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CYP2C8 polymorphisms in Caucasians and their relationship with paclitaxel 6α-hydroxylase activity in human liver microsomes

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Abstract

Published cDNA sequences suggest the existence of non-synonymous single nucleotide polymorphisms in the cytochrome P450 CYP2C8. To determine whether these polymorphisms could be confirmed in a Caucasian population and to investigate whether additional polymorphisms occur in the coding and upstream regions of this gene, we screened for previously described and for novel polymorphisms using PCR-RFLP and SSCP analysis. We confirmed the existence of two of the previously detected polymorphisms which give rise to the amino acid substitutions I264M and K399R, respectively, but failed to detect three others in our population. We also confirmed that a recently identified polymorphism (R139K) is linked to K399R (*CYP2C8*3*) in our study population. The allele frequencies for the I264M (*CYP2C8*4* allele) and the *CYP2C8*3* allele were 0.075 and 0.15, respectively. Three novel polymorphisms (T-370G, C-271A and T1196C/L390S) were also detected with the upstream polymorphisms showing allele frequencies of 0.061 and 0.196, respectively, but the L390S polymorphism detected only in a single subject. An additional single subject was heterozygous for a polymorphism recently described in African–Americans (A805T; *CYP2C8*2* allele). The functional significance of the two upstream polymorphisms and the *CYP2C8*3* and *CYP2C8*4* alleles was investigated in human liver microsomes. Samples heterozygous for *CYP2C8*3* showed significantly lower paclitaxel 6α-hydroxylase activity compared with wild-type samples. Median activity associated with *CYP2C8*4* also appeared lower than the wild-type but the difference was not significant. There was no evidence that either upstream polymorphism gave rise to altered CYP2C8 expression.

Keywords: Cytochrome P450; CYP2C8; Polymorphism; Paclitaxel; Human; Liver

1. Introduction

The cytochrome P450 CYP2C8‡ is present at relatively high levels in most human livers [1] and plays a major role in the metabolism of several therapeutically important drugs including paclitaxel, all-*trans* retinoic acid, verapamil, rosiglitazone, cerivastatin, amiodarone, dapsone and amodiaquine [2–10]. There is some evidence for a physiological role for CYP2C8 in arachidonic acid metabolism, especially in the oxidation of arachidonic acid to the putative endothelium-derived hyperpolarising factor 11,12-epoxyeicosatrienoic acid (11,12-EET) [11,12], though more recent reports suggest that CYP2C9 may be the major isoform involved [13]. CYP2C8 may also contribute to activation of toxicologically important compounds including benzo[*a*]pyrene and parathion [14,15]. CYP2C8 shows considerable homology with other members of the CYP2C family, particularly CYP2C9, but there is little overlap in substrate specificity with, for example CYP2C8 being considerably less efficient in the oxidative metabolism of tolbutamide compared with CYP2C9 [16], whereas all-*trans* retinoic acid is more efficiently hydroxylated by CYP2C8 compared with CYP2C9 [3]. A recent homology model for human CYP2C isoforms suggests that

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Abbreviations: CYP, cytochrome P450; SNP, single nucleotide polymorphism; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; SSCP, single strand conformational polymorphism.

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there are significant differences with regard to the key amino acids within the active site of CYP2C8 compared with other CYP2Cs [17] which is also consistent with differences in xenobiotic inhibitor profiles compared with CYP2C9 as well as a variety of other P450s [18]. Many of the drug substrates metabolised by CYP2C8 are also metabolised by CYP3A4 but in a number of cases including, for example paclitaxel, the products of metabolism by CYP2C8 and CYP3A4 are different. Unlike in the case of other members of the human CYP2C family, there is currently limited information on the existence of polymorphism in CYP2C8. However, there are reports of inter-individual variation in the metabolism of several CYP2C8 substrates particularly paclitaxel and rosiglitazone [2,6]. A number of independently determined cDNA sequences for CYP2C8 are available which suggest the existence of several genetic polymorphisms [11,19–23]. We have determined whether these polymorphisms occur *in vivo* and have in addition screened a Caucasian population for the existence of novel polymorphisms in the exons and part of the upstream sequence. We now describe the existence of a number of SNPs in the coding and upstream sequences of CYP2C8, their population frequencies and preliminary data on their possible functional significance by studies on the relationship between genotype and enzyme activity and protein expression in a human liver bank. Some of these SNPs have recently been detected in two independent studies [24,25].

2. Materials and methods

2.1. Blood and liver samples

Blood samples were obtained from apparently healthy British Caucasian volunteers resident in Northeast England. These individuals have been described in detail previously [26,27]. Sample collection was approved by the Newcastle University and Newcastle and North Tyneside joint ethical committee and all volunteers gave informed consent to their use in studies on cytochrome P450 polymorphisms. Three separate human liver banks were used. Bank 1 consisted of samples from 26 British Caucasian organ donors obtained from (i) Freeman Hospital, Newcastle upon Tyne with the approval of the Newcastle University and Newcastle and North Tyneside joint ethical committee and (ii) University of Aberdeen with the approval of the Grampian Health Board ethical committee. The livers were shown by histological investigation not to have liver disease. Samples were collected between 1985 and 1998 and flash-frozen and stored at −70° before extraction. The donors (10 females, 13 males, 3 sex unknown) ranged in age from 21 to 66 years. Complete drug, alcohol and smoking histories were not available. Bank 2 consisted of Caucasian (26 samples), Hispanic (2 samples) and African–American (1 sample) donor livers obtained from Gentest Corporation. The age range was 2–73 years with 18 samples from female donors and 11 from males. Complete drug, alcohol and smoking histories were not available. Bank 3 consisted of 28 histologically normal livers from Belgian kidney donors obtained from the Gasthuisberg Hospital (Leuven, Belgium) between 1990 and 1995 with the consent of donors and the hospital as described previously [28]. No additional information on age, sex or medical history was available.

