**Protein C Levels Are Regulated by a Quantitative Trait Locus on Chromosome 16**

**Results from the Genetic Analysis of Idiopathic Thrombophilia (GAIT) Project**

Alfonso Buil, José Manuel Soria, Juan Carlos Souto, Laura Almasy, Mark Lathrop, John Blangero, Jordi Fontcuberta

**Objective**—Protein C (PC) is a component of the protein C anticoagulant pathway. PC deficiency is a risk factor associated with venous thromboembolism. As part of the Genetic Analysis of Idiopathic Thrombophilia (GAIT) Project, we conducted a genome-wide linkage scan to localize genes that influence variation in PC plasma levels.

**Methods and Results**—PC levels were measured in 398 individuals belonging to 21 Spanish families. A total of 485 DNA microsatellite markers were genotyped to provide a 7.1-cM genetic map. Variance component linkage methods were used to evaluate linkage and to detect quantitative trait loci (QTL). A region on chromosome 16 (16q23), flanked by markers D16S3106 and D16S516, showed strong evidence of linkage with PC levels (LOD = 3.69). This region contains 1 positional candidate gene, the NAD(P)H:dehydrogenase quinone 1 (NQO1), involved in vitamin K metabolism. The association of 1 SNP of this gene with PC levels (P = 0.005) strongly supports the implication of NQO1 gene in the variability of PC levels.

**Conclusions**—These results illustrate the application of genomic scans to identify the genetic determinants of quantitative variation in a component of the hemostatic pathways. They provide strong evidence for a locus (QTL) on chromosome 16 that influences PC levels. (Arterioscler Thromb Vasc Biol. 2004;24:1-5.)

**Key Words:** protein C ■ linkage analysis ■ variance components ■ NQO1 gene ■ quantitative trait locus

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Protein C (PC) is a vitamin K-dependent protein that, after activation by the thrombin–thrombomodulin complex, produces an anticoagulant effect by inactivating activated factor V (FVa) and activated factor VIII (FVIIIa). Thus, PC is a major component of the PC anticoagulant pathway that is critical for the prevention of inadequate thrombus formation. Individuals with abnormalities in any of the components in this pathway, such as PC or protein S (PS) deficiencies, exhibit a dramatically increased incidence of thromboembolic disorders.

The PC structural gene (PROC) is located on chromosome 2. Although mutations have been identified in this gene that cause profound PC deficiencies associated with familial thrombophilia, mutations in the PROC gene are not detectable in 10% to 30% of families with PC deficiency. In addition, little is known about the determinants of normal variation in PC levels. Several polymorphisms in the promoter of the PROC gene have been identified that account only for only about 6% of the variability of PC levels. Therefore, the majority of the genetic components are unknown.

As part of the Genetic Analysis of Idiopathic Thrombophilia (GAIT) Project, we estimated that the additive genetic heritability of PC levels was 0.50, indicating that approximately half of the phenotypic variation in this trait is caused by the additive effects of genes. Following-up on these results, we conducted a genome-wide linkage scan of the GAIT sample to localize genes influencing variation in PC plasma levels. The identification of genes affecting quantitative risk factors is the first step in our attempt to elucidate the genetic mechanisms influencing risk of common thrombosis. Ultimately, we hope that our results will suggest preventive strategies to reduce thrombosis-related morbidity and mortality.

**Methods**

The GAIT Project has been described extensively in a previous publication. The GAIT sample consists of 398 individuals in 21 extended Spanish pedigrees composed of 3 to 5 generations. The subjects ranged in age from younger than 1 year to 88 years, with a mean of 37.7 years, with approximately equal numbers of males (46%) and females (54%). Twelve families were selected through a
proband with idiopathic thrombophilia; the remaining 9 families were randomly selected without regard to phenotype. Thrombophilia was defined as early-onset thrombosis or recurrent episodes of thrombosis, at least 1 of which was spontaneous. A proband’s thrombophilia was considered idiopathic because all known biological causes of thrombosis at the time of recruitment (1995) were excluded (ie, PS and PC deficiencies and activated PC resistance).

