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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.

Members of the *CYP2C* gene family in humans include *CYP2C8*, *CYP2C9*, *CYP2C18*, and *CYP2C19*. Among the four *CYP2C* genes, *CYP2C19* is a major polymorphic cytochrome P450 (P450) enzyme. CYP2C19 metabolizes a variety of clinically important drugs, which include mephenytoin, omeprazole, diazepam, proguanil, imipramine, and clopidogrel (Goldstein and de Morais, 1994; Evans and Relling, 1999; Ingelman-Sundberg et al., 2007). Many studies have reported interindividual variations in the metabolism of these drugs depending on *CYP2C19* genotype (Desta et al., 2002). Furuta et al. (1998) first described the influence of *CP2C19* genotype on the therapeutic outcome of gastric ulcer patients who were administered proton pump inhibitors. Although several *CYP2C19* variants have been shown to cause different metabolic phenotypes, major genetic determinants causing these differences have been attributed to *CYP2C19*2* and **3*. *CYP2C19*2* is a mutation in exon 5 that produces an aberrant splice site, leading to a truncated nonfunctional protein (de Morais et al., 1994b). *CYP2C19*3* is a mutation creating a stop codon in exon 4 (de Morais et al., 1994a). Population studies have indicated that ethnic differences exist in frequencies of *CYP2C19* polymorphisms (Desta et al., 2002; Ozawa et al., 2004). Different distributions of the subgroups of poor metabolizers (PMs), extensive metabolizers, and ultrarapid metabolizers have been observed for different racial groups, mostly using *CYP2C19*2*, **3*, and **17*. The frequency of *CYP2C19*2* in Asians has been estimated at approximately 30%, accounting for 70 to 80% of PM alleles in Asians (Xie et al., 2001). *CYP2C19*3* has been reported at a 5 to 7% frequency in Asians, but it is rare in black populations and almost nonexistent in white populations (Xie et al., 2001; Desta et al., 2002; Ozawa et al., 2004). *CYP2C19*17*, a recently reported allele, has a frequency of 17 to 23% in white populations (Sim et al., 2006). Based on an overall search of the literature, PMs represent 2 to 5% of the white population, 13 to 23% of the Asian population, and approximately 4% of African-American population (Bertilsson et al., 1992; Xie et al., 2001; Desta et al., 2002). However, many of the interindividual variations in the metabolism of CYP2C19

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ABBREVIATIONS: P450, cytochrome P450; PM, poor metabolizer; IPTG, isopropyl β-D-thiogalactopyranoside; MR, metabolic ratio; PCR, polymerase chain reaction; SNP, single-nucleotide polymorphism; bp, base pair; 5′-UTR, 5′-untranslated region.

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*-mephenytoin and omeprazole as prototype substrates in a recombinant enzyme system.

Materials and Methods

Chemicals and Materials. *S*-Mephenytoin and omeprazole were purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). Human NADPH-P450 oxidoreductase and human cytochrome b_5 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 QuikChange mutagenesis kit was purchased from Stratagene (La Jolla, CA). Ni-NTA affinity columns were obtained from QIAGEN (Valencia, CA). *Escherichia coli* DH5α competent cells, isopropyl 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 27 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 (Frerichs et al., 2005; Kim et al., 2005). The metabolic ratio (MR) was calculated as the ratio of omeprazole to 5-hydroxyomeprazole. Individuals carrying *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.cbrc.jp/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′- GGAGGTCATATGGCTCTGTTATTAGCAGTTTTTCTCTGTCTCTCATGTT-3′ and 5′-GCTGCCAAGCTTTCAATGGTGATGGTGATGATGACAGACAGGAATGAAGCACA-3′. Site-directed mutagenesis was performed by using a QuikChange XL site-directed mutagenesis kit (Stratagene) with mutagenesis primers as follows: 5′-CACCAAGAATCGATG<u>A</u>ACATCAACAACCCTC-3′ for CYP2C19 D256N; 5′-ACTTCCCTCACTTCT<u>A</u>TGCTACATGACAACA-3′ for CYP2C19 V394M; 5′-GAATTTCCCAACCCA<u>A</u>AGATGTTTGACCCTC-3′ for CYP2C19 E405K; and 5′-CATTGATTATTTCC<u>T</u>GGGAACCCATAAC-3′ for CYP2C19 P227L. The underlined nucleotides are mismatches with the *CYP2C19* reference sequence. The entire open reading frame region was sequenced 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. Detailed 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 interexperimental 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 second set of all five CYP2C19 proteins was prepared in the same procedure for the purpose of comparison.

