PHARMACOGENOMIC TESTING IN A CLINICAL LABORATORY: TECHNICAL AND INTERPRETATION CHALLENGES IN IMPLEMENTING VKORC1/CYP2C9 TESTING FOR WARFARIN DOSING

AWARD APPLICANT POSTER NUMBER

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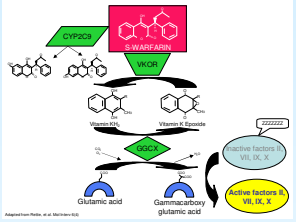
INTRODUCTION

Long-term warfarin (Coumadin) therapy prevents unwanted thrombus formation in a number of different conditions, from recurrent thrombophlebitis to implanted cardiac valves. Patients on warfarin therapy vary widely in the amount of drug needed to achieve desired results (target PT INR of 2-3). Excess dosage leads to dangerously elevated values, which can result in serious and potentially fatal consequences, including intracranial bleeds. Recent research has shown that single nucleotide polymorphisms (SNPs) in the genes VKORC1 and CYP2C9 can significantly affect the pharmacodynamics and pharmacokinetics of warfarin (Figure 1), altering the effective dosage required. Implementing clinical testing to address the effect of these SNPs includes instrument/method selection and validation, as well as choice of dosing algorithm.

FIGURE 1



Warfarin acts by inhibiting the enzyme VKOR, preventing the reduction of vitamin K epoxide, ultimately limiting the activation of factors II, VII, IX and X. Warfarin is inactivated through metabolism by CYP2C9. Polymorphisms in either gene (VKORC1 or CYP2C9) affect the pharmacodynamics or pharmacokinetics, respectively, of the drug.

MATERIALS AND METHODS

TECHNOLOGY EVAULATION

The UNC Molecular Genetics Laboratory evaluated three commercially available methods for detection of CYP2C9 and VKORC1 SNPs associated with warfarin dosage: Third Wave Invader CYP2C9 and VKORC1 Reagents, ParagonDx (formerly Gentrix) Rapid Genotyping Assay – CYP2C9 & VKORC1 on the Cepheid SmartCycler and AutoGenomics INFINITI 2C9-VKORC1 Assay. Each method was challenged with the same set of 20 samples of various genotypes, following the manufacturer's instructions for each reagent set. The samples were residual DNA from the study published in the Li paper, which were collected under IRB approval. Results were compared and discrepancies further evaluated. Turnaround time (TAT) and hands-on time were quantified by recording start and stop times for each step of the analysis process. Cost for labor was determined for each method by multiplying the hands-on time by an hourly rate of $25.00. Reagent and equipment costs were not included since contract pricing from a manufacturer will vary between institutions.

ALGORITHM EVALUATION

In order to select the most suitable dosing algorithm, we tested three (Gage, Linder and Sconce) retrospectively in a local patient population treated at the UNC coagulation clinic (n=114, 86% Caucasian, 14% African-American) and evaluated each algorithm for correlation with actual stable dosage (mean of last seven doses upon reaching stable target PT INR). All patient and SNP data used were those generated for the Li et al paper. In addition, selected samples from patients on outlier doses of warfarin were subjected to sequencing of the promoter and coding regions of the VKORC1 gene to investigate whether additional mutations could account for resistance. This data was gathered under an IRB-approved protocol. A total of 39 patients were eliminated due to inadequate patient information for one or more algorithms (n=75, 81% Caucasian, 19% African-American). Dosage predictions from each algorithm were plotted in Excel three different ways: all patients, Caucasians only and African-Americans only. Regression analysis was performed on all graphs and the R^2 values for each were compared. A trendline and identity line were added to each graph.

Required data was entered into the Gage algorithm at www.warfarindosing.com, using the May 2007 version.

