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Human CYP4F12 genetic polymorphism: identification and functional characterization of seven variant allozymes

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Abstract

The human cytochrome CYP4F12 has been shown to be metabolically active toward inflammatory mediators and exogenous compounds such as antihistaminic drugs. We recently identified a genetic polymorphism within the promoter region, associated with a decreased level of enzyme expression. In the present study, we report the further identification of single nucleotide polymorphisms in the coding sequence of the *CYP4F12* gene. A polymerase chain reaction–single strand conformational polymorphism (PCR–SSCP) analysis of DNA samples from 53 unrelated French Caucasians, allowed the identification of ten mutations, comprising seven missense mutations, 31C > T (Leu^{11}Phe), 38C > T (Pro^{13}Leu), 47C > T (Met^{16}Thr), 4759G > A (Asp^{76}Asn), 4801G > A (Val^{90}Leu), 8896C > T (Arg^{188}Cys) and 23545G > A (Gly^{522}Ser). Their functional impact toward ebastine hydroxylation was evaluated using heterologous expression in *Saccharomyces cerevisiae* cells of site-directed mutated cDNA variants. Five out seven variants did not exhibit any significant difference in CYP4F12 catalytic activity, whereas two variants, Val^{90}Ile and Arg^{188}Cys, displayed significant changes in their Michaelis–Menten (K_m, V_m) parameters. These data on CYP4F12 genetic polymorphism provide tools for further studies of association with pathological processes involving an inflammatory component and with variations in anti-histaminic drug response.

Keywords: CYP4F12; Genetic polymorphism; PCR–SSCP; Heterologous expression

1. Introduction

Cytochromes P450 (CYP) constitute a superfamily of haem-thiolate proteins which play a pivotal role in the metabolism of endogenous compounds and xenobiotics including drugs and environmental contaminants [1]. 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 [2].

The *CYP4F* subfamily was originally discovered in rat hepatic tumours by Chen and Hardwick [3]. In human, at least five *CYP4F* genes that encode functional enzymes have been described, i.e. the *CYP4F2* [4], the *CYP4F3* [5], the *CYP4F8* [6], the *CYP4F11* [7] and the *CYP4F12* [8–9]. In addition, a *CYP4F22* gene, which seems to encode a functional P450 protein, and eight *CYP4F* pseudogenes have been identified in genomic DNA databases (Dr. Nelson homepage: http://drnelson.utmen.edu/CytochromeP450.html).

Since human CYP4F enzymes play a role in the metabolism of endogenous compounds such as inflammatory mediators, they are likely involved in the regulation of inflammation processes and could exhibit a potent physiological function [10]. CYP4F12 has been shown to be highly catalytically active toward arachidonic acid and prostaglandin H2 (PGH2) analogs, namely U51605 and U46619. It also metabolises to a lesser extent PGE2, PGF2α and leukotriene B4 [8]. In contrast to the other CYP4Fs proteins, CYP4F12 has been shown to be highly efficient toward two antihistaminic drugs, ebastine (4′terbutyl-4-[4(diphenylmethoxy)piperidino]butyrophenone) [9] and terfenadine (www.gentest.com) [11].

This relatively narrow substrate specificity contrasts its wide tissue distribution [12]. Indeed, CYP4F12 was found

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to be highly expressed in liver, but was also detected at lower levels in several tissues such as kidney, colon and heart. Interestingly, it is the only CYP4F member expressed in the small intestine [8].

At the molecular level, the *CYP4F12* gene is located on chromosome 19p13, in a gene cluster spanning 320 kb, along with all other *CYP4F* genes. The *CYP4F12* gene, which encompasses about 25 kb, contains 13 exons (1694 nucleotides), with its open reading frame being encoded from exons 2 to 13. The gene product is an approximately 60 kDa protein of 524 amino acids [9], showing between 78 and 83% similarity with other human CYP4F enzymes amino acid sequences [8].

Variations in the coding and/or regulatory regions of the human *CYP4F12* gene could alter CYP4F12 expression and/or enzymatic activity and result in impaired metabolism of inflammatory mediators, as well as some drugs. We recently identified different polymorphisms in the promoter region of the *CYP4F12* gene, consisting in a large deletion located in intron 1 (*CYP4F12*v1*), two isolated substitutions −402G > A (*CYP4F12*v3*) and −188 T > C (*CYP4F12*v4*) and nine combined mutations, [−474T > C, −279A > C, −224A > G, −173G > A, −145C > G, −140T > C, −126T > C, −56T > C and −21T > G] (*CYP4F12*v2*) [GenBank Accession numbers AY544782–AY544785]. Using a gene reporter assay, we demonstrated that the *CYP4F12*v1* and **v2* alleles led to a significant decrease of *CYP4F12* gene expression in HepG2 cell line [13]. To date, even if no systematic analysis for variations in the *CYP4F12* coding sequence has been undergone, few polymorphisms have been reported at the cDNA level. Two cDNAs were isolated [8,9]. Compared to the wild-type cDNA [8], one cDNA harbours four substitutions located in exon 3 ($Val^{76}Asn$ and $Val^{90}Ile$), exon 4 ($Arg^{188}Cys$) and exon 13 ($Gly^{522}Ser$) [9].

