G169R MUTATION DIMINISHES THE METABOLIC ACTIVITY OF CYP2D6 IN CHINESE

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(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 (CYP2D6*14) instead of C188, G1846, C2938, and G4268 as in wild-type subjects. Xbal 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/N1946 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 (C1934 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 bufuralol 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 metabolic activity 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 Xhol 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 T 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 Xhol 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 c (GTG CAT 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 in the presence of 250 µM each of deoxynucleoside-5'-triphosphate, 100 pmol of primers c and f, 10 ng of genomic DNA as template, and 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 Gnon were given 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 T188 (exon 1) was prepared by PCR using primers a (CC A 159 TTT AGT GAG CCA GGA GTA AT 68–90), b (CAC CAT CCA TGG CTG CTC GTG 338–316), and the genomic DNA containing T188 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 T188 containing oligonucleotide from PCR at 22°C for 16 h. The recombinant plasmid was cloned and its nucleotide sequence was confirmed. The CYP2D6(T188)/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(T188)/pGEM was then ligated with CMV/pc plasmid using endonucleases HindIII and XbaI as in previous steps. The CYP2D6(T188)/CMV/pc plasmid was used for transfection.

A 866-bp DNA fragment containing C4268 (exon 9) was prepared by PCR using primers j (GAC ACA AAG CAG CAG CCT CCA 3632–3652), n (GOC TCA ACG TAC CCC TGT CTC 118-98 downstream), and the genomic DNA containing C4268 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(C4268)/pUC18 plasmid was ligated with pGEM, which was digested with endonucleases HindIII and EcoRI, to yield CYP2D6(C4268)/pGEM plasmid. The HindIII/XbaI-digested fragment of CYP2D6(C4268)/pGEM was then ligated into CMV/pc plasmid. The recombinant plasmid CYP2D6(C4268)/CMV/pc was used for transfection. Similarly, the 866-bp DNA fragment (exon 9) was ligated into CYP2D6(T188)/pUC18, which was digested with endonucleases SacI and KpnI, and the nucleotide sequence was confirmed. The recombinant CYP2D6(T188,C4268)/pUC18 plasmid was ligated with pGEM then CMV/pc plasmids to yield CYP2D6(T188,C4268)/CMV/pc as the construction of CYP2D6(C4268)/CMV/pc described above.

A 206-bp megaprimer was synthesized by PCR using CYP2D6 cdNA in pGEM plasmid as template. The sequence of primers were 5′-GACA CCA CTC CAC ACG CCC CTT-3′ (1846A) and 5′-GGA TAT GCA GGA GGA CGG GG-3′ (1846B). The PCR was carried out in a total volume of 100 µl in the presence of 250 µM each of deoxy nucleoside-5′-triphosphate, 100 pmol of primers, 10 ng of genomic DNA as template, and 2.5 mM 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′-GGA ATT AAT ACG ACT CAC TAT AGG-3′ to perform a second PCR to obtain a DNA fragment containing A1846. 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 A1846 was ligated with CYP2D6/pGEM, which was digested with endonucleases HindIII and BsoI to yield CYP2D6(A1846)/pGEM plasmid and the nucleotide sequence of the recombinant plasmid was confirmed. The CYP2D6(A1846)/pGEM plasmid was then ligated with CMV/pc plasmid, which was digested with endonucleases HindIII and XbaI to result in CYP2D6(A1846)/CMV/pc plasmid.

The recombinant CYP2D6(A1846)/CMV/pc was used for transfection.

**Expression and Metabolic Activity of CYP2D6 Variants.** CYP2D6/pCMV/pc, CYP2D6(T188)/CMV/pc, CYP2D6(C4268)/CMV/pc, CYP2D6(T188,C4268)/CMV/pc, and CYP2D6(A1846)/CMV/pc plasmids were transfected into Rat-1 cells using the calcium phosphate precipitation method. Six transfected cell lines, V74 (vector), W14 (wild-type CYP2D6), T69 (T188); lane 2, W14 (wild-type); lane 5, T69 (T188); lane 6, C63 (C4268). With densitometric quantitation, the cDNA levels were 155%, 0%, 100%, 108%, and 70% of lane 4 after being normalized with reference Rat-1 autologous DNA for lanes 1 to 6, respectively. Right: lane 1, W14 (wild-type); lane 2, A17 (A1846); lane 3, V74 (vector); lane 4, Rat-1; lane 5, W14. The cDNA levels were 100%, 181%, 0%, 0%, and 92% of lane 1 after being normalized with reference Rat-1 autologous DNA for lanes 1 to 5, respectively.

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-lyzing 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 and 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 (Gentest Co., Woburn, MA) along with 5 mM NADP (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 incubated 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 afterMspI digestion. Direct DNA sequencing indicated the nucleotide being A1846. 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 metabolic phenotype.

**MspI 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(A1846) 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'-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 decreased metoprolol a-hydroxylation approximately 50% as well (data not shown). G169R appeared to decrease debrisoquine hydroxylation from limited in vivo data. CYP2D6 metabolizes more...
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 pharmaco-
kinetics 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

Sjoqvist F (1992) Pronounced differences between native Chinese and Swedish populations in

for detection of known and new mutations of the CYP2D6 gene using single strand confor-

Daly AK, Broekmøller J, Broly F, Eichelbaum M, Evans WE, Gonzalez FJ, Huang JD, Idle JR,
Ingelman-Sundberg M, Ishizaki T, Jacqz-Aigrain E, Meyer UA, Nebert DW, Steen VM, Wolf

Johansson I, Oscarson M, Yue QY, Bertilsson L, Sjoqvist F and Ingelman-Sundberg M (1994)
Genetic analysis of the Chinese cytochrome P450 2D locus: Characterization of variant
CYP2D6 genes present in subjects with diminished capacity for debrisoquine hydroxylation.

Kagimoto M, Heim M, Kagimoto K, Zeugin T and Meyer UA (1990) Multiple mutations of the
human cytochrome P450IID6 gene (CYP2D6) in poor metabolizers of debrisoquine. J Biol
Chem 265:17209–17214.


Marez D, Legrand M, Sabbagh N, Lo Guidice JM, Spire C, Lafitte JI, Meyer UA and Broly F
(1997) Polymorphism of the cytochrome P450 CYP2D6 gene in a European population:
Characterization of 48 mutations and 53 alleles, their frequencies and evolution. Phar-

Matsunaga E, Zeugin T, Zanger UM, Aoyama T, Meyer UA and Gonzalez FJ (1990) Sequence

codeine in Chinese subjects of different CYP2D6 genotypes. Clin Pharmacol Ther 60:177–
182.

in debrisoquin hydroxylation in Chinese subjects: Polymorphism in RFLP and DNA sequence

Wang SL, Lai MD, Liu CH and Huang JD (1995) R296C and other CYP2D6 mutations in