Identification of New CYP2C19 Variants Exhibiting Decreased Enzyme Activity in the Metabolism of S-Mephenytoin and Omeprazole

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ABSTRACT:

Although many cases of interindividual variation in the metabolism of CYP2C19 drugs are explained by the CYP2C19*2, *3, and *17, a wide range of metabolic variation still occurs in people who do not carry these genetic variants. The objectives of this study were to identify new genetic variants and to characterize functional consequences of these variants in metabolism of CYP2C19 substrates. In total, 21 single-nucleotide polymorphisms including three new coding variants, V394M, E405K, and D256N, were identified by direct DNA sequencing in 50 randomly selected subjects and in individuals who exhibited an outlier phenotype response in the omeprazole study. Recombinant proteins produced from the coding variants V394M, E405K, and D256N were prepared by using an Escherichia coli expression system and purified. Metabolism of S-mephenytoin and omeprazole by V394M was comparable with that of the wild-type protein. E405K showed a moderate decrease in metabolism of the substrates. However, D256N exhibited a significantly decreased activity in S-mephenytoin metabolism, resulting in 50 and 76% decreases in V_{max} and intrinsic clearance, respectively, compared with the wild type. This variant also exhibited a significant decrease in omeprazole metabolism in vivo. CYP2C19 D256N and E405K were assigned as CYP2C19*26 and *2D, respectively, by the Cytochrome P450 Nomenclature Committee. In summary, this report characterizes the allele frequency and haplotype distribution of CYP2C19 in a Korean population and provides functional analysis of new coding variants of the CYP2C19 gene. Our findings suggest that individuals carrying CYP2C19*26 would have lower activity for metabolizing CYP2C19 substrate drugs.
drugs still remain unexplained in people who do not carry CYP2C19*2, *3, or other known variants. In the present study, we resequenced the CYP2C19 gene in 50 normal Korean subjects and in individuals exhibiting an outlier phenotype of omeprazole distribution to explain the possible mechanism in these subjects. In addition to the information on allele and haplotype distributions in a Korean population, identified functional variants were further characterized by using S-mephentoin and omeprazole as prototype substrates in a recombinant enzyme system.

Materials and Methods

Chemicals and Materials. S-Mephentoin and omeprazole were purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). Human NADPH-P450 oxidoreductase and human cytochrome b₅ were obtained from Oxford Biomedical Research (Rochester Hills, MI). Sodium cholate, β-nicotinamide adenine dinucleotide phosphate (reduced NADPH), δ-aminolevulinic acid, L-α-dilauroyl-sn-glycero-3-phosphocholine, L-α-dioleoyl-sn-glycero-3-phosphocholine, bovine brain phosphatidylserine, phenylmethylsulfonyl fluoride, and lysozyme were purchased from Sigma-Aldrich (St. Louis, MO). Protease mix was purchased from Roche Diagnostics (Indianapolis, IN). Oligonucleotide primers were obtained from Bioneer (Daejeon, Korea). A Protease mix was purchased from Roche Diagnostics (Indianapolis, IN). Oligonucleotide primers were obtained from Bioneer (Daejeon, Korea). A QuikChange mutagenesis kit was purchased from Stratagene (La Jolla, CA).

Protease mix was purchased from Roche Diagnostics (Indianapolis, IN). Oligonucleotide primers were obtained from Bioneer (Daejeon, Korea). A QuiChange mutagenesis kit was purchased from Stratagene (La Jolla, CA). Ni-NTA affinity columns were obtained from QIAGEN (Valencia, CA). Escherichia coli DH5α competent cells, isoropyl β-D-thiogalactopyranoside (IPTG), restriction enzymes, and T4 DNA ligase were obtained from Invitrogen (Carlsbad, CA). Imidazole was purchased from Sigma-Aldrich. All of the other chemicals and organic solvents were of the highest grade from commercial sources.

Subjects. Healthy volunteers, 113 Vietnamese aged 20 to 77 years and 94 Koreans aged 22 to 28 years, participated in the phenotyping study after providing written informed consent, which was approved by the Institutional Review Board of Busan Paik Hospital (Busan, Korea) (Lee et al., 2005b, 2007). After oral and written explanation of the study, written informed consent was obtained from all participants. Volunteers were asked by a physician to report their medical history, including any drugs they had taken in the past 6 months. None of the participants were alcoholics or were taking any medication, including herbal medicines or food supplements.