In this study, we examined the coding sequence of the *CYP4F12* gene in genomic DNA from 53 healthy volunteers using a polymerase chain reaction–single strand conformational polymorphism (PCR–SSCP) strategy. Ten polymorphisms were identified and the functional analysis of seven variant cDNAs, generated by site-directed mutagenesis, was performed using *Saccharomyces cerevisiae* cells and achieved by kinetic analysis of ebastine hydroxylation.

2. Materials and methods

2.1. Subjects

Fifty-three unrelated French subjects of Caucasian origin have been involved in the study after ethical committee

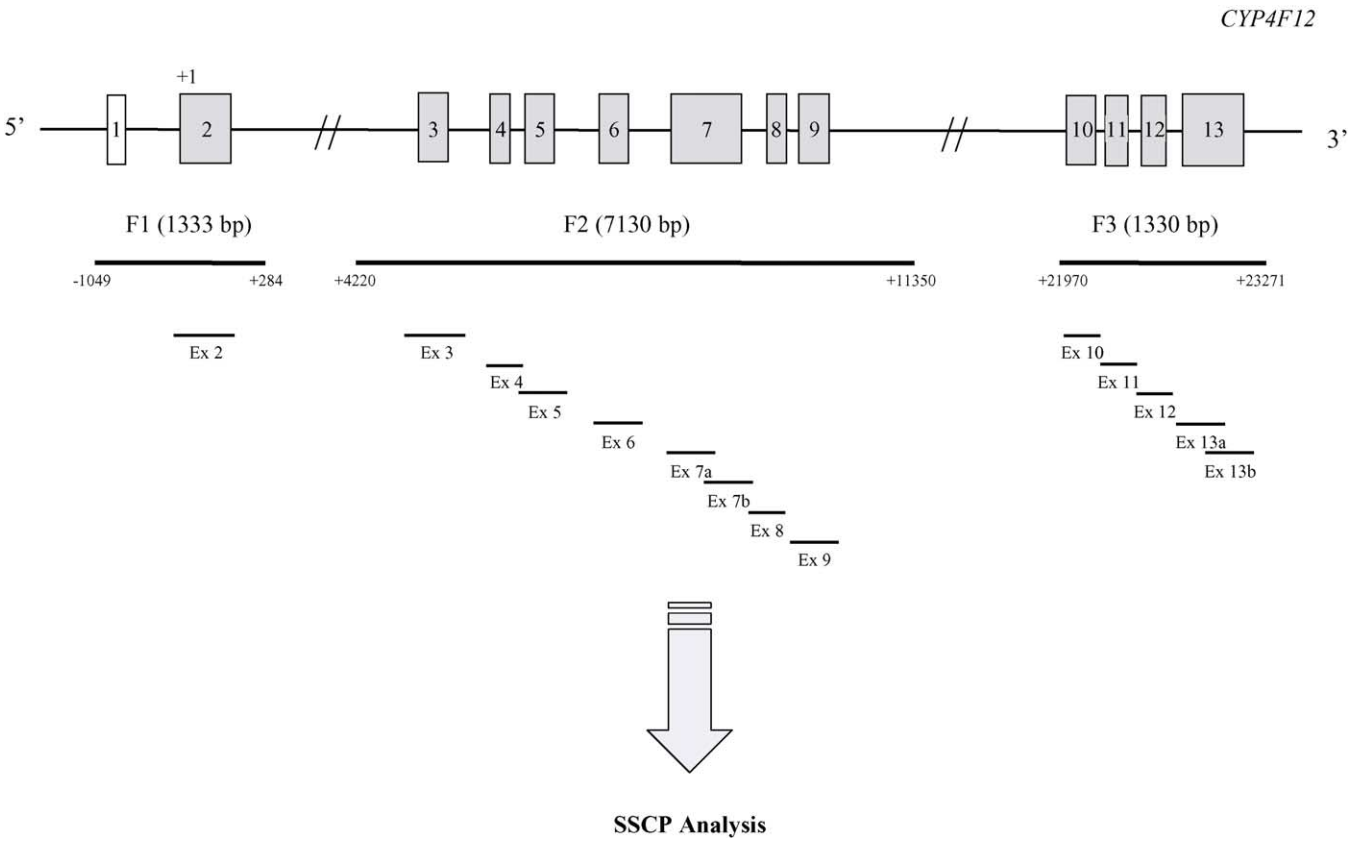

Fig. 1. Outline of the strategy for PCR–SSCP analysis of the *CYP4F12* gene in genomic DNA. In a first step, amplification of three specific fragments (F1, F2, F3) of the *CYP4F12* gene was performed. In a second step, F1, F2 and F3 were used as templates for 14 parallel PCRs using nested primers. *CYP4F12* exons are illustrated with solid boxes. A of ATG is numbered as +1.

approval and informed consent had been obtained. Genomic DNA was isolated from peripheral blood leucocytes using the Nucleon BACC3 kit (Amersham Pharmacia Biotech), according to manufacturer's instructions.

