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Novel cytochrome P450-2D6 promoter sequence variations in hepatitis C positive and negative subjects(◆)

Martin T. Ho,*,** Edward J. Kelly,* Miklos Bodor,*,*** Tot Bui,* Kris V. Kowdley,**,**** Rodney J.Y. Ho*

*Department of Pharmaceutics, University of Washington, Seattle, WA. USA. ** Department of Medicine, University of Washington, Seattle, WA. USA.
*** First Department of Internal Medicine, Medical Health and Science Center, University of Debrecen, Debrecen, Hungary.
**** Center for Liver Disease, Virginia Mason Medical Center and Benaroya Research Institute, Seattle, WA. USA.

ABSTRACT

Introduction. CYP2D6 is a liver enzyme that metabolizes more that 25% of drugs and thus may play a pivotal role in drug-drug interactions. The promoter sequences of cytochrome P450 2D6 (CYP2D6) gene could impact metabolic activity. **Methods.** We analyzed genetic variations in the promoter sequence of CYP2D6 gene for 71 hepatitis C negative and 15 hepatitis C positive subjects. **Results.** We found two novel genetic variants -1822A→G; -1740C→T, only in two patients with hepatitis C. Also, two linked new promoter sequence variations at -2060 G→A and -2053 T→G nucleotide positions that present in both hepatitis C negative and positive subjects are identified. The -2060 and -2053 variations are confirmed to be in linkage disequilibrium. The individuals with -2060A/A, and -2053G/G variation appeared to be associated with significantly lower levels of liver CYP2D6 mRNA. Analysis of CYP2D6 enzymatic activity in *1/*1 (wild type) subjects revealed that hepatitis C positive subjects expressed about 2.6-fold lower activity (24.0 ± 1.5 *vs.* 62.6 ± 3.7 pmol/min/mg; p = 0.0061) relative to hepatitis C negative. **Conclusion.** These data suggest that promoter variations -1822A→G and -1740C→T are present only in hepatitis C infected subjects. Hepatitis C positive individuals were associated with a lower liver CYP2D6 enzyme activity.

Key words. CYP450 enzyme. Genotype. Phenotype. Genetic variant. Hepatitis.

INTRODUCTION

Cytochrome P450 2D6 (CYP2D6) is responsible for the oxidative metabolism of more than 25% of the currently prescribed medications, including antiarrhythmic, β-adrenoceptor blocking agents, serotonin-selective reuptake inhibitors, tricyclic antidepressants and opioid analgesics.[1] It is one of the key metabolic enzymes central to variation in drug-drug interactions. The CYP2D6 gene consists of nine exons and eight introns, and is located on chromosome 22 with two pseudogenes, CYP2D7 and CYP2D8P.[2] CYP2D6 genes are highly polymorphic, and as a result, a broad range inter-individual variation is reported for hepatic CYP2D6 expression and function.[3] A continuously expanding database (www.imm.ki.se/CYPalleles) lists more than 80 allelic variants, including 15 null alleles encoding nonfunctional gene products with impaired enzymatic function. Other variants produce enzymes with increased, decreased or normal CYP2D6 activity.

Based on the metabolism of CYP2D6 probe drugs such as debrisoquine dextromethorphan or sparteine, four different phenotypes have been described. These phenotypes include the ultrarapid metabolizer (UM), extensive metabolizer (EM), intermediate metabolizer (IM) and poor metabolizer (PM) phenotype.[4-6]

Although many allelic variants within CYP2D6 have been reported, very little is known about genetic variations within the ~2 kb promoter of the CYP2D6 gene. Thus, we analyzed the CYP2D6 promoter sequences. We discovered new novel variants within the CYP2D6 promoter sequence, some of which are found only in hepatitis C positive, while others are found in both hepatitis C negative and positive subjects.

