applied biosystems
by life technologies

Genotyping of the *CYP2C9*, *VKORC1*, and *CYP4F2* Genes to Evaluate Warfarin-Metabolism Variants using the Applied Biosystems® ViiA™ 7 Real-Time PCR System

RESEARCHERS FROM NIGUARDA HOSPITAL, MILAN, ITALY

Dr. Piera Angelica Merlini is a cardiologist working in the Cardiovascular Department leading many clinical trials in ischemic cardiopathy. Silvio Veronese, Ph.D., is a senior biologist and director of the Pathology Laboratory where the main research interest is oncohematology and solid tumors. They are shown here with senior geneticist Nicola Marziliano, Ph.D., who focuses on cardiovascular diseases; junior molecular biologists Francesco Orsini, BD and Calogero Lauricela, BD; and laboratory technicians Marina Carniel, Francesca Leanza, and Paolo Agarossi.

APPLICATION

Genotyping

TECHNOLOGIES

Applied Biosystems® ViiA™ 7 Real-Time PCR System

Applied Biosystems® 7900HT Real-Time PCR System

TaqMan® Drug Metabolism Genotyping Assays

From the left (front row): Silvio Veronese, Ph.D., Marina Carniel, Francesca Leanza, Calogero Lauricella, BD, Nicola Marziliano, Ph.D., and Paolo Agarossi.

Back row: Dr. Piera Angelica Merlini and Francesco Orsini, BD.

Abstract

Real-time PCR–based genotyping analysis requires assays and instrument platforms that can deliver accurate and reproducible results. In this study, researchers from Niguarda Hospital in Milan, Italy, used TaqMan® Drug Metabolism Genotyping Assays in conjunction with either the Applied Biosystems® ViiA™ 7 Real-Time PCR System or the Applied Biosystems® 7900HT Real-Time PCR System in a research-only study to evaluate genotype variants affecting warfarin sensitivity. The data indicate that the ViiA™ 7 Real-Time PCR System produces equivalent results to those obtained with the 7900HT System. In addition, the ViiA™ 7 Real-Time PCR System offered several advantages to the workflow.

Introduction

Warfarin is the most frequently prescribed oral anticoagulant drug used for the prevention of, and treatment of individuals with, thromboembolic perturbances. However, it can cause severe bleeding resulting from significant inter-individual variability in dose requirements, with an estimate of events occurring at a rate of 1.3 to 2.7 per 100 individuals per year [1]. Ongoing pharmacogenetic research studies have estimated that the screening of warfarin-correlated allelic variants could reduce warfarin-related major bleeding events by 85,000 and reduce strokes by 17,000 annually in the United States, resulting in a reduction of $1.1 billion annually in health care spending [2]. A test that provides a simple, easy detection method for variants correlated with the warfarin metabolism is warranted.

The Role of *CYP2C9* and *VKORC1* in Warfarin Metabolism

Evidence of genetic influence in warfarin metabolism was first described by O'Reilly et al. [3]. The *CYP2C9* gene (*CYP2C9**3) and the *VKORC1* gene, encoding the warfarin "receptor" vitamin K epoxide reductase, have since been shown to play a role in individuals with warfarin resistance [4,5,6]. It is estimated that 9% of warfarin dose variance depends on *CYP2C9* variants, while genetic variability in *VKORC1* accounts for approximately 25% of dose sensitivity variance [5,6]. The influence of *CYP2C9* and *VKORC1* genotypes on warfarin dose requirements has been consistently demonstrated in diverse population groups in observational studies and randomized clinical trails [6,7] suggesting that individuals of Asian, European, and African ancestry require, on average, lower, intermediate, and higher doses of warfarin respectively.

