The CYP3A4*18 Allele, the Most Frequent Coding Variant in Asian Populations, Does Not Significantly Affect the Midazolam Disposition in Heterozygous Individuals

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ABSTRACT:

The objective of this study was to identify CYP3A4 variants in Koreans and to characterize their functional consequences in vitro and in vivo. Four single nucleotide polymorphisms were identified in 50 Koreans by direct DNA sequencing. In an additional genotyping using 248 subjects, CYP3A4*18 was confirmed as the most frequent coding variant in Koreans at 1.7%, and its frequency was similar to that of Asians, suggesting that CYP3A4*18 would be the highest coding variant in Asians. The recombinant CYP3A4*18 protein prepared in baculovirus expression system showed 67.4% lower V_max and 1.8-fold higher K_m for midazolam 1'-hydroxylation compared with the wild type. The mean values of C_max and area under the concentration curve (AUC) in the CYP3A4*1/*18 and CYP3A5*1/*3 subjects (n = 8) were 63% and 32% higher than in CYP3A4*1/*1 and CYP3A5*1/*3 carriers (n = 8), respectively. Although the in vitro assay exhibited a significant reduction of the enzyme activity for midazolam, the in vivo differences associated with the CYP3A4*1/*18 tend to be low (P < 0.07 in C_max and P < 0.09 in AUC). In summary, the heterozygous CYP3A4*1/*18 does not appear to cause a significant change of midazolam disposition in vivo; however, the clinical relevance of CYP3A4*18/*18 remains to be evaluated.

CYP3A is the most abundantly expressed subfamily of cytochrome P450 (P450) enzymes in the human liver (Shimada et al., 1994). The CYP3A enzymes are responsible for the metabolism of more than 50% of clinically used drugs (Komori et al., 1990; Guengerich, 1999; Lamba et al., 2002a). Four human CYP3A enzymes, CYP3A4, CYP3A5, CYP3A7, and CYP3A43, have been identified. CYP3A4 is regarded as the most dominant CYP3A enzyme in the liver and small intestine of humans. It has been reported that CYP3A4 expression shows large interindividual variation (Guengerich, 1999; Ozdemir et al., 2000; Lin et al., 2002). These variations can lead to different responses to human drugs that are substrates for CYP3A4. Because approximately 85% of this variability is attributed to genetic factors (Ozdemir et al., 2000), genetic analysis is needed to understand interindividual variability. To date, more than 39 allelic variants have been described (http://www.cypalleles.ki.se/cyp3a4.htm) (Dai et al., 2001; Eiselt et al., 2001; Kuehl et al., 2001; Lamba et al., 2002a; Fukushima-Uesaka et al., 2004). Among the CYP3A4 variants, alleles with nonsynonymous single nucleotide polymorphisms (SNPs), i.e., CYP3A4*2, *4, *5, *6, *17, and *18, have been shown to alter enzyme activity, compared with the wild type (Lee and Goldstein, 2005). Although some CYP3A SNPs exhibited an altered intrinsic clearance of CYP3A substrates in vitro, there have been few data explaining their meaningful influences on its substrate clearance in humans. The CYP3A4*1B promoter SNP has been extensively studied because of its role in transcriptional regulation in vitro. However, no significant change associated with CYP3A4*1B was observed in midazolam (MDZ) clearance (Wandel et al., 2000; Garcia-Martin et al., 2002; Eap et al., 2004; He et al., 2005) and in cyclosporine clearance (Rivory et al., 2000), supporting the idea that this SNP may not have a significant role in the CYP3A4 expression (Westlind et al., 1999). Genetic polymorphisms in CYP3A4 gene seem to be the reason for interindividual variation in the enzyme activity; however, no key variant alleles of CYP3A4 have been found to support these variations (Lamba et al., 2002a).