2.2. PCR–SSCP analysis

Fig. 1 depicts the strategy we developed for PCR–SSCP analysis of *CYP4F12* from genomic DNA samples. In a first step, for specific amplification, three PCRs were performed to generate three large fragments, F1 (1338 bp), F2 (7130 bp) and F3 (1330 bp), encompassing the 13 exons of the gene. In a second step, F1, F2 and F3 were used as templates in fourteen separate amplification reactions, each using a set of nested primers listed in Table 1. Primers were designed to allow detection of mutations located in the sequence of each coding exon and in their 3′- and 5′-splice site consensus sequences.

The largest fragment, F2, was generated by PCR of 200 ng genomic DNA using 2.5 U of TaKaRa Ex Taq polymerase and following the two-step cycling procedure, as described in the manufacturer's instructions (Bio Whittaker). For amplification of F1 and F3 fragments, and the nested PCRs, 200 ng genomic DNA or 0.6 μL of the large fragment were amplified in 25 μL of 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 0.01% gelatine, 0.1 mM of each dNTP, 0.25 μM of each primer and 0.2 U Taq polymerase (Invitrogen). The $MgCl_2$ concentration was optimised for each primer pair (Table 1). In addition, for nested PCR, 0.7 μCi of [α-^{32}P]dCTP (3000 Ci mmol^{-1}, Amersham

Table 1
Details of the primers used for the PCR–SSCP analysis of the *CYP4F12* gene

Amplified region	Primer	Sequence (5′ → 3′)	Size (bp)[a]	T_m (°C)[b]	$MgCl_2$ (mM)[c]
F1	Long1F	GCTGGCTCTTTATCCCCAGAATCTGACGA	1338	68	
	Long1R	CAATACCTAGCTCACTGCGCCCCATTCCTG			
F2	Long2F	TTCTCAAACACCCTCACAGACACACACAGC	7130	68	
	Long2R	CCTCCTCACTTGGGGAGCAGAACCAATGAT			
F3	Long3F	AAAGGTCTCATTTGCAAATAGAAAAGGTGG	1330	68	
	Long3R	AAGAGCCCCCTGCAGGCTGTCAGTGAGG			
Exon 2	Ex 2F	TCACCCTTGCATCCCCTCT	251	60	1
	Ex 2R	CAGACACATTTTGCTGTCACA			
Exon 3	Ex 3F	GTGACAGCCCTTGACTTGC	193	60	1.5
	Ex 3R	CCACCACAAGCTCTGCATG			
Exon 4	Ex 4F	CCCTGATGGTCCTCGTTCA	165	60	1.5
	Ex 4R	GGCAGCCAAGGACAGTGG			
Exon 5	Ex 5F	CCACTGTCCTTGGCTGCC	184	60	1.5
	Ex 5R	TCCATCTGGGACCCAGACT			
Exon 6	Ex 6F	TAGCTCTCTTCCCTTCTCTG	171	60	1.5
	Ex 6R	CCCAGGCCCTAGGAAGGA			
Exon 7a	Ex 7Af	TCCCTTTCTGCCCTTGCTC	236	60	1.5
	Ex 7aR	AAAAATCATCAATACCCTGAGT			
Exon 7b	Ex 7bF	GTATTACCTCTCCCATGACG	209	60	1.5
	Ex 7bR	ACCTAAAATCAGATCATAGTGAA			
Exon 8	Ex 8F	GGAGTCTCAGAGATAGTATAAT	154	55	1.5
	Ex 8R	TAGTCCCACACTGGGACC			
Exon 9	Ex 9F	CAAGCTTACCTGGCTGCTC	177	60	1.5
	Ex 9R	CAGGCCAGAAGAACTTGCA			
Exon 10	Ex 10F	CTGGGGCTGGGGTGTTTC	179	60	2
	Ex 10R	TCTCCCCCTGAGGCTGTG			
Exon 11	Ex 11F	TGACTGCCCCTTTCTCTCC	112	60	2
	Ex 11R	GGTGGTGAATGGGGAAGGC			
Exon 12	Ex 12F	GCAAACCTTCTTTGTCTCACC	131	58	2
	Ex 12R	CCTCAGACACAGGGCGCT			
Exon 13a	Ex 13aF	CAGTCCCCACTCCCGCC	215	60	2
	Ex 13aR	TGGATGGGTCAGAAAGTCAC			
Exon 13b	Ex 13bF	ATGGCGGAGATGAAAGTGGT	250	60	2
	Long 3R	AAGAGCCCCCTGCAGGCTGTCAGTGAGG			

[a] Size of amplified fragments.
[b] Optimized annealing temperature for each set of primers.
[c] Optimized $MgCl_2$ concentration for the PCR.

Biosciences) were added to label the amplified fragments. Standard cycling conditions were used. Size and specificity of PCR fragments were controlled in ethidium bromide-stained agarose gels.

For SSCP electrophoreses, 3 μL of each amplicon were mixed with 3 μL of denaturing dye (10 mM NaOH, 20 mM EDTA, 95% formamide, 0.05% bromophenol blue and 0.05% xylene cyanol), heated for 2 min at 92 °C and rapidly chilled on ice. Three microliters were then loaded on a non-denaturing MDE (Cambrex Biosciences) polyacrylamide gel (400 mm × 300 mm × 0.4 mm) containing 10% glycerol.

