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

Su-Jun Lee, Woo-Young Kim, Hyunmi Kim, Ji-Hong Shon, Sang Seop Lee, and Jae-Gook Shin

Department of Pharmacology and Pharmacogenomics Research Center (S.-J.L., W.-Y.K., H.K., S.S.L., J.-G.S.) and Department of Clinical Pharmacology (J.-H.S., J.-G.S.), Inje University College of Medicine, Inje University Busan Paik Hospital, Jin-gu, Busan, Korea

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

Materials and Methods

Chemicals and Materials. S-Methylenotin 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, $\beta$-nicotinamide adenine dinucleotide phosphate (reduced NADPH), $\delta$-aminolevulinic acid, 1-$\alpha$-di-arylsulfonyl-$\gamma$-glycerol-3-phosphocholine, 1-$\alpha$-di-oleoyl-$\gamma$-glycerol-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). N-terminally affinity columns were obtained from QIAGEN (Valencia, CA). Escherichia coli DH5α competent cells, isoaryl $\beta$-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 medications, 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 (Frei 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.cbrp.jrcp/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 $17α$-hydroxyalase (Barnes et al., 1991), and a 6×-His tag was added to the C-terminal for purification. Forward and reverse PCR primers for these modifications had the following sequences: 5′- GAG-GGTCAATATGTCCTTATAGGCAGTTTTCTTCTGTCCTAAGTT-3′ and 5′-GCTGCGCAATTTCTAGTGAGTGATGACAGACAAGTAAGAACACAACAC-3′. Site-directed mutagenesis was performed by using a QuikChange XL site-directed mutagenesis kit (Stratagene) with mutagenesis primers as follows: 5′-CACCACAAGATGCAGACATACACAAACCTTCC-3′ for CYP2C19 D256N, 5′-ACTCTTCCACTCCTTCTGCTACAGTGACAGACA-CAACA-3′ for CYP2C19 V394M, 5′-GATATTCCGACCCCAAGATCTTGTTACCCATC-3′ for CYP2C19 E231V, and 5′-CATGGAATTTTCTCCAGGAACCCATAAC-3′ for CYP2C19 P227L. The underlined nucleotides are mismatches with the CYP2C19 reference sequence. The entire open reading frame sequence was performed 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 purification and expression 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 $\delta$-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 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 set of four of all five CYP2C19 proteins was prepared in the same procedure for the purpose of comparison.

Reconstitution and Enzyme Activity Assays. For S-methylenotin 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 mM sodium cholate, and 2 μg of lipid mix (a 1:1:1 mixture of 1-$\alpha$-di-arylsulfonyl-$\gamma$-glycerol-3-phosphocholine, 1-$\alpha$-di-oleoyl-$\gamma$-glycerol-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 $S$-methylenotin reaction was 15 min at 37°C. Concentrations of $S$-methylenotin for kinetic analysis were 6.25, 12.5, 25, 50, 100, 200, and 400 μM. For the single-point assay, 400 μM $S$-methylenotin was used under the conditions described above. Reconstitution conditions for the metabolism of omeprazole were identical to those of the $S$-methylenotin 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
phenytoin and 5-hydroxy omeprazole with a Qtrap 4000 liquid chromatography with a Phenomenex Luna C18 column (5 μm, 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 CYP2C19, 4.6 mm; T. A linkage between V394M and A variant and a 990C 

Statistical Analysis. The allele frequencies were analyzed by using the Hardy-Weinberg ratio with SNPAllyze software (version 4.1; Dynacorn Co. Ltd., Yokohama, Japan). Haplotype inference was made using the software program PHASE (Stephens and Donnelly, 2003). Two independently purified 5450s 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-phenytoin 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*2 was identified as a heterozygous mutation in five individuals. A silent mutation at amino acid 417Gly in exon 8 was linked to CYP2C19*2. 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
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 reconstitution 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<sub>max</sub> (8.6 nmol/min/nmol P450) and an increased K<sub>m</sub> (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

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**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 (3 kb) 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_0000010. Frequencies of CYP2C19*2-containing haplotypes are underlined.

**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/S-hydroxymephenytoin). 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.

- **Korean** (n=94)
- **Vietnamese** (n=113)
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
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 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-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 68 kbp, 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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References


**Address correspondence to:** Dr. Jae-Gook Shin, Department of Pharmacology and Pharmacogenomics Research Center Department of Clinical Pharmacology, Inje University College of Medicine, 633-165 Gaegum-Dong, Jin-Gu, Busan 614-735, Korea. E-mail: phshinjg@inje.ac.kr