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Rapid Detection of *CYP2C18* Genotypes by Real-time Fluorescence Polymerase Chain Reaction

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Abstract

In man, CYP2C19, a liver enzyme, plays an important role in the metabolism of several drugs. Mutation of the *CYP2C19* gene results in a poor metaboliser phenotype. S-Mephenytoin hydroxylation genetic polymorphism is due to two mutations of the *CYP2C19* gene, namely *CYP2C19*2*, located in exon 5, and *CYP2C19*3*, located in exon 4. *CYP2C18* is also polymorphically expressed. The mutant alleles of this enzyme are *CYP2C18m1*, located in exon 2 and *CYP2C18m2*, located in the 5′-flanking region. We have developed an allele-specific TaqMan polymerase chain reaction (PCR) assay with which to detect *CYP2C18* mutant alleles. This assay combines hybridization of the TaqMan probe and allele-specific amplification primers to the target DNA. The TaqMan probe is labelled with 6-carboxyfluorescein at the 5′ end and 6-carboxytetramethylrhodamine together with a phosphate at the 3′ end. Genotypes are separated according to the different threshold cycles of the wild type and mutant primers.

We applied this procedure to DNA extracted from the blood or saliva of 144 healthy Japanese volunteers. The *wt/wt*, *wt/m1*, *wt/m2*, *m1/m1*, *m1/m2* and *m2/m2* genotypes of the *CYP2C18* alleles detected by the assay were consistent with the results obtained from restriction enzyme cleavage. In accordance with a previous report, the genotypes of *CYP2C18m1* and *CYP2C18m2* coincided with those of *CYP2C19*3* and *CYP2C19*2*, respectively. Therefore, detection of *CYP2C18* mutant alleles also allows that of *CYP2C19* mutant alleles. Among 19 poor metabolisers, eight showed the homozygous *CYP2C19*2/CYP2C19*2*, two the homozygous *CYP2C19*3/CYP2C19*3* and nine the compound heterozygous *CYP2C19*2/CYP2C19*3* genotype.

We found the allele-specific TaqMan PCR assay rapid, simple and cost-effective, as well as suitable for high-throughput applications in a routine laboratory. This assay allows the fast and reliable detection of inherited disorders that might influence diagnosis and treatment.

Human CYP2C is a major subfamily of enzymes in the liver, of which four members have been identified (CYP2C8, CYP2C9, CYP2C18 and CYP2C19; Goldstein & de Morais 1994). Of these, CYP2C19 plays the most important role in the metabolism of several drugs, such as omeprazole, lansoprazole, diazepam and proguanil (Goldstein & de Morais 1994). However, little is known about the functional role of this enzyme in-vivo, although it may contribute to the hydroxylation of warfarin (Goldstein & de Morais 1994), lansoprazole (Pichard et al 1995), tienilic acid (Jean et al 1996; Minoletti et al 1999) and diazepam (Jung et al 1997). CYP2C18 was also recently reported to be highly expressed in the human epidermis (Zaphiropoulos 1997).

The poor metaboliser phenotype is associated with *CYP2C19*2* and, less commonly, *CYP2C19*3* genetic mutations. A single G to A transition causes both mutations. The *CYP2C19*2* mutation is in exon 5 and that of *CYP2C19*3* is in exon 4 (de Morais et al 1994a, b). Poor metabolisers have two defective alleles, whereas those who are either homozygous or heterozygous for *CYP2C19*1* are

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almost always extensive metabolisers. The two defective alleles can be detected by a combination of PCR (polymerase chain reaction), cleavage with restriction enzymes and gel electrophoresis (de Morais et al 1994a, b). These two defects account for $>99\%$ of the defective alleles in the Japanese populations but only $\sim 87\%$ of Caucasian defective alleles (de Morais et al 1994a).

Komai et al (1996) identified a single nucleotide change (T to A) at position 204 of exon 2 (*CYP2C18m1*) that caused an amino-acid exchange at position 68 from Tyr to a TAA stop codon. This anomaly yielded a truncated 67-amino-acid CYP2C18 lacking a haem-binding region.