The equation used for the Linder algorithm (Zhu et al.) was:
Dose (mg/day) = ln(1.35-(0.009 X age in years)+(0.116 X gender code)+(0.004 X weight in lbs)-(0.376 X VKOR-AA code)+(0.271 X VKOR-GG code)-(0.307 X CYP2C9*2 code)-(0.318 X CYP2C9*3 code),
where gender code is 1 for male, 0 for female; VKOR-AA code is 1 for AA, 0 for AG and GG(-1639); VKOR-GG code is 1 for GG and 0 for AG and AA(-1639); CYP2C9*2 code is 1 for each *2 allele; and CYP2C9*3 code is 1 for each *3 allele.

The equation used for the Sconce algorithm (Sconce et al.) was:
Dose (mg/day) = (0.628-(0.0135 X age in years)-(0.24 X CYP2C9 code)-(0.37 X CYP2C9*3 code)-(0.241 X VKOR code)+(0.0162 X height in cm))^2
where CYP2C9*2 code is 1 for each *2 allele; CYP2C9*3 code is 1 for each *3 allele; and VKOR (-1639) code is 1 for GG, 2 for AG, and 3 for AA.

RESULTS

TECHNOLOGY EVALUATION

All three genotyping methods generated accurate genotype information, with 100% concordance (Table 1). The ParagonDx assay had the shortest TAT and least hands-on time (Table 2), and would be best suited for rapid analysis of a few (up to 6) samples. This assay is not commercially available at the time of publication. The Third Wave assay was more readily scalable for larger test volumes without significant impact on TAT at the cost of increased hands-on time. The AutoGenomics method had the longest TAT but is fully automated after the PCR is completed, and could assay up to 24 samples per run. All of the assays detect CYP2C9*2 and *3 and either VKORC1 -1639G>A or 1173C>T, all of which have been correlated with warfarin sensitivity and are included in the currently available dosing algorithms. The two VKORC1 SNPs (-1639G>A and 1173C>T) are in 100% linkage disequilibrium, so the presence of either was assumed to indicate the other as well. In addition, the ParagonDx assay detects VKORC1 3730G>A, which has been associated with resistance to warfarin, requiring higher dosages than standard. The AutoGenomics method detects a total of six CYP2C9 variants (*2, *3, *4, *5, *6, *11) and eight VKORC1 SNPs: (-1639G>A, 497T>G, 698C>T, 1173C>T, 1542G>C, 2255T>C, 3482C>T, 3730G>A). None of these additional variants is included in any of the currently available dosing algorithms and their impact on warfarin dosage is currently being investigated.

TABLE 1

VKORC1/CYP2C9 COMPARISON STUDY

	THIRD WAVE †		PARAGONDx		AUTOGENOMICS	
	CYP2C9	VKORC1	CYP2C9	VKORC1	CYP2C9	VKORC1
01	*3/*3	1173/ 1173	*3/*3	1173/ 1173	*3/*3	1173/ 1173
02	wt/ wt	wt/ wt	ND	3730/ 3730	wt/ wt	3730/ 3730
03	*2/wt	wt/ wt	*2/wt	ND	*2/wt	3730/ 3730
04	wt/ wt	wt/ wt	wt/ wt	ND	wt/ wt	3730/wt
05	wt/ wt	1173/wt	ND	1173/wt	wt/ wt	1173/wt
06	*2/wt	1173/ 1173	*2/wt	ND	IND	1173/ 1173
07	*3/wt	1173/wt	*3/wt	1173/wt	*3/wt	1173/wt
08	wt/ wt	1173/wt	ND	1173/ 3730	wt/ wt	1173/ 3730
09	*3/wt	1173/wt	*3/wt	1173/ 3730	*3/wt	1173/ 3730
10	*2/wt	1173/ 1173	*2/wt	ND	*2/wt	1173/ 1173
11	wt/ wt	wt/ wt	ND	3730/ 3730	wt/ wt	3730/ 3730
12	*3/ *3	1173/wt	*3/ *3	1173/ 3730	*3/ *3	1173/ 3730
13	wt/ wt	1173/ 1173	ND	1173/ 1173	wt/ wt	1173/ 1173
14	*2/wt	wt/ wt	ND	3730/ 3730	*2/wt	3730/ 3730
15	wt/ wt	wt/ wt	ND	3730/ 3730	wt/ wt	3730/ 3730
16	wt/ wt	1173/ 1173	ND	1173/ 1173	wt/ wt	1173/ 1173
17	*3/wt	1173/wt	*3/wt	ND	*3/wt	1173/ 3730
18	wt/ wt	wt/ wt	ND	3730/wt	wt/ wt	3730/wt
19	*2/wt	1173/wt	*2/wt	ND	*2/wt	1173/ 3730
20	wt/ wt	1173/ 1173	wt/ wt	1173/ 1173	wt/ wt	1173/ 1173