Phenotyping Procedures. Subjects received a single 20-mg oral dose of omeprazole (Yuhan Pharmaceutical, Seoul, Korea) after overnight fasting. Subjects did not eat any food before sample collection. Blood samples (5 ml) were collected 3 h after administration. Plasma was immediately separated by centrifugation and stored at −20°C until analysis. The separated plasma samples collected in Vietnam were transported by air to the Pharmacogenomics Research Center (Inje University College of Medicine, Busan, Korea). During transport, the blood samples were kept frozen on dry ice. Plasma concentrations of omeprazole and 5-hydroxyomeprazole were analyzed by high-performance liquid chromatography as described previously (Freidichs et al., 2005; Kim et al., 2005). The metabolic ratio (MR) was calculated as the ratio of omeprazole to 5-hydroxyomeprazole. Individual carriers of CYP2C19*1/*2 or *1/*3 genotypes who exhibited MR values higher than the mean MR values obtained from homozygous carriers of CYP2C19*2 and *3 were selected for direct DNA sequencing to identify unknown genetic variants.

Direct DNA Sequencing and Variant Identification. DNA samples were obtained from the DNA repository bank at INJE Pharmacogenomics Research Center (Inje University College of Medicine, Busan, Korea) (Lee et al., 2005b, 2007). The research protocol for the use of human DNA from blood samples was obtained from the Institutional Review Board and conformed to institutional guidelines. Genomic DNA was prepared from peripheral whole blood by using the QiAamp blood kit (QIAGEN). Direct sequencing of the CYP2C19 gene was performed in 50 randomly selected Korean subjects and from individuals who exhibited an outlier phenotype response in the omeprazole study. Another set of samples from 500 Koreans was also obtained from this DNA-repository bank, which was used for genotyping analysis as described under Materials and Methods. Primers for polymerase chain reaction (PCR) amplification were identical to those used in previous studies (Blaisdell et al., 2002; Fukushima-Uesaka et al., 2005). The amplified products were purified with a PCR purification kit (NucleoGen, Ansan, Korea), and sequencing was performed by using an ABI Prism 3700XL Genetic Analyzer (Applied Biosystems, Foster City, CA). A software package, PC Gene (Oxford Molecular, Campbell, CA), was used to identify variants with single-nucleotide substitutions in heterozygous or homozygous mutations. In the present study, the novel three nonsynonymous single-nucleotide polymorphisms (SNPs) were detected in one subject each. Therefore, the presence of these SNPs was confirmed by repeating the DNA amplification and sequencing. A sequence analysis program, www.fruitfly.org/seq_tools/splice.html, was used to predict alternative splice sites introduced by mutations. A software program (http://www.ncbi.nlm.nih.gov/research/db/TFSEARCH.html) was used to detect changes in transcription factor-binding elements introduced by mutations.

Site-Directed Mutagenesis for Expression Plasmids. CYP2C19 wild-type cDNA was constructed in the pCw vector, which was a kind gift from Dr. Goldstein (Human Metabolism Section, Laboratory of Pharmacology and Chemistry, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, NC). The N-terminal of CYP2C19 wild-type cDNA was modified as described for the 17α-hydroxylase (Barnes et al., 1991), and a 6x-His tag was added to the C-terminal for purification. Forward and reverse PCR primers for these modifications had the following sequences: 5'-GGA-GGTCTATGGTCGTGTTATGACGTTTTTCTCTGCTCATTGG-3' and 5'-GTCGCGCAAGTTTCAATGGATGTGGTATGACGACA-GAATGAAAGACA-3'. Site-directed mutagenesis was performed by using a QuikChange XL site-directed mutagenesis kit (Stratagene) with mutagenesis primers as follows: 5'-CACCAAGAATGTCATACATACAAACACCCTC-3' for CYP2C19 D256N, 5'-ACTTCCCTCACCCTCATGTAACCCACA-3' for CYP2C19 V394M, 5'-GAAATTC GGAGAATGTT TGACCCTCT-3' for CYP2C19 E456K, and 5'-CATGGATTATTCCTCAGGGAAACCTATAAC-3' for CYP2C19 P227L. The underlined nucleotides are mismatches with the CYP2C19 reference sequence. The entire open reading frame sequence was obtained in both directions, and changes were confirmed prior to expression.