Warfarin consists of a racemic mixture of two active enantiomers—R- and S-forms—each of which is cleared by different pathways. S-warfarin (the active enantiomer) has five times the potency of the R-isomer with respect to vitamin K antagonism. The *CYP2C9* cytochrome, the major determinant of S-warfarin metabolism, has two main genetic variants (*CYP2C9**2 and *CYP2C9**3) with reduced catalytic activity compared to the wild type gene (*CYP2C9**1). *CYP2C9* variant genotypes have been associated with a significantly increased risk of serious bleeding events. In addition, the *VKORC1* gene is also polymorphic and a variant (*VKORC1*-1639G>A) has been associated with low warfarin maintenance doses. The main genotypes of *CYP2C9* and *VKORC1* that correlate with warfarin testing are listed in Table 1. Research efforts designed to evaluate the effectiveness of genotype-guided therapy in improving outcomes are underway.

Genotyping Analysis of *CYP2C9*, *VKORC1*, and *CYP4F2* and Comparison of Instrument Platforms

In this study, allelic discrimination assays were performed to detect *CYP2C9*, *CYP4F2*, and *VKORC1* variants in 50 samples with acute coronary syndromes (ACS) and 50 samples with hypertrophic cardiomyopathy (HCM) using TaqMan® Drug Metabolism Genotyping Assays and the Applied Biosystems® 7900HT Real-Time System. The genotyping workflow was also tested using the new Applied Biosystems® ViiA™ 7 Real-Time PCR System to evaluate data reproducibility between the two systems.

Methods

DNA Isolation and Quantification

Genomic DNA (gDNA) was extracted from 50 acute coronary syndromes (ACS) and 50 hypertrophic cardiomyopathy (HCM) whole blood samples using the NucleoSpin™ Extraction Kit (Macherey-Nagel) according to the manufacturer's instructions. DNA quantification was performed using the Infinite® 200 NanoQuant Instrument (Tecan).

Genotyping

Genotypes for *CYP2C9*, *CYP4F2*, and *VKORC1* were determined using TaqMan® Drug Metabolism Genotyping Assays following the standard assay protocol on the Applied Biosystems® 7900HT Real-Time PCR System. Approximately 6–10 ng of gDNA were used in each reaction. The experiments were repeated on the new Applied Biosystems® ViiA™ 7 Real-Time PCR System.

Results

Accurate Genotyping Using TaqMan® Drug Metabolism Genotyping Assays

Figures 1–3 show genotyping results using TaqMan® Drug Metabolism Genotyping Assays. Genotypes were successfully determined in all ACS and HCM DNAs. Interestingly, in the ACS samples, the following polymorphisms were not in the Hardy-Weinberg equilibrium: rs2108622, rs1799853, and rs1057910 ($p<0.005$); while in the HCM samples, the rs1799853 and rs1057910 ($p<0.010$) SNPs were not in the Hardy-Weinberg equilibrium. In both cases, the frequency of the "adverse" allele was

Table 1. Genotypes Associated with Warfarin Dosing.

Gene Symbol and Mutation	dbSNP rs#	AB Assay ID
CYP2C9		
CYP2C9 2* p.Arg144Cys	rs28371674	C__25625805_10
CYP2C9 3* p.Ile359Leu	rs1057910	C__27104892_10
CYP4F2		
CYP4F2	rs2108622	C__16179493_40
VKORC1		
C1173T	rs9934438	C__30204875_10
-1639G>A	rs9923231	C__30403261_20
3730G>A	rs7294	C___7473918_10

Features	ViiA™ 7 Real-Time PCR System
Block configurations	96-well, Fast 96-well, 384-well, TaqMan® Array Micro Fluidic Cards
Run time	30 minutes expected (Fast 96-well) 35 minutes (384-well)
Resolution	Down to 1.5-fold changes for singleplex reaction
Excitation source	OptiFlex™ System with halogen lamp
Detection channels	Decoupled—6 emission, 6 excitation
21 CFR p11	Optional software module
Remote monitoring	Available to monitor up to 4 instruments in real time and the status of up to 15 instruments
Data export format	User configurable: *.xls, *.txt, and 7900 formats

more represented in the patient population than in the control population. This might be caused by selection bias from genotyping individuals who do not yet have an optimal anticoagulant therapy.

