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Genetic Polymorphisms and Haplotype Structures of the Human *CYP2W1* Gene in a Japanese Population

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Running Title: Genetic polymorphism of *CYP2W1*

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Abbreviations:SNP, single nucleotide polymorphism; DHET, 8,9-dihydroxyeicosatrienoic acid; DHPLC, denaturing high performance liquid chromatography; TEAA, triethylammonium acetate.

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ABSTRACT:

A novel human cytochrome P450, designated CYP2W1, has recently been identified and is found to be present mainly in tumor cells, particularly in colon cancer cells. In the present study, we report the first systematic investigation of polymorphisms in the human *CYP2W1* gene. Based on denaturing high performance liquid chromatography analyses of polymerase chain reaction products, we analyzed 9 exons and exon-intron junctions of the gene in DNA samples from 200 Japanese subjects and identified 6 single nucleotide polymorphisms (SNP). Three of the novel nonsynonymous SNPs were as follows: 173A>C (Glu58Ala) in exon 1, 5432G>A (Val432Ile) and 5584G>C (Gln482His) in exon 9. Two previously known nonsynonymous SNPs, i.e., 2008G>A (Ala181Thr) in exon 4 and 5601C>T (Pro488Leu) in exon 9, were also found. On haplotype analyses, in addition to the wild-type *CYP2W1*1A* (frequency: 0.295) allele, other alleles, namely, *CYP2W1*1B* (0.318), *CYP2W1*2* (0.005), *CYP2W1*3* (0.005), *CYP2W1*4* (0.008), *CYP2W1*5* (0.003), and *CYP2W1*6* (0.368), were also characterized. The most common allele *CYP2W1*6* exhibited the amino acid substitution Pro488Leu. These results were in good agreement with the expected genotype distributions that were calculated using the Hardy-Weinberg equation. The data on variant alleles and comprehensive haplotype structures would be useful for predicting the metabolic phenotypes of CYP2W1 substrates in the Japanese population.

Cytochrome P450s (P450s) are monooxygenases that play an important role in the oxidative metabolism of many therapeutic drugs and endogenous compounds such as fatty acids, vitamins, and steroids. The P450 enzymes are expressed in high levels in the liver, but these enzymes are also found in extrahepatic tissues. Extrahepatic tissues with high P450 expression levels are the respiratory and gastrointestinal tracts that are exposed to foreign compounds entering the body. The extrahepatic P450 enzymes can be important for tissue-specific metabolic activation or the inactivation of xenobiotics compounds.

CYP2W1, an extrahepatic P450, was recently identified. In adult human tissues, CYP2W1 mRNA was either not expressed or expressed at very low levels (Choudhary et al., 2005; Karlgren et al., 2006; Aung et al., 2006). In human tumor tissues, CYP2W1 mRNA is expressed in the colon and adrenal gland (Karlgren et al., 2006). In particular, high CYP2W1 mRNA and protein expressions were found in HepG2 cells (Karlgren et al., 2006). Several studies on CYP2W1 have recently investigated the activity of CYP2W1 heterologously expressed in *Escherichia coli* and mammalian cells. Karlgren *et al.* (2006) reported that the CYP2W1 expressed in HEK293 cells catalyzes the oxidative metabolic conversion of arachidonic acid into 8,9-dihydroxyeicosatrienoic acid (DHET), 11,12-DHET, and 14,15-DHET at a small but significant rate. Wu *et al.* (2006) reported that recombinant CYP2W1 expressed in *E. coli* metabolizes benzphetamine and catalyzes the activation of several procarcinogens, particularly polycyclic hydrocarbon diols. Yoshioka *et al.* (2006) also showed that CYP2W1 expressed in *E. coli* catalyzes the oxidation of indoles.

Wide interindividual differences in metabolic capacity have been detected in many CYP enzymes. For *CYP2W1*, several single nucleotide polymorphisms (SNPs) have been reported thus far, but 3 of them are located only within exons (Ensembl and International HapMap project). The SNP 166C>T that is located in exon 1 is silent, but both the other 2 SNPs, namely, 2008G>A in exon 4 and 5601C>T in exon 9, give rise to the amino acid exchanges

Ala181Thr and Pro488Leu, respectively.

