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Genotyping three SNPs affecting warfarin drug response by isothermal real-time HDA assays

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ABSTRACT

Background: The response to the anticoagulant drug warfarin is greatly affected by genetic polymorphisms in the VKORC1 and CYP2C9 genes. Genotyping these polymorphisms has been shown to be important in reducing the time of the trial and error process for finding the maintenance dose of warfarin thus reducing the risk of adverse effects of the drug.
Method: We developed a real-time isothermal DNA amplification system for genotyping three single nucleotide polymorphisms (SNPs) that influence warfarin response. For each SNP, real-time isothermal Helicase Dependent Amplification (HDA) reactions were performed to amplify a DNA fragment containing the SNP. Amplicons were detected by fluorescently labeled allele specific probes during real-time HDA amplification.
Results: Fifty clinical samples were analyzed by the HDA-based method, generating a total of 150 results. Of these, 148 were consistent between the HDA-based assays and a reference method. The two samples with unresolved HDA-based test results were repeated and found to be consistent with the reference method.
Conclusion: The HDA-based assays demonstrated a clinically acceptable performance for genotyping the VKORC1 −1639G>A SNP and two SNPs (430C>T and 1075A>C) for the CYP2C9 enzyme (CYP2C9*2 and CYP2C9*3), all of which are relevant in warfarin pharmacogenetics.

1. Introduction

Warfarin is the most widely prescribed anticoagulant drug in North America. Many studies have proven the effectiveness of warfarin in preventing recurrent stroke, ischemic stroke in patients with atrial fibrillation, thromboembolism in patients with mechanical prosthetic heart valves, and myocardial infarction in patients with coronary artery diseases. Warfarin also aids in the prevention of pulmonary embolism (PE) and deep venous thrombosis (DVT) in patients requiring orthopedic surgery, and in those with a history of venous or arterial thromboembolism [1–6].

Due to polymorphic variations influencing the elimination of and sensitivity to warfarin, and other patient to patient variability, wide ranges of warfarin doses can lead to a safe degree of anticoagulation. If a dose is too low, patients are at increased risk of stroke; if the dose is too high, they are at risk for bleeding events [7]. A 2006 study estimated that, of the greater than 700,000 patients treated for adverse drug events (ADEs) in emergency departments, anticoagulants such as warfarin are associated with about 5% (or more than 35,000) of those ADEs [8]. The challenge of warfarin therapy is finding the most appropriate dose for a given patient in what can be a lengthy, and sometimes dangerous, trial and error process.

More than 30 gene products contribute to the therapeutic effects of warfarin and genetic polymorphisms in these genes affect drug responses. Among these genes, the most widely studied are the vitamin K epoxide reductase (VKOR) complex subunit 1 (VKORC1) [9] and the cytochrome P450 family 2, subfamily C, polypeptide 9 (CYP2C9) [10]. Warfarin exerts its anticoagulant effect by inhibiting VKORC1, which depletes the pool of reduced vitamin K available, and prohibits the activation of the vitamin K-dependent clotting factors and, ultimately, thrombin formation [2,11,12]. One polymorphism within the VKORC1 gene, the guanine to adenine conversion at position −1639 (or 3673) (−1639G>A, rs9923231), is associated with increased warfarin sensitivity in Caucasian and Asian people. It has been suggested that this increased sensitivity is a result of decreased production of VKORC1 mRNA from the variant allele and thus reduced expression of the VKOR enzyme [13].

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Warfarin is made as a 50:50 racemic mixture of R- and S-enantiomers, each with differing pharmacokinetic profiles and pharmacodynamic potencies. S-warfarin exerts a three- to five-fold more potent inhibitory effect on the target enzyme, VKOR, and thus is the principal active form [14–17]. S-warfarin is metabolized almost exclusively by the hepatic cytochrome P450 2C9 (CYP2C9) enzyme to the predominant inactive 7- and 6-hydroxywarfarin in a 3:1 ratio [10]. Two polymorphisms within the CYP2C9 gene, 430C>T (CYP2C9*2, rs1799853) and 1075A>C (CYP2C9*3, rs1057910) have been associated with inferior warfarin clearance and account for most of the variability in warfarin response. Patients with a low warfarin dose requirement were found to often have one or more variant alleles compared to the normal population [18]. Consequently, bleeding complications were more frequent among patients with the CYP2C9*2 or *3 allele than in the patients with CYP2C9*1 allele [19–25]. Genotyping for polymorphisms affecting warfarin drug effects has been shown to be important in reducing the time of the trial and error process for finding the maintenance dose of warfarin, thus reducing the risk of adverse effects of the drug [26].