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Correspondence and reprint request: Rodney JY Ho, Ph.D.
Department of Pharmaceutics. University of Washington. Seattle, WA 98195-7610.
Tel.: 01 206 543-3796. Fax: 01 206 543-3204
E-mail: rodneyho@u.washington.edu

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METHODS

Tissue procurement

The 71 hepatitis C negative livers were collected from patients undergoing liver diagnosis procedures (n = 11) and from the liver bank (n = 60) at the University of Washington. The hepatitis C positive (n = 15) samples were collected by needle biopsy from patients undergoing similar liver diagnosis procedures. All the samples were collected with approval from the University of Washington Institutional Human Subjects Board. Liver tissue was immediately snap-frozen in liquid nitrogen and stored at -80 ºC prior to analysis.

DNA ANALYSIS

DNA was extracted from liver tissue using the Qiagen DNeasy Kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions. For sequence analysis, oligonucleotide primers were synthesized by Invitrogen Life Technologies (Carlsbad, CA). Primers were designed using the Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) using the CYP2D6 genomic sequence accession NG_003180 [Homo sapiens cytochrome P450, family 2, subfamily D, polypeptide 6 genomic region (CYP2D6 on chromosome 22)].

The forward PCR primer (5'GCAAAGAGCCAGGACTGGTA3') encompasses nucleotides (nt) 5441-5460 and the reverse (5'CCAGTGCTTCTAGCCCCATA3') nt7744-7725 for NG_003180; the transcription start site for CYP2D6 mRNA is nucleotide 7638. For PCR, 100 ng DNA was added to a reaction containing 200 nM oligonucleotides primers, 0.2 mM of each dNTP, 1X final concentration of PfuTurbo® Hotstart buffer and 1.25 units of PfuTurbo® Hotstart DNA polymerase (Stratagene, La Jolla, CA) in 25 μL. Following 2 min 95 ºC denaturation, a PCR sequence was initiated as follows: 30 s at 95 ºC, 30 s at 65ºC and 2.5 min at 72 ºC for 35 cycles with a final extension period of 10 min at 72 ºC.

The PCR reaction generated a single amplicon of 2.3 kb as verified by agarose gel electrophoresis; the product was purified using a QIAquick PCR purification kit (Qiagen Inc., Valencia, CA). The purified PCR product was sequenced using BigDye® Terminator v3.1 Cycle Sequencing kit and a 3100-Genetic Analyzer (Applied Biosystems, Foster City, CA). The sequencing primers used included both the forward and reverse primers as well as the following primers (position on NG_003180 indicated in parentheses):

- 5'CCATACAATCCACCTGTAGA3'(nt 5897-5916).
- 5'CAACAAGAGGAAATCTCCGT3'(nt 6439-6458).
- 5'ATCCAGGAAACCTCCGGCAT3'(nt 6893-6912).
- 5'CCACTGAAACCCTGGTTATC3'(nt 7365-7834).

CYP 2D6 RNA determination

Tissue levels of CYP2D6 mRNA were determined by a quantitative competitive RT-PCR method. Briefly, total RNA was isolated from the liver samples using the Totally RNA Microprep Kit (Stratagene, La Jolla, CA). RT-PCR was accomplished with the use of the Titan One-Tube RT-PCR System (Roche Diagnostics, Indianapolis IN). In brief, serial dilutions of total RNA (0-200 ng) from liver samples and a fixed copy number of Δ2D6 were mixed with 200 μM of each dNTP, 2.5 μL dithiothreitol, 1 μL of the enzyme mix (AMV reverse transcriptase and Expand High™ Fidelity enzymes), 0.4 μM of each primer, 6 units RNAase inhibitor, 10 μL 5X RT-PCR buffer, and sterile nuclease-free H_2O to a final volume of 50 μL.