In the present study, we systematically investigated the variants of *CYP2W1* in a population sample that comprised 200 Japanese subjects. In order to analyze the protein-coding region of all of the 9 exons and find novel genetic variations, we used a denaturing high performance liquid chromatography (DHPLC) system. In addition, cloning methods were used to determine these haplotypes of *CYP2W1*.

Materials and Methods

Subjects and DNA samples.

Venous blood was obtained from 200 unrelated healthy Japanese volunteers, and the patients were admitted to Tohoku University Hospital. Written informed consent was obtained from all blood donors, and the study was approved by the Local Ethics Committee of Tohoku University Hospital and Tohoku Pharmaceutical University. DNA was isolated from K_2EDTA-anticoagulated peripheral blood by using QIAamp DNA Mini Kits (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions.

PCR amplification.

Table 1 lists the primer pairs that were used to amplify the entire coding sequence and the exon-intron junctions of the *CYP2W1* gene. These primers were designed based on the genomic sequence reported in GenBank (NT_007819). Amplicons of exon 1 were generated using the AmpliTaq Gold PCR Master Mix (Applied Biosystems, Foster City, CA, USA). PCR reactions were performed using the iCycler (Bio-Rad, Hercules, CA, USA). Moreover, the method relied on the combination of the slowdown method (Bachmann et al., 2003) and the addition of betaine (Sigma-Aldrich, St.Louis, MO) for this region with high GC content (>70%). The PCR protocol was as follows: denaturation at 95ºC for 5 minutes, followed by 48 cycles of denaturation at 95ºC for 30 seconds, annealing for 30 seconds, extension at 72ºC for 40 seconds,

and finally, 15 additional cycles with an annealing temperature of 60ºC. The amplicons for exons 2–9 were generated using the AmpliTaq Gold PCR Master Mix and betaine addition. The PCR protocol was as follows: denaturation at 95ºC for 10 minutes, followed by 30 or 35 cycles of denaturation at 95ºC for 30 seconds, annealing for 30 seconds, extension at 72ºC for 30 seconds, and a final extension at 72ºC for 7 minutes. The annealing temperatures and PCR cycles for the screening of *CYP2W1* variants are summarized in Table 1.

DHPLC analysis.

The PCR products were analyzed using the DHPLC system (WAVE; Transgenomic Inc., Omaha, NE, USA) (Hiratsuka et al., 2004a; Hiratsuka et al., 2004b; Ebisawa et al., 2005; Hiratsuka et al., 2005; Hiratsuka et al., 2006; Sasaki et al., 2006; Hanzawa et al., 2007). Unpurified PCR samples (5 μL) were separated on a heated C18 reverse-phase column (DNAsep) by using 0.1 M triethylammonium acetate (TEAA) in water and 0.1 M TEAA in 25% acetonitrile at a flow rate of 0.9 mL/min. The software provided with the instrument selected the temperature for the heteroduplex separation in the heterozygous *CYP2W1* fragment. Table 1 summarizes the DHPLC running conditions for each amplicon. The linear acetonitrile gradient was adjusted to the retention time of the DNA peak at 4–5 minutes.

Homozygous nucleotide exchanges can occasionally be distinguished because of a slight shift in the elution time as compared with the reference. The addition of an approximately equal amount of wild-type DNA to the samples (1:1) before the denaturation step enabled the reliable detection of homozygous alterations. This was performed routinely for all samples to identify homozygous sequence variations. Therefore, all the samples were analyzed as follows: first, the sample alone to detect heterozygotes, and then, after mixing each sample with wild-type DNA to detect homozygous variants. The resultant chromatograms were compared with those of the wild-type DNA.

Direct sequencing.

Both strands of samples with variants as determined by DHPLC were analyzed using a CEQ8000 automated DNA sequencer (Beckman-Coulter Inc., Fullerton, CA, USA). We also sequenced all samples with chromatographic findings that differed from the wild type to establish links between mutations and specific profiles. We sequenced the PCR products by fluorescent dideoxy termination by using the DTCS DNA Sequencing Kit (Beckman-Coulter Inc.) in accordance with the manufacturer's instructions.

