A novel functional VKORC1 promoter polymorphism is associated with inter-individual and inter-ethnic differences in warfarin sensitivity

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Warfarin, a commonly prescribed anticoagulant, exhibited large inter-individual and inter-ethnic differences in the dose required for its anticoagulation effect. Asian populations, including Chinese, require a much lower maintenance dose than Caucasians, for which the mechanisms still remain unknown. We determined DNA sequence variants in CYP2C9 and VKORC1 in 16 Chinese patients having warfarin sensitivity (≤1.5 mg/day, n = 11) or resistance (≥6.0 mg/day, n = 5), 104 randomly selected Chinese patients receiving warfarin, 95 normal Chinese controls and 92 normal Caucasians. We identified three CYP2C9 variants, CYP2C9*3, T299A and P382L, in four warfarin-sensitive patients. A novel VKORC1 promoter polymorphism (−1639 G > A) presented in the homozygous form (genotype AA) was found in all warfarin-sensitive patients. The resistant patients were either AG or GG. Among the 104 randomly selected Chinese patients receiving warfarin, AA genotype also had lower dose than the AG/GG genotype (P < 0.0001). Frequencies of AA, AG and GG genotypes were comparable in Chinese patients receiving warfarin (79.7, 17.6 and 2.7%) and normal Chinese controls (82, 18 and 0%), but differed significantly from Caucasians (14, 47 and 39%) (P < 0.0001). The promoter polymorphism abolished the E-box consensus sequences and dual luciferase assay revealed that VKORC1 promoter with the G allele had a 44% increase of activity when compared with the A allele. The differences in allele frequencies of A/G allele and its levels of VKORC1 promoter activity may underscore the inter-individual differences in warfarin dosage as well as inter-ethnic differences between Chinese and Caucasians.

INTRODUCTION

Warfarin is a widely prescribed anticoagulant for the prevention of thromboembolic diseases for subjects with deep vein thrombosis, atrial fibrillation or mechanical heart valve replacement (1–4). However, warfarin treatment is problematic because the dose requirement for warfarin is highly variable, both inter-individually and inter-ethnically (5–7). Asian populations, including Chinese, require a much lower maintenance dose than Caucasians and Hispanics (6–9), for which the mechanisms still remain elusive. Bleeding is by far the most serious complication of warfarin treatment (10–12). Much effort has been devoted to monitor the safety of this oral anticoagulant. Currently, the dose has to be closely monitored by serial determinations of blood prothrombin time using standardized international normalized ratio (INR).

Cytochrome P450, subfamily IIC, polypeptide 9 (CYP2C9) is the principal drug metabolizing enzyme that catalyzes the
hydroxylation of warfarin (13–15). CYP2C9*2 and CYP2C9*3 are the polymorphisms most frequently found in the Caucasian population; using CYP2C9*1 as wild-type, the haplotype frequencies for CYP2C9*1/*2 and CYP2C9*1/*3 are ~20 and ~12%, respectively (16,17). The CYP2C9*2 and CYP2C9*3 variants have been shown to decrease the enzymatic activity which leads to warfarin sensitivity and in serious cases, bleeding complications (18–20). However, both CYP2C9*2 and CYP2C9*3 are either completely absent or rare in the Asian populations (9,21). Genetic variants discovered in CYP2C9 so far can only partially explain some of the inter-individual differences in warfarin dosage but they cannot explain the inter-ethnic differences (6,7,16).

Gamma-carboxylation of vitamin K-dependent clotting factors (factor II, VII, IX and X) is essential for blood clotting. The gamma-carboxylase uses the reduced form of vitamin K and oxygen to add a carbon dioxide molecule to the side chain of glutamic acid in the clotting factors. During carboxylation, the reduced vitamin K is oxidized to vitamin K 2,3-epoxide, from which the reduced vitamin K is regenerated by vitamin K epoxide reductase for another cycle of catalysis. Warfarin blocks clotting factor synthesis by inhibiting vitamin K epoxide reductase (22,23). Recently, the gene coding for vitamin K epoxide reductase complex, subunit 1 (VKORC1) has been cloned (24,25) and mutations in VKORC1 gene were found in warfarin-resistant patients (25,26). An intronic polymorphism was also discovered to associate with warfarin dose requirements in Italian patients (27). These suggest that genetic factors other than CYP2C9 can also alter warfarin sensitivity.

