GENETIC POLYMORPHISM OF CYTOCHROME P450 3A5 IN CHINESE

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ABSTRACT:

The CYP3A subfamily enzymes are the most abundant and important drug-metabolizing enzymes. Wide variation in the CYP3A5 expression has been well known. Recently, G_{-44} to A of CYP3AP1 was found to segregate with CYP3A5*3 defective allele. The homozygous A_{-44} subjects showed low expression of CYP3A5. In Caucasian, only 9.2% of CYP3AP1 alleles were with G_{-44} and associated with the wild-type CYP3A5*1 allele, which expressed CYP3A5 significantly. By using polymerase chain reaction and Faul endonuclease digestion, we found that 28% of CYP3AP1 alleles are G_{-44} in 110 Chinese subjects. The frequency is 3 times higher in Chinese than in Caucasian, implying more Chinese subjects are probably extensive CYP3A5 metabolizers. In two Chinese subjects, we also found a heterozygous G_{13048}gt-to-G_{13048}gc mutation at the intron 5 splicing donor site, leading to a splicing defect. A 6478-base pair minigene, including intron 4 to intron 7, was used for in vitro transcription. Both the wild-type and the mutated minigenes produced splicing variants. The wild-type minigene used G_{13050} as the splicing donor. The mutant minigene used G_{13054} in intron 4 or G_{13112} in intron 5 as the splicing donor for various splicing acceptors. The splicing defect may result in a shorter peptide or cause frameshift. In the other two Chinese subjects, we found A_{14763} to-G mutation in exon 7, resulting in the Q200R amino acid change. The consequence of the polymorphism site has not been known. In Caucasian, there is a reported T398N polymorphism. In these Chinese subjects, we did not find polymorphism at this site.

The CYP3A enzymes are the most important subfamily of cytochrome P450s in xenobiotic metabolism. Four CYP3A genes have been described in humans: CYP3A4, CYP3A5, CYP3A7, and CYP3A43 (Hashimoto et al., 1993; Gellner et al., 2001). Among them, CYP3A7 is a fetal protein. CYP3A4 and CYP3A5 are functional enzymes in adults and metabolize a wide range of xenobiotics. The sequences of CYP3A4 and CYP3A5 genes are available recently. In 2000, we published the CYP3A4 sequence in the GenBank database (accession no. AF209389). We have also aligned CYP3A5 cDNA sequence with the human genome database AC005020. The 15810 to 47610 bp of AC005020 was assigned as CYP3A5 gene, comprising the transcriptional initiation site (Jounaidi et al., 1994) to exon 13. In this study, we renumbered the position 15810 of AC005020 as position 1. The cDNA sequences of CYP3A4 and CYP3A5 have been characterized with 90% similarity. Interestingly, similarity is not only at the coding region; the intron 5 of CYP3A4 and CYP3A5 also show 90% identity. In a genetic polymorphism study, the intron sequence is usually used to design specific primers to amplify exon fragments by PCR. It was difficult to design specific primers to study the genetic polymorphism of CYP3A enzymes. This difficulty explains the late discovery of CYP3A4 and CYP3A5 polymorphism in the attempt of finding interindividual variation for major cytochrome P450s.

Overlapping substrate specificity between CYP3A4 and CYP3A5 has also made it difficult to separate the metabolism of these two enzymes. Although a wide interindividual variation of CYP3A metabolism has been known, little phenotypic data have been produced to reveal variation in CYP3A5 activity in humans. However, there is evidence for wide variation in the expression of CYP3A5. Both immunoblotting and Northern blot analysis have detected CYP3A5 expression in only 10 to 30% of human livers (Aoyama et al., 1989; Wrighton et al., 1990; Schuetz et al., 1994). Previously, a point mutation (10%) of Thr398Asn (CYP3A5*2) was found (Jounaidi et al., 1996). It was postulated that the amino acid change caused protein instability and the low level of CYP3A5 expression. The postulation was, however, not well evidenced. Paulussen et al. (2000) found two linked mutations, A/G_{-45} and T/G_{-369}. The mutations were well associated with the CYP3A5 expression. Recent publications indicate that A/G_{-45} polymorphism identified by Paulussen et al. (2000) is in fact the A/G_{-44} polymorphism in the promoter of the pseudogene CYP3AP1 (Finta and Zaphiropoulos, 2000; Gellner et al., 2001). Furthermore, Kuehl et al. (2001) found a complete concordance between A/G_{-44} polymorphism and CYP3A5*3 defect allele in Caucasians. Only the subjects with G_{-44} in CYP3AP1 had normal CYP3A5 expression.

