G169R MUTATION DIMINISHES THE METABOLIC ACTIVITY OF CYP2D6 IN CHINESE

SU-LAN WANG, MING-DERG LAI, AND JIN-DING HUANG

Departments of Pharmacology (J.D.H., S.L.W.) and Biochemistry (M.D.L.), Medical College, National Cheng Kung University, Tainan, Taiwan

(Received June 22, 1998)

This paper is available online at http://www.dmd.org

ABSTRACT:

The molecular basis of the reduced ability of a Chinese to metabolize debrisoquine was explored by sequencing all of the nine exons of the CYP2D6 gene. The subject has T188, A1846, T2938, and C4268 as in wild-type subjects. XbaI restriction fragment length polymorphism indicated that the subject has a 29-kb allele and a gene deletion (11.5 kb) in another allele (CYP2D6*5). A CYP2D6*14 allele together with a CYP2D6*5 allele may cause the poor metabolism of the subject. T188, T2938, and C4268 are common haplotypes in Chinese extensive metabolizers. The effect of G1846 to A mutation in CYP2D6 metabolism has not been reported. A polymerase chain reaction-based endonuclease digestion test was designed for the G/N1846 polymorphism and 124 Chinese subjects were screened.

With DNA sequencing, two other subjects showed the heterozygous G/A1846 and have a relatively high metabolic ratio of debrisoquine hydroxylation. The site-directed mutagenesis was used to create recombinant CYP2D6 cDNA with T188, A1846, or C2938. The cDNA was then transfected into Rat-1 cells. The transfection was confirmed by Southern, Northern, and Western blots. Based on the same microsomal protein level, the bufuralol 1'-hydroxylation activity of CYP2D6 (T188) or CYP2D6 (A1846) was significantly lower than that of the wild-type CYP2D6. P34S mutation (C188 to T) significantly decreased CYP2D6 activity. G169R mutation (G1846 to A) also decreased CYP2D6 activity and may further reduce the metabolic activity of CYP2D6 protein with P34S, R296C, and S486T mutations.

The cytochrome P-450 (CYP) enzyme CYP2D6 is involved in the metabolism of numerous drugs, including bufaralol and debrisoquine. The genetic polymorphism and clinical importance of CYP2D6 are now well known. There are more than 20 variants of CYP2D6 identified (Daly et al., 1996). Approximately 5 to 10% of Caucasians exhibit a poor metabolism phenotype characterized by reduced metabolism of debrisoquine, sparteine, dextromethorphan, or other drugs. The poor metabolism is most often due to a mutation at the intron-exon junction (G1934 to A), causing splicing defect and lack of functional CYP2D6 protein (Kagimoto et al., 1990).

In Chinese, the most frequent mutation sites of CYP2D6 are P34S, R296C, and S486T (Wang et al., 1993; Johansson et al., 1994; Wang et al., 1995). These mutations decrease, but do not abolish totally, the metabolic activity of CYP2D6 (Wang et al., 1993; Lai et al., 1995; Tseng et al., 1996). The poor metabolizer [metabolic ratio (MR) > 12.6 for debrisoquine] is rarely found in Chinese (Bertilsson et al., 1992; Wang et al., 1993). In our previous study, only 2 of 124 subjects were the poor metabolizers (Wang et al., 1993). The genotype of one of the poor metabolizers was shown as heterozygous CYP2D6*5 [CYP2D6 deleted, 11.5 kilobases (kb) in XbaI restriction fragment length polymorphism (RFLP)] and a new allele. The new allele was reported in a nomenclature article (Daly et al., 1996) and designated as CYP2D6*14. Nucleotide sequencing indicated that the new allele contained a mutation, G1846 to A, in addition to the T188, T2938, and C4268 mutations as in some extensive metabolizers. A mutation at G1846 was previously reported (Broly et al., 1995). The G1846 to T mutation created a stop codon. In this subject, the G1846 to A mutation caused an amino acid change, G169R. Because the new mutation is detected from a poor metabolizer, it is interesting to know how important the G169R amino acid change is. We have, therefore, studied the influence of G169R mutation, in addition to previously reported P34S and S486T mutations, on catalytic activity of CYP2D6 by site-directed mutagenesis in this study.

