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Original Article

Warfarin genotyping using three different platforms

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Abstract: Genetic testing for common variants in the *CYP2C9* and *VKORC1* genes may provide useful clinical information to guide dosing patients receiving oral warfarin. Specifically, the *CYP2C9**2, *CYP2C9**3 and either the *VKORC1* -1639 G>A or *VKORC1* 1173C>T polymorphisms can be used to help predict an approximate warfarin maintenance dose needed for a particular patient. Although clinical uptake and use of this genotyping has been slow, an increasing body of literature provides evidence of the clinical utility of supplementing traditional warfarin dosing algorithms with a pharmacogenetic approach. The availability of multiple methods for clinical genotyping provides the opportunity for molecular diagnostic laboratories to introduce genotyping assays tailored to their specific needs based on variables such as testing volumes, staffing, available instrumentation and needed turnaround times. Three assays (Invader, Verigene and TaqMan) designed to detect three genetic variations associated with warfarin dosing are evaluated and compared as potential clinical tests to assist in patient care. Identical genotypes were reported by each assay for all samples tested but the assays were found to differ in turnaround time, approval status by the U.S. Food and Drug Administration (FDA), requirements for amount of input genomic DNA and other logistical factors that might make each assay more favorable in different settings.

Keywords: warfarin, *CYP2C9*, *VKORC1*, genotyping methods, Verigene, Invader, TaqMan PCR

Introduction

Warfarin is well known both for being an effective oral anticoagulant and for its difficulties in dosing due to its narrow therapeutic range and the wide interindividual variability in dosing between patients. Although factors such as age, sex, race, and weight can contribute to this inter-patient variability, dosing algorithms that include genotyping variants in the cytochrome P450, family 2, subfamily C, polypeptide 9 (*CYP2C9*) and vitamin K epoxide reductase complex, subunit 1 (*VKORC1*) genes may aid in achieving the optimal warfarin dose [1, 2]. A number of laboratory developed tests and commercial products for genotyping for warfarin dose selection have been introduced in recent years [3]. Clinical practice has been slow to adopt genotyping in the care of patients being treated with warfarin due to clinical concerns over insufficient evidence supporting the clinical utility of such testing and a paucity of evidence-based guidelines on how to use genotyping results to optimize a patient's warfarin dosing. New genotype-based dosing guidelines in the warfarin product insert and results from completed and ongoing prospective studies may result in increased utilization of genotyping to predict warfarin sensitivity [4].

The specific variants genotyped for predicting warfarin sensitivity include the single nucleotide polymorphisms (SNPs) in *CYP2C9* and *VKORC1* can be performed in the clinical setting to aid in the dosing of the oral anticoagulant, warfarin. Both the R and S enantiomer of warfarin competitively inhibits *VKORC1* (the S enantiomer being the most potent) and the S-warfarin is mainly metabolized by *CYP2C9* making these two genes and their genetic variants key contributors, along with other factors such as age, weight, race and diet, to the wide variability in the optimal daily warfarin dose between patients. The *CYP2C9* and *VKORC1* SNPs that are

established as being clinically significant for determining warfarin dosing include *CYP2C9**2 (NP_000762.2:p.Arg144Cys, rs1799853), *CYP2C9**3 (NP_000762.2:p.Ile359Leu, rs1057910), which yield reduced enzyme activity, and two *VKORC1* SNPs in complete linkage disequilibrium, normally referred to as *VKORC1* 1173C>T (rs9934438; NT_010393.15: g.22417957G>A) and *VKORC1* -1639G>A (rs9923231; NT_010393.15: g.22420768C>T) [5]. The *VKORC1* SNPs are non-coding and therefore are thought to affect optimal warfarin dose by altering *VKORC1* expression [6].

Although methods for sequencing the regions of the *CYP2C9* and *VKORC1* genes containing the clinically significant SNPs, such as Sanger sequencing, could potentially be used in the clinical setting, most molecular diagnostic laboratories choose alternate methods for routine genotyping applications due to regulatory and quality assurance issues as well as financial constraints and a need for rapid turnaround times [7]. A large number of molecular testing strategies exist for determining the genotype of an individual at one or more loci [8]. The anticipated need for warfarin sensitivity genotyping led to numerous commercial and laboratory-developed clinical assays for *CYP2C9**2, *CYP2C9**3 and either *VKORC1* 1173C>T or -1639G>A genotyping. Here two commercially-developed assays and one research use only (RUO)-based in house laboratory-developed warfarin genotyping assay are evaluated for use in a molecular testing laboratory.

