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ACCELERATED COMMUNICATION

Identification of a New Genetic Defect Responsible for the Polymorphism of (*S*)-Mephenytoin Metabolism in Japanese

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SUMMARY

A genetic polymorphism in the metabolism of the anticonvulsant drug (*S*)-mephenytoin has been well documented in humans. There are marked interracial differences in the frequency of the poor metabolizer phenotype, which comprises 2–5% of Caucasian but 18–23% of Asian populations. We have recently reported that the principal genetic defect responsible for the poor metabolizer phenotype is a single-base pair mutation in exon 5 of *CYP2C19* (*CYP2C19*$_m$), which accounts for ~75–83% of the defective alleles in both Japanese and Caucasians subjects. In the present study, we have identified a new mutation (*CYP2C19*$_{m2}$) in Japanese poor metabolizers, consisting of a guanine to adenine mutation at position 636 of exon 4 of *CYP2C19*, which creates a premature stop codon. Genotyping of seven Japanese poor metabolizers who were not homozygous for the previously described *CYP2C19*$_m$ defect (now designated *CYP2C19*$_{m1}$) indicated that they were either homozygous for the new defect (*CYP2C19*$_{m2}$/*CYP2C19*$_{m2}$) or heterozygous (*CYP2C19*$_{m1}$/*CYP2C19*$_{m2}$) for the two defects. *CYP2C19*$_{m1}$ accounts for 25 of 34 alleles in Japanese poor metabolizers, whereas *CYP2C19*$_{m2}$ accounts for the remaining nine alleles. Hence, *CYP2C19*$_{m1}$ and *CYP2C19*$_{m2}$ explain 100% of the available Japanese poor metabolizers (34 alleles). In contrast, the *CYP2C19*$_{m2}$ defect was not detected in nine Caucasian poor metabolizers (83% of available poor metabolizer alleles were *CYP2C19*$_{m1}$), indicating the existence of another, as yet unidentified, mutation. Genetic testing of the families of two Japanese poor metabolizer probands showed that coinheritance of the *CYP2C19*$_{m1}$ and *CYP2C19*$_{m2}$ alleles was concordant with the autosomal recessive inheritance of the poor metabolizer phenotype.

A genetic polymorphism associated with the 4′-hydroxylation of (*S*)-mephenytoin is one of the most widely studied polymorphisms of the CYP enzymes in humans (1–3). Its characteristics include autosomal recessive inheritance of the PM trait (4, 5) and considerable racial heterogeneity in the frequency of the PM phenotype (3, 6). For example, in Caucasians of European descent the PM phenotype is present in only 2–5% of the population, but in Asians (Japanese, Chinese, and Koreans) the frequency is much higher (18–23%). The 4′-hydroxylation of (*S*)-mephenytoin has been shown to be mediated by CYP2C19 (7, 8). CYP2C19 is also important in the *in vivo* metabolism of a number of related hydantoins and barbiturates (3, 9, 10), as well as in that of structurally dissimilar drugs such as omeprazole (11), proguanil (12), and citalopram (13). As a result, large interphenotypic differences occur in the disposition of these drugs, which may affect their efficacy and toxicity. The metabolism of propranolol (14), certain tricyclic antidepressants (15–17), and possibly diazepam (18) is also mediated by CYP2C19, albeit to a lesser extent.

The impaired metabolism of (*S*)-mephenytoin in PMs results from the defective hepatic expression of CYP2C19 in human liver (7, 8). We have recently demonstrated that the major defect in PMs is a guanine to adenine mutation in exon 5 of *CYP2C19*, which produces an aberrant splice site and a truncated nonfunctional protein (19). This defect accounts for ~75–83% of PM alleles in both Japanese and Caucasian subjects. The present study identifies a new mutation that is responsible for the remainder of the defective alleles in Japanese PMs but apparently is not present or is rare in Caucasians.