All procedures were approved by the Institutional Review Board of the Hospital de la Santa Creu i Sant Pau (Barcelona). Adult subjects gave informed consent for themselves and for their children.

Blood was collected in 1/10 volume of 0.129 mol/L sodium citrate from fasting subjects. Thrombophilic participants were not using oral anticoagulants at the time of sampling. Plasma samples were stored at −80°C before assaying for PC levels (in duplicate) as previously described. Normal ranges for PC were obtained from the analysis of 100 healthy local blood donors (mean ± 2SD) and were 70% to 130%. Potential probands were excluded on the basis of PC deficiency when the plasma level of PC was below the lower limit of the normal range in at least 2 different samples. We also analyzed the other vitamin K-dependent proteins.

The subjects were genotyped for 485 microsatellite markers distributed through the autosomal genome. DNA was extracted using standard protocols. Microsatellites consisted primarily of the ABI-Prism genotyping set MD-10. Linkage mapping was undertaken with the PE LMS II fluorescent marker set with multiplex polymerase chain reaction as described; in a few instances, nearby Genethon markers were substituted for LMS II markers to improve robustness (http://www.cng.fr/). The polymerase chain reaction products were analyzed on PE 310, PE 377, and PE 3700 automated sequencers, and genotyped using the PE Genotype software. The average heterozygosity of the microsatellite markers was 0.79, and the average interval between markers was 7.1 cM. Markers in or near several hemostasis-related candidate genes were used to augment this genome scan.

The genotypic data were entered into a database and were analyzed for discrepancies (ie, violations of Mendelian inheritance) using the PEDSYS program INFER. Discrepancies were checked in the laboratory for mistyping, and markers for discrepant individuals were either corrected or excluded from the analysis. Allelic frequencies were estimated from the GAIT sample using maximum likelihood methods. Marker maps for multipoint analyses were obtained from ABI-Prism (http://www.appliedbiosystems.com/molecularbiology/) and from the Marshfield Medical Research Organization (http://research.marshfieldclinic.org/genetics/).

We genotyped 3 SNPs in the NQO1 gene using the SNP Genotyping Assays-on-Demand from Applied Biosystems, following the recommendation of the provider. Information on SNPs can be found in the public accessible myScience Applied Biosystems Database (http://myscience.appliedbiosystems.com) with the reference numbers hCV1035499, hCV16180947, and hCV2091258. Table 1 contains information about these SNPs.

Table 1. SNPs in the NQO1 Gene: Information and Association (P) With the Protein C Levels

<table>
<thead>
<tr>
<th>SNPs</th>
<th>Location in the Gene</th>
<th>Nucleotide Change</th>
<th>ID Celera Database</th>
<th>Position (bp)</th>
<th>Allelic Frequencies</th>
<th>Association (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Promoter</td>
<td>C/G</td>
<td>HCV16180947</td>
<td>69,473,262</td>
<td>0.12/0.88</td>
<td>0.93</td>
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<tr>
<td>2</td>
<td>Intronic</td>
<td>A/G</td>
<td>HCV2091258</td>
<td>69,493,244</td>
<td>0.22/0.78</td>
<td>0.005*</td>
</tr>
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<td>3</td>
<td>Intronic</td>
<td>A/G</td>
<td>HCV1035499</td>
<td>69,496,979</td>
<td>0.15/0.85</td>
<td>0.52</td>
</tr>
</tbody>
</table>

*Significant at level α=0.05.

The position is related to the assembly 33 of the NCBI public database.

Results

The range of PC values in the GAIT sample was 37% to 198%. Using the specific PC thresholds, no individual with thrombosis in the GAIT sample was PC-deficient. However, 4 healthy individuals had levels of PC <70%. Two of them belonged to families selected through a proband with thrombosis and the other 2 to families randomly selected. In the GAIT sample, the mean age- and sex-correlated PC plasma level was 118.3%, measured relative to a normal pooled sample. Levels of PC increased significantly with age in both sexes (5.3% every 10 years) and females exhibited levels slightly greater than males. Age and sex accounted for 22.5% of the variation in PC levels.