For samples displaying a change in electrophoretic mobility compared to a reference sample with a homozygous wild-type genotype, the PCR–SSCP procedure was repeated to eliminate any detection of mutations due to errors of amplification generated by the DNA polymerase. Amplicons were sequenced on both strands, using an automated DNA sequencer (Model 373A, Applied Biosystems) and labelled with the ABIPrism Dye Terminator Cycle Sequencing Reaction FS kit (Applied Biosystems).

2.3. Site-directed mutagenesis

Mutated cDNAs of *CYP4F12* were generated by in vitro mutagenesis using the Quick Change Site-Directed Mutagenesis kit (Stratagene) and the pYeD60 (V60) shuttle vector containing the wild-type full-length *CYP4F12* cDNA (a kind gift from Pr.E.H. Oliw, Department of Pharmaceutical Biosciences, BMC, Uppsala, Sweden). The oligonucleotide primers used to introduce amino acid substitutions are listed in Table 2. Sequencing of mutated cDNA constructs was carried out on both strands in order to check their identity with the expected mutated sequence and to confirm that no additional mutation was introduced.

2.4. Expression of recombinant wild-type and variant CYP4F12 proteins

The recombinant wild-type and mutant constructs were used for the transformation of the *S. cerevisiae* W(R) strain (a generous gift from Dr. Pompon, Centre de Génétique Moléculaire, CNRS, OPR 9061, Gif-sur-Yvette, France) by the lithium acetate method [14]. Recombinant yeast cells were cultured as described previously [15]. Microsomal fractions were prepared by centrifugation after mechanically disruption of yeast cells, as described by Peyronneau et al. [16]. Control microsomes were also prepared from yeast cells transfected with the V60 plasmid without an insert.

Protein concentrations of microsomal fractions were determined by the method of Lowry et al. [17] with the BCA Protein Reagent kit (Pierce). Cytochrome P450 content was quantified in each microsomal fraction according to the method of Omura and Sato [18] using a UV500 spectrophotometer (Spectronic Unicam) and with 91 mM^{-1} cm^{-1} as the absorption coefficient.

2.5. Kinetic analysis of ebastine hydroxylation

Hydroxylation of ebastine was assessed in microsomal preparations obtained from the recombinant yeast cells at eight different ebastine concentrations ranging from 1.5 to 100 μM. Incubations were performed at 37 °C for 10 min in a final volume of 250 μL Tris–HCl buffer (50 mM, pH 7.4) with 1 mM $NADPH_2$ and were started with the addition of 50 pmol P450. Reactions were terminated by the addition of 1 mL methanol and 100 μL of a solution of internal standard (methylclonazepam at 2.5 mg L^{-1}) in methanol, used for the quantitation. After centrifugation at 10,000 rpm for 5 min, the pellet was resuspended in

Table 2
Primers used for site-directed mutagenesis

Mutations	Sequence (5′ → 3′)[a]	Mutated codons	Amino acid substitution
31C > T + 38C > T	F CCTGGCTGGGC**T**TCAGA**CTG**GTGGCAATGTC R GACATTGCCAC**CAG**TCTGA**AG**CCCAGCCAGG	CCG → CTG CTG → TTC	$Leu^{11}Phe + Pro^{13}Leu$
38C > T	F CCTGGCTGGGCCTCAGA**CTG**GTGGCAATGTC R GACATTGCCAC**CAG**TCTGAGGCCCAGCCAGG	CCG → CTG	$Pro^{13}Leu$
47T > C	F GACCGGTCAA**CG**TCCCCATGGCTAC R GTAGCCATGGGGA**CGT**TGCCACCGGTC	ATG → ACG	$Met^{16}Thr$
4759G > A	F GAGGAGGGCTTGAAG**A**ACTCGACCCAGATG R CATCTGGGTCGAGT**T**CTTCAAGCCCTCCTC	GAC → AAC	$Asp^{76}Asn$
4801G > A	F CCAGGGCTTTACG**A**TATGGCTGGGTCCC R GGGACCCAGCCATA**T**CGTAAAGCCCTGGG	GTA → ATA	$Val^{90}Ile$
8896C > T	F CAGAGGGCAGCAGT**T**GTCTGGACATGTTTGAGC R GCTCAAACATGTCCAG**ACA**ACTGCTGCCCTCTG	CGT → TGT	$Arg^{188}Cys$
23545G > A	F GAGCCCCTGAATGTA**AGC**TTGCAGTGAGAATTC R GAATTCTCACTGCAA**GCT**TACATTCAGGGGCTC	GGC → AGC	$Gly^{522}Ser$

[a] F, forward primer; R, reverse primer. Underlined nucleotides correspond to the mutated codon. Bold nucleotides correspond to the introduced mutations by site-directed mutagenesis.

3 mL ether and submitted to evaporation under gentle nitrogen stream at room temperature. One hundred microliters of the mobile phase was finally added to the dry residue.

