Cytochrome P450 2C9 (CYP2C9) and vitamin K epoxide reductase (VKORC1) genotypes as determinants of acenocoumarol sensitivity

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Oral anticoagulants, including warfarin and acenocoumarol (AC), are the most widely prescribed drugs for the prevention and treatment of arterial and venous thromboembolic disorders. However, warfarin was recently identified as a causal agent in 10% of cases of all hospital admissions for adverse drug reactions in England.1 The management of anticoagulant therapy is challenging because patients exhibit a large variability in their dose-anticoagulant effect response. A narrow range for a therapeutic effect makes individual dosing necessary. This interindividual variability is known to depend on environmental factors, but a genetic influence has also been demonstrated.2-6 The cytochrome P450 2C9 (CYP2C9) is the main enzyme responsible for the hepatic metabolism of oral anticoagulants such as AC and warfarin. The CYP2C9 has been demonstrated to be polymorphic, and its genetic variability has been shown to be associated with variations in the levels of enzyme activity.4,7-8 In humans, 3 major alleles have been found: (1) CYP2C9*1 (wild-type or reference allele) Cys144/Leu359; (2) CYP2C9*2, Arg144/Leu359; and (3) CYP2C9*3, Cys144/Ile359.9-11 Due to an impaired metabolic capacity, patients having at least one CYP2C9*3 allele require, on average, a lower dose of AC than homozygotes for the CYP2C9*1 allele.8,11 Moreover, it has recently been shown in our laboratory that the CYP2C9*3 allele influences the AC response. Up to 14% of the interindividual variability of the AC pharmacodynamic response can be explained based on this genotypic-haplotypic approach among white subjects.12 Recent works have also suggested that genetic variations within vitamin K–dependent protein genes could also be useful for predicting anticoagulant response.13-16 The vitamin K epoxide reductase multiprotein complex 1 (VKORC1) gene has been hypothesized to play a role in the variability of the AC response based upon a seminal study showing that an intronic 1173C>T polymorphism was associated with warfarin dose requirement. Patients carrying the T allele required a lower dose of warfarin compared with those carrying the C allele (the mean daily dose decreased 43% for homozygous TT carriers and 22% for heterozygous).16

The aim of this study was to evaluate the contribution of genetic variability of the VKORC1 gene, in addition to the 1173C>T polymorphism, on the pharmacodynamic outcome after AC intake.
Patients, materials, and methods

Subjects

The study population was the same as the one used in our previous analysis of the CYP2C9 haplotype. Enrolled in this study were 263 healthy volunteers between 18 and 65 years of age. Among them, 230 were white and DNA samples were available for 222 (145 males and 77 females). In accordance with the Declaration of Helsinki, each subject provided written informed consent before participation. The study was approved by the Comité Consultatif pour la Protection des Personnes Participant à la Recherche Biomédicale of Pitie-Salpêtrière Hospital (Assistance Publique des Hôpitaux de Paris local ethics committee) and complies with French bioethics laws. This study took place at the Center for Clinical investigation des Hoˆpitaux de Paris local ethics committee and complies with French bioethics laws. This study took place at the Center for Clinical investigation des Hoˆpitaux de Paris local ethics committee and complies with French bioethics laws.

Methods

Selection of polymorphisms in the VKORC1 gene. The VKORC1 gene polymorphisms studied in this report were selected from public databases (http://pga.gs.washington.edu/data/vkorel/welcome.html). Of the polymorphisms, 29 have been identified from a population including 24 African individuals and 23 European individuals, and 13 haplotypes have been inferred (frequency ranged from 1% to 17%) from 17 single nucleotide polymorphisms (SNPs) with allele frequency of more than 4% (http://pga.gs.washington.edu/data/vkorel/welcome.html). All the polymorphisms described by D’Andrea et al16 with a frequency more than 1% were listed within this database (ie, 3462C>T in exon 3, 1173C>T in intron 1, 4451C>A in the 3’ untranslated region [UTR]) and among the 17 SNPs mentioned in this paragraph.

For the present study, we used the TAG’n’TELL software (http://spn.ebi.ac.uk/sfagetn/ctl) to identify and select the minimum number of SNPs that capture more than 95% of the haplotypic diversity referred to as the haplotype tagging SNPs (htSNPs). In this way, 6 SNPs were selected: 5 (-4931T>C, -4451C>A, -2659G>C, -1877A>G, -1639G>A) located in the 5’ flanking region of the VKORC1 gene and 1 (497C>G) in intron 1 (Table 1). In view of the recent findings suggesting a potential effect of the 1173C>T intronic polymorphism on the pharmacodynamic response, this SNP was also selected for genotyping even though it was not identified as an htSNP.