Materials and Methods

DNA was isolated (20) from peripheral blood obtained from selected Caucasian and Japanese subjects who had been previously phenotyped

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ABBREVIATIONS: CYP, cytochrome P450; PM, poor metabolizer; EM, extensive metabolizer; PCR, polymerase chain reaction; bp, base pair(s).

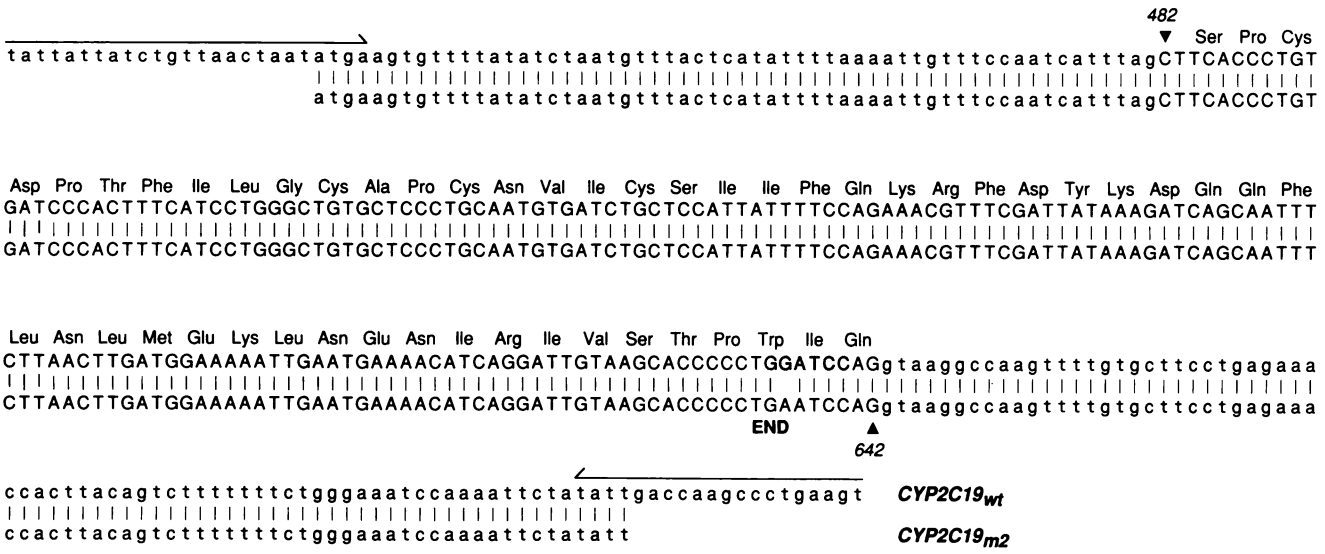

Fig. 1. Sequence alignment of the normal (*CYP2C19*$_{wt}$) and defective (*CYP2C19*$_{m2}$) genes, with the corresponding amino acids indicated above the nucleotide sequence. *END*, the new termination codon TGA in *CYP2C19*$_{m2}$. *Gray box*, the *Bam*HI site. *Solid horizontal arrows in the sequence*, PCR primers. *Vertical arrowheads*, predicted intron/exon junctions, based on comparison with the gene structure of *CYP2C9* and *CYP2C18* (22). *Numbers*, positions in the *CYP2C19* cDNA of the first and last nucleotides of exon 4. The sequence alignment was performed using GAP, of the Genetics Computer Group (Madison, WI) software package.