CYP3A4*18 has been identified in 3 of the 118 Japanese subjects...
(Yamamoto et al., 2003), 22 of the 416 Japanese subjects and 6 of the 302 Chinese subjects (Hu et al., 2005), and 1 of 60 subjects in a Chinese population (Du et al., 2006). However, there have been no discovery studies on CYP3A4 genetic polymorphisms and the report of CYP3A4*18 allele in the Korean population. Therefore, we sequenced the CYP3A4 gene in 50 subjects and evaluated the allele frequency in an additional 248 Korean subjects. Although the CYP3A4*18 is a high frequency allele in Asians, there have been no in vivo clinical reports associated with the CYP3A4*18 allele. Therefore, we evaluated for the first time the clinical relevance of CYP3A4*18 with respect to the disposition of MDZ in humans.

Materials and Methods

Chemicals and Materials. The QIAamp blood kit was obtained from QIAGEN (Valencia, CA). All of the restriction enzymes were purchased from TaKaRa Bio (Shiga, Japan), and the primers were synthesized by Bioneer (Daejon, Korea). MDZ and the two MDZ metabolites, 1'-OH-MDZ, and 4-OH-MDZ, were purchased from Ultrafine Chemical (Manchester, UK). Phenacetin was obtained from Sigma-Aldrich (St. Louis, MO). All other reagents and chemicals were of analytical or HPLC grade.

Subjects. In all, 298 Korean subjects were recruited for the CYP3A4 genotyping study. Genomic DNA samples prepared from 50 of the 298 subjects were sequenced directly to identify CYP3A4 sequence variants and the DNA samples from the remaining subjects were used to screen for the identified functional CYP3A4 SNPs by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) or pyrosequencing. All of the participants were healthy, according to medical history, physical examination, and routine laboratory tests. All subjects provided written informed consent before participating in the present study, which was approved by the Institutional Review Board of Busan Paik Hospital (Busan, Korea).

Direct DNA Sequencing and PCR-RFLP. Genomic DNA was extracted from the peripheral whole blood of 298 subjects using the QIAamp blood kit and routine laboratory tests. All subjects provided written informed consent. The PCR products were sequenced by pyrosequencing (Pyrosequencing AB, Uppsala, Sweden). The CYP3A5*3 allele was detected using a previously published method (Roy et al., 2005). The 293-bp DNA fragment that contains the CYP3A5*3 allele was amplified with the PCR primers 5'-CATGACTTAGTAGACAGATGA-3' and 5'-TGGTTGAATGAAA-3', and for CYP3A4*16, 5'-CTCACAGCATGGATGTGA-3'. The resulting mixture was analyzed on the PSQ 96MA Pyrosequencing platform.

Expression of Recombinant Wild-Type and Mutant CYP3A4 Proteins. The CYP3A4 and NADPH-CYP (P450) oxireductase (CPR) genes were cloned by PCR using the cDNA prepared from total liver RNA. The primers used for CYP3A4 amplification were: 5'-GTGCAGACTGCTCTCTATCCA-3' and 5'-GTGCCATAGTGCCTCTATCCA-3'. The primers used for CPR amplification were: 5'-CTCGGATGGGAGACTCCCAC-3' and 5'-GTGACCTCTATTGCACCCTGC-3'. The PCR products were ligated into the pGem T-Easy vector (Promega, Madison, WI), and the complete open reading frames for the cloned inserts were verified by sequencing. The full-length cDNA that encodes the CYP3A4.18 (L293P) protein was generated by an overlap extension procedure (Ho et al., 1989) using the specific primers 5'-CAAGACGTCCTGCGTGGAATC-3' and 5'-ACCATGGAAGGAGATGACTTCTGCCGC-3'. Introduction of the mutation was verified by DNA sequencing.