Reconstitution and Enzyme Activity Assays. For *S*-mephenytoin hydroxylation assays, purified and spectrally determined P450 protein (5 pmol) was reconstituted with human NADPH-P450 oxidoreductase (20 pmol), cytochrome b_5 (10 pmol), 0.05 μmol 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*-mephenytoin reaction was 15 min at 37°C. Concentrations of *S*-mephenytoin for kinetic analysis were 6.25, 12.5, 25, 50, 100, 200, and 400 μM. For the single-point assay, 400 μM *S*-mephenytoin was used under the conditions described above. Reconstitution conditions for the metabolism of omeprazole were identical to those of the *S*-mephenytoin reaction, with the exception of purified P450 protein (5 pmol), human NADPH-P450 oxidoreductase (10 pmol), and cytochrome b_5 (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-

TABLE 1

SNPs found in CYP2C19 gene in a Korean population

The reference sequence used was GenBank accession NC_000010.

Site	Position[a]	Amino Acid Change	Nucleotide Change	Subject Number			Frequency
				wt/wt	mt/wt	mt/mt	
					n		%
5-UTR	−2720T>C		cagtaaagctattt(T/C)atatgatacta	41	8	1	10
5-UTR	−2305G>A[b]		tgaactggga(G/A)ttgaaaaac	48	2	0	2
5-UTR	−2040C>T		taaagagagcaa(C/T)caagcttatctt	49	0	1	2
5-UTR	−1418C>T		gaacaaata(C/T)gatgatatct	35	14	1	16
5-UTR	−1312C>G[b]		aatgtcagctc(C/G)cgttaaggtcta	49	1	0	1
5-UTR	−889T>G		cagaataactaa(T/G)gtttggaagttg	35	14	1	16
5-UTR	−806C>T		ctgttctcaaag(C/T)atctctgatgta	48	2	0	2
5-UTR	−559T>C		tattgaagata(T/C)atgagttatgtta	49	1	0	1
5-UTR	−529G>C		atttcatgtttag(G/C)ctgctgtattttta	49	1	0	1
5-UTR	−98T>C		gattggccactt(T/C)atccatcaaaga	26	19	5	29
Exon 1	99C>T	P33P	ccctcctggccc(C/T)actcctctccca	1	14	35	84
Intron 2	IVS2–23A>G		gatctccctcct(A/G)gtttcgtttctc	26	19	5	29
Exon 4	636G>A	W212X	aagcaccccctg(G/A)atccaggtaagg	45	5	0	5
Exon 5	681G>A	Splice site	attatttccc(G/A)ggaacccata	26	19	5	29
Exon 5	766G>A[c]	D256N	caagaatcgatg(G/A)acatcaacaacc	113	1	0	0.3
Intron 5	IVS5–113 T>G		tttttctagtac(T/G)atactttacagt	41	8	1	10
Intron 5	IVS5–51 C>G		atttactgtcat(C/G)aaatatgctgtt	26	17	7	31
Exon 7	990 C>T	V330V	gattgaacgtgt(C/T)gttggcagaaac	26	19	5	29
Exon 7	991 A>G	I331V	attgaacgtgtc(A/G)ttggcagaaacc	0	8	42	92
Exon 7	1180 G>A[b]	V394M	ttccctcacttct(G/A)tgctacatgac	49	1	0	1
Exon 8	1213 G>A[b]	E405K	cccaaccca(G/A)agatgttt	49	1	0	1
Exon 8	1251 A>C	G417G	ggatgaaggtgg(A/C)aattttaagaaa	45	5	0	5

wt, wild type; mt, mutant.

