CYP3A4-V polymorphism detection by PCR-RFLP and its allelic frequency among 199 Dutch Caucasians

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Summary

Background
Interindividual variation in drug metabolism may complicate the treatment of patients in modern medicine. Detection of genetic polymorphisms in cytochrome P450 enzymes may contribute to an improvement of effective pharmacotherapy. Recently, a genetic polymorphism was identified in the nifedipine-responsive element in the promoter region of the abundantly expressed cytochrome P450 3A4 enzyme (CYP3A4).

Methods
We developed a simple and rapid assay for the specific detection of the CYP3A4-V polymorphism. The assay uses a polymerase chain reaction (PCR) with specifically designed oligonucleotide primers, followed by restriction fragment length polymorphism (RFLP) detection.

Results
Applying this assay on genomic DNA obtained from 199 healthy Dutch Caucasian volunteers, we identified 21 heterozygotes and no homozygotes for this polymorphism. This corresponds to an allelic frequency of 5.3% for the CYP3A4-V allele in the group studied.

Conclusion
A PCR-RFLP procedure is described which can be used for screening purposes for the CYP3A4-V allele. Using this assay, we found that the allelic frequency in Dutch Caucasians is significantly lower than the allelic frequency reported by Rebbeck et al. (J Natl Cancer Inst 1998; 9:1225-1229) in U.S. Caucasians.
Introduction

Interindividual variation in drug metabolism is a complicating factor in pharmacotherapy. Enzymes of the cytochrome P450 system are involved in the metabolism of several endogenous substrates and a broad range of foreign compounds, such as drugs, environmental pollutants and carcinogens (1). The cytochrome P450 enzyme family consists of several subfamilies, with CYP3A4 being the most abundant P450 enzyme in human liver (2). CYP3A4 is involved in the metabolism of >50% of all drugs used in humans (3, 4). Interindividual differences in CYP3A4 expression may account for the observed interindividual differences in pharmacokinetics of drugs metabolized by this enzyme (4-6). Variations in CYP3A4 expression may be caused by factors inhibiting or stimulating transcription and/or translation (e.g. concomitant drug administration), and by genetic polymorphisms.

In a recent study, an A (-290) G substitution was described in the 5'-regulatory region of the CYP3A4 gene (7, 8). This allele was termed CYP3A4-V, but was recently also referred to as CYP3A4*1B (9). The allelic frequency, determined by using conformation sensitive gel electrophoresis, was estimated to be 9.6% among U.S. Caucasians (7). In 94 healthy unrelated male volunteers, 3.2% were found to be homozygous and 12.8% heterozygous for the CYP3A4-V allele (7). In a recent publication, the allelic frequency was shown to display major interethnic differences, ranging from 0.0% among Chinese Americans to 54.6% among black Americans (10, 11). The CYP3A4-V allele has recently been associated with higher clinical stage and grade of prostate cancer (11-13).

In this study, a simple PCR-RFLP procedure was developed that can be used in routine screening procedures to detect the CYP3A4-V polymorphism. The assay was used on 199 DNA samples obtained from Dutch Caucasian volunteers to determine the allelic frequency of the CYP3A4-V allele in this group.