Nucleic acid amplification technologies can be divided between those that use thermocycling of the reaction mixtures, such as the Polymerase Chain Reaction (PCR) and the Ligase Chain Reaction (LCR), and those that use isothermal incubation conditions. Isothermal amplifications are clearly preferable for point of care applications because they offer the promise of reduced equipment costs and potentially decreased reaction time. Unfortunately most isothermal reactions rely on complicated sets of primers and biochemical manipulations [27–33]. We have developed the Helicase Dependent Amplification (HDA) chemistry [34,35], which uses helicases to separate DNA strands rather than heat. The HDA reaction relies on only one DNA polymerase to amplify DNA rather than on combinations of polymerases with other enzymes, as do most other isothermal reactions. This greatly simplifies the enzymology involved in the amplification process, and reduces the manufacturing cost. HDA reactions can be performed at 37 °C using DNA helicase and polymerase from mesophilic organisms or at a constantly elevated temperature between 60 and 65 °C using thermal stable DNA helicase and polymerase, thus improving the specificity of the primers. In this report, all HDA reactions were performed at 65 °C. Like traditional PCR, HDA can utilize fluorescently labeled probes. Similar to the TaqMan chemistry, digestion of the probes by the 5′–3′ exonuclease activity of the polymerase releases the fluorescent label from its quencher, enabling it to fluoresce when excited [36]. By this way, the HDA amplification can be monitored in real-time.

In this study, we have developed three real-time isothermal HDA amplification assays for genotyping the three SNPs most commonly associated with pharmacokinetics and pharmacodynamics of warfarin, VKORC1 −1639 (or 3673) G>A (rs9923231), CYP2C9 430C>T (CYP2C9*2, rs1799853), and 1075A>C (CYP2C9*3, rs1057910). In each assay, two primers specific to each target gene are used to amplify a short DNA fragment containing the SNP. Also included are two probes, labeled with either a FAM or a VIC fluorophore at the 5′ end and a quencher at the 3′ end. The two probes differ by only one nucleotide at the location of the SNP, where one probe complements the wild-type allele and one matches the variant. When the reaction is carried out in a real-time PCR machine, the amplification can be monitored by an increase in the fluorescent signal resulting from the cleavage of the probe(s) annealed to the allele specific amplicon(s).

HDA-based assays were performed on 50 clinical samples and the results obtained were compared to those obtained with a reference method. Out of the total 150 initially performed HDA-based tests, 148 were in agreement with the results obtained with the Luminex xTAG® (Luminex Corp, Austin, TX) genotyping methodology used in our clinical laboratory. The two HDA-based tests that failed to result in a clear genotype in a first attempt were repeated and resulted in genotypes that were in agreement with the reference method.

2. Material and methods

2.1. Control DNA

Commercially available human genomic DNA samples were obtained from the Coriell Institute Cell Repository (Camden, NJ). Each DNA sample is either wild-type or variant or a heterozygote for the desired SNP.

2.2. DNA extraction from blood samples

Human waste blood specimens were utilized to isolate genomic DNA. Briefly, buffy coats were collected and 200 μL used to extract DNA by the Qiagen EZ-1 BioRobot with Qiagen EZ-1 blood 200 μL kit (Qiagen, Valencia, CA). Samples were analyzed for absorbance and concentration, aliquotted and DNA was stored at −20 °C until use.