In this study, we investigated variants in the exonic and 5' flanking regions of CYP2C9 and VKORC1 in Chinese patients receiving low and high warfarin maintenance doses and compared their frequencies with Caucasians, in an attempt to determine the basis between inter-individual and inter-ethnic differences in warfarin dose requirements. An understanding of major genetic factors that contribute to the individuality of warfarin maintenance dose may help to control and prevent thromboembolic diseases during therapy and to avoid the serious bleeding complications.

RESULTS

CYP2C9 and VKORC1 DNA sequence variants in selected Chinese patients with warfarin sensitivity or resistance

The mean maintenance dose of warfarin in Chinese is 3.3 mg/day (8,9), therefore, we considered patients who received maintenance dose ≤1.5 mg/day as warfarin sensitive (Table 1, 11 patients) and patients on warfarin maintenance dose ≥6 mg/day as warfarin resistant (Table 1, five patients; see Materials and Methods for further details). Sequencing of the coding regions, exon–intron junctions and the promoter region of CYP2C9 revealed three sequence variants in four of the 11 warfarin-sensitive patients. The variants were: 1075 A > C (I359L known as CYP2C9*3), 895 A > G (T299A) and 1145 C > T (P382L). CYP2C9*3 was detected in three patients (subjects 1, 3 and 5, Table 1). Subject 5, in addition to CYP2C9*3, also had the 895 A > G (T299A) change as previously described (7). A novel exonic mutation, 1145 C > T (P382L), was detected in the fourth patient (subject 6). As shown in Table 1, CYP2C9 gene variants were not present in all warfarin-sensitive patients and were completely absent in all warfarin-resistant patients.

We also sequenced the promoter, coding regions and the exon–intron junctions of VKORC1. A variant (3730 G > A) which was located in 3' untranslated region (3' UTR) of VKORC1 was detected. In addition, a promoter polymorphism, −1639 G > A (or −1413 using transcription start site as +1) was detected in the upstream region of VKORC1. This polymorphism showed an association with warfarin sensitivity in that all warfarin-sensitive patients were homozygous AA at position −1639. The warfarin-resistant patients, in contrast, were either heterozygous AG or homozygous GG (Table 1). When warfarin doses were plotted against the −1639 genotypes, it demonstrated that patients with the AA genotype had the lowest dose requirements (mean 1.19 mg/day, range 0.71–1.50 mg/day) as compared to AG (mean 3.81 mg/day, range 3.07–10 mg/day) and the homozygous GG genotype had the highest dose (mean 9.11 mg/day, range 8.57–10 mg/day) (Fig. 1).

In addition to the −1639 G > A, an intron 1 polymorphism, 1173 C > T which has been described by D’Andrea et al. (27) was also identified in our patients. These two polymorphisms appeared to be in strong linkage disequilibrium (LD) (Table 1). Warfarin-sensitive patients with the −1639 AA genotype were found to be associated with the 1173 homozygous TT except for one patient (subject 6). In warfarin-resistant patients, patients with the −1639 heterozygous AG genotype were found to be associated with 1173 heterozygous CT and patients with the homozygous −1639 GG were found to be associated with 1173 homozygous CC (Table 1).

