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Haplotype Analysis and Single Nucleotide Polymorphism Frequency of Organic Cation Transporter Gene (*OCT1* and *2*) in Korean Subjects

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ABSTRACT – Organic cation transporters (*OCTs*) are important for absorption, elimination of many endogenous small organic cations as well as a wide array of drugs and environmental toxins. This gene is located in a cluster on chromosome 6 and *OCT*s are in major organs such as intestine, liver, kidney, brain and placenta. Therefore, expression levels and function of *OCT*s directly affect plasma levels and intracellular concentrations of drugs and thereby determine therapeutic response. The aim of this study was to investigate the frequency of the SNPs on *OCT1* (C181T and C1022T) and *OCT2* (G808T) to analyze haplotype frequency in healthy Korean population. Human subjects have been genotyped for *OCT1* (C181T for 195 subjects and C1022T for 825 subjects), using polymerase chain reaction-based diagnostic tests (RFLP). And for *OCT2* (G808T), a total of 861 subjects have been genotyped, using pyrosequencing method. Haplotype was statistically inferred using an algorithm based on the expectation-maximization (EM). *OCT1* C181T genotyping showed 100% homozygous wild-type (C/C). *OCT1* C1022T genotyping showed wild-type (C/C), heterozygous (C/T) and homozygous mutant-type (T/T) and each accounted for 72.1, 24.5 and 3.4%, respectively. *OCT2* G808T genotyping results also showed homozygous wild-type (G/G), heterozygous (G/T) and homozygous mutant-type (T/T) and each took 81.8, 17.9 and 0.3%, respectively. Based on these genotype data, haplotype analysis between *OCT1* C181T and *OCT1* C1022T has proceeded. The result has revealed that linkage disequilibrium between alleles is not obvious (P=0.0122).

Key words – *OCT*s, Haplotype, Genetic polymorphisms, Korean

Interindividual differences in drug response may be attributable to factors such as age, body weight, nutrition, liver and kidney function, disease and drug-drug interactions. Genetic factors are known to contribute to individual differences in bioavailability, drug transport, metabolism and drug action.[1)]

Transporters are important factor that acts widely from reaching the target site to eliminating out of the body. Organic cation transporters (*OCTs*), one of the major transporter families, are polyspecific transporter to transport multiple different organic cations. The *OCTs* are responsible for body absorption, distribution and elimination of small endogenic cations, environmental xenobiotics, plant or animal toxins, and drugs of clinical importance.

OCT gene is located in a cluster on chromosome 6 and *OCTs* are in major organs such as intestine, liver, kidney, brain and placenta. Especially, *OCT1* is a major hepatic transporter that is distributed in sinusoidal membrane of the liver cells. *OCT2* is in basolateral membrane of the proximal tubule in kidney and distributed in placenta and brain.[2,3)]

OCTs interact with a variety of structurally diverse organic cations, including clinically used drugs (e.g., metformin, procainamide, cimetidine), endogenous compounds (e.g., dopamine, norepinephrine) and toxic substances (e.g., tetraethylammonium bromide, 4-(4-chlorophenyl) -1-[4-(4-fluorophenyl)-4-oxobutyl]pyridinium ion, methyl-4-phenylpyridinium acetate). Especially, metformin acts as a selective substrate to *OCT2*.[4)]

Therefore, *OCTs* mediate the distribution and excretion of organic cations as well as the drug in the major organs including the liver, kidney and brain. Expression of the *OCTs* in normal cells affects the bioavailability and drug response to substrates of the *OCTs*. For example, when the metformin is administered to a person who has decreased expression of *OCTs* in epithelial cell of the kidney, its clearance is decreased, and then drug effect is prolonged. But the drug administered to a person who has increased expression of *OCTs* is eliminated from the body quickly.[5)]

The SNP is a DNA sequence variation occurring when a single nucleotide-A, T, C or G-in the gene differs between mem-

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bers of a species in 30 billion DNA base sequence of the human gene. The SNPs with a minor allele frequency of greater than or equal to 1% are given the title "SNP". The SNP is a very frequent variation which is the general case of the varied human base. The variations frequently occur in human DNA at a frequency of one every 100-300 bases.[6)]

The most of SNPs which exist in intron portion of chromosome (random SNP: rSNP) affect the expression of gene indirectly. But, if the SNP exists in exon portion of chromosome (coding SNP: cSNP), the expression of amino acid is affected directly to change or not. And it is important that the expression of SNP to the portions of promoter and enhancer, affecting splicing process to pre-mRNA and affecting the stability to mRNA affects expression and function of protein which is the last product of the gene.[7)]

