Warfarin Genotyping in a Single PCR Reaction for Microchip Electrophoresis

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BACKGROUND: Warfarin is the most commonly prescribed oral anticoagulant medication but also is the second leading cause of emergency room visits for adverse drug reactions. Genetic testing for warfarin sensitivity may reduce hospitalization rates, but prospective genotyping is impeded in part by the turnaround time and costs of genotyping. Microfluidics-based assays can reduce reagent consumption and analysis time; however, no current assay has integrated multiplexed allele-specific PCR for warfarin genotyping with microfluidic microfluidics hardware. Ideally, such an assay would use a single PCR reaction and, without further processing, a single microchip electrophoresis (ME) run to determine the 3 single-nucleotide polymorphisms (SNPs) affecting warfarin sensitivity [i.e., CYP2C9 (cytochrome P450, family 2, subfamily C, polypeptide 9) *2, CYP2C9 *3, and the VKORC1 (vitamin K epoxide reductase complex 1) A/B haplotype].

METHODS: We designed and optimized primers for a fully multiplexed assay to examine 3 biallelic SNPs with the tetraprimer amplification refractory mutation system (T-ARMS). The assay was developed with conventional PCR equipment and demonstrated for microfluidics infrared-mediated PCR. Genotypes were determined by ME on the basis of the pattern of PCR products.

RESULTS: Thirty-five samples of human genomic DNA were analyzed with this multiplex T-ARMS assay, and 100% of the genotype determinations agreed with the results obtained by other validated methods. The sample population included several genotypes conferring warfarin sensitivity, with both homozygous and heterozygous genotypes for each SNP. Total analysis times for the PCR and ME were approximately 75 min (1-sample run) and 90 min (12-sample run).

CONCLUSIONS: This multiplexed T-ARMS assay coupled with microfluidics hardware constitutes a promising avenue for an inexpensive and rapid platform for warfarin genotyping.

Warfarin (for example, Coumadin®) is the leading oral anticoagulant in the US, with >2×10⁶ new prescriptions every year (1, 2). Alarminglly, warfarin has been identified as the second leading cause of drug-related emergency room visits (3). Initiation of warfarin therapy is complicated by many factors, including a narrow therapeutic index and substantial interpatient variability in warfarin sensitivity. This variability is due in part to genetic factors. (5)-warfarin is metabolized by cytochrome P450 variant 2C9 (CYP2C9), and the *2 and *3 single-nucleotide polymorphisms (SNPs) in the CYP2C9 gene (cytochrome P450, family 2, subfamily C, polypeptide 9) gene cause impaired clearance of warfarin (1). A major haplotype of the pharmacologic target of warfarin, encoded by the VKORC1 (vitamin K epoxide reductase, complex 1) gene, is predictive of an increased sensitivity to warfarin, which is determined by SNP 6853 (−1639AG) or 6484 (1173CT) (4, 5). Other SNPs that have been evaluated for warfarin dosing include CYP2C9 alleles *4 and *5, APOE (apolipoprotein E) allele *E4, and GGCX (γ-glutamyl carboxylase), but their predictive value among patient populations has been modest and inconsistent (6). The CYP2C9 and VKORC1 genetic factors account for approximately 40% of interpatient warfarin dosing variation, and substantial portions of many patient populations carry one or both factors (1, 5).

An ongoing controversy exists regarding the clinical utility of warfarin genotyping, but recent prospective clinical trials have shown a statistically significant
improvement in patient outcomes when genotyping is performed before warfarin dosing (7–9). One study by Epstein et al. found that knowledge of the CYP2C9 and VKORC1 genotype reduced hospitalizations for bleeding or thromboembolism by 43% compared with non-genotyped control groups (7). Importantly, genotyping was most useful before initial dosing, and its clinical utility decreased once monitoring of the international normalized ratio had begun (7–9). Many testing methodologies, but certainly not all (see Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol58/issue4), have a lengthy turnaround time that precludes predosing genotyping in emergency cases. A rapid turnaround time is key to maximizing the utility of warfarin genotyping.

Cost considerations also affect the feasibility of routine warfarin genotyping. Tests for warfarin genotyping currently cost an average of $250 (10, 11), and many insurers will not reimbursement at this price (7, 8). Cost considerations for warfarin genotyping platforms include the cost of reagents and consumables, up-front instrumentation costs, and the amount of handling required. When fairly ubiquitous equipment is used for an assay, laboratories may be able to validate the method for their needs without purchasing a new genotyping platform. The economics of warfarin pharmacogenetic testing has created a demand for low-cost genotyping featuring low instrumentation costs and good economy for a relatively low volume of samples.

