Genetic analysis of the cytochrome P-450IIC18 (CYP2C18) gene and a novel member of the CYP2C subfamily

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Received 7 February 1996; revised version received 19 March 1996

Abstract The CYP2C18 gene was investigated in order to characterize its molecular basis in the CYP2C subfamily. A mutation of the CYP2C18 gene was identified at the 5′-flanking region of the gene, which could be detected by digestion with *Dde*I. The allele frequency of the mutant CYP2C18 gene was 21.4%. Genotypes of the polymorphic *Dde*I site of the CYP2C18 gene were found to be completely consistent with that of the polymorphic CYP2C19 gene (the m1 mutant). The CYP2C18 and CYP2C19 genes were suggested to be linked and located close together on the chromosome. Clones containing the 5′-flanking region of a member of the CYP2C subfamily were obtained from the PCR products from human genomic DNA. The nucleotide sequence of the clone proved to be 90.5% identical to the corresponding region of the CYP2C18 gene. This is very likely to be a novel member of the CYP2C subfamily.

Key words: CYP2C18; CYP2C19; Polymorphism; 5′-flanking region

1. Introduction

In human liver, many clinically used drugs such as mephenytoin and hexobarbital are metabolized by the CYP2C subfamily [1]. The human CYP2C subfamily consists of at least 4 members (CYP2C8, CYP2C9, CYP2C18, CYP2C19) [1,2]. Extensive metabolizer (EM) and poor metabolizer (PM) polymorphism for mephenytoin [3,4] is well known and has been studied extensively. It has been shown that CYP2C19 is a candidate for mephenytoin 4′-hydroxylation [5,6]. However, the role of CYP2C18 and the relationships between the other CYP2C subfamily members and the CYP2C18 gene have not been clearly demonstrated. Further study is required to understand the characteristics of the CYP2C subfamily.

In the present study, we investigated the CYP2C18 gene to characterize the molecular basis of the CYP2C18 gene and to search for other members of the CYP2C subfamily. A novel mutation was identified at the 5′-flanking region of the CYP2C18 gene and the mutation was found to be completely linked to the m1 mutation [7] of the polymorphic CYP2C19 gene. Moreover, the 5′-flanking region and each exon of the CYP2C18 gene were analyzed by the PCR method and SSCP analysis, and a novel variant of the 5′-flanking region of the CYP2C subfamily was obtained and sequenced.

2. Materials and methods

2.1. Genomic DNA extraction

Human genomic DNA was extracted from leukocyte nuclei of peripheral blood obtained from 56 healthy volunteers according to the method of Kan and Dozy [8].

2.2. PCR amplification for fragments of CYP2C18 genes

A PCR for the CYP2C18 gene was carried out using the following primers (Fig. 1): primers 1 and 2 for fragment 1, primers 3 and 4 for fragment 2, primers 5 and 6 for fragment 3, primers 7 and 8 for fragment 4, primers 9 and 10 for fragment 5, primers 11 and 12 for fragment 6, primers 13 and 14 for fragment 7, primers 15 and 16 for fragment 8, primers 17 and 18 for fragment 9. Each was conducted in a total volume of 50 μl in the presence of 1.25 mM magnesium chloride, 10 mM Tris-HCl (pH 8.3), 200 μM of each dNTP, 5 pmol of each primer [2,9] as shown below: primers 1 (5′-AGTCTAAGCCACTCCATATT-3′) and 2 (5′-TGACTCTGACTGTGACTAGT-3′), or 3 (5′-GCTCTGCTTCAGACTAGTTA-3′) and 4 (5′-ATGTCTGTACTTCCATGTTT-3′), or 5 (5′-ATATAGACCAAATACTGAGT-3′) and 6 (5′-GATCATTACACATTCTGTGC-3′), or 7 (5′-CCTCACCCTGTGATCCCACT-3′) and 8 (5′-CTGGATAATCCTGAGGTTTT-3′), or 9 (5′-CAGGTCTGCAATAATTTCCC-3′) and 10 (5′-CTGTTCCATTTTGATCAGGA-3′), or 11 (5′-GAAAAGCACAATCAACAGTC-3′) and 12 (5′-TGTGACCTCTGGGTACTTCA-3′), or 13 (5′-GCTAAAGTCCAGGAAGAGAT-3′) and 14 (5′-CTTGGGGATGAGGTAGTTTT-3′), or 15 (5′-GGCACGACCATAATAACATC-3′) and 16 (5′-TGCTGAGAAAGGCATGAAGT-3′), or 17 (5′-GGAAAACGGATGTGTATGGG-3′) and 18 (5′-TTGCAGGTGACAGCACAGGA-3′). To each was added 400 ng genomic DNA and 0.5 U Taq DNA polymerase (Promega, Co., Madison, WI, USA) or *Pfu* DNA polymerase (Stratagene, La Jolla, CA, USA) for cloning. The conditions for annealing, polymerization and denaturation were 52°C for 2 min, 72°C for 2 min, and 94°C for 1 min, respectively. The number of cycles amplified was 40.