Numerous SNPs have been detected in the *OCT1* gene, but functional activities of variants induced by these SNPs have not been characterized.[8,9,10)] We selected two cSNPs in *OCT1* and these are common nonsynonymous, resulting in the amino acid changes; Arg61Cys (C181T; rs12208357) and Pro341Leu (C1022T; rs2282143). And population genetic analysis of the variants and the biochemical characterization of four common non-synonymous variants suggest that *OCT2* plays a significant role in renal elimination.[11)] We selected one cSNPs in *OCT2* and it is common nonsynonymous, resulting in the amino acid change; Ala270Ser (G808T; rs316019). Moreover, we have data regarding to the influence of *OCTs* on the metformin and levosulpiride, which were not published yet. It is important to preliminarily investigate the allele frequency of *OCTs* for description of *OCTs* function. And there were many studies with respect to the ethnic difference of frequency. But these studies didn't include Korean data. So it is necessary to investigate the difference among ethnic groups including Korean.

Because of the problem which has high expense and low efficiency to study associations between the gene and drug effects by using the SNP analysis, haplotype analysis is preferred. Haplotype is a set of SNPs on a single chromatid that is statistically associated and has strong linkage disequilibrium. It is thought that these associations, and the identification of a few alleles of a haplotype block, can identify all other polymorphic sites in its region. Such information is very valuable for investigating the genetics behind common diseases. As analyzing the haplotye, we can estimate the associations between the gene and drug responses.[12)]

Like this, if we know the degree of *OCT* expressions, we can expect the activity and bioavailability of the drug which is the substrate of *OCTs*. Therefore, we investigated the polymorphisms of two *OCT* SNPs (*OCT1* C181T, C1022T and *OCT2* G808T) most frequently suggested to be associated with *OCT* function in humans. In addition, we determined the SNPs in *OCT1* C181T, C1022T and *OCT2* G808T and the haplotype frequencies in the Korean populations.

Experiments

Reagents and Instruments

Primers, 100 bp DNA ladder, 25/100 bp DNA ladder, 50X TAE buffer, 5X TBE buffer and AccuPower™ PCR PreMix (Bioneer Co., Cheongwon-Kun, Chungbuk, Korea), genomic DNA extraction kit (Promega Co., Medison, WI, USA), *Afe I, BstU I* (New England Biolabs, Inc., Beverly, MA, USA), agarose (USB™, Cleveland, OH, USA), Ethidium Bromide (EtBr), deionized water (D.I.W.), 30% glycerin, streptavidin-coated Sepharose beads (Amersham Biosciences, Uppsala, Sweden), NaOH, ethanol, Tris-acetate, Mg-acetate, Tris-HCl, EDTA, Tween 20 (Sigma Chemical Co., St Louis, MO, USA) and the reagent for PSQ (Solgent Co., Ltd., Daejeon, Korea) were used in the experiments. The primers used for amplifying the template are summarized in Table I.

PCR machine (Mastercycler Gradient, Eppendorf AG, Hamburg, Germany) was used for the amplification of target gene. Horizontal gel electrophoresis system (Toylab, Seoul, Korea), SL-20 DNA image visualizer (Seolin Scientific Co., Seoul, Korea), Pyrosequencing 96 MA (Pyrosequencing AB, Uppsala, Sweden), centrifuge (UNION 55R, Hanil Science Industrial Co., Ltd., Inchon, Korea), deep-freezer (DF9014, Il-Shin Lab., Seoul, Korea), vortex mixer (G560, Sciencific Co., Bohemia, NY, USA) and chemical balance (AJ100L, Mettler, Switzerland) were used.

