ORIGINAL ARTICLE

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Novel mutations in the cytochrome P450 2C19 gene: a pitfall of the PCR-RFLP method for identifying a common mutation

Abstract CYP2C19 is a clinically important enzyme involved in the metabolism of therapeutic drugs such as (S)-mephenytoin, omeprazole, proguanil, and diazepam. Individuals can be characterized as either extensive metabolizers (EM) or poor metabolizers (PM) on the basis of CYP2C19 enzyme activity. The PM phenotype occurs in 2–5% of Caucasian populations, but at higher frequencies (18–23%) in Asians. CYP2C19*2 and CYP2C19*3, which are single-nucleotide polymorphisms of CYP2C19, are the main cause of PM phenotyping in homozygotes or compound heterozygotes. We report two novel mutations in the CYP2C19 gene identified by direct sequencing and subcloning procedures. One of these mutations was considered to be CYP2C19*3 by polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP). This result suggests that mutations classed as CYP2C19*3 might include other mutations. Further studies are needed to clarify the relationship between these novel mutations and enzyme activity.

Keywords CYP2C19 · PCR-RFLP · Diazepam · Extensive metabolizers · Poor metabolizers

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Introduction

Polymorphisms of cytochrome P450 (CYP) enzymes in humans are the main cause of differences in the metabolic activities of therapeutic drugs. Genetic polymorphisms of CYP2C19, which are associated with 4′-hydroxylation of (S)-mephenytoin, have been studied extensively (Kupher and Preisig 1984; Wedlund et al. 1984; Wilkinsin et al. 1989). This 4′-hydroxylation of (S)-mephenytoin has been shown to be mediated by CYP2C19 (Goldstein et al. 1994; Wrighton et al. 1993). This polymorphism affects the metabolism of several other commonly used drugs, such as omeprazole (Andersson et al. 1992), proguanil (Ward et al. 1991), certain barbiturates (Adedoyin et al. 1994; Kupfer and Branch 1985), and citalopram (Sindrup et al. 1993); the oxidation of propranolol (Ward et al. 1989), certain tricyclic antidepressants (Skjelbo et al. 1991), and diazepam (Bertilsson et al. 1989) is also affected.

Individuals can be characterized as either extensive metabolizers (EM) or poor metabolizers (PM) on the basis of the enzymatic activity of CYP2C19. The PM phenotype is inherited in an autosomal recessive fashion in homozygotes with affected genes (Ward et al. 1987; Inaba et al. 1986). The EM phenotype has either homozygous-dominant or heterozygote genotypes. There are marked interracial differences in the frequency of this polymorphism. For example, the PM phenotype occurs in 2–5% of Caucasian populations, but at higher frequencies (18–23%) in Asians (Japanese, Chinese, and Koreans; Kupher and Preisig 1984; Nakamura et al. 1985). Large interphenotypic

differences occur in the disposition of these drugs, which may affect their efficacy and toxicity. PMs can experience undesirable side effects after the administration of diazepam, such as prolonged sedation and unconsciousness. In contrast, omeprazol has been reported to produce a greater cure rate for gastric ulcers and accompanying *Helicobacter pylori* infections in CYP2C19 PMs than in EMs, because blood levels are higher in PMs (Goldstein 2001). Moreover, the improvement rate in reflux oesophagitis after 8 weeks of lansoprazole medication varies from 77.4% (homozygous EM) to 100% (PM) (Kawamura et al. 2003).

De Morais et al. (1994a) reported that two principal genetic defects were responsible for the PM phenotype of (S)-mephenytoin 4′-hydroxylation in Japanese subjects. The first mutation is a single-base pair mutation (guanine to adenine) in exon 5 of CYP2C19, which creates an aberrant splice site (CYP2C19*2) (De Morais et al. 1994b). The second mutation is at position 636 of exon 4 of CYP2C19, which creates a premature stop codon (CYP2C19*3) (De Morais et al. 1994b). Fourteen different mutations in CYP2C19 have been described to date (Table 1).

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) is a simple and widely used technique. The restriction enzyme *Sma*I (recognizes CCCGGG) detects CYP2C19*2, and *Bam*HI (recognizes GGATCC) detects CYP2C19*3. However, if a mutation occurs in both recognition sequences, the two mutations cannot be distinguished.

We report novel two mutations in the CYP2C19 gene. These mutations show the same PCR-RFLP pattern as CYP2C19*3, which is one of the common mutations reported previously.

