MUTATION IN BRIEF

First Report of a Genetic Polymorphism of the Cytochrome P450 3A43 (*CYP3A43*) Gene: Identification of a Loss-of-function Variant

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In the present study, we report the first investigation of polymorphisms in the human *CYP3A43* gene. A screening for sequence variations in the 5'-flanking and protein coding regions of the *CYP3A43* gene was performed by a Polymerase Chain Reaction – Single Strand Conformational Polymorphism (PCR-SSCP) strategy, using DNA samples from 48 unrelated French individuals. Three polymorphisms in the coding region were identified, comprising two nucleotide substitutions, one silent (c.1047C>T) and one missense mutation (c.1018C>G / P340A), and a frame shift mutation (c.74delA), leading to a premature stop codon and, presumably, to a severely truncated protein. In order to evaluate the extent of the frame shift mutation in a larger population, 352 individuals were further genotyped. Thirty-four samples (4.83%) were found to be heterozygous and one homozygous (0.14%) for the nucleotide deletion, which suggests that, although the potential significance of this polymorphism remains to be further evaluated, some individuals are deficient for CYP3A43 activity.

KEY WORDS: CYP3A43; genetic polymorphism; SNP; PCR-SSCP

INTRODUCTION

Cytochromes P450 are heme-thiolate proteins that play a pivotal role in the biotransformation of endogenous compounds involved in intermediary metabolism and the breakdown of xenobiotics such as drugs and environmental contaminants (Nelson et al., 1996). To date, 57 cytochromes P450 genes and 24 pseudogenes, classified into families and subfamilies based on deduced protein sequences homology, have been characterized in humans (Dr Nelson homepage: http://drnelson.utmen.edu/CytochromeP450.html).

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Over the past three years, the ongoing Human Genome Project has allowed the discovery of 14 novel CYPs (Dr Nelson's website), including a new member of the CYP3A subfamily, CYP3A43 (MIM# 606534) (Domanski et al., 2001; Westlind et al., 2001). The CYP3A subfamily is of major importance since CYP3A enzymes are the most abundantly expressed cytochromes P450 in human liver and small intestine (Gonzalez, 1993; Thummel and Wilkinson, 1998). In addition, these enzymes exhibit a very broad substrate specificity; they metabolize more than 50% of all currently used drugs (Evans and Relling, 1999), numerous xenobiotics and some endogenous steroids such as testosterone, progesterone and cortisol (Danielson, 2002).

The human *CYP3A* genes, namely *CYP3A4*, *CYP3A5*, *CYP3A7* (Finta and Zaphiropoulos, 2000; Nelson et al., 1997) and the recently cloned *CYP3A43* (Domanski et al., 2001; Westlind et al., 2001), are organized in a cluster on chromosome 7 which spans about 200 kb, along with two pseudogenes, *CYP3AP1* and *CYP3AP2* (Finta and Zaphiropoulos, 2000). All members of the CYP3A subfamily share more than 80% homology in their cDNA sequence (Gellner et al., 2001). The human *CYP3A43* gene contains 13 exons (1509 nucleotides) encompassing 38 kb and encodes approximately a 57 kDa protein of 503 amino acids (Gellner et al., 2001).

CYP3A43 is expressed in several tissues that exhibit extensive hormone metabolism, predominantly in the prostate and in a less significative way in the testis, kidney and pancreas (Domanski et al., 2001). Compared to other CYP3A subfamily members, CYP3A43 is expressed at lower levels in the liver. Hepatic expression of CYP3A43 mRNA has been reported to be approximately 0.1-0.2% of CYP3A4 transcripts (Koch et al., 2002; Westlind et al., 2001), although in some livers it may be as high as 5% (Gellner et al., 2001). As with CYP3A4, CYP3A43 is induced by rifampicine in the liver (Gellner et al., 2001) and by dexamethasone in HepG2 cells (Krusekopf et al., 2003). A high frequency of alternatively spliced forms of *CYP3A43* (Domanski et al., 2001; Gellner et al., 2001) and intergenic *CYP3A43/CYP3A4* mRNA molecules resulting from *trans*-splicing (Finta and Zaphiropoulos, 2002) have been described. To date, little data on CYP3A43 function are available. However, CYP3A43 has been reported to exhibit *in vitro* activity toward testosterone (Domanski et al., 2001).