2.3. DNA extraction from buccal samples

Genomic DNA was extracted from human buccal swabs. Three swabs were combined into a microfuge tube and lysis carried out with proteinase K at 56 °C for 20 min according to the Qiagen EZ-1 tissue protocol. The lysates were further manipulated by the Qiagen EZ-1 BioRobot with the Qiagen EZ-1 tissue kit, and analyzed for absorbance and concentration. Samples were aliquotted and stored at −20 °C until use.

2.4. Reference genotyping

For reference genotyping, 30 ng of genomic DNA was analyzed with the Luminex xTAG CYP2C9 + VKORC1 genotyping kit per protocol. Briefly, a multiplex PCR produced amplicons containing the regions of interest. The amplicons were then treated with exonuclease 1 and shrimp alkaline phosphatase to eliminate remaining primers and dNTPs. An aliquot of the treated PCR product was used in an allele specific primer extension (ASPE) reaction containing universally-tagged primers. The ASPE products were then sorted by hybridization to the universal array (bead mix) in the presence of hybridization buffer, and incubated with Streptavidin, R-Phycoerythrin conjugate (reporter solution). Samples were read on the Luminex® 100 xMAP™ Instrument and signal was generated for each of the variants. The median fluorescence intensity values (MFI) were analyzed to determine whether the samples were wild-type, heterozygous or variant for each of the variants.

2.5. Real-time isothermal HDA assays

Three isothermal HDA-based assays were developed, each for the VKORC1, CYP2C9*2, and CYP2C9*3 SNP, respectively. Assays were performed using the IsoAmp® II Universal tHDA kit (BioHelix, Beverly, MA). Each 50 μL reaction contained 3.5 mM $MgSO_4$, 30 mM NaCl, 400 μM of each dTTP, dCTP, and dGTP, 3.4 mM dATP, 200 to 300 ng of the UvrD DNA helicase from *Thermoanaerobacter tengcongensis* (BioHelix), 50 ng of the Single Stranded DNA binding protein (SSB) from *Sulfolobus solfataricus* (BioHelix), and 40 to 80 units of the full-length DNA polymerase from *Bacillus stearothermophilus* (Bst) (New England Biolabs, Ipswich, MA). The primers were obtained from Operon (Huntsville, AL) or Integrated DNA Technologies (Coralville, IA). The probes were from Applied Biosystems (Carlsbad, CA) for minor groove binding domain (MGB) labeled probes or Integrated DNA Technologies for locked-nucleic acid (LNA) labeled probes. The primer/probe concentrations were optimized individually for each assay.

Table 1
Primers and probes for the isothermal HDA-based genotyping assays.

	Name	Length	Label/modification	Sequence (5′ to 3′)	Concentration (nM)
VKORC1					
Primers	VKORC1-primer1	27	None	CTCTGGGAAGTCAAGCAAGAGAAGACC	75
	VKORC1-primer2	26	None	CCAAAATGCTAGGATTATAGGCGTGA	75
Probes	VKORC1-probe-C	14	5′ VIC, 3′ MGB and NFQ[a]	CGCACCCGGCCAAT[c]	90
	VKORC1-probe-T	15	5′ FAM, 3′ MGB and NFQ	CCGCACCTGGCCAAT[c]	20
*CYP2C9*2*					
Primers	CYP2C9*2-primer1	27	None	TTTCTCCCTCATGACGCTGCGGAATTT	75
	CYP2C9*2-primer2-LNA	27	LNA	TCCAGTA + AGGTCAG + TGA + TATGG + AGTAG[d]	75
Probes	Star2-probe-G	20	5′ VIC, 3′ MGB and NFQ	CCTCTTGAACACGGTCCTCA[c]	80
	Star2-probe-LNA-A	18	5′ FAM, internal LNA, 3′ BHQ_1[b]	T + CTTGAA + CA + CAGTC + CT + CA[c,d]	20
*CYP2C9*3*					
Primers	CYP2C9*3-primer1	27	None	CTACACAGATGCTGTGGTGCACGAGGT	75
	CYP2C9*3-primer2	30	None	GTCACTGCATGGGGCAGGCTGGTGGGGAGA	75
Probes	Star3-probe-LNA-A	20	5′ FAM, internal LNA, 3′ BHQ_1	CA + GA + GA + TA + CAT + TGA + CCTTCT[c,d]	10
	Star3-probe-C	15	5′ VIC, 3′ MGB and NFQ	CCAGAGATACCTTGACCTTCT[c]	50

[a] NFQ: Non-Fluorescent Quencher.
[b] BHQ_1: Black Hole Quencher.
[c] The SNP site in the probe is underlined.
[d] LNA is indicated by the "+" in front of the nucleotide.