2.2. DNA preparation

DNA was prepared from leukocytes by the method described by Daly *et al.* [29]. DNA was prepared from human liver samples by proteinase K digestion followed by phenol–chloroform extraction using standard methods [30].

2.3. PCR and design of CYP2C8-specific primers

All PCR reactions involved amplification using approx 500 ng genomic DNA in a total volume of 25–50 μL in a buffer consisting either of 10 mM Tris–HCl, pH 8.8, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% (v/v) Triton X-100 or 50 mM Tris–HCl, pH 9.1, 16 mM ammonium sulphate, 3.5 mM $MgCl_2$, with 0.2 mM dNTPs (Roche or Promega), 0.025 U/μL BioTaq polymerase (BioLine or Promega) and 0.25 μM oligonucleotide primers (Newcastle University Central Molecular Biology Services or Invitrogen). For long PCR, a Long Template PCR kit (Roche) was used. Primer sequences are listed in Table 1. PCR reactions normally involved 30 or 35 cycles of amplification with 1 min denaturation at 94°, followed by annealing for 1 or 2 min at the temperature shown in Table 1 and synthesis for 2 min at 72°.

To design primers specific for CYP2C8 which did not also amplify other CYP2C genes, sequence alignments of CYP2C8 cDNA sequences, partial genomic sequence [31] and chromosome 10 BAC clone sequences (http://www.sanger.ac.uk) were performed using the program Geneworks (Intelligenetics). Primers specific for CYP2C8 at the 3′-end were then designed. PCR reactions were optimised so that the annealing temperature was as high as possible to minimise amplification of related genes. Confirmation that only CYP2C8 was being amplified in the PCR reactions for SSCP analysis (upstreams 1 and 2 and exons 1–9) was provided by sequencing PCR products from at least two separate individuals.

2.4. RFLP-based screening assays

2.4.1. C792G (codon 264) polymorphism

A forward primer specific for exon 5 of CYP2C8 (**3A**) and a reverse primer consisting of 13 bp from the 3′-end of exon 5 of CYP2C8 together with 7 bp from the start of intron 5 for CYP2C9/2C18 were used for amplification of a 125 bp fragment (**3B**). The product was digested with *Taq*I

Table 1
PCR primers

Reaction	Primers	Annealing temperature	Size of PCR product
T579A	**1A** GAGGACCGTGTTCAAGAGGAAGCTCA	2 min at 61°	12 kb
	1B AGTTCCTGGGAAACAATCAATGAGTA	2 min at 55°	115 bp
	1C CACCCTGTGATCCCACTTTC	2 min at 55°	115 bp
	1D CTTTTCATCAGGGTGAGAAA	2 min at 55°	115 bp
A1232T	**2A** GCACAACCATAATGGCATTA	2 min at 60°	1.8 kb
	2B GCGGGCAAGTCCTTCTCCTG	2 min at 60°	1.8 kb
	2C CTAATCCAAATATCTTTGACCCAGG	2 min at 61°	87 bp
	2D CTGCTGAGAAAGGCATGAAGTAGAC	2 min at 61°	87 bp
C792G	**3A** AAAAATGTTGCTCTTACACG	2 min at 55°	125 bp
	3B ATTTTACCTGCTCCATTTTG	2 min at 55°	125 bp
C389A	**6A** CCCCAATATCTCAAAGAATTACT	1 min at 55°	395 bp
	6B CCCCATCCCAAAATTCCGCCAG	1 min at 55°	395 bp
SSCP upstream 2 (−165 to −430)	**U2F** ACAATGTACATTTTTTATAT	1 min at 47°	266 bp
	U2R AAAATGTTCTTTTGTCCTACT	1 min at 47°	266 bp
SSCP upstream 1 (−235 to +15)	**U1F** AATCCAATACAATTAAACCA	1 min at 52°	250 bp
	U1R CACAAAAGGTTCCATTGAAG	1 min at 52°	250 bp
SSCP exon 1	**Ex1F** TTGGAGTGCAAGCTCACAGC	1 min at 59°	240 bp
	Ex1R GCTGGAGGAACATAAGGCAG	1 min at 59°	240 bp
SSCP exon 2	**Ex2F** CGACTTATTTTGCTGCTATT	1 min at 58°	248 bp
	Ex2R CCCCCTCACCCCAGTTACCA	1 min at 58°	248 bp
SSCP exon 3	**Ex3F** AATTCTCCCAGTTTCTGCCC	1 min at 64°	241 bp
	Ex3R CGTCACTAGTGAAGACAGGT	1 min at 64°	241 bp
SSCP exon 4	**Ex4F** CTTTATGTCTTAACAAATGC	1 min at 58°	295 bp
	Ex4R ACCAAGTCTTCCCTACAACC	1 min at 58°	295 bp
SSCP exon 5	**Ex5F** TGGTGTAAGATACATATATC	1 min at 54°	265 bp
	Ex5R GACAAGAAATCAAAATACTG	1 min at 54°	265 bp
SSCP exon 6	**Ex6F** GTATTTAGATATACTGGCAC	1 min at 56°	267 bp
	Ex6R GTGGAGGATACTGGCACCAT	1 min at 56°	267 bp
SSCP exon 7	**Ex7F** TGTGGCCATGAATTGCTATG	1 min at 58°	316 bp
	Ex7R CAGCACTATGGAAATTTCAG	1 min at 58°	316 bp
SSCP exon 8	**Ex8F** ACTACTTCTCCTCACTTCTG	1 min at 58°	277 bp
	Ex8R TGCCATGTAAATTCCAACTA	1 min at 58°	277 bp
SSCP exon 9	**Ex9F** TGTGCAAGTCACAAATGACT	1 min at 64°	305 bp
	Ex9R TTGCAGGTGATAGCAGATCG	1 min at 64°	305 bp

which digests the wild-type sequence to fragments of 90 and 35 bp. Digestion products were analysed by electrophoresis on a 10% polyacrylamide gel.