VKORC1 −1639 G > A polymorphism in random Chinese patients receiving warfarin

To further investigate whether the −1639 G > A polymorphism was associated with the inter-individual differences in warfarin dosage, we genotyped 104 patients receiving warfarin regardless of the dose, using MALDI-TOF mass spectrometry. The AA and AG/GG groups did not differ with respect to age, sex and INR (Table 2). Only two patients were found to be homozygous for GG and they were grouped together with AG for statistic analysis. We analyzed data regardless of the presence of other confounding variables such as diet or other medications. As shown in Table 2, the AA group had significant lower dose requirements (2.61 mg/day) than the AG/GG group (3.81 mg/day). The differences were significant by either t-test (P < 0.0001) or Wilcoxon–Mann–Whitney test (P = 0.0002) between the AA and AG/ GG groups.

Genotype frequencies of VKORC1 −1639 G > A polymorphism and CYP2C9 variants in Chinese and Caucasians

The Chinese population has been known to require a much lower warfarin maintenance dose than the Caucasians.
To test whether differences in the VKORC1 \(-1639\) genotype frequencies could account for the inter-ethnic differences in warfarin dose, 95 normal Han Chinese subjects and 92 normal Caucasian subjects were genotyped. In the Caucasian population, homozygous AA had the lowest frequency, whereas the AG and GG genotypes made up the majority of the population (14.2, 46.7 and 39.1%, respectively, Table 3). In the Chinese population, on the contrary, homozygous AA had the lowest frequency, whereas the AG and GG genotypes made up the majority of the population (17.9%). No homozygous GG was detected in this randomly selected population. This ratio was also similar in the 104 warfarin patients in which 79.8% were found to be homozygous GG. The differences in genotype frequencies between the Caucasian group and the two Chinese groups were significant with the \(P\) values \(<0.0001). These differences between the two ethnic groups (Caucasians and Chinese) and the correlation of AA with warfarin sensitivity are in concordance with what has been observed clinically that Chinese population requires smaller dose than the Caucasians.

In addition to the \(-1639\) G > A, we screened three intronic polymorphisms (rs9934438, intron 1 1173 C > T; rs8050894, intron 2 g.509 + 124C and rs2359612, intron 2 g.509 + 837C) and one 3'UTR polymorphism (rs7294, 3730 G > A) in the Chinese population. All these four polymorphisms were in LD with the \(-1639\) promoter polymorphism (Inter-marker \(D'\) and \(r^2\) values = 1.0).

CYP2C9 variants were also genotyped in the Chinese groups. The frequency of CYP2C9*1/*3 was 7.3% in Chinese population and 5.4% in the randomly selected Chinese patients receiving warfarin. CYP2C9*2 variant was not detected in the Chinese patients and controls. When compared with the published data on the CYP2C9 variant frequency in Caucasians, the Caucasian population had a much higher frequency of CYP2C9 variants than the Chinese (\(~30\ versus \(7\%), yet Caucasians are more resistant to warfarin. Other missense mutations detected in the warfarin-sensitive patients, 895 A > G (T299A) and 1145 C > T (P382L) (Table 1) were not found in any of the randomly selected patients and controls suggesting these were rare mutations.

**VKORC1 promoter activity**

The differences in the warfarin sensitivity between the AA and the GG genotype patients could be explained by changes in the VKORC1 promoter activity. The \(-1639\) promoter single nucleotide polymorphism (SNP) was located in the second nucleotide of an E-Box (CANNTG), and, therefore, this polymorphism would alter the E-box consensus sequence and could lead to changes in the VKORC1 promoter activity. To test this hypothesis, the VKORC1 promoter encompassing the \(-1639\) A or G was polymerase chain reaction (PCR)-amplified from patients with the \(-1639\) AA or GG genotype and cloned into the pGL-3 vector. HepG2 cell (a human hepatoma cell line) was chosen for the promoter assay because highest expression of VKORC1 is in the liver. A total of nine experiments were performed and all demonstrated consistent results (Fig. 2). The \(-1639\) G VKORC1 promoter exhibited higher luciferase activity (\(~44\%\) higher) compared with the \(-1639\) A promoter. The pGL-3 basic vector did not have any promoter inserted in the multiple cloning site and had a negligible amount of the luciferase activity.

