Background—The haplotypes in the gene vitamin K epoxide reductase complex subunit 1 (VKORC1) have been found to affect warfarin dose response through effects on the formation of reduced-form vitamin K, a cofactor for γ-carboxylation of vitamin K–dependent proteins, which is involved in the coagulation cascade and has a potential impact on atherosclerosis. We hypothesized that VKORC1-dependent effects on the coagulation cascade and atherosclerosis would contribute to susceptibility for vascular diseases.

Methods and Results—To test the hypothesis, we studied the association of polymorphisms of VKORC1 with stroke (1811 patients), coronary heart disease (740 patients), and aortic dissection (253 patients) compared with matched controls (n=1811, 740, and 416, respectively). Five common noncoding single-nucleotide polymorphisms of VKORC1 were identified in a natural haplotype block with strong linkage disequilibrium (D²=0.9, r²=0.9), then single-nucleotide polymorphism (SNP) +2255 in the block was selected for the association study. We found that the presence of the C allele of the +2255 locus conferred almost twice the risk of vascular disease (odds ratio [OR] 1.95, 95% confidence interval [CI] .58 to 2.41, P<0.001 for stroke; OR 1.72, 95% CI 1.24 to 2.38, P<0.01 for coronary heart disease; and OR 1.90, 95% CI 1.04 to 3.48, P<0.05 for aortic dissection). We also observed that subjects with the CC and CT genotypes had lower levels of undercarboxylated osteocalcin (a regulator for the bone), probably vascular calcification, and lower levels of protein induced in vitamin K absence or antagonist II (PIVKA-II, a des-γ-carboxy prothrombin) than those with TT genotypes.

Conclusions—The haplotype of VKORC1 may serve as a novel genetic marker for the risk of stroke, coronary heart disease, and aortic dissection. (Circulation. 2006;113:1615-1621.)

Key Words: vitamin K epoxide reductase ■ haplotypes ■ stroke ■ coronary disease ■ aortic dissection

Stroke and coronary heart disease (CHD) are the leading causes of morbidity and mortality in China.1 Each year, more than 2.5 million Chinese have strokes, 1 million have heart attacks, and 2 million die of stroke- and CHD-related causes. Furthermore, 7 million patients have survived a stroke and are disabled (http://www.moh.gov.cn). Elucidation of the pathogenesis of these diseases and identification of subjects at risk for these events are major challenges to medical society. A high degree of comorbidity and common risk factors have been observed among myocardial infarction (MI), stroke, and aortic dissection. Most of these cases result from atherosclerotic disease that is characterized by lesions in large and medium-sized elastic and muscular arteries.2–4

Platelet adhesion and mural thrombosis are ubiquitous in the initiation and generation of lesions of atherosclerosis in animals and in humans.5,6 Plaque adhesion and thrombosis formation result from activation of the coagulation cascade, initiated by atherosclerotic plaque erosion or rupture contributing to propagation of thrombosis.7–9

Vitamin K–dependent proteins play very important roles in activation of the coagulation cascade and in maintaining blood flow and integrity of the vasculature.10–13 The biological activity of all known vitamin K–dependent proteins is
highly dependent on correct γ-carboxylation. Vitamin K hydroquinone, a cofactor for γ-carboxylation, is converted to vitamin K epoxide, which in turn is recycled to vitamin K hydroquinone by vitamin K epoxide reductase. Undercarboxylation of vitamin K–dependent proteins in patients with a specific mutation in the γ-carboxylase results in bleeding disorders. Common polymorphisms in the gene vitamin K epoxide reductase complex subunit 1 (VKORC1) affect warfarin dose response and blood clotting through effects on the formation of the reduced form of vitamin K, which subsequently alters carboxylation of vitamin K–dependent hemostatic and nonhemostatic proteins. Numerous studies indicate that vitamin K–dependent proteins have additional activities that extend their roles beyond hemostatic and bone metabolism, perhaps in vascular calcification and atherosclerotic complications. Price et al showed that inhibition of VKORC1 by warfarin results in undercarboxylation of matrix Gla protein (MGP) and subsequent medial calcification of the arterial vessel wall. Gene-deletion studies in mice have shown that MGP is an inhibitor of calcification.