To address both costs and turnaround-time concerns, we set out to design an assay that would use widely available reagents, any PCR thermocycling equipment, and rapid microchip gel electrophoresis (ME) instrumentation. Published methods for warfarin genotyping include allele-specific PCR (12, 13), PCR with ligation (14), single-base extension (15), PCR with restriction enzyme digestion (16), melt analysis (17), and quantitative PCR (18, 19). Despite the wealth of published genotyping methods, multiple PCR reactions are required, specialized equipment is needed, or the assays call for sample cleanup steps that complicate analyses. In particular, we sought a genotyping method that would require only a single PCR amplification followed by ME, because such a method would be well suited to an integrated microfluidics device (20). One such method is the amplification refractory mutation system (ARMS) (21), in which primers anneal to the SNP site at their 3’ end. Under the right conditions, Taq polymerase will not extend a primer that does not match the DNA template and exhibit the canonical Watson–Crick base pairing with the SNP site (22). ARMS assays provide a digital “yes” or “no” answer for 1 allele according to the presence or absence of the allele-specific amplicon. When 2 pairs of ARMS primers are applied to a single SNP, unambiguous genotyping of biallelic SNPs is achieved in what is called “tetraprimer” ARMS (T-ARMS) (23–25). Both ARMS (12) and T-ARMS (13, 26, 27) have been applied to pharmacogenetic testing, but a fully multiplexed T-ARMS assay for warfarin genotyping has not been described. We describe our efforts to multiplex 3 T-ARMS primer sets together for a definitive determination, in a single PCR reaction, of CYP2C9 *2, CYP2C9 *3, and VKORC1 genotypes used in warfarin dosing algorithms.

Materials and Methods

ASSAY SCHEME

Our multiplex T-ARMS assay is presented schematically in Fig. 1. As with the ARMS assay, allele specificity is derived from the primers (Fig. 1A). Either of 2 “inner” allele-specific PCR products may be formed, along with an “outer” PCR product as an internal positive control (Fig. 1, B and C). Three SNP sites were genotyped by developing nonoverlapping T-ARMS PCR products (Fig. 1D). When the PCR products were separated by size via ME, each genotype generated a characteristic pattern of peaks (Fig. 1E).

SEQUENCE ANALYSIS, PRIMER DESIGN, AND PCR SIMULATION

Sequences for the SNPs were obtained from the National Center for Biotechnology Information (NCBI): CYP2C9 *2 (430C>T, rs1799853), CYP2C9 *3 (1075A>C, rs1057910), and VKORC1 6484 (1173C>T, rs9934438). Comparisons with homologous sequences were made with the NCBI basic local alignment search tool (BLAST) for nucleic acid sequences. Outer primers for the VKORC1 6484 primer set were designed by using the Primer3 online primer-design tool (28). PCR was simulated in silico with AmpliFiX [by Nicolas Jullien; version 1.44 (http://itfrj.nord.univ-mrs.fr/AmpliFiX-Home-page)] and Amplify 3 [for Mac OS X; B. Engels (http://engels.genetics.wisc.edu/amplify/)].

To apply the T-ARMS assay methodology to warfarin pharmacogenetic testing, we designed 3 compatible primer sets to determine the genotypes of the CYP2C9 *2, CYP2C9 *3, and VKORC1 loci in a single multiplexed PCR reaction. Highly homologous genes (CYP2C19, cytochrome P450, family 2, subfamily C, polypeptide 19; CYP2C8, cytochrome P450, family 2, subfamily C, polypeptide 8; CYP2C18, cytochrome P450, family 2, subfamily C, polypeptide 18) necessitate CYP2C9 subtype–specific outer primers as well. A library of potential primers was generated from the sequences of the multiple candidate-primer binding sites, and a list of theoretical amplicon sizes was assembled. T-ARMS primer sets consisting of 2 outer primers and 2 inner primers were chosen for each of the 3 SNP
Frequency was given to primers that yielded products with unique sizes within the assay so that PCR products could be identified by size via ME.

