Influence of cytochrome P-450 CYP2C9 polymorphisms on warfarin sensitivity and risk of over-anticoagulation in patients on long-term treatment

Janis Taube, David Halsall, and Trevor Baglin

Cytochrome P-450 2C9 is the principle enzyme that terminates the anticoagulant effect of warfarin. Genetic polymorphisms in CYP2C9 producing variants with altered catalytic properties have been identified. Patients (n = 561) with a target international normalized ratio (INR) of 2.5 who had been treated with warfarin for more than 2 months were anonymously genotyped for the wild-type CYP2C9*1 allele and the 2C9*2 and 2C9*3 variants. The mean maintenance dose of warfarin in patients who were wild-type for both alleles was 5.01 mg. The maintenance dose of warfarin was significantly related to genotype (Kruskall-Wallis, $\chi^2 = 17.985$, $P = .001$) with mean maintenance doses in patients with variant alleles between 61% and 86% of that in wild-type patients. The odds ratio for the 2C9*2 allele in patients with a maintenance dose of 1.5 mg or less was 5.42 (95% CI 1.68-17.4). The odds ratio for one or more variant alleles in patients developing an INR of 8.0 or greater was 1.52 (95% CI 0.64-3.58). The SD of the mean INR, percentage of high INRs, and person-time spent in range were determined as parameters of stability. There was no difference between patients grouped according to genotype for any parameter of stability. This study confirms an association between CYP2C9 genotype and warfarin sensitivity. However, the possession of a variant allele does not increase the likelihood of severe over-anticoagulation or stability of anticoagulation during long-term therapy. (Blood. 2000;96:1816-1819)

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Introduction

The clinical effectiveness of warfarin is established. However, because of a low therapeutic index, bleeding frequently complicates anticoagulation with warfarin. Overall, the bleeding rate is 7.6 to 16.5 per hundred patient-years. Major or life-threatening bleeds occur at a rate of 1.3 to 2.7 per hundred patient-years. Although major bleeding can occur at therapeutic levels the risk of bleeding rises with increasing intensity of anticoagulation. The international normalized ratio (INR) is commonly adopted for laboratory monitoring of oral anticoagulant therapy and as a guide to warfarin dose adjustment. Because the risk of bleeding increases with increasing intensity of anticoagulation, identification of risk factors for the development of a high INR may identify patients at high risk of bleeding. Cytochrome P-450 2C9 is the principle enzyme that terminates the anticoagulant effect of warfarin by catalyzing the conversion of the pharmacologically more potent S-enantiomer to its inactive metabolites. Genetic polymorphisms in CYP2C9 producing variants with altered catalytic properties have been identified. In addition to the wild-type CYP2C9*1 allele, 2 allelic variants, 2C9*2 (R144C) and 2C9*3 (I359L), with approximately 12% and 5% enzymatic activity, respectively, have been identified. Patients with variant alleles might be susceptible to over-anticoagulation during intermittent illness or during a change of medication, particularly if an additional drug is competitively metabolized by the cytochrome P-450 system. In a previous study the 2C9*2 and 2C9*3 alleles were more frequent in patients with a daily warfarin dose requirement of 1.5 mg or less immediately after initiation of therapy. Patients with a low-dose requirement were more likely to have an INR more than 4.0 during induction of therapy (OR 5.97, 95% CI 2.26-15.82) and have an increased risk of major bleeding complications during induction (OR 3.68, 95% CI 1.43-9.50). The aim of this study was to determine the pharmacogenetic effect of CYP2C9 polymorphisms on warfarin sensitivity and the risk of over-anticoagulation in patients receiving long-term warfarin treatment.