Tsuneoka et al (1996) discovered a single nucleotide change (T to C) at position −478 of the 5′-flanking region (*CYP2C18m2*). Mamiya et al (1998) recently reported that, in a Japanese population, genotypes of *CYP2C18m1* and *CYP2C18m2* were completely coincident with those of *CYP2C19*3* and *CYP2C19*2*, respectively. Inoue et al (1998) independently found this linkage both in Japanese and Caucasians. Kubota et al (1998) also confirmed this linkage in the Japanese population.

Routine *CYP2C19* genotyping, before treatment with drugs metabolised by this enzyme and large-scale research studies, requires a simple and reliable diagnostic procedure. The principal aim of our study was to develop a simple, rapid and high-throughput assay. TaqMan (P. E. Applied Biosystems, Foster City, CA) is a homogeneous amplicon detection system that uses Taq DNA polymerase and generates a fluorescent signal. Taq DNA polymerase lacks 3′–5′ exonuclease activity (Tindall & Kunkel 1988) but retains 5′–3′ exonuclease activity (Longley et al 1990). These properties form the basis of a 5′ exonuclease assay that detects target DNA as PCR proceeds in real time (Holland et al 1991; Lee et al 1993; Livak et al 1995; Gibson et al 1996; Heid et al 1996). TaqMan probes are blocked from extension at the 3′ terminus and are labelled with a fluorescent reporter at the 5′ end. The probes are also conjugated to another fluorescent molecule that quenches the fluorescence emitted by the reporter when both labels are in close proximity. Degradation of the probes from their 5′ ends liberates the label. TaqMan specificity therefore results from probes annealing to their amplicons followed by cleavage to separate the reporter and quencher fluorophores. This separation of the fluorophores causes an increase in fluorescence under appropriate illumination.

We have developed an assay with which to detect *CYP2C19*2* and *CYP2C19*3* by detecting *CYP2C18m1* and *CYP2C18m2*. Our assay consists of allele-specific amplification and TaqMan PCR. Thus, cleavage with restriction enzymes and gel electrophoresis is no longer necessary.

Materials and Methods

The institutional ethics committee of the Tohoku University School of Medicine, Sendai, Japan, approved this study protocol.

Oligonucleotides

The primers were synthesized by Nihon Gene Research Laboratories Inc. (Sendai, Japan). The TaqMan probes synthesized by Perkin–Elmer contained 6-carboxyfluorescein (FAM) at the 5′ end and 6-carboxytetramethylrhodamine (TAMRA) with a phosphate molecule at the 3′ end (Table 1).

DNA samples

Venous blood (100 μL) or saliva (1 mL) was obtained from 144 unrelated healthy Japanese volunteers. DNA was isolated from peripheral blood anticoagulated with K_2EDTA using a DNA Extractor WB-Rapid Kit (Wako Pure Chemical Industries, Osaka, Japan) and from saliva using a QIAamp Tissue Kit (QIAGEN, Hilden, Germany)

Table 1. Summary of primer pairs and TaqMan probes.

Name	Sequences	5′–3′ position
2C18m1 wt primer	5′-atg gcc ctg tgt tca ctg tgt G(a)t-3′	182–204
2C18m1 mt primer	5′-atg gcc ctg tgt tca ctg tgt G(a)a-3′	182–204
2C18m1 TaqMan probe	5′-ttg gcc tga agc cca ttg tgg tgt-3′	206–229
2C18m1 reverse primer	5′-cct tca ctg ctt cat atc cat gc-3′	253–231
2C18m2 wt primer	5′-tca tgc ctg taa tcc cag caT(c) t-3′	−499 to −478
2C18m2 mt primer	5′-tca tgc ctg taa tcc cag caA(c) c-3′	−499 to −478
2C18m2 TaqMan probe	5′-tgg cca aca tgg tga aac cct gtc t-3′	−463 to −439
2C18m2 reverse primer	5′-aat gca caa cta cgc cca ggt-3′	−399 to −419

Nucleotides generating a mismatch in the template DNA are printed as capital letters. TaqMan probe consists of an oligonucleotide with 5′-reporter dye (FAM) and a downstream, 3′-quencher dye (TAMRA).

according to the manufacturer's recommendations. *CYP2C19*1* and the mutated alleles, *CYP2C19*2* and *CYP2C19*3*, were identified by PCR amplification with specific primers and by the restriction fragment length polymorphism (RFLP) procedure described by de Morais et al (1994a, b). *CYP2C18wt* and the mutated alleles, *CYP2C18m1* and *CYP2C18m2*, were identified by PCR amplification using specific primers and by the RFLP methods described by Komai et al (1996) and by Tsuneoka et al (1996), respectively.