Expression and Purification of CYP2C19 Variants. CYP2C19.1 and variant proteins were expressed in E. coli DH5α cells. Details of procedures for expression and purification of P450 proteins were described in a previous report (Lee et al., 2003, 2005a). In brief, overnight cultures of E. coli containing the constructs were diluted 10-fold into 500 ml of Terrific Broth. Optimal expression was obtained with 0.5 mM IPTG and 0.5 mM δ-aminolevulinic acid for 72 h at 23°C with gentle shaking at 150 rpm. The P450 content in intact cells was monitored by CO difference spectra measured by using an UV-visible spectrophotometer (Omura and Sato, 1964). To minimize interexperi- mental variations in functional studies, all five CYP2C19 constructs including the wild type were simultaneously expressed, harvested, and purified under the same conditions. The P450 was eluted through a Ni-NTA-affinity column and dialyzed twice for 48 h in two changes of dialysis buffer (100 mM potassium phosphate, pH 7.4, and 20% glycerol). A negative control of all of the five CYP2C19 proteins was prepared in the same procedure for the purpose of comparison.

Reconstitution and Enzyme Activity Assays. For S-mephentoin hydroxylation assays, purified and spectrally determined P450 protein (5 pmol) was reconstituted with human NADPH-P450 oxidoreductase (20 pmol), cytochrome b₅ (10 pmol), 0.05 mM sodium cholate, and 2 μg of lipid mix (a 1:1:1 mixture of L-α-dilauroyl-sn-glycero-3-phosphocholine, L-α-dioleoyl-sn-glycero-3-phosphocholine, and bovine brain phosphatidylserine) in 20 mM HEPES buffer (pH 7.4). The reaction was preincubated at room temperature for 5 min in a volume of 0.1 ml. The linear range of enzyme activity was determined by using wild-type protein, and based on this result, the duration of the S-mephentoin reaction was 15 min at 37°C. Concentrations of S-mephentoin for kinetic analysis were 6.25, 12.5, 25, 50, 100, 200, and 400 μM. For the single-point assay, 400 μM S-mephentoin was used under the conditions described above. Reconstitution conditions for the metabolism of omeprazole were identical to those of the S-mephentoin reaction, with the exception of purified P450 protein (5 pmol), human NADPH-P450 oxidoreductase (10 pmol), and cytochrome b₅ (2 pmol). The reaction was initiated with 10 mM NADPH and incubated for 15 min at 37°C. Omeprazole concentrations for kinetic analysis were 3.125, 6.25, 12.5, 25, 50, 100, 200, and 400 μM. For the single-point assay, 200 μM omeprazole was used as described above. No catalytic activity was detected in the absence of NADPH. Liquid chromatography-mass spectrometry was used to quantify 4'-hydroxy me-
phenytoin and 5-hydroxy omeprazole with a Qtrap 4000 liquid chromatography/
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CYP2C19*2
*3
presence of
were performed according to the previous report (Lee et al., 2007). The
pyrosequencing method (Sim et al., 2006). For the detection of
After biotin was attached to the 5
/H11032
-end of the reverse primer, the 409-base pair
amplification was validated by direct DNA sequencing by using the same
quencer (Biotage, Uppsala, Sweden). The accuracy of pyrosequencing and
of sequencing primer and template were analyzed on a PSQ 96MA Pyrose-
used to detect the 766G
A change via pyrosequencing. The resulting mixtures
newly designed sequencing primer, 5-AACACCAAGAATCGATG-3
previously (Lee et al., 2007). In brief, after amplifying the DNA fragment, a
2C9
CYP2C8

Statistical Analysis.
The allele frequencies were analyzed by using the
Hardy-Weinberg equation with SNPAlyze software (version 4.1; Dynacom
Corporation, College Station, TX). Differences were considered to be statistical
significantly when P values were less than 0.05.