The reactions were performed on a 7300 Real-Time PCR system (Applied Biosystems) with 60 (VKORC1 and CYP2C9*2) or 45 (CYP2C9*3) cycles of 66 °C for 5 s and 65 °C for 115 s (VKORC1 and CYP2C9*3) or 175 s (CYP2C9*2). The reaction setup for cycling between two different temperatures was due to the requirement of the ABI 7300 real-time instrument. The reaction itself could be carried out at a constant temperature of 65 °C.

2.6. Determination of genotypes

The genotypes of each of the three tested SNPs were determined using two complementary methods of analyzing the real-time fluorescence data. The first method is based on the C_t (cycle number for the fluorescent signal to cross the threshold for detection) difference between the FAM and VIC signals. Thresholds were set automatically by the SDS software (ABI). For a given sample, if the C_ts of the wild-type and variant probes differed by less than 5 cycles, the sample was designated as heterozygous. Homozygous samples were designated as such if only one probe (corresponding to either wild-type or variant) gave signal beyond the threshold value.

The second method of genotyping is based on the difference between fluorescence intensity changes of the FAM and VIC signals at the end of each reaction compared to that at the beginning. In each real-time run, a reaction containing 10 ng of the heterozygous DNA was performed as the standard control reaction. The fluorescence intensity change from all samples was normalized against that of the control reaction. For each SNP, the normalized fluorescence intensity for the wild-type (y axis) probe for each sample was plotted against that for the variant (x axis) probe. The plot area was divided into three sections by the $y = 2x$ and $y = 0.5x$ lines. The genotype for each sample regarding a particular SNP was determined based on the position of the sample on the above described plot. If a sample fell between the $y = 2x$ and $y = 0.5x$ lines, meaning that the normalized intensity changes between the two reporter probes differed by less than two fold, it was designated as a heterozygous sample. If a sample fell between the y axis and the $y = 2x$ line, meaning that the normalized intensity change of the wild-type reporter probe was greater than two fold of that of the variant probe, the sample was designated as a homozygous wild-type. Similarly, samples between the x axis and the $y = 0.5x$ line were designated as homozygous variant.

3. Results

3.1. Real-time isothermal HDA-based SNP genotyping assays

3.1.1. VKORC1

The VKORC1 assay uses a pair of primers that amplify an 84 base pair fragment containing the −1639G>A SNP. The HDA amplification utilizes two probes, each labeled with either FAM or VIC at the 5′ end and MGB and a quencher at the 3′ end. Each probe hybridizes to either the "G" (wild-type) or "A" (variant) allele. The primer and probe sequences are listed in Table 1. MGB labels were used in the probes to increase the melting temperature (T_m). The reaction mix was incubated at 65–66 °C for 120 min with 10 ng of the input DNA template in the ABI 7300 real-time PCR machine. The probes showed the expected specificity for the three control DNA templates (see Table 2 for the genotypes of each control DNA regarding the three SNPs). For the wild-type (GG) template (NA17207), amplification signal from only the VIC probe (specific to wild-type) was observed and that for the FAM probe (specific to variant) remained at background level through the 60 cycles (2 min per cycle) of incubation (Fig. 1A). The opposite was observed for the variant (AA) template (NA17285) (Fig. 1B). For the heterozygous (GA) template (NA17222), amplification signals from both VIC and FAM probes were observed (Fig. 1C). Although both probes have a calculated T_m of 73 °C, the FAM fluorescence was stronger than VIC at the same probe concentration, due to the shorter wavelength of FAM compared to VIC. The probe concentrations were adjusted so that the VIC probe concentration was 4.5 times higher than that of the FAM probe (90 nM over 20 nM) to balance the uneven fluorescent strength of the two fluorophores.