Genotyping of VKORC1 htSNPs (-4931T>C, -4451C>A, -2659G>C, -1877A>G, -1639G>A, 497C>G) and 1173C>T SNP. For the -4931T>C polymorphism, a real-time polymerase chain reaction (PCR) allelic discrimination assay was designed using Primer Express software (Applied Biosystems, Courtaboeuf, France) and used a forward primer (5’GCAACATACAAACACATCATCTAATG 3’), a reverse primer (5’GGCCAGCTTCTCCTCAAC 3’), and 2 minor groove-binding (MGB) probes, namely 5’Fam-ATCGCACCTTTGTGG (3’ as the wild-type genotype) and 5’Fam-ATGATCCGGGCTCCCT 3’ (as the mutated genotype). Amplification was performed using a 7900HT Applied Biosystems real-time thermal cycler (Applied Biosystems, Courtaboeuf, France). All probe/primer sets were designed to function using universal reaction and cycling conditions.

Direct sequencing after PCR was performed for the detection of all other SNPs. The primer sequences are available on request. Sequencing was performed on an ABI Prism Genetic Analyzer System 9700 (Applied Biosystems).

Pharmacodynamics. International normalized ratio (INR) was determined using Thromborel S (Dade Behring, Marburg, Germany). Factor VII coagulant activity was determined by a one-stage assay using factor VII-deficient plasma (Dade Behring) and Thrombol S.

To evaluate AC pharmacodynamics, factor VII ratio ([factor VII 24 hours after AC intake/factor VII before AC intake] × 100) and INR change (ie, [(INR 24 hours after AC intake – INR before AC intake)/INR before AC intake] × 100)) were chosen to evaluate AC pharmacodynamics.

Plasmid construction

To study the effect on the promoter activity of the VKORC1 -1639G>A polymorphism, a 2171-bp fragment containing either the G (wild-type) or A (mutant) allele was initially amplified from genomic DNA (isolated from subjects homozygous either for the G or A allele) with VKORC1 specific primers (5’-AGAGGATGGTGCAGTTATCCATC-3’ and 5’-TTTACATCTGTTATCCCTGGC-3’). Finally, a 1678-bp subfragment (−1756, −12; the nucleotides are numbered from the A in the AGT initiating translation) was obtained from the first PCR product with primers incorporating NheI and HindIII restriction sites (5’-GGGAGAATCCGAGGAGGCGG-3’ and 5’-TATCTTAAGGCTCGGCCGG-3’). The 2 purified PCR products (eg, one containing the G allele; the other, the A allele) were then digested with HindIII and NheI and cloned in the pGL3 basic vector, upstream of the Firefly luciferase reporter gene (Promega, Charbonnière, France). The PCR reactions were run with proofreading PfuTurbo DNA polymerase (Stratagene, Charbonnière, France). All constructs were checked by direct sequencing on an ABI PRISM 7700 Genetic Analyser sequencer (Applied Biosystems). The pglbo-RL plasmid, a gift of Dr Y. Morel, contains a promoter derived from the proximal part of the human α-globin promoter upstream of the Rennilla luciferase reporter gene (used as internal control), which allows control of the transfection efficiency.

Cell culture and transfection

Hept2 cells were grown in 50% Dulbecco modified Eagle medium (DMEM) and 50% HamF12 medium supplemented with 10% fetal bovine serum (FBS), 200 U/mL penicillin, and 0.5 mg/mL fungizone in a 5%-humidified atmosphere. Transfection experiments were performed in Hept2 cells using the calcium-phosphate method. Briefly, the day prior to the transfection, cells were trypsinized and seeded (2 × 10^4 in 12-well plates) into the usual culture medium. For each series of transfections, 1 μg pG3 basic plasmid, containing either the VKORC1 wild-type or mutated promoter construct, and 100 ng pgo-RL plasmid were added to 31 μL 2-M CaCl2, and mixed with an equal volume of 2 × HBS (HEPES [N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid]-buffered saline) buffer. After a 20-minute incubation, 500 μL of this mixture was added to each well containing 2 mL media. The media was changed 4 hours after transfection. The luciferase activity was measured using the Dual luciferase (Firefly and Renilla) assay kit (Promega) 48 hours later, according to the manufacturer’s instructions. Results of wild-type and mutant constructs were analyzed by expressing the ratio of the 2 luciferase activities. Renilla luciferase activity was used to normalize the transfection efficiency in all culture dishes. Each assay was repeated in 4 independent experiments including 6 replicates for the 2 constructs.