[a] Position is indicated in relation to the start codon ATG of the *CYP2C19* gene; the A in ATG is +1.

[b] New variants found in the present study.

[c] Variant identified in a Vietnamese individual (Fig. 2) and genotyped in 114 Vietnamese subjects.

phenytoin and 5-hydroxy omeprazole with a Qtrap 4000 liquid chromatography/tandem mass spectrometry system (Applied Biosystems) equipped with electrospray ionization as described previously (Frerichs et al., 2005; Kim et al., 2005). The oxidized metabolite of *S*-mephenytoin was analyzed by high-performance liquid chromatography with a Phenomenex Luna C_{18} column (5 μ, 250 × 4.6 mm; ANSYS Technologies, Inc., Lake Forest, CA) as described previously (Ryu et al., 2007).

Genotyping for *CYP2C19*2*, **3*, **17*, and **26* (D256N). The presence of *CYP2C19*2*, **3*, **17*, and **26* was analyzed in 94 Korean and 113 Vietnamese subjects in the present omeprazole study. Additional genotyping for *CYP2C19*17* and *CYP2C19*26* was performed in an extended set of 500 Koreans available from the DNA repository bank at INJE Pharmacogenomics Research Center (Lee et al., 2005b, 2007). Genotyping for *CYP2C19*2* and **3* were performed according to the previous report (Lee et al., 2007). The presence of *CYP2C19*17* was determined by using a previously described pyrosequencing method (Sim et al., 2006). For the detection of *CYP2C19*26*, genomic DNA sequences for *CYP2C8*, *2C9*, *2C18*, and *2C19* were obtained from GenBank and aligned to design a pair of specific primers for amplifying the fragment that contained the CYP2C19 D256 site using Vector NTI 8.0. After biotin was attached to the 5′-end of the reverse primer, the 409-base pair (bp) DNA fragment containing *CYP2C19 D256N* was amplified by using primers with the following sequences: forward primer, 5′-CAACCAGAGCTTGGCATATTG-3′; reverse primer, 5′-TGATGCTTACTGGATATTCATGC-3′. Details of sample preparation for pyrosequencing were described previously (Lee et al., 2007). In brief, after amplifying the DNA fragment, a newly designed sequencing primer, 5-AACACCAAGAATCGATG-3′, was used to detect the 766G>A change via pyrosequencing. The resulting mixtures of sequencing primer and template were analyzed on a PSQ 96MA Pyrosequencer (Biotage, Uppsala, Sweden). The accuracy of pyrosequencing and amplification was validated by direct DNA sequencing by using the same genomic DNA.

Statistical Analysis. The allele frequencies were analyzed by using the Hardy-Weinberg equation with SNPAlyze software (version 4.1; Dynacom Co. Ltd., Yokohama, Japan). Haplotype inference was made by using the software program PHASE (Stephens and Donnelly, 2003). Two independently purified P450s for each allele were assayed in triplicate twice. Kinetic data were analyzed by using SigmaPlot (version 8.0; SAS 9.1.3, Chicago, IL). The kinetics observed for *S*-mephenytoin hydroxylation and 5′-hydroxyomeprazole exhibited the best fit with the Michaelis-Menten equation. All of the data are presented as means ± S.D. Statistical analysis was performed by one-way analysis of variance and Bonferroni post hoc test by using STATA 9.0 (Stata Corporation, College Station, TX). Differences were considered to be statistically significant 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, IVS2–23 A>G, IVS5–51C>G, and 990C>T, in six homozygous individuals and 19 heterozygous individuals, comprising a haplotype structure (Fig. 1). *CYP2C19*3* was identified as a heterozygous mutation in five individuals. A silent mutation at amino acid 417Gly in exon 8 was linked to *CYP2C19*3* as reported previously (Blaisdell et al., 2002). Two new coding variants, E405K and V394M, were found in a Korean population via direct DNA sequencing. V394M was found in an individual with a heterozygous mutation of the *CYP2C19*2* splice variant and a 990C>T. A linkage between V394M and *CYP2C19*2* remains to be confirmed due to the fairly long distance between these sites. E405K was identified as a homozygous mutation of *CYP2C19*2* in an individual, suggesting that this variant may be linked to *CYP2C19*2* (*CYP2C19*2D*). However, this evidence may be inconclusive because this variant is rare and *CYP2C19*2* is a commonly detected variant. Therefore, whether the variant is linked with *CYP2C19*2* by chance remains unknown. In the omeprazole MR study, six individuals carrying *CYP2C19*1/*2* or *CYP2C19*1/*3*