Haplotype analysis.

In order to determine the linkage among the polymorphisms identified in this study, PCR reactions were used to amplify long fragments that were obtained from individuals who were heterozygous at sites of interest. Long amplicons (5701bp) were generated with LA-Taq DNA polymerase (TaKaRa Co., Kyoto, Japan). The PCR protocol was as follows; denaturation at 95ºC for 5 minutes, followed by 30 cycles of denaturation at 95ºC for 1 minute, annealing at 60ºC for 1 minute, extension at 68ºC for 5 minutes, and a final extension at 72ºC for 7 minutes. The forward and reverse primers were 5′-ggacggggcccaggaggggagtgga-3′ and 5′-gctgggaggggagtggtcaggagga-3′, respectively. The fragment was run on a low-melting gel, gel-purified, and ligated to the pCR-XL-TOPO vector (Invitrogen Co., CA, USA). After ligation, the plasmid was transfected into *E. coli* strain TOP10 (Invitrogen Co., CA, USA), and single colonies (each containing a plasmid with only 1 of the 2 alleles) were grown and subjected to plasmid isolation and sequencing using the CEQ8000 automated DNA sequencer. In cases of samples that having both heterozygous 5601C>T and 166C>T, eight samples were cloned and sequenced to ascertain whether or not they are found together on the same chromosome.

Results

DHPLC and sequence analysis.

DHPLC analysis of the *CYP2W1* gene for the 200 DNA samples obtained from Japanese individuals revealed chromatographic profiles that were distinct from those of the wild type for exons 1, 4, and 9. Direct sequencing analysis of the deviant DNAs detected by DHPLC revealed a total of 6 different polymorphisms, including 3 novel nonsynonymous (Glu58Ala, Val432Ile, and Gln482His) polymorphisms, 2 known nonsynonymous (Ala181Thr and Pro488Leu) polymorphisms, and 1 silent SNP (Leu56Leu). The locations and frequencies of these polymorphisms are described in Table 2. We did not observe any variants affecting the recognition sequences of the exon-intron splice site or any variants that would create new putative splice sites near the exon-intron boundaries.

Haplotype analysis.

Based on the concomitant occurrence of the SNPs among the individuals studied and the linkage analysis by the cloning method, the different SNPs can be deduced to comprise 7 haplotypes, as described in Fig. 1. A genomic reference sequence of accession number NT_007819 was defined as the wild-type allele *CYP2W1*1A*. The other alleles were named according to the recommendations of the CYP allele nomenclature committee (http://www.cypalleles.ki.se/). The allelic variants (*CYP2W1*1B*–*CYP2W1*6*) discovered in this study have been submitted to the CYP alleles web page.

Allele and genotype frequencies.

The frequency of the alleles and genotypes discovered are listed in Fig. 1 and Table 3, respectively. *CYP2W1*6* was the most common among all the alleles. It occurred at a frequency of 36.8% in our study population (Fig. 1). The frequencies of the various genotypes observed in our Japanese population followed those predicted by the Hardy-Weinberg law (Table 3). These results were in good agreement with the expected genotype distributions that were calculated using the Hardy-Weinberg equation.

Discussion

In the present study, we performed a comprehensive investigation of the genetic variations in *CYP2W1* gene. In order to screen for SNPs in the coding region of this gene, we developed a PCR-DHPLC assay that allows molecular analysis of each exon of the gene. The sequence designated in NCBI as the genome reference sequence with the accession number NT_007819 was defined as the wild-type allele *CYP2W1*1A*. Five of the 6 SNPs detected in the *CYP2W1* gene in the DNA samples of 200 Japanese subjects resulted in amino acid substitutions. The alleles carrying these alterations were named *CYP2W1*2– CYP2W1*6* by the CYP allele nomenclature committee. The variant allele carrying the silent SNP (166C>T, Leu56Leu) was defined as *CYP2W1*1B*. The most frequent variant allele was *CYP2W1*6*, followed by *CYP2W1*1B* and *CYP2W1*1A*, and their frequencies were observed to be 0.368, 0.318, and 0.295, respectively. Three novel nonsynonymous SNPs, 173A>C (Glu58Ala), 5432G>A (Val432Ile) and 5584G>C (Gln482His), identified in this study were rare, with allele frequencies <1%.