† Does not test for VKORC1 3730
ND – sample not tested at this locus
IND – indeterminate results, sample inadequate to repeat

Comparison of genotyping results from all three methods.
Third Wave method characterizes VKORC1 -1639. Reported here as 1173 assuming 100% LD for clarity.
Limited testing with ParagonDx method due to time and samples available.
All results not confirmed by the other two assays were confirmed against the original study (Li, et al).

TABLE 2

VKORC1/CYP2C9 GENOTYPING TAT AND LABOR ANALYSIS

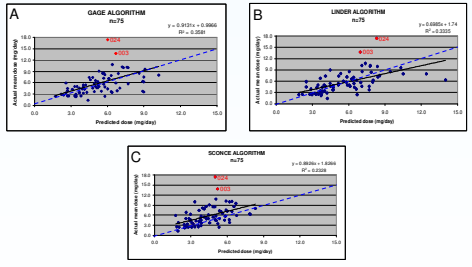
SUPPLIER	TAT (hrs)	LABOR TIME (hrs)	LABOR COST*
Third Wave	3.2	1.42	$5.92
ParagonDx	1.5	0.75	$3.13
AutoGenomics	10.6	0.83	$3.46

Time and cost figures based on 6 patient samples per run. Extraction time and costs not included.
*Labor cost = Labor time X $25/hour / # of patient samples per run

ALGORITHM EVALUATION

In the comparison of the three dosing algorithms (Figure 2, Table 3), the Gage algorithm of May 2007 demonstrated slightly better correlation with stable warfarin dose in our study population (Gage n=75, R^2=0.36; Linder n=75, R^2=0.33; Sconce n=75, R^2=0.23). The drawback to the Gage algorithm is collecting the clinical information necessary.

FIGURE 2



Regression analysis of three algorithms for warfarin dosage. **A** Gage algorithm. **B** Linder algorithm. **C** Sconce algorithm.
Solid black line = trendline generated by Excel; dashed blue line = identity line (y = x)
Patient population seen at UNC Hospitals (n=75, 81% Caucasian, 19% African-American)
Racial group data not graphed due to space constraints and low statistical significance

TABLE 3

ALGORITHM COMPARISON

ALGORITHM NAME	ALL PATIENTS	CAUCASIANS	AFRICAN-AMERICANS
Gage	n = 75 R^2 = 0.36	n = 61 R^2 = 0.46	n = 14 R^2 = 0.13
Linder	n=75 R^2 = 0.33	n = 61 R^2 = 0.39	n = 14 R^2 = 0.09
Sconce	n=75 R^2 = 0.23	n = 61 R^2 = 0.29	n = 14 R^2 = 0.12

Comparison of three algorithms for determining warfarin dosage vs. actual mean stable dose
Statistics shown for each racial group; sample size too small for statistical significance
Low R^2 values in African-Americans affected by two possible outliers

DISCUSSION

TECHNOLOGY EVALUATION

The three methods tested (Third Wave, ParagonDx and AutoGenomics) performed equally well at identifying SNPs in VKORC1 and CYP2C9 in the 20 patient samples. Reagent costs for all three assays are comparable. Each has its unique strengths and weaknesses that must be considered.