The primers were derived from the CYP2D6 sequence data from Genbank (M33388.1) as follows: forward 5'- (nt 1655) AGTGGCCATCTTCCTGCTCC, and reverse 5'- (nt 5832) CGGGGCACAGCACAAAGC. Reactions were conducted in standard thin-walled tubes using a Perkin-Elmer model 9600 Thermocycler (PE, Foster City CA). The first step, reverse transcription, was performed for 45 minutes at 47 ºC. This was immediately followed by PCR: initial denaturation at 94 ºC for 2 min, 10 cycles of 94 ºC for 30 s, 59 ºC for 30 s, and 68 ºC for 1.5 min. This was followed by 25 cycles of 94 ºC for 30 s, 62 ºC for 30 s, and 68 ºC for 1.5 min with an incremental increase of 5 s per cycle. The last of the 25 cycles was followed by a final extension at 68ºC for 7 min. Products were separated with a 2.0% agarose gel stained and analyzed on digitized fluorophotographs to provide CYP2D6 RNA levels based on per microgram of total liver as described previously.[7]

CYP2D6 enzyme activity

The *in vitro* enzymatic activity of CYP2D6 in the human liver specimens was determined by O-demethylation of dextromethorphan to dextrorphan as described previously.[8] The data presented for CYP2D6 enzyme activity for normal liver biopsies (as well as genotype for the 6 most common CYP2D6 genetic variants i.e. *3, *4, *5, *6, *7 and *8) were determined with a CYP2D6 substrate as previously described.[7] For each sample, 0.25 mg/mL total tissue homogenate protein in 0.5 mL was pre-incubated

Table 1. The profile of hepatitis C negative and positive study subjects.

Hepatitis	N	Age (year)		Sex		Ethnicity*			
		Median	Range	M	F	C	B	A	O
Negative	71	49	7-76	41	30	67	3	1	0
Positive	15	46	25-57	9	6	10	0	1	4

* Ethnicity abbreviations. C: Caucasian. B: Black. A: Asian. O: Other or undisclosed.

with 12 μM dextromethorphan for 5 min in 37 °C water bath. Reactions were initiated with the addition of 1 mM NADPH and terminated at 10 min by the addition of 2 mL acetonitrile. Internal standard (16 ng butorphanol) was added followed by centrifugation to remove protein. After drying under a gentle stream of air, samples were injected onto the liquid chromatograph-mass spectrophotometer (LC-MS).

Chromatographic separation of dextrorphan, dextromethorphan and butorphanol was accomplished with a Zorbax C-18 column, and mobile phase consisting of 70% 10 mM ammonium acetate (pH 4.0) and 30% acetonitrile at a flow rate of 0.2 mL/min. The retention times were approximately 4.4 min for dextrorphan and 8.3 min for butorphanol. The mass spectrometer (Agilent Model # G1946B) was set on the positive ion electrospray mode, and mass ions at *m/z* ratios of 258 and 328 were monitored that corresponded to the MH^+ dextrorphan and butorphanol, respectively. CYP2D6 activity was expressed as dextrophan formation in pmol/min/mg protein.

Linkage disequilibrium and statistical analysis of the RNA and CYP2D6 activity data:

Linkage disequilibrium was analyzed using an online Cubex program to estimate pairwise haplotype frequencies.[9] These data were further analyzed with a Hardy-Weinberg equilibrium calculator that includes analysis for bias. One-way ANOVA with Tukey post test was performed using GraphPad InStat version 3.06 (San Diego, CA) for each individual SNP and where applicable, two-tailed unpaired t-tests and *p* values were calculated. A p value of 0.05 is considered statistically significant.

RESULTS AND DISCUSSION

To determine sequence variation in the CYP2D6 promoter, we examined the entire 2.3 kb region upstream of the transcriptional start site. The demographic and ethnicity characteristics of the hepatitis C positive and negative subjects are listed in table 1. The age range of hepatitis C negative subjects (n = 71) was 7-76 years (median = 49); 41 M and 30 F; 3 Black, 1 Asian and the remaining 67 were Caucasian. The age range of hepatitis C positive subjects (n = 15) was 27-57 years (median = 46); 9 M and 6 F; 10 Caucasian; 1 Asian and 4 undisclosed. In the 86 subjects tested, we found 11 sites of genetic variations within the 2.3 kb CYP2D6 promoter. As shown in table 2, we discovered four novel allelic variations, at:

- -2060 G→A.
- -2053 T→G.
- -1822 A→G.
- -1740 C→T.