Table I–*Primers Used for Amplifying the Template*

Name	Sequence
(1) *OCT1*(C181T) Forward	5'-CGTGGATGACATTCTGGAGC-3'
(2) *OCT1*(C181T) Reverse	5'-GTGAAGTATCGCGGACGTG-3'
(3) *OCT1*(C1022T) Forward	5'-ATGCTTTCCCTCGAAGAGGATGT-3'
(4) *OCT1*(C1022T) Reverse	5'-AGTTAGGGGTTCCCTCTTTG-3'
(5) *OCT2*(G808T) Forward	5'-CGGAGAACAGTGGGGATTTTTTAC-3'
(6) *OCT2*(G808T) Reverse	5'-CACGTAATTCCTTCCGTCTGAAGA-3'
(7) *OCT2*(G808T) Sequencing	5'-GGTGGTTGCAGTTCACA-3'

Genotyping Procedures

DNA extraction

After receiving informed consents, we obtained a 3 ml blood sample from each subjects in a vial containing EDTA solution (Becton Dickinson, Franklin Lakes, NJ, USA). For genotype determination, genomic DNA was isolated from peripheral leukocytes by Wizard Genomic DNA purification kit (Promega Co., Medison, WI, USA), according to the guidelines of the manufacturer. The DNA was dissolved in 100 μl of DNA hydration solution and stored at -70°C.

Genotype analysis

(1) *OCT1* C181T and C1022T

195 and 825 subjects were genotyped for *OCT1* C181T and C1022T, respectively, and the genotypes were identified by polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) analysis. PCR was carried out with minor modifications to amplify the portions of *OCT1* as described by http://www.pharmgkb.org. For *OCT1* genotype, PCR was carried out in a total volume of 20 μl reaction mixture containing PCR PreMix, 200-300 ng genomic DNA and 10 pmol each primer. The PCR conditions consisted of a denaturation step at 94°C for 2 min followed by 34 cycles, each consisting of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 30 sec. Final extension was performed at 72°C for 5 min. The reaction conditions used for amplifying *OCT1* (C181T and C1022T) are summarized in Table II. After amplification of the specific fragments, SNPs were detected by digestion with specific restriction enzymes: *Afe I* for *OCT1* C181T and *BstU I* for *OCT1* C1022T, capable of discriminating between wild-type and mutant alleles (Table III). The digested PCR products were then separated on 2.5% agarose gels of varying concentrations depending on size, and visualized over ultraviolet transilluminator after ethidium bromide staining (Figure 1 and 2).

C and T allele frequencies were calculated by these equations.

$$\text{C allele frequency (\%)} = \frac{W + 0.5H}{\text{the Number of Sample}}$$

$$\text{T allele frequency (\%)} = \frac{W + 0.5H}{\text{the Number of Sample}}$$

(2) *OCT2* G808T

864 subjects were genotyped for OCT2 G808T and the genotypes were identified by Pyrosequencing analysis. A pair of primers was designed to amplify one region the OCT2 gene by the software PSQ assay program (version 1.0.6). Forward primer has a 5' biotin-triethylene glycol label necessary for post-PCR processing. PCR was carried out in a total volume of 20 μl reaction mixture containing PCR PreMix, 200-300 ng genomic DNA and 10 pmol each primer. The PCR conditions consisted of a denaturation step at 94°C for 5 min followed by 45 cycles, each consisting of denaturation at 94 for 20 sec, annealing at 52.1°C for 30 sec, and extension at 72°C for 20 sec. Final extension was performed at 72°C for 5 min. The reaction conditions used for amplifying *OCT2* G808T are summarized in Table II. Biotinylated PCR products were immobilized on streptavidin-coated Sepharose beads (Amersham Biosciences, Uppsala, Sweden). 20 μl of water, 37 μl of binding buffer (10 mM Tris-HCl, 2 M NaCl, 1 mM EDTA, 0.1% Tween 20, pH 7.6, Pyrosequencing AB, Uppsala, Sweden) and 3 μl of streptavidin-coated Sepharose beads were added to 20 μl PCR product; then the solution was vigorously shaked at room temperature for 10 min. A 96-pin magnetic tool (Pyrosequencing AB, Uppsala, Sweden) was used to transfer up to 96 samples at a time to solutions as follows. The beads with bound template were first transferred to 70% ethanol solution and 0.2 N NaOH solution, then to 1X Washing buffer (Pyrosequencing AB, Uppsala, Sweden), and finally into a solution of 1X annealing buffer (20 mM Tris-acetate, 2 mM Mg-acetate, pH 7.6) containing 10 pmol of the appropriate sequencing primer. Lately, this mixture was heated to 95°C for 1 min and then cooled to and incubated at room temperature for at least 5 min to bind the sequencing primer to the template.