Results
In the present study, 21 variants of CYP2C19 were identified by resequencing of the CYP2C19 gene in 50 Koreans (Table 1). Ten SNPs were detected in the 5'-untranslated region (5'-UTR), 9 SNPs were detected in the exons, and 3 SNPs were detected in the introns. CYP2C19*2, the variant reported to have the highest frequency in other Asian populations (Xiao et al., 1997; Fukushima-Uesaka et al., 2005), was found in 29% of the Korean subjects. CYP2C19*2 was identified along with four other mutations, 98T>C, 2720T>C, and 990C>G, and 990C>G, in six homozygous individuals and 19 heterozygous individuals, comprising a haplotype structure (Fig. 1). A linkage between V394M and T. A linkage between V394M and

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exhibited unusually high log MR values, similar to the levels of subjects with PM genotypes. These individuals were included in the direct DNA sequencing analysis, which revealed that one individual had a coding change for CYP2C19 D256N (Fig. 2). In addition, CYP2C19*17 was found in individuals having lower log MR values, suggesting its role in the increased CYP2C19 activity for omeprazole metabolism (Sim et al., 2006). With the exception of the D256N mutation in exon 5, no other mutations were identified in our sequenc-
ing analysis, which included approximately 3 kb of the 5'-UTR, all 9 exons and intron/exon junctions, and 300 bp of the 3'-UTR. D256N was identified as a heterozygous mutation of CYP2C19*2. PCR amplification of the region covering the D256N and CYP2C19*2 mutation was performed, and the product was sequenced. The sequencing result indicated that D256N was located at 681Gly, suggesting that D256N was not linked to CYP2C19*2 in this individual.

Functional studies for these coding variants were conducted in a recombination system. CYP2C19 wild type and CYP2C19 P227L (CYP2C19.10) were included in the expression system as controls, because the CYP2C19 P227L variant has been demonstrated to have decreased enzyme activity (Blaisdell et al., 2002). All cDNAs coding for CYP2C19.1, P227L, D256N, E405K, and V394M were cloned into the pCW expression vector and expressed in an E. coli system. Two independent purification procedures were performed for functional assessment. All variants exhibited a maximum CO-reduced spectrum at 450 nm, and no P420 forms were detected (Fig. 3). Recombinant CYP2C19 proteins metabolized S-mephenytoin (Fig. 4) and omeprazole (Fig. 5) in the reconstituted system. Kinetic parameters for these two substrates are summarized in Table 2. At a fixed dose of 400 μM S-mephenytoin, the activity of the D256N and E405K variants was reduced to 50 and 30% of the wild type, respectively. The activity of V394M was comparable with that of the wild type. P227L, used as a control, also showed a 5-fold decrease in activity compared with the wild type. In kinetic studies of S-mephenytoin 4'-hydroxylase activity, D256N showed a 2-fold decrease in Vmax (8.6 nmol/min/nmol P450) and an increased Km (159 μM) compared with the wild type, resulting in a 4.2-fold decrease in intrinsic clearance. The E405K variant also exhibited a 2-fold decrease in intrinsic clearance compared with the wild type. At a fixed dose of 200 μM omeprazole, both D256N and P227L exhibited 40 and 39% decrease in activity, respectively, compared with the wild type. E405K showed slightly decreased activity, but the activity of V394M was comparable...
Fig. 3. Reduced CO-difference spectrum of CYP2C19 wild-type and variant proteins expressed in an E. coli system. Details are explained under Materials and Methods.

Fig. 4. S-Mephenytoin 4'-hydroxylase activity of CYP2C19 wild-type and variant proteins. A. S-mephenytoin metabolism measured at a high concentration of 400 µM. B. kinetic assessment of S-mephenytoin 4'-hydroxylase by CYP2C19 wild-type and variant proteins. Enzyme reconstitution included purified P450 (10 pmol), human reductase (40 pmol), and cytochrome b₅ (20 pmol) as described under Materials and Methods. To reduce intra-assay variations, expression, purification and enzymatic assays were performed simultaneously for all proteins. Results represent one independent data set from two separate purifications of proteins. Values plotted are the mean ± S.D. of triplicates. P values for differences between CYP2C19.1 and variant proteins were determined by using Bonferroni’s post hoc test. *, P < 0.05 and **, P < 0.001.
with that of the wild type. P227L was included for the kinetic study of omeprazole metabolism, because no report on metabolism was available for this allele using omeprazole. In kinetic studies of 5-hydroxy omeprazole activity, the wild type exhibited strong activity, followed by E405K, V394M, D256N, and P227L in the intrinsic clearance values. D256N exhibited a decrease of approximately 2.6-fold in $V_{\text{max}}$ compared with the wild type. P227L showed a decreased activity of approximately 1.6-fold in $V_{\text{max}}$ and 5-fold in intrinsic clearance values compared with those of the wild type. E405K also showed slightly decreased activity in $V_{\text{max}}$ compared with the wild type. In summary, D256N exhibited a significantly decreased activity for the metabolism of $S$-mephenytoin and omeprazole. In particular, the in vitro metabolism study for D256N supported the finding of decreased omeprazole metabolism in vivo shown in Fig. 2. Genotyping for $CYP2C19*17$ and $CYP2C19*26$ in an extended set of 500 Koreans revealed a 1.4% frequency of $CYP2C19*17$ and no additional individuals with the $CYP2C19*26$ allele.