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### Table 1. Position and frequencies of VKORC1 genetic polymorphisms and SNPs used for haplotype typing

<table>
<thead>
<tr>
<th>SNP Gene Location</th>
<th>Frequency‡</th>
<th>NCBI</th>
<th>Our Study</th>
<th>Region</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relative to A*</td>
<td>Relative to first nucleotide†</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-4931T&gt;C</td>
<td>381</td>
<td>0.47</td>
<td>0.42</td>
<td>5’ flanking</td>
</tr>
<tr>
<td>-4451C&gt;A</td>
<td>861</td>
<td>0.26</td>
<td>0.33</td>
<td>5’ flanking</td>
</tr>
<tr>
<td>-2659G&gt;C</td>
<td>2653</td>
<td>0.17</td>
<td>0.21</td>
<td>5’ flanking</td>
</tr>
<tr>
<td>-1877A&gt;G</td>
<td>3435</td>
<td>0.14</td>
<td>0.02</td>
<td>5’ flanking</td>
</tr>
<tr>
<td>-1639G&gt;A</td>
<td>3673</td>
<td>0.29</td>
<td>0.42</td>
<td>5’ flanking</td>
</tr>
<tr>
<td>497T&gt;G</td>
<td>5808</td>
<td>0.18</td>
<td>0.30</td>
<td>Intron 1</td>
</tr>
<tr>
<td>1173C&gt;T</td>
<td>6484</td>
<td>0.31</td>
<td>0.42</td>
<td>Intron 1</td>
</tr>
</tbody>
</table>

*The position of the SNP is relative to the A of the AGT initiation codon (denoted nucleotide +1).
†Designation of the SNP relative to the first nucleotide referred to as accession number AF587020 (National Center for Biotechnology Information [NCBI]) or in http://pga.gs.washington.edu/data/vkorel/welcome.html. The length of the analyzed VKORC1 sequence (including 5’ flanking, exonic, intronic, and 3’ untranslated regions) is of 12 megabases (Mb), and the bases are numbered from 1 to 12,000.
‡The indicated frequencies correspond to those of the minor allele.
Statistics

One-way analysis of variance (ANOVA) with Dunnett post test was performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA). Allele frequencies were estimated by gene counting. Departure from Hardy-Weinberg equilibrium was tested by a Chi² test with 1 degree of freedom (df). ANOVA analysis was first performed to test for the association of each VKORC1 polymorphism with AC response and then followed by a haplotype-based association analysis to take into account the simultaneous information of all VKORC1 polymorphisms.

Linkage disequilibrium (LD) and haplotype analyses were performed by use of the THESIAS software (http://ecgene.net/genecanvas/modules/mydownloads/singlefile.php?cid = 1&lid = 4) based on the SEM algorithm.15 This method allows the estimation of haplotype frequencies and phenotypic means according to the carried haplotypes under the assumption of additive haplotypic effects. Phenotypic changes, expressed as a percentage compared with baseline values, before AC intake were reported with their corresponding 95% confidence intervals. Finally, in order to test whether the CYP2C9*3 allele previously shown to be associated with factor VII variability could interact or act additively with VKORC1 SNPs, interaction was investigated through multiple regression analysis by introducing genotype × genotype interaction terms in the regression model.

Results

Allele frequencies of the VKORC1 gene among 222 healthy volunteers

Allele frequencies of the 7 SNPs studied are reported in Table 1. No significant deviation from Hardy-Weinberg equilibrium was observed for any polymorphism (P > .05). The observed frequencies of minor alleles ranged from 0.21 to 0.42, except for the −1877A>G that appears with a lower frequency (0.02).

Effect of VKORC1 genotypes on AC pharmacodynamic response

All polymorphisms studied were strongly associated with the factor VII ratio and INR change (P < 10⁻⁴). The factor VII ratio phenotypic variance explained by genotype ranged from 8% for the −2659G>C polymorphism to 37% for the −1639G>A polymorphism. Corresponding values for INR change ranged from 7% to 30% for the same polymorphisms. The factor VII ratio (Figure 1A) and the INR change (%) (Figure 1B) are plotted against VKORC1 genotype (only the −1639G>A SNP, which corresponds to the more informative SNP, is shown as detailed in the next paragraph). The factor VII ratio after AC intake decreased in the following order for VKORC1 −1639G>A genotype: 70% ± 14 for VKORC1 −1639GG, 56% ± 15 for VKORC1 −1639GA, and 35% ± 17 for VKORC1 −1639AA (P < .01). The same effect was observed for INR change, with an increase of INR more marked in subjects carrying the VKORC1 −1639A allele (12% ± 9 for VKORC1 −1639AA, 21% ± 14 for VKORC1 −1639GA, and 42% ± 22 for VKORC1 −1639GG; P < .01).

