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Review

Phenotype–genotype variability in the human *CYP3A* locus as assessed by the probe drug quinine and analyses of variant *CYP3A4* alleles ☆

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Abstract

The human cytochrome P450 3A (CYP3A) enzymes, which metabolize 50% of currently used therapeutic drugs, exhibit great interindividual differences in activity that have a major impact on drug treatment outcome, but hitherto no genetic background importantly contributing to this variation has been identified. In this study we show that *CYP3A4* mRNA and hnRNA contents with a few exceptions vary in parallel in human liver, suggesting that mechanisms affecting *CYP3A4* transcription, such as promoter polymorphisms, are relevant for interindividual differences in CYP3A4 expression. Tanzanian ($n = 143$) healthy volunteers were phenotyped using quinine as a CYP3A probe and the results were used for association studies with *CYP3A4* genotypes. Carriers of *CYP3A4*1B* had a significantly lower activity than those with *CYP3A4*1* whereas no differences were seen for five other SNPs investigated. Nuclear proteins from the B16A2 hepatoma cells were found to bind with less affinity to the *CYP3A4*1B* element around −392 bp as compared to *CYP3A4*1*. The data indicate the existence of a genetic *CYP3A4* polymorphism with functional importance for interindividual differences in enzyme expression.

Keywords: Cytochrome P450 3A; Haplotype; Polymorphism; *CYP3A4*1B*; Phenotype–genotype associations; Quinine; Interethnic differences

The cytochrome P450s (CYPs) in families 1–3 are the most relevant enzymes catalyzing the biotransformation of xenobiotics. As a consequence, many profound adverse reactions of drugs and poor therapeutic response are due to atypical CYP activities [1–3]. Among the P450s, the CYP3A subfamily is involved in the metabolism of more than 50% of currently used therapeutic drugs, representing the most abundant P450s in human fetal and adult liver. Consequently, variations in CYP3A activity have a major impact on pharmacokinetics and metabolic fate of drugs, drug–drug interactions, and toxic effects of drugs [1,4,5]. The expression of the CYP3A enzymes, which is regulated by developmental mechanisms, is considerably different between fetus and adults, fetal liver shows a predominant expression of CYP3A7 while adult liver mainly expresses CYP3A4 [6]. By contrast, CYP3A5 can be present at relevant levels in both fetal and adult liver, in particular in Black populations where the functional *CYP3A5*1* allele is common [7].

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There are important interethnic differences in the constitution of the *CYP3A* locus. Two major haplotypes that influence the fetal liver capacity for CYP3A dependent hepatic metabolism have been described. In Caucasians the most common allelic combination, *CYP3A7*1/CYP3A7_39256T/CYP3A5*3*, causes a lower detoxification capacity than the most abundant haplotype in Africans, *CYP3A7*2/CYP3A7_39256A/CYP3A5*1* [8]. The genetic basis causing the interindividual differences in CYP3A4 expression in adult liver is not known. Several studies have, however, indicated that CYP3A4 inter-individual variability is genetically determined and extensive studies searching for allelic variants have been carried out [9]. However, the low frequency of functional polymorphisms found in the coding regions of *CYP3A4* cannot account for the variation observed [10,11]. Some promoter and intronic polymorphisms have been proposed to contribute to the observed CYP3A4 inter-individual variability, but the results are unclear [12,13].

In the present investigation, we have phenotyped 143 Tanzanian healthy volunteers using the CYP3A probe quinine and carried out association studies with *CYP3A4* polymorphisms and electrophoretic mobility shift assays (EMSAs) with hepatic nuclear proteins in order to identify molecular mechanisms influencing CYP3A4 expression. We found that *CYP3A4*1B* was associated with a significantly lower hydroxylation of quinine and less binding of nuclear proteins, suggesting that *CYP3A4*1B* (−392A > G) is a functional SNP.

Materials and methods

Materials. [γ-^{32}P]dATP was purchased from Amersham and poly(dI-dC) was from Pharmacia (Peapack, NJ). The antibodies used in this work were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Human liver material. Liver samples from 45 individuals were from three different sources as previously described [14]. All livers, except one (HL46), were documented to be of Caucasian origin. The tissue was frozen in liquid nitrogen, stored at −70 °C and used to isolate total RNA and genomic DNA.

Real-time quantitative RT-PCR. Total RNA was extracted from the human livers using the QuickPrepTotal RNA Extraction Kit (Amersham Biosciences, Freiburg, Germany) according to the manufacturer's instructions. Two and a half microgram of total RNA that had been previously subjected to DNaseI treatment was reverse-transcribed using Super-script II Reverse Transcriptase (Invitrogen) and an oligo(dT)$_{14}$ primer together with a CYP3A4-specific primer located in intron 2: 5′-CCTTCTTTATGGCGCTCACA-3′. Quantitative real-time PCR was carried out with the Smart Cycler (Cepheid) and using the SyberGreen PCR master mix (PE Applied Biosystems). The following primers were used to amplify CYP3A4 mRNA: 5′-CCTTACATATACACACCC TTTGGAAGT-3′ and 5′-AGCTCAATGCATGTACAGAATCCCCG GTTA-3′; CYP3A4 heteronuclear RNA (hnRNA): 5′-ATGGACTTTTT AAGAAGCTTGGAATTCCA-3′ and 5′-GAGCCCTTGGGTAAACA TTG-3′; and β-actin: 5′-CGTACCACTGGCATCGTGAT-3′ and 5′-GT GTTGGCGTACAGGTCTTTGCG-3′. Aliquots of the PCRs were subjected to electrophoresis, and purified and sequenced to confirm that the amplified DNA corresponded to CYP3A4. For quantification, standard curves were constructed with serial dilutions. For CYP3A4 hnRNA measurements, due to the possibility of genomic DNA amplification, RTs without reverse transcriptase were prepared and measured using identical PCR conditions. These values, representing genomic DNA amplification, were subtracted from the amounts of total CYP3A4 hnRNA (average genomic amplification was 2.8% of hnRNA levels). Normalization for variation in reverse transcriptase efficiency and small differences in the amount of RNA in each reaction were carried out with the internal standard β-actin.