Instrument Comparison

Results obtained on the 7900HT Real-Time PCR System were reproducible on the ViiA™ 7 Real-Time PCR System. The accuracy of the genotyping calls and genotyping clusters was equivalent on both systems.

The ViiA™ 7 Real-Time PCR System combines the pre-PCR read, PCR amplification, and the post-PCR read into a single run, making the genotyping run convenient and easy to set up. Availability of the PCR amplification data provided an additional level of optimization and troubleshooting that was previously unavailable. The ability to use real-time data to view the traces of the cluster progression provided more confidence in making unambiguous calls. In addition, the software interface of the ViiA™ 7 System was user-friendly and made plate setup easy.

TaqMan® Drug Metabolism Genotyping Assays

Applied Biosystems® TaqMan® Drug Metabolism Genotyping Assays are a comprehensive collection of over 2,700 unique assays for detecting polymorphisms located in regulatory elements and coding regions of 220 drug metabolism and transporter genes. The assays are optimized for genotyping single nucleotide polymorphisms (SNPs), insertions and deletions (indels), and multinucleotide polymorphisms (MNPs).

The assay uses the 5´ nuclease chemistry for amplifying and detecting specific polymorphisms in purified genomic DNA samples. Each assay allows researchers to genotype individuals for a single polymorphism.

The assays were developed with Applied Biosystems' validated bioinformatics mapping, design, and *in silico* QC. Where possible, all assays have been mapped to the common public allele nomenclature. All TaqMan® DME Genotyping Assays have proven performance across 180 unique DNA samples, and work under the same amplification conditions. Search for your gene of interest at www.appliedbiosystems.com/dme.

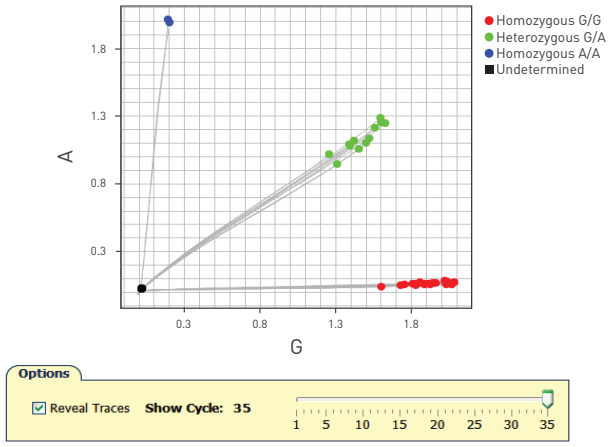

Figure 1. Allelic Discrimination Plot of *CYP4F2*. Cluster plot of 50 gDNA samples genotyped using TaqMan® Drug Metabolism Genotyping Assay C__16179493_40 on the Applied Biosystems® ViiA™ 7 Real-Time PCR System. In ASC samples, the rs2108622 SNP is not in Hardy-Weinberg equilibrium.

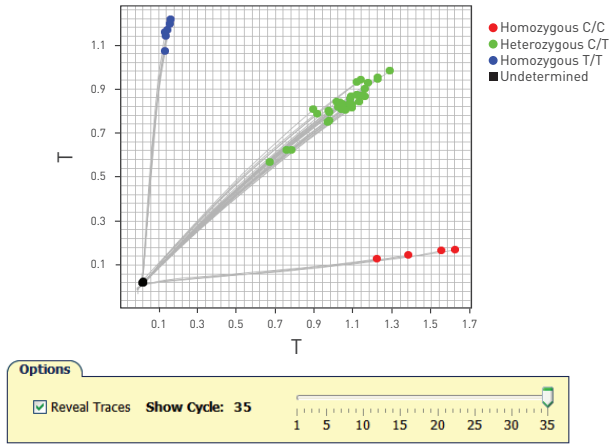

Figure 2. Allelic Discrimination Plot of *VKORC1* -1639G>A. Cluster plot of 50 gDNA samples genotyped using TaqMan® Drug Metabolism Genotyping Assay C__30403261_20 on the Applied Biosystems® ViiA™ 7 Real-Time PCR System. The results show genetic variability in *VKORC1*, which accounts for approximately 25% of dose sensitivity variance to warfarin. Homozygous C/C individuals are less sensitive to warfarin and less represented in the population.