After template preparation, a 96-well plate containing the samples was loaded into the instrument (PSQ 96MA; Pyrosequencing AB, Uppsala, Sweden) along with the appropriate

Table II–*Reaction condition of PCR for OCT1 (C181T and C1022T) and OCT2 (G808T)*

PCR condition	OCT1		OCT2	
	Temperature	Time	Temperature	Time
Initial denaturation	94°C	2 min	94°C	5 min
Denaturation	94°C	30 sec	94°C	20 sec
Annealing	55°C	30 sec	52.1°C	30 sec
Extention	72°C	30 sec	72°C	20 sec
Final extention	72°C	5 min	72°C	5 min

Table III–*Restriction enzyme and digestion products*

	SNP	Enzyme	Digestion products
OCT1	C181T	Afe *I*	W : 173, 98 M : 271
	C1022T	BstU *I*	W : 210, 69 M : 279

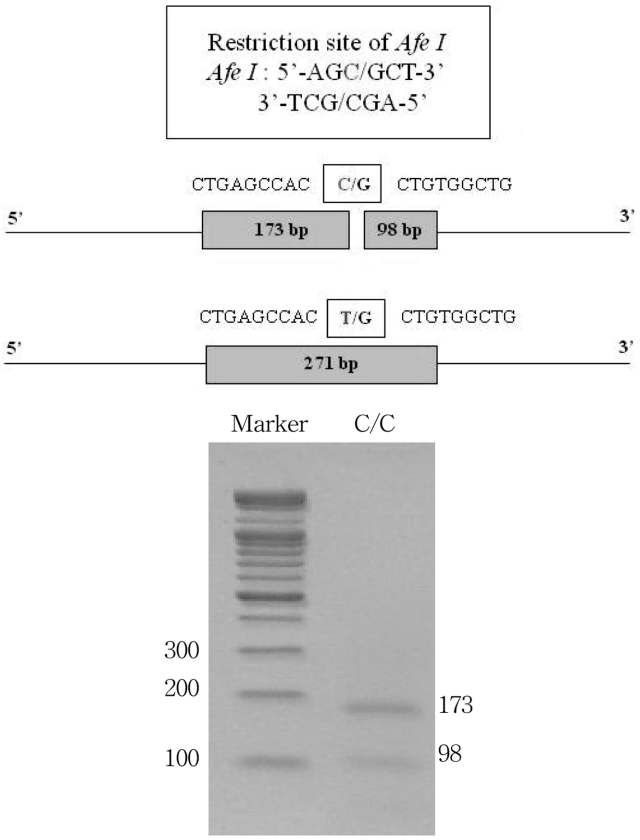

Figure 1–PCR-based diagnostic test for *OCT1* C181T mutation.

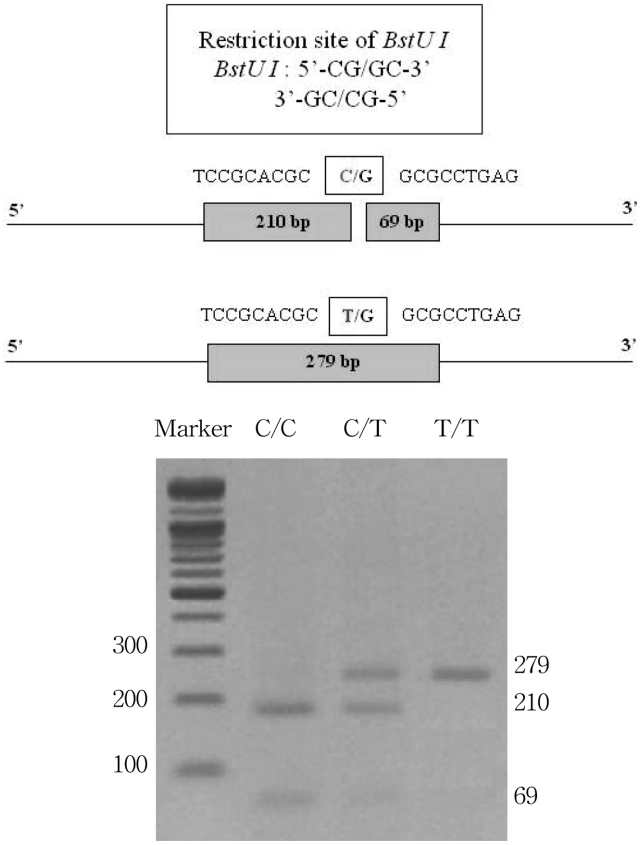

Figure 2–PCR-based diagnostic test for *OCT1* C1022T mutation.

reagents (Pyro Gold; Biotage AB, Uppsala, Sweden). This instrument sequences the templates by dispensing reagents and deoxynucleotide triphosphates in a user-defined order, achieving real-time sequencing by synthesis in an automated fashion. This is achieved by creating and monitoring an enzyme cascade initiated by nucleotide incorporation that produces light emission. Pyrosequencing data were evaluated using Peak Height Determination Software v2.1 (Pyrosequencing AB, Uppsala, Sweden) (Figure 3).

