 protein is present in the small intestine of Japanese individuals, we immunostained jejunal sections for CYP2D6. CYP2D6 was detected in enterocytes of the jejunal mucosal epithelium and lamina propria (Fig. 1).

**Effect of CYP2D6*10 Genotype on CYP2D6 mRNA Levels in the Small Intestine** To certify the quality of the intestinal samples, we analyzed CYP3A4 mRNA expression in the same samples as those used for CYP3A4 mRNA analysis. CYP3A4 mRNA levels (353.3±341.8×10⁶) were 13-fold higher than CYP2D6 mRNA levels (26.8±25.4×10⁶) (Fig. 2A).

The frequencies of the CYP2D6*1/*1, *1/*10, and *10/*10 genotypes in the small intestine samples were 29.0% (n=9),
35.5% (n=11), and 35.5% (n=11), respectively. The CYP2D6*5 allele was not detected in any sample. To clarify the effect of these CYP2D6 genotypes on CYP2D6 transcription, we analyzed CYP2D6 mRNA levels. The mean CYP2D6 mRNA level in the CYP2D6*10 homozygotic tissue (45.6±19.9×10⁴) was significantly (p<0.01) higher than that in the CYP2D6*1 homozygotic tissue (5.8±3.7×10⁴) or the CYP2D6*1/*10 heterozygotic tissue (19.6±12.0×10⁴), although individual differences in CYP2D6 mRNA levels were observed within each genotype (Fig. 2B).

**CYP2D6 Protein Levels and Activity in the Small Intestine** Next, we evaluated the effect of CYP2D6 polymorphisms on CYP2D6 protein levels and activity. When we measured the quantity of protein in some intestinal samples using recombinant CYP2D6 (BD Biosciences) as standard, we confirmed that there was correlation between the quantified protein levels and band density (R=0.864). The correlation (R=0.625, p=0.011) was found between CYP2D6 protein levels and enzymatic activity (Fig. 3A). CYP2D6 protein levels did not differ significantly between the genotypes (Fig. 3B).

The mean CYP2D6 activity associated with CYP2D6*1/*1 (2.6±1.4 pmol/mg protein/min) tended to be higher than that associated with CYP2D6*1/*10 (0.8±0.4 pmol/mg protein/min, p=0.106) and with CYP2D6*10/*10 (1.3±0.4 pmol/mg protein/min, p=0.348) (Fig. 3C).

**CYP2D6 Protein Levels and Activity in Small Intestine vs. Liver** Results for Western blotting for CYP2D6 in the small intestine and liver of Japanese are shown in Fig. 4A. CYP2D6 protein levels were significantly lower in the small intestine (5.5±2.7×10⁴ counts/mg protein) than in the liver (16.2±3.2×10⁴ counts/mg protein) (Fig. 4B). Specific CYP2D6 genotypes were not determined in this experiment because of the limited volume of the liver sample.

CYP2D6 activity in the small intestine (1.6±1.2 pmol/mg protein/min) was much lower than that in the liver (67.9±43.6 pmol/mg protein/min) (Fig. 4C).

**DISCUSSION**

Human small intestine epithelial cells are the site of initial biotransformation of orally ingested xenobiotics. Recent studies have shown that metabolism by small intestine CYP proteins can affect the toxicity and therapeutic efficacy of xenobiotics. In this study, we evaluated the metabolic capacity of CYP2D6 in the small intestine of Japanese patients based on CYP2D6 protein level and activity, and investigated the effect of genetic polymorphisms on CYP2D6 metabolic capacity.

Previous studies have shown that CYP2D6 is expressed in the human small intestine. Using immunohistochemistry, we found that CYP2D6 protein was localized in the enterocytes and lamina propria of the small intestine of Japanese individuals. Although CYP2D6 mRNA levels were lower than the CYP3A4 mRNA levels in the samples, we did find that CYP2D6 mRNA was expressed in the small intestine of Japanese individuals. Previous studies have reported that CYP3A4 mRNA levels were quantified in human intestine. The present results of CYP3A4 mRNA stability in the small intestine is preserved. We verified that CYP3A4 mRNA levels were expressed on average at high levels. Further, these findings are consistent with those of previous studies.