Third Wave requires little investment in equipment outside what is usually found in a molecular pathology laboratory (thermal cycler, fluorescent plate reader). It is easy to learn and has a very reasonable turnaround time (TAT) for a six-sample run (3.2 hrs vs. 1.5 and 10.6 hrs), but the most hands-on time and thus highest labor cost per sample ($5.92 vs. $3.13 and $3.46). The larger number of pipetting steps requires strict attention to protocol to prevent errors.

ParagonDx is the technically simplest assay, with the fastest TAT of the three (1.5 hrs). It also tests for the VKORC1 3730G>A SNP that may prove important in warfarin resistance. No algorithm incorporates 3730 status at this time, but investigations are ongoing. It requires a dedicated instrument, the Cepheid SmartCycler. The potential for moving this assay to the Cepheid GeneXpert would change it to moderate complexity and potentially allow for genotyping directly from blood samples. At the present time, this assay is not commercially available.

The AutoGenomics method requires the largest upfront instrument investment, the INFINITI. Hands-on time and labor cost are similar to the ParagonDx ($3.46 vs. $3.13) and the test is completely hands-off after loading the PCR product onto the instrument. The onboard ASPE/hybridization does include removing the caps from the PCR tray, a potential source of contamination. It also offers a much larger number of SNPs in both genes, thus providing additional information which may impact warfarin response. However, none of the additional SNPs are incorporated into currently available dosing algorithms. In addition, some of the VKORC1 SNPs are in linkage disequilibrium and thus do not provide additional information. The ingenious chip-based approach allows for rapid inclusion of additional SNPs or development of unique assays, and the platform works for both proteomics and genomics.

ALGORITHM EVALUATION

The three algorithms for predicting dosage varied somewhat in predictive power in our patient population (n = 75), which was 81% Caucasian and 19% African-American. The Gage algorithm performed best (R^2 = 0.36 vs. 0.33 for Linder and 0.23 for Sconce), but requires additional patient information that may be difficult to acquire, especially on referred samples. All three algorithms performed poorly in the African-American population (R^2 = 0.13, 0.09 and 0.12 for Gage, Linder and Sconce, respectively), but the sample size is too small (n = 14) to make firm conclusions. Two of the patients (003 and 024), both African-American females, have much higher actual doses than predicted by any of the algorithms. Patient 003 has a novel single nucleotide change, VKORC1 -477(4835)G/A in addition to SNPs 2255(7566)T/C and 3730(9041)A/G (haplotype H4/H7 or H8), both of which have been associated with Warfarin resistance in the Reider et al paper. Patient 024 has SNPs at VKORC1 1542(6853)C/C and 2255(7566)T/C (haplotype H1/H3). The significance of these changes is not known at this time.

The Gage algorithm currently requires that anonymized patient information be entered on a website, which may be an impediment to some. In addition, this algorithm has undergone changes over time and the equation is not provided online nor published. The other two algorithms are in the public domain and can be built into a simple spreadsheet in Excel to calculate dosage based on input data.

CONCLUSIONS

Implementation of testing for mutations in VKORC1 and CYP2C9 demonstrates many of the challenges inherent in pharmacogenomics. Beyond the usual evaluation and validation of instruments and methods, how the information generated will be handled and reported presents unique challenges. Advantages of each of the assays evaluated are: ease of use and shortest TAT (ParagonDx, not commercially available at present); scalability and equipment costs (Third Wave); or automation and number of additional SNPs detected (AutoGenomics).

Much work remains to be done to bring warfarin dosage testing into the mainstream. The impact of additional SNPs and mutations needs to be evaluated and relevant ones included in algorithms. Other significant patient information needs to be defined and incorporated as well. Most importantly, concerns about TAT and clinical validity must be addressed through process improvements and prospective clinical trials, many of which are ongoing. The process used for establishing this assay will serve as our model for evaluating future pharmacogenomic testing.

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