To enhance allele or subtype specificity, we gave primers an intentional mismatch to destabilize the 3′ end to ensure that effective priming would occur only if the terminal base matched the template. The thermodynamic stabilities of mismatched bases (e.g., A:A, A:C, A:G) are not uniform, and thus the position and base of each intentional mismatch were selected to normalize the stability of the 3′ end of the primers. Primer dimers predicted to be very stable were averted by adding an additional intentional mismatch in the middle of the primer where dimerization was expected. To avoid overlapping product sizes, we increased the lengths of the \textit{VKORC1} products by adding a string of poly(AT) bases at the 5′ end of the primers. Finally, the length of each primer was adjusted to yield primers with compatible annealing temperatures (\(T_m\)). The sequences of the final set of primers are shown in Table 2 of the online Data Supplement.

**PRIMER PREPARATION AND CONVENTIONAL PCR**

Primers were synthesized by Eurofins MWG Operon. Lyophilized primers were suspended in deionized water, and 20-\(\mu\)mol/L working stocks were prepared in deionized water or in 100 \(\mu\)mol/L Tris, pH 8.0, and stored at \(-20^\circ\)C. Tris buffer, deoxynucleoside triphosphates, and BSA were obtained from Fisher BioReagents. AmpliTaq Gold polymerase, GeneAmp PCR Buffer II, and 25 mmol/L MgCl\(_2\) were purchased from Applied Biosystems. SpeedSTAR polymerase was obtained from Takara Bio. Cheetah Taq hot-start polymerase was obtained from Biotium. Conventional PCR reactions were performed in a Bio-Rad MyCycler thermal cycler (Bio-Rad Laboratories). As with all rapid PCR assays, thermal cyclers should be validated for rapid thermocycling before use for this assay, because nonideal thermocycling may adversely affect the specificity and yield. The Agilent 2100 Bioanalyzer DNA 1000 Series II kits and instrumentation (Agilent Technologies) were used for ME analysis of post-PCR samples.
Primer were tested in conventional PCR amplification reactions at increasing levels of multiplexing—first in pairs, then the 4 primers for each SNP site, and then all 12 primers together. Systematic studies of the MgCl₂ concentration and thermostability annealing temperatures were conducted to identify the conditions that produced a satisfactory yield. Samples of known genotype were used as template to verify the allele specificity of the inner primers. Verification of the CYP2C9 subtype specificity was possible because the closely related genes (CYP2C19, CYP2C18, and CYP2C8) contain a wild-type base at the site homologous to the CYP2C9 *2 and *3 SNPs. To streamline analyses, we wrote an algorithm for the Agilent 2100 Expert software (and developed a custom LabVIEW program) to interpret the pattern of PCR product peaks and to automatically report the genotyping results in standard notation.

The optimized thermocycling conditions were as follows: 2 min at 95 °C; 10 cycles of 5 s at 95 °C, 5 s at 62 °C, and 5 s at 72 °C; 25 cycles of 5 s at 95 °C, 5 s at 60 °C, and 5 s at 72 °C; and 2 min at 72 °C. The optimized composition of the reaction mixture was as follows: 1× GeneAmp PCR Buffer II, 3 mmol/L MgCl₂, 0.075 μg/μL BSA, 0.2 mmol/L of each deoxynucleoside triphosphate, 1 ng/μL human genomic DNA (hgDNA), primers at the concentrations listed in Table 2 in the online Data Supplement, and 0.05 U/μL of Cheetah Taq polymerase in a total volume of 20 μL.

MICROCHIP PCR

The PCR microdevices used in this work were fabricated from Borofloat glass. Fabrication and operation processes have previously been described in greater detail (31). The PCR reaction mixture was identical to that described in the previous section, except for higher concentrations of BSA (0.4 μg/μL) and Taq polymerase (0.1 U/μL). Microchip PCR reactions required a 4-μL total volume, with a PCR chamber volume of <1 μL.

The dual-module microfluidics apparatus consisted of an infrared-mediated PCR system, (see Fig. 1A in the online Data Supplement) and the Agilent 2100 Bioanalyzer ME system. The hardware of the infrared PCR system has been described previously (31), but the present study was the first implementation of dual-stage thermocycling on this system.