**DISCUSSION**

In this Han Chinese study, we found that CYP2C9 DNA sequence variants were present in only 5.4−7.3% of the Chinese population. The variants were primarily CYP2C9*3; two missense mutations identified in this study, including a novel mutation (P382L) which was not reported previously, were all rare and present only in warfarin-sensitive patients. The CYP2C9 variants also showed different prevalence in different ethnic populations. CYP2C9*1/*2 was present in

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**Table 1.** Patient demographics and DNA sequence variants identified

<table>
<thead>
<tr>
<th>Case number</th>
<th>Dose (mg/day)</th>
<th>INR</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Weight (kg)</th>
<th>VKORC1 (-1639)</th>
<th>VKORC1 (1173)</th>
<th>#CYP2C9 variants</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.71</td>
<td>F</td>
<td>65</td>
<td></td>
<td>42</td>
<td>AA</td>
<td>TT</td>
<td>CYP2C9*3</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>2.69</td>
<td>74</td>
<td>M</td>
<td>65</td>
<td>AA</td>
<td>TT</td>
<td>Normal</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>3.23</td>
<td>70</td>
<td>M</td>
<td>66</td>
<td>AA</td>
<td>TT</td>
<td>CYP2C9*3</td>
</tr>
<tr>
<td>4</td>
<td>1.25</td>
<td>1.5</td>
<td>84</td>
<td>F</td>
<td>50</td>
<td>AA</td>
<td>TT</td>
<td>Normal</td>
</tr>
<tr>
<td>5</td>
<td>1.25</td>
<td>1.96</td>
<td>68</td>
<td>M</td>
<td>75</td>
<td>AA</td>
<td>TT</td>
<td>CYP2C9*3</td>
</tr>
<tr>
<td>6</td>
<td>1.25</td>
<td>2.5</td>
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<td>F</td>
<td>70</td>
<td>AA</td>
<td>CT</td>
<td>895 A &gt; G (T299A)</td>
</tr>
<tr>
<td>7</td>
<td>1.25</td>
<td>2</td>
<td>71</td>
<td>F</td>
<td>80</td>
<td>AA</td>
<td>TT</td>
<td>Normal</td>
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<td>8</td>
<td>1.25</td>
<td>2.9</td>
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<td>AA</td>
<td>TT</td>
<td>Normal</td>
</tr>
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<td>9</td>
<td>1.25</td>
<td>1.59</td>
<td>67</td>
<td>M</td>
<td>58</td>
<td>AA</td>
<td>TT</td>
<td>Normal</td>
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<tr>
<td>10</td>
<td>1.43</td>
<td>2.05</td>
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<td>AA</td>
<td>TT</td>
<td>Normal</td>
</tr>
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<td>11</td>
<td>1.5</td>
<td>2.24</td>
<td>61</td>
<td>M</td>
<td>65</td>
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<td>TT</td>
<td>Normal</td>
</tr>
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<td>12</td>
<td>6.07</td>
<td>2.82</td>
<td>48</td>
<td>F</td>
<td>52</td>
<td>AG</td>
<td>CT</td>
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<td>8.57</td>
<td>2.09</td>
<td>63</td>
<td>F</td>
<td>58</td>
<td>GG</td>
<td>CC</td>
<td>Normal</td>
</tr>
<tr>
<td>14</td>
<td>8.75</td>
<td>2.32</td>
<td>26</td>
<td>M</td>
<td>88</td>
<td>GG</td>
<td>CC</td>
<td>Normal</td>
</tr>
<tr>
<td>15</td>
<td>10</td>
<td>1.3</td>
<td>46</td>
<td>M</td>
<td>64</td>
<td>AG</td>
<td>CT</td>
<td>Normal</td>
</tr>
<tr>
<td>16</td>
<td>10</td>
<td>2.33</td>
<td>58</td>
<td>F</td>
<td>61</td>
<td>GG</td>
<td>CC</td>
<td>Normal</td>
</tr>
</tbody>
</table>

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et al. was in LD with the VKORC1 1173 C
G or CC genotype. Our study also showed that the 3730 TT genotype had the lower average daily dose than the CT
may explain their results that Italian patients with the 1173
other ethnic groups were not available for our study, we do
dance with what has been observed clinically that Chinese
populations between the Caucasians and the Chinese and
higher difference (6,7,16).

