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Detection of MDR1 Single Nucleotide Polymorphisms C3435T and G2677T Using Real-Time Polymerase Chain Reaction: MDR1 Single Nucleotide Polymorphism Genotyping Assay

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ABSTRACT The objective of this study was to develop a real-time polymerase chain reaction (PCR) method to detect *MDR1* (human multidrug resistance gene) single nucleotide polymorphisms (SNPs) C3435T and G2677T. C3435T and G2677T are linked to *MDR1*2* , which is associated with enhanced efflux activity in vitro. Using the Smart Cycler, an allele-specific real-time PCR-based genotyping method was developed to detect C3435T and G2677T. The *MDR1* genotype of human genomic DNA templates was determined by direct DNA sequencing. PCR reactions for genotyping C3435T and G2677T by using allele-specific primers were conducted in separate tubes. An additional nucleotide mismatch at the third position from the 3' end of each allele-specific primer was used to abrogate nonspecific PCR amplification. The fluorescence emitted by SYBR Green I was monitored to detect formation of specific PCR products. PCR growth curves exceeding the threshold cycle were considered positive. Fluorescence melt-curve analysis was used to corroborate results from PCR growth curves. Using PCR growth curves, our assay accurately determined hetero- and homozygosity for C3435T and G2677T. Genotype assignments based on PCR growth curve, melt-curve analysis, agarose gel electrophoresis, and direct DNA sequencing results of PCR products were in perfect agreement. We have developed a rapid *MDR1* genotyping method that can be used to assess the contribution of *MDR1*2* to pharmacokinetic and pharmacodynamic variability of P-glycoprotein substrates.

KEYWORDS: P-glycoprotein, single nucleotide polymorphism, real-time polymerase chain reaction.

INTRODUCTION P-glycoprotein (P-gp), a member of the large adenosine triphosphate-binding (ATP-binding) cassette superfamily of transport proteins also called traffic ATPases, is the product of the human multidrug resistance gene (*MDR1*). [1] P-gp is highly expressed on the apical (luminal) surface of organs that have excretory functions, such as the bile canalicular membrane of hepatocytes and the renal proximal tubule. [2,3] Moreover, P-gp is significantly expressed on the luminal surface of tissues that serve as barriers, such as the brush border of the small intestine and the capillary endothelial cells of the blood-brain barrier. [2,4-6] Tissue distribution suggests that P-gp protects the body from toxic xenobiotics by secreting them into the bile, urine, and intestinal lumen and by reducing their accumulation in the brain and testes. As a result, interindividual variability in the disposition of numerous drugs has been ascribed to differences in P-gp expression. Lown et al reported that intestinal P-gp expression accounted for approximately 30% of interindividual variability in the maximal plasma concentration after oral administration of cyclosporine. [7]

A novel P-gp aberrant allele, *MDR1*2* , linked to 2 synonymous single nucleotide polymorphisms (SNPs) (C1236T in exon 12 and C3435T in exon 26) and a nonsynonymous SNP in exon 21 (G2677T, Ala893Ser) was recently described. [8] The SNPs found on exons 12, 21, and 26 are not strictly allelic; however, they exhibit strong linkage disequilibrium and account for a majority of the described haplotypes. [8,9] *MDR1*2* was found to be associated with altered fexofenadine disposition. Individuals carrying 2 wild-type alleles (**1/*1*) had a 40% greater fexofenadine systemic exposure after oral administration compared with individuals heterozygous or homozygous for *MDR1*2* . [8] Reduced fexofenadine systemic exposure in carriers of the *MDR1*2* allele potentially results in reduced therapeutic benefit after oral administration of fexofenadine. Unfortunately, attempts to determine the association between polymorphic P-gp expression and drug disposition have yielded equivocal results. [10-12] Interestingly, Kim et al reported significant ethnic differences in *MDR1*2* allelic frequency, with 62% and 13% of European Americans and African Americans, respectively, carrying at least one *MDR1*2* allele. [8] Thus, polymorphic MDR1

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Table 1. Oligonucleotide Sequences*