Allele-specific TaqMan PCR conditions

The allele-specific TaqMan PCR assay used here represents the further development of two established methods: the allele-specific oligonucleotide (Newton et al 1989; Wu et al 1989) and the TaqMan PCR (Holland et al 1991; Lee et al 1993; Livak et al 1995) methods. Allele-specific primers were designed with an additional mismatch at position −2 from the 3′ end of each primer. Details concerning the design of allele-specific primers are described in Table 1. TaqMan PCR reactions proceeded in 30 μL of TaqMan Universal PCR Master Mix (P. E. Applied Biosystems) containing 0·4 μmol L^{-1} of forward primers, 0·4 μmol L^{-1} of reverse primers, 0·1 μmol L^{-1} of TaqMan probes and 20–100 ng of genomic DNA.

Thermal cycling was performed in 0·2-mL thin-walled optical caps using the PRISM 7700 sequence detection system (P. E. Applied Biosystems). This instrument monitors fluorescent emissions during the reaction by measuring the ratio of the signal of the FAM reporters against that of the internal standard, 6-carboxyrhodamine. Amplification and detection were performed using the PRISM 7700 system with the following profile: 1 cycle of 50°C for 2 min, 1 cycle of 95°C for 5 min and 50 cycles each of 95°C for 15 s and 60°C for 1 min. Samples were deemed positive at any given cycle when the value of the emitted fluorescence was greater than that of the threshold calculated by the instrument's software, Sequence Detector Ver. 1.6.3. To test the reliability of the allele-specific TaqMan PCR in each experiment, each sample was amplified three times using the same master-mix. The threshold cycle (Ct), which is defined as the cycle at which PCR amplification reaches a significant value (i.e., usually 10 times the standard deviation of the baseline), is given as a mean. The Ct values linearly correlated with relative DNA copy numbers (Heid et al 1996).

Statistical analysis

Multiple comparisons for the medians of differences between Ct values obtained using wild and mutant primers among the three groups with different *CYP2C18m1* or *CYP2C18m2* genotypes (*wt/wt*, *wt/m1* and *m1/m1* or *wt/wt*, *wt/m2* and *m2/m2*, respectively) were made using the Kruskal–Wallis test. A P value below 0·001 was considered statistically significant throughout the study.

Results

Since we were initially unable to develop an allele-specific TaqMan PCR assay for *CYP2C19*2* and *CYP2C19*3* genotyping, due to difficulties in the construction of the appropriate PCR primers and TaqMan probes, we tried to construct primers and probe sets for detecting *CYP2C18m1* and *CYP2C18m2*. Allele-specific PCR usually relies on the presence of a 3′-end mismatch (at position −1) between a PCR oligonucleotide primer and a target sequence. We improved the specificity of *CYP2C18* mutation detection by introducing additional mismatches at position −2, which further destabilized the allele-specific PCR primers. *CYP2C18* genotypes were specifically detected by the PRISM 7700. By performing two PCR reactions per sample, with one tube containing the normal and the other tube the mutant allele-specific PCR primer, we reasoned that an accurate diagnosis could be made based on the presence or absence of a PCR product in either tube. The instrument software, Sequence Detector Ver. 1.6.3, calculates Rn (normalised reporter) by determining the contribution of the reporter FAM and dividing that by the contribution of the internal reference dye included in the reaction. ΔRn is calculated as the value of Rn in any cycle minus the Rn before PCR amplification. Ct is the functional cycle number at which ΔRn crosses a fixed threshold. The threshold values for detecting *CYP2C18m1* and *CYP2C18m2* mutations were fixed at 0·009 and 0·200, respectively. The specific profiles of all *CYP2C18* genotypes are shown in Figure 1. Figure 2 shows that the differences between the Ct values obtained using wild-type and mutant primers in the assay of *CYP2C18m1* from individuals with the genotypes *CYP2C18wt/CYP2C18wt*, *CYP2C18wt/CYP2C18m1* and *CYP2C18m1/CYP2C18m1* were below −3·0, from −3·0 to 3·0 and above 3·0, respectively. The differences between Ct values using wild-type and mutant primers in the assay of *CYP2C18m2* from individuals with the genotypes *CYP2C18wt/CYP2C18wt*, *CYP2C18wt/CYP2C18m2* and *CYP2C18m2/CYP2C18m2*, were below −3·0, from −3·0 to 3·0 and above 3·0, respectively. The med-

ian values of the differences in Ct values between wild-type and mutant primers among the three *CYP2C18m1* or *CYP2C18m2* genotypes differed significantly ($P < 0{\cdot}001$).