Materials and methods

DNA samples

A total of fifty human genomic DNA samples isolated from de-identified peripheral blood specimens were separated into two sets for use in this study. DNA sample set A consisted of 24 samples isolated using the MagNA Pure Compact (Roche Diagnostics, Indianapolis, IN) and set B consisted of 26 DNA samples isolated using the EZ1 BioRobot (QIAGEN Inc, Valencia, CA). DNA concentration was measured using spectrophotometry (A_{260}) and appropriate dilutions were made when necessary for the Invader and TaqMan assays.

Genotyping methods

Common SNPs in *CYP2C9* and *VKORC1* were analyzed using three different methodologies: the Verigene® System (Nanosphere, Inc., Northbrook, IL), Invader chemistry (Third Wave Technologies, Madison, WI; now owned by Hologic), and TaqMan PCR assays performed on the 7500 Fast Real-time PCR System (Applied Biosystems, Carlsbad, CA). Specifically, *CYP2C9* was genotyped with respect to the *CYP2C9**1 (wild-type), *CYP2C9**2 and *CYP2C9**3 alleles and one of two *VKORC1* SNPs in strong linkage disequilibrium by each method. Because of the strong linkage disequilibrium between the two *VKORC1* variants and to allow for inter-assay comparisons, when a variant allele was detected at one locus it was assumed that a variant allele was also present at the other locus. DNA samples in set A were genotyped by all three methods and samples in set B were only genotyped using the Invader and TaqMan assays.

Verigene® genotyping

The Verigene® Warfarin Metabolism Nucleic Acid Test (IVD) (Nanosphere, Inc., Northbrook, IL) is an in vitro diagnostic (IVD) assay approved by the FDA for clinical use. Each single-use, self-contained test cartridge can be run in a random access format and contains a set of fluidic chambers with reagents for processing the attached low-density microarray with replicate features of oligonucleotide probes designed to hybridize to the specific alleles to identify *CYP2C9**2, *CYP2C9**3 and the *VKORC1* polymorphism 1173C>T. Genomic DNA samples were analyzed without any PCR amplification steps by pipetting 25 µL of genomic DNA (recommended concentration of 40-400 ng/µL) into a single test cartridge with 25 µL of sample buffer. Cartridges were then inserted into a Verigene® Processor until the automated hybridization, detection and wash steps were completed. After removing the reagent pack from each cartridge, the microarrays were then analyzed in a Verigene® Analyzer which automatically makes genotyping calls of wild-type (homozygous), heterozygous or mutant (homozygous) for each of the polymorphisms [9-11].

Invader ®genotyping

Invader® Warfarin Analyte-Specific Reagents (ASRs) were used for *CYP2C9**2, *CYP2C9**3 and *VKORC1* -1639G>A genotyping. These reagents were discontinued after the increased enforcement by the FDA of policies defining and

regulating the use of ASRs in diagnostic laboratories but individual researchers may be able to develop similar Invader® testing strategies using custom designed reagents with general purpose reagents. Each genomic DNA sample (5 μL; 10-80 ng/μL) was first subjected to three separate 15-cycle PCRs (one for each SNP) and the resulting DNA was then used in isothermal Invader® reactions containing Invader® probes and FRET cassettes on a 96-well plate[12]. Signal amplification during each Invader® reaction resulted in increased fluorescent signal for one or both alleles being interrogated in each reaction. Fluorescence for each allele was then measured on a Tecan GENiosFL plate reader and calls of either heterozygous or homozygous for one of the two alleles for each SNP were determined based on the relative fluorescent signals. Synthetic DNA controls representing heterozygous samples and "no template" water blanks were included for each SNP.