Primer	5?Position†	Sequence‡§	3?Position†	T_m‖
C3435T				84°C
3435W	43288	GTGGTGTCACAGGAAGAG**G**TC	43268	
3435M	43288	GTGGTGTCACAGGAAGAG**GT**T	43268	
3435R	43155	ACTATAGGCCAGAGAGGCTGC	43175	
G2677T				80°C
2677W	65221	AGTTTGACTCACCTTCCC**T**GC	65241	
2677M	65221	AGTTTGACTCACCTTCCC**TG****A**	65241	
2677C	65436	GCTATAGGTTCCAGGCTTGCT	65416	

*W indicates wild-type specific primer; M, mutant-specific primer; R and C, common primers used in allelic discrimination assays.
†Based on published *MDR1* sequence (AC005068).
‡Sequence shown as 5?to 3?
§Nucleotides in bold indicate nucleotide mismatches from published wild-type sequence.
‖Amplicon melt temperature (T_m) obtained from melt curve analysis.

expression may contribute to interracial variability in drug disposition.

To facilitate clarification of the significance of commonly occurring MDR1 SNPs and their ethnic frequency on drug disposition, we sought to develop a rapid and robust polymerase chain reaction-based (PCR-based) screening method for the SNPs C3435T and G2677T.

MATERIALS AND METHODS

Primer Design

PCR primers are listed in **Table 1** . Oligonucleotide primers were designed based on the published *MDR1* sequence (AC005068) using the online program Primer3 (www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi), and hairpin structures and primer-dimers were predicted with Oligo Toolkit (www.operon.com) and then synthesized by Integrated DNA Technologies (Coralville, IA). Expected amplicon lengths were 216 base pairs (bp) and 134 bp for G2677T and C3435T, respectively. Discrimination between wild-type and mutant alleles was achieved using PCR amplification of specific alleles modified to prevent non-Watson Crick base pairing. [13-16] Briefly, the first nucleotide difference (C or T) between sense primers (3435W and 3435M) used to discriminate between wild-type and mutant 3435 alleles is located at the 3' terminal base. The second primer base change (A to G) located 3 bases from the 3' end generates an internal primer/template mismatch, and this prevents amplification of the nonmatching primer. These changes were made to prevent the generation of possible spurious products, which could otherwise occur by the annealing and extension of the 3435W primer to the first-round product of 3435M. A similar strategy was used to achieve allelic discrimination for G2677T (**Table 1**).

Real-Time PCR Amplification

The Smart Cycler (Cepheid, Sunnyvale, CA) was used to monitor PCR amplification using SYBR Green I (Molecular Probes, Eugene, OR), a nonspecific double-stranded DNA intercalating fluorescent dye. Thus, to achieve allelic discrimination between wild-type and mutant alleles, 2 physically separate PCR reactions containing either wild-type or mutant-specific primers were performed. All reactions were carried out in a total volume of 25 µL. Reaction conditions were identical for G2677T and C3435T except where noted. Each reaction mixture contained a 1:12 500 dilution of SYBR Green I nucleic acid gel stain 10 000X in dimethyl sulfoxide (DMSO) (Molecular Probes); 0.2mM of dATP, dCTP, dGTP, and dTTP; 200nM of both forward and reverse primers; 1.0 U of Taq DNA polymerase (Promega, Madison, WI); 6% DMSO; and 20 to 120 ng of genomic DNA in 1X PCR buffer (pH 8.3, 10X solution containing 100mM Tris-HCl, 500mM KCl, and 15mM $MgCl_2$ and 0.01% gelatin) (Sigma, St. Louis, MO). Genomic DNA was obtained from the Human Genetic Cell Repository, sponsored by the National Institute of General Medical Sciences (http://locus.umdnj.edu/nigms/). The use of Human Genetic Cell Repository samples was approved by the University of Tennessee Institutional Review Board.