Two of these variations (at -2060 G→A, -2053 T→G) appeared to occur with similar frequency between hepatitis C negative and positive subjects. The remaining two new variations, -1822 A→G and -1740 C→T were found only in two Caucasian hepatitis C positive subjects (Table 2). We did not find any of the 71 (of which 67 Caucasian) hepatitis C negative subjects that exhibited these two variants.

In addition to the above novel variants, we have identified seven promoter variants that have been reported by others (Table 2); namely:[10]

- -1770 A→G.
- -1584 C→G.
- -1426 C→T.
- -1253 A→G.
- -1000 G→A.
- -740 C→T.
- -678 G→T.

Comparing the frequency of these variants between the two groups indicates that there about 61.5% higher (21.8 *vs.* 83.3%) occurrences of -1253 A→G in hepatitis C positive than negative groups. The remaining six variants (listed above) expressed less than 18% difference between the two study groups (Table 2).

We next determined whether any of these variant alleles are linked. We found that the three set of CYP2D6 variations [-2060, -2053], [-1426, -1000] and [-740, -678] appeared to be linked. Based on the

Table 2. Analysis of CYP2D6 promoter sequence variant allele frequency in hepatitis C positive and negative subjects.*

Hepatitis C	N	5661 -2060	5668 -2053	5899 -1822	5951 -1770	5981 -1740	6137 -1584	6295 -1426	6491 -1235	6726 -1000	6986 -740	7048 -678
		G > A†‡	T > G†‡	A > G†,§	A > G	C > T†,§	C > G	C > T‡	A > G	G > A‡	C > T‡	G > A‡
Negative	71	35.2%	35.2%	0%	64.7%	0%	19.7%	33.8%	21.8%	33.8%	22.5%	22.5%
Positive	15	40.0%	40.0%	6.7%	60.0%	3.3%	30.0%	16.7%	83.3%	16.7%	36.7%	40.0%
HW Equilibrium‖ (χ^2 test for deviation)		2.32	0.59	86	40.58	0	54.67	8.71	34.5	8.71	70.74	66.27

*DNA samples were extracted from the livers of hepatits C positive patients and hepatits C negative individuals. The DNA was analyzed for allelic variance at the indicated promoter site on CYP2D6. The data, expressed in the form of a percentage of individuals out of the sample expressing the variant allele for the hepatits C negative and positive group. The sequence positions are also listed in reference to the CYP2D6 transcription start site, as well as that listed in the Genbank # NG_00318 sequence. †Indicates a previously unreported allele. ‡Indicates an allele that is linked to another allele at a different locus: [-2060, -2053] [-1426, -1000] [-740, -678]. Variations between one locus of the pair of linked alleles are reciprocated in the other for all sequence analysis performed. §Indicates an allelic variation where the variant allele appears only in the hepatitis C positive group. ‖ Hardy-Weinberg equilibrium analysis is done for both test groups and expressed as Chi-squared data. With 1 degree of freedom for 86 samples, the p value of 0.05 is equivalent to Chi-squared value of 3.84.

linkage disequilibrium analysis with the Cubex program to estimate pairwise haplotype frequencies,[8] followed by Chi-squared test, the above three set of alleles are linked. Hardy-Weinberg equilibrium analysis revealed a significant disequilibrium ($p < 0.001$) observed for all three heplotypes.

We subsequently determined whether any of the three haplotypes are associated with CYP2D6 RNA expression. We found no apparent association of the [-1426, -1000] and [-740, -678] CYP2D6 promoter haplotype with RNA expression. However for [-2060, -2053], we found that, compared to [G/G, T/T] or [G/A, T/G], the [-2060A/A, -2053 G/G] haplotype was associated with lower CYP2D6 RNA regardless of the hepatitis C status of subjects tested (Figure 1).