2.4.2. T579A (codon 193) polymorphism

A two-step amplification involving long PCR to give a 12 kb product using primers from exons 3 and 5 (**1A** and **1B**) followed by reamplification with nested primers (**1C** and **1D**) to give a 115 bp fragment in which an *Eco*RI site for the wild-type sequence had been inserted by use of a mismatched primer (**1D**) was used. Digestion products were analysed by electrophoresis on a 10% polyacrylamide gel.

2.4.3. A1232T (codon 411) polymorphism

A two-step amplification involving long PCR to give a 1.8 kb product using primers from exons 8 and 9 (**2A** and **2B**) followed by reamplification with nested primers (**2C** and **2D**) to give a 87 bp fragment in which an *Stu*I site for the variant sequence had been inserted by use of a mismatched primer (**2C**) was used. Digestion products were analysed by electrophoresis on a 10% polyacrylamide gel.

2.4.4. C389A (codon 130) polymorphism

A forward primer from exon 2 (**6A**) and a reverse primer from exon 3 with a single mismatch to introduce a *Bst*NI site (**6B**) were used for amplification of a 395 bp fragment. The product was digested with *Bst*NI which digests the wild-type sequence to fragments of 371 and 24 bp but should not digest the variant sequence. Digestion products were analysed by electrophoresis on a 10% gel.

2.5. SSCP analysis

SSCP analysis was carried out using 1× Mutation Detection Enhancement (MDE; FMC) gel solution. The gel

consisted of 1× MDE gel solution, 1× TME buffer (0.3 M Tris, 0.35 M MES, 10 mM EDTA, pH 6.8), 2.5 M urea, 0.3 mg/mL ammonium persulphate and 0.1% (v/v) TEMED and the dimensions were 200 mm × 200 mm × 0.75 mm. Denaturation of PCR products was achieved by adding 8 μL PCR product to 25 μL denaturation solution (95% (v/v) formamide, 10 mM NaOH, 0.05% bromophenol blue, 0.05% xylene cyanol) and incubating at 94° for 3 min. The samples were then chilled on ice and the entire volume applied to the gel. Electrophoresis was performed in 1× TME at a constant voltage of 200 V for 16–20 hr. Two gels were run in parallel for each sample, one at 4° and one at 20°. Following electrophoresis, DNA was detected by silver staining [32].

2.6. DNA sequencing

To purify PCR products for DNA sequencing, approx. 300 μL of PCR product was mixed with 700 μL binding solution (0.1% (w/v) diatomaceous earth in 8 M guanidine HCl). The suspension was transferred to a mini spin filter in a 1.5 mL microcentrifuge tube and centrifuged at 1000 *g* for 2 min. The filtrate at the bottom of the tube was discarded and the filter replaced in the same tube. Six hundred microlitres of wash buffer (200 mM NaCl, 10 mM EDTA, 50 mM Tris–HCl, pH 7.4, in 50% (v/v) ethanol) was added and pellet was washed by centrifugation at 2200 *g* for 2 min. This wash step was repeated followed by a final centrifugation for 2 min at 2200 *g* to remove all traces of wash. Finally, the filter was transferred to a 1.5 mL collection tube and the bound DNA was eluted in 35 μL water by centrifugation at 9000 *g* for 5 min. The DNA concentration was estimated by analysis on an agarose gel and approx. 100–500 ng sequenced on an ABI PRISM 377 Genetic Analyzer (Perkin–Elmer Corporation) using fluorescently labelled terminators.

2.7. Preparation of human liver microsomes

Human liver microsomes were prepared according to previously described protocols [15,33]. For liver banks 2 and 3, protein content was determined by the method of Lowry as modified by Miller [34] and for liver bank 1 using the bicinchoninic method [35].

2.8. Immunoblotting

Human liver microsome proteins from liver bank 1 were size fractionated by electrophoresis on a 10% (w/v) SDS–polyacrylamide gel [36]. Proteins were transferred from the gel to Hybond-C Extra nitrocellulose membrane (Amersham-Pharmacia) using a Transblot cell (Biorad) for 4 hr at 60 V and 4° in accordance with the manufacturer's recommendations. The filter was blocked in 0.14 M NaCl, 25 mM Tris–HCl, pH 7.4, containing 10% (w/v) dried milk and 0.5% (v/v) Tween 20 for at least 1 hr. The filter was then incubated with an anti-CYP2C8 serum (1/3000) (anti-peptide antiserum from Dr. R. Edwards, Imperial College, London, UK [37]), followed by washing and incubation with peroxidase-conjugated goat anti-rabbit serum. After washing, peroxidase was detected by ECL detection (Amersham-Pharmacia). To quantify band densities, the film was photographed with a DC40 camera and band density was determined using 1D Image Analysis Software (Kodak Digital Science™). The relevant bands were identified and the image analysed to express results as net intensity, defined as the sum of the pixel values in the band rectangle minus the background pixel values. A standard curve for control samples with known CYP2C8 content (CYP2C8 supersomes, Gentest) was constructed for determination of CYP2C8 content of individual livers.