The amplification program for both G2677T and C3435T consisted of 1 cycle of 95ºC with 120-second hold followed by 27 cycles of 95ºC with 6-second hold, specified annealing temperature of 62ºC with 15-second hold, and 72ºC with 20-second hold. After amplification, melt analysis was performed by heating the reaction mixture from 60ºC to 95ºC at the rate of 0.2ºC/s. A negative control without DNA template was run with every assay to assess the overall specificity. PCR products for sequencing the 2677 locus were generated using the sense primer (5'-AAGATTGCTTTGAGGAATGGT-3') and the antisense primer (5'- GCTATAGGTTCCAGGCTTGCT-3'). PCR products for sequencing the 3435 locus were generated using the sense primer (5'-

GAGCCCATCCTGTTGACTG-3') and the antisense primer (5'-ACTATAGGCCAGAGAGGCTGC-3').

Product Analysis

The real-time fluorescence signal generated by the nonspecific double-stranded DNA binding dye SYBR Green I was analyzed using the Smart Cycler software. A threshold cycle (C_t) was determined for each sample using the exponential growth phase and the baseline signal from fluorescence versus cycle number plots. A sample was deemed positive if fluorescence exceeded the threshold. Threshold fluorescence level was automatically set by the Smart Cycler software. Melt curves were transformed to the negative first derivative melt curves ([-dF/dt] vs temperature). In the melt analysis, the negative first derivative peaks, which are characteristic of the PCR product melt temperature, were used to identify specific PCR products. Amplification reactions were routinely checked for the presence of nonspecific products by agarose gel electrophoresis. PCR products were isolated by QIAquick (Qiagen, Valencia, CA) after separation by agarose gel electrophoresis and subjected to direct sequencing using the ABI Prism Model 3100 (Applied Biosystems, Foster City, CA). Genomic DNA, obtained from individuals determined by sequencing to be homo-, hetero-, and nullizygous for the 2677T and 3435T alleles, was used for genotyping assay development and validation.

RESULTS

Allele-specific primers containing an additional nucleotide mismatch 3 bases from their 3' termini had little effect on specific PCR product yield. However, nonspecific PCR product yield was drastically reduced to undetectable levels. Consequently, PCR conditions were optimized such that the Ct was exceeded only when specific amplification occurred (ie, only in the presence of a primer:template match). **Figure 1A** illustrates the results of the MDR1 C3435T allelic discrimination assay using homozygous 3435C genomic DNA amplified with a common primer 3435R and either the wild-type specific primer 3435W or the mutant-specific primer 3435M. When primers 3435R and 3435W were used to amplify homozygous 3435C genomic DNA, the PCR growth curve exceeded the Ct value at approximately 21 cycles (**Figure 1A**), and the melt analysis (negative first derivative) yielded a characteristic sharp peak at approximately 84°C for the product (**Figure 2A**). However, PCR growth curves remained at approximately background fluorescence, and no distinct melt analysis peak was noted when primers 3435R and 3435M were used to amplify homozygous 3435C genomic DNA (**Figures 1A** and **2A**). Agarose gel electrophoresis yielded the expected 134-bp fragment when homozygous 3435C DNA was amplified with primers 3435R and 3435W (**Figure 3**). However, no bands were visualized after homozygous 3435C DNA was amplified using primers 3435R and 3435M (**Figure 3**). Similarly, allelic discrimination was achieved after amplification of homozygous 3435T DNA using primers 3435R, 3435M, and 3435W (**Figures 1B** , **2B** , and **3**).