Enzyme Kinetics. The enzymatic activities of the wild-type and mutant CYP3A4 proteins were determined using MDZ as a prototype substrate. The incubation mixtures (0.25 ml) were composed of 5 pmol of cytochrome P450 in 50 mM potassium phosphate buffer (pH 7.4). The MDZ concentration range for the kinetic analysis was 0.5 to 25 μM. After a 5-min preincubation step at 37°C, the reactions were initiated by the addition of the NADPH-generating system (3.3 mM glucose 6-phosphate, 1.3 mM NADPH, and 3.3 mM MgCl2, 1 U/ml glucose-6-phosphate dehydrogenase), and the reactions were incubated at 37°C for 30 min. The reaction was stopped by placing the incubation tube on ice and adding 100 μl of ice-cold acetonitrile. The incubation mixtures were then centrifuged at 20,000g for 10 min at 4°C. Aliquots (5 μl) of the supernatants were injected into the API 3000 LC-MS/MS system (Applied Biosystems), which comprises the Agilent 1100 series HPLC (Agilent, Wilmington, DE) and the API 3000 tandem mass spectrometer (Applied Biosystems). The liquid chromatograms were obtained by separation on a Luna C18 column (2 × 50 mm, 3 μm; Phenomenex, Torrance, CA) in an isocratic mobile phase of acetonitrile and water (3:7, v/v) at a flow rate of 0.2 ml/min. Detection of 1'-OH-MDZ was performed by monitoring the transitions of m/z 342 to 203. The apparent kinetic parameters of 1'-OH-MDZ were determined by fitting the unweighted kinetic data to the one-enzyme Michaelis-Menten equation (WinNonlin; Pharsight; Mountain View, CA). The kinetics observed for 1'-OH-MDZ exhibited the best fit with the Michaelis-Menten equation, as reported previously (Williams et al., 2002). All of the data are presented as mean ± S.D. The biotinylated PCR product was immobilized onto streptavidin-coated beads (Streptavidin Sepharose High Performance; Amersham Biosciences, Uppsala, Sweden). The beads were transferred to a filter plate; then, the liquid was removed by vacuum filtration (Multiscreen Resist Vacuum Manifold; Millipore, Bilérica, MA), and the double-stranded DNAs were separated in denaturation solution (0.5 M NaOH). The immobilized template was washed with 10 mM Tris-acetate (pH 7.6), transferred to a PSQ 96 plate, and resuspended in 20 mM Tris-acetate (pH 7.6) annealing buffer that contained the following sequencing primers: for CYP3A4*11, 5'-GACATG-7k to 5'-GTTGGAATGAAA-3'; and for CYP3A4*16, 5'-CTCACAGCATGGATGTGA-3'. The resulting mixture was analyzed on the PSQ 96MA Pyrosequencing platform.

Pharmacokinetics of MDZ. Eight subjects who carried the CYP3A4 1/*1 and CYP3A5*1/*3 alleles, seven who carried the CYP3A4*1/*1 and
**EFFECTS OF CYP3A4*18 ON MIDAZOLAM DISPOSITION**

### TABLE 1

**CYP3A4 genetic variants identified in a Korean population**

<table>
<thead>
<tr>
<th>SNP</th>
<th>Number of Subjects</th>
<th>Location</th>
<th>Effect</th>
<th>Allele</th>
<th>Activity (in vitro)</th>
<th>Number of Alleles</th>
<th>Observed Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>3857C&gt;T</td>
<td>50</td>
<td>Intron 2</td>
<td></td>
<td></td>
<td></td>
<td>4</td>
<td>4*</td>
</tr>
<tr>
<td>13880A&gt;G</td>
<td>298</td>
<td>Exon 5</td>
<td></td>
<td>I118V</td>
<td>*d</td>
<td>Decreased</td>
<td>0</td>
</tr>
<tr>
<td>1571C&gt;G</td>
<td>298</td>
<td>Exon 7</td>
<td></td>
<td>P218R</td>
<td>*5</td>
<td>Decreased</td>
<td>1</td>
</tr>
<tr>
<td>17670Ains</td>
<td>298</td>
<td>Exon 9</td>
<td></td>
<td>Frameshift</td>
<td>*6</td>
<td>None</td>
<td>5</td>
</tr>
<tr>
<td>21876C&gt;T</td>
<td>298</td>
<td>Exon 11</td>
<td></td>
<td>T363M</td>
<td>*11</td>
<td>Decreased</td>
<td>1</td>
</tr>
<tr>
<td>1561C&gt;G</td>
<td>298</td>
<td>Exon 7</td>
<td></td>
<td>T185S</td>
<td>*16</td>
<td>Decreased</td>
<td>1</td>
</tr>
<tr>
<td>20097T&gt;C</td>
<td>298</td>
<td>Exon 10</td>
<td></td>
<td>L293P</td>
<td>*18</td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>20239G&gt;A</td>
<td>50</td>
<td>Intron 10</td>
<td></td>
<td></td>
<td></td>
<td>13</td>
<td>13*</td>
</tr>
</tbody>
</table>

* Nucleotides are numbered according to the reference sequence NC_000007.

