Warfarin Genotyping Using Three Different Platforms

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Recommended Citation
Lefferts, Joel A.; Schwab, Mary C.; Dandamudi, Uday B.; Lee, Hong-Kee; Lewis, Lionel D.; and Tsongalis, Gregory J., "Warfarin Genotyping Using Three Different Platforms" (2010). Open Dartmouth: Faculty Open Access Articles. 1198.
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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. 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),
yield reduced enzyme activity,
etween 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 se-
quencing, could potentially be used in the clinical setting,
most molecular diagnostic laboratories choose alternate methods for routine geno-
typing applications due to regulatory and quality assur-
ance 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 war-
farin 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 (A260) and appropriate dilu-
tions 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 de-
tected at one locus it was assumed that a vari-
ant 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 ass-
says.

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 at-
tached 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 poly-
morphism 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 Veri-
gene® Processor until the automated hybridiza-
tion, detection and wash steps were completed.
After removing the reagent pack from each car-
tridge, the microarrays were then analyzed in a
Verigene® Analyzer which automatically makes
genotyping calls of wild-type (homozygous), het-
erozygous 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 re-
agents 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 were included for each SNP.

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

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

ASR=Analyte Specific reagent; IVD=in vitro diagnostic

respectively 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.

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 allele 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 under-represented 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.
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/Safety Information/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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References


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