2.9. Measurement of paclitaxel 6α-hydroxylation in human liver microsomes

For liver bank 1, the levels of paclitaxel 6α-hydroxylase activity in human liver microsomes were determined by minor modification of the method described by Sonnichsen *et al*. [38]. For liver bank 2, microsomes were incubated with 20 μM paclitaxel and a NADPH generating system for 10 min. Analysis of metabolites was on a C18 HPLC column maintained at 45° with a mobile phase initially of 60% methanol increasing to 70% methanol over 20 min and at a flow rate of 1.0 mL/min. The product was detected by its absorbance at 230 nm and quantitated by comparing to the absorbance of a standard curve for 6α-hydroxypaclitaxel. For liver bank 3, levels of paclitaxel 6α-hydroxylase activity were determined by the method described by Desai *et al*. [39], which involved incubation of microsomes with 25 μM paclitaxel and a NADPH generating system for 20 min and analysis of metabolites by reverse-phase HPLC using a C18 ODS column. Elution started at 1.0 mL/min with a gradient from 95% of an aqueous solution of 0.1 M ammonium acetate adjusted to pH 7.5 (solvent system A) and 5% of a mixture of an aqueous solution of 1 M ammonium acetate, adjusted to pH 7.5/methanol/acetonitrile (10/45/45; v/v/v) (solvent system B), to 30% of solvent system A and 70% solvent system B over 1 min. Then a gradient of 5 min to 100% of solvent system B was applied. This solvent composition was held for 2 min before returning to the starting conditions. Levels of paclitaxel and 6α-hydroxypaclitaxel were determined by monitoring absorbance at 230 nm.

2.10. Statistical analysis

Overall differences between groups were assessed using the Mann–Whitney and Kruskal–Wallis non-parametric tests with differences between subgroups assessed by Dunn's multiple comparisons test.

3. Results

3.1. Studies on known CYP2C8 cDNA sequences

At least six separate full length or partial CYP2C8 cDNA sequences have been reported as summarised in Fig. 1. Since the sequences indicated the existence of five different alleles, we developed PCR-RFLP or SSCP assays for at least one non-synonymous SNP associated with each sequence to confirm their existence and determine their population frequencies. Using these assays, we screened for the various polymorphisms in at least 100 British Caucasian DNA samples. The results obtained are summarised in Table 2. In the case of the T579A and A1232T polymorphisms, no subjects positive for the variant alleles were detected. However, in the case of the C792G polymorphism, which predicts a substitution of Met for Ile at residue 264, out of 100 DNA samples, 8 were heterozygous (Fig. 2). Okino *et al.* [20] also suggest a second non-synonymous polymorphism (C389A) occurs in association with C792G, but this base change was not detected in any samples screened in a PCR-RFLP assay with the enzyme *Bst*NI and sequencing of exon 3 from three samples positive for the C792G polymorphism did not show any evidence of a polymorphism at position 389. In the case of the A1196G polymorphism, we confirmed the existence of the A1196G variant at a frequency of 0.15 (see Table 2 and Fig. 2). Since this polymorphism has recently been detected in tight linkage disequilibrium with a polymorphism (G416A) in exon 3 [24], an area which did not form part of the original sequence cloned by Kolyada [23], we also screened for G416A. In the initial screen, all samples heterozygous for the A1238G variant were also heterozygous for G416A. This allele has been designated *CYP2C8*3* [24] and with the agreement of the CYP alleles nomenclature committee (see http://www.imm.ki.se/CYPalleles), alleles positive for C792G are designated *CYP2C8*4*.

3.2. Screening for novel polymorphisms by SSCP

Primers were designed to amplify each exon and at least 20 bp of flanking intron sequence by alignment of a CYP2C8 BAC sequence with those for other members of the CYP2C family so that amplification of non-CYP2C8 sequences would not occur. In addition, 407 bp of sequence upstream of the translation start site was amplified in two separate PCR reactions. A total of 40 random Caucasian DNA samples were screened for novel polymorphisms by amplifying each exon and the upstream regions with these primers and performing SSCP analysis on the PCR product. All SSCP analysis was performed at both 20° and 4° using TME buffer which has been reported to give better sensitivity in SSCP detection that the more widely used 0.5× TBE [40]. In addition, gels contained 2.5 M urea which has been reported to improve resolution without preventing intra-strand renaturation [41]. SSCP analysis followed by DNA sequencing of samples showing mobility shifts resulted in the detection of an additional four polymorphisms in coding and upstream sequences to those already confirmed by PCR-RFLP. Some typical results are shown in Fig. 2. As summarised in Table 3, the two coding region polymorphisms were non-synonymous, but one of these was a polymorphism recently detected in an African–American population [24] which has been given the designation *CYP2C8*2* and two were in the upstream sequence. One of the upstream polymorphisms (C-271A) resulted in the creation of a consensus sequence for the transcription factor C/EBPα but for the second (G-370T), neither the variant or the wild-type sequence was within a sequence homologous to any known transcription factor binding elements. The frequencies of all the novel polymorphisms were determined in a control Caucasian population. The results obtained are summarised in Table 3. Apart from the tight linkage previously reported between the G416A and A1196G polymorphisms (*CYP2C8*3* allele), we found no evidence that any of the novel polymorphisms are linked to one another or to common polymorphisms in the adjacent CYP2C9 and CYP2C19 genes. Sequencing of all exons, exon–intron boundaries and at least 400 bp of upstream sequence in DNA from single individuals heterozygous for the various novel polymorphisms (*CYP2C8*4*, G-370T, C-271A and T1169C) did not result in the detection of any additional linked polymorphisms. Alleles positive for C-271A and G-370T have been designated *CYP2C8*1B* and *CYP2C8*1C*, respectively.