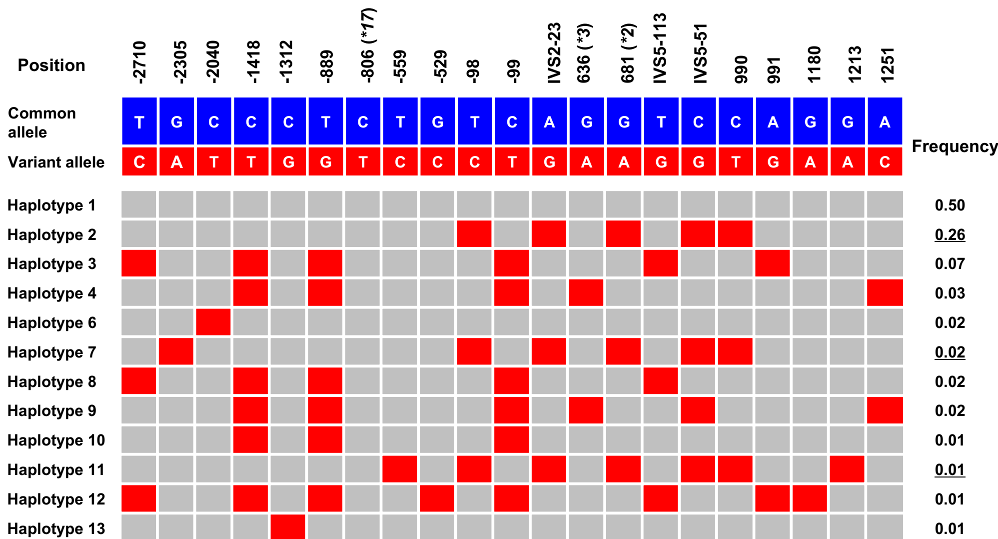

FIG. 1. SNPs in the *CYP2C19* gene and their occurrences in common haplotype structures. Haplotypes were estimated from 21 SNPs found in the 5′-UTR (3k bp) and all exons and intron/exon junction areas of *CYP2C19* from 50 Koreans by using the software program PHASE (Stephens and Donnelly, 2003). Position numbers refer to +1 ATG start codon of GenBank accession number NC_000010. Frequencies of *CYP2C19*2*-containing haplotypes are underlined.

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 sequencing 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 V_{max} (8.6 nmol/min/nmol P450) and an increased K_m (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