PATIENT SAMPLES

Clinical samples of prepurified hgDNA from 10 deidentified individuals were obtained from the University of Virginia Pathology Department and the Mayo Clinic. Some samples were split and blinded to the experimenter in order to assess reproducibility. The CYP2C9 genotypes of the samples were determined with the Invader® assay (University of Virginia pathology samples) or by allele-specific PCR assay (Mayo Clinic samples). The VKORC1 genotype at the 6484 locus was determined by bidirectional sequencing of PCR products encompassing the SNP site. Thirteen samples of deidentified hgDNA were prepared from buccal swabs, evaluated with the T-ARMS assay while genotypes were unknown, and then genotyped at all 3 loci via Sanger sequencing. Some samples were obtained from patients already undergoing warfarin genotyping; ergo, the population may contain a high proportion of warfarin-sensitive genotypes relative to the general population. One dual heterozygote was encountered (*1*2/AB) and was readily genotyped with the T-ARMS assay, but other compound heterozygotes were not part of this sample set. This collection of genotypes had at least 1 homozygote for both wild-type and mutant alleles and a heterozygote for each locus, thus allowing verification of allele specificity at each of the 3 SNP sites.

RESULTS

The results of this study were initially computational, with a substantial amount of in silico optimization with a library of potential primers. This step was followed by primer synthesis and in vitro testing and optimization. A set of 12 primers and specific PCR reaction conditions were evaluated empirically for accurate and reproducible genotyping of clinical samples. After optimization with conventional equipment, this assay was adapted to a dual-microchip apparatus for more rapid analysis. Blinded samples of diverse genotypes were genotyped by this method.

STUDY OF SNP SITE SUITABILITY FOR THE T-ARMS ASSAY

The sequences surrounding the *2 and *3 CYP2C9 SNP sites and the 5 SNPs constituting the VKORC1 haplotype were analyzed to find primer sequences with the subtype specificity, allele specificity, and product sizes required for this unique, single-tube T-ARMS assay. The sequence flanking the CYP2C9 *2 and *3 SNPs contained a few regions unique to the CYP2C9 subtype, and some of these regions were exploited as subtype-specific outer priming sites. One example of a CYP2C9-specific primer-binding site found through these computational studies is shown in Fig. 2. Sequence analysis of 5 VKORC1 SNPs diagnostic of the low- or high-dose haplotype (4) revealed that 4 of the 5 sites (381, 3673, 6853, and 7566) contained repetitive sequence elements problematic for a T-ARMS primer-binding arrangement. The VKORC1 6484 (1175C>T) SNP site was found to lack repetitive sequence or highly homologous sequence within the footprint of the T-ARMS binding sites. This suitability is opportune because the
6484 site (like its counterpart 3673) serves as a definitive SNP for assigning the VKORC1 haplotype.

ASSAY TROUBLESHOOTING AND OPTIMIZATION

Even after careful computational design, substantial empirical optimization was required. Each of the 9 PCR products constituting this assay were successfully amplified individually, and samples of homozygous mutant and wild-type genotypes were used to verify allele specificity of all products and, in the case of CYP2C9 sites, subtype specificity as well. In cases in which a wild-type background would be expected from non–subtype-specific amplification, conditions were identified that gave no callable product by ME of the incorrect CYP2C9 subtype or the incorrect genotype. The VKORC1 gene was similarly assessed for allele specificity.

To expedite analysis, we evaluated faster polymerases to replace AmpliTaq Gold. The SpeedSTAR polymerase gave high reaction efficiency but was unsuitable for this assay because the 3’→5′ exonuclease activity degraded the allele-specific ends of the primers. We ultimately selected the Cheetah Taq polymerase for its 5-fold reduction in the initial denaturation time (from 10 min to 2 min) while maintaining subtype and allele specificity.

In some cases, the efficiency or specificity of a specific product was suboptimal, and troubleshooting was required. Primer redesign was required when, for example, a prototype of the *1-specific inner primer at the *3 site proved to be stable against *3 template owing to relatively stable G:G mismatches. This stable mismatch led to nonspecific amplification of the *1-specific product (indicated with a dashed box in Fig. 2A of the online Data Supplement) to render a false *1*3 result for a *3*3 template. Selection of a different intentional mismatch resolved this problem, however, and produced acceptable allele specificity (see Fig. 2B in the online Data Supplement).

Next, the primer sets were multiplexed together, first by locus and then with the complete ensemble, and the troubleshooting continued. Competition between primers was problematic because unbalanced early proliferation of one of the products stunted the formation of other products (especially products sharing a common primer, such as the *2 inner product and the *2 outer product). To offset competition effects, we empirically adjusted the relative primer concentrations to those listed in Table 2 in the online Data Supplement. Furthermore, lowering the annealing temperatures led to enhancements in the formation of larger products (see the dashed boxes in Fig. 2, B and C, in the online Data Supplement). Degraded template DNA has been shown to reduce the intensity of the outer products (Fig. 3, samples 23–35), but the allele-specific inner products show ample intensities for accurate genotype calls.