* The recombinant CYP3A4.18 enzyme expressed in *Escherichia coli* showed enhanced metabolism of testosterone and chlorpyrifos (Dai et al., 2001). However, the same variant, when expressed in HepG2 cells, does not affect testosterone hydroxylation (Murayama et al., 2002).

### Results

From the sequencing data of all the exon regions, proximal promoter (from +71 bp to –1.1 kb), and a distal region containing the XREM at –7836 through –7607 bp of the CYP3A4 gene, four CYP3A4 variants were identified (Table 1). No novel variants were identified in the present study. The four variants are g.3857C>T (found in four individuals as all heterozygous mutation in intron 2), g.17670Ains (CYP3A4*6) and g.20097T>C (CYP3A4*18) (found in one individual each as a heterozygous mutation), and g.20239G>A (found in 13 individuals as all heterozygous mutation in intron 10). To determine the allelic frequency of CYP3A4 variants in a Korean population, the presence of CYP3A4*18, *5, *6, *11, *16, or *18 was detected by PCR-RFLP or pyrosequencing in 248 additional subjects. We included CYP3A4*4 for genotyping because this allele has been reported at a frequency of 1.5% in a Chinese population (Hsieh et al., 2001) and was expected to be observed in Koreans. However, we could not find the CYP3A4*4 allele in the present 298 Korean subjects. Except for the CYP3A4*14 allele, there were no significant differences in the allele frequency when compared with those of Asians. The overall allelic frequencies of the CYP3A4 variants are summarized in Table 1. From the PCR-RFLP, we identified three CYP3A4*6 carriers and eight CYP3A4*18 carriers. The most frequent mutant allele was CYP3A4*18, which showed a frequency of 1.7% in 298 Korean subjects. All eight individuals who carried the CYP3A4*18 allele were heterozygous for the CYP3A5*3 allele. The frequencies of all the other variants were <1%, and none of the coding variants were linked to other alleles as homozygote. Interestingly, all eight individuals who carried the CYP3A4*18 allele were heterozygous for the CYP3A5*1 allele.

The wild-type CYP3A4.1 and mutant CYP3A4.18 proteins were expressed together with CPR in S9 cells. The immunoblot of CYP3A4 showed similar levels of expression for the CYP3A4.1 and CYP3A4.18 proteins in our expression system (Fig. 1A). The expression levels of CPR in both the CYP3A4.1 and CYP3A4.18 expression systems were also similar (data not shown). A typical CO-difference spectrum with an absorbance maximum at 450 nm was observed for both the CYP3A4.1 and CYP3A4.18 proteins (Fig. 1B). The kinetics of metabolite formation was examined for the wild-type and mutant CYP3A4.18 using MDZ as the probe substrate. The wild-type CYP3A4.1 protein catalyzed the formation of substantial amounts of 1'-OH-MDZ (Fig. 2). Relative to the wild-type enzyme, CYP3A4.18 produced lower levels of 1'-OH-MDZ at all the tested concentrations of MDZ (Fig. 2). The kinetic parameters for 1'-OH-MDZ were determined for the CYP3A4.1 and CYP3A4.18 enzymes. The *Km* value of 1'-OH-MDZ in our reconstitution system was low compared with the values obtained from the *Escherichia coli* expression system.
Comparisons of the kinetic parameters of midazolam 1'-hydroxylation by the CYP3A4.1 and CYP3A4.18 proteins

The values are estimated from nonlinear least-squares regression analysis using WinNonlin software (Pharsight). The reactions include equal amounts of P450 (5 pmol) from the CO spectrum analysis, not P450 + P420. Detailed procedures are described under Materials and Methods.