A wide interindividual variation in the content and catalytic activity of CYP3A has been reported in the general population (Daly et al., 1993). Although the basis of this variation is not yet well understood, combined effects of environmental and therapeutic chemicals, as well as genetic factors, are often suggested. Because of their importance in human drug metabolism, the CYP3As have been the subject of recent extensive genetic investigations. To date, numerous allelic variants of *CYP3A4*, *CYP3A5* and *CYP3A7* have been identified (http://www.imm.ki.se/CYPalleles/). However, only one mutation, harbored by the *CYP3A4*17* allele, has been correlated with an altered CYP3A4 catalytic activity (Dai et al., 2001). Even if the functional consequences of all these different alleles in the human CYP3A subfamily are far from completion, impact of some of these genetic polymorphisms in interindividual variation in human CYP3A expression has begun to be evaluated (King et al, 2003; Kuehl et al., 2001; Saeki et al., 2003; Yeh et al., 2003). The aim of the present study was to analyze the extent of *CYP3A43* genetic polymorphisms in a French population. Based on a Polymerase Chain Reaction – Single Strand Conformational Polymorphism (PCR-SSCP) strategy, we screened genomic DNA samples from 48 individuals for mutations in the 13 exons and the 5'-flanking region of the *CYP3A43* gene.

MATERIAL AND METHODS

Subjects

Genomic DNA from 48 unrelated healthy volunteers of French origin was collected after ethical committee approval and informed consents had been obtained. Nucleic acid extraction from peripheral blood leucocytes was performed using a Nucleon BACC3 kit (Amersham Pharmacia Biotech, Saclay, France), according to the manufacturer's instructions. Additionally, genotyping was conducted with DNA from a total of 352 patients of various pathologies for the A74 deletion polymorphism.

DNA amplification and primers

Fifteen separate PCRs were performed on each DNA sample to individually amplify the 13 exons and parts of the 5'-flanking region of *CYP3A43*. Specific primers (Table 1) were designed based on the nucleotide sequence of the *CYP3A43* gene published in GenBank (Accession Number NG_000004.2). PCRs were carried out in a final volume of 25 µL of 10 mM Tris-HCl buffer (pH 8.5), containing 200 ng of DNA, 0.4 µM of each primer and 0.5 U of AmpliTaq polymerase (Applied Biosystem, Courtaboeuf, France). The $MgCl_2$ concentration and annealing

temperature were optimized for each primer pair (Table 1). Amplicons were generated for 35 cycles of 1 min at 94°C, 1 min at an optimized annealing temperature and 45 s at 72°C, followed by a 7 min final extension at 72°C. Size and specificity of PCR fragments were evaluated on a 1.2% agarose gel in the presence of ethidium bromide.

Table 1: Details of the Primers Used for PCR-SSCP Analysis of the *CYP3A43* Gene

Primer	Primer sequence (5'®3')	Location[a]	Tm (°C)[b]	Size (bp)[c]	Amplified region[d]
Pro2F	TTATTCAGGTAGGTAAAGTAAGA	-525 –499	55	290	5'-UTR
Pro2R	TGGCAAATAATTATGGCTGTCC	-235 –256			
Pro1F	CAGAATAGGAAATCAAAGGAGG	-288 –266	58	274	5'-UTR
Pro1R	TTCAGCTCTGTCTTCTGAGTT	-35 –15			
P1F	CAGCAAAGAGCAGCACACAG	-65 –46	60	196	Exon 1
P1R	GGGCCCAATTAGCACTTCAG	Intron I			
P2F	GTCTGGCCAACCCCTTATC	Intron I	60	192	Exon 2
P2R	AGAAGCAAAAGAGGGAGCTC	Intron II			
P3F	TGCCCTGGTTAAATGTAGCG	Intron II	60	162	Exon 3
P3R	GCAACAGCTCTATCCAATGC	Intron III			
P4F	GCGAGGTTTAGTCAGCTCTG	Intron III	60	255	Exon 4
P4R	TTGGGGGAAATAGGACGAAG	Intron IV			
P5F	GTAGGACAAACTGCTTCTGC	Intron IV	60	255	Exon 5
P5R	ACCTGTCTCCAGATTCATCC	Intron V			
P6F	GCGTAGTTAACTATGGGTGG	Intron V	60	140	Exon 6
P6R	TCAGAACCTCAAGACTGTGC	Intron VI			
P7F	GGTGCTGGTTTTAATTTTC	Intron VI	52	210	Exon 7
P7R	GAAATAACATAAAGTCCAC	Intron VII			
P8F	GATCCTGGTTGAGAACCCTG	Intron VII	60	269	Exon 8
P8R	TCTCATGTCACCACCACCAG	Intron VIII			
P9F	GGACTCCTGATTCACTTCTG	Intron VIII	60	157	Exon 9
P9R	CCCCAGATGCAGTTCTTGG	Intron IX			
P10F	GATTTGGGAGCTTCACTGG	Intron IX	60	261	Exon 10
P10R	CTTCTCCCTCCTTCTCCAG	Intron X			
P11F	TCTGGAGCTCCTAACACTTC	Intron X	60	353	Exon 11
P11R	TGAGGGAAGGCTCCTTTCC	Intron XI			
P12F	AGTTTTTATGTACTACTGTG	Intron XI	52	262	Exon 12
P12R	TCTCAGTTTAAAAAACTCCC	Intron XII			
P13F	GCATTACCCTTGATGTCATC	Intron XII	60	210	Exon 13
P13R	CAAAGTGGAAGTCCTTAGGG	3'-flanking			