Homology modeling of the human CYP2 family enzymes based on the CYP102 crystal structure (Lewis 1998) lead to speculation that Val432Ile is located at the heme-binding region. The important region called “P450 signature motif region” comprises the 10-residue P450 signature motif that contains the invariant cysteine that forms the proximal heme ligand. This region and the following L-helix represent one of the most highly conserved sections. This homologous segment is associated with the binding of both heme and redox partners with the common motif Phe-X-X-Gly-X-Arg-X-Cys-X-Gly. In wild-type *CYP2W1*1A*, this motif is Phe-Ser-Ala-Gly-Arg-Arg-Val-Cys-Val-Gly; in *CYP2W1*4* and *CYP2W1*5*, including Val432Ile, the first valine is changed to isoleucine (Phe-Ser-Ala-Gly-Arg-Arg-Ile-Cys-Val-Gly). For example, the Pro428Thr substitution of CYP2B6 (*CYP2B6*21*) in the same region has been clearly identified as being responsible for the decreased protein stability (Klein et al., 2005).

Therefore, the homologous segment of the CYP2 family, including CYP2W1, might be closely associated with enzyme activity. Further studies, such as the use of the recombinant CYP2W1 protein, are required to confirm the function of these variant enzymes.

Karlgren *et al.* (2006) have reported that CYP2W1 mRNA was detected by real-time PCR of human tumor cells; the highest expression was in colon tumors, but moderate expression was also observed in several adrenal gland tumor cells. They also suggest that CYP2W1 is potentially an important drug target or a useful molecular marker for cancer therapy and diagnosis (Karlgren et al., 2007). If this is the case, further understanding of the nature of CYP2W1 would be important for cancer therapy.

In summary, this comprehensive investigation of the polymorphisms in the coding region of the *CYP2W1* gene identified 6 variant *CYP2W1* alleles (*CYP2W1*1A–CYP2W1*6*). In vitro analysis of recombinant mutated cDNAs as well as phenotyping studies will help in determining the functions of the identified variants.

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Footnotes

On September 18, 2007, these SNPs detected in this study were not present in dbSNP in the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/SNP/), GeneSNPs at the Utah Genome Center (http://www.genome.utah.edu/genesnps/), or the Human CYP Allele Nomenclature Committee database (http://www.cypalleles.ki.se/).

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Legends for figures

Fig. 1. Allelic variants of the human *CYP2W1* gene.

Schematic representation of "wild-type" allele for *CYP2W1* (*CYP2W1*1A*) and 6 variant alleles. The numbering is based on the first nucleotide of the initiation codon that is represented as 1. *CYP2W1** alleles were named in accordance with the established nomenclature for the P450 alleles (http://www.cypalleles.ki.se/). The frequency of each *CYP2W1** allele has been estimated on the population of 200 Japanese volunteers.

Table 1

Amplification and DHPLC conditions for *CYP2W1* SNP analysis of genomic DNA

Exon	Size (bp)	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	Annealing Temp. (ºC)	PCR cycles	DHPLC Temp. (ºC)
1	264	ggacggggcccaggaggggagtgga	ggcagctgtccaagcggcaagagct	Slowdown[a] 70.0-55.0	63	64.8, 67.5
2	253	cttgtgggtgagggctgcccgggtg	tgcccccacacccagtaggccccgt	60.0	35	66.5
3	240	ctggggtgggaacctgggctcacca	ggcacgtccaggcccgggggaggggc	60.0	35	65.0, 67.5
4	248	ccccctccccgggcctggacgtgcct	actccaggctccaccccaccccaag	60.0	35	64.0
5	264	cctggggctgcgtccttatctccgc	caggacccctacaggccttcaagga	60.0	35	65.5
6	229	acagaccccagatcatcccacgagc	ccccgggggcagaaggagccgtctc	60.0	30	66.4
7	275	acgagggatggcgctgccacccaag	cctaccccagaggagatggaaggggg	60.0	35	66.8
8	232	atcttccccggggcccctctctctg	gagccctggaggtgccgccccaccc	60.0	35	65.4
9	278	agcaggcctggtgcagcccactctg	gctgggaggggagtggtcaggagga	60.0	35	66.8