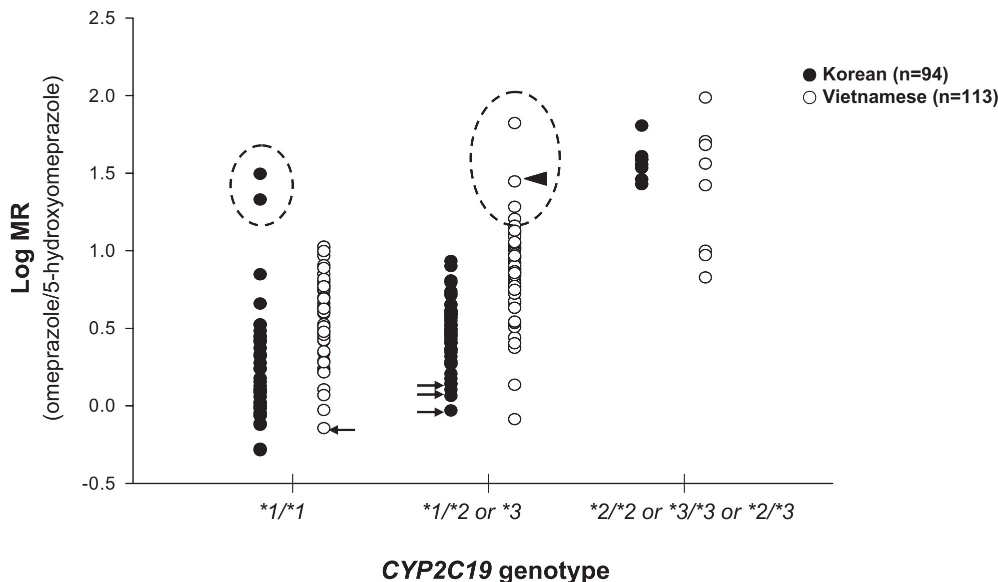

FIG. 2. Distribution of MRs of omeprazole in relation to *CYP2C19* genotypes. Blood samples drawn 3 h after omeprazole intake were used to calculate the omeprazole MR (omeprazole/5-hydroxyomeprazole). Subjects, as represented by the dashed circles, were used for direct DNA sequencing analysis. Detailed methods for DNA sequencing, genotyping, and the detection of omeprazole and its metabolite are described under *Materials and Methods*. Four arrows (bottom) and an arrow head in the dashed circle depict the location on the plot of the *CYP2C19*17* allele and new *CYP2C19*26* variant, respectively.