Materials and methods

Venous blood samples and DNA purification

From each subject, 1 ml venous blood was collected. The 125 healthy adult volunteers had no diazepam administration. Seventy-eight samples were collected from pediatric patients treated with diazepam because of febrile convulsion or epilepsy (14 patients) and sedation for cardioangiography (64 patients); only one of the pediatric patients experienced dizziness after receiving diazepam in his food. Informed consent was obtained from all volunteers and patients. Samples were divided into 200 μl aliquots in Eppendorf tubes, and kept at −20°C until required. Leukocyte genomic DNA was extracted directly from the blood samples using a QIAamp Blood Mini Kit (Qiagen, Tokyo, Japan) according to the manufacturer's protocol.

PCR amplification of CYP2C19*2 and CYP2C19*3 regions

Genomic DNA (100 ng) was amplified in 2× PCR Master Mix (50 U/ml *Taq* DNA polymerase; 400 μM dATP, dCTP, dGTP, and dTTP; 3.0 mM $MgCl_2$; Promega, Madison, WI) containing nuclease-free water in a total volume of 50 μl, with PCR primers at a concentration of 0.8 μM. A previously reported PCR primer set for CYP2C19*2 (De Morais et al. 1994b; Romkes et al. 1991) and our PCR primer set for CYP2C19*3 were used in this study (Table 2). Amplification of these two regions was performed in a separate tube with a Gene Amp PCR system 9700 (Applied Biosystems, Foster City, CA) using an initial denaturation step of 94°C for 10 min; 40 cycles

Table 1 CYP2C19 alleles reported to date. CYP2C19*1A, CYP2C19*1B, and CYP2C19*1C are wild type; CYP2C19*2A–CYP2C19*15 are mutation alleles

Allele	Trival name	Enzyme activity	Effect	Reference
CYP2C19*1A	wt1	Active		Romkes et al. (1991)
CYP2C19*1B	wt2	Active	I331V	Richardson et al. (1995)
CYP2C19*1C		Active	I331V	Blaisdell et al. (2002)
CYP2C19*2A	m1;m1A	Inactive	Splicing defect	De Morais et al. (1994b)
CYP2C19*2B	m1B	Inactive	Splicing defect; E92D	Ibeanu et al. (1998a, b)
CYP2C19*3	m2	Inactive	Stop codon	De Morais et al. (1994b)
CYP2C19*4	m3	Inactive	GTG initiation codon	Ferguson et al. (1998)
CYP2C19*5A	m4	Inactive	R433W	Xiao et al. (1997)
CYP2C19*5B		Inactive	I331V; R433W	Ibeanu et al. (1998a, b)
CYP2C19*6	m5		R1329; I331V	Ibeanu et al. (1998a, b)
CYP2C19*7			Splicing defect	Ibeanu et al. (1999)
CYP2C19*8			W120R	Ibeanu et al. (1999)
CYP2C19*9			R144H; I331V	Blaisdell et al. (2002)
CYP2C19*10			P227L; I331V	Blaisdell et al. (2002)
CYP2C19*11			R150H; I331V	Blaisdell et al. (2002)
CYP2C19*12			I331V; X491C	Blaisdell et al. (2002)
CYP2C19*13			I331V; R410C	Blaisdell et al. (2002)
CYP2C19*14			L17P; I331V	Blaisdell et al. (2002)
CYP2C19*15			I19L; I331V	Blaisdell et al. (2002)

Table 2 PCR primer set used in this study. The primer set used for CYP2C19*2 was previously reported (De Morais et al. 1994b; Romkes et al. 1991), and we created a primer set for CYP2C19*3

	Forward primer
CYP2C19*2	5′-ATTACAACCAGAGCTTGGC-3′
CYP2C19*3	5′-ACTTTCATCCTGGGCTGTGC-3′
	Reverse primer
CYP2C19*2	5′-TATCACTTTCCATAAAAGCAAG-3′
CYP2C19*3	5′-ACTTCAGGGCTTGGTCAATA-3′

of 94°C for 30 s, 53°C for 30 s, and 72°C for 30 s; and a final extension step of 72°C for 5 min. The amplified PCR products (167 bp for the CYP2C19*2 region and 233 bp for the CYP2C19*3 region) were analyzed on a 2% agarose gel with a 50 bp ladder (Invitrogen, Carlsbad, CA) as a molecular weight marker.