Materials, methods, and methods

Patient population and study methodology

The Cambridge Anticoagulant Service combines a hospital-based anticoagulant clinic with decentralized care by offsite blood sampling for more than two thirds of patients. A computerized decision support system is used for warfarin dose calculations and scheduling of INR monitoring. The Rapid Anticoagulation Interpretation and Dosing (RAID) software uses an established algorithm and enables extensive statistical analysis of patient data (software distributed by Organon Teknika, Durham, NC). The SD of the mean INR, percentage of high INRs, and proportion of person-time in range were determined as parameters of stability. High INRs were those more than 0.5 INR units above target. The percentage of person-time spent in range was calculated by the method of Rosendaal and coworkers. In September 1999, 1433 patients managed by the RAID computerized decision support system were registered as active patients and had been on treatment with warfarin for more than 2 months. A total of 28,152 INRs had been measured on this patient population. The median duration of treatment was 2.38 years, equating to 3417 patient-years. Eighty-five INRs in 74 patients were more than 8.0. Nine of the 74 patients had an INR more than 8.0 on 2 occasions and 1 patient on 3 occasions. Thus, 85 of 28,152 INRs (0.3% of INRs) were more than 8.0 and these were recorded in 74 of 1433 patients (5.2% of patients). A cohort consisting of all patients who had an
INR measured in October and November 1999 formed the study group. The INR was measured and following routine dose recommendation the blood sample was discarded. The RAID statistics file for the patient was electronically downloaded onto an Excel worksheet and patient details were replaced by an anonymous code. The discarded blood sample was given the same code and DNA was extracted and stored at −80°C until analysis. Genotyping was performed on completion of sample collection. After analysis the CYP2C9 genotypes were matched to the coded anonymous database. No additional blood or tissue was taken from any patient for the purpose of this study.

Measurement of INR
Venous blood was obtained by venipuncture and collected into Sarstedt monovette tubes containing 0.109 mol/L sodium citrate (9:1 vol/vol). Plasma was separated by centrifugation at 2000g for 10 minutes. Plasma prothrombin times were measured on an MDA-180 coagulometer using Simplastin Excel S as thromboplastin (Organon Teknika). The INRs were calculated according to the formula INR = (patient PT/GMNPT)1/2. The GMNPT (geometric mean normal prothrombin time) was calculated using 20 normal plasmas. The manufacturer’s International Sensitivity Index (ISI) was used for INR calculation. A satisfactory performance in the United Kingdom National Quality Assurance (UK NEQAS) and Central Quality Assurance Schemes (CQAS) was obtained throughout the study.

Genotyping
Genotyping was performed by multiplex polymerase chain reaction (PCR) and restriction enzyme digestion with AvaII and NsiI. The 2C9*2 allele is the result of a C416T transition in exon 3. This results in the loss of an AvaII restriction digest site. PCR was performed with primers, 5′-CAA TGG AAA GAA ATG GAA GGA GGT-3′ (location 331-354) and 5′-AGA AAG TAA TAC TCA GAC CAA CAA TCG-3′ (location 555-581). A forced mismatch was included in the penultimate base of the forward primer to create a control site for AvaII digestion. The PCR product from this amplification is 251 bp in length. After AvaII digestion, patients homozygous for the wild-type allele have 170- and 60-bp fragments, patients homozygous for the 2C9*2 allele have 229-bp fragments, and those heterozygous for 2C9*2 have 229-, 170-, and 60-bp fragments. The 2C9*3 allele does neither naturally create or destroy a restriction site. A restriction site was forced into the forward primer 5′-TGC ACG AGG TTC AGA GAT GC-3′ (location 1041-1060), such that A1061 in combination with the mismatch creates a restriction site for the NsiI restriction enzyme. The 2C9*3 A1061C substitution removes this restriction site. This primer also naturally includes an AvaII restriction sequence (GGTCC). The reverse primer, 5′-AGC TTC AGG GTC ATT GTA TCA TAG ATT A-3′ (location 1172-1198) also has a forced mismatch at 1186 to provide a control restriction site for the NsiI restriction enzyme. The PCR product for this set of primers prior to restriction enzyme digestion is 160 bp in length. Following restriction enzyme digestion with both NsiI and AvaII, patients homozygous for the wild-type allele have restriction fragments 130 bp in length. Those homozygous for the 2C9*3 allele have 140 bp fragments, and those heterozygous for the 2C9*3 allele have 130- and 140-bp fragments. Restriction products were resolved by electrophoresis on a 3% agarose gel. Each amplification reaction (25 μL) contained 50 ng of genomic DNA, 200 μmol/L of each deoxynucleotide triphosphate, 6.25 pmol of each amplification primer, and 0.2 U of Biotaq DNA polymerase in 2.5 μL of 10× ammonium buffer (Boiline, London, UK) and 3.0 mmol/L MgCl2. The amplification reactions were carried out in a Perkin Elmer Cetus DNA thermal cycler with an initial denaturation step of 5 minutes at 94°C, followed by 35 cycles of 30 seconds denaturation at 94°C, annealing at 58°C for 30 seconds, and extension at 72°C for 30 seconds with a final extension for 5 minutes at 72°C. After generation of the multiplex PCR products, 0.5μL of AvaII and 1.0μL of NsiI (New England Biolabs, Hitchin, Herts, UK) were added and incubated at 37°C overnight. Restriction products (8 μL) were analyzed by electrophoresis on a 3% agarose gel.