[a]Slowdown protocol: The annealing temperature was decreased after cycle 3 by 1.0ºC every 3 cycles beginning at 70ºC and decreased to a "slowdown" annealing temperature of 55ºC, followed by 15 additional cycles with an annealing temperature of 60ºC. The PCR was used with a ramp rate at 2.5ºC/s and reaching annealing temperature at 1.5ºC/s.

Table 2

Location of SNPs and frequencies of the *CYP2W1* gene in 200 DNA samples of Japanese individuals

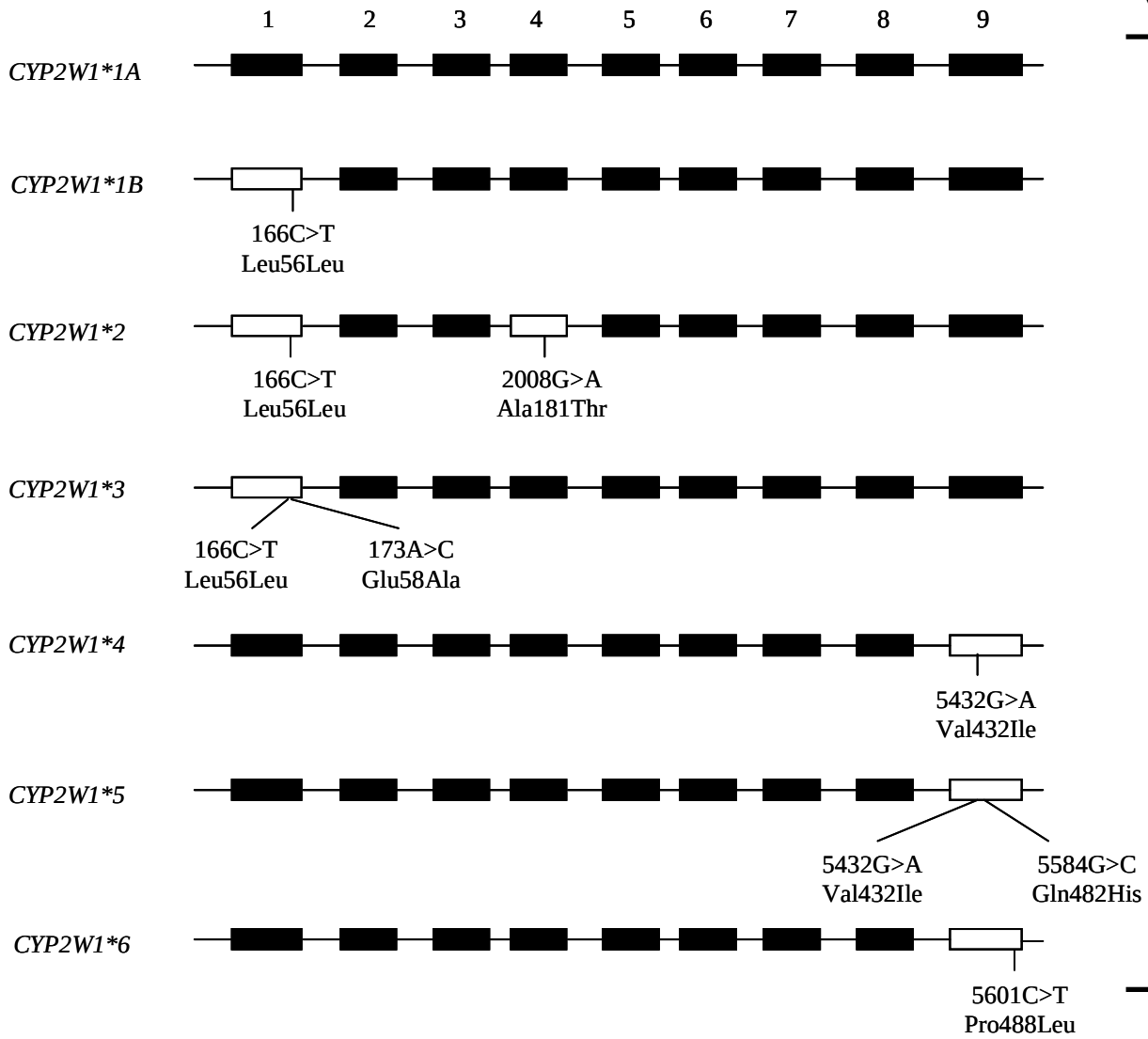
Exon	Location	Genotype	No. of Subjects (n=200)	Observed Frequency (95% CI)	Predicted Frequency by Hardy-Weinberg's law
1	166 C>T	C/C	92	46.0 (39.1-52.9)	44.9
	(Leu56Leu)	C/T	85	42.5 (35.6-49.4)	44.2
		T/T	23	11.5 (7.1-15.9)	10.9
1	173 A>C	A/A	198	99.0 (97.6-100)	99.0
	(Glu58Ala)	A/C	2	(0-2.4)	1.0
		C/C	0	0 (0)	0
4	2008 G>A	G/G	198	99.0 (97.6-100)	99.0
	(Ala181Thr)	G/A	2	1.0 (0-2.4)	1.0
		A/A	0	0 (0)	0
9	5432 G>A	G/G	196	98.0 (96.1-99.9)	98.0
	(Val432Ile)	G/A	4	2.0 (0.1-3.9)	2.0
		A/A	0	0 (0)	0
9	5584 G>C	G/G	199	99.5 (98.5-100)	99.5
	(Gln482His)	G/C	1	0.5 (0-1.5)	0.5
		C/C	0	0 (0)	0
9	5601 C>T	C/C	80	40.0 (33.3-46.7)	40.1
	(Pro488Leu)	C/T	93	46.5 (39.7-53.3)	46.4
		T/T	27	13.5 (8.7-18.3)	13.5