Unlike the previous studies, this work provided the evidence that changes in the VKORC1 gene could alter warfarin
requirements both inter-individually in Chinese and inter-ethnically between Chinese and Caucasians. Patients
with the −1639 promoter polymorphism AA genotype had
higher dose requirements, whereas the AG/GG genotypes had
higher dose requirements. The differences in the genotype
frequencies between the Caucasians and the Chinese and the correlation of AA with warfarin sensitivity are in concordance with what has been observed clinically that Chinese
population requires smaller dose than the Caucasians. As
other ethnic groups were not available for our study, we do
not yet know how they apply to other populations.

The VKORC1 −1639 A > G promoter polymorphism was in LD with the VKORC1 1173 C > T intronic polymorphism recently reported by D’Andrea et al. (27) and
may explain their results that Italian patients with the 1173
TT genotype had the lower average daily dose than the CT
or CC genotype. Our study also showed that the 3730
G > A polymorphism, which was located in the 3′-UTR, was
in LD with −1639 A > G and 1173 C > T in the Chinese population. The 3730 G allele was associated with −1639A allele and 1173T allele. This LD, however, was not observed in the
Italian study (27), probably due to the difference in haplotype structures in different populations.

D’Andrea et al. (27) could not detect any alternative mRNA splicing caused by the VKORC1 1173 SNP and concluded that the 1173 SNP might be in LD with other variants that altered the VKOR activity. Our data suggested that −1639 A > G could be that variant. The −1639 promoter SNP was located in an E-Box (CA/GNNTG) and within a short distance (200 bp), there were three additional E-boxes in the region. The consensus sequence of E-box is CANNTG. E-box sites have been shown to be important elements for mediating cell/tissue type specific transcription, such as in muscle, neuron, liver and pancreas (28,29). Changing the second base A to G as observed in the −1639 site would abolish the E-box consensus and would alter the promoter activity. This was clearly demonstrated by the promoter assay that in HepG2 cells the promoter activity was increased by 44% when the consensus sequences were abolished (Fig. 2). This suggested that E-box in HepG2 could function as a repressor binding site and because HepG2 was derived from hepatoma, it was likely that the −1639 E-box could also repress transcription in the liver.

Recently, Bodin et al. (30) suggested that the −1639 polymorphism was located in an NF1 binding site motif (TTGGCCCA). However, the complete NF1 consensus sequence TTGG(A/C)N6GCCAA (31) was not present around −1639, and the −1639 polymorphism was located in the second N where any nucleotide can be accepted for transcription factor binding. The authors also did not detect differences in the promoter activity between the −1639 A and G alleles. Several factors could explain this discrepancy. The promoter regions cloned were differed by several base pairs (−35 to −1798, this study versus −12 to −1756). The methods of transfection used were also different; we used lipofectamine, whereas they applied calcium phosphate. In our experience, calcium phosphate method has much lower transfection efficiency than the lipofectamine.

The fact that people with the −1639 G/G genotype require higher warfarin dose can be explained by that when VKORC1 promoter activity is increased, the VKORC1 mRNA expression would raise as a result. This would translate into an increased translation of the VKORC1 protein. An elevated level of VKORC1 mRNA can lead to a higher VKOR activity (25) and thus enhance the efficiency of the regeneration of the reduced vitamin K which ultimately would produce more gamma-carboxylation of the vitamin K dependent clotting factors. Having more active clotting factors would render the