Restriction enzyme digestion of PCR products

To detect the CYP2C19*2 defect, 15 μl PCR product was digested with 5 U *Sma*I (TOYOBO, Tokyo, Japan) in a supplemented reaction buffer in a total volume of 20 μl at 30°C for 1 h. To detect the CYP2C19*3 defect, 15 μl PCR product was digested with 5 U *Bam*HI (TOYOBO, Tokyo, Japan) in a supplemented reaction buffer in a total volume of 20 μl at 37°C for 1 h. Both digested products were analyzed on a 4% agarose gel. The wild type appears as two bands of digestion products (117 and 50 bp for CYP2C19*2, and 137 and 96 bp for CYP2C19*3). On the other hand, the homozygous mutated type appears as a single band of undigested product (167 bp for CYP2C19*2, and 233 bp for CYP2C19*3). If all products (undigested and digested) appeared on the gel, the subject was a heterozygote.

Direct sequencing

All samples from homozygous or heterozygous subjects, as detected by PCR-RFLP, were amplified for direct sequencing. Amplification was performed using a Gene Amp PCR System 9700, for 40 cycles consisting of denaturation at 94°C for 30 s, annealing at 53°C for 30 s, and extension at 72°C for 30 s. An initial denaturation step at 94°C for 10 min and a final extension step at 72°C for 5 min were also performed. PCR products were analyzed on 2% agarose gels. For sequencing, the PCR product was purified and an aliquot was used in the cycle sequencing reaction by a DNA sequencing kit (Applied Biosystems), the same primer used in the PCR (Table 2), and an automated sequencer (Applied Biosystems).

Subcloning

CYP2C19 cDNA was amplified by PCR using the primers listed in Table 2. The PCR products were purified by a GENECLEAN SPIN Kit (Qbiogene, Irvine, CA), ligated into the pGEM-T Easy Vector (Promega), and transformed into *Escherichia coli* JM109 (Promega). Transformed samples were plated on LB-agar (Becton Dickinson, Franklin Lakes, NJ), and cultivated at 37°C overnight. A white colony was inoculated into LB-broth (Becton Dickinson) and shaken in a bio-shaker at 200 rpm at 37°C overnight. Plasmid was then extracted using the Wizard Plus SV Minipreps DNA purification system (Promega). A sequencing reaction was performed, and the sample was applied to an automated sequencer (Applied Biosystems).

Results

In the wild type gene, cleavage of PCR products yields fragments of 117 and 50 bp for CYP2C19*2, and 137 and 96 bp for CYP2C19*3. With individuals homozygous for CYP2C19*2, the *Sma*I site in exon 5 is destroyed and the 167 bp fragment is not cut, whereas in CYP2C19*3, the *Bam*HI site in exon 4 is destroyed and

Fig. 1 a PCR amplification of exon 5 followed by *Sma*I digestion. Lanes: *1* 50-bp ladder; *2* wild type (CYP2C19*1)—bands at 50 and 117 bp; *3* homozygous CYP2C19*2/CYP2C19*2—a single 167 bp band. **b** PCR amplification of exon 4 of the same subject as in **a**, followed by *Bam*HI digestion. Lanes: *1* 50 bp ladder; *2* wild type (two bands: 96 and 137 bp); *3* heterozygote determined as CYP2C19*3 by electrophoresis (three bands: 96, 137, and 233 bp)
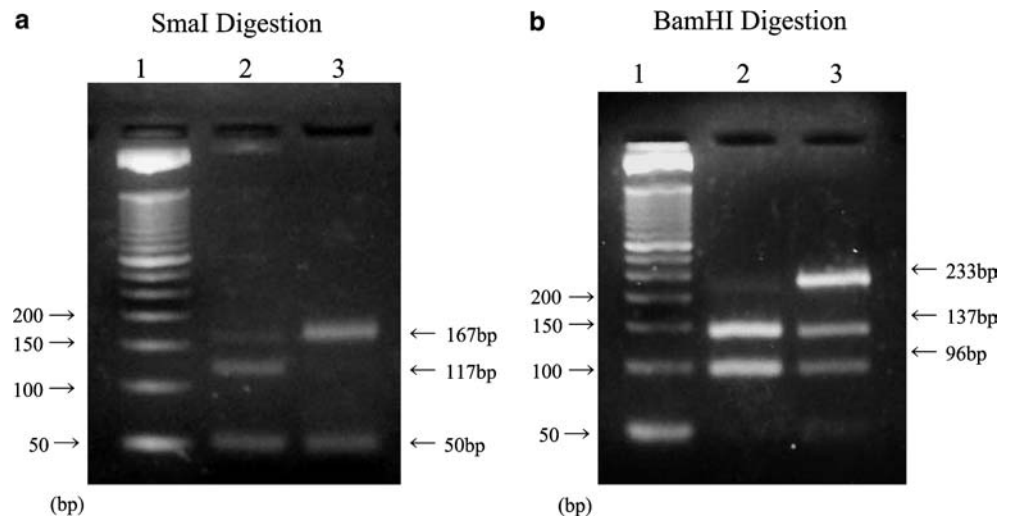
the 233 bp fragment is not cut. With heterozygous individuals, all three bands (50, 117, and 167 bp for CYP2C19*2; and 96, 137, and 233 bp for CYP2C19*3) are evident. Allele frequencies of CYP2C19*2 and CYP2C19*3 were 0.296 and 0.128 (120 and 52 of the total of 406 alleles), respectively.