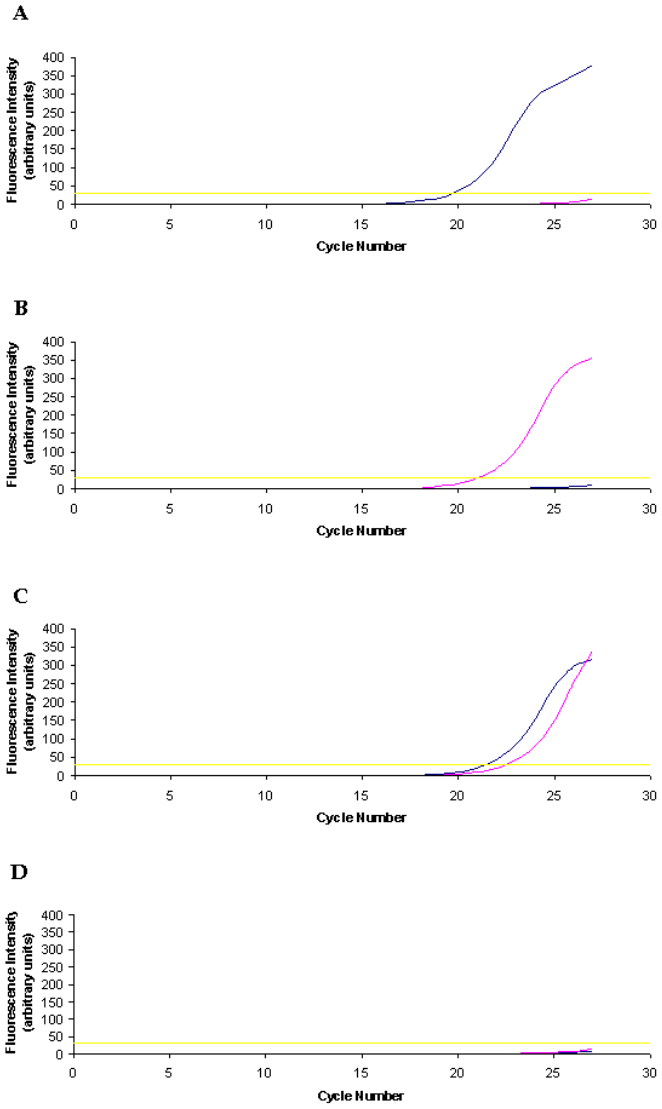

Figure 1. *MDR1* C3435T allelic discrimination by real-time analysis using the Smart Cycler. Plot of fluorescence versus cycle number using human genomic DNA obtained from individuals with CC (Panel A), TT (Panel B), or CT (Panel C) genotypes and nontemplate control (Panel D). Interrogation for the presence of either the C or T allele was conducted in physically separate tubes using the common reverse primer 3435R coupled with either the wild-type specific primer 3435W (—◆—) or the mutant-specific primer 3435M (—■—). PCR growth curves that exceed the threshold fluorescence indicate specific PCR product formation.