Statistics
Descriptive statistics were calculated using SPSS software (version 9). The Kruskall-Wallis test was used to compare groups of continuous data for significant differences. A cross-tabulation procedure with calculation of Pearson χ2 value was used to compare groups of categorical data. Odds ratios were calculated by the logit method using Confidence Interval Analysis software (British Medical Association, Tavistock Square, London, UK).

Results

Study group
The 683 patients who had a sample sent to the laboratory for INR measurement during the study period were anonymously genotyped for both the 2C9*2 and 2C9*3 polymorphisms. These patients constituted 48% of the total patient population. The gene frequencies were 84% for 2C9*1, 11% for 2C9*2, and 5% for 2C9*3. Allele frequencies were in Hardy-Weinberg equilibrium.

Effect of polymorphisms on maintenance dose of warfarin
To study the influence of polymorphisms on the maintenance dose of warfarin all anonymous patient data sets with a target INR of 2.5 were selected from the study group. The 561 patients with a target INR of 2.5 had been genotyped. The only criteria for selection was the target INR. The selection had to be made from the coded data set after genotyping to maintain patient anonymity. The mean doses of warfarin in relation to each combination of alleles are shown in Table 1. The mean maintenance dose of warfarin in patients who were wild type for both alleles was 5.01 mg. The maintenance dose of warfarin was significantly related to genotype (Kruskall-Wallis, χ2 = 17.985, P = .001). As a percentage of the mean dose in patients with wild-type alleles only, the mean warfarin maintenance doses were 86% in 2C9*2 heterozygotes, 79% in 2C9*3 heterozygotes, 82% in compound heterozygotes for 2C9*2 and 2C9*3, and 61% in 2C9*2 homozygotes.

The frequencies of genotypes in patients with a mean maintenance warfarin dose of 1.5 mg or less compared to patients with a maintenance dose more than 1.5 mg are shown in Table 2. The odds ratio for one or more variant alleles in patients with a maintenance dose of 1.5 mg or less was 3.85 (95% CI 1.24-11.9). This result was due to the influence of heterozygosity for the 2C9*2 allele (OR 5.42, 95% CI 1.68-17.4). None of 9 patients with 2 variant alleles (6 compound heterozygotes and 3 2C9*2 homozygotes) had a maintenance dose of 1.5 mg or less.

Effect of CYP2C9 polymorphisms on risk of INR of 8.0 or greater
First analysis was restricted to the 561 patients with a target INR of 2.5. Patients with an INR of 8.0 or greater on at least one occasion

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No.</th>
<th>Mean dose (mg)</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type (<em>1</em>1)</td>
<td>392</td>
<td>5.01</td>
<td>2.43</td>
</tr>
<tr>
<td>2C9*2 heterozygote (<em>1</em>2)</td>
<td>107</td>
<td>4.31</td>
<td>1.94</td>
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<tr>
<td>2C9*2 homozygote (<em>2</em>2)</td>
<td>3</td>
<td>3.04</td>
<td>1.29</td>
</tr>
<tr>
<td>2C9*3 heterozygote (<em>1</em>3)</td>
<td>53</td>
<td>3.97</td>
<td>1.79</td>
</tr>
<tr>
<td>2C9*3 homozygote (<em>3</em>3)</td>
<td>0</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>2C9<em>2</em>3 compound heterozygote (<em>2</em>3)</td>
<td>6</td>
<td>4.09</td>
<td>2.09</td>
</tr>
</tbody>
</table>

The mean maintenance dose of warfarin was significantly related to genotype (Kruskall-Wallis, χ2 = 17.985, P = .001).
were compared to patients with all INRs less than 8.0. The distribution of genotypes is shown in Table 2. There was no difference in the frequency of genotypes between patients with and without INRs of 8.0 or greater (Pearson $\chi^2 = 4.427, P = .351$). The odds ratios for one variant allele in patients developing an INR of 8.0 or greater was 1.52 (95% CI 0.64-3.58). None of the 9 patients with 2 variant alleles developed an INR of 8.0 or greater.