VKORC1 haplotype analysis

All polymorphisms were in strong LD with each other. In particular, the 1173C>T and −1639G>A polymorphisms were in complete disequilibrium. As a consequence of this LD pattern, 7 haplotypes were inferred, among which 5 appeared with a frequency more than 0.05 and accounted for about 95% of all chromosomes (Table 2).

The rare −1877G allele seemed to be carried out by several haplotypes with very low frequency (0.007) and is therefore not depicted in Table 2. VKORC1 gene haplotypes were highly associated with factor VII ratio (χ² = 95.43 with 6 df, P < 10⁻⁴) and explained 37% of its variability. Interestingly, all 3 haplotypes carrying the −1639A (or the 1173T) allele were associated with lower factor VII ratio levels (~19%) or higher INR change (~19%), while all those carrying the −1639G (or the 1173C) allele were associated with increased levels of factor VII ratio (~36%) or lower INR change (~5%), suggesting that the data could be compatible with the sole effect of the −1639G>A (or 1173C>T) polymorphism. This hypothesis was not rejected (χ² = 2.40 with 5 df, P = .79). Thus, in subjects with the −1639A, AC has a significantly higher pharmacologic effect than in subjects with the −1639G.

The same analysis, including INR change as quantitative variable, led to results similar to those obtained with factor VII ratio (eg, 30% of variability for VKORC1).

Multiple linear regression model

CYP2C9*3 allele was demonstrated in a previous paper12 to account for 14% of variability in percentage of remaining factor VII. Multiple regression analysis revealed that the CYP2C9*3 and the −1639G>A polymorphisms were independently associated with AC response and explained up to 50% of the variability in the AC response. When the subject’s body weight was combined with CYP2C9 and VKORC1 SNPs, an additive effect was observed, increasing the part of variability explained by these 3 parameters to 54%.

Results similar to those obtained with factor VII ratio were observed when using INR change as the pharmacologic index (eg, 40% of variability when combining subject’s weight with CYP2C9 and VKORC1 SNPs).

In Tables 3-4, the combined effects of the CYP2C9 and VKORC1 genotypes are presented and plotted against the factor VII
Table 2. Haplotypic analysis of the VKORC1 gene among the 222 healthy volunteers in relation to AC response

<table>
<thead>
<tr>
<th>VKORC1 SNPs</th>
<th>Factor VII ratio</th>
<th>INR change</th>
</tr>
</thead>
<tbody>
<tr>
<td>T/C A/G T/G</td>
<td>17 (15-19)</td>
<td>18 (16-20)</td>
</tr>
<tr>
<td>T/A G/C T/C</td>
<td>14 (12-16)</td>
<td>16 (14-18)</td>
</tr>
<tr>
<td>A/T G/A T/C</td>
<td>15 (13-17)</td>
<td>17 (15-19)</td>
</tr>
</tbody>
</table>

Table 3. Combined effect of CYP2C9 and VKORC1 genotypes on the percentage of factor VII ratio after a single-dose AC intake

<table>
<thead>
<tr>
<th>CYP2C9 genotype</th>
<th>VKORC1 genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G/G</td>
</tr>
<tr>
<td>*1/*1</td>
<td>74</td>
</tr>
<tr>
<td>*1/*3</td>
<td>53</td>
</tr>
</tbody>
</table>

Table 4. Combined effect of CYP2C9 and VKORC1 genotypes on INR change (%) after a single-dose AC intake

<table>
<thead>
<tr>
<th>CYP2C9 genotype</th>
<th>VKORC1 genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G/G</td>
</tr>
<tr>
<td>*1/*1</td>
<td>10</td>
</tr>
<tr>
<td>*1/*3</td>
<td>21</td>
</tr>
</tbody>
</table>

The combined genotypes of VKORC1 (G/A and A/A) and CYP2C9 (CYP2C9*3 allele) represent 25% of the population. Average of percentage of remaining factor VII and INR change is represented within different CYP2C9 and VKORC1 genotypes. The numbers of the VKORC1 G/G, G/A, and A/A genotypes among the CYP2C9*1/*1 individuals were 59, 95, and 30, respectively. The numbers of the VKORC1 G/G, G/A, and A/A genotypes among the heterozygous carriers of CYP2C9*3 allele were 13, 15, and 10, respectively.
coagulation factors (eg, factors II, IX, and X) are more than 48 hours (up to 60 hours for factor II). Since the pharmacologic response was calculated over 24 hours, the association between VKORC1 genotype and the INR change might have been underestimated.