Ebastine (Pharmafarm) and its metabolite, 4-hydroxyebastine, were quantified using a HPLC method, consisting of a Waters 717 Plus Autosampler injector, a 600E multisolvent pump, equipped with a C85 μm column. Elution was performed at a flow rate of 1 mL/min with a mobile phase (65% acetonitrile/35% 12 mM ammonium acetate) and monitored at 254 nm using a Waters 2487 Dual λ absorbance detector (Waters). The retention times for methylclonazepam, ebastine and hydroxyebastine were 5.4, 7.8 and 4.1 min, respectively.

Apparent Michaelis–Menten constants K_m and V_{max} were calculated by use of GRAFIT (version 3.0, Erithacus Software). The intrinsic clearance (Clint) was evaluated as the ratio of V_m to K_m.

2.6. Statistical analysis

Kinetic parameters were treated statistically with ANOVA and the Tukey post hoc test for multiple parameters. A value of $p < 0.05$ was considered as statistically significant.

3. Results

3.1. Identification of CYP4F12 mutations by PCR–SSCP

We analysed the entire coding sequence of the human *CYP4F12* gene by developing a simple and rapid method based on SSCP analysis of PCR fragments. Three large fragments (F1, F2 and F3) were first generated and used as templates for the amplification of fourteen nested fragments. Sequencing of all PCR products confirmed the specificity of PCR primers since there was no evidence of non-specific cross-amplification of other *CYP4F* genes.

For SSCP analysis, one DNA sample homozygote for a wild-type allele of *CYP4F12*, as confirmed by sequencing, was used as a reference sample. At least one alternative profile was found in exon 2 (Fig. 2a), exon 3 (Fig. 2b), exon 6 (Fig. 2d), exon 12 (Fig. 2e) or exon 13 (Fig. 2f) and in intron 4 (Fig. 2c), suggesting the presence of mutations. Sequencing of the corresponding regions allowed the characterization of ten polymorphisms in a heterozygous or in a homozygous state.

For exon 2 (Fig. 2a), five abnormal SSCP patterns were observed and correspond to different combinations of three nucleotide substitutions, 31C > T (Leu^{11}Leu), 38C > T (Pro^{13}Leu) and 47T > C (Met^{16}Thr). In the tested samples,

