Detection and Identification of Cytochrome P-450 2C9 Alleles *1, *2, and *3 by High-Resolution Melting Curve Analysis of PCR Amplicons

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Abstract

High-resolution melting curve analysis using a fluorescent DNA binding dye can detect sequence variations in a closed-tube system without labeled primers or probes. We developed and verified a melting analysis assay for common single nucleotide polymorphisms of cytochrome P-450 (CYP) 2C9 that affect warfarin metabolism. We used this method to genotype 84 patients receiving warfarin. For wild-type, *1/*1, 50% fluorescence corresponded to a mean ± SD of 87.17 ± 0.05°C, whereas *2/*2 was 0.4°C lower. The *1/*2 melting curve was easily distinguished from *1/*1 and *2/*2 based on transition temperature and shape. Exon 7 showed a more complex melting curve; however, genotypes *1/*1, *1/*3, and *3/*3 were easily distinguishable. Melting curves were highly reproducible (SD of temperature for multiple fluorescence values 0.04°C-0.11°C; mean, 0.06°C). Heterozygotes (*1/*2 or *1/*3) required significantly lower mean maintenance warfarin doses compared with wild-type (30.67 and 29.56 vs 42.81 mg/wk; P < .05). High-resolution melting analysis provides a simple and accurate method for genotyping of CYP2C9.

The cytochrome P-450 system (CYP) is responsible for the metabolism of a vast array of naturally occurring and synthetic compounds. The protein family is large with numerous functional enzymes and several nonproductive pseudogenes. The CYP2C9 isoenzyme is primarily responsible for the oxidative metabolism of several medically important compounds, including warfarin, phenytoin, losartan, irbesartan, tolbutamide, glipizide, torsemide, and various nonsteroidal anti-inflammatory drugs.1

Multiple single nucleotide polymorphisms within the gene for CYP2C9 have been identified, and at least 6 of these encode CYP2C9 alleles.1 According to the Human Cytochrome P450 Allele Nomenclature Committee, “To be assigned as a unique allele it [the gene] should contain nucleotide changes that have been shown to affect transcription, splicing, translation, posttranscriptional or posttranslational modifications or result in at least one amino acid change.”2 The most common allele is designated *1 and is considered the wild-type allele. The *2 allele results from a single base substitution (430C>T), which is located in exon 3. The *3 (1075A>C), *4 (1076T>C), and *5 (1080C>G) alleles result from base changes located in exon 7. In addition, a null polymorphism containing an adenine base pair deletion at n*t 818 (allele *6) recently has been identified.3 The *2 and *3 alleles encode a protein with reduced enzymatic activity in vitro, and patients with these alleles have been designated “poor metabolizers” of warfarin [3-(2-acetyl-1-phenylethyl)-4-hydroxycoumarin]. The genotype distribution among individuals varies with ethnic background. In Caucasian populations, approximately two thirds of individuals express the wild-type genotype, *1/*1; one third express the *1/*2 or *1/*3 genotype; and fewer than 2.5% express the *2/*2, *2/*3, or *3/*3 genotype.4
Because warfarin has a low therapeutic index, the *2 and *3 alleles, which encode P-450 enzymes with decreased activity, are associated with an increased risk of overanticoagulation and bleeding events. Alternate genotypes composed of 1 or more low-activity alleles also may account for the difficult-to-control anticoagulation status in some patients. The accumulated evidence suggests that the CYP2C9 genotype can be used prospectively to develop warfarin dosing strategies to avoid the morbidity associated with overanticoagulation. Previously described methods for CYP2C9 genotyping include direct nucleic acid sequencing, polymerase chain reaction (PCR) endonuclease analysis, and a commercially available melting curve analysis kit that uses fluorescence energy transfer probes (Roche Applied Science, Indianapolis, IN).

The earliest and most commonly used methods for genotyping P-450 2C9 involve PCR followed by restriction enzyme digestion. PCR products then are analyzed by gel electrophoresis with ethidium bromide staining. Although these methods use inexpensive, commonly available reagents, they involve risks of cross-contamination owing to the necessity of opening PCR tubes containing billions of amplicons. An ideal method would require inexpensive reagents, a closed-tube system to reduce contamination risks, and a minimal amount of labor.