Table 3

Frequencies of *CYP2W1* genotypes in Japanese individuals

CYP2W1 genotype	No. of subjects (n=200)	Observed frequency (%) (95% CI)	Frequency (%) predicted by Hardy-Weinberg law
*CYP2W1*1A/*1A*	21	10.5 (6.2-14.8)	8.7
*CYP2W1*1A/*1B*	33	16.5 (11.5-21.6)	18.8
*CYP2W1*1A/*4*	2	1.0 (0-2.4)	0.5
*CYP2W1*1A/*5*	1	0.5 (0-1.5)	0.2
*CYP2W1*1A/*6*	40	20.0 (14.5-25.5)	21.7
*CYP2W1*1B/*1B*	22	11.0 (6.7-15.3)	10.1
*CYP2W1*1B/*3*	1	0.5 (0-1.5)	0.3
*CYP2W1*1B/*6*	49	24.5 (18.5-30.5)	23.4
*CYP2W1*2/*6*	2	1.0 (0-2.4)	0.4
*CYP2W1*3/*6*	1	0.5 (0-1.5)	0.4
*CYP2W1*4/*6*	1	0.5 (0-1.5)	0.6
*CYP2W1*6/*6*	27	13.5 (8.8-18.2)	13.5

Figure 1



	No. of alleles (n=400)	Ratio (%) of alleles (95% CI)
*CYP2W1*1A*	118	29.5 (25.0-34.0)
*CYP2W1*1B*	127	31.8 (27.2-36.4)
*CYP2W1*2*	2	0.5 (0-1.2)
*CYP2W1*3*	2	0.5 (0-1.2)
*CYP2W1*4*	3	0.8 (0-1.7)
*CYP2W1*5*	1	0.3 (0-0.8)
*CYP2W1*6*	147	36.8 (32.1-41.5)