2.3. Restriction enzyme digestion of the PCR products

DNA fragments of the CYP2C18 gene were digested by the following restriction enzymes for 2 h at 37°C to find RFLP, and analyzed by agarose gel electrophoresis: *Apa*I, *Acc*I, *Alu*I, *Ava*I, *Afa*I, *Afl*III, *Bfa*I, *Bgl*II, *Bcl*I, *Bam*HI, *Ban*III, *Dde*I, *Eco*RI, *Eco*RV, *Fok*I, *Hae*III, *Hinc*II, *Hpa*II, *Hph*I, *Hin*fI, *Hha*I, *Hin*dIII, *Kpn*I, *Mbo*II, *Mnl*I, *Not*I, *Nco*I, *Nru*I, *Pst*I, *Pvu*II, *Sal*I, *Scr*FI, *Sau*3A, *Spe*I, *Sph*I, *Sma*I, *Ssp*I, *Sac*I, *Sac*II, *Taq*I, *Xho*I and *Xba*I.

2.4. Determination for genotypes of the CYP2C18 gene and CYP2C19 gene

The genotype at the *Dde*I site in the 5′-flanking region of the CYP2C18 gene was determined by digesting the PCR amplified frag-

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Abbreviations: CYP2C18, cytochrome P-450IIC18; CYP2C19, cytochrome P-450IIC19; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; SSCP, single-stranded conformational polymorphism; w, wild type gene; m, mutant type gene.

PII S0014-5793(96)00329-8

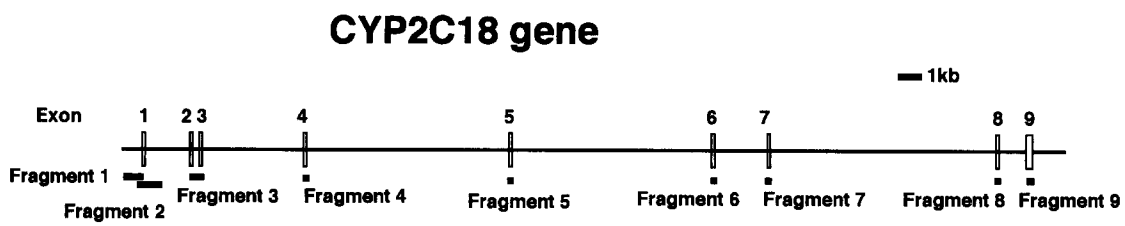

Fig. 1. Amplification for the fragments of the CYP2C18 gene. Locations of the fragments amplified by PCR are shown.

ment 1 with *Dde*I and analyzed by agarose gel electrophoresis as shown in Fig. 2.

Genotype analysis of the polymorphic CYP2C19 gene was also performed to investigate the relationship of the genotype between the polymorphic CYP2C18 gene and the polymorphic CYP2C19 gene by a modification of the method of de Morais et al. [7]. Primers sets used in the study were primers 1 (5′-AATTACAACCAGAGCTTGGC-3′) and 2 (5′-TATCACTTTCCATAAAAGCAAG-3′) in the CYP2C19 gene. A PCR reaction was performed in a total volume of 50 μl in the presence of 10 pmol of each primer, 1.25 mM magnesium chloride, 10 mM Tris-HCl (pH 8.3), 200 μM of each dNTP, 400 ng of genomic DNA and 0.5 U of Taq DNA polymerase (Promega Co., Madison, WI, USA). The conditions for annealing, polymerization and denaturation were 55°C for 1 min, 72°C for 1 min, and 94°C for 1 min, respectively. The number of cycles amplified was 30. The amplified fragment was digested with *Sma*I (New England Biolab, Beverly, MA, USA) and was electrophoresed on 2% agarose gel.

2.5. Cloning of the PCR products and SSCP analysis

About 30 ng of the PCR products of the CYP2C18 gene were ligated with 50 ng of pT7Blue T-vector (Novagen, Madison, WI, USA) and transformed with NovaBlue cell. Plasmid DNA from the white colony was analyzed for restriction map and SSCP.