A second analysis was performed in patients with a target INR more than 2.5 because these patients are more likely to become over-anticoagulated. A total of 117 patients with a target INR more than 2.5 had been genotyped. Again, there was no difference in the frequency of genotypes between patients with and without INRs of 8.0 or greater (Pearson $\chi^2 = 3.648, P = .302$). The odds ratios for one or more variant alleles in these patients developing an INR of 8.0 or greater was 0.19 (95% CI 0.02-1.53).

Five of the 683 patients had a target INR of less than 2.5 at the request of the referring clinician. This was not due to a history of bleeding but simply variable practice. Because the target INR is a major determinant of likelihood of over-anticoagulation, these patients would be at lower risk than patients with a target INR of 2.5 or 3.5 and were therefore excluded from the analysis.

Effect of CYP2C9 polymorphisms on stability of anticoagulant therapy

To study the effect of polymorphisms on warfarin stability, patients with a target INR of 2.5 were selected from the study group. The SD of the mean INR, percentage of high INRs (> 0.5 INR units above target), and person-time in range were determined as parameters of stability. There was no difference between patients grouped according to polymorphism for any parameter. The results are shown in Table 3. A total of 1188 of the original patient population of 1433 had a target INR of 2.5. Overall the mean INR was 2.55, SD 0.85, 19.9% of INRs were more than 0.5 INR units above target and patients were in range 68.2% of the time. Therefore, stability in each of the study groups was comparable to that in the whole patient population.

Discussion

This study has confirmed an association between CYP2C9 genotype and warfarin sensitivity. However, the possession of a variant allele does not increase the likelihood of severe over-anticoagulation or stability of anticoagulation during long-term therapy. In a previous study we identified a target INR of 3.5 and antibiotic therapy as major risk factors for over-anticoagulation. This present study indicates that there is no apparent gene-environment influence from the CYP2C9 locus.

In the study of Aithal and colleagues, patients with a warfarin dose requirement of 1.5 mg or less immediately after induction of anticoagulation were 6 times more likely to have a variant allele compared to the general population. For the purpose of comparison of studies we also used a warfarin dose requirement of 1.5 mg or less as a parameter of warfarin sensitivity. In our study, patients established on warfarin for more than 2 months were 5 times more likely to have the 2C9*2 allele if their maintenance dose was 1.5 mg or less. There was also a definite dose-response effect due to both variant alleles. The lowest warfarin dose requirement was in 2C9*2 homozygotes whose mean maintenance dose was 40% lower than in wild-type patients. No 2C9*3 homozygotes were identified in our low-dose warfarin group. The expected number based on a 2C9*3 gene frequency of 0.05% is 1.55 with a 95% CI overlapping zero. Therefore, the absence of homozygotes may not be significant. Aithal and coworkers also found no 2C9*3 homozygotes. The authors suggested that 2C9*3 homozygotes might have such a low warfarin dose requirement that stabilization is not possible and treatment with warfarin is abandoned. Prospective studies are required to identify such patients and determine if the finding is significant and if this is indeed the explanation. It remains to be determined if early genotyping and dose modification would significantly reduce over-anticoagulation and bleeding in patients during the first few weeks of warfarin therapy. The mechanistic relationship between 2C9 genotype and INR is a function of the catalytic efficiency of the 2C9 enzymatic variants produced by the polymorphic alleles. As a consequence of reduced catalytic activity the dose of warfarin required to produce a standardized intensity of anticoagulation is lower. Thus, when a group of patients with the same target INR, and hence intensity of anticoagulation, was selected, those with 2C9 variants had a lower warfarin maintenance dose requirement.