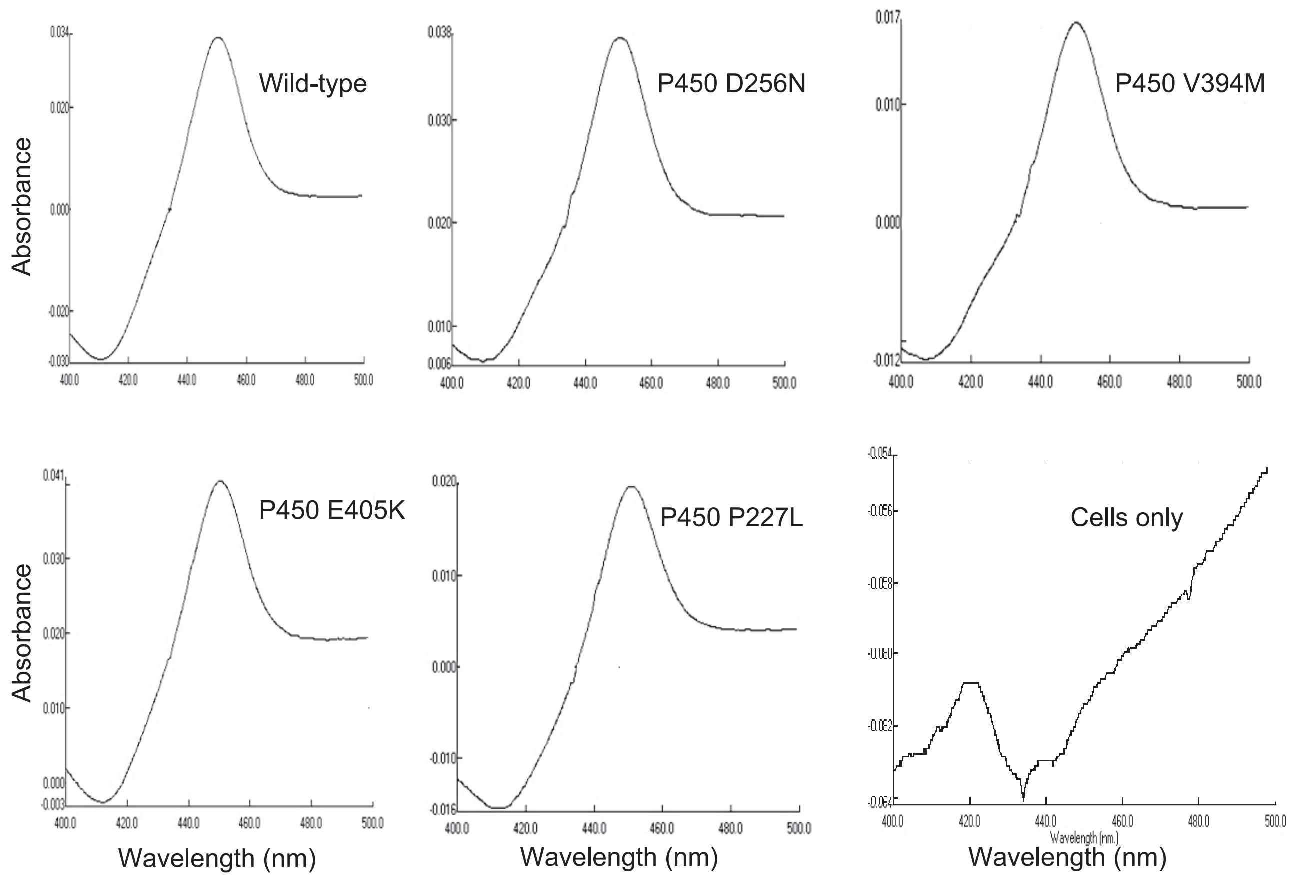

FIG. 3. Reduced CO-difference spectrum of CYP2C19 wild-type and variant proteins expressed in an *E. coli* system. Details are explained under *Materials and Methods.*