<table>
<thead>
<tr>
<th></th>
<th>Midazolam 1'-Hydroxylation</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$V_{\text{max}}$ ($\mu\text{mol/min/pmol}$)</td>
<td>$K_m$ ($\mu\text{M}$)</td>
</tr>
<tr>
<td>CYP3A4.1</td>
<td>4.3 ± 0.3</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>CYP3A4.18</td>
<td>1.4 ± 0.1</td>
<td>0.7 ± 0.4</td>
</tr>
</tbody>
</table>

Discussion

It is well known that the CYP3A4 enzyme shows large interindividual variability in activity and expression level. This polymorphic activity of CYP3A4 may generate interindividual variations in drug responses. One possible cause for the polymorphic activity is genetic variation of the CYP3A4 gene (Sata et al., 2000; Murayama et al., 2002; Amirimani et al., 2003; Min and Ellingrod, 2003; Matsumura et al., 2004). In the present study, we identified for the first time, to our knowledge, genetic variants of CYP3A4 in a Korean population. Four variants, two in the exon (CYP3A4*6, *18) and two in the intron, were identified in the direct DNA sequencing. The frequency of alleles with coding SNPs (from CYP3A4*2 to CYP3A4*20) was <5% in all of the ethnic groups studied (Eiselt et al., 2001; Hsieh et al., 2001; Lamba et al., 2002b; Cavaco et al., 2003; Fukushima-Uesaka et al., 2004; Westlind-Johansson et al., 2006). Despite their low frequencies, certain allelic variants have been observed exclusively in a specific subset of ethnic groups. For example, CYP3A4*4, *5, *6, and *16 have been found exclusively in Asians, including Chinese (Eiselt et al., 2001), Japanese (Lamba et al., 2002b), and Korean populations (except *4 in the present study), but not in Caucasians. On the other hand, CYP3A4*7, *8, *9, and *10 have been observed exclusively in Caucasians (Eiselt et al., 2001). CYP3A4*17 has been found in the Adygei ethnic group (Lee et al., 2005a). Ethnic differences in CYP3A4 polymorphisms are highlighted by the fact that the allelic frequency of CYP3A4*1B in blacks (66.7%) is much higher than in Caucasians (4–6.5%) and Asians (0%) (Sata et al., 2000; Lamba et al., 2002b; Cavaco et al., 2003). Of the 22 alleles...
with coding SNPs, nine nonsynonymous CYP3A4 variants have been shown to be associated with altered catalytic activities (http://www.cypalleles.ki.se/cyp3a4.htm). In brief, CYP3A4.2 has lower enzymatic activity for nifedipine, but there is no effect on 6β-hydroxytestosterone activity (Sata et al., 2000). CYP3A4.12 shows greater activity for 15β- and 2β-hydroxytestosterone than does the wild type (Eiselt et al., 2001). CYP3A4.17 reduces testosterone 6β-hydroxylation, chlorpyrifos desulfuration, and nifedipine oxidation (Dai et al., 2001; Lee et al., 2005a), whereas CYP3A4.18 enhances the catalytic activities for testosterone and chlorpyrifos (Dai et al., 2001). The in vitro functionality of the CYP3A4.18 protein has proven to be controversial. One report has revealed that CYP3A4.18 expressed in E. coli shows a 2-fold increase in catalytic activity for testosterone. However, according to another report, the CYP3A4.18 protein, when transiently expressed in HepG2 cells, catalyzes 6β-hydroxytestosterone to an extent similar