Arterial calcification has been correlated with an increased probability of dissection and a higher incidence of future ischemic episodes in patients undergoing angioplasty. Intimal calcification of atherosclerotic plaques correlates with plaque burden and high risk of cardiovascular events. Medial calcification has been shown to be associated with diabetes mellitus and end-stage renal disease and as a prognostic marker for cardiovascular mortality in patients requiring hemodialysis. Despite the fact that VKORC1 mediates vitamin K–dependent γ-carboxylation, which is involved in calcification, it remains unknown whether this could translate into higher risk for arterial disease. We hypothesized that VKORC1–dependent effects on the coagulation cascade and vascular calcification would contribute to susceptibility to vascular diseases. To test the hypothesis, we investigated the association of VKORC1 polymorphisms with stroke, CHD, or aortic dissection.

Methods
The study was approved by both the ethics committee of Fuwai Hospital and the local ethics committee of the collaborative hospitals. All subjects who participated in the study were provided written informed consent and reported themselves to be of Han nationality.

Stroke Cohort
The primary study population has previously been used to investigate risk factors of stroke. Briefly, case and control subjects were recruited from 7 clinical centers located in 7 provinces from the same demographic area and at the same time from November 2000 to November 2001. Only 3 subtypes of stroke—cerebral atherosclerosis, lacunar infarction, and intracerebral hemorrhage—were included. Diagnosis of stroke was based on strict neurological examination, computed tomography, or MRI according to the nosology. None of the control subjects had a history or symptoms of cardiovascular disease.

Biological Variable Determination and Clinical Data Collection
Blood samples were collected after a 12-hour overnight fast before cardiovascular procedures. In subjects with an acute event, the drawing of blood was delayed for at least 6 weeks. The plasma and cell buffert coat were kept at −70°C. Genomic DNA was extracted, and biological variables were determined within 3 months. A complete clinical history was obtained from all subjects. In addition to neurological history and family history of hypertension, CHD, and diabetes mellitus (DM), the following vascular risk factors were also recorded: history of vascular disease, cigarette smoking, alcohol consumption, body mass index, systolic blood pressure (SBP), diastolic blood pressure (DBP), blood glucose, HDL cholesterol (HDL-C), non–HDL-C lipids, total plasma cholesterol (TC), and triglycerides (TG). Plasma biochemical parameters were assayed by an automatic analyzer (Hitachi 7060, Hitachi, Tokyo, Japan). Non-HDL-C was calculated by the Friedewald formula. Hypertension was defined as a mean of 3 independent measures of blood pressure >140/90 mm Hg or the use of antihypertensive drugs. DM was diagnosed when the subject had a fasting glucose level >7.8 mmol/L, >11.1 mmol/L at 2 hours after oral glucose challenge, or both. All lipids were determined in a Centers for Disease Control and Prevention (CDC)–qualified laboratory in Fuwai hospital.

Screening for Single-Nucleotide Polymorphisms
The entire gene region of VKORC1 and the 2-kilobase (kb) 5′ upstream promoter region and 2-kb 3′ downstream region were screened by sequencing (ABI Prism 377, Perkin-Elmer Applied Biosystems, Foster City, Calif) in 50 stroke patients and 50 control subjects. No polymorphism was found in the entire coding sequence. The alleles with frequencies <0.05 were excluded; 5 common noncoding polymorphisms were identified in the VKORC1 gene, including −1639A/G (rs9923231) in the promoter region, +1173T/C (rs9934438) in the first intron, +1542C/G (rs8050894) and +2255T/C (rs2359612) in the second intron, and +3730G/A (rs7294) in the 3′ downstream region (defined by the nucleotide position from the translational start site). These single-nucleotide polymorphisms (SNPs) are located at positions 3673, 6484, 6853, 7566, and 9801 of the VKORC1 reference sequence (GenBank
accession number AF246702). We calculated linkage disequilibrium (LD) between pairs of SNPs using the standard definition of D' and r² and found all 5 SNPs were in strong LD, with D'>0.9 and r²>0.9, which indicates that any 1 of the 5 SNPs could reflect the natural haplotype block of VKORC1. SNP +2255 was selected for genotyping in all studied subjects because its frequency was slightly higher than the other 4 SNPs, and there is a natural digestion site of NcoI in the SNP +2255 fragment.