All samples were tested for *CYP2C18* and *CYP2C19* genotypes by restriction enzyme cleavage and by allele-specific TaqMan PCR. All the genotypes determined by both methods were consistent. The genotypes of *CYP2C18m1* and *CYP2C18m2* were completely coincident with those of *CYP2C19*3* and *CYP2C19*2*, respectively (data not shown), in agreement with the reports of

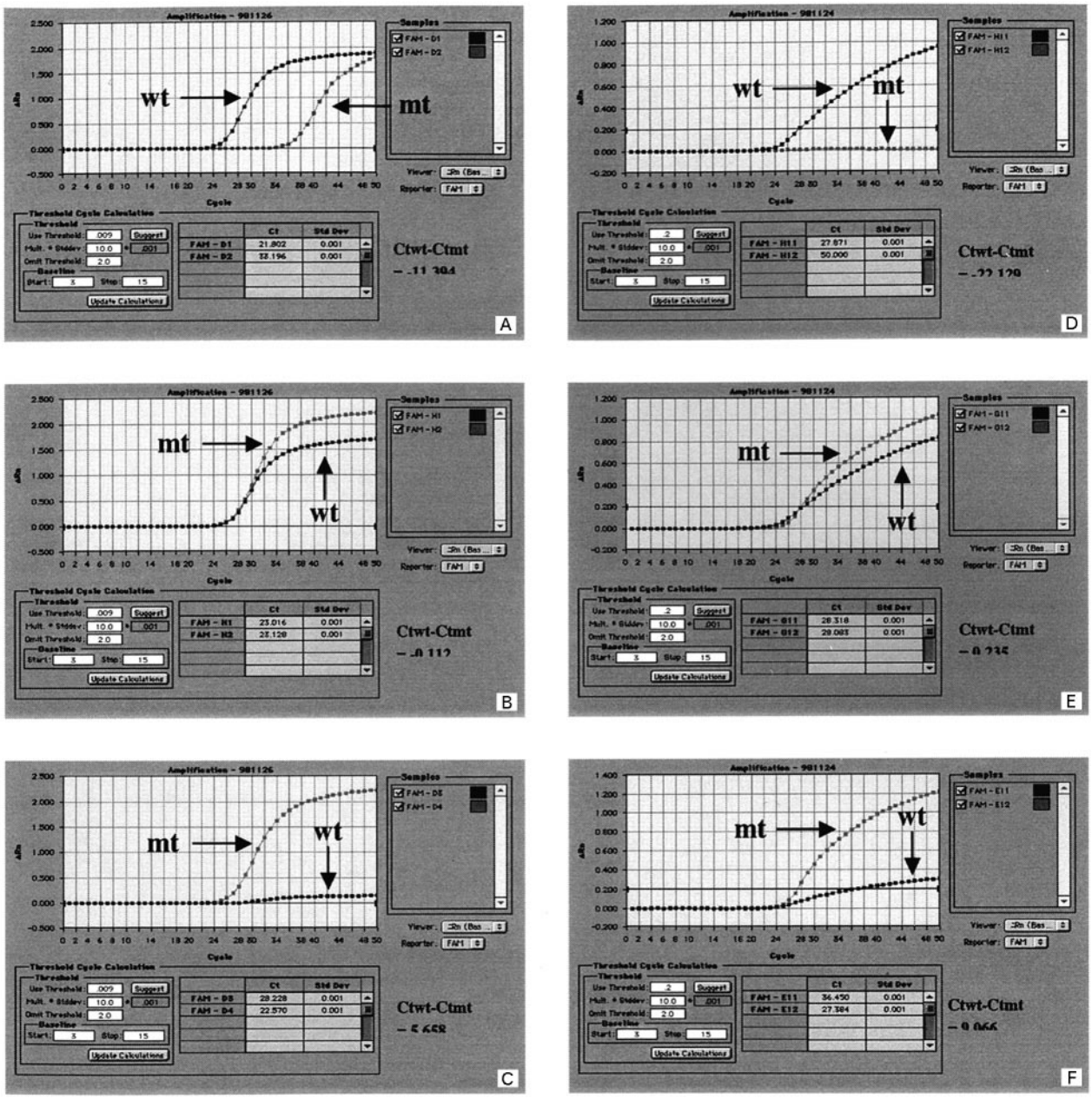

Figure 1. *CYP2C18* genotyping. Amplification plot of allele-specific TaqMan PCR assay for detecting *CYP2C18* genotypes generated by the PRISM 7700 Sequence Detector. The graph shows fluorescence emission data (ΔRn) collected during the extension phase of each cycle of the PCR. DNA from a healthy volunteer was subjected to allele-specific TaqMan PCR assay as described in Materials and Methods. PCR were analysed and plotted on a linear scale. Ct