Table 2
Genotypes of the control DNA templates for the target SNPs.

	Genotypes[a]		
Coriell Cat. #	VKORC1	CYP2C9*3	CYP2C9*2
NA17207	Wild-type	Wild-type	Wild-type
NA17285	Variant	Heterozygous	Heterozygous
NA17247	–	Variant	–
NA17222	Heterozygous	–	Variant

[a] Genotype of a DNA sample for a particular SNP is listed only when that sample was used as a control in the genotyping assay for that SNP.

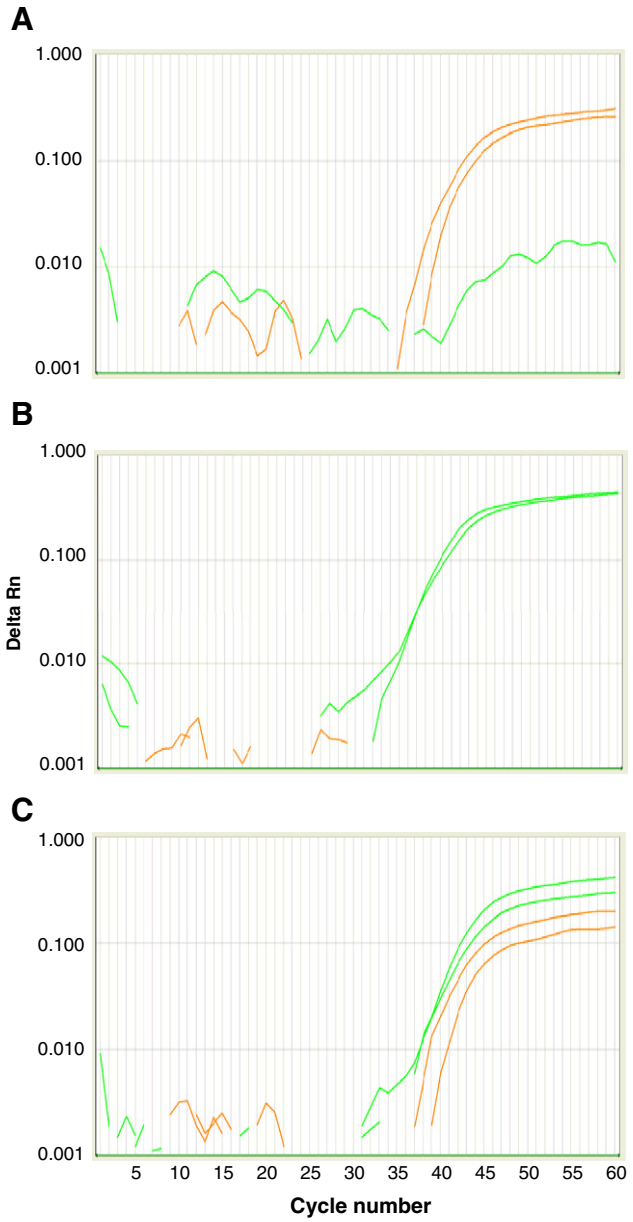

Fig. 1. Real-time isothermal HDA-based VKORC1 assay. Human genomic DNA (10 ng) bearing the wild-type (Coriell cat. # NA17207), variant (NA17285), or heterozygous (NA17222) allele of the VKORC1 SNP was added to 50 μL HDA reactions containing 75 nM primers, 90 nM VKORC1-probe-C (VIC labeled), and 20 nM of the VKORC1-probe-T (FAM labeled) and incubated at 65–66 °C for 60 cycles (2 min per cycle). Each reaction was performed in duplicate. The real-time signal for VIC is shown in orange and that for FAM in green. A. Real-time amplification plot for the wild-type (NA17207) template. Distinct amplification signal was only observed for the VIC probe, indicating that only wild-type amplicon was generated. B. Real-time amplification plot for the variant template (NA17285). Distinct amplification signal was only observed for the FAM probe, indicating that only variant amplicon was generated. C. Real-time HDA amplification plot for the heterozygous template (NA17222). Distinct amplification signals were observed for both the VIC and FAM probe, indicating that both wild-type and variant amplicons were generated.