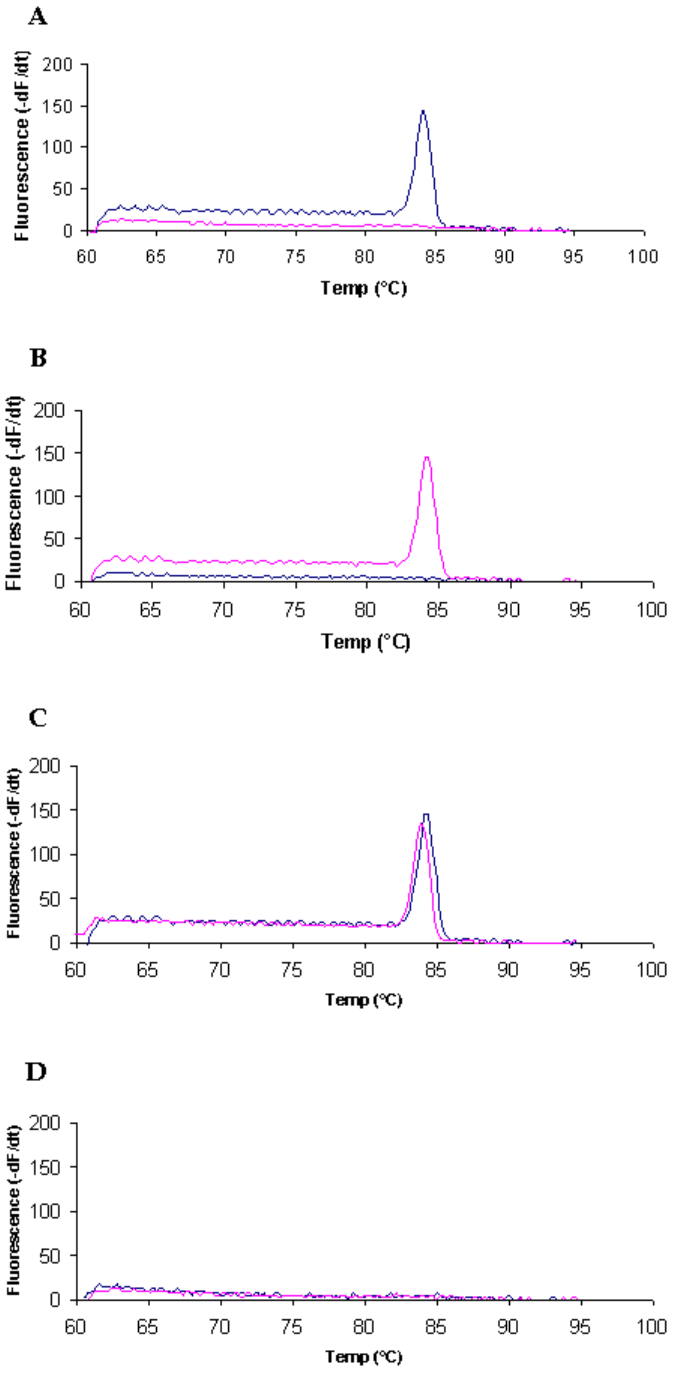

Figure 2. Melt curve analysis of PCR products using SYBR Green I. Melt curves were converted to melt peaks by plotting the negative first derivative of the fluorescence versus temperature ([-dF/dt]). Plot of [-dF/dt] versus temperature obtained after amplification of CC (Panel A), TT (Panel B), CT (Panel C) genomic DNA and nontemplate control (Panel D) using the common reverse primer 3435R coupled with either the wild-type specific primer 3435W (—♦—) or the mutant-specific primer 3435M (—●—). The melt temperature (Tm = 84°C) was identical for PCR products formed using either the wild-type or mutant-specific primers.

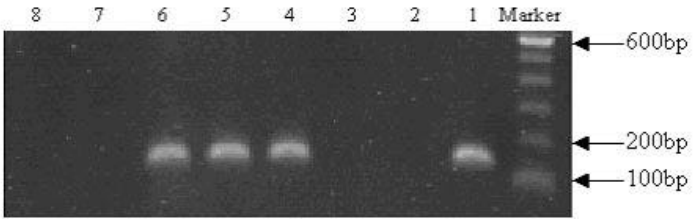

Figure 3. *MDR1* C3435T allelic discrimination by conventional modified allele-specific PCR. Anethidium bromide-stained 2.0% agarose gel containing PCR fragments (134 bp) Odd-numbered lanes contain PCR fragments after amplification with 3435R and 3435W. Even-numbered lanes contain PCR fragments after amplification with 3435R and 3435M. PCR products amplified from genomic DNA with different 3435 genotypes were loaded as follows: CC (lanes 1 and 2), TT (lanes 3 and 4), and CT (lanes 5 and 6). Lanes 7 and 8 represent nontemplate controls. Lane marker contains a 100-bp DNA ladder.

Overlapping PCR growth curves yielding similar Ct values were obtained when CT genomic DNA was amplified using wild-type and mutant-specific primers (**Figure 1C**). In addition, a distinct melt analysis peak was present after amplification with both wild-type and mutant-specific primers (**Figure 2C**). Results from real-time PCR corroborate conventional PCR results (**Figure 3**) and accurately predict the presence of both wild-type and mutant 3435 alleles in the heterozygote control. **Figures 1D** and **2D** illustrate results from nontemplate control reaction. Results obtained from optimization and application of the G2677T genotyping assay to individuals with GG, GT, and TT genotypes were similar to those reported for C3435T (data not shown). Melt analysis yielded a characteristic sharp peak at approximately 80°C (**Table 1**).