Genotyping of SNP +2255
The polymorphism +2255 was analyzed by amplification of a 198-bp sequence with the use of the following primers: 5'-TCTGAAACATGTGTCAGCAGACC-3' and 5'-GAAACAGAGAGAGAACTACGGTGA-3'. The resultant polymerase chain reaction products were digested with NcoI (New England Biolabs, Beverly, Mass), which yielded 2 DNA fragments of 26 and 172 bp for the T allele on 4% agarose gel and only 1 band for the C allele. Reproducibility of genotyping was confirmed by bidirectional sequencing in 500 samples, and the reproducibility was 100%.

Testing of Genetic Stratification of the Populations
The possible unequal genetic admixture or population subdivision in the control and patient populations could have resulted in a spurious association between a marker and disease. We additionally typed 7 unlinked microsatellite markers: TNNT2 (1q32; D15S262), MYL3 (3p21.3-p21.2; D3S3560), NEXLIN (1p31.1; D15S2876), MYH7 (14q12; D14S990), TPM1 (15q22.1; D15S993), PRK2 (7q35-q36; D7S483), and TNNT3 (19q13.4; D19S927). The allele frequencies at those markers were tested for association with phenotypes. The primer sequences were obtained from the Human Genome Database (http://www.gdb.org/), and the primers were synthesized and fluorescently labeled commercially. Genotype results were analyzed with GeneScan and Genotyper software (Applied Biosystems), with the positive control DNA 1347-02 (Centre d'Etude du Polymorphisme Humain [CEPH]) and the negative control. χ² Tests were used to compare allele frequencies of the microsatellite loci in patients and controls. Alleles with frequencies <0.05 were combined at a given microsatellite locus in both groups.

Undercarboxylated Osteocalcin and Protein Induced in Vitamin K Absence or Antagonism II Measurements
To investigate the impact of the gene polymorphisms on γ-carboxylation of vitamin K–dependent proteins, we determined the levels of undercarboxylated osteocalcin and PIVKA-II (protein induced in vitamin K absence or antagonism II) antigen in the serum of 49 subjects aged 45 to 65 years. To avoid potential confounding effects of risk factors on the serum levels of undercarboxylated osteocalcin and PIVKA-II, subjects without conventional risk factors were selected, including 26 with homozygotes, 18 with a heterozygote for the T allele, and 5 with a homozygote for the C allele of SNP +2255. The assay was performed with ELISA kits, undercarboxylated osteocalcin from Takara Shuzo (Tokyo, Japan), and PIVKA-II from Diagnostica Stago (Asnieres Sur Seine, France).

Statistical Analysis
The distribution of quantitative variables was tested for normality by use of a 1-sample Kolmogorov-Smirnov test. Because the TG level was highly skewed, we compared the difference between cases and controls with a Mann-Whitney nonparametric test. Quantitative variables, including age, body mass index, SBP and DBP, glucose, HDL-C, non-HDL-C, and TC, were compared with the 1-way ANOVA test. A χ² test was used to test for qualitative variables, genotype/allele frequencies, and Hardy-Weinberg equilibrium of the polymorphisms. Association of SNP +2255 with vascular disease was analyzed by multivariable logistic regression adjusted by age, sex, body mass index, blood pressure, smoking, alcohol consumption, glucose, HDL-C, non-HDL-C, and TC. The association was expressed as an odds ratio. The Student t test and Spearman correlation were used to assess the difference and correlation between serum levels of undercarboxylated osteocalcin and PIVKA-II in 3 genotypes of SNP +2255. A 2-tailed probability value of ≤0.05 was considered significant. Analyses were performed with SPSS 11.0 (SPSS Inc, Chicago, Ill) for Windows (Microsoft Corp, Redmond, Wash). The χ² measurement was used to determine LD with the software EMILD (http://request.mdacc.tmc.edu/qhuang/Software/pub.htm). The D' and r² were used to indicate the strength of LD.

