Detection of a CYP3A5 Allelic Variant: A Candidate for the Polymorphic Expression of the Protein?

Y. Jounaïdi, Valérie Hyrailles, Laurence Gervot, and Patrick Maurel

INSERM U 128, CNRS, BP5051, 1919 Route de Mende, 34033 Montpellier, France

Received March 4, 1996

In liver samples from 19 Caucasian subjects, the CYP3A5 protein was detected in 74% of individuals (14/19), while the messenger was shown to be expressed in 100% of individuals, assessed by the RT-PCR method. In order to characterise the putative mutation(s) in the messenger, accounting for the absence of protein accumulation, the full coding region of the CYP3A5 cDNA was sequenced for two unrelated individuals, one expressing the protein at a high level and one being defective. A point mutation in exon 11 at position 1280 (C→A) was found to cosegregate with the absence of protein accumulation in 2 of the 5 defective individuals. This mutation produces a change in the aminoacid at position 398 (Thr→Asn). Whether this mutation affects the stability of the protein or whether it is in linkage desequilibrium with other mutation(s) in the non-coding region of the messenger is not known. The C→A change at 1280 generates a Tsp509 I site which can be used for routine evaluation of the frequency of this mutation in population studies.

The human CYP3A family contains at least three functional genes including CYP3A3/3A4, CYP3A5 and CYP3A7 (1–3). CYP3A4 is the major CYP protein expressed in the adult human liver where it may represent up to 60% of the total amount of CYP proteins (4). It is also expressed at a high level in the duodenum, jejunum and ileon (5–7). CYP3A7 is the major CYP protein detected in the human fetal liver (8, 9) and kidney (10), and may be expressed as well, although at a very low level, in the adult liver (11). This gene was recently found to be expressed in the adult endometrium and in the placenta (12).

CYP3A5 appears to be expressed polymorphically in both the adult and fetal liver and in the kidney and intestine. Several studies have been devoted to this polymorphism with, however, conflicting results. For example, in adult Caucasians, the mRNA and the protein were detected in the liver of 10 to 30% of subjects (13–16), while the protein was detected in the kidney and intestine of approximately 70% of subjects (7, 10). Interestingly, Wrighton et al. (15) observed that in the liver of young Caucasians under 19, the protein was expressed in a greater percentage of individuals (47%). The molecular basis for this polymorphism has not yet been clearly established. The aim of this work was to re-examine the expression of CYP3A5 in a bank of liver tissue from 19 adult Caucasians, at the levels of mRNA and protein, using highly sensitive methods including RT-PCR and enhanced chemiluminescence immunoblotting, respectively.

MATERIALS AND METHODS

Liver tissue samples. Liver samples used in this work were obtained either from organ donors (the liver being not used for transplantation for elevated transaminase or steatosis) or from lobectomy resections for medical purposes (primary or secondary tumors, adenoma, angioma). Ten to fifty grams of tissue were frozen in liquid nitrogen immediately after collection.

Protein analysis. Microsomes were prepared by differential centrifugation. CYP3A5 protein was quantitated by immunoblotting using form-specific anti-CYP3A5 antibodies (14, 15) kindly provided by Dr. SA Wrighton (Lilly Research Laboratories, Indianapolis IN). The blots were revealed with the enhanced chemiluminescence method (ECL, Amersham, Amersham England).
RNA analysis. RNA was prepared according to the method of Chirguin (17). CYP3A5 and CYP3A4 mRNA were analysed by Northern blot using a full length CYP3A5 cDNA probe. Expression of CYP3A5 mRNA was also assessed by RT-PCR. For this purpose the following oligonucleotides were used: 3A5(S) from nucleotide 45 to 74: 5’- AAAAGGAAGACTCACAGAACACAGTTGAAG-3’ (13) 3A5(R) from nucleotide 1620 to 1592: 5’- CAAAGGTAGAAGTCTTAGAATTTGAGTA-3’. Oligonucleotide 3A5(S) has 8 and 10 mismatches with the corresponding sequences on CYP3A4 and CYP3A7, respectively. Oligonucleotide 3A5(R) has 9 mismatches with both corresponding sequences on CYP3A4 and CYP3A7.