TaqMan® genotyping

For each genomic DNA samples three separate TaqMan® PCR reactions were carried out on a 96-well plate in a 7500 Fast Real-time PCR System using 1 μL of DNA (5-20 ng/μL). Each PCR contained a pair of oligonucleotide primers and two TaqMan® probes predesigned to hybridize specifically to the two alleles for each SNP (CYP2C9*2, Assay ID: C__25625805_10; CYP2C9*3, Assay ID: C__27104892_10; VKORC1 1173C>T, Assay ID: C__30204875_10) (Applied Biosystems, Carlsbad, CA). After 40 PCR cycles the relative fluorescence values for each allele were plotted against each other on an allelic discrimination plot with the 7500 version 2 software to determine *CYP2C9**2, *CYP2C9**3 and *VKORC1* 1173C>T genotypes. Each run included previously genotyped genomic DNA controls representing each allele and "no template" water blanks for each of the three loci tested.

Nomenclature

*CYP2C9**1 is reference or wild-type allele and in this study refers to *CYP2C9* alleles other than *2 (s1799853) or *3 (rs1057910) (www.cypalleles.ki.se/cyp2c9.htm). Due to the complete linkage disequilibrium between the 1173C>T (rs9934438) and -1639G>A (rs9923231) SNPs for *VKORC1*, haplotype designations (A or B) described elsewhere [1] are used to simplify comparisons between assays targeting different *VKORC1* loci.

Results

Genotyping of MagNA pure DNA samples

CYP2C9 and *VKORC1* genotypes were obtained by the Verigene®, Invader® and TaqMan® assays for all 24 genomic DNA samples extracted using the MagNA Pure Compact System(set A). One of these samples, however, had to be repeated with the Verigene® assay due to an initial "no call" result. 100% concordance in genotype results was observed between the three assays for all three loci examined. With respect to *CYP2C9,* the samples in set A included 16 (66.7%) with a *1/*1 genotype, 4 (16.7%) with a *1/*2 genotype, 3 (12.5%) with a *1/*3 genotype and a single sample (4%) homozygous for *CYP2C9**2 (*2/*2). No *2/*3 or *3/*3 genotypes were observed. *VKORC1* haplotypes in this set included 3 AA (12.5%), 12 AB (50%) and 9 BB (37.5%) DNA samples.

Genotyping of EZ1 DNA samples

DNA samples isolated using the EZ1 BioRobot (set B) were tested using the Invader® and TaqMan® assays (**Table 1**). Genotypes were defined for all 26 samples without the need for any repeat testing. Samples in set B were also found to have 100% concordance between the two genotyping methods for all three loci in *CYP2C9* and *VKORC1*. Only *1/*1, *1/*2 and *1/*3 genotypes were detected in *CYP2C9* in 16 (61.5%), 8 (30.8%) and 2 (7.7%) of the 26 samples, respectively. *VKORC1* haplotypes AA, AB and BB were detected in 2 (7.7%), 16 (61.5%) and 8 (30.8%) of the 26 samples, respectively.

Assay comparisons

Selected features for each assay were evaluated and compared in **Table 1**. The Verigene® assay required the most DNA by volume and concentration (10-80 ng/μL, 25 μL) the largest volume and total amount while the TaqMan® assay required much less DNA (5-20 ng/μL,1uL). Other comparisons were complicated due to lack of sample batching needed for the Verigene assay. The approximate turnaround times (TATs) for the Verigene®, Invader® and TaqMan® assays of 1.5, 4.0 and 2.0 hours, re-

Table 1. Comparative Features of the Verigene®, Invader®, and TaqMan® Genotyping Assays.

	Verigene®	Invader®	TaqMan®
DNA Requirements	10-80 ng/μL (25 μL)	10-80 ng/μL (5 μL)	5-20 ng/μL (1 μL)
Post DNA Extraction Turn-around Time	1.5 hr	4 hr	2 hr
Manipulations Needed	Minimal	Many	Intermediate
Maximum Batch Size	No need for batching	26	28
Unique Features	Random Access; No PCR; Automated Calls; IVD Status	ASR Reagents; Discontinued	No post-PCR Manipulation

ASR=Analyte Specific reagent; IVD=*in vitro* diagnostic

spectively were based on running a single sample with appropriate controls. Additional samples (up to the maximum batch sizes shown) were found to have very little effect on the turnaround times for the Invader® and TaqMan® assays but each additional sample run at the same time using the Verigene® assay added 5-10 minutes to the turnaround time for the entire batch. The relative number of manipulations shown for each assay reflects the need for only two pipetting steps per sample for the Verigene® assay, three different PCRs per sample/control for the TaqMan® assay and three PCRs and three Invader® reactions per sample/control for the Invader® assay.