### Table 3

<table>
<thead>
<tr>
<th>Genotype</th>
<th>CYP3A4*1/<em>1/CYP3A5</em>1/*3 n=8</th>
<th>CYP3A4*1/<em>1/CYP3A5</em>1/*3 n=7</th>
<th>CYP3A4*1/<em>18/CYP3A5</em>1/*3 n=8</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MDZ</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>t1/2 (h)</td>
<td>2.5 ± 0.6</td>
<td>2.5 ± 0.6</td>
<td>2.9 ± 0.8</td>
</tr>
<tr>
<td>T_max (h)</td>
<td>1.3 ± 0.8</td>
<td>0.6 ± 0.2</td>
<td>0.9 ± 0.9</td>
</tr>
<tr>
<td>C_max (ng/ml)</td>
<td>35.4 ± 11</td>
<td>56.6 ± 29</td>
<td>57.7 ± 30</td>
</tr>
<tr>
<td>CL/F (l/h/kg)</td>
<td>1.0 ± 0.4</td>
<td>0.8 ± 0.1</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>AUCinf (ng/h/ml)</td>
<td>112 ± 36</td>
<td>132 ± 63</td>
<td>148 ± 43</td>
</tr>
<tr>
<td>1′-OH-MDZ</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>t1/2 (h)</td>
<td>2.3 ± 0.6</td>
<td>2.5 ± 0.5</td>
<td>2.2 ± 0.5</td>
</tr>
<tr>
<td>T_max (h)</td>
<td>1.2 ± 0.8</td>
<td>0.6 ± 0.2</td>
<td>1.2 ± 1.1</td>
</tr>
<tr>
<td>C_max (ng/ml)</td>
<td>18.2 ± 7.8</td>
<td>27.2 ± 14</td>
<td>27.7 ± 12</td>
</tr>
<tr>
<td>AUCinf (ng/h/ml)</td>
<td>46.9 ± 12</td>
<td>51.7 ± 17</td>
<td>61.9 ± 20</td>
</tr>
<tr>
<td>4-OH-MDZ</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>t1/2 (h)</td>
<td>2.0 ± 0.5</td>
<td>2.8 ± 0.7*</td>
<td>2.5 ± 0.6</td>
</tr>
<tr>
<td>T_max (h)</td>
<td>1.2 ± 1.0</td>
<td>0.71 ± 0.3</td>
<td>1.3 ± 1.1</td>
</tr>
<tr>
<td>C_max (ng/ml)</td>
<td>2.1 ± 0.6</td>
<td>2.9 ± 0.8*</td>
<td>2.8 ± 1.2</td>
</tr>
<tr>
<td>AUCinf (ng/h/ml)</td>
<td>6.5 ± 1.6</td>
<td>8.9 ± 3.1</td>
<td>9.2 ± 3.0†</td>
</tr>
</tbody>
</table>

* Tmax, time to maximum plasma concentration; t1/2, terminal elimination half-life; CL/F, total oral clearance; AUCinf, area under the concentration time curve from zero to last observation.

* P < 0.05, Student’s t test for 3A4*1/*1 and 5A5*1/*3 versus 3A4*1/*1 and 5A5*1/*3 groups.

† P < 0.05, Student’s t test for 3A4*1/*1 and 5A5*1/*3 versus 3A4*1/*18 and 5A5*1/*3 groups.

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**Fig. 3.** Time profiles of the MDZ plasma concentrations and hydroxy metabolites. A, plasma MDZ; B, plasma 1′-OH-MDZ; C, plasma 4-OH-MDZ. After oral administration of 7.5 mg of MDZ, plasma was serially collected and analyzed for MDZ content, as described under Materials and Methods. Closed circle, CYP3A4*1/*1 and CYP3A5*1/*3 subjects; closed square, CYP3A4*1/*1 and CYP3A4*1/*18 subjects; open square, CYP3A4*1/*1 and CYP3A5*3/*3 subjects. Each point represents the mean ± S.D.