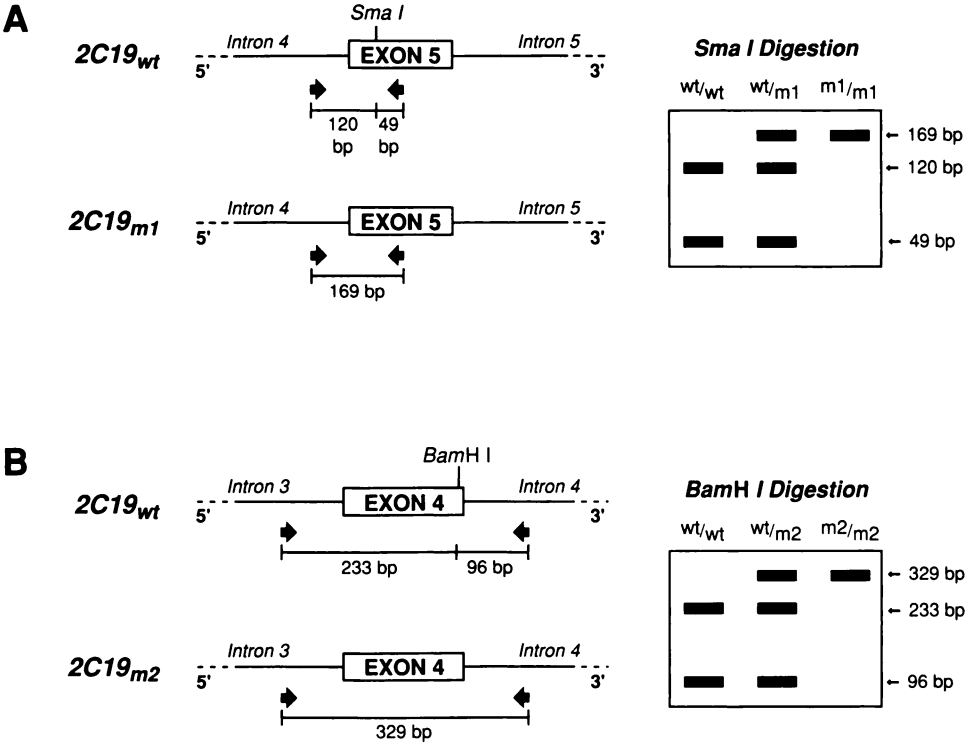

Fig. 2. Strategy used to genotype genomic DNA from human blood, utilizing PCR amplification of exon 5 followed by *Sma*I digestion (*CYP2C19*$_{m1}$) (A) and amplification of exon 4 followed by *Bam*HI digestion (*CYP2C19*$_{m2}$) (B). The predicted sizes of the digested DNA fragments for the various genotypes are shown.

(19). In American and Japanese subjects, this phenotyping was based on the urinary *S/R* ratio, as described previously (2), with PMs being characterized by a value above 0.9. Swiss subjects were phenotyped using the "hydroxylation index," with an hydroxylation index value of >5.6 identifing the deficient trait (1). All of these populations contained an intended over-representation of PMs. One American subject (subject 511) previously reported to be a PM (19) had, in fact, been misidentified, and subsequent phenotyping found him to be an EM. Therefore, the populations contained 18 Caucasian EMs, nine Caucasian PMs, 12 Japanese EMs, and 17 Japanese PMs.

To identify possible new genetic defects, several exons and adjoining introns of *CYP2C19* were amplified using intron-specific primers[1] for *CYP2C19* and DNA from a Japanese PM subject (subject 43) that did not contain the mutation *CYP2C19*$_m$ described in our previous study (19). Accordingly, this individual was presumed to have a second, unidentified, defect in *CYP2C19*. Genomic DNA (200 ng) was amplified in 1× PCR buffer [67 mM Tris·HCl, pH 8.8, 17 mM $(NH4)_2SO_4$, 10 mM β-mercaptoethanol, 7 μM EDTA, 0.2 mg/ml bovine serum albumin] containing 50 μM concentrations of dATP, dCTP, dGTP, and dTTP, 0.25 μM concentrations of PCR primers, 2.5 units of AmpliTaq DNA polymerase (Perkin Elmer Cetus), and 3.0 mM $MgCl_2$. For exon 4, the

[1] S. M. F. de Morais, J. Blaisdell, and J. A. Goldstein, unpublished observations.