In this study, we examine the genetic polymorphism in Chinese by SSCP analysis. We found a novel missense mutation, an intron mutation causing splicing defect, and an ethnic difference in the polymorphism at the reported sites in CYP3AP1. Furthermore, we also demonstrated that the splicing defect at intron 5 could result in many splicing variants by in vitro transcription.

Materials and Methods

Genomic DNA Isolation. Blood samples were obtained from 75 healthy unrelated subjects and 35 stroke patients from Chinese (Han) population living in Taiwan. The samples were from two previous studies of CYP2D6 and CYP3A4 polymorphism. DNA was isolated from peripheral leukocytes using a DNA isolation kit (Puregene; Genta System Inc., Minneapolis, MN).

PCR-SSCP and Sequencing Analysis. Exons (including the exon-intron boundaries) and part of the 5' upstream region from −438 to +105 bp were amplified by PCR in separate reactions. The primer sets and PCR conditions used are shown in Table 1. The PCR reaction was carried out in 50 μl of
solution consisting of 5 μl of 10× Taq buffer, 0.2 μM dNTPs, 0.06 to 0.3 μM of each primer, 0.1 μl of genomic DNA as template, and 2.5 U of Taq polymerase (Takara, Kyoto, Japan). To carry out SSCP gel electrophoresis, a solution consisting of 5 μl of 10× Taq buffer, 0.2 μM of each primer, 0.06 μl of primer ET5(R) and ET7(R) (Table 1), 0.1 μg of genomic DNA as template, and 2.5 U of Taq polymerase. After PCR amplification, the DNA fragments were digested with BsmAI before electrophoresis using a 12.5% polyacrylamide gel. Samples with A14763 gave 317-bp band, while samples with G14763 gave 149- and 168-bp bands (Fig. 1).

PCR-RFLP for G13048G → ge Splicing Defect (CYP3A5*5) in 5’ End of Intron 5. A PCR-based test of G13048G → ge was developed. The G13048G → ge change in the sequence TATG creates the recognition site of Hsp92II (CATG). The PCR reaction was carried out in 50 μl of solution consisting of 5 μl of 10× Taq buffer, 0.2 μM dNTPs, 0.3 μM primer EX5(S) and EX5(R) (Table 1), 0.1 μg of genomic DNA as template, and 2.5 U of Taq polymerase. After PCR amplification, the DNA fragments were digested with Hsp92II before electrophoresis using a 12.5% polyacrylamide gel. Samples with G13048G gave 4- and 248-bp bands, while samples with G13048G gave 149- and 168-bp bands (Fig. 1).

CYP3A5 Minigene Construction. Genomic DNA samples of G13048G were cloned into TA cloning kit (Invitrogen, San Diego, CA) and subcloned into the pcDNA3.1/V5/His-TOPO vector. The pcDNA3.1/V5/His-TOPO vector was designed to direct sequencing using ABI PRISM BigDye terminator cycle sequencing ready reaction kit and ABI PRISM 377-96 DNA sequencer (Applied Biosystems, San Francisco, CA).

PCR-RFLP for Gln200Arg (CYP3A5*4) in Exon 7. A PCR-based test of Gln200Arg was developed. The A14763 → G change in the sequence ΔGAG creates the recognition site of BsmAI (G2AGC). The PCR reaction was carried out in 50 μl of solution consisting of 5 μl of 10× Taq buffer, 0.2 μM dNTPs, 0.06 μl of primer ET7(R) and EX7(R) (Table 1), 0.1 μg of genomic DNA as template, and 2.5 U of Taq polymerase. After PCR amplification, the DNA fragments were digested with BsmAI before electrophoresis using a 12.5% polyacrylamide gel. Samples with A14763 gave 317-bp band, while samples with G14763 gave 149- and 168-bp bands (Fig. 1).

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Following PCR and digestion with the appropriate enzyme, oligonucleotides were analyzed by gel electrophoresis. A, detection of A14763G by BsmAI digestion. Lanes 1 and 2 [without (−) and with (+) BsmAI digestion] are from a sample of homozygous A14763G. Lanes 3 to 7 [without (−) and with (+) BsmAI digestion] are from samples of heterozygous A14763G/G13048Gt. Detection of G13048Gt digestion] are from samples of heterozygous G13048Gt/c.