Materials and Methods

EDTA- whole blood was collected from 199 healthy Caucasian volunteers after informed consent. The Medical Ethical Committee of the University Hospital Rotterdam approved the study. From 300 µL of blood, genomic DNA was isolated using the GenomicPrep Blood DNA Isolation Kit (Amersham Pharmacia Biotech, Buckinghamshire, UK). DNA yields were estimated by measuring the absorbance at 260 nm (A260). For the polymerase chain reaction (PCR), approximately 50 ng of genomic DNA was used in a total 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 0.01 g/L (w/v) gelatin) (Perkin-Elmer, Norwalk, CT, USA), 0.2 mmol/L of each dNTP (Boehringer Mannheim, Germany), 1.25 U AmpliTaq Gold (Perkin-Elmer), 40 pmol forward primer 5’-GGA CAG CCA TAG AGA CAA CTG CA-3’ (position -315 to -291 (8)) and 40 pmol reverse primer 5’-CTT TCC TGC CCT GCA CAG-3’ (position +22 to +5 (8)). The underlined nucleotides are mismatches with the CYP3A4 sequence, creating a PstI restriction site in the CYP3A4-V PCR product. The 3’ end of the forward primer lies in the nifedipine-responsive element, a sequence that is characteristic for CYP3A4 and is absent in the homologous CYP3A7 sequence, ensuring specific amplification of CYP3A4 (8). PCR conditions were as follows: 7 min 94 °C, 35 cycles of [1 min 94 °C, 1 min 55 °C, 1 min 72 °C] and finally 7 min 72 °C. A 334-bp fragment was amplified using the CYP3A4-V primers described
(Fig 1, Lane 0). Fifteen µL of PCR product was digested for 1 1/2 hours at 37 °C by adding 10 U PstI (Boehringer Mannheim). The digested product was analyzed on a 3% agarose/Tris-borate-EDTA gel with ethidium bromide staining. The DNA sequences of the nifedipine-responsive element were verified by direct sequencing. Genomic DNA was amplified by PCR: 7 min 94 °C, 35 cycles of [30 sec 94 °C, 30 sec 55 °C, 30 sec 72 °C], followed by 7 min 72 °C using forward primer 5’-AAC AGG GGT GGA AAC ACA AT-3’ and reverse primer 5’-CTT TCC TGC CCT GCA CAG-3’ (7). Direct sequencing was performed on an automated ABI 310 capillary sequencer (Perkin-Elmer) using the Big Dye Terminator Cycle Sequencing Kit (Perkin-Elmer).

Results

PCR-RFLP for CYP3A-V
A 334 bp fragment was amplified; using the CYP3A4-V primers described (Fig. 1B, lane 0). Digestion of the PCR product with PstI results in 220, 81 and 33 bp fragments for the wild-type sequence (Fig. 1B, lane wt/wt), or 199, 81, 33 and 21 bp fragments for the CYP3A4-V allele (Fig. 1B, lane V/V). Two internal PstI sites in this fragment serve as an internal control for digestion (Fig. 1A). In case of heterozygosity, both the 220 bp and the 199 bp fragments are present (Fig. 1B, lane wt/V). The DNA sequences of the nifedipine-responsive element of these controls were verified by sequencing.

Allelic frequency in a group of 199 Dutch Caucasians
Using the described PCR-RFLP, we screened 199 unselected and unrelated healthy Caucasians for the CYP3A4-V allele using the wt/wt, wt/V and V/V samples described above as controls. We identified 21 CYP3A4-V alleles, resulting in an allelic frequency of 5.3%. In total, 21 individuals were found to be heterozygous for this allele, giving a
heterozygote frequency of 10.6%. No individuals were found that were homozygous for this polymorphism, indicating that the homozygote frequency is likely to be under 2% in this population. DNA sequencing of all heterozygous individuals confirmed the A(-290)G substitution in all cases; no additional mutations were detected in the nifedipine-responsive element. As a control, 10 wild-type samples were also sequenced, confirming that the wild-type DNA sequence was present in the nifedipine-responsive element in all cases.