is the fractional cycle number at which ΔRn crosses some fixed threshold above baseline. The threshold is indicated by a solid line. In this case the threshold values for detecting *CYP2C18m1* and *CYP2C18m2* mutations were fixed at 0·009 and 0·200, respectively. Detection: of *CYP2C18m1*, A–C; of *CYP2C18m2*, D–F. *CYP2C18 wt/wt* (A), *CYP2C18 wt/m1* (B), *CYP2C18 m1/m1* (C), *CYP2C18 wt/wt* (D), *CYP2C18 wt/m2* (E) and *CYP2C18 m2/m2* (F). Wild primer (wt), mutant primer (mt).

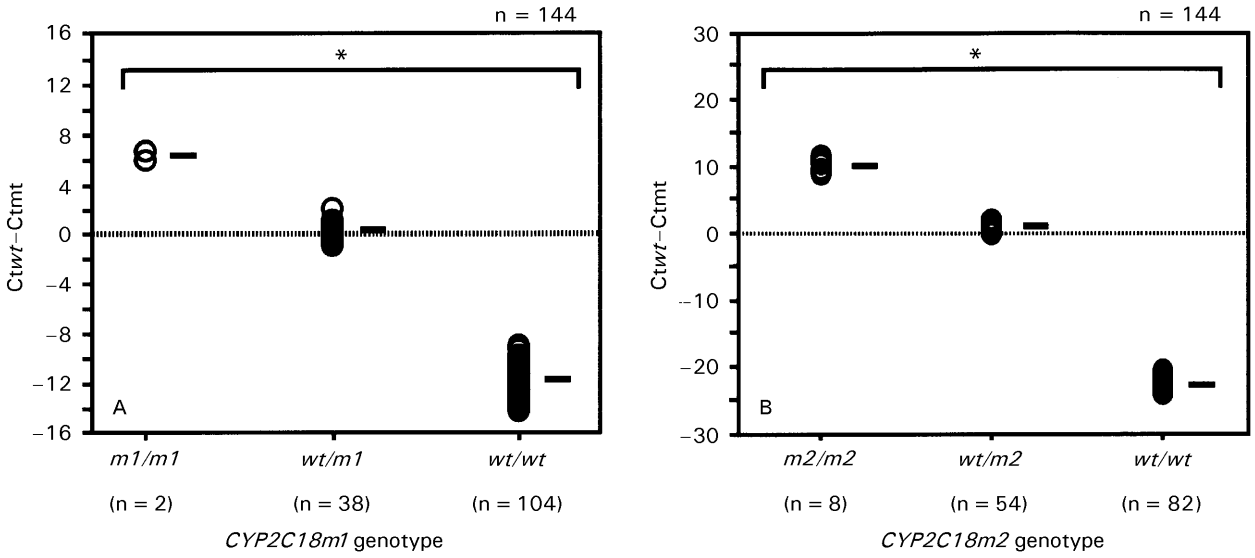

Figure 2. The relation between the difference of threshold cycle (Ct*wt*−Ctmt) and genotypes of *CYP2C18m1* (A) or *CYP2C18m2* (B) in the Japanese population. $*P < 0{\cdot}001$ by the Kruskal–Wallis test for comparison among the three groups with different genotypes of *CYP2C18m1* or *CYP2C18m2*. Median values (horizontal bars).