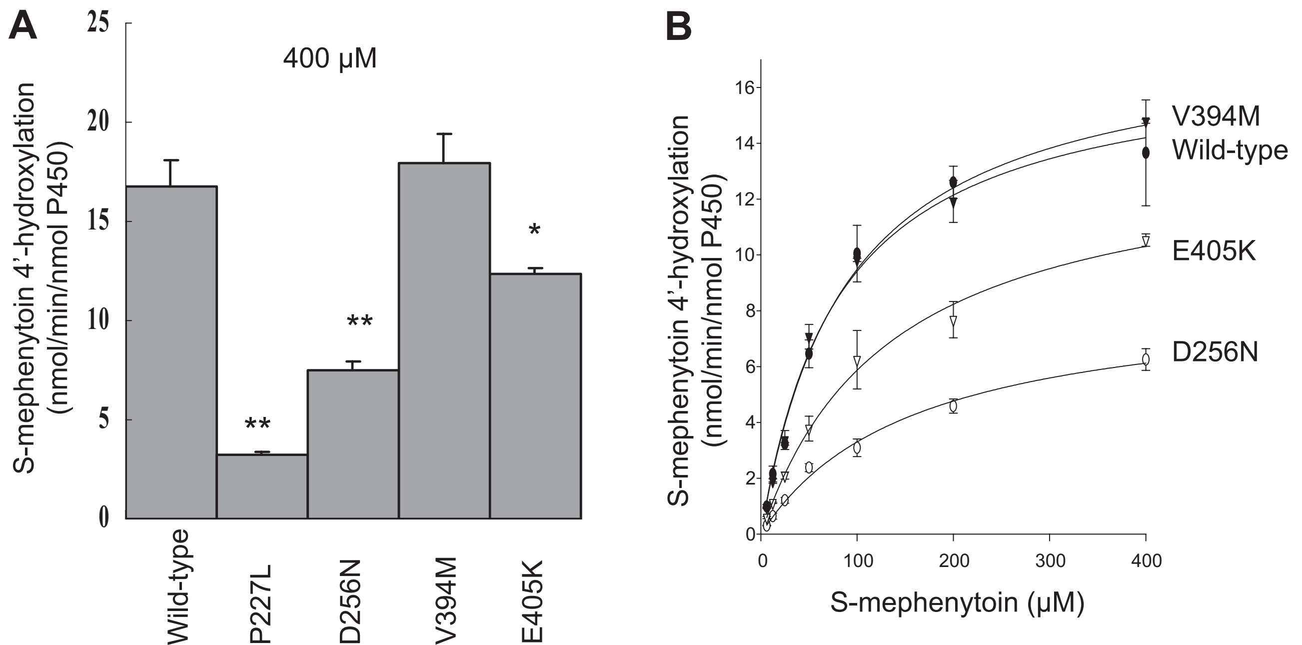

FIG. 4. *S*-Mephenytoin 4′-hydroxylase activity of CYP2C19 wild-type and variant proteins. A, *S*-mephenytoin metabolism measured at a high concentration of 400 μM. B, kinetic assessment of *S*-mephenytoin 4′-hydroxylase by CYP2C19 wild-type and variant proteins. Enzyme reconstitution included purified P450 (10 pmol), human reductase (40 pmol), and cytochrome b_5 (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 **, $P < 0.001$.