[a] Location of primers referred to the ATG translation start site (GenBank accesssion number AF319634).
[b] Optimized annealing temperature for each set of primers.
[c] Size of amplified fragments.
[d] Amplified region of *CYP3A43.*

Silver-stained SSCP

Non-isotopic SSCP analyses were performed on a GenePhor Electrophoresis unit (Amersham Pharmacia Biotech), according to the manufacturer's instructions. Three µL of PCR products were mixed with 3 µL of denaturing dye which consists of formamide containing 0.05% (w/v) of bromophenol blue and 0.05% (w/v) of xylene cyanol. This mixture was heat-denatured for 4 min at 95°C and after rapid cooling on ice, directly loaded into the wells of a precasted acrylamide gel (GeneGel Excel 12,5/24 gel, Amersham Pharmacia Biotech). After 1 h

45 min of electrophoresis at 25 mA, 600 V, 15 W and at 15°C, the gels were stained using the DNA silver staining kit (Amersham Pharmacia Biotech).

For samples displaying a change in electrophoretic mobility when compared to a reference with a wild-type sequence, the PCR-SSCP procedure was repeated to eliminate any detection of mutations due to errors of amplification generated by the DNA polymerase.

Nucleotide sequence analysis

PCR products showing altered SSCP patterns were re-amplified from genomic DNA with the primers used for the PCR-SSCP analysis, and purified with the QIAquick PCR Purification kit (Qiagen, Courtaboeuf, France). Samples were then used directly for DNA sequencing of both strands with the ABIPrism Dye Terminator Cycle Sequencing Reaction FS kit (Applied Biosystems, Courtaboeuf, France), using an automated DNA sequencer (Model 373A, Applied Biosystems).

RESULTS

Sequence analysis of *CYP3A43*

We analyzed the human *CYP3A43* gene by developing a simple and rapid method based on SSCP analysis of PCR fragments. We screened the entire coding region (the 13 exons with their consensus splice sites) and two fragments of the 5'-flanking region of the gene in 48 unrelated individuals. PCR primers (Table 1) were checked initially for specificity by sequencing all PCR products. There was no evidence to indicate non-specific cross-amplification with other *CYP3A* members. For SSCP analysis, one DNA sample homozygote for a wild-type allele of *CYP3A43*, as confirmed by sequencing, was used as a reference sample.

Thirty-four DNA samples showed a similar SSCP profile to the reference wild-type (Wt) sample. Analysis of the remaining 14 individuals showed at least one alternative profile in exons 2 (Fig. 1a), 10 (Fig. 1b), or 11 (Fig. 1c), suggesting the presence of mutations. Sequencing of the corresponding regions allowed the characterization of three polymorphisms in a heterozygous state. No polymorphisms were detected in the other exons or in the 5'-flanking region.