Materials and Methods

Subjects. DNA samples were obtained from a previous debrisoquine study including both phenotyping and genotyping (Wang et al., 1993). The study was approved by the Ethics Committee of the National Cheng Kung University Medical College. There are two poor metabolizers in 124 subjects. In XbaI RFLP, the two poor metabolizers showed 29/11.5-kb and 29/16-kb bands (Wang et al., 1993). The 29/16-kb subject has CYP2D6*4A and CYP2D6*10C alleles (S.-L.W., unpublished data). The 29/11.5-kb subject has CYP2D6*5 and CYP2D6*14 alleles.

Diagnosis of G1846 to A Mutation. Human genomic DNA from leukocytes was amplified with primers e (GTG GAT GGT GGG GCT AAT GCC TT 1637–1659) and f (CAG AGA CTC CTC GGT CTC TCG CT 2124 –2102) for CYP2D6 exon 3 according to the method of Wang et al. (1993). The polymerase chain reaction (PCR) was carried out in a total volume of 100 μl with 2.5 U Taq (Thermus aquaticus) polymerase (Promega, Madison, WI). After initial denaturation at 95°C for 5 min, 30 cycles of 30 s at 95°C, 30 s at 61°C, and 3 min at 72°C were repeated. The 486-base pair (bp) PCR product was examined by digestion with endonuclease MspI (CCG1846G; Bio-Labs, Beverly, MA) to determine the G1846 or other genotypes. For samples with homozygous G1846, bands at 207...
and 279 bp were obtained. Samples of heterozygous GN	1846 gave 207-, 279-, and 486-bp bands in 2.5% agarose electrophoresis. The polymorphism was determined in 124 Chinese subjects who participated in the debrisoquine phenotyping study.

**Site-Directed Mutagenesis of CYP2D6 cDNA.** A 272-bp DNA fragment containing T_{188} (exon 1) was prepared by a PCR using primers a (CCA TTT AGT GAG GCA GGT AT 68–90), b (GCC TCA ACG TAT CCC TGT CTG CTC 118–98 downstream), and the genomic DNA containing C_{4268} as the template (Wang et al., 1993). The DNA fragment was ligated with CMV/pRC as the construction of CYP2D6(T_{188})/CMV/pRC plasmid. The CYP2D6(T_{188})/CMV/pRC plasmid was then digested with endonucleases HindIII and EcoRI to yield a 1.6-kb fragment. The fragment was then digested with HindIII/EcoRI-digested pGEM pUC18 plasmid and ligated with CMV/pRC plasmid using endonucleases HindIII and XbaI as in previous steps. The CYP2D6(T_{188})/CMV/pRC plasmid was used for transfection.

A 866-bp DNA fragment containing C_{4268} (exon 9) was prepared by PCR using primers j (GAG ACA AAC CAG CAG CTG CCA 3632–3652), n (GCC TCA ACG TAT CCC TGT CTG CTC 118–98 downstream), and the genomic DNA containing C_{4268} as the template (Wang et al., 1993). The DNA fragment was ligated with CYP2D6/pUC18, which was digested with endonucleases SacI and KpnI. The nucleotide sequence of the recombinant DNA was confirmed. The CYP2D6(C_{4268})/pUC18 plasmid was ligated with pGEM, which was digested with endonucleases HindIII and EcoRI, to yield CYP2D6(C_{4268})/pGEM plasmid. The HindIII/XbaI-digested DNA fragment of CYP2D6(C_{4268})/pGEM plasmid was then digested into CMV/pRC plasmid. The recombinant plasmid CYP2D6(C_{4268})/CMV/pRC was used for transfection. Similarly, the 866-bp DNA fragment (exon 9) was ligated into CYP2D6(T_{188})/pUC18, which was digested with endonucleases SacI and KpnI, and the nucleotide sequence was confirmed. The recombinant CYP2D6(T_{188}, C_{4268})/pUC18 plasmid was ligated with pGEM then CMV/pRC plasmids to yield CYP2D6(T_{188}, C_{4268})/CMV/pRC as the construction of CYP2D6(C_{4268})/CMV/pRC described above.