Table 2. Mean doses and other clinical characteristics of randomly selected patients on warfarin stratified according to the genotypes.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Warfarin dose (mg/day)</th>
<th>INR</th>
<th>Age (years)</th>
<th>Sex (M/F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VKORC1-1639</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA (n = 83)</td>
<td>2.61 ± 1.10 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>57.5</td>
<td>14.8</td>
<td>43/40</td>
</tr>
<tr>
<td>AG + GG (n = 21)</td>
<td>3.81 ± 1.24</td>
<td>60.4</td>
<td>13.1</td>
<td>13/8</td>
</tr>
</tbody>
</table>

<sup>a</sup>P value comparison between AA and AG + GG groups; P < 0.0001 using t-test. P = 0.0002 using Wilcoxon–Mann–Whitney test. Data represent mean ± SD.

![Figure 1. Scatter plot of warfarin dose against the VKORC1 −1639 genotype. Warfarin doses in selected patients with warfarin sensitivity or resistance (see Table 1 for details) were plotted against different genotypes at the VKORC1 promoter −1639 locus. Asterisk denotes individuals with CYP2C9 variants including CYP2C9*3, T299A and P382L.](Image)
Table 3. Genotype frequencies of VKORC1 promoter polymorphism (−1639 G>A) and CYP2C9 variants in Chinese and Caucasians

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Caucasian (n = 92)</th>
<th>Chinese (n = 95)</th>
<th>Randomly selected Chinese warfarin patients (n = 104)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VKORC1 −1639</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>13 (14.2%)</td>
<td>78 (82.1%)</td>
<td>83 (79.8%)</td>
</tr>
<tr>
<td>AG</td>
<td>43 (46.7%)</td>
<td>17 (17.9%)</td>
<td>19 (18.3%)</td>
</tr>
<tr>
<td>GG</td>
<td>36 (39.1%)</td>
<td>0 (0%)</td>
<td>2 (1.9%)</td>
</tr>
<tr>
<td>CYP2C9 variants(^a)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2C9(^a)2'2</td>
<td>20.4%</td>
<td>0 (0%)</td>
<td>0 (0%)(^b)</td>
</tr>
<tr>
<td>2C9(^a)2'2</td>
<td>0.9%</td>
<td>0 (0%)</td>
<td>0 (0%)(^b)</td>
</tr>
<tr>
<td>2C9(^a)1'3</td>
<td>11.6%</td>
<td>7 (7.3%)</td>
<td>4 (5.4%)(^b)</td>
</tr>
<tr>
<td>2C9(^a)3'3</td>
<td>0.4%</td>
<td>0 (0%)</td>
<td>0 (0%)(^b)</td>
</tr>
</tbody>
</table>

\(^a\)CYP2C9\(^a\)1 is the wild-type of CYP2C9. CYP2C9\(^a\)2 and CYP2C9\(^a\)3 are variants with cystine substitutes for arginine at residue 144 and leucine substitutes for isoleucine at residue 359, respectively. Frequencies in Chinese were obtained from published data (17).

\(^b\)CYP2C9\(^a\)3 frequency was derived from genotyping 74 warfarin patients.

\(^P<0.00001\) when compared between Caucasian and Chinese population.

\(^P<0.00001\) when compared between Caucasian and Chinese randomly selected warfarin patients.

\(^P=0.817\) when compared between Chinese and randomly selected warfarin patients.

\(^\)CYP2C9\(^a\) variants were inserted.

\(^\)pGL3-basic was used as control without any promoter sequence inserted.

requirement of higher dose of warfarin for its anticoagulation effect. Because liver is the primary organ for the synthesis of vitamin K dependent clotting factors and VKORC1’s expression is highest in the liver, a 44% change in the level of VKORC1 in the liver is likely to have a significant impact on the blood clotting processes. This repressor binding abolition in the liver is also vital to explain the inter-ethnic differences observed clinically. In the Caucasian population, >85% of the population are either heterozygous AG or homozygous GG. Therefore, these individuals would have more VKOR activity and this would explain why higher dose of warfarin is required to inhibit VKOR activity in Caucasians.