The validity of our methods was verified by testing 20 individuals (10 Caucasians and 10 African Americans) comprising all 3 G2677T and C3435T genotypes. The genotype distribution was in Hardy-Weinberg equilibrium. The allele frequency for 2677T was 0.50 and 0.15 for Caucasians and African Americans, respectively. The allele frequency for 3435T was 0.55 and 0.20 for Caucasians and African Americans. The allele frequencies for 2677T and 3435T were similar to those previously reported for Caucasians and African Americans. [8, 17-19] Eighteen samples (3 individuals; homo-, hetero-, and nullizygous for either 2677T or 3435T) were sequenced. Sequencing results were in perfect agreement with real-time PCR results.

DISCUSSION Fluorescence-based SNP detection assays offer several important advantages over traditional PCR approaches used to determine genotype (eg, sequencing of PCR products and restriction fragment length polymorphism [RFLP]).

First, RFLP can in some instances result in significant false positive rates as a result of incomplete restriction enzyme digestion or the presence of other mutations close to the mutation of interest.[20,21] Second, fluorescence-based genotyping assays are more amenable to high-throughput screening, as they do not require extensive postamplification manipulation. Commonly used fluorescence-based PCR techniques for SNP detection include the use of either the nonspecific DNA intercalating dye SYBR Green I or an allele-specific fluorogenic probe (ie, Taqman). In many instances, the use of SYBR Green I is more cost-effective when applied to haplotype analysis of genes with numerous allelic variants since it does not require the synthesis of numerous allele-specific fluorogenic probes. Third, the use of allele-specific primers containing an additional internal mismatch obviates the need for extensive optimization of PCR amplification conditions associated with traditional PCR amplification of specific alleles. Our laboratory has successfully applied the approach described here to genotype 11 other SNPs using, in most instances, identical PCR amplification conditions.

Current methods for genotyping *MDR1* include PCR amplification followed by sequencing and fluorogenic probe-based PCR assays.[8, 22,23] The simple, rapid, inexpensive, reproducible, and reliable real-time PCR genotyping methods presented here constitute a significant improvement over current techniques. Using this approach, genotyping results can be obtained within 2 hours of whole blood or tissue procurement. Importantly, these techniques are also applicable in laboratories lacking access to real-time PCR equipment since allelic discrimination can be determined using traditional PCR and agarose gel electrophoresis. In conclusion, we present real-time PCR assays for the rapid detection of the *MDR1* SNPs C3435T and G2677T. These methods can be readily applied to investigate the effect of *MDR1* polymorphic expression on pharmacokinetic and pharmacodynamic variability of P-gp substrates.

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REFERENCES

1. Higgins CF. ABC transporters: from microorganisms to man. Annu Rev Cell Biol. 1992;8:67-113.

2. Thiebaut F, Tsuruo T, Hamada H, Gottesman MM, Pastan I, Willingham MC. Cellular localization of the multidrug-resistance gene product P-glycoprotein in normal human tissues. Proc Natl Acad Sci U S A. 1987;84(21):7735-7738.

3. Cordon-Cardo C, O'Brien JP, Casals D, et al. Multidrug-resistance gene (P-glycoprotein) is expressed by endothelial cells at blood-brain barrier sites. Proc Natl Acad Sci U S A. 1989;86(2):695-698.

4. Leveille-Webster CR, Arias IM. The biology of the P-glycoproteins. J Membr Biol. 1995;143(2):89-102.

5. Schinkel AH, Wagenaar E, van Deemter L, Mol CA, Borst P. Absence of the mdr1a P-glycoprotein in mice affects tissue distribution and pharmacokinetics of dexamethasone, digoxin, and cyclosporin A. J Clin Invest. 1995;96(4):1698-1705.

6. Schinkel AH, Wagenaar E, Mol CA, van Deemter L. P-glycoprotein in the blood-brain barrier of mice influences the brain penetration and pharmacological activity of many drugs. J Clin Invest. 1996;97(11):2517-2524.

7. Lown KS, Mayo RR, Leichtman AB, et al. Role of intestinal P-glycoprotein (mdr1) in interpatient variation in the oral bioavailability of cyclosporine. Clin Pharmacol Ther. 1997;62(3):248-260.