A 206-bp megaprimer was synthesized by PCR using CYP2D6 cDNA in pGEM plasmid as template. The sequence of primers was 5'-CAA CCA CCA CAG CAG CCC CTT-3' (1846A) and 5'-GGA TAT GCA GGA GGA CAGGG-3' (1846B). The PCR was carried out in a total volume of 100 µl in the presence of 250 µM each of deoxyadenosine-5'-triphosphate, 100 pmol of primers, 10 ng of genomic DNA as template, and 2.5 µl Taq polymerase. After initial condition at 94°C for 5 min, 61°C for 2 min, 72°C for 3 min, 25 cycles of 45 s at 95°C, 30 s at 61°C and 3 min at 72°C were repeated. The 206-bp PCR product was used as a megaprimer along with another T7 primer 5'-GAA ATT AAT AAC AGC ACT CAC TAT AGG-3' to perform a second PCR to obtain a DNA fragment containing A_{1846}. The PCR conditions were the same as the previous one to form the megaprimer, except that the annealing time was 1 min and primer amount was 20 pmol. The DNA fragment containing A_{1846} was ligated with CYP2D6/pGEM, which was digested with endonucleases HindIII and BsmI to yield CYP2D6(A_{1846})/pGEM plasmid and the nucleotide sequence of the recombinant plasmid was confirmed. The CYP2D6(A_{1846})/pGEM plasmid was then digested with CMV/pRC plasmid, which was digested with endonucleases HindIII and XbaI to result a CYP2D6(A_{1846})/CMV/pRC plasmid. The recombinant CYP2D6(A_{1846})/CMV/pRC plasmids were transfected into Rat-1 cells using the calcium phosphate precipitation method. Six transfected cell lines, V74 (vector), W14 (wild-type CYP2D6), T69 (CYP2D6 with T_{188}), C63 (CYP2D6 with C_{4268}), T/C4 (CYP2D6 with T_{188} and C_{4268}), and A17 (CYP2D6 with A_{1846}) were selected. Genomic DNA was extracted from cell lines and the mutations were confirmed with PCR and endonuclease reactions (Wang et al., 1993). The C/T_{188}, G/A_{1846}, and C/C_{4268} sites in W14, T69, C63, T/C4, and A17 were further confirmed by direct DNA sequencing after PCR amplification. The approximate copy number of CYP2D6 in cell lines was determined by Southern blotting (Fig. 1). The total RNA of transfected cells was isolated by the guanidium thiocyanate method.

RNA was fractionated by agarose-formaldehyde gel electrophoresis and then blotted onto a nylon membrane. The membrane was hybridized with ³²P-labeled CYP2D6 cDNA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe for quantification (Fig. 2). The transfected cells were lysed with SDS-lysing buffer. The CYP2D6 protein expression in the lysates was determined by Western blotting using anti-CYP2D6 antibody (BRL, Burlington, Ontario, Canada) and anti-rabbit IgG peroxidase (Fig. 3). Microsomal fraction was prepared from cell lysates in 1.15% KCl by ultracentrifugation. Microsomes of Rat-1, V74, W14, A17, C63, T69, T/C4, and a human liver sample (surgical waste from a patient with gall bladder cancer and liver metastasis; Department of Surgery, National Cheng Kung University Medical Center) were prepared. Metabolic activity of bufuralol 1'-hydroxylation in microsomal fractions was compared by a HPLC method. The microsomal preparation was incubated with 1 mM bufuralol (Genest Co., Woburn, MA) along with 5 mM NADPH (E. Merck, Darmstadt, Germany) and 17 mM MgCl₂ at 37°C for 30 min. After protein precipitation with 10% perchloric acid, 50 µl of the supernatant was injected onto a reverse-phase LiChrosphere 100 RP-18 column (5-µm particle, 250 x 4 mm; E. Merck) and detected with a fluorescence detector (excitation, 225 nm; emission, 330 nm). The HPLC eluent was 30% acetonitrile with 0.086% perchloric acid at 1 ml/min. The retention time of 1'-hydroxy-bufuralol (Genest) was 6.6 min. The CYP2D6 content in the microsomes was also determined by Western blotting. The metabolic activity as the formation of 1'-hydroxy-bufuralol was normalized by the same CYP2D6 protein level of human hepatic microsomes (Fig. 4).