In summary, our data, for the first time, demonstrated that the inter-ethnic differences in warfarin dose requirement could be explained by the differences in VKORC1 gene polymorphism and its promoter activity. The VKORC1 −1639 A > G polymorphism was also associated with inter-individual variability in warfarin dose requirements. A large, prospective study will be required to address whether screening for this polymorphism will help physicians to determine more accurate dosages for the patients and reduce the chances of bleeding for the warfarin treatment.

MATERIALS AND METHODS

Patients

During October 2003 to December 2004, we recruited 16 patients who received warfarin either at low or at high dose from cardiovascular clinics of four major medical centers in Taiwan (National Taiwan University Hospital, Kaohsiung Medical University Hospital, Taipei General Veteran Hospital and Shin-Kong Wu Ho-Su Memorial Hospital). The mean maintenance dose of warfarin in Chinese is 3.3 mg/day (8,9), therefore, we considered patients who received maintenance dose ≤1.5 mg/day as warfarin sensitive (Table 1, 11 patients) and patients on warfarin maintenance dose ≥6 mg/day as warfarin resistant (Table 1, five patients). The definition of the warfarin-resistant patients was supported by our own data that none of the randomly selected patients (Table 2) had warfarin dose > 6 mg/day. This is contrast to the Caucasian patients who had average maintenance dose between 5.1 and 5.5 mg/day (20,27).

The average daily dose of warfarin was calculated from 1 week period, and latest INR was recorded. The randomly recruited 104 patients who received warfarin, regardless of the dose, had target INR of 1.4–3 from the same four hospitals (Table 2). The indications of warfarin were: valve replacement (90 patients), deep vein thrombosis (five patients), atrial fibrillation (five patients) and stroke (four patients). Clinical information (including age, sex, weight and average daily maintenance dose) was obtained from every participant. At the time of blood draw, every patient had a constant maintenance dose for at least 3 weeks. Patients with liver, kidney, gastro-intestinal cancer or abnormal bleeding before warfarin therapy were excluded.

In addition, DNA from 92 unrelated Caucasians [Cat. No, HD100CAU, National Institute of General Medical Sciences (NIGMS) Human Genetic Cell Repository, Camden, NJ, USA] were used as Caucasian controls, and DNA from 95 healthy subjects randomly selected from a biobank under a nation-wide population study in which 3312 Han Chinese descendants were recruited based on the geographic distribution across Taiwan were used as Chinese controls. The random Chinese controls and all participating patients receiving warfarin therapy were unrelated Han Chinese residing in Taiwan. The Han Chinese forms the largest ethnic group in Taiwan, making up roughly 98% of the population. None of the participants was aboriginal Taiwanese, which account for the remaining 2% of the Taiwan’s population.
The study was approved by the institutional review board, and informed consent was obtained from all of the participants.

**DNA sequencing and SNP genotyping**

Genomic DNA was isolated using PUREGENE™ DNA purification system (Gentra systems, Minnesota, USA). CYP2C9 and VKORC1 DNA sequence variants were first determined by direct sequencing (Applied Biosystems 3730 DNA analyzer, Applied Biosystems, Foster City, CA, USA) in 16 Han Chinese patients having warfarin sensitivity (≤1.5 mg/day, 11 patients) and resistance (≥6.0 mg/day, five patients). Primers were specifically designed for the intron–exon junctions, exons and 2 kb upstream of the transcription start site for both CYP2C9 and VKORC1. Primers were designed by Primer3 PCR primer program (http://fokker.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The primers used to detect variants in both CYP2C9 and VKORC1. Primers were designed by Primer3 PCR primer program (http://fokker.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The primers used to detect variants in the VKORC1 promoter were: 5' -CCACTGCAACTTGTTTCTCTTTCC, anti-sense strand located 0.9 kb upstream of the transcription start site and 5' -CACTGCAACTTGTTTCTCTTTCC, sense strand located 1.5 kb upstream of the transcription start site.