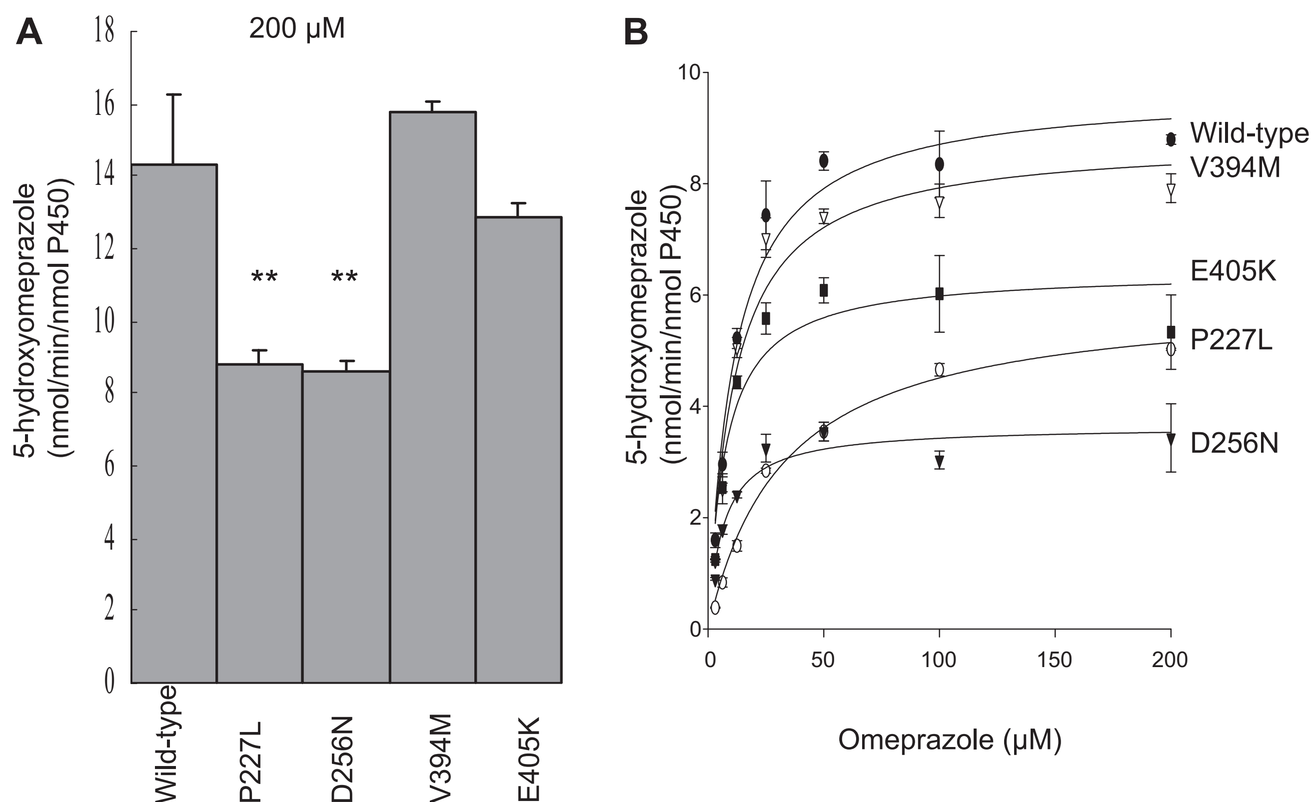

FIG. 5. 5-Hydroxyomeprazole activity of recombinant CYP2C19 wild-type and variant proteins. A, omeprazole metabolism was measured at a high concentration of 200 μM (near V_{max}). B, kinetic assessment of 5-hydroxyomeprazole by CYP2C19 wild-type and variant proteins. Reconstituted reactions included purified P450 protein (5 pmol), human NADPH-P450 oxidoreductase (10 pmol), and cytochrome b_5 (2 pmol) in 20 mM HEPES buffer (pH 7.4). Details of reactions and kinetic studies are described under *Materials and Methods*. The data represent one of two independent results from separately purified proteins. Values plotted are the means ± S.D. of triplicates. **, significantly lower than the CYP2C19.1 activity, $P < 0.001$ using Bonferroni post hoc test.

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.

CYP2C19	*S*-Mephenytoin 4′-Hydroxylation			5-Hydroxyomeprazole		
	V_{max}	K_m	CL_{int}	V_{max}	K_m	CL_{int}
	nmol/min/nmol P450	*μM*		*nmol/min/nmol P450*	*μM*	
Wild-type	17.1 ± 0.89	81.6 ± 11.70	0.21	9.68 ± 0.48	11.2 ± 2.135	0.88
D256N	8.60 ± 0.55*	159 ± 22.97	0.05**	3.65 ± 0.22**	6.45 ± 1.73	0.56**
V394M	17.9 ± 0.83	88.8 ± 10.99	0.20	8.80 ± 0.56	11.0 ± 2.70	0.80
E405K	13.8 ± 0.75	134 ± 17.16	0.10	6.43 ± 0.48**	7.45 ± 2.38	0.86
P227L	N.D.	N.D.	N.D.	5.99 ± 0.25**	32.9 ± 4.04**	0.18**

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.

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_{max} compared with the wild type. P227L showed a decreased activity of approximately 1.6-fold in V_{max} and 5-fold in intrinsic clearance values compared with those of the wild type. E405K also showed slightly decreased activity in V_{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

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 reproduced in the present study. Metabolism of omeprazole by CYP2C19.10, a variant protein previously reported to have 3% frequency 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 heterozygous mutation of *CYP2C19*2* in one individual. The distance from V394M (1180G>A) to CYP2C19*2 (681G>A) was approximately 68k bp, making linkage analysis difficult. In the present study, V394M appears to play an insignificant role in the structure and functional activity of CYP2C19, because this variant exhibited a similar CO spectrum and similar activity compared with those of the wild type. The location of V394M was between the K and L helices, using a model system for comparison. E405K was identified as a homozygous mutation of the *CYP2C19*2* variant, suggesting that the E405K allele is linked to *CYP2C19*2* in this individual. Although one might assume that a functional study of E405K is unnecessary due to its linkage with *CYP2C19*2*, the linkage may be inconclusive because this variant is found along with the high-frequency allele of *CYP2C19*2* in only one individual. Further study is needed to determine whether this linkage is due to chance. For these two reasons, we included the E405K variant in the functional study. Although this variant was linked to the *CYP2C19*2* allele in this individual, the characterization of the functional role of E and K at 405 would be helpful in a structure-function study.

In summary, our results confirmed that in the Korean population, *CYP2C19*2* and **3* are the most common nonsynonymous functional variants, and other nonsynonymous functional variants are rare. The frequency distribution of *CYP2C19* polymorphisms in a Korean population further extends fundamental information for Asian populations, which may be useful for genotyping or functional analysis in the future. Although the three variants identified are low-frequency alleles in the present study, functional characterization of these alleles would provide additional information to increase the accuracy of phenotype prediction by the genotype in the related Asian populations because a wide range of metabolic variation still occurs in people who do not carry *CYP2C19*2*, **3*, and **17*.

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