A Multiplex Assay for Detecting Genetic Variations in CYP2C9, VKORC1, and GGCX Involved in Warfarin Metabolism

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BACKGROUND: Patients differ in responses to warfarin, which is commonly prescribed to treat thromboembolic events. Genetic variations in the cytochrome P450, family 2, subfamily C, polypeptide 9 (CYP2C9), vitamin K epoxide reductase complex, subunit 1 (VKORC1), and gamma-glutamyl carboxylase (GGCX) genes have been shown to contribute to impaired metabolism of warfarin.

METHODS: We designed a custom multiplex single-nucleotide polymorphism (SNP) panel to interrogate the CYP2C9 *2, *3, VKORC1 (–1639G→A), and GGCX (1181T→G) alleles simultaneously in a single sample by use of single-base extension and capillary electrophoresis after genomic DNA extraction and PCR amplification.

RESULTS: Our assay successfully detected various genotypes from known controls and 24 unknown samples. It was found to be 100% concordant with sequencing results.

CONCLUSIONS: Our multiplexed SNP panel can be successfully used in genotyping of patient blood samples. Results can be combined with other clinical parameters in an algorithm for warfarin dosing. These data provide a proof-in-principle of multiplexed SNP analysis using rapid single-base extension and capillary electrophoresis, and warrant additional validation using a larger cohort of patient samples.

Pharmacogenetics is the study of genetic differences and their effects on drug metabolism. The application of pharmacogenetics in identifying individuals with the polymorphisms that produce various responses in patients and the concomitant adjustment of their warfarin dose is expected to confer substantial benefit to patients and to the healthcare system overall. Improved patient safety and reduction in healthcare costs can be realized by decreasing the time required to achieve a stable international normalized ratio, thereby decreasing the risk of adverse events and the length of hospitalization for some patients. The importance of this initiative is underscored by its inclusion in the Critical Path Initiative of the US Food and Drug Administration as an example of patient-tailored therapies. In addition, the US Food and Drug Administration recently mandated the inclusion of a warning label on warfarin packaging recommending pharmacogenetic testing for patients starting such therapy.

Studies of heterogeneity in patient sensitivity to warfarin have led to identification of at least 2 genes that contribute to differential responses in patients. These genes, cytochrome P450, family 2, subfamily C, polypeptide 9 (CYP2C9) and vitamin K epoxide reductase subunit protein 1 (VKORC1), contribute approximately 60% to this difference. There are 2 clinically important alleles of CYP2C9 [2C9*2 (430C→T) and 2C9*3 (1075A→C)] and 1 for VKORC1 (–1639 G→A) with demonstrated effects on warfarin metabolism. In addition, another recently described polymorphism in the gamma-glutamyl carboxylase (GGCX) gene, 1181T→G, has demonstrated a modest yet significant effect on warfarin metabolism. Because the GGCX gene product is immediately downstream of the VKORC1 gene product in warfarin metabolism, and its genetic variation contributes mildly to the warfarin response, we included this in our panel.

We sought to design a multiplexed assay allowing for the interrogation of these 4 single-nucleotide polymorphisms (SNPs) in a single sample. Primers for PCR of the appropriate gene fragments were used for amplification, in a single plex or multiplex format. Subsequently, SNP interrogation oligonucleotides were used to query the allele of interest, with the antisense strands being investigated for the 4 SNPs. Unlabelled oligonucleotides of varying sizes were designed to flank the SNP of interest and were subsequently extended by 1 base with base terminator. This entire reaction was per-

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5 Human genes: CYP2C9, cytochrome P450, family 2, subfamily C; VKORC1, vitamin K epoxide reductase subunit protein 1; GGCX, gamma-glutamyl carboxylase.
formed in solution. In the final step, the multiplexed SNP reaction was separated by capillary electrophoresis, and a window of 10–90 bp was visualized. Because each of the bases was labeled with a different color fluorophore, we readily detected the allele on the basis of peak size and color.

Genomic DNA was isolated from whole blood by using the QIAamp DNA mini kit (Qiagen) according to manufacturer's instructions. Primer3 software (http://frodo.wi.mit.edu) was used to design primers to amplify fragments of 200–700 bp surrounding the SNP of interest. Details for each of the PCR primers are listed in Table 1; they were amplified simultaneously in a single multiplex reaction. In a total reaction volume of 40 μL, the final concentration for each component was as follows: 50 nmol/L of each forward and reverse primer, 200 nmol/L dNTPs, 100 ng genomic DNA, 10× reaction buffer [100 mmol/L Tris-HCl (pH 8.6), 500 mmol/L KCl, 15 mmol/L MgCl2], nuclease-free water, and 0.5 μL Taq Polymerase (5 U/μL; USB). Samples were subject to these cycling parameters: denaturation at 95 °C for 10 min, 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 68 °C for 1 min, followed by 4 °C hold. Each primer set amplified a single fragment, and the multiplex reaction produced 4 bands comigrating with the appropriate singlet bands (Fig. 1). The SNP oligonucleotide extension reaction was performed in a 10-μL reaction volume comprised of 4 μL SNPStart MasterMix, 1 μL of nuclease-free PCR-grade water, 5 μmol/L (final concentration) of each SNP interrogation primer (from 100 μmol/L mix), and 2 μL PCR product. The SNP interrogation oligonucleotides were of different lengths (Table 1), and after capillary electrophoresis, resolved and manifested as distinct peaks of the appropriate size (Fig. 1).