Mamiya et al (1998), Inoue et al (1998) and Kubota et al (1998). Allele-specific TaqMan PCR distinguished all six *CYP2C18* or *CYP2C19* genotypes. We identified 19 poor metabolisers, of whom 8 had homozygous *CYP2C19*2/CYP2C19*2*, 2 had homozygous *CYP2C19*3/CYP2C19*3* and 9 had compound heterozygous *CYP2C19*2/CYP2C19*3* genotypes.

Discussion

We developed the allele-specific TaqMan PCR assay to rapidly identify two point mutations in the *CYP2C18* gene. This assay combines the hybridization of TaqMan probes, labelled with FAM at the 5′ end and with TAMRA at the 3′ end, with allele-specific amplification primers to the target DNA. This assay is simple, reliable and reproducible. It does not require either restriction enzyme cleavage or purification of PCR products. Furthermore, the assay does not include electrophoresis and is therefore highly specific as it is unaffected by the formation of spurious PCR amplification products. In contrast to traditional assays, which often depend on analysis of ethidium bromide-stained gels, the results are presented exclusively in numerical form. The interpretation in this study is based on the difference between Ct values generated using wild-type and mutant primers. As shown in Figure 1D, no signal was detected in the *CYP2C18wt/wt* by *CYP2C18m2* genotyping using 2C18*m2* as mt primer. The plotted mutant curve did not reach a threshold (0·200). In this case, the Ct values were fixed at 50. The Ct*wt*−Ctmt score for *CYP2C18wt/wt* ranged from −20·29 to −23·52. This had no influence on the results of the *CYP2C18m2* genotyping itself. The evaluation does not require specialized laboratory personnel because it is not visual and the results can be interpreted by a computer. The assay does require a PRISM 7700 Sequence Detector, which is usually found in large departments of clinical biochemistry.

Allele-specific PCR can therefore be used to genotype DNA samples. We exploited a genetic version of the TaqMan detection system and coupled it with allele-specific PCR, which allowed high specificity. Allele-specific PCR is a method for selectively amplifying specific alleles (Newton et al 1989). However, when developing allele-specific amplification primers with which to detect *CYP2C18m1* or *CYP2C18m2* mutations, we found that mutant alleles were amplified even in the presence of the wild-type primer. We therefore included an extra mismatch at position −2 to further destabilize the annealing of primers to target sequences. A similar strategy was successfully used to screen for mutations responsible for glucose-6-phosphatase deficiency and medium-chain acyl-CoA dehydrogenase deficiency (Matsubara et al, unpublished experiments).

Bathum et al (1998) developed an oligonucleotide ligation assay with which to detect *CYP2C19* mutant alleles. This assay combines the ability of a

DNA ligase to distinguish mismatched nucleotides with the hybridization of one common, biotinylated capture probe and two allele-specific probes to the target DNA. The allele-specific probes are labelled with either europium or samarium, both of whose emissions can be simultaneously measured. Our method analyses samples more rapidly than either this method or standard PCR–RFLP. In our laboratory, it would take one person a minimum of one day to analyse, by standard PCR–RFLP, a set of serial samples collected from one individual, whereas the allele-specific TaqMan PCR assay takes less than 2·5 h.

The genotypes of the *CYP2C18* gene were completely consistent with those of the *CYP2C19* gene, in agreement with the reports of Mamiya et al (1998), Inoue et al (1998) and Kubota et al (1998). Therefore, the method presented here should be directly extensible to detection of not only *CYP2C18* mutant alleles, but also *CYP2C19* mutant alleles in Japanese populations. This assay should provide a useful tool with which to study drug metabolism. Additional studies, such as a direct detection assay of *CYP2C19* polymorphisms, are presently under investigation in our laboratory.

Routine *CYP2C19* genotyping before treatment would be of relevance to any treatment strategy involving a drug that is eliminated primarily by CYP2C19 and for which plasma level monitoring is recommended. We found the allele-specific TaqMan PCR assay to be rapid, simple and cost-effective, as well as suitable for high-throughput applications in a routine laboratory. This assay allows the fast and reliable detection of inherited disorders that might influence diagnosis and treatment.

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