SSCP analysis was conducted as follows [10]. The insert of plasmid DNA was amplified by the secondary PCR with the same primers used as in the first amplification from genomic DNA. The secondary PCR was performed in a total volume of 25 μl in the presence of 1.5 mM magnesium chloride, 10 mM Tris-HCl (pH 8.3), 200 μM of each dNTP, 5 pmol of each primer, about 1 ng of plasmid DNA and 0.25 U *Pfu* DNA polymerase (Stratagene, La Jolla, CA, USA). The conditions for annealing, polymerization and denaturation were the same as in the first PCR. The number of amplified cycles was 15. 2 μl of the PCR reaction was mixed with 30 μl of formamide-dye buffer (0.1% bromophenol blue, 0.1% xylene cyanol FF, 20 mM EDTA in formamide) and denatured for 5 min at 80°C. 2 μl of the mixture was electrophoresed on a 5% acrylamide gel at 150 V for 8 to 10 h at 4°C. Separated DNA was stained with a Silver Stain Plus Kit (Bio-Rad, Tokyo, Japan).

2.6. Sequencing

Determination of the nucleotide sequence was performed by the dideoxy chain-termination method [11] with a Bcabest sequencing primer (5′-CGCCAGGGTTTTCCCAGTCACGAC-3′) using a Sequenase Version 2.0 kit (United States Biochemical, Cleveland, OH, USA) on both strands.

3. Results and discussion

3.1. Searching for a polymorphic allele of the CYP2C18 gene

The 5′-flanking region and 9 exons of the CYP2C18 gene were amplified by PCR (Fig. 1) and a polymorphic allele was surveyed by 42 restriction enzymes. For each fragment, PCR products from more than 16 individuals were analyzed for RFLP. Only a *Dde*I site, which is situated 479 bp upstream

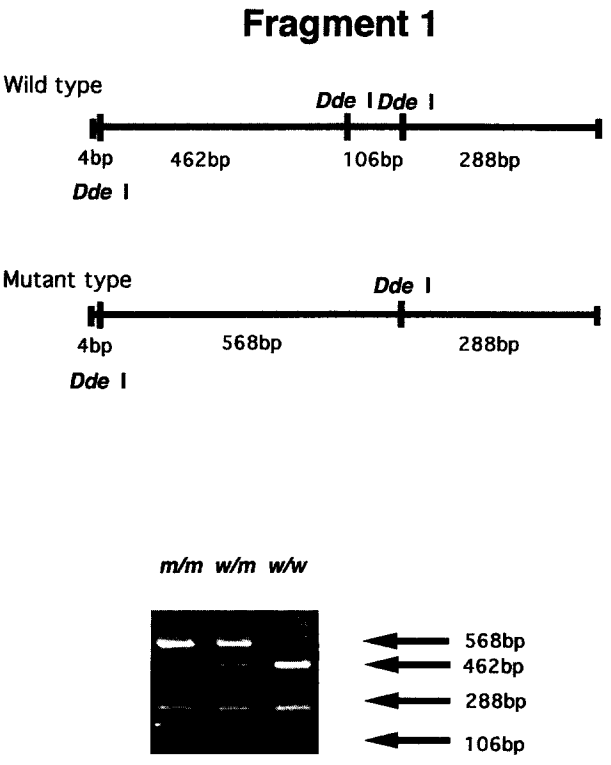

Fig. 2. Restriction maps for fragment 1 of the CYP2C18 gene for wild type and mutant type. The *Dde*I digestion patterns for three genotypes are shown in the lower panel.

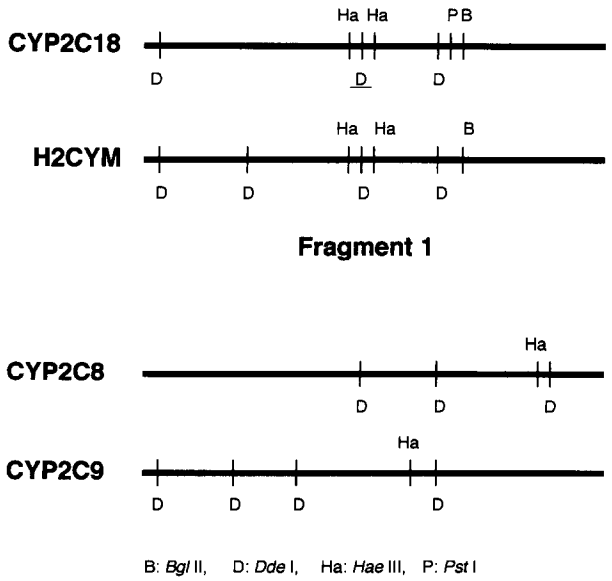

Fig. 3. Restriction map for the H2CYM clone. The restriction maps of the corresponding regions for CYP2C18, CYP2C8 and CYP2C9 are shown for comparison. A polymorphic *Dde*I site in the CYP2C18 gene is underlined.