Melting curve analysis using real-time PCR instruments and the double-stranded (ds) DNA dye, SYBR Green I (Invitrogen, Carlsbad, CA), was described in 1997 and can be used to roughly characterize sequence variation in PCR amplicons. Subsequently, fluorescent hybridization probes have been used in melting curve analysis to provide more detailed information about sequence variation in PCR products.

If PCR is performed with a 5'-labeled primer, high-resolution melting analysis can distinguish different homozygotes and heterozygotes and can genotype single nucleotide polymorphisms in amplicons up to 304 base pairs (bp) long. Genotyping and mutation scanning also can be accomplished by high-resolution melting analysis of PCR products amplified in the presence of a dsDNA dye, LCGreen I (Idaho Technology, Salt Lake City, UT). Unlike other fluorescent dyes used in real-time PCR, LCGreen I can identify heterozygous and homozygous sequence variants owing to its high molar binding ratio with DNA. Melting curve analysis with LCGreen I obviates the need for labeled probes or primers. These methods are significantly faster than direct sequencing and are performed in a closed-tube system alleviating some of the amplicon cross-contamination risks associated with PCR endonuclease-based assays. Compared with fluorescent hybridization probe assays, the use of LCGreen I allows interrogation of the entire amplicon for sequence variation rather than a short segment.

We report the development and verification of a simple assay for CYP2C9 genotyping based on high-resolution amplicon melting analysis. The assay uses unlabeled PCR primers, a LightCycler (Roche Applied Science), LCGreen I dye, and a high-resolution melting instrument (HR-1, Idaho Technology). We used this method to conduct a retrospective population genotype study of patients receiving warfarin in a tertiary care center anticoagulation management service.

Materials and Methods

Standards

Previously genotyped DNA standards for *1/*1, *1/*2, *1/*3, *2/*2, and *3/*3 were provided by Mark Linder, PhD, University of Louisville, Louisville, KY. Standards were genotyped in Dr Linder’s laboratory by PCR–restriction fragment length polymorphism methods as previously described.

Patient Specimens

Patients gave consent and were enrolled from the Emory Healthcare Anticoagulation Management Service (EHC-AMS, Atlanta, GA) with the approval of the Emory University Institutional Review Board (IRB No. 1068-2003). All patients seen in the EHC-AMS were eligible for enrollment. Genotyping was performed on dried filter paper blood spots obtained during a regular visit for prothrombin time international normalized ratio (INR) measurement and warfarin management. Outpatient INRs were measured by finger-stick blood specimens, and filter paper blood spots were obtained without additional procedures (venipuncture) or discomfort to the patient. Patient records were reviewed for clinical parameters, INR, and warfarin dose. Data were analyzed only for the period before and including the date that the specimen was obtained for genotyping. All patients had warfarin therapy initiated before enrollment in the EHC-AMS and had been monitored for a minimum of 1 month in the EHC-AMS.

Sample Preparation

Dried filter paper blood spots were transferred to screw-capped microcentrifuge tubes using a singe hole paper punch (5 mm). The paper spots were hydrated with 250 µL of phosphate-buffered saline at room temperature and mixed vigorously. Microfuge tubes were transferred to a 100°C heat block for 10 minutes. Samples again were mixed vigorously. Samples were allowed to cool to room temperature before extraction. Genomic DNA was extracted from the filter paper eluate or 200 µL of whole blood with the MagnaPure LC instrument (Roche Applied Science) using the total nucleic acid reagent kit according to the manufacturer’s instructions. DNA was eluted from the magnetic beads in 65 µL of elution buffer and used without quantification.
Polymerase Chain Reaction