3.3. Studies on functional significance using human liver microsomes

To assess the functional significance of the various polymorphisms, three separate liver banks were genotyped

Table 2
Allele frequencies of CYP2C8 SNPs originally detected in cDNA clones

Variant	Codon	Number of subjects	Allele frequency (±95% CI)
C389A	T130N	100	0
T579A	N193K	100	0
C792G	I264M	107	0.075 ± 0.035
A1196G	K399R	107	0.150 ± 0.024
A1232T	H411L	100	0

Table 3
Additional coding sequence and upstream polymorphisms detected by SSCP analysis and their frequencies

Variant	Codon	Number of subjects	Allele frequency (±95% CI)
T-370G	na	107	0.061 ± 0.032
C-271A	na	107	0.196 ± 0.052
A805T	Ile269Phe	116	0.004 ± 0.008
T1169C	Leu390Ser	116	0.004 ± 0.008

na: not applicable.

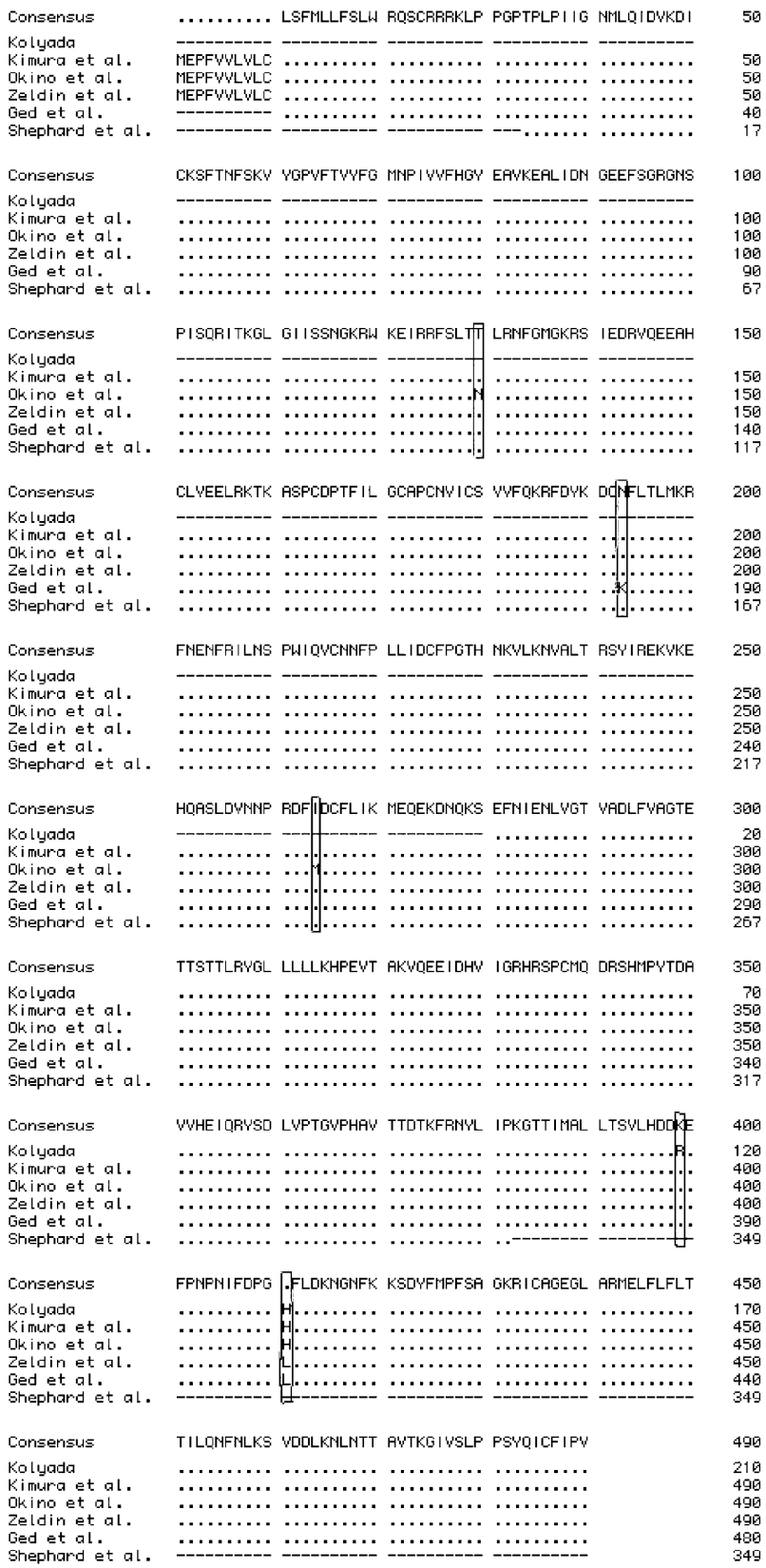

Fig. 1. Alignment of previously published CYP2C8 amino acid sequences. The consensus sequence represents that for which there is agreement between at least four of the six published sequences. Individual sequences are given for positions where fewer than four independent sequences are available or where there is a sequence difference. The sequences are taken from Kolyada [23], Kimura *et al.* [19], Okino *et al.* [20], Zeldin *et al.* [11], Ged *et al.* [21] and Shephard *et al.* [22].