**Fig. 4.** Scatter plots of C_max (A), AUCinf (B), and oral clearance (CL/F) (C), as estimated after oral administration of a single dose of 7.5 mg of MDZ. ●, CYP3A4*1/*1 and CYP3A5*1/*3 subjects; ○, CYP3A4*1/*1 and CYP3A5*3/*3 subjects; □, CYP3A4*1/*18 and CYP3A5*1/*3 subjects. Each symbol with a bar indicates the mean ± S.E. of a group. The P values were determined by Student’s t test for differences between the CYP3A4 and CYP3A5 genotype groups.
to that of wild type. These contradictory in vitro results could be explained by the different expression systems used in the different laboratories. In the present study, CYP3A4.18 expressed in baculovirus-infected SF9 cells showed a small decrease in MDZ metabolism. The different effects of CYP3A4.18 on MDZ metabolism may be explained by the presence of multiple substrate recognition sites on CYP3A4, resulting in the variable activities depending on different substrates. According to Dai et al. (2001), the side-chain of the L293 residue is largely masked, being packed into the interior of the CYP3A4 protein. Owing to the nonconservative nature of the L293P amino acid substitution, this mutation may affect protein conformation, substrate access, and catalytic activity. Gotoh (1992) has proposed the presence of six substrate recognition sites in mammalian P450s. According to this proposition, the L293P substitution is located within the fourth substrate recognition site, which is highly conserved in a variety of animal species and is thought to be associated with substrate specificity. Several confounding factors have caused the difficulty in the extrapolation of the in vitro result into the phenotype observation, although the variant is proven to have an altered function in vitro. These factors may include unknown mutations in CYP3A4 gene, unknown endogenous and exogenous compounds affecting its expression or activity, substrate overlapping with other enzymes, variation in nuclear receptors, different assay systems in different laboratories, and complexity in the kinetics, depending on the substrate. In the present study, we detected the CYP3A4*18 variant at a frequency of 1.7% in 298 Korean subjects. The frequencies of the CYP3A4*18 variant among Far East Asian populations show no significant differences. This variant has also been observed at a frequency of 2% in a Chinese population (Dai et al., 2001) and 1.3% in a Japanese population (Yamamoto et al., 2003), suggesting that it is not a rare allele in Asians and is an important allele to be tested in vivo. Therefore, we evaluated the clinical relevance of CYP3A4*18 for MDZ disposition. To our knowledge, the present study is the first to evaluate the effect of CYP3A4*18 on therapeutic drug disposition in humans. Recently, it has been reported that CYP3A4*20 exhibits a clear genotype-phenotype correlation with MDZ pharmacokinetics (Westlind-Johnsson et al., 2006). MDZ has been reported to be extensively metabolized by CYP3A in the liver and intestine (Thummel and Wilkinson, 1998) but is not a substrate for P-glycoprotein (Kim et al., 1999). However, the estimation of the relative contribution of CYP3A5 to total CYP3A activity has been a matter of debate, ranging from up to 50% of total CYP3A protein in the liver (Kuehl et al., 2001) to a minor contributor (Westlind-Johnsson et al., 2003). Therefore, the clinical impact of CYP3A5*3 on the CYP3A substrates in vivo has been also controversial. The presence or absence of the CYP3A5*3 allele has affected tacrolimus disposition (Hesselink et al., 2003; Thervet et al., 2003; Haufroid et al., 2004), but not in cyclosporine (Hesselink et al., 2003; Yu et al., 2004). The impact of the decreased activity against CYP3A5*3 is 1.09 ± 0.07, 0.42, and 0.51, respectively, when analyzed by SNP Alyze (version 4.1.1; Dynacom, Yokohama, Japan). All eight individuals in the present study contained at least one CYP3A5*1, and two subjects were identified on the background of CYP3A5*1/*1 (Y. Saito, personal communication). Fukushima-Uesaka et al. (2004) reported a haplotype between CYP3A4*18 and CYP3A5*1E. This haplotype finding is also supported in the present results of our genotyping study. In the present study the expected haplotype frequency of CYP3A4*18/CYP3A5*3 was 1.09 × 10⁻⁷, whereas CYP3A4*18/CYP3A5*3, CYP3A4*1/*1, and CYP3A4*1/CYP3A5*3 were 0.07, 0.42, and 0.51, respectively, when analyzed by SNP Alyze (version 4.1.1; Dynacom, Yokohama, Japan). Eight individuals in the present study contained at least one CYP3A5*1 allele. No individuals having CYP3A4*18 have been reported to carry the homozygous mutation of CYP3A5*3 allele so far (Yamamoto et al., 2003; Fukushima-Uesaka et al., 2004; Hu et al., 2005). The impact of the decreased activity against MDZ caused by the CYP3A4*18 allele may be compensated by the strong expresser genotype CYP3A5*1 in terms of overall CYP3A activity, even in the case of the homozygous CYP3A4*18/*18.