We then analyzed liver samples that did not contain CYP2D6 genetic variance, and coding regions that are reported to influence CYP2D6 enzyme activity. Due to the limited tissue size, only 11 of 15 hepatitis C negative and 13 of 15 hepatitis C positive samples were available for CYP2D6 analysis. The data were summarized in figure 2 as a box-Whisker plot. We found that CYP2D6 enzyme activity between hepatitis C positive and negative subjects expressing a wild-type (*1/*1) genotype exhibited about a three-fold difference between the two groups (24.0 ± 1.5 *vs.* 62.6 ± 3.7 pmol/min/mg protein: p = 0061). These data suggest that hepatitis C positive subjects are associated with significantly lower hepatic CYP2D6 activity.

With evaluation of 2.3kb DNA sequences upstream of the CYP2D6 start site, we have identified four novel promoter variants. These novel variants include 2 alleles that are linked, -2060 G→A and -2053 T→G and are found in both hepatitis C positive and negative subjects with similar frequency. In addition to [-2060 C→T, -2053 T→G] haplotype, our data also confirmed the haplotype of the following set of variations, [-1426 C→T, -1000 G→A] and [-740 C→T, -678 G→A]. The other two novel variants, -1822 A→G and -1740 C→T were found only in hepatits C positive subjects. Finally, we analyzed the enzymatic activity of the two subject groups. We found, in a subset of *1/*1 phenotype (wild type) individuals, that the CYP2D6 enzymatic activity was three-fold higher in the hepatitis C negative than that in hepatitis C positive subjects.

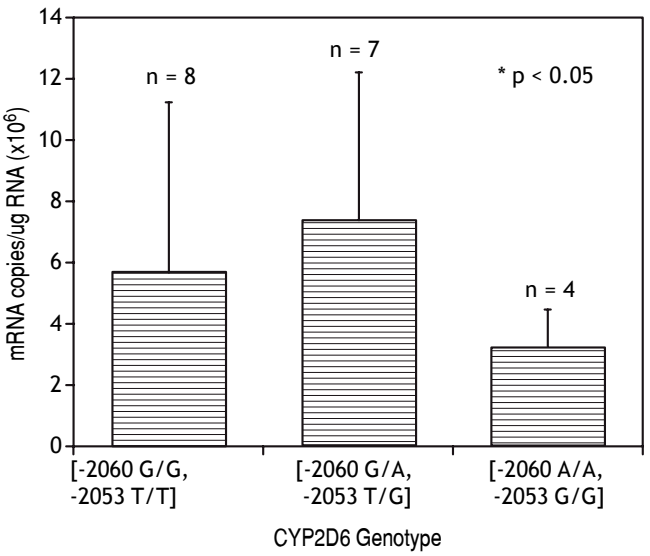
Within the 2.3 kb CYP2D6 promoter sequences, we also determined the allele frequencies for 11 single nucleotide polymorphisms (SNPs). These SNPs are common, with allele frequencies of 3% or greater. While allele frequencies for polymorphisms within the coding region and introns of the

CYP2D6 gene are well documented[11,12] we know of only one report citing allele frequencies for CYP2D6 promoter polymorphisms.[12] Other data were reported in the Genbank but was not available in a formal publication. Gaedigk, et al.,[13] reported that the -1584 C→G SNP was not associated with CYP2D6 poor metabolizer phenotype, but it was present in Caucasians at a frequency of 25.4%. The reported -1584 C→G allelic frequency was similar to our data 19.7 and 30% for hepatitis C negative and positive groups (Table 2).

None of the variants appeared to have a significant impact on liver CYP2D6 enzyme activity. However, in our preliminary study, we found a significantly lower activity of CYP2D6 in hepatitis C positive subjects regardless of their promoter genotype or haplotype. The hepatitis C subjects reflected a mean enzyme activity of 24.0 ± 1.5 pmol/min/mg protein, while the hepatits C negative subjects exhibited an average enzyme activity of 62.6 ± 3.7 pmol/min/mg protein (Figure 2). While these results remained to be verified with larger population studies, these data are consistent with our previous report.[7] Although it is probable that chronic liver injury associated with hepatitis C infection may have impact on CYP2D6 activity, we cannot exclude the possibility the indirect effects of hepatitis C viral infection. These and other possibilities remained to be evaluated in the future studies.