CYP2D6 genetic polymorphisms have been shown to be the major cause of variation in the metabolism of tamoxifen, and the polymorphisms can thus lead to adverse effects or lack of therapeutic efficacy. CYP2D6 genotypes include CYP2D6*1 (wild-type), *2, *4, *5, and *10. Of the possible CYP2D6 alleles, CYP2D6*10 occurs at a frequency of 34.6–71.0% in
Japanese individuals, but only 1–2% in Caucasians.\textsuperscript{10,11,15,19) Because the IM phenotype produced by CYP2D6*10 exhibits lower catalytic activity than that of the phenotype produced by CYP2D6*1,\textsuperscript{32,33) CYP2D6*10 is an important allele in the Japanese population, as it confers interindividual differences in the pharmacokinetics of CYP2D6 substrates. The allele frequency CYP2D6*10 was 53.2%. The allele frequency for CYP2D6*10 we observed was similar to that reported previously (34.6–71.0%).\textsuperscript{10,11,15,19) Additionally, the mean CYP2D6 mRNA levels in patients with the CYP2D6*10/*10 genotype were significantly higher than those in patients with CYP2D6*1/*1 and CYP2D6*1/*10 genotypes. Consistent with this, CYP2D6*10 is believed to be the allele that encodes CYP2D6 protein with lowered stability, and the higher mRNA levels in patients with CYP2D6*10/*10 may be due to a tandem-type CYP2D6*36-*10/*10 allele, which produces four copies of CYP2D6 mRNA.\textsuperscript{34) Further study is needed to evaluate whether the other CYP2D6 alleles, including the tandem-type *36-*10 might be included in the examined Japanese subjects.

In contrast to the mRNA results, there was no statistical difference in CYP2D6 protein or activity levels between the genotypes, though the protein and activity levels in patients with the CYP2D6*10 allele tended to be lower than those in the other groups. These findings indicate that the CYP2D6*10 allele has only a small influence on CYP2D6 activity levels in the small intestine. Additionally, CYP2D6 activity levels in the small intestine of the subjects were significantly lower than those in the liver. Therefore, CYP2D6 in the small intestine of Japanese individuals does not appear to contribute as much to drug metabolism as does CYP2D6 in the liver. We showed that CYP2D6 protein levels in the liver were two-fold higher than that in the small intestine, whereas the activity in the liver was 42-fold higher than that in the small intestine. For example, the presence of enteric bacteria is known to alter the mRNA or protein expression and activity of hepatic drug metabolizing enzymes.\textsuperscript{35) The difference in the enzyme activity in the small intestine and liver may be related to induction or inhibition factors included in each tissue microsome.\textsuperscript{36) This will have to be investigated in detail because the cause has not been clarified yet in human tissues.

We found no correlation between CYP2D6 mRNA levels and CYP2D6 protein levels. This seemingly paradoxical finding is attributable to mechanisms that are involved in post-translational processing steps of CYP2D6 in the small intestine. For example, CYP3A4 protein expression is partly regulated by post-translational machinery.\textsuperscript{37) Additionally, glycoprotein 78 (gp78) and C-terminal heat shock protein 70 (Hsp70)-interacting protein
(CHIP) E3 ligases cooperatively degrade CYP3A4 protein via its ubiquitination in human liver. This mechanism is believed to be independent of the transcriptional steps; therefore, the discrepancy between CYP2D6 mRNA and CYP2D6 protein levels might be the result of inter-individual differences in post-translational regulation. Furthermore, it remains unclear whether CYP2D6 more rapidly degraded in the small intestine than in the liver. Further investigation is necessary to clarify this issue.

A limitation of this study is that we did not examine other CYP2D6 alleles besides the CYP2D6*10 allele in the small intestine samples we obtained. Additionally, we used jejunal tissues for the study, but did not examine all parts of the small intestine. Further studies are needed to fully characterize CYP2D6 genetics in the small intestine of the Japanese population.

In conclusion, we found that CYP2D6*10 polymorphism did not influence the metabolic capacity of CYP2D6 in the small intestine. Our findings suggest that the metabolic capacity of CYP2D6 in the small intestine of Japanese individuals has a relatively small effect on the metabolism of orally administered drugs in comparison to that of CYP2D6 in the liver.

**Conflict of Interest** The authors declare no conflict of interest.

**REFERENCES**