CYP3A5 cDNA sequencing. The full coding cDNA of CYP3A5 was prepared by RT-PCR amplification using the RNA extracted from each liver sample, as indicated above. The CYP3A5 cDNA prepared from subjects FH45, FT32 and FH37, was used as a matrix to generate by PCR 9 fragments of approximately 300 bp. Each fragment was designed in such a way as to overlap with the immediate upstream and downstream fragments along the 5’–3’ sequence. These fragments were then subcloned in pBKS+ plasmid (Stratagene, La Jolla, CA). Nucleotide sequencing was carried out with a BioRad sequencer (Hercules, CA), using the sequenase 7 (version 2) kit (US Biochemicals, Cleveland OH). Every sequence was confirmed at least twice. The sequence alignments were made with the Clustal V program (EMBL, Heidelberg, Germany).

RT-PCR analysis of the C1280→A mutation. The fragment from nucleotide 1192 to 1375 was amplified by PCR from the full coding cDNA of CYP3A5 prepared from each subject as indicated above, using the following oligonucleotides: MUT1280(S): from nucleotide 1192 to 1215: 5’- GTTGCTATTAGACTTGAGAGGACT-3’ (13) MUT1280(R): from nucleotide 1375 to 1356: 5’-TGTAGAAGTCTTAGAATTTGAGTA-3’. The amplified product was digested for 2 hours with 10 units of Tsp509 I (which digests DNA at the site 5’–AATT–3’), and the products of digestion were analysed on agarose gel. In some experiments, the fragments separated by electrophoresis were further analysed by Southern blot using the full length CYP3A5 cDNA as a probe.

RESULTS AND DISCUSSION

Microsomes and total RNA were extracted from liver samples of 19 Caucasian individuals. For each individual, the levels of CYP3A5 protein and mRNA were determined by immunoblot and Northern blot or RT-PCR, respectively. The results of an immunoblot developed with form-specific anti-CYP3A5 antibodies (14, 15) and revealed using the enhanced chemiluminescence (ECL) method are presented in Figure 1 for 8 of the 19 individuals examined. To our surprise, the CYP3A5 protein was detected in 14 of the 19 subjects, that is with a frequency of 74%. This frequency is greater than that observed for the liver by previous investigators (11, 13–16). We believe that this is due to the greater sensitivity of the method of detection (ECL) used in this work. As it may be seen from the immunoblot presented in Figure 1, the interindividual variability in the accumulation of CYP3A5 is very large (1 to 100 for extreme cases in individuals for whom the protein was detectable). In contrast, CYP3A4 was detected in all individuals, although here again with a wide interindividual variability; its level did not correlate with that of CYP3A5 (not shown).

It is interesting that recently, Schuetz et al. (10) using the streptavidin-biotin detection method and Lown et al. (7) using the ECL method, detected the CYP3A5 protein in the kidney and intestine of approximately 70% of subjects (5/7 and 14/20), respectively. This percentage is close to the one obtained here. The CYP3A5 protein is therefore not more commonly expressed in the kidney or intestine than in the liver, as previously suspected (7, 10).

When CYP3A5 mRNA was quantitated by Northern blot with a CYP3A5 full length cDNA probe, both CYP3A4 (2.3 and 3.0 kb) and CYP3A5 (1.9 kb) messengers were revealed although CYP3A4 mRNA was always expressed in greater amount. Using this method, the CYP3A5 mRNA was detected only in the samples from those subjects for whom the protein was expressed at a high

![FIG. 1. Immunoblot analysis of CYP3A5 protein accumulation in the liver of adult Caucasians. Microsomes (100 µg per lane) were submitted to polyacrylamide gel electrophoresis and the resolved proteins were transferred to a nitrocellulose filter for immunoquantitation with form-specific anti-CYP3A5 antibodies. The blot was developed by the enhanced chemiluminescence method.](image-url)
level (approximately 30% of individuals, not shown). This is in agreement with recent results from Schuetz et al. (11). We therefore decided to assess the expression of this mRNA by the more sensitive RT-PCR method, using two CYP3A5-specific oligonucleotides 3A5(S) and 3A5(R) (see Materials and Methods). These oligonucleotides were designed in such a way as to exhibit more than 8 mismatches with the corresponding sequences of CYP3A4 (18) and CYP3A7 (9). Indeed, with this assay, CYP3A5 mRNA was detected in all individuals examined (19/19). Obviously we verified, on all samples, that the amplified cDNA was indeed CYP3A5 by restriction analysis, according to the published sequence (13). This suggests therefore that the absence of CYP3A5 protein in the 5 defective subjects (FT32, FH37, FT79, FH176 and FH61) is likely to result from one or several mutations in the coding sequence of the mRNA, but is not the consequence of a defect in the expression of the messenger.

