

Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Rieder MJ, Reiner AP, Gage BF, et al. Effect of *VKORC1* haplotypes on transcriptional Regulation and Warfarin Dose. *N Engl J Med* 2005;352:2285-93.

Methods

Genotyping

For genotyping at each SNP site, PCR primers were designed using Primer Express version 1.5 (ABI, Foster City, CA). Pyrosequencing primers were designed using the Pyrosequencing SNP Primer Design Version 1.01 software (<http://www.pyrosequencing.com>). Unique localization of the PCR primers was verified using NCBI Blast (<http://www.ncbi.nlm.nih.gov/blast/>). PCR was carried out using Amplitaq Gold PCR master mix (ABI, Foster City, CA), 5 pmole of each primer (IDT, Coralville, IA), and 1 ng DNA. Pyrosequencing was carried out as previously described¹ using the following primers (5' – 3') for each SNP: 861 (A/C), forward = TCTTGAGTGAGGAAGGCAAT, reverse = Biotin-GACAGGTCTGGACAACGTGG, internal = CTCAGGTGATCCA; 5808 (G/T), forward = Biotin - GGATGCCAGATGATTATTCTGGAGT, reverse = TCATTATGCTAACGCCTGGCC, internal = CAACACCCCCCTTC; 6853 (G/C), forward = CTTGGTGATCCACACAGCTGA, reverse = Biotin - AAAAGACTCCTGTTAGTTACCTCCCC, internal = AGCTAGCTGCTCATCAC; 9041 (A/G), forward = TACCCCTCCTCCTGCCATA, reverse = Biotin - CCAGCAGGCCCTCCACTC, internal = TCCTCCTGCCATACC. Samples of each genotype were randomly selected and repeated to confirm the genotype assignment.

Assay of *VKORC1* mRNA

1.2 uL of total cDNA from each sample was used as template for the quantitative PCR (using 9 uL reactions) in the presence of SYBR green reporter (Applied Biosystems, Foster City, CA). PCR primers (5' to 3' - forward = ATCAGCTGTTTCGCGCGTC, reverse = AGAGCACGAAGAACAGGATC) were selected from sequences in exon 1 and 3 of the *VKORC1* coding sequence (Accession No. NM_024006). All quantitative PCR was performed on an Applied Biosystems 7900HT and real-time data collected during the entire thermocycling period (cycling conditions: 95°C – 15 minutes for initial denaturation and 40 cycles of 94°C – 30 sec., 60 °C – 30 sec, 72°C – 30 sec and a final extension of 72 °C – 5 minutes). Each sample was measured in duplicate and the results from two independent experiments were averaged. All *VKORC1* mRNA levels were normalized to GAPDH expression levels (primers (5' to 3'): forward = ACAGTCAGCCGCATCTTCTT, reverse = ATGGGTGGAATCATATTGGAAC), and scaled relative to the A/A haplotype group (Mean value = 1.49).

Statistical Methods

For the 186 patients in the UW sample, only a single patient was assigned an ambiguous haplotype pair. This reflects the simple genetic structure underlying this small genomic segment encompassing *VKORC1*, and the lack of observed recombinant haplotypes in this region. For direct comparison of the *VKORC1* haplotypes between the primary clinical subjects and the replication subjects, only the four informative SNPs were used when estimating haplotypes.

For the multiple linear regression analysis we used the most likely pair of haplotypes estimated for each patient and the association between number of copies of each *VKORC1* haplotype (coded 0, 1, 2) and maintenance warfarin dose was assessed on an additive scale. Additional multivariable adjustment for other known factors (including reason for anticoagulation, target INR, additional medications) did not alter the highly significant association between *VKORC1* haplotypes and warfarin maintenance dose in any of our analyses. In separate regression analyses to determine the dose effect of *VKORC1* haplotypes, we used a generalized linear model score test method² that additionally estimates and takes into account the uncertainty of haplotype assignments. Significant covariates were used to adjust warfarin doses (and 95% confidence intervals) associated with each additional haplotype copy (and single SNP sites) and were estimated by exponentiation of the mean fitted values and standard errors of the linear prediction.

For the survival analysis, a hazard ratio (and 95% confidence interval) was computed to compare patients between haplotype groups. A Cox proportional hazards model was used to adjust for warfarin daily dose in all analyses except for time to bleeding event; covariates such as sex, age, warfarin indication, comorbid diseases, prescription medications, and over-the-counter products were not included as these were not found to be significant in our previous study³. To account for changes in prescribed dose of warfarin, we programmed mean daily dose as a time-varying covariate. When there was a change in warfarin dose or a new INR value, we updated the regression model's covariates accordingly. Consequently, the regression model always used patients' most recent mean dose to adjust the hazard ratio for haplotype group. Individuals could contribute more than one event to the above-range INR outcome.

References:

1. Rose CM, Marsh S, Ameyaw MM, McLeod HL. Pharmacogenetic analysis of clinically relevant genetic polymorphisms. *Methods Mol Med* 2003; 85:225-37.
2. Lake SL, Lyon H, Tantisira K, et al. Estimation and tests of haplotype-environment interaction when linkage phase is ambiguous. *Hum Hered* 2003; 55:56-65.
3. Higashi MK, Veenstra DL, Kondo LM, et al. Association between CYP2C9 genetic variants and anticoagulation-related outcomes during warfarin therapy. *Jama* 2002; 287:1690-8.