TABLE 1

Genotypes of Japanese and Caucasian individuals phenotyped (EM or PM) for *S*)-mephenytoin 4′-hydroxylation

All samples were genotyped for both mutations.

	n*	No. of individuals with genotype					
		wt/wt	*wt/m1*	*m1/m1*	*wt/m2*	*m1/m2*	*m2/m2*
Japanese							
EM	12	5	4	0	3	0	0
PM	17	0	0	10	0	5	2
Caucasians							
Swiss							
EM	9	5	4	0	0	0	0
PM	6	0	1	5	0	0	0
American							
EM	9	7	2	0	0	0	0
PM	3	1	0	2	0	0	0

* *n*, Total number of individuals belonging to each phenotype.

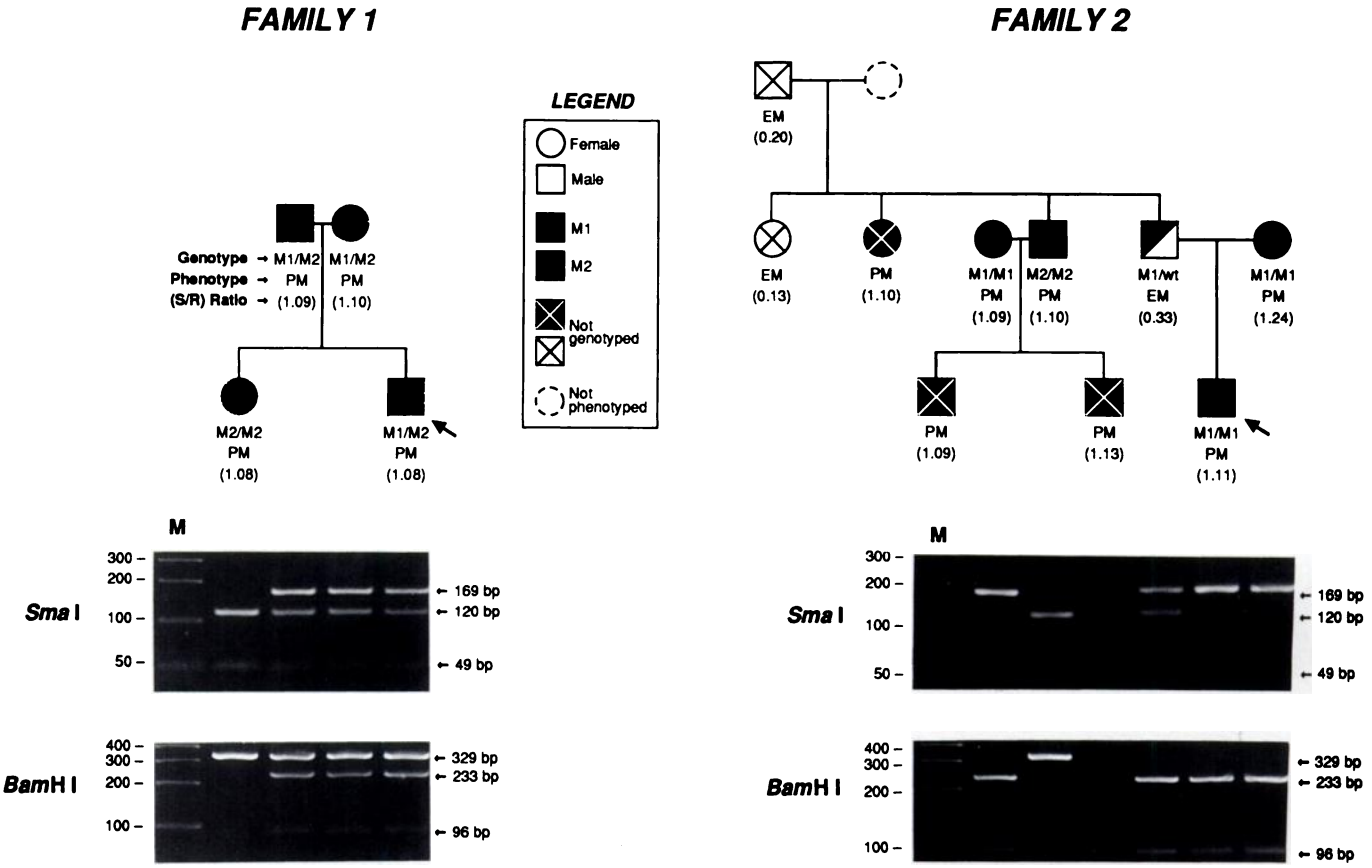

Fig. 3. Diagram of the family trees of two probands (*arrows*), showing their genotypes, as analyzed in gels of *Sma*I-digested exon 5 PCR products (detection of $CYP2C19_{m1}$) and *Bam*HI-digested exon 4 PCR products (detection of $CYP2C19_{m2}$). The expected sizes of the *Bam*HI-digested exon 4 PCR products and the *Sma*I-digested exon 5 PCR products are indicated in Fig. 2. The sizes of the molecular weight markers (*M*) are shown on the left. Heterozygous $CYP2C19_{m1}/CYP2C19_{m2}$ individuals from family 1 have all three bands for both the *Sma*I and *Bam*HI digestions. The genotypes agree with the previously published phenotypes of families B and D (4).

forward primer (5′-TATTATTATCTGTTAACTAATATGA-3′) anneals in intron 3, 78 bp upstream from the intron 3/exon 4 junction, and the reverse primer (5′-ACTTCAGGGCTTGGTCAATA-3′) anneals in intron 4, 88 bp downstream from the exon 4/intron 4 junction. Amplification was performed using a Perkin Elmer thermocycler, for 35 cycles consisting of denaturation at 94° for 1 min, annealing at 53° for 30 sec, and extension at 72° for 30 sec. An initial denaturation step at 94° for 5 min and a final extension step at 72° for 5 min were also performed. For sequencing, the PCR product was purified using Microcon (Amicon) columns, and an aliquot was used in the cycle sequencing reaction employing fluorescence-tagged dye terminators (PRISM; Applied Biosystems), the same forward primer used in the PCR, and an automated sequencer (Applied Biosystems).

