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.

As DNA sequencing, two 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 (G169 to A) also decreased CYP2D6 activity and may further reduce the metabolic activity of CYP2D6 protein with P34S, R296C, and S486T mutations.
and 279 bp were obtained. Samples of heterozygous G\textsuperscript{1446}A 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\textsubscript{188} (exon 1) was prepared by a PCR using primers a (CCAC TTG GAT GAG GCA GGT AT 68–90), b (CAC CAT CCA TGT TGG CTG GT 338–316), and the genomic DNA containing T\textsubscript{188} as the template (Wang et al., 1993). The CYP2D6/pUC18 plasmid (0.3 μg; obtained from Frank J. Gonzalez’s laboratory, National Institutes of Health) was digested with endonucleases BalIII and XmaI for 2 h and a 4-kb fragment was purified. The DNA fragment was ligated with T\textsubscript{188} containing oligonucleotide from PCR at 22°C for 16 h. The recombinant plasmid was cloned and its nucleotide sequence was confirmed. The CYP2D6/T\textsubscript{188}/pUC18 plasmid was then digested with endonucleases HindIII and EcoRI for 2 h to obtain a 1.6-kb fragment. The fragment was then ligated with HindIII/EcoRI-digested pGEM at 22°C for 16 h. The CYP2D6/T\textsubscript{188}/pGEM was then ligated with CMV/pRC plasmid using endonucleases HindIII and XbaI as in previous steps. The CYP2D6/T\textsubscript{188}/CMV/pRC plasmid was used for transfection.

A 866-bp DNA fragment containing C\textsubscript{4268} (exon 9) was prepared by PCR using primers j (GAG ACA AAC CAG GAC CTC CCA 363–3652), n (GCC TCA ACG TAC CCC TGT CTC 118–98 downstream), and the genomic DNA containing C\textsubscript{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\textsubscript{4268}/pUC18 plasmid was ligated with pGEM, which was digested with endonucleases HindIII and EcoRI, to yield CYP2D6/C\textsubscript{4268}/pGEM plasmid. The HindIII/XbaI-digested DNA fragment of CYP2D6/C\textsubscript{4268}/pGEM was then ligated into CMV/pRC plasmid. The recombinant plasmid CYP2D6/C\textsubscript{4268}/CMV/pRC was used for transfection.

Similarly, the 866-bp DNA fragment (exon 9) was ligated into CYP2D6/T\textsubscript{188}/pUC18, which was digested with endonucleases SacI and KpnI, and the nucleotide sequence was confirmed. The recombinant CYP2D6/T\textsubscript{188}/C\textsubscript{4268}/pUC18 plasmid was ligated with pGEM then CMV/pRC plasmids to yield CYP2D6/T\textsubscript{188}/C\textsubscript{4268}/CMV/pRC as the construction of CYP2D6/C\textsubscript{188}/C\textsubscript{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 were 5′-CAC GAT CAT CCA TGC TCT 118–98 downstream) and the nucleotide sequence was confirmed. The CYP2D6/T\textsubscript{188}/C\textsubscript{4268}/pUC18 plasmid was digested with CMV/pRC plasmid and the nucleotide sequence was confirmed. The recombinant CYP2D6/T\textsubscript{188}/C\textsubscript{4268}/CMV/pRC was used for transfection.

**Expression and Metabolic Activity of CYP2D6 Variants.** CYP2D6/pGEM was then ligated with pGEM then CMV/pRC plasmid was used for transfection. CYP2D6(A\textsubscript{1846})/CMV/pRC plasmid was used for transfection.

**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 quantitation (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, 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 μM bufuralol (Gentest Co., Woburn, MA) along with 5 mM NADPH (E. Merck, Darmstadt, Germany) and 17 mM MgCl\textsubscript{2} at 37°C for 30 min. After protein precipitation with 10% perchloric acid, 50 μl of the supernatant was injected onto a reverse-phase LiChrospher 100 RP-18 column (5-μm particle, 250 × 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 (Gentest) 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 after Mspl digestion. Direct DNA sequencing indicated the nucleotide being A\textsubscript{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\textsubscript{188}, G/A\textsubscript{1846}, G/G\textsubscript{1934}, and C/C\textsubscript{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).

Mspl endonuclease test and direct sequencing confirmed the site-directed mutagenesis of A17 cells. Southern blots indicated a higher copy number of CYP2D6 cdNA in A17 than in W14 cells (181%, Fig. 1). Northern blotting indicated that CYP2D6 mRNA level in A17 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\textsubscript{1846}) in A17 was significantly lower than the wild-type CYP2D6 in W14 cells (Fig. 4).
Southern, Northern, and Western blots also indicated the expression of CYP2D6 in T69, C63, and T/C4 cell lines (Figs. 1–3). The normalized metabolic activity of CYP2D6(T188) and CYP2D6(T188,C4268) was much lower than the wild-type CYP2D6 or human hepatic microsomes, whereas the G4268 to C mutation did not affect the rate of bufuralol 1-9-hydroxylation significantly (Fig. 4).

Discussion

The effect of P34S and S486T mutations on 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 CYP2D6(Ch1) had only 2.5% of that obtained in homogenates from cells with wild-type 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 CYP2D6*14.

The G169R mutation caused poor metabolism for one subject and may have decreased CYP2D6 metabolic activity for the other two extensive metabolizers. However, in the expression system, the G169R mutation in 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 CYP2D6. Multiple mutations may explain the high debrisoquine MR of the poor metabolizer (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 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 CYP2D6*8 (G1846 to T) was detected in 146 poor metabolizers. If the 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 CYP2D1 decreased bufuralol hydroxylation, but not debrisoquine hydroxylation (Matsunaga et al., 1990). G169R of CYP2D6 decreases metoprolol \(\alpha\)-hydroxylation approximately 50% as well (data not shown). G169R appeared to decrease debrisoquine hydroxylation from limited in vivo data. CYP2D6 metabolizes more

*Fig. 2. Northern blots of 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.

*Fig. 3. Western blots of CYP2D6-expressed cell lines.*

A polyclonal antibody against 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.

Southern, Northern, and Western blots also indicated the expression of CYP2D6 in T69, C63, and T/C4 cell lines (Figs. 1–3). The normalized metabolic activity of CYP2D6(T188) and CYP2D6(T188,C4268) was much lower than the wild-type CYP2D6 or human hepatic microsomes, whereas the G4268 to C mutation did not affect the rate of bufuralol 1'-hydroxylation significantly (Fig. 4).
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