Primer design was performed using Primer3 software (Whitehead Institute/MIT Center for Genomic Research, Cambridge, MA, http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). Owing to the significant amount of homology within the CYP system, the primers for exons 3 and 7 were designed so that one primer was homologous to the exon sequence and the second primer was in an adjacent intron. The amplicon was designed with the single nucleotide polymorphism as close to the middle of the amplicon as possible. The predicted amplicon lengths are 122 and 160 bp for exons 3 and 7, respectively. Primers were synthesized by the Emory University Microchemical Facility using standard phosphoramidite chemistry. Primer sequences were as follows: exon 3 sense, C9E3-F1, 5'-AAT TTT GGG ATG GGG AAG AG-3'; exon 3 antisense, C9E3-R1, 5'-TCC AGT AAT GTC AGT GAT ATG GA-3'; exon 7 sense, C9E7-F1, 5'-CCA CAT GCC CTA CAC AGA TG-3'; and exon 7 antisense, C9E7-R1, 5'-TGG AAA ACA TGG AGT TGC AG-3'.

Separate PCR reactions were performed for each exon in 10-µL volumes with the following constituents: 200 µmol/L of each deoxynucleoside triphosphate, 0.4 U KlenTaq1 polymerase (AB Peptides, St Louis, MO), 88 ng TaqStart Antibody (Clontech, Mountain View, CA), 250 mmol/L of each primer, LCGreen I, and 3 mmol/L of magnesium chloride, 50 mmol/L of tris(hydroxymethyl)aminomethane (pH 8.3), 500 ng/µL of bovine serum albumin (as 30 mmol/L MgCl₂ PCR Buffer, IT Biochem, Salt Lake City, UT), and 1 µL of sample DNA. Thermal cycling was performed in a LightCycler instrument for 45 cycles of 94°C for 0 seconds, 60°C for 5 seconds, and 72°C for 7 seconds followed by a single cycle of 94°C for 30 seconds. The samples then were cooled rapidly to 40°C and held for 30 seconds.

Melting Curve Acquisition

Melting analysis was performed in an HR-1 instrument. It is a single-sample instrument that surrounds 1 LightCycler capillary with an aluminum cylinder that is heated by a coil wound around the outside. Sample temperature and fluorescence signals are converted to 16-bit digital signals, ideally providing resolution down to 0.002°C and 0.002% of normalized fluorescence. Approximately 50 data points are acquired for every 1°C. Following amplification, each capillary was transferred to the HR-1 instrument and heated at 0.3°C/s. Data were acquired from 80°C to 90°C and 70°C to 95°C for exon 3 and exon 7 melts, respectively.

Melting Curve Analysis

Data were analyzed using software provided with the HR-1 instrument. Fluorescence intensity values were normalized by defining linear baselines before and after melting transitions, which were designated values of 100% and 0%, respectively. Within each sample, the fluorescence of each acquisition was calculated as a percentage of fluorescence between the top and bottom baselines at each acquisition temperature.

For the exon 7 melting analysis, the curves were temperature-shifted such that the mid portions of all curves were superimposed over a short interval. Melting temperature maps of the exon 3 and exon 7 amplicons using the wild-type sequences were obtained using the Poland and Fixman-Freire algorithms with MELT94 software (available at http://web.mit.edu/osp/www/melt.html).

Reproducibility Studies

Reproducibility of melting for exon 3 was assessed by analyzing 15 samples each of *1/*1 (5 samples in triplicate), *1/*2 (5 samples in triplicate), and *2/*2 (15 replicates of 1 sample) DNA. Normalized data were used to examine the temperatures corresponding to 90%, 70%, 50%, 30%, and 10% fluorescence for each curve. Reproducibility of melting for the exon 7 amplicon was examined by analyzing 15 samples each of *1/*1 (5 samples in triplicate), *1/*3 (5 samples in triplicate), and *3/*3 (15 replicates of 1 sample) DNA. Normalized and temperature-shifted data were used to examine the temperatures corresponding to 50%, 40%, 30%, 20%, and 10% fluorescence.