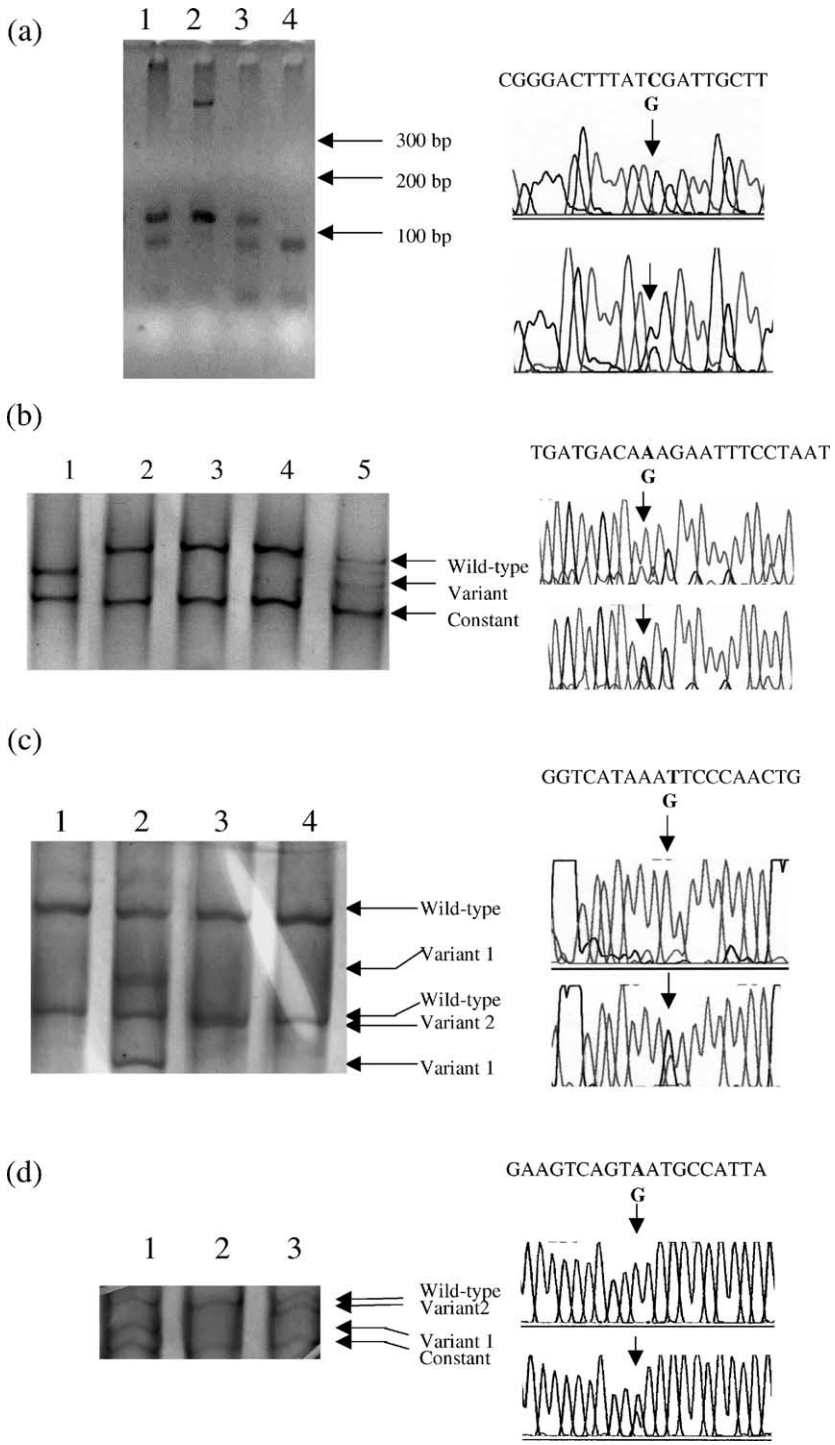

Fig. 2. Typical results for mutation screening by PCR-RFLP, SSCP analysis and DNA sequencing. (a) Detection of C792G by PCR-RFLP and DNA sequencing. Agarose gel of *Taq*I-digested PCR products is shown on the left. Lanes 1 and 3 represent heterozygous samples, lane 2 homozygous wild-type and lane 4 homozygous mutant. Sequencing traces of the region for a homozygous wild-type sample (top) and a heterozygous sample (bottom) are shown on the right. (b) Detection of A1196G by SSCP analysis and DNA sequencing. A silver-stained SSCP gel run at 4° is shown on the left. Lane 1 represents a homozygous mutant sample, lanes 2–4 homozygous wild-types and lane 5 a heterozygote. Sequencing traces of the region for a homozygous wild-type sample (top) and a heterozygous sample (bottom) are shown on the right. (c) Detection of T-370G and C-271A by SSCP analysis and DNA sequencing. A silver-stained SSCP gel run at 20° is shown on the left. Lanes 1 and 4 represent homozygous wild-types, lane 2 a heterozygote for C-271A and lane 3 a heterozygote for T-370G. Sequencing traces of the region for a homozygous wild-type sample (top) and a sample heterozygous for T-370G (bottom) are shown on the right. (d) Detection of T1169C by SSCP analysis and DNA sequencing. A silver-stained SSCP gel run at 4° is shown on the left. Lane 1 represents a sample heterozygous for A1196G, lane 2 a homozygous wild-type and lane 3 a heterozygote for T1169C. Variant 1 is the band characteristic of A1196G and variant 2 the T1169C-specific band. Sequencing traces of the region for a homozygous wild-type sample (top) and a sample heterozygous for T1169C (bottom) are shown on the right.

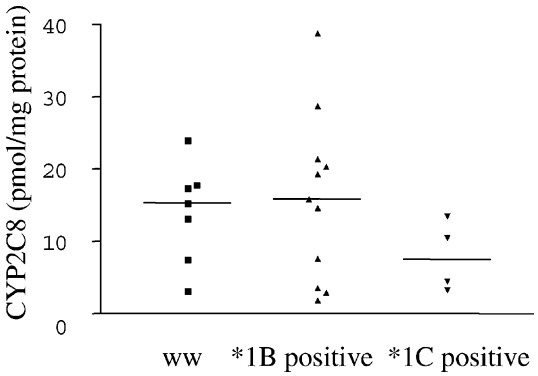

Fig. 3. Effect of upstream polymorphisms on CYP2C8 expression. Levels of CYP2C8 expression detected by immunoblotting for each upstream genotype detected are shown. Median levels are indicated by horizontal bars; ww indicates samples negative for *CYP2C8*1B* and *CYP2C8*1C*. Statistical significance was evaluated by the Kruskal–Wallis test with no significant difference between groups detected.