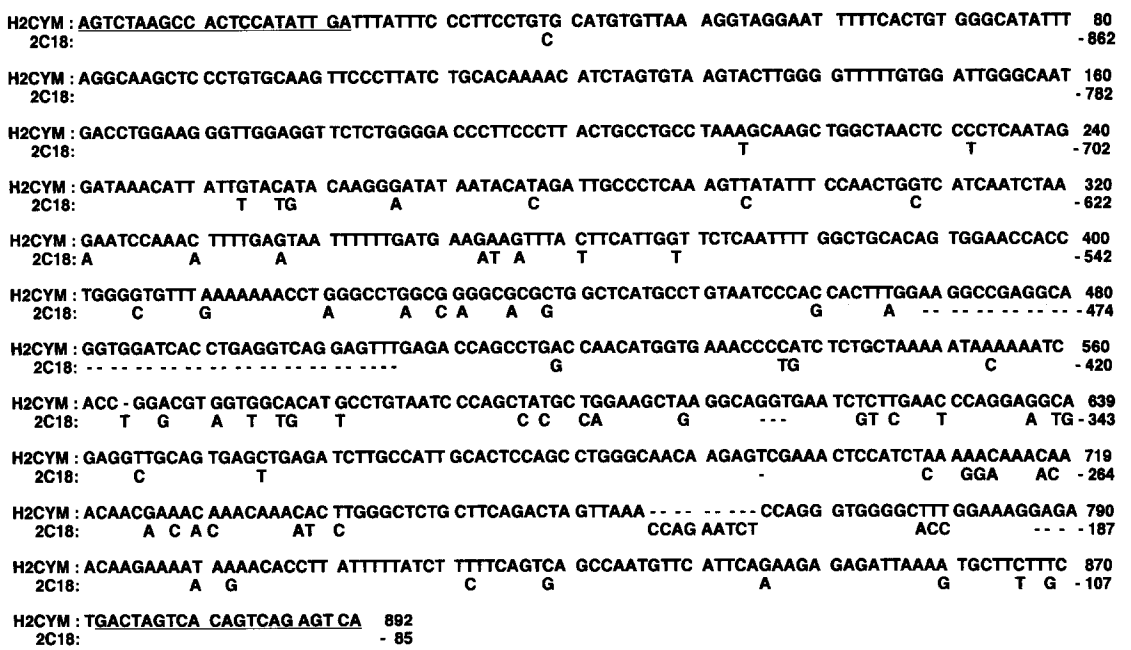

Fig. 4. Nucleotide sequence of the clone H2CYM. Nucleotide sequence for the corresponding region of CYP2C18 is shown for comparison. Only the nucleotide sequences that are different from H2CYM are indicated. The locations of the nucleotide sequences used for synthesizing primers are underlined. −: gap site. The nucleotide sequence of H2CYM has been deposited in the GenBank under the accession no. D 78379.

from the first codon of the CYP2C18 gene, was found to be polymorphic while other sites were monomorphic. The restriction enzyme map and the *Dde*I digestion patterns of the 5′-flanking fragment (fragment 1) are shown in Fig. 2. In the wild type homozygote (w/w), heterozygote (w/m) and mutant homozygote (m/m), three bands (462 bp, 288 bp and 106 bp), four bands (568 bp, 462 bp, 288 bp and 106 bp) and two bands (568 bp and 288 bp) were seen, respectively. Ibeanu and Goldstein [12] investigated the promoter region of the CYP2C9 and CYP2C18 genes, and showed that the 5′-flanking region of the CYP2C18 gene had low promotional activity. Although the polymorphic *Dde*I site of the CYP2C18 gene is located close to the potential AP-2 site [13], the functional property of the mutation such as in the expression of the gene is not clear and should be analyzed by site-directed mutagenesis.

3.2. *Analysis for genotypes of the CYP2C18 gene and the CYP2C19 gene*

The polymorphic *Dde*I site in the 5′-flanking region of the CYP2C18 gene was analyzed for 56 healthy controls and the genotypes are shown in Table 1. The allele frequency of the mutant CYP2C18 gene was 21.4%. The genotype of the polymorphic CYP2C18 gene was consistent with that of the m1 mutation [7] of the CYP2C19 gene. This result suggests that the CYP2C18 and CYP2C19 genes are linked and closely located on the chromosome.