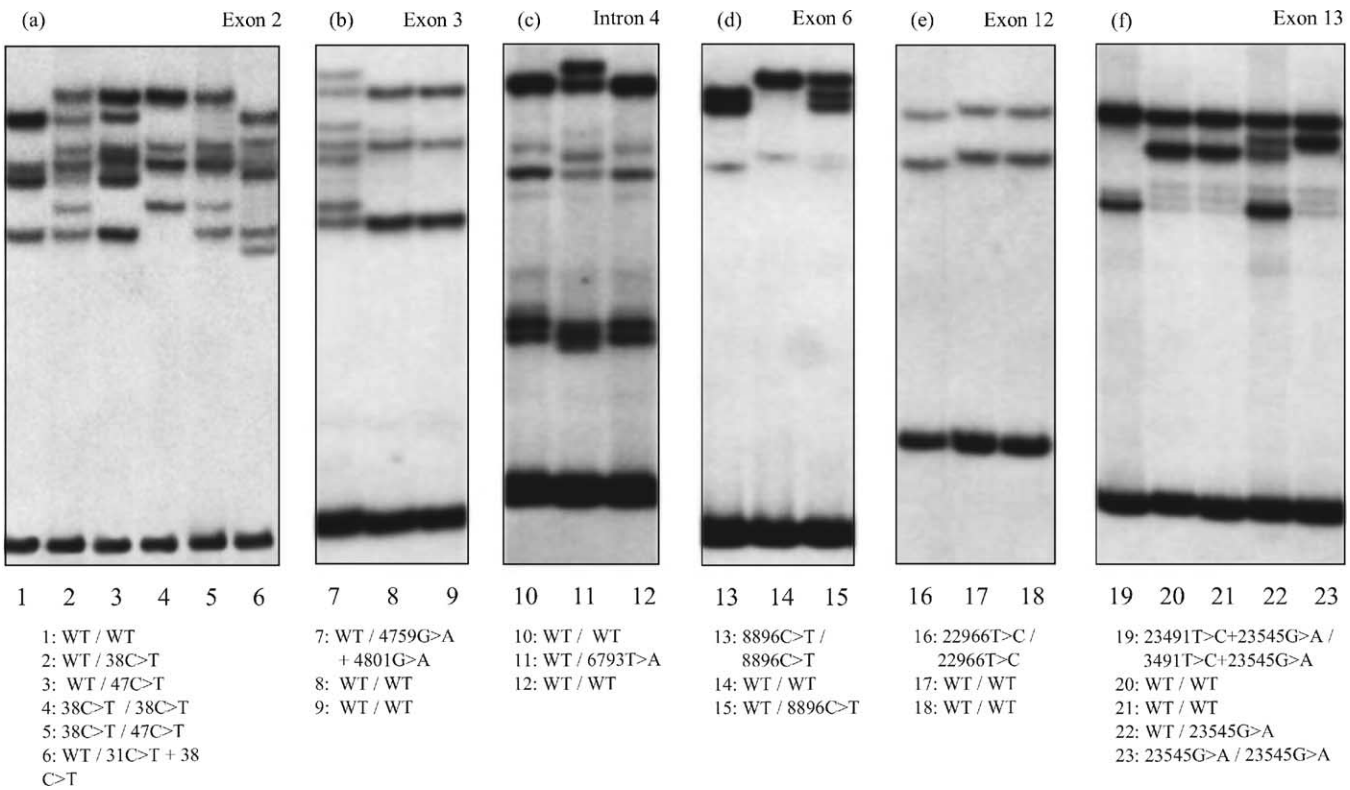

Fig. 2. PCR–SSCP analysis of the human *CYP4F12* gene. Fourteen nested fragments were separately amplified from 53 unrelated French individuals, using the specific fragments F1, F2 and F3 as templates. The single-stranded PCR products were separated by non-denaturing electrophoresis. One sample with a wild-type sequence was used as a reference sample for SSCP analysis. Abnormal SSCP patterns were observed with exons 2 (a) exons 3 (b) intron 4 (c) exons 6 (d) exons 12 (e) and in exons 13 (f). Results from sequencing of the corresponding fragments are reported at the bottom of the gels.

the 31C > T mutation appears to be systematically combined with the 38C > T mutation on the same allele. For exon 3 (Fig. 2b), only one SSCP pattern differed from that of the wild-type. Sequencing of the corresponding DNA sample allowed the identification of two heterozygous missense mutations, 4759G > A (Asp^{76}Asn) and 4801G > A (Val^{90}Ile), located on the same allele. Analysis of exon 4 allowed the detection of a 6793T > C mutation in intron 4 (Fig. 2c), located 24 nucleotides downstream the end of the exon. For exon 6, two alternative profiles were observed and correspond to a missense substitution, 8896C > T (Arg^{188}Cys), either in a heterozygous or in a homozygous state (Fig. 2d). For exon 12 (Fig. 2e), one abnormal profile was detected and corresponds to a 22966T > C silent substitution (Pro^{460}Pro) in a homozygous state. Finally, for exon 13 (Fig. 2f), three types of SSCP patterns differing from that of the wild-type one were observed. Two of them correspond to the presence of a 23545G > A (Gly^{522}Ser) substitution in a heterozygous or in a homozygous state. The last profile allowed the identification of a 23491T > C silent substitution (Leu^{503}Leu) and was correlated to a (23545G > A + 23491T > C)/(23545G > A + 23491T > C) genotype. Frequency of all these mutations identified in our sample population is listed in Table 3.

3.2. Heterologous expression of CYP4F12 in Saccharomyces cerevisiae

Seven variants, consisting in six variants carrying one missense mutation, 38C > T (Pro^{13}Leu), 47T > C (Met^{16}Thr), 4759G > A (Asp^{76}Asn), 4801G > A (Val^{90}Ile), 8896C > T (Arg^{188}Phe) or 23545G > A (Gly^{522}Ser), and one variant carrying both 31C > T (Leu^{11}Phe) and 38C > T (Pro^{13}Leu) substitutions, which were found in combination, were analysed. Wild-type and mutant *CYP4F12* cDNAs were expressed in *S. cerevisiae* cells using the pYeD60 expression vector. Typical CO-difference spectra with Soret maxima at 452 nm were observed for all CYP4F12 microsomal preparations, as illustrated for the wild-type in Fig. 3. The contents of recombinant CYP4F12 proteins were ranging from about 120 to 320 pmol P450/mg microsomal protein. No P450 was detected in control yeast cells (data not shown).

Table 3
Distribution of the *CYP4F12* mutations identified in 53 French individuals

Nucleotide changes	Effect	Location	Frequency (%)
31C > T	Leu^{11}Phe	Exon 2	4
38C > T	Pro^{13}Leu	Exon 2	25
47C > T	Met^{16}Thr	Exon 2	9
4759G > A	Asp^{76}Asn	Exon 3	8
4801G > A	Val^{90}Ile	Exon 3	8
6793T > A	–	Intron 4	17
8896C > T	Arg^{188}Cys	Exon 6	47
22966T > C	Pro^{460}Pro	Exon 12	10
23491T > C	Leu^{503}Leu	Exon 13	10
23545G > A	Gly^{522}Ser	Exon 13	41

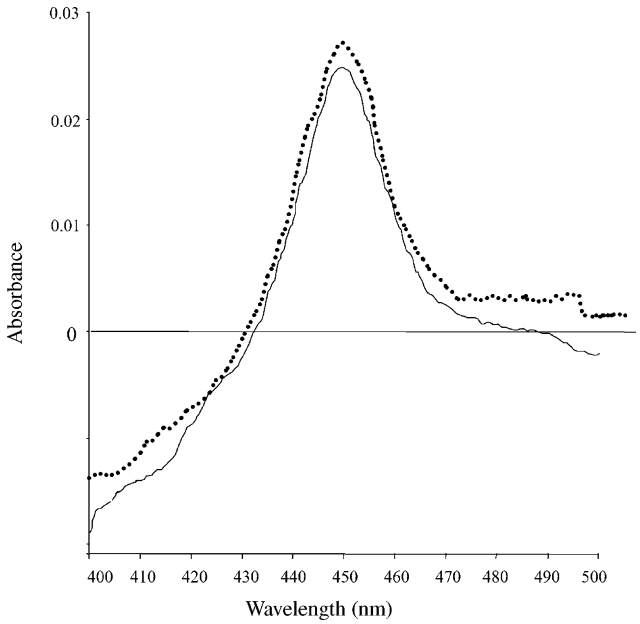

Fig. 3. Typical reduced-CO difference spectra of CYP4F12 expressed in yeast microsomes. No absorption around 450 nm was observed for the control microsomes from yeast cells transformed with the vector only. (—) wild-type CYP4F12, (···) Asp^{76}Asn CYP4F12 variant.