The authors had full access to the data and take full responsibility for its integrity. All authors have read and agree to the manuscript as written.

Results
Characteristics of the Subjects
Clinical characteristics of the subjects in the present study are shown in Table 1. In the stroke study, of the 4000 subjects initially recruited for that study, 189 cases and 189 controls were excluded because of lack of definite diagnosis, insufficient DNA, or failure of genotyping. Significantly higher levels of SBP and DBP and a higher incidence of cigarette smoking and hypertension were found in cases than in controls (all P<0.01). In the CHD study, 740 cases and 740 controls were enrolled; higher frequencies of DM, hypertension, and cigarette smoking were found in cases than in controls (all P<0.01). In the aortic dissection study, 253 cases and 416 controls were recruited. Significantly higher levels of SBP and HDL-C, lower levels of non-HDL-C, TC, and TG, and a lower drinking frequency were found in patients than in controls (all P<0.05).

The C Allele of +2255 Was Associated With Stroke, CHD, and Aortic Dissection
The distribution of VKORC1 genotypes of +2255 is shown in Tables 2, 3, and 4, respectively, and fulfilled expectations of Hardy-Weinberg equilibrium in both cases and controls. The estimated risk of vascular disease for subjects with the CT genotype (1 copy of the risk allele C) was significantly higher than for those with the TT genotype (no copy of the risk allele C) but was comparable to those with the CC genotype, which indicates a dominant effect of the at-risk allele C. We used the dominant model to analyze the association of the SNP with vascular disease. The frequency of the CC+CT genotype was significantly higher in patients than in controls (stroke 19.7% versus controls 11.7%, P<0.001; CHD 16.9% versus controls 11.2%, P<0.01; aortic dissection 17.0% versus controls 9.9%, P<0.05). The association remained after adjustment for age, sex, and other conventional risk factors with multiple logistic regression analysis; the OR was 1.95 (95% CI 1.58 to 2.41) for stroke (P<0.001), 1.72 (95% CI 1.24 to 2.38) for CHD (P<0.01), and 1.90 (95% CI 1.04 to 3.48) for aortic dissection (P<0.05). In subtypes of stroke patients, the frequencies of CC+CT were 18.4% for thrombosis (OR 1.75, 95% CI 1.34 to 2.29, P<0.001), 23.2% for lacunar stroke (OR 2.33, 95% CI 1.09 to 2.16, P<0.05), and 18.2% for hemorrhage (OR 1.53, 95% CI 1.76 to 3.06, P<0.001). The presence of the C allele of the +2255 locus conferred an almost 2-fold increase in vascular disease risk within these studied populations. Because the C allele of +2255 can reflect the G-C-G-C-A (3673-6484-6853-7566-9041) haplotype of VKORC1 gene, one can reasonably expect that the
No Evidence of Strong Population Stratification

Among the 7 highly polymorphic markers that we genotyped, no significant allele-frequency differences were detected between controls and patients within each clinic center and among these centers, which indicates that there was no obvious evidence for genetic stratification in the cohort. The levels of both undercarboxylated osteocalcin and PIVKA-II antigen were lower in carriers of the C allele than TT allele carriers (Figure). The Spearman coefficient for the existing correlation was \( r = -0.219 \) for undercarboxylated osteocalcin and \(-0.567\) for PIVKA-II with genotype, respectively. The differences were also significant in undercarboxylated osteocalcin and in PIVKA-II between TT and CC carriers (\( P = 0.021 \) and \( P = 0.001 \), respectively), which suggests that the C allele contributes to a higher functional efficiency of the \( VKORC1 \) complex.