In one of the samples the following were present simultaneously: one band of 167 bp in gel electrophoresis of exon 5, and three bands (233, 137, and 96 bp) in gel electrophoresis of exon 4 (Fig. 1). The homozygous mutation of CYP2C19*2 (guanine to adenine in exon 5) was detected by direct sequencing as reported previously. In exon 4, instead of the CYP2C19*3 mutation, two cytosine-to-guanine changes at positions 612 and 639 were detected, corresponding to amino acid substitutions Asn204Lys and Ile213Met . One of these substitutions, at position 639, overlaps with the *Bam*HI recognition site. We also performed subcloning so as to determine which of the new mutations was heterozygous: two mutations were simultaneously present in one allele (Fig. 2). The frequency of this allele was 0.0025 (1 of the total of 406 alleles).

Discussion

CYP2C19 plays an important role in the metabolism of many types of drug (Kupher and Preisig 1984; Wedlund et al. 1984; Wilkinsin et al. 1989). Polymorphisms in CYP2C19 are known to affect the metabolic activity of some commonly used drugs, such as omeprazol (Andersson et al. 1992), proguanil (Sindrup et al. 1993), and certain tricyclic antidepressants (Skjelbo et al. 1991). The relation between diazepam and CYP2C19 is especially important in pediatric patients. Diazepam is a widely used as an anticonvulsant drug because of its

rapid effect on convulsions with intravenous or per rectum administration. PMs of CYP2C19 can experience undesirable side effects after the administration of diazepam, such as prolonged sedation and unconsciousness. Occasionally, respiratory suppression and distraction occur. If a CYP2C19 polymorphism can be identified before medication, the most appropriate drug regime can be determined for each individual. A modified PCR-RFLP technique was used to determine the details of CYP2C19 polymorphism in a Japanese population. The PCR-RFLP method is a rapid and simple technique for detecting genetic defects (Itoh et al. 1999).

Generally, homozygotes and compound heterozygotes are termed PM, and homozygous dominant and heterozygotes are termed EM. In this study, CYP2C19*2/CYP2C19*2, CYP2C19*3/CYP2C19*3, and CYP2C19*2/CYP2C19*3 were designated as PM phenotypes, and CYP2C19*1/CYP2C19*1, CYP2C19*1/CYP2C19*2, and CYP2C19*1/CYP2C19*3 were designated as EM phenotypes. Incidence of PM phenotypes was 18.2% (homozygous for CYP2C19*2/CYP2C19*2:15, homozygous for CYP2C19*3/CYP2C19*3:3, compound heterozygous for CYP2C19*2/CYP2C19*3:19), which is in good agreement with a previous report (Kubota et al. 1996)

De Morais et al. (1994a) reported that all Japanese EMs have at least one wild-type allele, and that CYP2C19m1(CYP2C19*2) and CYP2C19m2(CYP2C19*3) are allelic and segregate as two independent mutated alleles. However, in one of our cases a homozygote of CYP2C19*2 and heterozygote of CYP2C19*3 were detected by PCR-RFLP. This indicates that it is possible for CYP 2C19*2 and CYP 2C19*3 to exist on the same allele. Direct sequencing of CYP2C19*2 and CYP2C19*3 was performed, and CYP2C19*3 of this subject was subcloned. As a result of subcloning, we