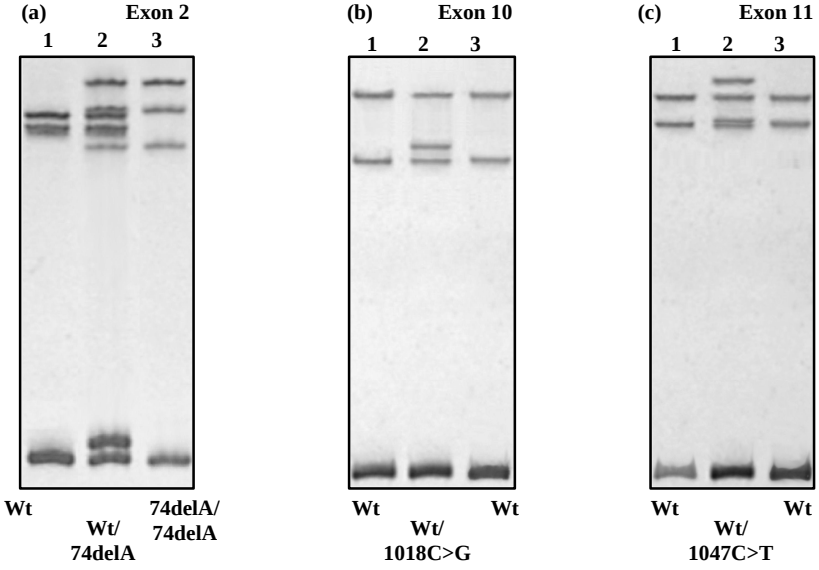

Figure 1: PCR-SSCP analysis of the human *CYP3A43* gene. The 13 exons and two fragments encompassing the 5'-flanking region were separately amplified from 48 unrelated French individuals. The single-stranded products were separated in non-denaturing gels. One sample with a wild type sequence (lanes 1, wt) was used as a reference for SSCP analysis. Abnormal SSCP patterns were observed with exons 2 (a), 10 (b) and 11(c). Results from sequencing of the corresponding fragments are reported at the bottom of the gels.

One variant, which contains a silent substitution (c.1047C>T) in exon 11, was found in ten out of 48 subjects (Fig. 1c). For exon 2, an abnormal SSCP pattern was observed in five individuals (Fig. 1a, lane 2), and corresponds to a nucleotide deletion (c.74delA), leading to a frame shift and creating a premature stop codon located 64 codons downstream. The last variant, identified in four individuals, carries a missense mutation (c.1018C>G) in exon 10 (Fig. 1b), leading to the substitution of a proline with an alanine at position 340 (P340A).

Overall, of the 48 French volunteers analyzed, ten DNA samples harbored one mutation (c.74delA or c.1047C>T), three samples carried two mutations (c.74delA and c.1018C>G), and one individual carried all three identified mutations. The distribution of the *CYP3A43* mutations identified in our population is shown in Table 2.

Table 2: Distribution of the *CYP3A43* Mutations Identified in 48 French Individuals

Nucleotide changes	Effect	n[a]	%
c.1047C>T	none	10	10.4
c.74delA	frameshift	5	5.2
c.1018C>G	P340A	4	4.2

[a] Number of mutated alleles out of 96 alleles.

In order to further investigate the frequency of the c.74delA deletion in a larger population, 352 individuals were genotyped by PCR-SSCP. The deletion was detected in 35 samples either in a heterozygous (34/352) or homozygous state (1/352) (Fig. 1a, lane 3), giving rise to a frequency (5.1%) similar to that determined previously. In addition, the complete sequencing of the homozygous sample revealed the presence of the c.1018C>G substitution on one allele.

Characterization of *CYP3A43* alleles

Analysis of the mutation distribution in the two population samples allowed the characterization of five different *CYP3A43** alleles. They were designated according to the Human Cytochrome P450 Allele Nomenclature (http://www.imm.ki.se/CYPalleles/). The wild-type allele, *CYP3A43*1A*, is the most frequent. The allele carrying the silent c.1047C>T mutation was considered as a functional allele and was consequently named *CYP3A43*1B*. The *CYP3A43*2A* and *CYP3A43*3* alleles harbor the c.74delA mutation and the missense c.1018C>G mutation, respectively. Finally, the *CYP3A43*2B* allele harbors a combination of two mutations, c.74delA and c.1018C>G. All these novel alleles have been submitted to GenBank with accession numbers AY390423, AY390424, AY390425 and AY390426.

DISCUSSION

Numerous human cytochromes P450 are highly polymorphic, causing interindividual variability in the metabolic reactions that they catalyze. Since CYP3A members are involved in the biotransformation of numerous commonly prescribed drugs (Ingelman-Sundberg et al., 1999), the discovery of functional SNPs in their genes could allow the individualization of drug dosage and improve therapeutics efficiency. Even if evidence is beginning to emerge of the existence of functional genetic polymorphisms in human CYP3As that may partially contribute to interindividual variations in drug biotransformation, previous studies on this subfamily have mainly focused on CYP3A4, the predominant isoform expressed in human liver. However, it now seems clear that all CYP3A members may be relevant to drug disposition, although the function of CYP3A43 needs further investigation.