Several studies have established a clear association between levels of vitamin K-dependent proteins and lipid metabolism. For this reason, in addition to sex and age, high-density lipoprotein (HDL) and triglycerides have been used as covariates in all of the analyses. Their effects were estimated simultaneously with the genetic effects. When HDL and triglycerides were included as covariates in the model, the amount of variance accounted for the covariates increased to 29%. We have performed the linkage analysis with 2 models. First including as covariates only sex and age, and second adding HDL and triglycerides. With this expanded model, the maximum LOD score increased from 2.41 to 3.69.

The results of the genome scan for QTLs influencing PC levels with the model including HDL and triglycerides as covariates are shown in Figure 1. Three areas showed...
suggestive or significant evidence of linkage. A LOD score of 1.68 (nominal $P=0.0027$) was observed on chromosome 9q and a LOD of 2.27 (nominal $P=0.0006$) on chromosome 10q. Such LOD scores would be expected to occur by chance approximately once every genome scan and must be considered only suggestive. In addition to these 2 linkage signals, the highest LOD score observed in the genome screen was 3.69 (nominal $P=0.00002$, genome-wide $P=0.009$) on chromosome 16q. Adjusting the evidence for linkage at this peak for nonnormality in the underlying distribution yields LOD$_R=3.28$ (nominal $P=0.00005$, genome-wide $P=0.024$).

The genome-wide $P$ value is a measure of the global test accounting for multiple testing. It is calculated following the method described by Feingold et al.18

The peak LOD score occurred in the interval flanked by markers D16S3106 and D16S516 in a region that maps to 16q22–16q23 (Figure 2). This is strong evidence for linkage and suggests that a gene in this region influences plasma levels of PC. The 1-unit support interval surrounding this peak ranges on chromosomal location from 88 to 100 cM from the p terminus. In this region, the gene NAD(P)H:menadione oxidoreductase 1 (NQO1), which codes for a quinone reductase involved in the metabolism of vitamin K, has been mapped. Because vitamin K is essential for the synthesis of PC, NQO1 gene becomes a plausible candidate gene to explain the linkage signal in 16q. We have genotyped 3 polymorphisms located across this gene in the GAIT sample and have performed association analysis with PC levels. One of the SNPs (hCV2091258) revealed significant association with PC levels (Table 1), supporting the presence of a QTL in the region of the NQO1 gene.

In addition, we performed a combined linkage and association analysis to evaluate whether the effect of the SNP hCV2091258 explains the linkage signal on 16q. The LOD score of the model including this SNP as a covariate is 3.29, only 0.4 U lower than the score obtained without the covariate.

We also performed a linkage analysis on 16q of another 5 vitamin K-related proteins measured in the GAIT Project, and association analysis between the 3 SNPs and these proteins. Table 2 shows these results.

**LOD scores in the region of the PC structural locus on chromosome 2q were <1, providing little support for a PROC QTL influencing normal variation in PC levels.**

**Discussion**

PC plays a central role in the delicate balance between coagulant and anticoagulant forces, and deficiency of PC is a well-known cause of inherited venous thrombophilia.7 Genetic mutations in the PROC structural gene explain many
cases of PC deficiency, but the genetic components determining normal variation in PC levels are largely unknown. In our sample, variation at the PROC structural locus is not a primary determinant of the observed quantitative variation on PC levels. Instead, the results of our genome scan suggest that the major QTL influencing variation in PC levels is located near the 16q23 chromosomal region.

We performed the linkage analysis under 2 models. First we included only sex and age as covariates, and second we added HDL and triglycerides, 2 phenotypes that affect the levels of PC.20–21 With this expanded model, the maximum LOD score increased from 2.41 to 3.69. This is an example of how the incorporation of covariates may improve the genetic signal-to-noise ratio and refine the phenotype so that there is a higher probability of finding a QTL.