This is the first study that reports the effect of genetic polymorphisms of the VKORC1 gene by haplotype analysis in the variability to the AC response. The htSNPs were identified from data obtained from sequencing of a panel of 47 selected individuals (http://pga.gs.washington.edu/data/vkorc1/welcome.html) in order to perform haplotype construction, and then to determine the haplotype effect on the pharmacodynamic response to AC. As shown in Table 1, the frequencies observed in our population (222 subjects) are similar to those observed in the panel of 47 samples for all SNPs, except for the −1877T>G mutation (0.02 versus 0.14). This difference could be explained by the genetic heterogeneity between the ethnic groups. Further, the association of each SNP individually with the percentage of remaining factor VII and INR 0.14). This difference could be explained by the genetic heterogeneity between the ethnic groups. Further, the association of each SNP individually with the percentage of remaining factor VII and INR change was tested. These SNPs are significantly correlated with the AC response and allowed the identification of 7 major VKORC1 haplotypes (Table 2). Haplotype analysis revealed that the −1639G>A polymorphism (or the 1173C>T polymorphism in complete association with it) is sufficient to explain all of the pharmacologic variability (~37%). It is noteworthy that the htSNPs are currently used as genetic markers (for haplotype information) independently of the functional impact. In this case, the use of MathInspector (Genomatix Software, Munich, Germany) has revealed a potential nuclear factor 1 (NFI) binding-site motif (TTG GCCA) in the VKORC1 gene at position −1639 upstream of ATG initiation codon. NFI is a ubiquitous transcription factor known to play an important role in viral and cellular regulation and is involved in the control of constitutive and inducible gene expression. Thus, a possible explanation would be that the mutation −1639G>A modifies a putative binding site for NFI affecting VKORC1 transcription. To test this hypothesis, a luciferase reporter gene assay was performed. Our results showed no difference between the wild-type and the mutant promoter. D’Andrea et al found that the 1173C>T intronic mutation, associated with low-dose requirement of warfarin, did not affect the processing of VKORC1 mRNA. Altogether, these results suggested that a clear functional SNP in the VKORC1 gene was not yet identified, although both SNPs were linked in the pharmacologic response to anticoagulant.

The multiple linear regression analysis indicated that the genetic polymorphisms of CYP2C9 and VKORC1 strongly modulate the pharmacologic response to AC with an additive effect, and, based upon our previously published data, body weight, CYP2C9, and VKORC1 accounted for about 54% for the variability. The results concerning these factors will likely have implications not only for AC but also for other oral anticoagulant treatments such as warfarin and phenprocoumon,22,26 which are metabolized by the same enzymes and have the same targets (vitamin K-dependent enzymes). Thus, the inclusion of these parameters in computer-generated algorithms to determine the initial doses of these drugs after a simple genotyping (the 2 SNPs described in this study for AC and the additional CYP2C9*2 for warfarin and phenprocoumon). Our results should be confirmed by prospective clinical studies including treated patient populations. The knowledge of the pharmacogenetics of drug metabolism and targets for vitamin K antagonists raises the possibility of individualizing the initial dose and, thus, limiting the frequent complications observed with the use of these drugs.

In conclusion, the joint genotyping of 2 SNPs (CYP2C9*3 and −1639G>A or 1173C>T on the VKORC1 gene), which is easy and inexpensive, predicts about 50% of the interindividual variability of the AC pharmacodynamic response, providing the opportunity for safer anticoagulation therapy. For the sake of clarity, a consensus choice should be made between −1639G>A and 1173C>T as a definitive Tag SNP. Pharmacogenetic testing could predict a high risk of overdose among 25% of the population before initiation of anticoagulation. This would correspond to the subjects carrying either the VKORC1 variant allele (−1639A or 1173T) or the CYP2C9*3 allele. Some authors have found a weak effect of CYP2C9*2 on the dose of AC necessary to provide the expected anticoagulation.8,20,27 In the current paper, we did not find such a difference, as did some other authors28 and as discussed in our previous paper.12 The improvement of coumarin drug (acenocoumarol, warfarin) management is clinically relevant because of the wide use of these drugs and because of the difficulties encountered during the development of the novel anticoagulants.

Acknowledgments

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