Discussion
The initial publication describing the \( \text{CYP3A4-V} \) polymorphism made use of PCR of the -571 to +22 bp region of the \( \text{CYP3A4} \) allele in which the PCR product was analyzed using conformation-sensitive gel electrophoresis (7). In order to simplify the procedure for large scale screening purposes, and to make screening specific for the described \( \text{CYP3A4-V} \) mutation, we developed a PCR in which a simple digestion with PstI reveals the presence of this polymorphism. The forward primer has two mismatches, creating a PstI restriction site when the \( \text{CYP3A4-V} \) allele is amplified. Due to the fact that two additional PstI sites are present in the PCR-product, an internal control of digestion is automatically included. The 3’ end of the forward primer lies in the nifedipine-responsive element, a sequence which is characteristic for \( \text{CYP3A4} \) and which is absent in the homologous \( \text{CYP3A7} \) sequence, ensuring specific amplification of \( \text{CYP3A4} \) (8). DNA sequencing of 31 samples confirmed the reliability of the assay. Interestingly, in the heterozygous samples, the 220 bp wild-type derived band was usually less intense when compared to the 199 bp \( \text{CYP3A4-V} \) derived band. We assume that the first base to be added by the Taq-polymerase, being a G for the \( \text{CYP3A4-V} \) sequence and an A for the wild-type sequence, increases the stability of the forward primer-genomic DNA complex better for the \( \text{CYP3A4-V} \) allele when compared to the wild-type sequence. We tried to minimize this effect by having a forward primer synthesized missing the 3’ A base, making the stability of the primer-DNA complex less dependent on the first base to be added by the Taq-polymerase. PCR-RFLP with this alternative forward primer in combination with the described reverse primer, yielded 220 bp and 199 bp bands of equal intensity (results not shown). However, the overall efficiency of the PCR reaction was dramatically decreased, probably because the two mismatches with the \( \text{CYP3A4} \) sequence are now in too close proximity of the 3’ end of the primer, decreasing efficiency of the PCR. Application of this alternative primer therefore needed 45 cycles of PCR and 25 µL of PCR product for digestion and subsequent detection on gel.

Recently, another PCR-RFLP procedure was described for detection of the \( \text{CYP3A4-V} \) allele, which is based on creating a MboII restriction site (15). This procedure makes use of nested PCR, which is more laborious and has an increased risk of sample contamination. Moreover, the \( \text{CYP3A4-V} \) allele PCR products cannot be digested, which means that this assay lacks an important internal control on digestion efficiency.

Applying the assay on 199 samples obtained from Dutch Caucasian volunteers identified 21 heterozygotes for the \( \text{CYP3A4-V} \) allele, resulting in an allelic frequency of 5.3%. This is significantly different (\( p<0.05 \), Fisher’s Exact Test) from the reported allelic frequency of 9.6% (18 \( \text{CYP3A4-V} \) alleles in 94 individuals) by Rebbeck et al. for U.S. Caucasians (7). In that specific study on 94 healthy volunteers, 3.2% appeared to be
homozygous for this mutation. We did not find any CYP3A4-V homozygotes among the 199 individuals studied. The allele and genotype frequencies were in Hardy-Weinberg equilibrium (P=0.342); the absence of homozygotes in our study population of 199 individuals is consistent with a Hardy-Weinberg distribution. In Swedish Caucasians, 3 CYP3A4-V alleles were recently found when studying 39 individuals, giving an allelic frequency of 3.8% (16), whereas Sata et al. (9) reported an allelic frequency of 4.2% in 59 white subjects. These data are more in agreement with our results.

CYP3A4, the most abundant form of the cytochrome P450 enzyme family present in human liver, is involved in the metabolism of a large number of drugs (3, 4, 14). Recently, a genetic polymorphism in the 5' regulatory region was discovered, in which an A(-290)G substitution changes the nifedipine-responsive element (7). This mutation potentially alters the transcription efficiency and thus the overall enzymatic activity of CYP3A4. Initially, the effect of this mutation on transcription was believed to be a decrease, based upon the clinical presentation of prostate cancer (7) and drug-induced leukemia (12). Later experiments, in which protein expression and enzymatic activity using liver samples were compared, suggested that the CYP3A4-V mutation had no effect on transcription (15). This was supported by experiments on the 6ß-hydroxylation of testosterone in three microsomal liver samples from individuals heterozygous for the CYP3A4-V allele (16), although this conclusion was questioned later by others (17). Experiments, in which the promoter region of CYP3A4 was fused to the luciferase reporter gene, followed by expression of these constructs in HepG2 and MCF7 cells, showed that the CYP3A4-V allele actually gives rise in an increase in CYP3A4 transcription compared to the CYP3A4 wild-type allele (18).

In conclusion, we have described and validated a fast and simple PCR analysis, which can be applied for specific screening for the CYP3A4-V allele. This assay will greatly facilitate studies on the effect of this polymorphism in endogenous processes, environmental susceptibility to cancer and individual ability to metabolize drugs.

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Chapter 7 - CYP3A4-V polymorphism detection by PCR-RFLP and its allelic frequency among 199 Dutch Caucasians

References