Fig. 2 a Direct sequencing was performed in the case of the subject considered to be a mutation of CYP2C19*3 by PCR-RFLP. As a result of the sequencing, this subject was considered heterozygous and subcloning of the fragment was performed. **b** Sequencing of wild type CYP2C19. The *Bam*HI recognition site is underlined. An *arrow* indicates the new mutation discovered in this study. Cytosine-to-guanine changes occurred at positions 612 and 639, corresponding to amino acid substitutions Asn204Lys and Ile213Met. Of these, the mutation at position 639 lies within the *Bam*HI recognition sequence
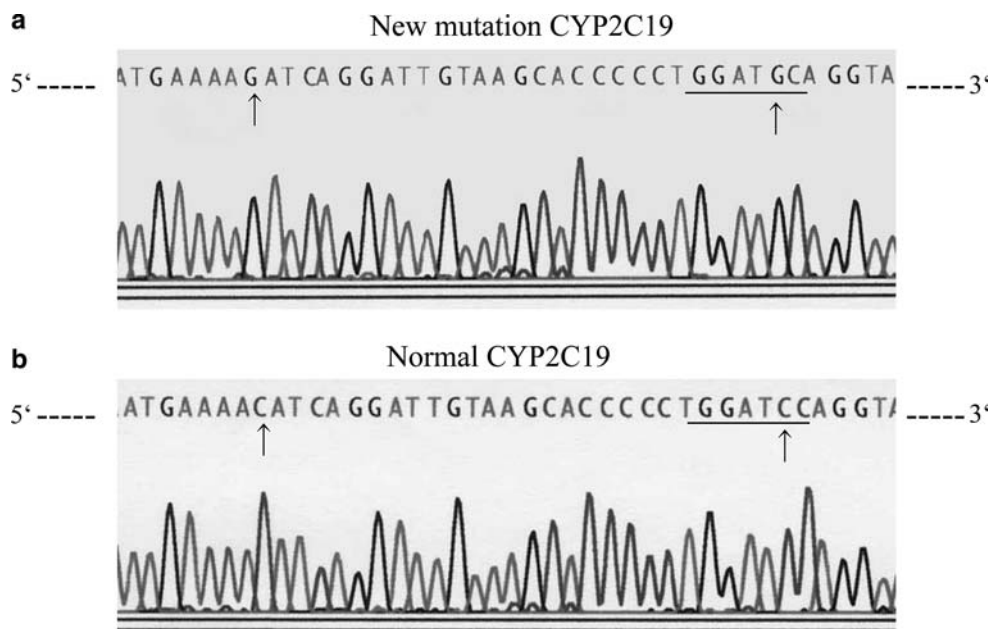
found a new mutation on the allele considered to be CYP2C19*3, and another mutation on the same allele (Fig. 2).

In this case, the phenotype is expected to be PM due to the homozygous CYP2C19*2 genotype. This volunteer subject experienced dizziness after a normal pediatric dose of diazepam. Enzyme activity was not measured because it requires a liver biopsy. The two mutations—at positions 612 and 639—in the same allele indicated that amino acid substitution had occurred in two almost adjacent locations, which may result in decreased activity. However, we have not yet clarified the effects of the new mutation.

If two mutations (CYP2C19*2 and the new mutation reported here) exist on the same allele, even if found with a compound heterozygote (CYP2C19*2/CYP2C19*3) by RFLP, the wild-type allele will still exist. Previous authors have stated that where EMs have at least one wild-type allele, such cases will have EM phenotype. However, a compound heterozygote is usually PM phenotype, indicating an inconsistency of phenotype arising here.

Our results warn of a pitfall of the PCR-RFLP method. In recent years, many population studies have used only PCR-RFLP. Although PCR-RFLP is a simple and very useful screening method, mutations should be confirmed by sequence analysis, especially in compound heterozygotes. Our results indicate that the mutation considered previously to be CYP2C19*3 through PCR-RFLP, may actually be a new mutation in practice. In polymorphisms of CYP2C19, mutations of CYP2C19*2 and CYP2C19*3 are very important. Although the case reported here is probably rare, reconsideration of past CYP2C19*3 subjects is warranted.

The effects of CYP2C19 on drug metabolism are very interesting, and there is a need for further research—including how to positively identify the phenotype—to obtain further information on how best to supply safe and effective medical treatment. Moreover, characterizing the phenotype will contribute greatly to tailor-made medical treatment.

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