Allelic frequencies

The genotypes of all fifty samples, set A and B combined (**Table 2**), were used to calculate observed allelic frequencies which were found to be similar to the expected frequencies for the *CYP2C9* and *VKORC1* SNPs, further confirming the accuracy of the assays used. The *CYP2C9* *2 and *3 allelic frequencies based on these 50 samples were 0.14 and 0.05, respectively. The combined allelic frequencies for the *VKORC1* haplotypes A and B were 0.38 and 0.62, respectively.

Table 2. CYP2C9 Genotypes and VKORC1 Haplotypes identified

Gene	Genotype or Haplotype	Number of samples
CYP2C9	*1/*1	32
	*1/*2	12
	*1/*3	5
	*2/*2	1
VKORC1	AA	5
	AB	28
	BB	17

Discussion

The Verigene®, Invader®, and TaqMan® assays used in this study were all suitable methods for genotyping the *CYP2C9**2, *CYP2C9**3 and *VKORC1* 1173C>T or -1639G>A SNPs that contribute to patient-to-patient variability in optimal warfarin dosing as demonstrated in the 100% concordance observed between assays. Additionally, the allelic frequencies for *CYP2C9**2, *CYP2C9**3 and the *VKORC1* variant were comparable to previous reports and other public information (www.ncbi.nlm.nih.gov/projects/SNP/) for predominantly caucasian populations, supporting the accuracy of the genotyping results [2, 5, 8].

Although some *CYP2C9* genotypes were underrepresented or not present in this study (homozygous for *CYP2C9* *2 or *3), the reproducibility of results for control samples included in each run for the Invader® and TaqMan® assays or run previously for the Verigene® assay (vendor-supplied DNA used during initial performance verification) indicate excellent performance across genotypes for all three assays.

The IVD status of the Verigene® assay may make it more favorable to many laboratories for clinical diagnostic use in the United States. De-

pending on the specific application or testing algorithm, the shorter turnaround time (TAT) of this assay (approximately 90 min after DNA isolation) may also be a influential factor for laboratories performing testing for physicians who would like to use the genotype information to guide optimal warfarin dosing for patients beginning warfarin therapy. The Verigene® assay, which can be performed with only two pipetting steps and no data analysis on the part of the user, requires less technical expertise than the other assays and could be implemented with little difficulty in clinical laboratories without extensive experience in molecular techniques. Although calculated costs per sample can be much higher for the Verigene® assay, the added technologist 'hands-on' time and dependence on multiple controls per run for the Invader® and TaqMan® assay could make the cost of performing the Verigene® assay more reasonable, especially if single samples or small batches are run [3, 13].

The current non-availability of commercial reagents for a warfarin genotyping Invader® assay would make the implementation of clinical warfarin genotyping with Invader® chemistry difficult for most laboratories without experience in designing this type of assay. Although other comparisons of warfarin genotyping methodologies using the Verigene® and Invader® assays exist in the literature [3, 13] this study also evaluates TaqMan® PCR genotyping assays. In the research setting or when larger batches are processed, the TaqMan® assay could be a more feasible choice for warfarin genotyping, especially in laboratories already equipped with real-time PCR instruments, such as the 7500 instrument, capable of genotyping with TaqMan® PCR assays.

The debate and uncertainty, since the initial 2007 warfarin relabeling, surrounding the interpretation and use of warfarin genotype in managing patients being treated with this anticoagulant, has resulted in minimal adoption of routine clinical genotyping for warfarin dosing guidance. In January 2010, the FDA further modified the warfarin product inserts to not only mention the potential effect of *CYP2C9* and *VKORC1* polymorphisms on warfarin dosing but to also include more detailed dosing guidance for the various genotypes in the form of a table (www.fda.gov/Safety/MedWatch/SafetyInformation/ucm201100.htm) (packageinserts.bms.com/pi/pi_coumadin.pdf). Whether or not warfarin genotyping becomes a routine clinical laboratory test, the time and effort invested by various clinical molecular diagnostic companies and molecular laboratories into genotyping assays such as those described in this study may serve to prepare the health care industry for future genotyping applications within and outside the field of pharmacogenetics.

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