Population studied. One hundred forty-three healthy black Tanzanians, 39% of whom were women, participated in the study, they had a mean ± SD age of 35 ± 9 years and mean weight ± SD of 65 ± 12 kg. The volunteers were instructed not to take antimalarial drugs up for 4 weeks before and during the period of the study. Other medications (including contraceptive pills) and/or herbal entities were not taken up for 1 week before and during the period of the study. The subjects were advised to refrain from beverages or food containing grapefruit 2 days before and during the period of the study. All subjects were nonsmokers. The study was performed according to the Declaration of Helsinki. All subjects gave their written informed consent on the basis of verbal and written information in Swahili. The Human Ethics Committees at Muhimbili University College of Health Sciences, Dar es Salaam, Tanzania, and at Huddinge University Hospital, Karolinska Institutet, Sweden, approved the study.

Phenotyping with quinine. In vitro and in vivo studies have proved that the conversion of quinine to 3-hydroxyquinine is mainly catalyzed by CYP3A enzymes [15–18]. The details for the phenotyping procedure have been described in detail [19]. Briefly, the Tanzanian subjects received a single oral dose of 250 mg quinine hydrochloride and a blood sample was collected after 16 h. The blood samples were centrifuged at 1500*g* for 10 min, plasma was separated and stored at −20 °C until analysis. Quinine and its major metabolite 3-hydroxyquinine were quantified by high performance liquid chromatography as described [20]. The quinine metabolic ratio (MR), which reflects the CYP3A activity, was determined as the ratio of the molar concentration of quinine to that of 3-hydroxyquinine in the blood samples collected 16 h post-quinine intake. To ensure reliable phenotyping data, all laboratory work was pursued in the same laboratory with the same method.

CYP3A genotyping. The genomic DNA from the human livers was prepared according to the manufacturer's protocol using a QIAamp tissue kit (Qiagen GmbH, Hilden, Germany) and the genomic DNA from the volunteers was obtained from 5 ml of a venous blood and isolated using the QIAamp DNA Blood Midi Kit (Qiagen GmbH). The oligonucleotides and restriction enzymes used for genotyping the different *CYP3A4* SNPs by restriction fragment length polymorphisms (RFLP) are shown in Table 1. The genotyping accuracy was ascertained through direct sequencing of random samples using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit and analyzed on an ABI PRISM 377 DNA Sequencer (Applied Biosystems). The frequency found for the *CYP3A4* SNPs in the Tanzanian population was: 0.88 ($n = 89$), 0.80 ($n = 142$), 0.73 ($n = 142$), 0.33 ($n = 141$), 0.81 ($n = 137$), and 0.82 ($n = 130$) for −6790G, −1232T, −392G, In 7 + 268C, In 10 + 12A, and 3′UTR + 2477T, respectively. Genotyping for *CYP3A5*3*, **6* or **7* was performed as described by Mirghani et al. [19]. Lack of *CYP3A5*3*, **6*, and **7* alleles was interpreted as the presence of the functional *CYP3A5*1* genotype.

Preparation of nuclear extracts and electrophoretic mobility shift assay analysis. Nuclear extracts from B16A2 cells that had been in confluence during 4 weeks were prepared as described previously [21]. The oligonucleotides 5′-GAGACAAGGGCAAGAGAGAGGCGAT-3′ and 5′-GAG ACAAGGGCAGGAGAGAGGCGAT-3′ were annealed with their inverse/complementary oligonucleotide to produce, respectively, *CYP3A4*1* and *CYP3A4*1B* double-stranded probes covering the −404/−380 region of *CYP3A4* promoter. The oligonucleotide 5′-CACTTGATAACAG AAAGTGATAACTCT-3′ annealed with its inverse/complementary sequence was used as GATA consensus. For (EMSA), 12 μg of nuclear extracts were preincubated at 37 °C for 20 min with 2 μg poly(dI/dC), 100 mM NaCl, 15 mM Hepes, pH 7.9, 0.25 mM EDTA, 0.25 mM EGTA, 0.25 mM dithiothreitol, and 5% glycerol. When competition experiments were performed, double-stranded oligonucleotides were also added to the binding reaction. The probes were radiolabeled using [γ-^{32}P]dATP and T4 polynucleotide kinase (Invitrogen) and were added to the reaction mixture