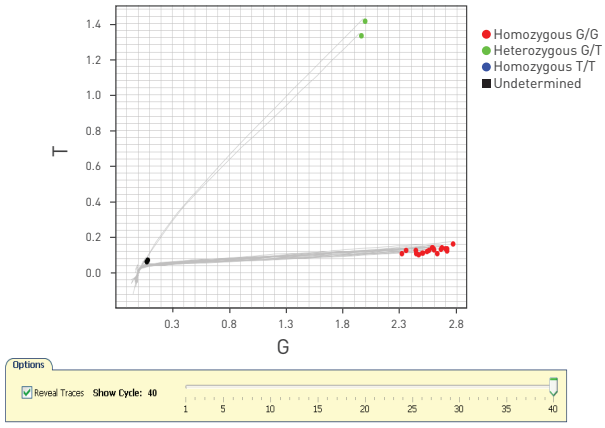

Figure 3. Allelic Discrimination Plot of *CYP2C9*. Cluster plot of 50 gDNA samples genotyped using TaqMan® Drug Metabolism Genotyping Assay C__27104892_10 on the Applied Biosystems® ViiA™ 7 Real-Time PCR System. In HCM samples, the rs1799853 and rs1057910 SNPs are not in Hardy-Weinberg equilibrium.

Conclusion

TaqMan® Drug Metabolism Genotyping Assays in conjunction with Applied Biosystems® real-time PCR instrument platforms provided a robust genotyping workflow to evaluate warfarin-correlated allelic variants. The workflow generated reproducible and reliable results on the Applied Biosystems® 7900HT Real-Time PCR System and on the ViiA™ 7 Real-Time PCR System. In addition, performing the genotyping run on the ViiA™ 7 System was convenient, and the instrument was user-friendly. This efficient, easy-to-use workflow may provide benefits to investigators operating in the clinical research environment.

References

1. Palareti G, Leali N, Coccheri S et al. (1996) Italian study on complications on oral anticoagulant therapy. Bleeding complications of oral anticoagulant treatment: An inception-cohort, prospective collaborative study (ISCOAT). *Lancet* 348:423–428.
2. Kirkwood TB (1983) Calibration of reference thromboplastins and standardization of the prothrombin time ratio. *Thromb Haemost* 49:238–244.
3. O'Reilly RA (1964) Hereditary transmission of exceptional resistance to coumarin anticoagulant drugs. The first reported kindred. *New Eng J Med* 271:809–815.
4. Rost S, Fregin A, Ivaskevicius V et al. (2004) Mutations in *VKORC1* cause warfarin resistance and multiple coagulation factor deficiency type 2. *Nature* 427:537–541.
5. Shikata E, Ieiri I, Ishiguro S et al. (2004) Association of pharmacokinetic (*CYP2C9*) and pharmacodynamic (factors II; VII, IX, and X; protein S and C; and gamma-glutamyl carboxylase) gene variants with warfarin sensitivity. *Blood* 103:2630–2635.
6. Cooper GM, Johnson JA, Langaee TY et al. (2008) A genome-wide scan for common genetic variants with a large influence on warfarin maintenance dose. *Blood* 112:1022–1027.
7. Shurin SB and Nabel EG. (2007) Pharmacogenetics: Ready for prime time? *New Eng J Med* 358:1061–1063.

Headquarters

5791 Van Allen Way | Carlsbad, CA 92008 USA | Phone +1 760 603 7200 | Toll Free in North America 800 955 6288

www.lifetechnologies.com

life technologies™