In order to characterise and identify these putative mutations, we carried out a comparative analysis of the sequence of the coding region of CYP3A5 cDNA isolated from individual FH45, expressing the protein at a high level, and from individual FT32 in the liver of whom the protein was not detectable (Figure 1). The cDNA from another individual (FH37) was also partially sequenced. In both FT32 and FH37 individuals, one point mutation was detected in exon 11 where nucleotide C1280 is replaced by an A as shown in Figure 2. In terms of the primary sequence of the protein, this mutation only produces a change in one aminoacid residue, i.e. Thr398 to Asn. It has been reported that Asn residues are sites at which proteins are particularly susceptible to nonenzymatic degradation through succinimide-forming reactions (19). Whether this aminoacid change results indeed in a decreased stability of the CYP3A5 protein, accounting for the absence of detectable accumulation, or whether the mutation at position 1280 is in strong linkage disequilibrium with another defective mutation in the non coding region of the mRNA, is not known at present.

Interestingly, the C1280→A mutation generates a Tsp509 I site in the cDNA, which therefore provides a means for routine detection of this allele in population studies. To verify this approach, two oligonucleotides, MUT1280(S) and MUT1280(R), were used to amplify by PCR the region

![FIG. 2. Partial nucleotide sequence of CYP3A5 exon 11. A cDNA fragment from nucleotide 1192 to 1375 was amplified by PCR from the full length cDNA template of three individuals: FH45 (high protein level) and FT32 and FH37 (no detectable protein, see Figure 1). The nucleotide sequence gel is displayed from nucleotides 1272 to 1288 and the arrow head indicates the mutation at position 1280.]

between nucleotides 1192 to 1375 from the CYP3A5 cDNA prepared for each subject of our bank. The amplified fragment of 184bp was then digested with Tsp509 I and the products (two subfragments of 84 and 95 bp when mutation is present) were analysed by electrophoresis on agarose gel. The results are reported in Figure 3 for the 19 individuals tested in this work. Clearly, the amplified fragment from all subjects, excepted FT32 and FH37, has not been cleaved, while in contrast and as expected, a band of approximately 100 bp (revealing the 84 and 95 bp subfragments) is observed with the digests from subjects FT32 and FH37. That these fragments are indeed harboring a CYP3A5 sequence was definitely proven by a Southern blot analysis with a CYP3A5 cDNA probe (not shown).

Three points have to be emphasized here. First, the percentage of individuals having this mutated allele is approximately 10% in our bank (2/19, only found in subjects FT32 and FH37). Second, the presence of a band migrating at 190 bp in the lanes analysing the digests from subjects FT32 and FH37 suggests that these individuals are heterozygous: they are likely to possess this mutated allele and another defective allele in which this mutation is not present. We obviously verified that this was not due to incomplete digestion by Tsp509 I; neither increasing the duration of digestion nor augmenting the amount of the endonuclease affected the pattern of digestion of the PCR amplified fragment. Third, the findings that FT32 and FH37 are heterozygous for the allele having the mutation 1280, while the three other deficient individuals (FT79, FH176 and FH61) do not possess this allele suggest that there are other(s) mutated defective allele(s) of CYP3A5 responsible for the absence of protein accumulation.

ACKNOWLEDGMENTS

We wish to express our thanks to Dr. S. A. Wrighton for kindly providing form-specific anti-CYP3A5 antibodies, and to Dr. Colin Young for careful reading of the manuscript.

**FIG. 3.** Detection by PCR of the 1280 mutation in the cDNA of 19 Caucasian individuals. A cDNA fragment from nucleotide 1192 to 1375 was amplified by PCR from the full length CYP3A5 cDNA template prepared from 19 individuals. The fragment was then digested with Tsp509 I and the mixture was loaded on an agarose minigel. After migration, the DNA fragments were revealed by BET staining.
REFERENCES