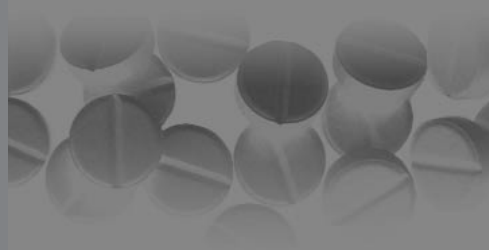


The CYP₃A₄*₃ allele: is it really rare?

Chapter 8



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Summary

- Introduction** Cytochrome P₄₅₀ 3A₄ (CYP_{3A4}) is the major cytochrome involved in drug metabolism. Genetic polymorphisms in the CYP_{3A4} allele, affecting enzymatic activity, may contribute to the observed interindividual variation in drug metabolism. Two genetic polymorphisms have been described: CYP_{3A4}*_{1B} and CYP_{3A4}*₂. A third variant allele, CYP_{3A4}*₃, was found only in one individual and was therefore referred to as a rare allele.
- Methods** We developed a PCR restriction fragment length polymorphism (RFLP) assay for detection of the CYP_{3A4}*₃ allele. A total of 762 individuals was screened for this variant allele.
- Results** Applying this assay on genomic DNA obtained from 499 Caucasians, we identified 13 heterozygote and no homozygote individuals for CYP_{3A4}*₃. When 263 patient samples were screened, 3 additional heterozygote individuals were identified.
- Conclusion** We conclude that CYP_{3A4}*₃ is not a rare allele, but can be found in 1.1 % of the population studied. Therefore, the CYP_{3A4}*₃ allele may represent a genuine polymorphism. A PCR-RFLP procedure is described which can be used to screen for the CYP_{3A4}*₃ variant allele.

Introduction

Enzymes of the cytochrome P450 system are involved in the metabolism of a broad range of foreign compounds, such as drugs, environmental pollutants and carcinogens (1). The most abundant enzyme in the human liver is cytochrome P450 3A4 (CYP3A4) (2). This enzyme is involved in the metabolism of over 50% of all drugs used in humans (3, 4) and the interindividual differences in the pharmacokinetics of these drugs are thought to be related to variations in CYP3A4 activity (4-6). These variations may be caused by age and disease related differences, by drugs inducing or repressing transcription/translation, or from genetic polymorphisms. Although the CYP3A4 gene was initially thought not to be polymorphic, recent reports described three genetic variants of this gene: CYP3A4*1B, CYP3A4*2 and CYP3A4*3 (7, 8). The allelic frequency for the CYP3A4*1B allele, which contains an A(-290)G substitution in the promoter region of CYP3A4, ranges from 0.0% in Chinese and Japanese Americans to over 54% in African Americans (8, 9). White Americans and European Caucasians were reported to have an allelic frequency of approximately 4-5% (8-11). The CYP3A4*2 allele, which encodes a Ser222Pro change, has an allelic frequency of 2.7% in the white (Finnish) population (8). Because variant alleles that are found in more than 1% of the population are defined as genetic polymorphisms (12), both the CYP3A4*1B and the CYP3A4*2 allele are considered to be genetic polymorphisms of CYP3A4. In addition, a variant allele found in the DNA of a single Chinese subject contained a T1437C substitution (8). Because this allele, encoding a Met445Thr change, was not found in any other of the 91 subjects investigated in that study, it was referred to as a rare allele.

Methods

In this study, we developed a PCR-restriction fragment length polymorphism (RFLP) procedure for the detection of the CYP3A4*3 allele. We used this assay to determine the allelic frequency of CYP3A4*3. EDTA-whole blood was obtained from 499 healthy Dutch Caucasian volunteers and from 66 pediatric patients involved in a midazolam pharmacokinetic study after informed consent. The study was approved by the Medical Ethical Committee of the University Hospital Rotterdam. We isolated genomic DNA from 300 µL of blood, using the GenomicPrep Blood DNA Isolation Kit (Amersham Pharmacia Biotech). DNA yields were estimated by measuring the absorbance at 260 nm (A_{260}). A total of approximately 50 ng of genomic DNA was used in a PCR volume of 50 µL. The PCR mixture contained 1X buffer (10 mmol/L Tris-HCl pH 8.3, 1.5 mmol/L MgCl₂, 50 mmol/L KCl and 0.001% (w/v) gelatin (Perkin-Elmer)), 0.2 mmol/L each dNTP (Roche), 1.25 U of AmpliTaq Gold (Perkin-Elmer), and 40 pmol of each of forward primer [5'-TGG ACC CAG AAA CTG CAT ATG C-3' (nucleotide 23,255-23,276; GenBank sequence AF209389)] and reverse primer [5'-GAT CAC AGA TGG GCC TAA TTG-3' (nucleotide 23,483-23,503; GenBank sequence AF209389)]. The nucleotides underlined are mismatches with the CYP3A4 sequence, creating a NsiI restriction site in the wild-type CYP3A4 PCR product. When the CYP3A4*3 allele is amplified, this NsiI site is disrupted. PCR conditions were as follows: 7 min at 94 °C; 35 cycles of 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C; and finally 7 min at 72 °C. The 249 bp PCR product of 5 DNA samples were sequenced, confirming that indeed only the CYP3A4 gene was amplified. The PCR product (15 µL) was digested with 10 U NsiI (Roche) for

2 hours at 37 °C, and analyzed on a 3% MP agarose/Tris-borate-EDTA gel with ethidium bromide staining. Samples that produced a heterozygote signal were re-analyzed by PCR-RFLP. Subsequently, heterozygosity for CYP3A4*3 was confirmed by sequencing of the PCR product with the reverse primer on an automated ABI 310 capillary sequencer (Perkin-Elmer) using the Big Dye Terminator Cycle Sequencing Kit (Perkin-Elmer). To check reproducibility, the CYP3A4*3 assay was performed 10 times at a wild-type and 10 times at a heterozygote sample, which resulted each time in the correct genotype.