**TABLE 1. Clinical Characteristics**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Controls (n=1811)</th>
<th>Total (n=1811)</th>
<th>Thrombosis (n=798)</th>
<th>Lacunar Stroke (n=514)</th>
<th>Hemorrhage (n=499)</th>
<th>Cases (n=740)</th>
<th>CHD (n=740)</th>
<th>Aortic Dissection (n=416)</th>
<th>Cases (n=253)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>59.6 (8.5)</td>
<td>60.3 (9.4)†</td>
<td>61.5 (9.3)†</td>
<td>61.1 (8.5)†</td>
<td>58.1 (9.7)†</td>
<td>58.5 (9.2)</td>
<td>58.6 (10.7)†</td>
<td>57.9 (8.6)</td>
<td>48.9 (13.3)†</td>
</tr>
<tr>
<td>Men, %</td>
<td>57.4</td>
<td>63.5†</td>
<td>63.7†</td>
<td>62.8‡</td>
<td>63.5†</td>
<td>85.3</td>
<td>84.7</td>
<td>83.2</td>
<td>82.2</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>24.2 (3.3)</td>
<td>24.3 (3.5)</td>
<td>24.4 (3.5)</td>
<td>24.4 (3.2)</td>
<td>24.0 (3.5)</td>
<td>24.5 (3.2)</td>
<td>25.2 (3.1)†</td>
<td>24.7 (3.1)</td>
<td>24.6 (3.5)†</td>
</tr>
<tr>
<td>SBP, mm Hg</td>
<td>128.6 (17.3)</td>
<td>147.0 (22.5)†</td>
<td>147.0 (23.0)†</td>
<td>142.6 (20.1)†</td>
<td>151.7 (23.2)†</td>
<td>125.8 (14.9)</td>
<td>124.5 (17.7)</td>
<td>132.2 (16.2)</td>
<td>143.7 (16.6)†</td>
</tr>
<tr>
<td>DBP, mm Hg</td>
<td>79.4 (9.7)</td>
<td>87.9 (12.9)†</td>
<td>86.7 (12.9)†</td>
<td>85.7 (11.7)†</td>
<td>92.0 (13.3)†</td>
<td>78.9 (9.3)</td>
<td>76.5 (10.7)</td>
<td>82.0 (9.7)</td>
<td>84.3 (24.1)‡</td>
</tr>
<tr>
<td>HDL-C, mmol/L</td>
<td>1.05 (0.28)</td>
<td>0.91 (0.26)‡</td>
<td>0.90 (0.27)‡</td>
<td>0.93 (0.27)‡</td>
<td>0.89 (0.32)‡</td>
<td>1.05 (0.29)</td>
<td>1.08 (0.26)‡</td>
<td>1.03 (0.27)</td>
<td>1.19 (0.35)‡</td>
</tr>
<tr>
<td>Non-HDL-C, mmol/L</td>
<td>2.94 (0.97)</td>
<td>2.87 (0.92)‡</td>
<td>3.06 (0.96)‡</td>
<td>2.92 (0.95)‡</td>
<td>2.87 (0.96)‡</td>
<td>2.89 (0.95)</td>
<td>3.16 (1.07)‡</td>
<td>3.10 (0.91)</td>
<td>2.49 (0.84)‡</td>
</tr>
<tr>
<td>TC, mmol/L</td>
<td>4.97 (1.00)</td>
<td>4.74 (1.02)‡</td>
<td>4.86 (1.04)‡</td>
<td>4.78 (0.99)‡</td>
<td>4.54 (0.99)‡</td>
<td>4.78 (0.92)</td>
<td>5.08 (1.11)†</td>
<td>4.96 (0.93)</td>
<td>4.69 (1.07)‡</td>
</tr>
<tr>
<td>TG, mmol/L</td>
<td>5.86 (1.74)</td>
<td>6.61 (2.64)†</td>
<td>6.76 (2.81)†</td>
<td>6.39 (2.60)†</td>
<td>6.58 (2.37)†</td>
<td>5.70 (1.70)</td>
<td>5.77 (2.11)</td>
<td>5.95 (1.66)</td>
<td>5.90 (1.94)†</td>
</tr>
<tr>
<td>Glucose, mmol/L</td>
<td>5.00 (1.2)</td>
<td>5.38 (1.5)</td>
<td>5.24 (1.6)</td>
<td>5.58 (1.9)</td>
<td>5.44 (1.6)</td>
<td>5.38 (1.5)</td>
<td>5.34 (1.3)</td>
<td>5.36 (1.4)</td>
<td>5.42 (1.5)‡</td>
</tr>
<tr>
<td>Cigarette smoking, %</td>
<td>37.2</td>
<td>48.7†</td>
<td>50.9†</td>
<td>44.9†</td>
<td>48.5†</td>
<td>50.0</td>
<td>56.9†</td>
<td>52.5</td>
<td>46.6</td>
</tr>
<tr>
<td>Alcohol intake, %</td>
<td>31.0</td>
<td>37.8†</td>
<td>37.9†</td>
<td>34.3†</td>
<td>41.2†</td>
<td>42.6</td>
<td>35.8†</td>
<td>35.1</td>
<td>20.9†</td>
</tr>
<tr>
<td>Hypertension history, %</td>
<td>26.5</td>
<td>63.2</td>
<td>63.8</td>
<td>59.8</td>
<td>65.3</td>
<td>25.8</td>
<td>44.4</td>
<td>60.0</td>
<td>59.3</td>
</tr>
<tr>
<td>DM history, %</td>
<td>5.2</td>
<td>12.4</td>
<td>16.6</td>
<td>12.3</td>
<td>5.9</td>
<td>5.7</td>
<td>15.3</td>
<td>4.9</td>
<td>2.5</td>
</tr>
</tbody>
</table>