8. Kim RB, Leake BF, Choo EF, et al. Identification of functionally variant MDR1 alleles among European Americans and African Americans. Clin Pharmacol Ther. 2001;70(2):189-199.

9. Tang K, Ngoi SM, Gwee PC, et al. Distinct haplotype profiles and strong linkage disequilibrium at the MDR1 multidrug transporter gene locus in three ethnic Asian populations. Pharmacogenetics. 2002;12(6):437-450.

10. von Ahsen N, Richter M, Grupp C, Ringe B, Oellerich M, Armstrong VW. No influence of the MDR-1 C3435T polymorphism or a CYP3A4 promoter polymorphism (CYP3A4-V allele) on dose-adjusted cyclosporin A trough concentrations or rejection incidence in stable renal transplant recipients. Clin Chem. 2001;47(6):1048-1052.

11. Hoffmeyer S, Burk O, von Richter O, et al. Functional polymorphisms of the human multidrug-resistance gene: multiple sequence variations and correlation of one allele with P-glycoprotein expression and activity in vivo. Proc Natl Acad Sci U S A. 2000;97(7):3473-3478.

12. Sakaeda T, Nakamura T, Horinouchi M, et al. MDR1 genotype-related pharmacokinetics of digoxin after single oral administration in healthy Japanese subjects. Pharm Res. 2001;18(10):1400-1404.

13. Okimoto R, Dodgson JB. Improved PCR amplification of multiple specific alleles (PAMSA) using internally mismatched primers. Biotechniques. 1996;21(1):20-22, 24, 26.

14. Sommer SS, Groszbach AR, Bottema CD. PCR amplification of specific alleles (PASA) is a general method for rapidly detecting known single-base changes. Biotechniques. 1992;12(1):82-87.

15. Bottema CD, Sarkar G, Cassady JD, Ii S, Dutton CM, Sommer SS. Polymerase chain reaction amplification of specific alleles: a

general method of detection of mutations, polymorphisms, and haplotypes. Methods Enzymol. 1993;218:388-402.

16. Newton CR, Graham A, Heptinstall LE, et al. Analysis of any point mutation in DNA: the amplification refractory mutation system (ARMS). Nucleic Acids Res. 1989;17(7):2503-2516.

17. Cascorbi I, Gerloff T, Johne A, et al. Frequency of single nucleotide polymorphisms in the P-glycoprotein drug transporter MDR1 gene in white subjects. Clin Pharmacol Ther. 2001;69(3):169-174.

18. Schaeffeler E, Eichelbaum M, Brinkmann U, et al. Frequency of C3435T polymorphism of MDR1 gene in African people. Lancet. 2001;358(9279):383-384.

19. Ameyaw MM, Regateiro F, Li T, et al. MDR1 pharmacogenetics: frequency of the C3435T mutation in exon 26 is significantly influenced by ethnicity. Pharmacogenetics. 2001;11(3):217-221.

20. Liebman HA, Sutherland D, Bacon R, McGehee W. Evaluation of a tissue factor dependent factor V assay to detect factor V Leiden: demonstration of high sensitivity and specificity for a generally applicable assay for activated protein C resistance. Br J Haematol. 1996;95(3):550-553.

21. Harth V, Bruning T, Abel J, et al. Real-time genotyping of cytochrome P4501A1 A4889G and T6235C polymorphisms. Mol Cell Probes. 2001;15(2):93-97.

22. Hoffmeyer S, Burk O, von Richter O, et al. Functional polymorphisms of the human multidrug-resistance gene: multiple sequence variations and correlation of one allele with P-glycoprotein expression and activity in vivo. Proc Natl Acad Sci U S A. 2000;97(7):3473-3478.

23. Nakamura T, Sakaeda T, Ohmoto N, et al. Real-time quantitative polymerase chain reaction for MDR1, MRP1, MRP2, and CYP3A-mRNA levels in Caco-2 cell lines, human duodenal enterocytes, normal colorectal tissues, and colorectal adenocarcinomas. Drug Metab Dispos. 2002;30(1):4-6.