### Discussion

The present study describes, for the first time, the distribution of $CYP2C19$ genetic polymorphisms in a Korean population, and it provides functional studies for three newly identified variants by using $S$-mephenytoin and omeprazole. Among the new variants, one allele, designated as $CYP2C19*26$ by the Cytochrome P450 Nomenclature Committee, exhibited significantly decreased activity in the metabolism of $S$-mephenytoin and omeprazole. Although direct DNA sequencing was performed in six subjects exhibiting outlier phenotype in omeprazole MR assays, only D256N was detected in an individual

### Table 2

**Kinetic parameters for $S$-mephenytoin and omeprazole metabolism by $CYP2C19.1$ and $CYP2C19$ variant proteins**

Best fit of the formation of $S$-mephenytoin 4'-hydroxylation and 5-hydroxyomeprazole was determined by required parameter estimates by using SigmaPlot 8.0, and kinetic parameters for both metabolism were determined by Michaelis-Menten kinetics. Details of reaction and kinetic study are described under Materials and Methods. All data are the mean ± S.D. of triplicates.

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<td>$K_m$ (µM)</td>
<td>$CL_{int}$ (µM)</td>
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<td>Wild-type</td>
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<td>D256N</td>
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<td>V394M</td>
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<td>E405K</td>
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$CL_{int}$, intrinsic clearance; N.D., not determined.

Significantly lower than the $CYP2C19.1$ activity, * $P < 0.05$ and ** $P < 0.001$, using Bonferroni’s post hoc test.
as a possible variant responsible for the phenotype. For the other outliers, there could be other factors or unknown variants in intron areas or regulatory regions beyond the region we analyzed. Newly identified coding variants were further studied to understand functional differences compared with the wild-type protein. Because the degree of decreased enzyme activity is affected by P450 protein stability, we compared the CO spectrum of these variants with that of the wild type. Although it was produced in the prokaryotic E. coli expression system, D256N exhibited levels of P450 without the P420 form similar to those of the wild type, suggesting that substitution of D256N may not affect the heme-binding property of the enzyme. The D256N variant was not located in the putative substrate recognition site (Gotoh, 1992). With the availability of the X-ray crystal structure of CYP2C5, the role of CYP2C19 256D was deduced by using CYP2C5-generated human CYP2C modeling (Williams et al., 2000).

The D256N variant was located in the junction area between the G helix and the longest I helix based on the crystal structure. It is possible that the change from an acidic amino acid (Asp) to a neutral amino acid (Asn) in this junction area may cause a structural change responsible for the decreased enzyme activity of the variant. It is noteworthy that Asp is highly conserved in positional alignment comparisons of 20 CYP2C peptide sequences (Lewis, 2003), suggesting that Asp in this position is important for maintenance of CYP2C activity or structural stability across the species. CYP2C19.10 (P227L) was included in the enzyme functional study as a reference allele that exhibited significantly decreased S-mephenytoin 4'-hydroxylase activity compared with the wild type (Blaisdell et al., 2002), and this decrease was reported in the present study. Metabolism of omeprazole by CYP2C19.10, a variant protein previously reported to have 3% activity in African-Americans, was investigated for the first time in the present study. D256N and CYP2C19.10 exhibited a similar extent of decreased activity in the metabolism of omeprazole. CYP2C19 has been shown to metabolize several structurally different substrates with different kinetic profiles. Although we found no evidence of further mutations in the 5'UTR, 3'UTR, or other intron regions, our in vitro results suggest that increased log MR ratios for omeprazole may be attributable to the D256N change together with the CYP2C19*2 mutation. Because the D256N allele showed impaired metabolism for omeprazole and mephenytoin, we predict that D256N represents a functional variant for CYP2C19 substrates in humans. V394M was identified as a possible variant responsible for the phenotype by the genotypic related variation still occurs in people who do not carry CYP2C19*2, *3, and *17.

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