BMI indicates body mass index. Clinical characteristics of age, BMI, SBP, DBP, glucose, HDL-C, non-HDL-C, and TC values are given as mean (SD); TG values as median (range); and other values as number of individuals (%).

**TABLE 2. Association of SNP +2255 in VKORC1 Gene With Stroke**

<table>
<thead>
<tr>
<th>Genotype, n (%)</th>
<th>Controls (n=1811)</th>
<th>Total (n=1811)</th>
<th>Thrombosis (n=798)</th>
<th>Lacunar Stroke (n=514)</th>
<th>Hemorrhage (n=499)</th>
<th>Cases (n=740)</th>
<th>CHD (n=740)</th>
<th>Aortic Dissection (n=416)</th>
<th>Cases (n=253)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude OR (95% CI)</td>
<td>11.7</td>
<td>1.00</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT+CC</td>
<td>1.85 (1.54–2.23)†</td>
<td>1.95 (1.58–2.41)†</td>
<td>1.70 (1.35–2.14)†</td>
<td>1.75 (1.34–2.29)†</td>
<td>1.68 (1.29–2.20)†</td>
<td>1.53 (1.09–2.16)*</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ORs and 95% CIs were calculated with the use of multivariate logistic regression analyses. Adjusted ORs were stratified by age, sex, body mass index, blood pressure, cigarette smoking, alcohol consumption, glucose, HDL-C, non-HDL-C, TC, and TG.

G-C-G-C-A haplotype of \( VKORC1 \) is also associated with a high risk of stroke, CHD, and aortic dissection.

**Discussion**

The present study is the first clinical investigation of the significance of the polymorphisms of \( VKORC1 \) in vascular disease, including stroke, CHD, and aortic dissection. We found that the variants of \( VKORC1 \) are associated with almost double the risk of stroke, CHD, and aortic dissection. Next, we examined whether the disease-associated allele was related to specific vascular risk factors, such as hypertension, diabetes, age, and sex. No significant association was observed between the +2255 allele and these risk factors, which suggests that the contribution of SNP +2255 to the risk of vascular disease is independent of those conventional vascular risk factors. We examined the population stratification effect by genotyping 7 unlinked highly polymorphic microsatellite markers in the case subjects and in control subjects, and no association was found between these markers and vascular disease.