Analysis of Splicing of Pre-RNA Transcribed from Minigene. The minigene constructs were transfected as LipofectAMINE complexes into Caco-2 cells. After 4.5 h, the transfection medium was removed and fresh medium was added. Cells were grown for another 36 h before harvesting. Total RNA was isolated by use of Ultraspec-II RNA isolation system (Promega, Madison, WI) and reverse transcription PCR (RT-PCR) was performed. To characterize the expression of the mRNA from the minigenes that contained the splice-donor site mutation, we used the two pCDNA 3.1 sense (pCDNA 3.1S) and reverse (pCDNA 3.1R) primers to perform the PCR reaction, using a GeneAmp PCR kit (Table 1 and Fig. 2). Aliquots from cells transfected with the vector alone, wild-type, and mutated minigenes were analyzed by RT-PCR and electrophoresis on 1.5% agarose gel. The multiple splicing variants from the wild-type and mutated minigenes were further subcloned and the PCR products were sequenced. The primers located at CYP3A5 cDNA XL-4kb(S) and XL-4kb(R) (Table 1) were used to amplify the endogenously expressed CYP3A5 at Caco-2 cells as the internal control.

PCR-RFLP Detection Assay for the G144→A Mutation in CYP3A1. All PCR assays were performed using a 1 in 50 dilution of the original 3A51F/3A52R PCR product as template. PCR conditions used are shown in Table 1. For G144→A mutation, the PCR reactions was carried out in 50 μl of solution consisting of 5 μl of 10× Taq buffer, 0.2 μM dNTPs, 0.3 μM primer A–44G(S) and A–44G(R) (Table 1), and 2 U of γTaq polymerase. A PCR-based test of A–44G was developed. The G144→A mutation changed the recognition site of FauI (CCCGC) into the sequence CCCAC. After PCR amplification, the DNA fragments were digested with FauI before electrophoresis using a 2.5% agarose gel. Samples with A–44 gave 335-bp band, while samples with G–44 gave 147- and 188-bp bands (Fig. 3).

Results

In addition to A–44G polymorphism of CYP3A1, we have screened exons 4, 5, 6, 7, 8, 10, 11, and 12 of CYP3A5 for possible genetic polymorphism by SSCP (Fig. 4). Two mutations were found (Table 2). One is Q200R (CYP3A5*4) and the other is at intron 5 splicing donor site (CYP3A5*5). The T398N mutation (CYP3A5*2) reported in the literature was not found in this study. By using PCR-RFLP with appropriate endonuclease (Fig. 1), the incidence rate of each mutation was found to be 1% (two heterozygous subjects among 110 subjects, 2 of 220). The two rare mutations do not explain the wide interindividual variation of CYP3A5 expression. The association of A–44G polymorphism with defective CYP3A5*3 allele is particularly interesting. The samples were screened with FauI endonuclease (Fig. 3). The percentage of G–44 allele in Chinese is much higher than in white subjects (Table 3).

A 6447-bp minigene (nt 8458–14935) containing intron 4, exon 5, intron 5, exon 6, intron 6, exon 7, and intron 7 was inserted into an expression vector pcDNA 3.1 (Fig. 2). The mRNA produced by in vitro transcription was detected by RT-PCR (Fig. 5). The endogenous CYP3A5 expression in Caco-2 cells was also amplified as a positive control using primers from exon 5 to exon 9 and gave a 503-bp band. After in vitro transcription, the pcDNA3.1 vector produced a 257-nt cDNA fragment. After inserting the wild-type or mutated minigenes, the cryptic splice sites are at g8458c in intron 4 and a4149g in intron 7. The wild-type minigene containing G13048Gt produced two cDNA bands by RT-PCR in Caco-2 cells. One is a 644-bp product (the 352-nt exon 5, 6, 7, the 257-nt vector, the first 45 nt from the intron 4 before the cryptic site, and the last 10 nt from the intron 7 after the cryptic site), and the other is 575-bp product (89-bp exon 6 less). The mutated minigene containing G13048Gt produced multiple splicing products in Caco-2 cells (Fig. 5). The cDNA sequence indicated that one of them used the second gt13009 in intron 5 as the splicing donor for intron 5. The size of intron 5 became 200 nt. The PCR product was 726 bp, which contains the additional 62-nt intron 5 fragment. The splicing variant is out of the open reading frame. The other three
transcripts all used the second g13050 in intron 4 to replace g13050, as the splicing donor site. The second product is to splice g13050 with a g13330 of intron 6, resulting the cDNA product without exon 5 (550 bp; 104 nt less, in-frame product). The third product used the a1814685 of intron 6 to delete exon 5 and 6 and gave a 461-bp product (203 nt deleted, out of frame). The last product is to splice using the a14925 of intron 7 to give the product containing no exon (312 bp; 352 nt less, out of frame). They represent various possibilities to use alternative splicing donor and acceptor sites (Fig. 6). Regardless, the G13048gc produced defective mRNA, which may produce a shorter peptide or out of reading frame.