3.3. Catalytic properties of recombinant CYP4F12 proteins

To examine whether the amino acid substitutions were associated with alterations of the CYP4F12 catalytic activity, microsomes expressing recombinant wild-type and variant proteins were incubated at 37 °C for 10 min with ebastine in the presence of NADPH. Production of the metabolite, 4-hydroxyebastine, was analysed by HPLC. Under the chosen incubation and analysis conditions, no other metabolite could be detected in the reaction medium. Monophasic Michaelis–Menten kinetics were observed for the formation of hydroxyebastine. Results from three independent experiments by using two different enzyme preparations for each recombinant protein are shown in Table 4. The kinetic parameters (K_m, V_m), as well as the in vitro intrinsic clearance (V_m/K_m) of ebastine hydroxylation of each recombinant microsome are shown in Table 4. Kinetic analysis showed that the wild-type CYP4F12 metabolised ebastine with an apparent V_m of about 4.8 pmol/min/pmol P450 and an apparent K_m of 7.8 μM. As expected, control microsomes (vector transfected without an insert) did not convert ebastine into any metabolite. Kinetics parameters related to the Leu^{11}Phe + Pro^{13}Leu, Pro^{13}leu, Met^{16}Thr, Asp^{76}Asn and Gly^{522}Ser variants did not differ significantly from those of the wild-type protein. The Val^{90}Ile variant encoded a protein with a significant increase in K_m and V_m values (about 3-fold that of the wild-type enzyme). However, this did not reach statistical significance when comparing intrinsic clearances (V_m/K_m: 1.03 versus 0.62). Similarly, whereas the Arg^{188}Cys

Table 4
Kinetic parameters of ebastine hydroxylation by recombinant CYP4F12 variants expressed in yeast

CYP4F12 variant	K_m (μM)	V_m (pmol/min/pmol P450)	V_m/K_m (μL (solidus) min/pmol P450)
Wild-type	7.75 ± 0.36	4.80 ± 0.68	0.62 ± 0.11
Leu[11]Phe + Pro[13]Leu	4.90 ± 2.70	3.01 ± 0.80	0.61 ± 0.44
Pro[13]Leu	4.40 ± 0.64	3.30 ± 0.42	0.75 ± 0.11
Met[16]Thr	6.80 ± 1.90	3.50 ± 0.25	0.51 ± 0.20
Asp[76]Asn	9.60 ± 1.10	4.80 ± 0.42	0.50 ± 0.10
Val[90]Ile	15.70 ± 3.08[a]	16.20 ± 0.47[a]	1.03 ± 0.18
Arg[188]Cys	3.00 ± 1.57[a]	1.90 ± 0.85[a]	0.63 ± 0.30
Gly[522]Ser	5.30 ± 1.79	4.40 ± 0.59	0.83 ± 0.19

[a] $p < 0.05$.

had a significantly reduced enzymatic activity (less than 50% of the wild-type CYP4F12 activity), the catalytic efficiency appeared to be similar to that of the wild-type protein. All CYP4F12 variants seemed to metabolise ebastine at comparable rates to that of the wild-type CYP4F12 protein.

4. Discussion

Cytochromes P450 (CYP) catalyse the oxidation of numerous xenobiotics and endogenous compounds [19]. A large majority of CYP genes have turned out to be extremely polymorphic (http://www.imm.ki.se/CYPalleles/) and, consequently, to be involved in interindividual variability in response to drugs [20,21]. The CYP4F12 enzyme, whose gene has been recently cloned [8,9], metabolises antihistaminic drugs [9], as well as endogenous compounds such as arachidonic acid and PGH2 analogs [8]. The aim of this study was to explore the extent of the *CYP4F12* genetic polymorphism in a French population of Caucasian origin, using a PCR–SSCP strategy, a method that had been successfully used for the detection of mutations in several other cytochrome P450 genes [22–25]. In addition, as highly homologous *CYP4F* genes are present in the *CYP4F12* containing gene cluster, a preliminary amplification step, using *CYP4F12* specific primers, has been added to the PCR–SSCP strategy.