Significantly, we found a positional candidate gene in the linkage signal region at position 16q22.1, the NAD(P)H:menadione oxidoreductase 1 (NQO1) gene, which codes for a quinone reductase involved in the metabolism of vitamin K.22 Vitamin K is required for the adequate synthesis of PC. Reduced vitamin K is a cofactor of the enzyme γ-glutamyl carboxylase, an integral membrane microsomal protein located in the rough endoplasmic reticulum, which carboxylates glutamate residues located in the Gla domain of the vitamin K-dependent proteins, like PC.23 These carboxylated amino acids are critical for calcium-ion binding and are necessary for the interaction of PC with the cell membranes.24 The carboxylation reaction is dependent on reduced vitamin K, which is converted to vitamin K-epoxide, and must be regenerated by vitamin K-epoxide reductase and vitamin K-quinone reductase (NQO1) in consecutive reactions for carboxylation to continue.23,25

We looked for more evidence in favor of the NQO1 gene as a good candidate to explain the linkage signal in 16q. So, we evaluated the possibility of typing additional microsatellites in this region to restrict the region and localize the peaks better. However, once a linkage signal is obtained as high as LOD=3.69 (defining an interval of 10 to 15 cM), the fine mapping of this area would add little information.26 Thus, we considered it more appropriate to exploit the linkage disequilibrium by association analysis. Among the 3 SNPs that we have analyzed, 1 showed significant association with PC levels (Table 1), supporting the fact that the NQO1 locus has an effect on PC levels variability. Although we do not have information about the functional role of this SNP, the most likely possibility is that it is in linkage disequilibrium with another putative functional polymorphisms of the NQO1 gene. Other evidence in that respect is the fact that the combined linkage and association analysis showed that this SNP only explains a small part of the linkage on chromosome 16q. In any case, we wish to emphasize that we are not looking for functional SNPs. We are just looking for association between genetic variants in this gene and PC levels to support the hypothesis that there is a relation between the NQO1 gene and PC levels.

Given that this gene encodes for a component of the vitamin K pathway, we postulated that other vitamin K-dependent phenotypes measured in the GAIT Project would show evidence of linkage in this region. Souto et al27 reported that plasma levels of the vitamin K-dependent phenotypes were highly genetically correlated. This implies that they are influenced co-jointly by a set of common genes, such as NQO1. Table 2 shows the linkage analyses between the region of the 16q23 markers and the remaining vitamin K-dependent phenotypes measured in the GAIT Project, adjusted by sex, age, and lipids. The estimated LOD scores, although weak, were not negligible. Moreover, Table 2 shows the association of these vitamin K-dependent proteins with the 3 SNPs typed on the NQO1 gene. Interestingly, 3 of the 5 proteins show significant association with some of the SNPs. All these results support our hypothesis that the NQO1 gene is a candidate gene to explain the phenotypic variability of PC plasma levels.

It is interesting to note that when we analyzed the families recruited through a proband with thrombosis and the families recruited through a random proband separately, we observed a difference of the LOD score between the thrombophilic and the random families. The thrombophilic families were responsible for almost 100% of the linkage signal on chromosome 16q. This could be chance, but it is more likely that whatever the functional variant is, it is rare in the general population but more prevalent in the thrombophilic families. So, the families selected through a proband with thrombosis would be enriched for this PC variant. This implies that there is a connection between this unknown variant and thrombosis. However, to confirm this point would require more experiments and the collection of a new sample.

We believe that our study is a good example of why one should not limit the search for genetic variation in a phenotype to known candidate genes only (such as the structural

<table>
<thead>
<tr>
<th>Protein</th>
<th>LOD Score</th>
<th>P</th>
<th>HCV16180947</th>
<th>HCV2091258</th>
<th>HCV1035499</th>
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<td>FII</td>
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<tr>
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<td>0.43</td>
<td>0.080†</td>
<td>0.04*</td>
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<tr>
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<td>0.272</td>
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<td>0.004*</td>
<td>0.17</td>
<td>0.87</td>
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</tr>
</tbody>
</table>

*Significant at level 0.05.
†Significant at level 0.05.© Significance at level 0.1.
gene that encodes PC). The advantage of the full genome scan is that it searches the entire autosomal genome and consequently it is able to find new causative genes.

In summary, our results demonstrate that a QTL on chromosome 16 is the major genetic factor affecting the variability of normal PC plasma levels and that the NQO1 gene is a very good candidate to explain the effect of this QTL.

Acknowledgments

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References

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