In the genotyping procedure for the new defect ($CYP2C19_{m2}$), the primers used to amplify exon 4 were identical to those used to identify the defect, as described above. PCR products were digested with *Bam*HI in the PCR buffer, without purification. Digested PCR products were analyzed on 3% agarose gels and stained with ethidium bromide. The genotyping procedure for the previously described defect ($CYP2C19_{m1}$) was performed as described previously (19). Briefly, exon 5 was amplified using intron 4- and exon 5-specific primers for *CYP2C19*, and the PCR products were digested with *Sma*I and analyzed by gel electrophoresis. In the wild-type gene, the 169-bp PCR products are cut to

yield fragments of 120 and 49 bp. With individuals homozygous for $CYP2C19_{m1}$, the *Sma*I site in exon 5 is destroyed and the 169-bp fragment is not cut. With heterozygous individuals, all three bands (49 bp, 120 bp, and 169 bp) are observed.

Results and Discussion

Comparison of the DNA sequence from Japanese PM subject 43 with that of *CYP2C19* (21) identified a guanine to adenine mutation in exon 4, corresponding to position 636 of the cDNA, which creates a premature stop codon instead of amino acid 212 (Fig. 1).[2] This mutation would yield a truncated 211-amino acid CYP2C19 protein that lacks the heme-binding region as well as the majority of the putative substrate-binding regions and would, therefore, be inactive. The guanine to adenine mutation at position 636 destroys a *Bam*HI site. This feature was used to design a PCR-based genotyping procedure to identify this new defect, which was termed $CYP2C19_{m2}$ to distinguish it from a previously described defect (19) that is now designated $CYP2C19_{m1}$. Amplification of exon 4 of *CYP2C19* was performed using primers that anneal to introns 3 and 4 (Fig. 1), and the PCR products were then cut with *Bam*HI. The genotyping procedures for both $CYP2C19_{m1}$ and $CYP2C19_{m2}$ are shown in Fig. 2.