The major complication of long-term oral anticoagulant therapy is over-anticoagulation and bleeding. The risk of bleeding is associated with increasing INR and so identification of genetic and

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Table 2. Frequencies of CYP2C9 genotypes in 561 patients with a mean maintenance warfarin dose of $1.5 \text{mg}$ compared to patients with a maintenance dose $> 1.5 \text{mg}$ and in patients developing an INR $> 8.0$

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Maintenance dose $\leq 1.5 \text{mg}$</th>
<th>Maintenance dose $&gt; 1.5 \text{mg}$</th>
<th>Odds ratios compared to wild type to wild type</th>
<th>Odds ratios compared to wild type</th>
<th>Patients with INR $&lt; 8.0$ on all occasions</th>
<th>Patients with INR $&gt; 8.0$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>5</td>
<td>378</td>
<td>4.52 (1.66-17.4)</td>
<td>14</td>
<td>378</td>
<td>2.18 (0.89-5.35)</td>
</tr>
<tr>
<td>2C9*2 heterozygote</td>
<td>7</td>
<td>100</td>
<td>1.49 (0.17-13.0)</td>
<td>1</td>
<td>52</td>
<td>0.52 (0.07-4.03)</td>
</tr>
<tr>
<td>2C9*3 homozygote</td>
<td>0</td>
<td>3</td>
<td>—</td>
<td>0</td>
<td>3</td>
<td>—</td>
</tr>
<tr>
<td>2C9*3 heterozygote</td>
<td>1</td>
<td>52</td>
<td>—</td>
<td>1</td>
<td>52</td>
<td>—</td>
</tr>
<tr>
<td>2C9*2 homozygote</td>
<td>0</td>
<td>0</td>
<td>—</td>
<td>0</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td>2C9<em>2</em>3 compound heterozygote</td>
<td>0</td>
<td>6</td>
<td>—</td>
<td>0</td>
<td>6</td>
<td>—</td>
</tr>
</tbody>
</table>

Figures in parentheses are 95% confidence intervals for odds ratios.

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Table 3. SD of the mean INR, percentage of INRs $> 5.0$, and percentage of time in range in relation to CYP2C9 genotype in patients with a target INR of 2.5

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No.</th>
<th>Mean INR</th>
<th>SD</th>
<th>% of high INRs</th>
<th>% of INRs in range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>392</td>
<td>2.55</td>
<td>0.85</td>
<td>19.0</td>
<td>68.9</td>
</tr>
<tr>
<td>2C9*2 heterozygote</td>
<td>107</td>
<td>2.54</td>
<td>0.82</td>
<td>20.7</td>
<td>68.3</td>
</tr>
<tr>
<td>2C9*3 homozygote</td>
<td>4</td>
<td>2.64</td>
<td>0.88</td>
<td>30.1</td>
<td>64.5</td>
</tr>
<tr>
<td>2C9*3 heterozygote</td>
<td>53</td>
<td>2.49</td>
<td>0.76</td>
<td>19.5</td>
<td>66.8</td>
</tr>
<tr>
<td>2C9*3 homozygote</td>
<td>0</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>2C9<em>2</em>3 compound heterozygote</td>
<td>6</td>
<td>2.57</td>
<td>0.75</td>
<td>20.7</td>
<td>62.6</td>
</tr>
</tbody>
</table>

There was no difference between patients grouped according to polymorphism for any parameter.
environmental factors that predict the development of a high INR might identify high-risk patients who would be candidates for more frequent monitoring or treatment with an alternative drug. In this study CYP2C9 variant alleles did not increase the likelihood of developing either an INR more than 8.0 or the frequency of an INR more than 0.5 INR units above target. In the primary patient population with a target INR of 2.5, patients were within range 68% of the time. Because warfarin sensitivity is influenced by genotype it seems that once a stable anticoagulant effect is established in patients with variant alleles, though their warfarin dose requirement is lower, their responsiveness is stable and they are not more prone to over-anticoagulation.

Our computerized decision support system, which uses an established algorithm, has reduced the incidence of over-anticoagulation in our clinical practice. Only 0.3% of INRs were more than 8.0. However, over an average treatment period of 2.5 years 5% of the patients had at least one INR more than 8.0. The CYP2C9 locus is not associated with the development of a high INR during long-term therapy. A missense mutation at ALA-10 in the factor IX propeptide increases the risk of bleeding without affecting the INR. However, this mutation is rare and prospective genotyping of patients starting oral anticoagulant therapy cannot be justified. As the pharmogenomics of warfarin-drug interactions is unraveled, genetic analysis may yet prove to have a role in the assessment of risk and management decisions relating to type of anticoagulant, frequency of monitoring, and optimum duration of therapy.

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References
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