It is interesting to note, however, that -2060 G→A and -2053 T→G CYP2D6 promoter variations were linked and associated with lower mRNA levels for individuals with -2060AA/-2053GG haplotype (Figure 1). We did not find a difference in CYP2D6 enzyme activity in the three groups. This could be due to the small sample size that did not provide sufficient statistical power or other confounding factors. The molecular mechanisms leading to the change in mRNA levels remain to be explored.

In summary, we have analyzed the sequence variations in 2.3 kb of upstream CYP2D6 promoter. We found two novel variants 1822 A→G and -1740 C→T in hepatitis C subjects, and the other two linked variants, -2060 G→A and -2053 T→G in all subjects. The liver CYP2D6 activity appeared to be



[-2053 T→G]	[-2060 G→A] G/G	G/A	A/A
T/T	**32**	2	4
T/G	2	**27**	0
G/G	2	5	**12**

Figure 1. CYP2D6 RNA transcript copy number for -2060/-2053 haplotype. Subjects with specific CYP2D6 SNPs were grouped according to their genotype at nucleotides 2060/-2053. The data are presented as mean ± SD. The * denotes statistically significant differences between the -2060 A/A, -2053 G/G (n = 4) genotype and either other genotype (G/G, T/G: n = 7 or G/G, T/T: n = 8) as determined by Student's t-test. The right panel presents the individual diplotype for the two linked polymorphism at -2060 and -2053.

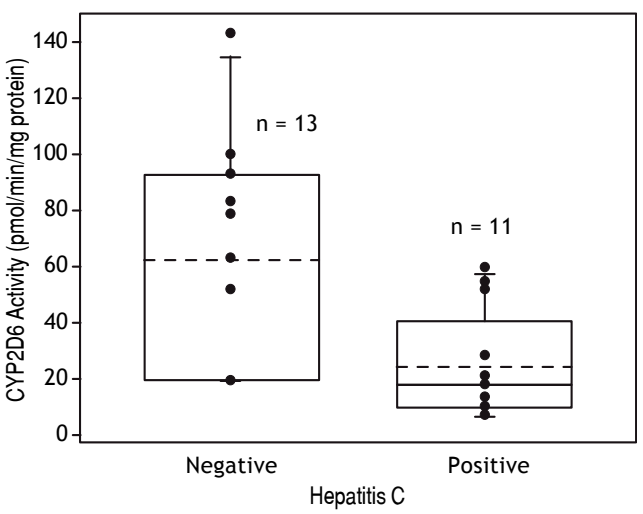


Figure 2. Hepatic CYP2D6 enzyme activity for hepatitis C negative and positive samples. The CYP2D6 acitivity was determined based on the formation of dextrophan and expressed as pmol/min/mg liver protein. Data were plotted as individual point that overlay the box-whisker plot that capture the median (solid lines) and mean (dashed lines) within the 5-95th percentile box. These data represent the individuals with *1/*1 genotype within hepatitis C negative (n = 13) and positive (n = 11) subjects. A two-tailed t-test analysis of the two groups yields a p value of 0.0061.

lower in hepatitis C postive subjects irregardless of the promoter genotype.

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ABBREVIATIONS

- **CYP2D6:** Cytochrome P450 2D6 gene.
- **UM:** Ultrarapid metabolizer.
- **EM:** Extensive metabolizer.
- **IM:** Intermediate metabolizer.
- **PM:** Poor metabolizer.
- **nt:** nucleotides.
- **LC-MS:** Liquid chromatograph-mass spectrophotometer.
- **SNPs:** Single nucleotide polymorphisms.

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