As a proof of principle for microchip PCR, we amplified 2 of the known genotype samples on the microchip format and generated full profiles with the correct genotype. For this assay, we reduced the required thermocycling time from 45 min in a benchtop thermal cycler (of which approximately 70% is ramping time) to 30 min in a microchip (of which approximately 30% is ramping time). At excessively low annealing temperatures, we observed a nonspecific 400-bp peak, but the robust peak-sizing capability of the ME instrument allowed reproducible peak identification and genotype calls. The determined assay conditions gave a mean signal-to-noise ratio for undesired allele- or subtype-nonspecific peaks of 3.62 (SD, 0.45) relative fluoro-
For allele-specific peaks (products of the inner primers), the assay gave a minimum mean signal-to-noise ratio of 52.6 (7.82) relative fluorescence units and a maximum mean signal-to-noise ratio of 414 (67.2) relative fluorescence units. The turnaround time for a single hgDNA sample was as short as 75 min for conventional PCR and ME, and 60 min for the microchip PCR and ME setup. Given that 12 conventional PCR reactions may be analyzed on a single Agilent 2100 microchip, batches of 12 samples were run; any increases in processing time were due only to the additional pipetting. We tested 14 samples of known genotype with the full complement of primers and, after a period of empirical optimization, observed 100% agreement between the predetermined genotypes and the experimental assay results.

**ANALYSIS OF BLINDED SAMPLES**

After troubleshooting and optimization, the new assay was further validated by the analysis of 21 blinded samples (8 split samples, 17 unique genomes in all). Genotyping results were concordant with the previously determined genotypes for the patient samples. Fig. 3 summarizes the analysis of the electrophoretic data and presents results for 35 samples genotyped with this assay. Of the 35 genotype determinations attempted, 100% were concordant with the results obtained via other validated methods. Analysis of the blinded split samples showed the split samples to be in complete agreement with respect to peak patterns. When taken together with the unblinded samples, our multiplex T-ARMS assay correctly and reproducibly genotyped 35 clinical samples (23 unique donors) for the following genotypes: *1*1/AA (21.7%), *1*1/BB (21.7%), *1*1/AB (13%), *1*3/BB (8.7%), *3*3/AB (8.7%), *1*2/AB (4.3%), *2*2/AA (4.3%), *2*2/AB (4.3%), *3*1/BB (4.3%), and *3*1/AA (4.3%). Optimized conditions and preparation of the reagents in advance allowed batch genotyping via conventional thermocycling in 45 min and single-sample ME analysis in 30 min, or approximately 90 min for 12 samples.

**Discussion**

In response to the economics and time sensitivity of warfarin pharmacogenetic testing, we developed a simple PCR-based CYP2C9/VKORC1 genotyping assay and implemented it on a microchip platform for rapid testing. A system of 12 primers was designed de novo to unambiguously determine a patient’s genotype at the CYP2C9*2, CYP2C9*3, and VKORC11173CT SNP sites in a single multiplexed PCR reaction. To our knowledge, this method is the first instance of 3 separate T-ARMS assays conducted in a single reaction. Moreover, it is the first single-reaction PCR-based genotyping method with single-color ME for simultaneously determining wild-type, heterozygote, and mutant genotypes at the CYP2C9*2, CYP2C9*3, and VKORC11173CT SNPs. Automated allele calling from the electrophoretic data was implemented to streamline the genotyping process.

After optimizing the assay in conventional-tube PCR with ME, we conducted proof-of-principle experiments on a dual-microchip platform to improve speed and reagent consumption. The combined analytical processing time from input of a single hgDNA sample to genotyping result was 75 min for conventional PCR.
with ME and 60 min for the modular microfluidics infrared PCR and ME platform. This assay was validated with 35 patient samples consisting of a range of CYP2C9 and VKORC1 genotypes, and 100% concordance with results obtained via other validated genotyping methods was achieved. This novel multiplex PCR assay and a future optimized microfluidics analysis platform should constitute a rapid, low-cost option for warfarin genotyping.

**Author Contributions:** All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

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**References**