Table 1. Genotype distributions of the *Dde*I site in the CYP2C18 gene of the healthy controls

	w/w	w/m	m/m	Total
Healthy controls	35 (62.5)	18 (32.1)	3 (5.4)	56

Within parentheses are the percentages of the total. w and m indicate the wild type gene and mutant type gene, respectively.

3.3. *Searching for a member of the CYP2C subfamily in the human genome*

The faint bands, which were visible when the PCR products were digested with several restriction enzymes, could be derived from the CYP2C subfamily members that are different from CYP2C18. The products of PCR were examined in order to determine which member of the CYP2C subfamily had been amplified. The PCR products from 9 regions were cloned into the plasmid, and more than 10 clones from each fragment were analyzed by restriction enzyme mapping and SSCP (Table 2). All except for 5 clones from the 5′-flanking fragment showed identical restriction maps and SSCP patterns to those of CYP2C18. A restriction map of the 5 clones, which was different from the corresponding regions of the CYP2C8 or CYP2C9 genes, was similar to the CYP2C18 gene as shown in Fig. 3. Since the nucleotide sequences of the corresponding region for CYP2C19 have not been reported, these 5 clones contain the 5′-flanking region which is different from any reported genes of the CYP2C subfamily.

The nucleotide sequence of H2CYM, one of the 5 clones

Table 2. Number of clones analyzed for restriction enzyme mapping and SSCP

Fragment No.	1	2	3	4	5	6	7	8	9
Length (bp)	860	1092	703	159	180	141	189	141	226
No. of clones analyzed	37 (5)	21	15	10	28	27	9	18	20

Within parentheses are the numbers of clones different from CYP2C18.

described above, has been determined (Fig. 4). The CYP2C18 gene showed the highest homology to H2CYM. Excluding the insertions and deletions, the number of nucleotides that are different between CYP2C18 and H2CYM was 76 out of 804 sites. Therefore, the nucleotide sequence of H2CYM was 90.5% identical to the corresponding region of the CYP2C18 gene. Several conserved sites could be aligned in the corresponding regions of the CYP2C8 and CYP2C9 genes. Since the CYP2C18 gene and H2CYM are 90.5% homologous in the 5′-flanking region, there should be no nucleotide difference or only minimal differences in the regions of 9 exons. Alternatively, the exon regions for H2CYM were not amplified with the PCR conditions used here. The nucleotide sequence of H2CYM is different from either CYP2C8 or CYP2C9, however, it is not clear whether H2CYM is CYP2C19 or not. Further sequence analysis of the CYP2C19 gene or gene corresponding to H2CYM should be conducted.

Acknowledgements: We are grateful to Dr. K. Iwahashi for collecting peripheral blood from healthy controls. Our thanks are also due to K. Ooi for helping with the experiments.

References

[1] Goldstein, J.A. and de Morais, S.M.F. (1994) Pharmacogenetics 4, 285–299.
[2] Romkes, M., Faletto, M.B., Blaisdell, J.A., Raucy, J.L. and Goldstein, J.A. (1991) Biochemistry 30, 3247–3255.
[3] Kupfer, A. and Preisig, R. (1984) Eur. J. Clin. Pharmacol. 26, 753–759.
[4] Wedlund, P.J., Aslanian, W.S., McAllister, C.B., Wilkinson, G.R. and Branch, R.A. (1984) Clin. Pharmacol. Ther. 36, 773–780.
[5] Wrighton, S.A., Stevens, J.C., Becker, G.W. and VandenBranden, M. (1993) Arch. Biochem. Biophys. 306, 240–245.
[6] Goldstein, J.A., Faletto, M.B., Romkes-Sparks, M., Sullivan, T., Kitareewan, S., Raucy, J.L., Lasker, J.M. and Ghanayem, B.I. (1994) Biochemistry 33, 1743–1752.
[7] de Morais, S.M.F., Wilkinson, G.R., Blaisdell, J., Meyer, U.A., Nakamura, K. and Goldstein, J.A. (1994) Mol. Pharmacol. 46, 594–598.
[8] Kan, Y.W. and Dozy, A.M. (1978) Proc. Natl. Acad. Sci. USA 75, 5631–5635.
[9] Ged, C. and Beaaune, P. (1992) Pharmacogenetics 2, 109–115.
[10] Orita, M., Suzuki, Y., Sekiya, T. and Hayashi, K. (1989) Genomics 5, 874–879.
[11] Sanger, F., Nicklen, S. and Coulsen, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[12] Ibeanu, G.C. and Goldstein, J.A. (1995) Biochemistry 34, 8028–8036.
[13] de Morais, S.M.F., Schweikl, H., Blaisdell, J. and Goldstein, J.A. (1993) Biochem. Biophys. Res. Commun. 194, 194–201.