Statistical Analysis

Mean patient doses were calculated based on each patient’s dosing regimen in place at the time of the genotyping visit. Differences in means were assessed by 2-tailed, 2-sample t test assuming equal variances. Data are given as mean ± SD unless noted otherwise.

Results

The design of PCR primers selective for CYP2C9 was made difficult by the high degree of homology between members of the CYP family. To ensure specific amplification, primers were selected such that one primer was in an intron (the introns in the CYP family are not highly conserved), while amplicon length was kept to a minimum to facilitate genotyping.

The amplicon for exon 3 produced a single transition melting curve using unlabeled primers and the dsDNA binding dye, LCGreen I. The 50% fluorescence value corresponded to 87.17°C ± 0.05°C for homozygous *1/*1 (Figure 11). Allele *2 results from a single base change at position 430 from C to T. In the 122 bp amplicon produced for exon 3, this single base change shifted the melting curve approximately 0.4°C lower at all points (50% fluorescence = 86.76°C ± 0.04°C, Figure 1). Although the heterozygote *1/*2 has a 50%
The amplicon for exon 7 produced a complex melting curve with multiple transitions [Figure 2A]. The single nucleotide polymorphism responsible for allele *3 was clearly visible in the highest temperature transition in the homozygous and heterozygous states. Because the melting curve region of interest existed in this high temperature transition, further analysis was restricted to the range of 10% to 50% fluorescence [Figure 2B].

The homozygous *1/*1 and *3/*3 curves differ by 0.5°C at the middle of the informative region (10%-20% fluorescence). The *1/*3 heterozygote crossed 30% fluorescence at approximately the same temperature as the wild type; however, the melting curve for the heterozygote had a less steep slope than the wild type and initiated the transition at a lower temperature, analogous to that observed for exon 3.

As was the case for exon 3, the melting curves for exon 7 were highly reproducible. The SD of temperature ranged from 0.01°C to 0.04°C over the informative region. The different genotypes were easily discerned based on the melting temperature and curve shape.

To determine whether smaller, nonspecific amplicons were responsible for the lower temperature transitions, the amplicons were analyzed by agarose gel electrophoresis. Single products of the predicted lengths of 122 bp for exon 3 and 160 bp for exon 7 were produced from each PCR of the 5 different genotypes tested (data not shown).

A single amplicon may produce multiple transitions if individual domains within the amplicon melt differently. By applying nearest neighbor thermodynamic modeling using the Poland and Fixman-Freire algorithms calculated with
MELT94 software, the exon 3 amplicon was predicted to melt evenly over most of its length [Figure 3A]. The lowest line in each graph represents the temperature at which 80% of that base is annealed and 20% melted. The middle and upper lines represent 50% annealed–50% melted and 20% annealed–80% melted, respectively. The small region in the middle of exon 3, which seems to require a higher temperature to melt, probably does not contribute to the overall melting owing to its short length.

The analysis of exon 7 indicated that there are multiple possible melting domains within the amplicon [Figure 3B]. A portion of the amplicon that is A/T rich was predicted to melt at a temperature slightly below 70°C. There are 2 additional domains that were predicted to require more heat to melt. The predicted melting transition for the region containing the base responsible for allele *3 is much higher than a large portion of the amplicon.

The genotypes were discerned easily for the 84 patient samples. Patients spanned a wide range of ages and many indications for warfarin therapy [Table 1]. The distribution of genotypes is provided in [Table 2] and is consistent with previously published distributions for Caucasian populations. Limited sequencing of representative patient specimens with wild-type and heterozygous melting curves was consistent with the assigned genotypes (data not shown).

Patients with non–wild-type genotypes did not have an increase in supratherapeutic INRs (Table 2), and no catastrophic bleeding events were documented for any patients tested. However, patients heterozygous for allele 2 or allele 3 required a significantly lower maintenance dose of warfarin ($P < .05$). Only 1 patient each demonstrated homozygosity for allele 2 or allele 3; however, both required low maintenance doses of warfarin at approximately 30% of the mean maintenance dose required for patients with wild-type alleles. No significant differences were noted for initial warfarin dose, initial INR, or INR at the genotype visit.