G and T allele frequencies were calculated by these equations.

$$\text{G allele frequency (\%)} = \frac{W+0.5H}{\textit{the Number of Sample}}$$

$$\text{T allele frequency (\%)} = \frac{W+0.5H}{\textit{the Number of Sample}}$$

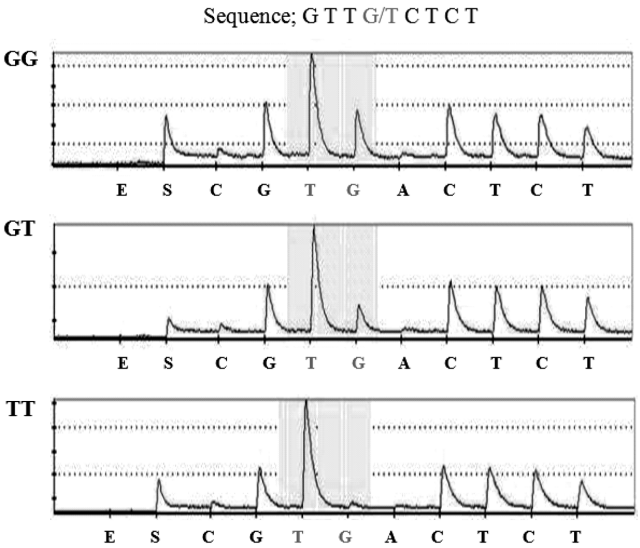

Figure 3–PCR-based diagnostic test for *OCT2* G808T mutation.

Haplotype and Statistical Analysis

Allele and genotype frequencies for the various SNPs were assessed for deviation from Hardy-Weinberg equilibrium using Fisher's exact test. *P* values less than 0.05 were considered statistically significant. Samples in which one or two loci could not be genotyped were excluded from haplotype frequency calculations. Haplotype frequencies were estimated based on the Expectation-Maximization (EM) algorithm using the population genetics data analysis program HapAnalyzer.[13] To control for validity of genotyping, all genotype analyses were

performed three times, and only the genotyping results that were 100% in concordance were used in the actual genotype run.

Results and Discussions

Genotyping analysis of *OCT1* C181T, C1022T and *OCT2* G808T

(1) *OCT1* (C181T)

195 healthy subjects were genotyped for *OCT1* (C181T) from their DNA, respectively and the genotypes were identified by polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) analysis. Primers were bound to *OCT1* (C181T) at 8~27 bp (F: 5'-CGT GGA TGA CAT TCT GGA GC-3') and 170~190 bp (R: 5'-GTG AAG TAT CGC GGA CGT G-3'), respectively. The amplified PCR products were 271 base pair sizes.

SNPs were detected by digestion with specific restriction enzyme: *Afe I* for *OCT1* (C181T), capable of discriminating between wild-type and mutant alleles.

The portions of *OCT1* (C181T) was 5'···AGCCAG**C(T)**GCT GTGG···3' and the restriction site (5'···*AGC*▼*GCT*···3', 3'···*TCG*▲*CGA*···5') was observed in C allele. The gene sequence for the wild type was described by http://www.pharmgkb.org. The C allele was only digested by *Afe I*, but T allele was not digested by restriction enzyme for they did not have restriction site. After being digested, the wild-types were observed to 173 and 98 bp size of DNA fractions and the mutant-types were observed to 271 bp size of DNA fractions. And the hetero-types were observed to 271, 176, 98 bp size of fractions for their C allele and T allele (Figure 1).

The digested PCR products were separated on 2.5% agarose gels depending on size. For the *OCT1* (C181T) polymorphism, the frequency of wild-types (C/C) genotype was 100.0% in 195 subjects (Table IV and V). The frequency of C allele was 100.0% and *OCT1* (C181T) had the same genotype (Table VI).