Discussion

The CYP3A5*3 allele is probably the most important mutant allele of CYP3A5. Kuehl et al. (2001) showed that A/G-44 polymorphism in CYP3AP1 is linked to the splicing defect of CYP3A5*3. Subjects with G-44 in CYP3AP1 have CYP3A5 protein expression in Western blot analysis, but not for the A-44 subjects (CYP3A5*3). Accounting for both homozygous G-44 and heterozygous A-44G subjects, there will be 54 of 300 in white subjects (Paulussen et al., 2000) and 57 of 110 in Chinese with CYP3A5 expression. The percentage in Chinese (52%) who might have CYP3A5 expression is much higher than the percentage in white subjects (18%). Because of overlapping substrate specificity of CYP3A4 and CYP3A5, this discrepancy has not been documented in the literature. Few substrates can be used to delineate CYP3A4 and CYP3A5 metabolism. Midazolam can be a probe drug (Gorski et al., 1994; Haehner et al., 1996). CYP3A5 metabolizes midazolam preferably through 1-hydroxylation rather than 4-hydroxylation. The level of CYP3A4 expression in Chinese is still unknown. Although the average clearance of midazolam is not necessarily higher in Chinese, the average ratio of 1-hydroxy-midazolam to midazolam can be expected to be higher in Chinese.

Jounaidi et al. (1994) sequenced two human CYP3A5 clones with different 5′-flanking sequences. Clone 1 contained A-44 and T-369, and clone 2 contained G-44, G-369, and a large number of other nucleotide changes. The A/G-44 was assigned as A/G-45 by Paulussen et al. (2000), while the T/G-369 was the same. It is now clear that these sequences are not from the promoter of CYP3A5, but from the pseudogene CYP3AP1 (Finta and Zaphiropoulos, 2000; Gellner et al., 2001). It was postulated that the A/G-44 and T/G-369 polymorphism is derived from the two clones (Paulussen et al., 2000). We have sequenced the region from five subjects with homozygous A-44 and five subjects with homozygous G-44. They all showed corresponding linked -369 mutation, but their sequence is identical with that of clone 1. None of the 10 subjects showed sequence of clone 2. Apparently, A/G-44 and T/G-369 sites are polymorphic sites of CYP3AP1, not the PCR product from the other CYP3A locus sequence.

Jounaidi et al. (1996) reported a T398N mutation in 2 of 38 alleles in Caucasian. In this study, we found none in 110 subjects. Instead, we found a point mutation of Q200R in 2 of 220 alleles. The percentage of this mutation in other ethnic groups is still unknown. It was postulated the T398N is associated with CYP3A5 expression level. The postulation remains to be verified. The effect of both T398N and Q200R mutation on CYP3A5 activity is to be studied by site-directed mutagenesis.
In this study, we also found 2 of 220 alleles showed a mutation at the intron 5 splicing donor site. We have prepared a minigene containing nearby exons and introns (Fig. 2). In vitro transcription has shown various splicing variants. The shift of splicing donor and acceptor sites created different mRNAs. Some of them are out of reading frame. For example, the introduction of 62-nt intron 5 fragment into mRNA sequence will cause a frame shift and create an early stop codon. One possible mRNA product is 114 nt shorter but is still in the open reading frame. The in vitro transcription revealed different possibilities; which product represents the splicing of CYP3A5*5 in vivo is unclear. The mutation is also worth studying in other ethnic groups to explain the wide interindividual variation of CYP3A activities.

References