Even at this ratio, the amplitude of the VIC signal did not reach that of the FAM, which caused the C_t value for the VIC signal to be approximate 2 cycles later than that of FAM signal (Fig. 1C).

3.1.2. CYP2C9*2 and CYP2C9*3

A BLAST search found several closely homologous sequences to the CYP2C9 gene, the most homologous ones being CYP2C18, CYP2C19, and CYP2C8. The existence of these homologous sequences limits the choice of primers with specificity for CYP2C9. For each of the CYP2C9*2 and CYP2C9*3 assays, the 3′ end of one primer was specially designed to match only the CYP2C9 sequence but not the closely homologous CYP2C8, CYP2C18 or CYP2C19 sequence, allowing for specific amplification of CYP2C9 only. Because of the low GC content (~42%) of such a primer in the CYP2C9*2 assay, internal modifications with LNA were introduced to this primer in order to produce the desired T_m (63.9 °C vs. 58.5 °C without LNA modification) optimal for the HDA reaction (Table 1). FAM and VIC labeled probes were designed the same way as those for VKORC1. However, for two of the probes, a single MGB label at the 3′ end was not sufficient to provide T_m high enough (~65 °C for the reaction temperature of HDA) for specific binding of the probe to the "A" alleles. Therefore, modification of internal nucleotides with LNA was employed for these two probes (Table 1, Star2-probe-LNA-A and Star3-probe-LNA-A) in lieu of MGB labeling.

Due to differences in amplicon length and primer efficiency, the CYP2C9*2 and CYP2C9*3 assays required 3 and 1.5 h to complete, respectively. Similar to the VKORC1 assay, the VIC probe concentration was set at 4 to 5 times higher than that of the FAM probe to balance the uneven fluorescence intensity between the two probes. In each assay, the probes showed the expected specificity for the three control DNA templates (Data not shown).

3.2. Pre-clinical assay verification with 50 clinical samples

We validated the performance of these HDA-based genotyping assays on 50 clinical samples which had been previously genotyped with the Luminex xTAG CYP2C9 + VKORC1 kit (Table 3). Out of the 50 samples, 48 originated from blood specimens, while 2 were from buccal swabs. The DNA concentration used in each 50 μL reaction ranged from 17.8 to 37.9 ng.

3.2.1. VKORC1

Review of the C_t values (Fig. 2A) for the VKORC1 assay determined that 20 samples were wild-type, containing only amplification signals for the wild-type VIC probe (C_t values represented by orange bars in Fig. 2A, for example, BHW-2, BHW-10, and BHW-11). Among the rest, 9 samples were determined to be homozygous variant, containing only amplification signals for the variant FAM probe (C_t values represented by green bars in Fig. 2A, for example, BHW-13, BHW-23, and BHW-24), and 20 heterozygous containing amplification signals for both probes (Fig. 2A, for example, BHW-1, BHW-4, and BHW-7). One sample, BHW-17, did not give amplification signal for either FAM or VIC, therefore, it was determined as "unresolved" by the HDA-based assay.

The HDA data were also analyzed using the normalized fluorescence intensity plot (Fig. 2B), which yielded genotyping results consistent with those from the C_t values. All the wild-type samples determined by the C_t values fell in the wild-type cluster on the fluorescence intensity plot (Fig. 2B, orange diamonds between y axis and the $y = 2x$ line). In fact, the fluorescence intensity changes for

Table 3
Verification of the isothermal tHDA-based genotyping assays.

	Genotypes by tHDA assays			Genotypes by reference method		
	VKORC1	CYP2C9*2	CYP2C9*3	VKORC1	CYP2C9*2	CYP2C9*3
Wild-type	20[a]	42	43[a]	21	42	44
Variant	9	1	1	9	1	1
Heterozygous	20	7	5	20	7	5
Invalid	1		1			

[a] Discrepancies between the HDA-based tests and the results determined by the Luminex xTAG CYP2C9 + VKORC1 assay. Invalid samples were found to be wild-type when the HDA-based VKORC1 and CYP2C9*3 tests were repeated.