(2) *OCT1* (C1022T)

825 healthy subjects were genotyped for *OCT1* (C1022T) from their DNA, respectively and the genotypes were identified by polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) analysis. Primers were bound to *OCT1* (C1022T) at 955~977 bp (F: 5'-ATG CTT TCC CTC GAA GAG GAT GT-3') and 1214~1233 bp (R: 5'-AGT TAG GGG TTC CCT CTT TG-3'), respectively. The amplified PCR products were 279 base pair sizes.

SNPs were detected by digestion with specific restriction enzyme: *BstU I* for *OCT1* (C1022T), capable of discriminating between wild-type and mutant alleles.

Table IV–*A number of genotyping subjects in OCT1 (C181T and C1022T) and OCT2 (G808T)*

	Wild	Hetero	Mutant	Total
C181T	195	0	0	195
C1022T	595	202	28	825
G808T	704	154	3	861

Table V–*Frequencies of genotyping subjects in OCT1 (C181T and C1022T) and OCT2 (G808T)*

	Wild	Hetero	Mutant	Total
C181T	100.0	0	0	100
C1022T	72.1	24.5	3.4	100
G808T	81.8	17.9	0.3	100

The portions of *OCT1* (C1022T) was 5'···CGCACGC**C(T)**G CGCCTGAGGA···3' and the restriction site (5'···*CG*▼*CG*···3', 3'···*GC*▼*GC*···5') was observed in C allele. The gene sequence for the wild type was described by http://www.pharmgkb.org. The C allele was only digested by *BstU I*, but T allele was not digested by restriction enzyme for they did not have restriction site. After being digested, the wild-types were observed to 210 and 69 bp size of DNA fractions and the mutant-types were observed to 279 bp size of DNA fractions. And the hetero-types were observed to 279, 210, 69 bp size of fractions for their C allele and T allele (Figure 2).

The digested PCR product were separated on 2.5% agarose gels depending on size. For the *OCT1* (C1022T) polymorphism, the frequency of wild-types (C/C) genotype was 72.1% in 595 out of 825 subjects. The frequency of hetero-type (C/T) genotype was 24.5% in 202 out of 825 subjects and the frequency of mutant-type (T/T) genotype was 3.4% in 28 out of 825 subjects (Table IV and V). The frequency of C and T allele was about 84.4% and 15.6%, respectively. The frequency of C allele was more higher than T allele (Table VI).

(3) *OCT2* (G808T)

861 healthy subjects were genotyped for *OCT2* (G808T) and the genotypes were identified by Pyrosequencing analysis. A pair of primers was designed to amplify one region the *OCT2* gene by the software PSQ assay program (version 1.0.6). Primers were bound to *OCT2* (G808T) at 700~723 bp (F: 5'-CGG AGA ACA GTG GGG ATT TTT TAC -3') and 895~918 bp (R: 5'-AAG GCA TGT ATG TTG GCC TC -3'), respectively. The portions of *OCT2* (G808T) was 5'···GTTCA-CAGTT**G(T)**CTCTGCCCAA···3' The gene sequence for the

Table VI–*Allele frequencies of OCT1 (C181T and C1022T) and OCT2 (G808T)*

Positions of SNP	Allele	Allele frequency(%)
C181T	C allele	100.0
	T allele	0
C1022T	C allele	84.4
	T allele	15.6
G808T	G allele	90.7
	T allele	9.3

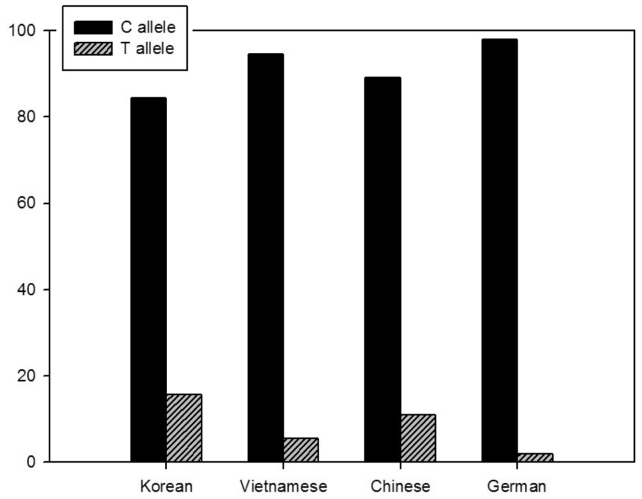

Figure 4–Comparison of *OCT1* C1022T SNP between Korean and other ethnic groups.