for *CYP2C8*3*, *CYP2C8*4* and the two upstream alleles (*CYP2C8*1B* and *CYP2C8*1C*). All three liver banks were screened for paclitaxel 6α-hydroxylase activity and in addition, the level of CYP2C8 expression for bank 1 was determined by immunoblotting. There was a generally good correlation between enzyme activity and levels of expression for these samples ($r = 0.53$, $P = 0.006$). As the two upstream polymorphisms had the highest population frequencies of all the various polymorphisms, we initially determined their relationship to CYP2C8 protein levels. However, as summarised in Fig. 3, no significant difference in protein levels was detected between samples with a homozygous wild-type genotype and those positive for either the *CYP2C8*1B* or *CYP2C8*1C* alleles. There was also no indication that levels of CYP2C8 in samples homozygous for *CYP2C8*3* or doubly heterozygous for *CYP2C8*3* and *CYP2C8*4* were different from those in homozygous wild-type samples. In view of the apparent lack of effect of the upstream polymorphisms on levels of expression, we decided to compare the effects of the coding region SNPs with both entirely wild-type samples and those positive for upstream polymorphisms. As shown in Fig. 4, there were no significant different in median paclitaxel 6α-hydroxylase activity between homozygous wild-type samples and those heterozygous for *CYP2C8*3* or *CYP2C8*4* for two of the liver banks (1 and 3), but in the case of the bank 2 and the combined data, significant differences were detected with heterozygotes for *CYP2C8*3* showing a significant difference from wild-type individuals. In the case of the *CYP2C8*4* allele, a significant difference between heterozygotes and wild-type subjects was not detected possibly due to small sample numbers though median activity values were of a similar magnitude to those for *CYP2C8*3* heterozygotes. Two liver samples homozygous for *CYP2C8*3* were detected, one each in two of the three liver banks. For these samples, paclitaxel 6α-hydroxylase levels of 68 and 95 pmol/min/mg were obtained which represented 31 and 84% of the

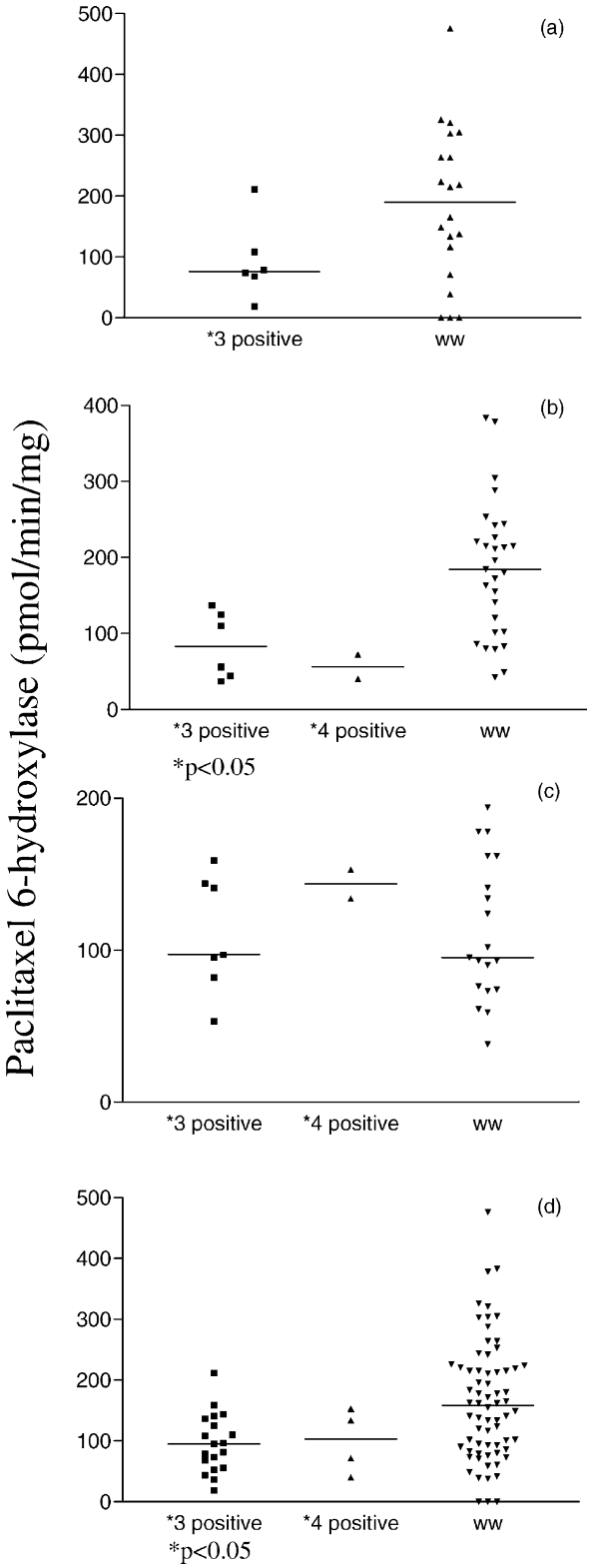

Fig. 4. Effect of coding region polymorphisms on paclitaxel 6α-hydroxylase activity in liver banks. Panels (a) to (c) show the relationship between genotype and phenotype for each of the three separate liver banks 1–3, respectively. Panel (d) shows the pooled data; ww indicates samples negative for *CYP2C8*2*, *CYP2C8*3* and *CYP2C8*4*. Median levels are indicated by horizontal bars. Statistical significance was evaluated either by the Mann–Whitney *U*-test or by the Kruskal–Wallis test with subgroup analysis performed by Dunn's multiple comparison test. Significant differences from the wild-type are indicated as follows: $^{*}P < 0.05$.

corresponding median homozygous wild-type activities. These levels appeared similar to the median values obtained for heterozygous samples rather than being lower as might be predicted from a previous study [24]. A single sample was heterozygous for both *CYP2C8*3* and *CYP2C8*4* and showed an activity of 19 pmol/min/mg compared with a median of 190 pmol/min/mg for homozygous wild-type livers in this bank.