Our multiplexed SNP interrogation assay was used for the simultaneous detection of these 4 alleles in a single reaction, and entailed these steps: (a) PCR amplification of the target gene fragment and removal of deoxynucleotides and primers by using exonuclease–shrimp alkaline phosphatase (ExoSAP-IT, GE Healthcare); (b) inactivation of exonuclease–shrimp alkaline phosphatase; (c) hybridization of SNP oligonucleotide ending 1 base from the SNP of interest and single-base extension to allow incorporation of a fluorophore-specific chain-terminating nucleotide, by using the SNPStart Kit (Beckman Coulter), and subsequent removal of any excess dye with additional shrimp alkaline phosphatase (SAP, GE Healthcare); and (d) capillary electrophoresis separation of the fragments on a GeXP genetic analysis system (Beckman Coulter).

| Table 1. DNA sequence for all PCR primers and single-nucleotide polymorphism (SNP) oligonucleotides used in the assay, 5’ → 3’ direction. |
| PCR Primer | Sequence | Tm, °C* | Expected size, nt | Observed size, nt | Application |
| 2C9*2-f | TTGCTTGGAGATCTCCCTCCTAGTTTCG | 63 | 62 | N/A | sequencing |
| 2C9*2-r | TGGCCACCCCTGAAGTGTTCACAGAA | 60 | 278 | N/A | PCR |
| 2C9*3-f | TGTCTACACAAATGTGCGCATTTTTCCTT | 58 | 338 | N/A | PCR |
| 2C9*3-r | CACCCGGTGATGTAAGGTIGTTAAATGAT | 60 | 64 | N/A | PCR |
| VKORC1-f | CGCCAGAGGAAAGAGAGTTCAGGAGG | 64 | 64 | N/A | PCR |
| VKORC1-r | CTGCTTGCCGATGGCTGACACCTA | 64 | 42 | N/A | PCR |
| GGX-f | TGGAGGACTGCTTCACCCCGTCCGAT | 66 | 66 | N/A | PCR |
| GGX-r | AATGGAACAAAGAGAGTTCCTCGGCTG | 63 | 668 | N/A | PCR |
| SNP Primer | Sequence | Tm, °C* | Expected size, nt | Observed size, nt | Application |
| 2C9*2-SNP-f | GGATGGGAGAGGAGCTTGAAGAGAC | 63 | 27 | N/A | sequencing |
| 2C9*2-SNP-r | GGCAGCCGGCTCTCTCTTGAC | 63 | 25 | N/A | SNP |
| 2C9*3-SNP-f | AAAAAATGCTGTGTCGAGAGTCACTCGAGATAC | 64 | 35 | N/A | SNP |
| 2C9*3-SNP-r | AAAAAAAAAGCCAGCTGCTGATGGAAGGAAGTCAAA | 64 | 35 | N/A | SNP |
| VKORC1-SNP-f | AAAAAAAAAAAAAAAAAAGAGAAGACCTGAAAACAACGTTGGCC | 64 | 42 | N/A | SNP/sequencing |
| GGX-SNP-r | AAAAAAAAAAAAAAAAAAAAAAAAAAGACCACTCGATGCCCAGGAATGACG | 64 | 52 | N/A | SNP/sequencing |

* Melting temperature (Tm) was calculated with the Oligo Calc: Oligonucleotide Properties Calculator (http://www.basic.northwestern.edu/biotools/oligocalc.html).
We tested samples containing the various polymorphisms of interest (2C9*2 and 2C9*3, VKORC1, and GGCX) to confirm the correct genotype, and a single peak at the expected size was detected. We assayed all samples using this reaction and compared the results with results from automated sequencing performed with an ABI 3730 (University of California at Los Angeles Sequencing Facility), with the appropriate primers (Table 1). We tested a set of known positive samples and also a set of 24 unknown samples. The GGCX SNP exhibits a low allele frequency and therefore, as expected, was not detected in our small cohort.

Our assay can be performed within a 5-h turnaround time with minimal hands-on effort, after extraction of genomic DNA. We observed 100% concordance on 24 samples when we compared our assay with traditional DNA sequencing (see Supplemental Table 1 in the Data Supplement that accompanies the online version of this Brief Communication at http://www.clinchem.org/content/vol55/issue4). This assay can be used for high-throughput screening of patient samples, allowing for analysis of two 96-well plates on a single overnight run. It can be employed to assess a patient’s genotype with regard to each of these alleles. Because these alleles have demonstrated clinical significance in warfarin metabolism, it is important to identify patients with these polymorphisms who will be enrolled for treatment in warfarin-containing regimens.

Genotyping patients before initiation of therapy or during the first few days of starting a regimen would help determine the appropriate drug dose and improve the likelihood of achieving a stable international normalized ratio, thus reducing adverse events. Our mul-

Fig. 1. Representative electropherogram with the following genotype: CYP2C9 heterozygous 2C9*2 and wild-type 2C9*3, VKORC1 homozygous mutant, and GGCX wild-type.

Peaks at 13 and 88 bp correspond to size standards. The other peaks correspond to the 4 SNPs being interrogated, with peak size and color used for identification (A, red; C, black; G, green; T, blue). Inset shows singlet and multiplexed PCR reactions run on 2% agarose gel: lane 1, 100-bp molecular weight ladder; lane 2, 2C9*2 fragment amplification; lane 3, 2C9*3 fragment amplification; lane 4, VKORC1 fragment amplification; lane 5, GGCX fragment amplification; lane 6, multiplexed PCR reaction with all 4 products.
tiplex SNP panel can be used as a stand-alone test for patients starting warfarin therapy, or its results can be combined in an algorithm with additional parameters such as weight, height, age, and sex to provide dosing adjustment recommendations (9).

Our data indicate that this assay warrants further analysis and application with larger cohorts of patient samples to correlate these polymorphisms with requirements for maintenance doses of warfarin.

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