**Results**

In the 124 DNA samples that we analyzed (Wang et al., 1993), only three alleles showed the 486-bp band afterMspI digestion. Direct DNA sequencing indicated the nucleotide being A_{1846}. One was in the poor metabolizer. The other two heterozygous subjects had debrisoquine hydroxylation MR of 2.54 and 2.99. Both subjects have a XbaI RFLP pattern of 44/29 kb and T/T_{188}, G/A_{1846}, G/G_{1934}, and C/C_{4268}. No other mutation has been found in these two subjects. Their MR values were all much higher than the mean value of the extensive metabolizers (0.57). MspI endonuclease test and direct sequencing confirmed the site-directed mutagenesis of A_{1846}. Southern blots indicated a higher copy number of CYP2D6 cDNA in A_{1846} than in W14 cells (181%, Fig. 1). Northern blotting indicated that CYP2D6 mRNA level in A_{1846} cells was higher than in W14 cells (260%, Fig. 2). Western blotting also indicated a nearly 2-fold expression (Fig. 3). The protein level-normalized metabolic activity of CYP2D6(A_{1846}) in A_{1846} was significantly lower than the wild-type CYP2D6 in W14 cells (Fig. 4).
Southern, Northern, and Western blots also indicated the expression of \textit{CYP2D6} in T69, C63, and T/C4 cell lines (Figs. 1–3). The normalized metabolic activity of \textit{CYP2D6} (T188) and \textit{CYP2D6} (T188, C4268) was much lower than the wild-type \textit{CYP2D6} or human hepatic microsomes, whereas the G4268 to C mutation did not affect the rate of bufuralol 1\textsuperscript{9}-hydroxylation significantly (Fig. 4).

\section*{Discussion}

The effect of P34S and S486T mutations on \textit{CYP2D6} activity has been studied by expression experiments. Kagimoto et al. (1990) reported that the P34S mutation abolished the activity of expressed protein, but S486T mutation increased the activity. Johansson et al. (1994) constructed a chimeric gene with both C188 to T and G4268 to C mutations (Ch1) and expressed in COS-1 cells. Homogenates from cells transfected with \textit{CYP2D6} (Ch1) had only 2.5\% of that obtained in homogenates from cells with wild-type \textit{CYP2D6} gene. Our results of P34S and S486T mutations in Rat-1 cells basically supported the previous findings. We have also studied the significance of another mutation (G1846 to A) in \textit{CYP2D6*14}.

The G169R mutation caused poor metabolism for one subject and may have decreased \textit{CYP2D6} metabolic activity for the other two extensive metabolizers. However, in the expression system, the G169R mutation in \textit{CYP2D6} decreased, but not totally abolished, the metabolic activity in transfected Rat-1 cells (43\% of the wild-type). It appeared that the decrement in metabolic activity by G169R mutation is not as dramatic as P34S mutation. When the G169R mutation is combined with P34S, R296C, and S486T mutations, it may have further diminished the metabolic activity of \textit{CYP2D6}. Multiple mutations may explain the high debrisoquine MR of the poor metabolizer (\textit{CYP2D6*5/CYP2D6*14}). It is very interesting to note the possible additive effect of these four mutation sites in this subject.