4. Discussion

The current investigation has shown that at least four additional CYP2C8 variant alleles including two involving non-synonymous mutations occur and has also confirmed the existence of a previously described variant [24,25]. In addition, we have surveyed existing database sequence data on CYP2C8 and shown that while this correctly predicts the existence of two allelic variants (*CYP2C8*3* and *CYP2C8*4*), the existence of an additional three non-synonymous polymorphisms predicted from published cDNA sequences could not be confirmed experimentally in a Caucasian population. It remains possible that the polymorphisms we failed to detect exist at a very low frequency among Caucasians or are found in other ethnic groups only but they could also represent sequencing errors or cloning artefacts.

Some evidence for decreased paclitaxel 6α-hydroxylase activity associated with both the *CYP2C8*3* and *CYP2C8*4* alleles was obtained in the study but these differences are small and seem unlikely to be of major biological significance. Though two separate previous *in vitro* expression studies on the *CYP2C8*3* gene product suggested that turnover of paclitaxel was decreased compared with the wild-type, there is disagreement as to the extent of this decrease with one study reporting that the variant allele is associated with only approx. 10% of that seen for the wild-type gene product [24] while the second reports an activity of 75% of the wild-type for the variant [25]. The results of the present study are consistent with a small decrease in enzyme activity associated with the variant allele with the data, especially that for microsomes from homozygous mutants, more consistent with the 75% of normal seen by Soyama *et al*. [25] than the larger effect reported by Dai *et al*. [24]. It is also possible that the relatively high activity seen in the homozygous mutant samples could be due to a contribution by other P450 isoforms to the 6α-hydroxylase reaction. Though CYP2C8 has been shown to be the major P450 isoform catalysing 6α-hydroxylation in two independent studies, neither of these studies used a full range of expressed P450 isoforms with, for example CYP2C19 not included in either study [2,38]. A separate study, however, found that 6α-hydroxylation by human liver microsomes was inhibited by diazepam and that activity also correlated with diazepam demethylation which would be suggestive of a contribution by CYP2C19 [42]. Diazepam is a CYP2C8 inhibitor at high concentrations [18], but the extent of its inhibition of paclitaxel 6α-hydroxylase seemed higher than would be expected if the entire reaction was catalysed by CYP2C8. In both the current study and the previous studies on *CYP2C8*3*, the substitutions were not detected separately but the possibility that this occurs in other ethnic groups should be investigated further especially since there is some evidence that both amino acid substitutions individually are also functionally significant *in vitro* [25].

In general, microsomes from livers heterozygous for *CYP2C8*4* showed activities similar to those for *CYP2C8*3* heterozygotes. This suggests that the I264M change may also be functionally significant though further investigation is required. The L390S substitution was only observed in one subject and no liver microsomes with this genotype were available for functional characterisation.

None of the observed coding region mutations give rise to substitutions in amino acids predicted to have major roles in CYP2C8-mediated catalysis [17,43]. However, several of the amino acid substitutions lie within areas conserved among CYP2C isoforms increasing the possibility that they may be associated with altered enzyme activity or protein stability. The I264M is a non-conservative amino acid substitution at a position which is conserved among both CYP2C members and in CYP2A and CYP2B isoforms. The I269P substitution (*CYP2C8*2* allele) is adjacent but I269 appears less well conserved among CYP2C isoforms than I264 with methionine at the 269 position in CYP2C9. In the case of the *CYP2C8*3* allele and the K399R polymorphism, lysine is conserved among CYP2C genes from a number of species. For the linked R139K substitution, arginine is conserved within human CYP2C genes but the lysine substitution is seen in rabbit and dog CYP2C genes. In the case of the L390S substitution, most other CYP2C family members have serine in this position rather than leucine. This finding raised the possibility that the polymorphism was not genuine but due to co-amplification of another CYP2C gene. We believe this is very unlikely since only one base substitution was detected and CYP2C9, CYP2C18 and CYP2C19 all have additional sequence differences to CYP2C8 in this area of exon 8.

In addition to the two novel polymorphisms in the coding region, we detected two upstream polymorphisms. Though several low frequency upstream polymorphisms have recently been detected by Dai *et al*. [24], the two polymorphisms detected in the present study appear different from these and also to occur at higher frequencies. In the case of the *CYP2C8*1B* allele, a new consensus sequence for CEBP is created suggesting that the polymorphism could be associated with differential expression. There was, however, no evidence from immunoblotting studies for any difference in CYP2C8 expression in those heterozygous or homozygous for the variant allele.

As with most other xenobiotic metabolising P450 genes, it is now clear that CYP2C8 is subject to genetic polymorphism with approx. 20% of Caucasians heterozygous for at least one non-synonymous polymorphism and an additional 20% positive for upstream SNPs. The failure to detect three of the four common Caucasian alleles in a recent study is interesting [24]. In that study, a panel of 72 DNA samples from a variety of ethnic groups were used but only 24 of these were Caucasian, including 10 of probable Western European descent and comparable to the panel used in the present study. It is generally accepted that resequencing and other types of mutation detection studies are an important sequel to the sequencing of the human genome [44]. However, to enable polymorphisms that occur at relatively low frequencies (in the range 0.01–0.1) but may still make an important contribution to disease susceptibility to be detected, it seems likely that larger numbers of samples from particular ethnic groups will need to be examined as, for example has been done in the case of the *SULT1C1* gene where 89 Caucasian samples were sequenced [45].

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