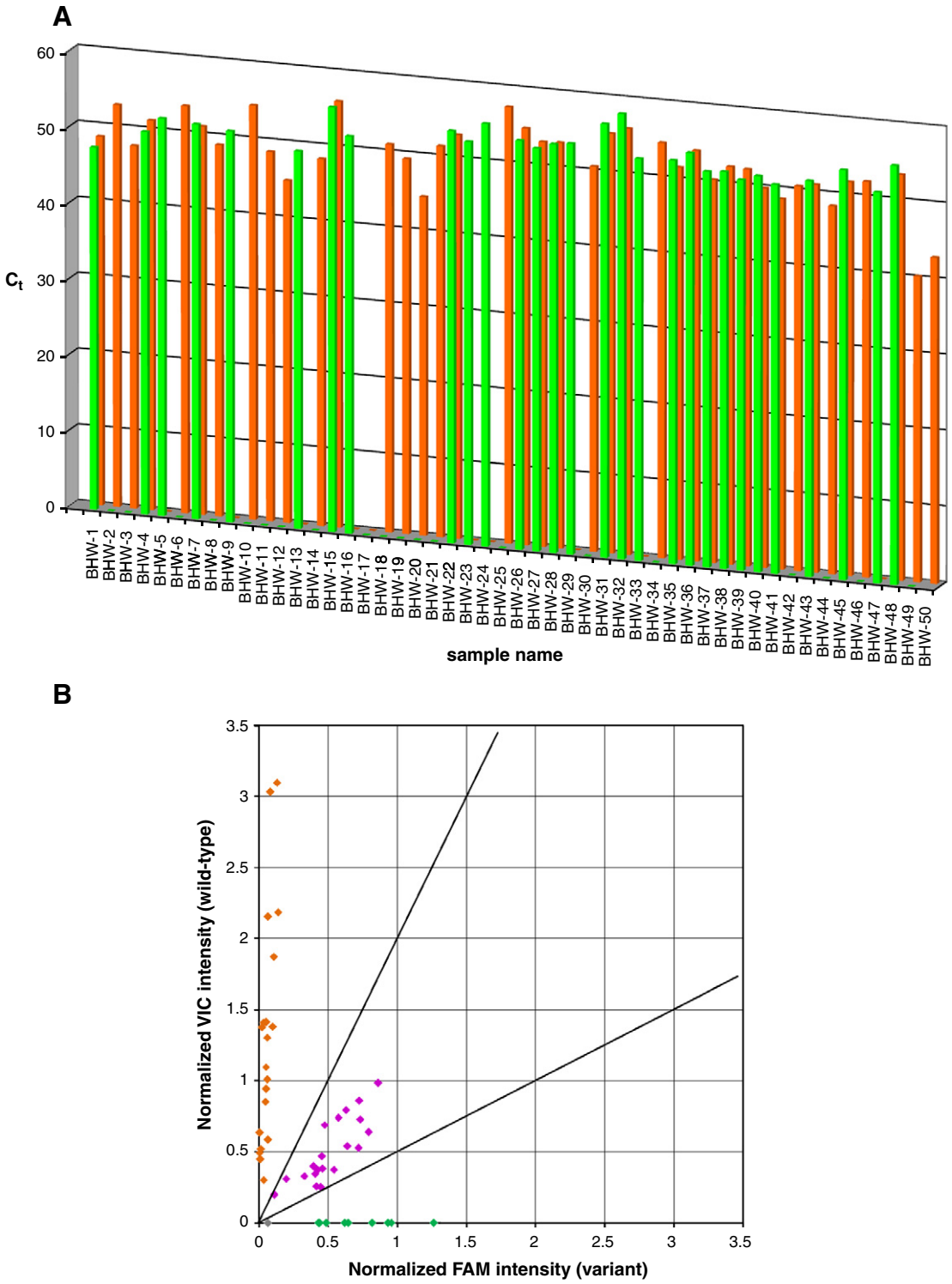

Fig. 2. Genotype determination for the 50 clinical samples. Only VKORC1 assay results were shown here. In A, C_t numbers (2 min per cycle) from the VIC signal (orange) and FAM signal (green) for each sample were plotted against the sample number. In B, Normalized fluorescence intensity changes for VIC (y axis) and FAM (x axis) for each sample were plotted against each other. The y = 2x and y = 0.5x lines are shown on the plot. Samples are grouped as wild-type (orange), variant (green), and heterozygous (magenta), respectively. The unresolved sample (BHW-17) is in grey.