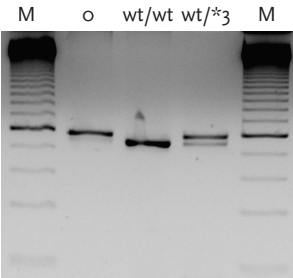
Results

A 249 bp fragment was amplified, using the primers described (Fig 1b, lane o). Digestion of the PCR product with NsiI resulted in 226 and 23 bp fragments for the wild-type sequence (Fig. 1, lane wt/wt) and 249, 226 and 23 bp for heterozygote sequences (Fig. 1, lane wt/*3). The 23-bp fragments are not visible on gel. To check reproducibility, the CYP3A4*3 assay was performed 10 times at a wild-type and 10 times at a heterozygote sample, which resulted each time in the correct genotype.

The CYP3A4*3 PCR-RFLP assay was applied to 499 genomic DNA samples obtained from Dutch Caucasian volunteers and 66 samples from pediatric patients who participated in a midazolam pharmacokinetic study. This resulted for the 499 volunteers in 488 cases in digestion of the 249 bp PCR product into 226 and 23 bp (not visible) fragments, as expected for wild-type samples and in 11 cases (2.2%) in the heterozygote signal. No homozygotes were detected. The allelic frequency of CYP3A4*3 in these Caucasians is therefore 1.1%. In the 66 pediatric patients, one heterozygous individual for CYP3A4*3 was detected. These allele and genotype frequencies are in Hardy Weinberg equilibrium ($p=0.80$). In the heterozygote samples, direct sequencing showed a mixed T/C peak corresponding to position 1437, indicating that the nucleotide change was indeed T1437C in all cases.

Because only one pediatric patient was heterozygous for CYP3A4*3, it was not possible to determine the effect of this mutation on midazolam pharmacokinetics in pediatric patients.

Figure 1 PCR-RFLP procedure for the CYP3A4*3 allele. Undigested PCR product (o), and NsiI-digested PCR fragments of 226 and 23 bp for a wild-type (wt/wt) and 249, 226 and 23 bp for a heterozygote DNA sample (wt/*3). The 23 bp fragment is not visible. Analysis on a 3% agarose/TBE gel; picture is printed negative. M= basepair marker (50 bp ladder).



Discussion

Variant CYP3A4 alleles in the population may contribute to interindividual variability in CYP3A4 activity and detecting genetic polymorphisms may help to predict an individual's ability to respond to certain drugs. The CYP3A4*3 allele, which has a T1473C change leading to a Met445Thr substitution in exon 12, was found only in one Chinese subject from Shanghai and could not be detected in 91 other individuals (8). The investigators concluded that CYP3A4*3 is a rare allele, which may result in low priority in performing functional studies on this allele. Our data indicate that the CYP3A4*3 allele is not restricted to a single individual, but has an allelic frequency of 1.1% in Caucasians. This implies that the variant CYP3A4*3 allele is not a rare allele, but instead represents a genetic polymorphism which can be found in a substantial part of the population. The identification of the CYP3A4*3 variant allele as a genetic polymorphism adds up to the two other described genetic polymorphisms for CYP3A4. The CYP3A4*1B allele potentially alters the transcription efficiency and thus the overall enzymatic activity of CYP3A4: although initial reports suggested decreased activity *in vivo* (7, 13, 14), increased activity *in vitro* (15, 16) and no effect (10, 14, 17). For the variant allele CYP3A4*2, a decreased enzymatic activity was observed for nifedipine, but not testosterone (8). For CYP3A4*3, the location of the amino acid which is changed in the CYP3A4 protein is near the cysteine that is involved in the active site of the enzyme (8). This might induce structural differences leading to alteration in enzymatic activity. However, expression studies need to be performed to prove this. Taking into account the allelic frequencies of the genetic polymorphisms in CYP3A4 (10% heterozygotes for CYP3A4*1B, 2.7% heterozygotes for CYP3A4*2 and 2.2% heterozygotes for CYP3A4*3), this implies that approximately 10-15% of the (Caucasian) population may carry a genetic polymorphism in this allele. Since genetic polymorphisms may exhibit strong variation in occurrence among different ethnic groups, other populations need to be investigated to determine the allelic frequency of CYP3A4*3.

In conclusion, we have described and validated a PCR-RFLP analysis for the CYP3A4*3 allele. The frequency of this variant allele in the Caucasian population of 1.1% indicates that it might be important when predicting CYP3A4 activity based on genotype. Future research should be directed upon elucidating the effect of this polymorphism on the CYP3A4 enzymatic activity and to establish whether we are dealing with only a genetic, or also a functional polymorphism.

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