Stroke, CHD, and aortic dissection are all vascular diseases. The pathologies of these diseases are quite different. Atherothrombotic stroke mainly results from large-artery atherosclerosis, intracerebral hemorrhages may be due to...
microaneurysms in intracerebral arteries (whether this is the main cause is still in debate), and lacunar infarction is usually caused by lipohyalinosis or microatheromata with thrombosis of the vascular lumen (≈30 to 100 μm). Although stroke results from a number of different pathological processes, predisposing factors for each stroke subtype are surprisingly similar, such as increased age, hypertension, smoking, and DM. These are risk factors for CHD and aortic dissection as well. Some of the same factors are manifested in Marfan syndrome, which is caused primarily by mutations in the FBN1 gene. However, other factors are clearly important in the expression of Marfan syndrome; relatives who share the same mutations show dramatically different phenotypes. Therefore, all mechanisms that weaken the aorta’s medial layers will contribute to higher aortic wall stress. This can induce aortic dilation and aneurysm formation and eventually cause intramural hemorrhage, aortic dissection, or rupture.

VKORC1 variation could serve as a common genetic risk factor for all vascular diseases. It involves mediation of γ-carboxylation of hemostatic and nonhemostatic proteins by control of the vitamin K cycle. Polymorphisms of VKORC1 have been shown to affect the expression and activity of VKORC1 and thus warfarin dose response and blood clotting. Vitamin K–dependent nonhemostatic proteins mediate calcification. Vessel calcification decreases vessel elasticity and increases shear stress and is associated with the increased morbidity and mortality of arterial disease.

D’Andrea et al recently found that patients with the TT genotype of SNP rs9934438 require a lower dose of warfarin, which indicates a lower activity of the coagulation system in these patients, due at least in part to a lower activity of VKORC1. This leads to less conversion of vitamin K epoxide back to its reduced form, which results in less carboxylation of vitamin K–dependent proteins. The T allele at rs9934438 is on the same haplotype as the T allele of SNP 2255.

Rieder et al reported that mRNA levels in the group with the G-C-G-C-A (3673-6484-6853-7566-9041) haplotype were 3 times as high as those in the wild-type group. Yuan et al reported that the VKORC1 promoter with the G allele yielded a 44% increase of activity compared with the A allele of -1639. Consistent with these results, we found that subjects with the TT genotype of +2255 had higher levels of undercarboxylated vitamin K–dependent proteins, osteocalcin and PIVKA-II. We proposed that the VKORC1 haplotype G-C-G-C-A (3673-6484-6853-7566-9041) would increase the promoter activity and higher expression of VKORC1 mRNA and protein. This would result in increased γ-carboxylation, as indicated by the decreased levels of the undercarboxylated vitamin K–dependent proteins osteocalcin and PIVKA-II and a potential need for increased warfarin dosage.

The biological activity of all known vitamin K–dependent proteins is highly dependent on correct γ-carboxylation, which affects not only procoagulatory (factor II, VII, IX, and X) but also anticoagulatory (protein C, S, and Z) clotting factors and osteocalcin in the same way as MGP. The greater functional activity of the C allele may also lead to higher levels of MGP and less vascular calcification. Therefore, the observed association may not be related to the effect on the γ-carboxylation of the vitamin K–dependent proteins but may be caused by another yet-unknown function of VKORC1.

One possibility is that VKORC1 haplotypes are only markers for LD, and some genes in the linkage region independent of or in conjunction with VKORC1 confer susceptibility to arterial disease. At least 6 other genes are found in the natural haplotype block over the 68 000 bases around VKORC1. ZNF668 (zinc finger protein), ZNF646, BCKDK (branched chain ketoacid dehydrogenase kinase), MYST1 (histone acetyltransferase1), PRSS8 (protease serine