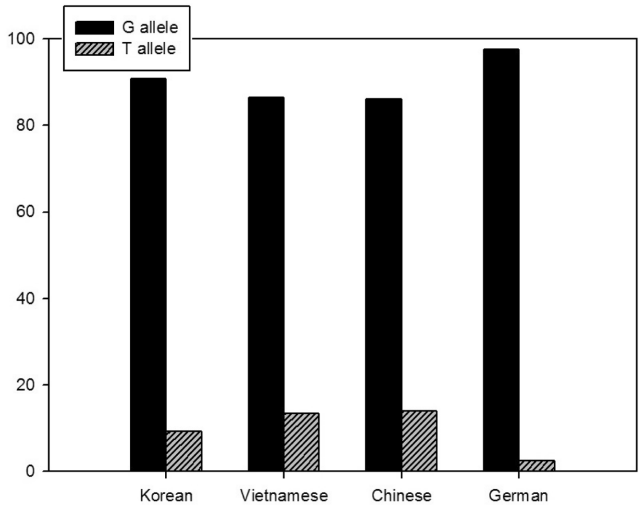

Figure 5–Comparison of *OCT2* G808T SNP between Korean and other ethnic groups.

wild type was described by http://www.pharmgkb.org. The amplified PCR products were 219 base pair sizes. Biotinylated PCR products were genotyped by pyrosequencing instrument (PSQ 96MA; Pyrosequencing AB, Uppsala, Sweden). For the *OCT2* (G808T) polymorphism, the frequency of wild-types (G/G) genotype was 81.8% in 704 out of 861 subjects. The frequency of hetero-type (G/T) genotype was 17.9% in 154 out of 861 subjects and the frequency of mutant-type (T/T) genotype was 0.3% in 3 out of 861 subjects (Table IV and V). The frequency of G and T allele was about 90.7% and 9.3%, respectively. The frequency of G allele was more higher than T allele (Table VI).

Haplotype Analysis

Genotyped 789 subjects in *OCT1* C1022T and *OCT2* G808T were analyzed to gene-based haplotype. According to the analysis results, estimated P value was 0.0122. The close linkage disequilibrium was not detected in *OCT1* C1022T and *OCT2* G808T.

Comparison of the *OCT* SNPs from Different Ethnic Groups

Genoyping results of the *OCT* variants had a number of differences with not only each person but also ethnic groups. We compared the allele frequencies of *OCT1* C1022T and *OCT2* G808T in Korean subjects with them in Chinese, Vietnamese and German already reported.[14] In *OCT1* C1022T, the frequencies of C allele were 84.4, 94.5, 89.0 and 98.0% in Korean subjects, Vietnamese, Chinese and German, respectively. C allele had high frequency rates in four ethnic groups. And especially, the frequency of C allele in German was higher than any other ethnic groups. In *OCT2* G808T, the frequencies of G allele were 90.7, 86.5, 86.0 and 97.5% in Korean subjects, Vietnamese, Chinese and German, respectively. G allele had high frequency rates in four ethnic groups. And also, the frequency of G allele in German was higher than any other ethnic groups. From these results, the wild-type allele was predominant in four ethnic groups. But the difference of frequency rate in ethnic groups was also observed.

Conclusion

Organic cation transporters (*OCTs*), a member of the solute carrier (SLC) 22 family, are one of the major transporter families responsible for body absorption, distribution and elimination of environmental xenobiotics, plant or animal toxins, and drugs of clinical importance. These transporters are especially important for elimination of many endogenous small organic cations. It is expressed in tissues as varied as kidney, liver, intestine, brain, and et al. Therefore, expression levels and function of *OCTs* directly affect plasma levels and intra-

cellular concentrations of drugs and thereby determine therapeutic response. In this study, the frequency of the SNPs on *OCT1* (C181T and C1022T) and *OCT2* (G808T) were investigated by PCR-RFLP and PCR-Pyrosequencing method for analyzing the DNA of Korean subjects, respectively.

From the results, the frequency of T allele in *OCT1* C181T, *OCT1* C1022T and *OCT*2 G808T was 0, 15.6 and 9.3%, respectively. Haplotype frequencies in *OCT1* C1022T and *OCT2* G808T were estimated based on the Expectation-Maximization (EM) algorithm using the investigated genetics data. Estimated P value was 0.0122. The result has revealed that linkage disequilibrium between alleles is not obvious.

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