In the Chinese population, we found three alleles of A1846 in 124 subjects. The existence of this polymorphism site in Caucasians has not been specified. However, no \textit{CYP2D6*14} allele was reported by Marez et al. (1997) in their extensive screening of a European population by PCR/single-strand conformational polymorphism and DNA sequencing, whereas one allele of \textit{CYP2D6*8} (G1846 to T) was detected in 146 poor metabolizers. If the \textit{CYP2D6*14} allele does exist in Caucasians in a significant percentage, the polymorphism must have been detected. The genetic polymorphism we detected in the Chinese population is probably not very important in Caucasians. The importance of this mutation in subjects of other ethnic origins remains unknown.

A single amino acid change may specifically decrease the metabolic activity of one substrate, but not another. For example, the I380P mutation of \textit{CYP2D1} decreased bufuralol hydroxylation, but not debrisoquine hydroxylation (Matsunaga et al., 1990). G169R of \textit{CYP2D6} decreases metoprolol \textalpha{}-hydroxylation approximately 50\% as well (data not shown). G169R appeared to decrease debrisoquine hydroxylation from limited in vivo data. \textit{CYP2D6} metabolizes more

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2}
\caption{**Figure 2.** Northern blots of \textit{CYP2D6} mRNA in expressed cell lines. Left: lane 1, Rat-1; lane 2, A17 (A1846); lane 3, V74 (vector); lane 4, W14 (wild type). With densitometric quantitation, the mRNA levels relative to GAPDH mRNA level were 0\%, 260\%, 0\%, and 100\% of lane 4 for lanes 1 to 4, respectively. Right: lane 1, Rat-1; lane 2, W14 (wild type); lane 3, V74 (vector); lane 4, C63 (C4268); lane 5, T69 (T188); lane 6, T/C4 (T188, C4268). The mRNA levels relative to GAPDH mRNA level were 0\%, 100\%, 0\%, 66\%, 140\%, and 208\% of lane 2 for lanes 1 to 6, respectively.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3}
\caption{**Figure 3.** Western blots of \textit{CYP2D6}-expressed cell lines. A polyclonal antibody against \textit{CYP2D6} and a monoclonal antibody against actin were used for hybridization, and the enhanced chemiluminescence method was used for visualization. Top: lane 1, Rat-1; lane 2, V74 (vector); lane 3, W14 (wild type); lane 4, C63 (C4268); lane 5, T/C4 (T188, C4268); lane 6, T68 (T188). With densitometric quantitation, the expression levels relative to actin level were 0\%, 0\%, 100\%, 72\%, 150\%, and 121\% of lane 3 for lanes 1 to 6, respectively. Bottom: lane 1, W14 (wild type); lane 2, W14; lane 3, Rat-1; lane 4, V74 (vector); lane 5, A17 (A1846); lane 6, A17. The expression levels relative to actin level were 100\%, 86\%, 0\%, 0\%, 158\%, and 224\% of lane 1 for lanes 1 to 6, respectively.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4}
\caption{**Figure 4.** Comparison of the bufuralol 1\textsuperscript{9}-hydroxylation in microsomes of various transfected Rat-1 cells: HHM (human hepatic microsomes), W14 (wild type), V74 (vector), C63 (C4268), T69 (T188), T/C4 (T188, C4268), and A17 (A1846). The formation of 1\textsuperscript{9}-hydroxy-bufuralol was normalized by the same \textit{CYP2D6} protein level of human hepatic microsome (HHM).}
\end{figure}
than 40 drugs. The influence of G169R mutation on CYP2D6 activity for other substrates also needs further study.

Influence of a single amino acid change in CYP2D6 in pharmacokinetics of propranolol and morphine formation from codeine has been documented in the literature (Lai et al., 1995; Tseng et al., 1996). Based on the limited data, the G169R mutation is possibly rare. Nevertheless, the clinical significance of this mutation cannot be totally ignored. With increasing knowledge of pharmacogenetics, subjects of different ethnicity are known to have different genetic polymorphisms. In an effort to establish the application of genotyping in drug therapy, it is important to explore all of the possible mutation sites for any designated ethnicity.

References