<table>
<thead>
<tr>
<th>Groups</th>
<th>Genotype, n (%)</th>
<th>Frequency, %</th>
<th>Crude OR (95% CI)</th>
<th>Adjusted OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (n=740)</td>
<td>CC 2 (0.3), CT 81 (10.9), TT 657 (88.8)</td>
<td>11.2</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Cases (n=740)</td>
<td>CC 5 (0.7), CT 120 (16.2), TT 615 (83.1)</td>
<td>16.9</td>
<td>1.61 (1.19–2.17)*</td>
<td>1.72 (1.24–2.38)*</td>
</tr>
</tbody>
</table>

ORs and 95% CIs were computed with the use of multivariate logistic regression analyses. Adjusted ORs were stratified by age, sex, body mass index, blood pressure, cigarette smoking, alcohol consumption, glucose, HDL-C, non-HDL-C, TC, and TG.

χ² Test vs control, *P<0.01.
Undercarboxylated osteocalcin and PIVKA-II antigen serum levels in carriers of different genotypes of the VKORC1 gene SNP +2255. Levels of the 2 proteins are presented as means; T bars represent SDs. PIVKA-II antigen serum levels were significantly correlated with SNP +2255 (P<0.001). Five samples in the CC group, 18 in the CT group, and 26 in the TT group were analyzed. ***P<0.001 for the comparison between genotype TT and CC groups.

8), and PRSS36. No evidence through a search of the PubMed database shows that these genes have any definite biological function. Previously, we found that VKORC1 is involved in angiogenesis\(^{10}\); others have shown that polymorphisms of VKORC1 are associated with differences in dose requirements in warfarin sensitivity among patients of different ancestries. In the present report, lower levels of undercarboxylated osteocalcin and PIVKA-II antigen were associated with the C-allele of SNP +2255 of the VKORC1, which supports the idea that the association between the haplotype of the VKORC1 block and vascular disease is attributable to VKORC1.

Furthermore, vascular calcification is more complex in humans than in rodents. In women, progression of atherosclerotic calcification is associated with increased bone loss during menopause.\(^4^1\) In patients with mutations in the MGP gene, arterial calcification is not a common feature.\(^4^2\) In severely atherosclerotic patients, circulating MGP is significantly increased, as is osteocalcin in women with aortic atherosclerosis.\(^4^3\) These have been proposed as feedback mechanisms to attempt calcium clearance. Thus far, the pathophysiology behind this exciting finding is not clear and needs to be addressed by further studies.

In conclusion, the prevalence of the haplotype G-C-G-C-A of VKORC1 was significantly more frequent in patients with vascular disease than in controls. The haplotype may serve as a novel genetic marker for the risk of stroke, CHD, and aortic dissection.

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Disclosures
None.

References
**CLINICAL PERSPECTIVE**

Epidemiological data indicate that coronary atherosclerosis is an important cause of morbidity and mortality worldwide. To minimize the devastating consequence of vascular disease, we must reliably distinguish those individuals who will experience an event from those who will not. The haplotypes in VKORC1 affect warfarin dose response through effects on the formation of the reduced form of vitamin K, a cofactor for γ-carboxylation of vitamin K–dependent proteins. Vitamin K–dependent proteins play a critical role in the coagulation cascade and may potentially impact atherosclerosis. We tested the association of the single-nucleotide polymorphisms of the VKORC1 gene and arterial vascular diseases including coronary heart disease, stroke, and aortic dissection. We observed that VKORC1 variation is significantly associated with each of these diseases. Although the pathophysiology behind the present findings is not entirely clear, confirmation of these observations in additional studies is warranted. Additional research is necessary to elucidate the potential role of VKORC1 and its downstream products in the pathogenesis of vascular disease.
VKORC1 Haplotypes Are Associated With Arterial Vascular Diseases (Stroke, Coronary Heart Disease, and Aortic Dissection)
Yibo Wang, Weili Zhang, Yuhui Zhang, Yuejin Yang, Lizhong Sun, Shengshou Hu, Jilin Chen, Channa Zhang, Yi Zheng, Yisong Zhen, Kai Sun, Chunyan Fu, Tao Yang, Jianwei Wang, Jing Sun, Haiying Wu, Wayne C. Glasgow and Rutai Hui

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