**Discussion**

The CYP2C9 enzyme is responsible for the metabolism of warfarin and numerous other drugs. Knowledge of a patient’s genotype may allow lower loading doses and slower regimen changes for poor metabolizers. High-resolution
amplicon melting analysis is ideally suited for CYP2C9 genotyping. This method is rapid and allows testing to occur without opening the PCR reaction vessel, essentially eliminating the risks of amplicon contamination. It also provides the possibility of identifying novel and rare alleles owing to the ability to acquire information about the entire amplicon. The dye, LCGreen I, is key to this analysis. Unlike other dsDNA dyes used in real-time PCR, it can detect heteroduplexes and accurately genotype short PCR amplicons.

The process is easy to perform and interpret and requires only 25 minutes for amplification and 1 to 2 minutes for generation of each amplicon melting profile. A batch of 15 patient samples can be genotyped at both exons in approximately 1.5 hours. Because labeled primers or probes are not used, the reagents are inexpensive. However, the analysis requires a LightCycler and the recently developed HR-1 instrument to generate the PCR amplicons and to acquire and analyze the amplicon melting curves, respectively. The single-sample HR-1 instrument used in this study costs less than $10,000, a modest additional investment for laboratories that want to expand their genotyping capabilities of the LightCycler. Although the LightCycler is capable of generating basic melting curves, the resolution is not sufficient to detect the small differences in melting of the amplicons from the different genotypes, even with the use of LCGreen I (data not shown).

The ease and speed of this assay method allow relatively rapid clinical validation, if appropriate control samples are available. The simplest algorithm involves split-sample testing with a reference laboratory and comparisons of results. However, owing to the different distributions of genotypes in various populations, many clinical laboratories genotyping CYP2C9 have performed population studies to define the genotype distributions within their respective patient populations. It is exceptionally important to provide a carefully constructed report that includes guidance regarding the implications of the patient genotype and the limitations of using genotype data to predict phenotype.

The melting curves generated in this study allowed differentiation of the homozygous and heterozygous states with ease. Based on the melting temperature and melting curve shape, it was possible to unambiguously assign a genotype in all samples tested thus far. Although the heterozygous samples produced melting curves with midpoint temperatures similar to those of a homozygous sample, in each case, the melting curve was sufficiently different in shape to allow distinction from the homozygote. All specimens tested thus far have been very similar to one of the control samples that were genotyped by a reference method allowing assignment of genotype to the patient specimen. Furthermore, by using this method, the population distribution of genotypes is similar to the previously reported distribution for a Caucasian population.

The melting curves produced for all genotypes are highly reproducible. The SD of temperature at the tested levels of fluorescence ranged from 0.01°C to 0.06°C. These values represent total variability because within-run and run-to-run variability are included. Because the distinction between the various homozygote states was at least 0.4°C, all genotypes could be distinguished easily.

Although the melting curve for exon 7 was complex, the various genotypes could be identified in the highest temperature transition. The complexity of the melting curve was likely the result of melting domains present within the amplicon. At least 2 distinct regions of the amplicon were identified with disparate A/T compositions. The portion of the amplicon that is most G/C rich contains the base responsible for allele *3. This is in complete agreement with the observation that the third exon 7 contained the base responsible for allele *3. This result was consistent with the observation that the third exon 7 contained the base responsible for allele *3.

### Table 2

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. (%)</th>
<th>Therapeutic (%)</th>
<th>Subtherapeutic (%)</th>
<th>Supratherapeutic (%)</th>
<th>Mean No. of Measurements per Patient</th>
<th>Mean at Genotyping Visit</th>
<th>Mean Warfarin Maintenance Dose (mg/wk)</th>
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<tr>
<td>*1/*1</td>
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<td>50.09</td>
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<td>19.08</td>
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<td>2.51</td>
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<td>45.45</td>
<td>18.18</td>
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<td>72.09</td>
<td>18.60</td>
<td>43</td>
<td>3.20</td>
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INR, international normalized ratio.