The screening of 53 DNA samples has allowed the identification of ten single nucleotide polymorphisms. Nine of them are located in the coding sequence of *CYP4F12* and comprise two silent substitutions, 22966C > T (Pro[460]Pro) and 23491T > C (Leu[503]Leu) and seven missense mutations, 31C > T (Leu[11]Phe), 38C > T (Pro[13]Leu) and 47C > T (Met[16]Thr) in exon 2, 4759G > A (Asp[76]Asn) and 4801G > A (Val[90]Leu) in exon 3, 8896C > T (Arg[188]Cys) in exon 6 and 23545G > A (Gly[522]Ser) in exon 13. Another polymorphism, a 6793T > A substitution, was detected in intron 4. Even if no previous systematic screening of the *CYP4F12* gene sequence had been undergone, some of the identified polymorphisms, namely the Pro[13]Leu, Asp[76]Asn, Val[90]Leu, Arg[188]Cys and Gly[522]Ser mutations, have already been described at the cDNA level by Hashizume et al. [9]. In our population, the 38C > T, 8896C > T and 23545G > A mutations are the most frequent polymorphisms with a mutation frequency of 25, 47 and 41%, respectively. In contrast, all other mutations represent less than 10% of the tested alleles (Table 3). Interestingly, no sample contained the 22966T > C and 23491T > C mutations in a heterozygous state, whereas 10% of the subjects appeared to be homozygous for both mutations. Further experiments are then needed in order to assess if this disequilibrium with the Hardy–Weinberg law could be the consequence of an abnormal locus, such as partial or complete deletion of the gene, as seen for other cytochrome P450 genes [26,27].

In a previous study, we identified polymorphisms in the promoter region of the *CYP4F12* gene, consisting in a large deletion located in intron 1 (*CYP4F12*v1*), two isolated substitutions −402G > A (*CYP4F12*v3*) and −188 T > C (*CYP4F12*v4*) and nine combined mutations, [−474T > C, −279A > C, −224A > G, −173G > A, −145C > G, −140T > C, −126T > C, −56T > C and −21T > G] (*CYP4F12*v2*) [13]. As the present study and the former one were performed on the same DNA samples, we were able to target some allelic combination between polymorphisms located in the promoter and the coding sequence. Nevertheless, at this stage of the work, complete haplotypes could not be fully characterized and, consequently, *CYP4F12* alleles could not be defined. Screening of the identified mutations in a larger population, family pedigree studies or mutation specific PCRs would allow the accurate determination of the *CYP4F12* alleles.

In order to evaluate their catalytic activities toward ebastine, wild-type and mutant CYP4F12 proteins were heterologously expressed in a yeast strain that had been optimised for functional expression of different CYPs [15]. This particular expression system has been successfully used to functionally characterize various naturally-occurring mutations of CYPs [16,28–30]. In this system, a CYP4F12 wild-type enzyme with CYP characteristic spectral properties was produced and was found to metabolise ebastine with an apparent K_m of 7.8 μM and a V_m of 4.8 pmol hydroxyebastine/min/nmol P450. A recombinant wild-type CYP4F12 enzyme, expressed in *S. cerevisiae*, has been reported [9], as capable of converting ebastine to hydroxyebastine at a rate of 0.38 pmol hydroxyebastine/

min/nmol P450, which is approximately 10 times lower than that obtained in our system. This discrepancy could be due to the use of a different yeast strain (AH22), a different expression vector (pGYR1), as well as different yeast cultivation.

Our results did not indicate any statistical difference between kinetic parameters related to the Leu[11]Phe + Pro[13]Leu, Pro[13]leu, Met[16]Thr, Asp[76]Asn and Gly[522]Ser variants compared to those of the wild-type protein. Furthermore, we confirmed that the Pro[13]Leu substitution entails little effect on the catalytic properties of CYP4F12 toward ebastine hydroxylation, as shown previously (Hashizume et al., 2001). The Val[90]Ile and Arg[188]Cys variants gave rise to an increase or a decrease in K_m and V_m values, respectively, but they did not lead to any significantly drift in their intrinsic clearances (V_m/K_m). Overall, all CYP4F12 wild-type and variant proteins seem to catalyse ebastine with the same efficiency. The lack of functional consequences of the tested mutations on CYP4F12 catalytic activity could be attributed to the fact that they are not located in putative domains known to influence enzymatic properties, such as the highly conserved sequence among CYP4F members, from amino acids 317 to 333, or the haem binding domain, from amino acid 461 to 474 [8,9]. Nevertheless, functional impact of combined mutations remains to be evaluated in further studies. In addition, missense mutations may entail substrate specificity modifications, as it has already been reported for other human CYPs [31]. Consequently, it would be interesting to evaluate the hydroxylation of terfenadine, another antihistaminic drug, or toward inflammatory mediators, by recombinant CYP4F12 proteins.

In conclusion, we report here the identification of ten polymorphisms affecting the *CYP4F12* sequence, including seven missense mutations, in 53 DNA samples from a French population of Caucasian origin. Furthermore, their functional impact toward ebastine hydroxylation was evaluated. Although no significant variation in the catalytic activity of recombinant CYP4F12 proteins was observed, this study gave insight in the knowledge of this particular extra-hepatic cytochrome P450. Furthermore, complementary studies could help in determining the in vivo functional impact of the identified variants and will provide more definite data on the role of *CYP4F12* genetic polymorphism in the metabolism of some inflammatory mediators and drugs.

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