In summary, the CYP3A4*18 allele, which leads to decreased MDZ metabolism in vitro, is often observed in Koreans as a particular phenotype, especially with respect to metabolite disposition, since the metabolite can be further metabolized to the conjugated products by phase II enzymes, thereby masking the primary effect of CYP3A4*18 on MDZ metabolism. Genetic polymorphisms of phase II enzymes may define further confounding factors that will increase our understanding the role of the CYP3A4 genotype in phenotypic variations, especially with regard to multiple metabolite formation, as in the case of MDZ. In this context, CYP3A4*18 may be associated with functionally altered SNPs of the phase II enzymes that are responsible for the conjugation of MDZ metabolites for plasma clearance. This may partly explain the lack of reduction of plasma MDZ metabolites in those CYP3A4*18/*1 subjects who exhibited a slightly prolonged plasma concentration of the parent MDZ compound. Human pregnane X receptor polymorphisms have been shown to influence the MDZ clearance particularly in African Americans (He et al., 2006), suggesting that pregnane X receptor polymorphism would be another confounding factor in the MDZ clearance variation in the present study.

The CYP3A4*18 carriers exhibited increases in C_{max} and AUC_{0-Inf} for the parent MDZ compound, compared with CYP3A4*1 carriers with the same genotypic background of CYP3A5*3/*3. Although these results were not statistically significant, CYP3A4*18 seems to slightly affect MDZ clearance in vivo. From the post hoc power analysis for the AUC data obtained in the present study, at least 26 subjects per group are required to detect statistical significance in a t test comparison of mean value at the 0.05 significance level with 80% power. The evaluation of the CYP3A4*18 on MDZ disposition using a larger number of subjects would be necessary for better assessment. However, it may be difficult to conduct the investigation using such a large number of subjects with consent because of the low frequency of this allele in Asians, and its near-nonexistence in Caucasians.

The extent of the increases in C_{max} and AUC_{0-Inf} for the parent MDZ compound in CYP3A4*18/*1-CYP3A5*3/*1 carriers was similar to that in CYP3A4*1/*1-CYP3A5*3/*3 carriers. Kuehl et al. (2001) have also reported decreased MDZ clearance in CYP3A5*3/*3 carriers, compared with CYP3A5*1/*3 carriers. All of the CYP3A4*18 carriers examined in the present study were heterozygous for CYP3A5*3. Subjects who are homozygous for CYP3A4*18 are relatively rare in the Korean population. According to the Hardy-Weinberg equation, 1 in approximately 3000 Koreans would be expected to have the homozygous CYP3A4*18 genotype. Actually, an individual having homozygous mutation for CYP3A4*18 was identified in 418 Japanese subjects (Fukushima-Uesaka et al., 2004). Recently, Fukushima-Uesaka and coworkers found an additional individual having homozygous mutation for CYP3A4*18 and two subjects were identified on the background of CYP3A5*1/*1 (Y. Saito, personal communication). Fukushima-Uesaka et al. (2004) reported a haplotype between CYP3A4*18 and CYP3A5*1E. This haplotype finding is also supported in the present results of our genotyping study. In the present study the expected haplotype frequency of CYP3A4*18/CYP3A5*3 was 1.09 × 10⁻⁷, whereas CYP3A4*18/CYP3A5*1, CYP3A4*1/*1, and CYP3A4*1/CYP3A5*3 were 0.07, 0.42, and 0.51, respectively, when analyzed by SNP Alyze (version 4.1.1; Dynacom, Yokohama, Japan). All eight individuals in the present study contained at least one CYP3A5*1 allele. No individuals having CYP3A4*18 have been reported to carry the homozygous mutation of CYP3A5*3 allele so far (Yamamoto et al., 2003; Fukushima-Uesaka et al., 2004; Hu et al., 2005). The impact of the decreased activity against MDZ caused by the CYP3A4*18 allele may be compensated by the strong expresser genotype CYP3A5*1 in terms of overall CYP3A activity, even in the case of the homozygous CYP3A4*18/*18.
heterozygous mutation. We suggest that the altered plasma concentration of MDZ in vivo caused by CYP3A4*18/*1 is not clinically significant. One of the reasons for this may be the linkage between CYP3A4*18 and CYP3A5*1 (Fukushima-Uesaka et al., 2004), compromising the lack of CYP3A4 activity toward MDZ. Further clinical studies would be helpful to elucidate the role of the CYP3A4 allele in the disposition of other CYP3A substrates.

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References


EFFECTS OF CYP3A4*18 ON MIDAZOLAM DISPOSITION

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