To date, no genetic polymorphism affecting the *CYP3A43* gene has been reported. In order to carry out a screening for sequence variations in the coding region of this gene, a PCR-SSCP strategy was developed. This method has been successfully used for the detection of mutations in several other cytochrome P450 genes (Broly et

al., 1995; Chevalier et al., 2001a, 2001b, 2001c, 2001d; Lo-Guidice et al., 2002; Marez et al., 1997). Following optimization of several parameters that affect single-strand separation, such as temperature for migration or acrylamide concentration, we observed clear differences in band patterns when a CYP3A43-derived amplicon contained a mutation.

Out of the 48 unrelated individuals we studied, 34 (71%) appeared to exhibit a homozygous wild-type genotype (*CYP3A43*1A/*1A*). Four variant alleles, confirmed by sequencing, were identified from the remaining fourteen individuals. One allele contained the synonymous substitution c.1047C>T and was designated *CYP3A43*1B*. Nine out of 44 subjects were heterozygous for this allele, giving rise to an allele frequency of 10% in the tested population. The *CYP3A43*3* allele is characterized by a c.1018C>G substitution in exon 10, resulting in an amino acid exchange P340A. Functional significance of the P340A substitution remains to be determined and could be evaluated using a heterologous expression system, as previously described for other P450s (Crespi et al., 1999; Masimirembwa et al., 1999). The third allele, *CYP3A43*2A*, harbors a single nucleotide deletion (c.74delA). The frame shift caused by this deletion, located in the beginning of exon 2, creates an early TGA stop codon at nucleotides 265-267. This mutation is likely to produce an extremely truncated protein of only 88 amino acids. These data suggest that at least one null allele of *CYP3A43* exists, as described previously for other P450s such as *CYP2A6* (Fernandez-Salguero et al., 1995), *CYP2D6* (Marez et al., 1997) and *CYP2C19* (Meyer et al., 1997). Further genotyping in a larger group of 352 individuals allowed the detection of the c.74delA mutation in a homozygous state in one individual (1/352), suggesting that a deficiency in CYP3A43 activity does not appear to be lethal. In this sample, a combination of two mutations, c.74delA and c.1017C>G was identified on one of the alleles (*CYP3A43*2B)*. Therefore, an accurate determination of alleles *CYP3A43*2A*, **2B* and **3* frequency cannot be achieved, since for individuals heterozygous for both c.74delA and c.1018C>G mutations, it was not possible to determine their genotype: *CYP3A43*1A/*2B* or *CYP3A43*2A/*3*. Contemplating these data, it appears that even if the *CYP3A43* gene is not affected by a large number of polymorphisms, the identified mutations are not rare. However, combinations of these different mutations affecting the *CYP3A43* sequence might exist in the French population or in other ethnic groups. In addition, despite many precautions, some additional mutations might have been undetected under the chosen SSCP conditions.

Data related to the function of CYP3A43 is sparse. No successful *in vitro* expression of CYP3A43 has been achieved in yeast cells, or mouse hepatoma H2.35, human embryonic kidney HEK 293 or African green monkey kidney COS-1 cell lines (Westlind et al., 2001). However, transient expression of CYP3A43 in an *Escherichia coli* system has been reported with low specific activity toward testosterone hydroxylation (Domanski et al., 2001). In the same system, a reduced CO difference peak at 450 nm was highlighted, suggesting that CYP3A43 might be functional. Meanwhile, more suitable expression conditions or more specific substrates remain to be investigated, as suggested by others (Westlind et al., 2001). Indeed, even if the CYP3A isoforms share high sequence homology, they differ in substrate specificity and expression level (Koch et al., 2002). Consequently, the lack of activity observed with conventional CYP3A-substrates might result from the fact that CYP3A43 differs from CYP3A4 by 29 amino acid residues located in the putative substrate-recognition sites, SRS-1 to SRS-6 (Wang et al., 1998). For example, SRS-4 has been shown to influence both the rate and stereoselectivity of endogenous steroid hydroxylation (Domanski et al., 1998).

In conclusion, we report here the first investigation of sequence variations in the human *CYP3A43* gene. In addition to the previously described wild-type allele, four allelic variants have been identified in a French population, two of which are characterized by a nucleotide deletion, leading to a premature stop codon and likely to the synthesis of an inactive enzyme. The identification of such a mutation suggests that a polymorphism affecting CYP3A43 activity exists. Furthermore, as the highest expression of CYP3A43 has been observed in prostate and other organs with extensive hormone metabolism, the consequences of its polymorphic expression on the metabolism of endogenous compounds in these particular tissues should be considered.

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