The $CYP2C19_{m2}$ genotype was determined in 29 Japanese subjects (12 EMs and 17 PMs) and 27 Caucasian subjects (18 EMs and nine PMs) previously genotyped for $CYP2C19_{m1}$ (19), and the combined results are shown in Table 1. All seven Japanese PMs whose phenotype could not previously be explained by the $CYP2C19_{m1}$ defect were found to be either homozygous for the new defect ($CYP2C19_{m2}/CYP2C19_{m2}$) (subjects 41 and 43) or heterozygous for the two defects ($CYP2C19_{m1}/CYP2C19_{m2}$) (subjects 11, 36, 48, 69, and 100). Accordingly, the mutations $CYP2C19_{m1}$ and $CYP2C19_{m2}$ explained all of the available 34 alleles in Japanese PMs. All Japanese EMs had at least one wild-type allele. The $CYP2C19_{m2}$ allele was not found in any individuals previously genotyped as being homozygous for $CYP2C19_{m1}$. Interestingly, the $CYP2C19_{m2}$ mutation was not found in any of the Caucasian subjects (Table 1). These included 18 EMs, one PM with an apparent $CYP2C19_{wt}/CYP2C19_{wt}$, genotype and one PM with an apparent $CYP2C19_{m1}/CYP2C19_{wt}$ genotype. It thus appears likely that at least one additional mutation that results in the PM phenotype occurs in Caucasians. Moreover, the $CYP2C19_{m2}$ allele appears to be rare or absent in Caucasians (none of 18 PM alleles and none of 36 EM alleles), compared with the high frequency (~25%) in Japanese subjects (nine of 34 PM alleles and three of 24 EM alleles).

The inheritance of $CYP2C19_{m1}$ in a Japanese family was concordant with the phenotype reported in our previous study (19). In the present investigation, genetic analysis of two other Japanese families from the original study of Ward *et al.* (4) showed that coinheritance of the $CYP2C19_{m1}$ and $CYP2C19_{m2}$ alleles accounted for the PM phenotype (Fig. 3). The inheritance of the genotypes was in complete agreement with the recessive inheritance of the PM phenotype. The results in Fig. 3 and Table 1 therefore confirm that $CYP2C19_{m1}$ and $CYP2C19_{m2}$ are allelic and segregate as two independent mutated alleles.

[2] The nucleotide sequences reported in this paper have been submitted to the GenBank/EMBL DataBank, with accession numbers L32982 and L32983.

In summary, a second mutation in *CYP2C19* that is responsible for the genetic polymorphism of (*S*)-mephenytoin 4′-hydroxylation in Japanese subjects has been identified. The defect consists of a single-base pair substitution in exon 4 of *CYP2C19*, which results in an early stop codon and produces a truncated and inactive protein. This mutation ($CYP2C19_{m2}$) accounts for approximately 25% of Japanese PM alleles. $CYP2C19_{m2}$ and the previously identified defect in exon 5 ($CYP2C19_{m1}$) thus account for 100% of the defective alleles for the available Japanese PMs. In contrast, approximately 83% of the available Caucasian PM alleles have been identified, and these all consist of the $CYP2C19_{m1}$ mutation in exon 5. The $CYP2C19_{m2}$ mutation is apparently rare or absent in this racial group. It will be of interest not only to establish the identity of the other mutations that account for the unidentified 15–20% of Caucasian PMs but also to determine the worldwide distribution frequencies of the different *CYP2C19* mutations in various populations. The PCR/restriction enzyme procedures developed to detect the $CYP2C19_{m2}$ and $CYP2C19_{m1}$ mutations can now be used for this purpose. These genotyping procedures will also be useful in clinical studies investigating the importance of the *CYP2C19* polymorphism in the metabolism of various drugs and its possible involvement in susceptibility to various disease states, such as cancer.

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