* Values refer to the mean percentage of INR measurements that fall within the therapeutic range. Total INR measurements per patient ranged from 2 to 115.

† Values for *1/*1, *1/*2, and *1/*3 are given as mean ± SD.

‡ *P < .05 compared with *1/*1.
*1/*1 is 89.1°C compared with 88.6°C and 89.4°C for *1/*3 and *3/*3, respectively. These values approximate those observed in the highest temperature transition in actual melting curves. Allele *4 is predicted to produce amplicons with 89.0°C and 89.3°C for heterozygote and homozygote specimens, respectively. Although the *1/*4 heterozygote amplicon is predicted to melt at a similar temperature to the wild type, the heterozygote is expected to produce heteroduplexes with a flatter melting transition than either homozygote. In a similar way, the *5 allele is predicted to yield amplicons with melting temperatures of 88.3°C and 89.1°C in heterozygote and homozygote forms, respectively. These are values predicted by modeling and may not represent the actual discriminatory power of high-resolution amplicon melting.

In the present assay, it is clear that different domains melt at different temperatures; therefore, the relative contribution of a single base substitution may be greater than that predicted by modeling, which assumes a single melting transition. Recent work has demonstrated that this system can discern heterozygous single base substitutions in short amplicons (<400 bases long) with near 100% sensitivity.\(^1\) Detection of homozygous substitutions may be enhanced by mixing with wild-type amplicon before melting analysis.

Detection of allele *6 would require the use of another primer pair because the adenine base pair deletion at nt 818 is outside the target regions of the primers for exons 3 and 7. Unlike other approaches that use fluorescent primers or probes, melting curve analysis with LCGreen I interrogates the entire amplicon and may be useful for mutation scanning. Melting curves of amplicons from different heterozygotes should trace different paths defined by the unique heteroduplexes and homoduplexes.

Our data also demonstrate that for patients whose anticoagulation is being managed carefully, individuals require lower maintenance warfarin doses in proportion to the number of non–wild-type alleles. The 2 homozygous poor-metabolizer patients required very low doses to maintain a therapeutic INR. Although none were identified during this study, this assay should also be capable of identifying *2/*3 compound heterozygotes, who are predicted to behave similarly to homozygote poor metabolizers. Because these patients are participants in a specialized antiocoagulation clinic and are being monitored closely, it is not surprising that bleeding events and poor outcomes are rare. All genotypes demonstrated a similar profile for subtherapeutic and supratherapeutic INRs, consistent with close monitoring of the coagulation status of the patients. This study was not designed to capture adverse events related to initiation of warfarin therapy because this usually occurs in a hospital setting before enrollment in the outpatient anticoagulation management clinic. Previous data support the proposition that adverse events occur most frequently during the initiation phase of therapy.\(^1\)

Genotyping patients before initiation of therapy might allow more careful titration of dosing in those with poor-metabolizer genotypes. As more data become available, it may be possible to incorporate genotype in the dosing model for warfarin. Although the CYP2C9 genotype contributes to the observed dosing variability for warfarin, genotype does not account for all of the patient-to-patient variability. As with all P-450s, 2C9 is inducible, and numerous endogenous and exogenous compounds interact with the enzyme. The CYP2C9 genotype provides useful information, but other factors also contribute to individual responses to warfarin.

Previous studies have shown that patients with variant CYP2C9 genotypes experience a higher rate of above-range INRs, less stability with maintenance warfarin therapy, and a higher risk of life-threatening bleeding events.\(^4\) Understanding the pharmacogenetics contributing to the variability in the warfarin dose responses might help tailor therapy to patients in a more safe and effective manner. The use of CYP2C9 genotyping tests may identify high-risk patients who are candidates for lower warfarin doses, more frequent monitoring, or treatment with alternative drugs as they become available. However, the clinical effectiveness of prospective CYP2C9 genotyping in reducing adverse bleeding events is yet to be proven. The availability of a rapid and simple test for CYP2C9 genotyping should make more widespread testing possible and facilitate clinical trials.

References