The PCR reactions were performed in a final volume of 25 μl, containing 0.4 μM of each primer, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM dNTPs and 1 U HotStart Taq™ (Qiagen Inc., Valencia, CA, USA). Amplification conditions consisted of an initial denaturation of 12 min at 96°C, followed by 34 PCR cycles in 30 s at 96°C, 30 s at 60°C, 40 s at 72°C.

MALDI-TOF mass spectrometry (SEQUENOM MassARRAY system, Sequenom, San Diego, CA, USA) was then used to screen for the identified variants in 104 randomly selected Chinese patients receiving warfarin, 95 normal Chinese controls and 92 normal Caucasians. Briefly, primers and probes were designed using the SpectroDESIGNER software (Sequenom). Multiplex PCR was performed, and unincorporated dNTPs were dephosphorylated using shrimp alkaline phosphatase (Hoffman-LaRoche, Basel, Switzerland) followed by primer extension. The purified primer extension reactions were spotted onto a 384-element silicon chip (SpectroCHIP, Sequenom), analyzed in the Bruker Biflex III MALDI-TOF SpectroREADER mass spectrometer (Sequenom) and the resulting spectra was processed with SpectroTYPER (Sequenom).

**Luciferase reporter assay**

*Plasmid construction.* To assay for the VKORC1 promoter activity, the VKORC1 promoter encompassing the −1639 polymorphism (from −1798 to −35) from patients with −1639 AA and −1639 GG genotypes was first cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA) with the forward primer: 5' -ccgctcgagtagatgtgagaaacagcatctgg (contained XhoI restriction site) and the reverse primer: 5' -ccttggagtttaagccgaaacctgag (contained HindIII restriction site). The fragment containing the VKORC1 promoter was released from the pGEM-T Easy vector by digesting the vector with XhoI and HindIII restriction enzymes and was then sub-cloned into the multiple cloning sites (XhoI and HindIII) of pGL3-basic vector (Promega). The pGL-3 vector contains the cDNA encoded for firefly luciferase which when fused with a promoter, can be used to analyze the inserted promoter activity once transfected into mammalian cells. The vector containing the −1639 G/G genotype was designated pGL3-G and the vector containing the −1639 A/A genotype was designated pGL3-A. VKORC1 promoter sequences in both vectors were confirmed by direct sequencing analysis.

**Cell culture and dual luciferase reporter assay.** HepG2 cells were grown in Dulbecco’s modified Eagle medium (DMEM) and 10% fetal calf serum supplemented with 100 U/ml Penicillin, 100 μg/ml Streptomycin and 2 mM l-Glutamine. Twenty-four hours prior to transfection, 1.5 x 10⁵ cells were seeded in each well of a 12-well plate. On the day of transfection, each well was co-transfected with 1.5 μg of the pGL3 vector and 50 ng of the pRL-TK vector (Promega) using lipofectamine 2000 (Invitrogen Corporation, Carlsbad, CA, USA). The pRL-TK vector encoded for the Renilla luciferase transcribed by a HSV-TK promoter which was used as internal controls to normalize firefly luciferase expression. Forty-eight hours after transfection, the cells were lysed in passive lysis buffer (Promega). Cell lysate was added to the luciferase substrate (dual luciferase reporter system, Promega) and firefly and Renilla luciferase activity was measured with a luminometer (SIRIUS, Pforzheim, Germany).

**Statistical analysis**

The genotype frequencies at each SNP were counted. Chi-square test was used to compare genotype frequencies at each SNP for the three sample groups. The t-test and the Wilcoxon–Mann–Whitney test were performed for multiple comparisons of mean dose levels among the different genotype groups. Inter-marker LD was assessed by two measures, D̲ and r², calculated using Graphical Overview of Linkage Disequilibrium (GOLD, http://www.sph.umich.edu/csg/abecasis/GOLD/).

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**Conflict of Interest statement.** None declared.

**REFERENCES**