the FAM labeled variant probe for these homozygous wild-type samples were very close to 0 and the samples were mostly distributed along the y axis. Likewise, the variant samples determined by C_t values were in the variant clusters (green diamonds in Fig. 2B) and distributed mostly along the x axis on the intensity plot. The heterozygous samples fell between the y = 2x and y = 0.5x lines (Fig. 2B, magenta). Sample BHW-17, which did not give amplification signal for either FAM or VIC, resulted in no significant fluorescence intensity change for either of the probes, and was close to the (0,0) point of the plot (Fig. 2B, grey diamond).

The genotyping results from HDA-based assays were consistent with the genotypes determined with the reference method (Table 3),

except for one sample, BHW-17. When the assay was repeated for sample BHW-17, it gave the expected result as a wild-type, suggesting that when this sample was not resolved with the first attempt, it was probably due to an operator error.

*3.2.2. CYP2C9*2 and CYP2C9*3*

Review of the fluorescence intensity plot and C_t values for the CYP2C9*2 assay (data not shown) determined forty-two samples were wild-type for the CYP2C9 430C>T SNP, 1 was variant and 7 were heterozygous. The HDA-based assay results were consistent with the reference genotypes for all 50 samples (Table 3).

The C_t and fluorescence plots showed that the HDA-based assay resolved 1 sample as the variant and 5 as heterozygous for the CYP2C9*3 SNP, which was in agreement with the genotypes obtained with the reference method. For the rest 44 samples, the HDA-based assay resolved 42 as unambiguously wild-type, consistent with those from the reference method. One sample, BHW-39, showed both amplification signals for FAM and VIC; however, the VIC signal appeared much later ($C_t = 37.5$) than FAM ($C_t = 25.9$). Furthermore, the intensity plot clearly showed that this sample is on the VIC axis with FAM intensity close to 0. Therefore, this sample was determined as homozygous wild-type by the HDA-based assay, consistent with the reference method. One sample, BHW-31, was unresolved due to lack of both FAM and VIC signals. Similar to the VKORC1 assay, when this sample was tested again, it was resolved as wild-type for the CYP2C9*3 SNP, suggesting operator error was responsible for the error.

4. Discussion

Numerous studies have demonstrated the correlation between the genetic polymorphisms in VKORC1 and CYP2C9 and warfarin dosing [20,37–39]. In 2010, the FDA updated the label for warfarin to include information in the "Dosage and Administration" section to remind physicians that "The patient's CYP2C9 and VKORC1 genotype information, when available, can assist in selection of the starting dose" (http://www.accessdata.fda.gov/drugsatfda_docs/label/2010/009218s108lbl.pdf). A number of warfarin genotyping assays have obtained FDA clearance. Although FDA clearance will greatly facilitate the adoption of these warfarin assays by clinical laboratories, all these tests require costly instrumentation for performing the test.

We have developed in this study isothermal genotyping assays for the three SNPs found most commonly associated with the dosage and drug response of the anticoagulant drug warfarin. The real-time genotyping of SNPs using HDA technology is very robust, and can handle a wide range of DNA concentrations. Although the three assays were carried out for different length of time, we found that extending the reaction time beyond the necessary length did not affect the test results (data not shown). Therefore, the three assays can be performed simultaneously on one machine for the time required for the slowest reaction. Because of the isothermal nature of these assays, they can be performed on a low-cost instrument instead of a real-time PCR thermocycler. This, in combination with a low-cost, instrument free method of DNA extraction, has the potential of bringing such genotyping assays to the doctor's office, thus greatly improving the speed of getting the results while at the same time lowering the cost of such tests.

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