Venous and arterial thrombosis : associations and risk factors

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NQO1: Candidate gene in a quantitative trait locus affecting factor V and prothrombin levels

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Abstract

Background

Earlier genome-wide linkage analyses of coagulation factor levels in a large Dutch pedigree (GENES Family 8) yielded suggestive linkage signals on chromosome (chr) 16 for Factor (F) V (LOD score: 3.9 at 110 cM) and FII levels (LOD score: 3.4 at 97 cM).

Objectives

NQO1, which encodes an enzyme involved in vitamin K metabolism, is a candidate gene in the LOD-1 region of the linkage signals on chr16 and therefore we evaluated the influence of five *NQO1* haplotypes on coagulation factor levels (i.e. protein C, protein S, FII, FV, FVII, FVIII and FIX) in the pedigree and in controls of a population-based case-control study on venous thrombosis, the Leiden Thrombophilia Study (LETS). We also assessed the risk of venous thrombosis for each haplotype in the LETS.

Results

NQO1 haplotype 4 (H4) carriers in Family 8 had lower FV levels than non-carriers while H4 had no effect on FV levels in the LETS controls. Each H4 copy was associated with 11.8 U/dl decrease in FV level (95% CI: -21.4, -2.3) in the family. In the LETS controls, H4 was associated with lower levels of vitamin K-dependent coagulation factors. The strongest association was observed with the levels of FII (-2.6; 95% CI: -5.2, -0.1) and total protein S (-4.3; 95% CI: -7.6, -1.0). We did not observe a similar pattern in the Family 8. NQO1 haplotypes do not influence the risk of thrombosis in the LETS.

Conclusions

Haplotype 4 carriers of *NQO1* have lower levels of vitamin K-dependent coagulation factors especially lower FII and total protein S. None of the haplotypes affect the risk of venous thrombosis.

Introduction

The levels of many coagulation factors are in part genetically determined [1]. However, the genes influencing the levels are not all known.

Previously, we conducted genome-wide linkage analyses of coagulation factor levels and of the outcome of global coagulation assays in a large Dutch pedigree (Family 8) from the GENES study [2]. Four statistically significant linkage signals were observed: on chromosome (chr) 20 for protein C levels (LOD score: 4.8), on chr 17 for prothrombin time (LOD score: 3.8) and on chr 16 for factor (F) V (LOD score: 3.9) and FII (LOD score: 3.4) levels. The protein C linkage signal and the signal on chr 17 will be presented elsewhere (in preparation). In the current study, we set out to explore explanations for the positive linkage signal for factor V and prothrombin (FII) on chr 16 (figure 1).

Candidate genes were searched in the LOD-1 region using Biomart (www.biomart.org). Only one gene, *NQO1,* was identified with a plausible role in blood coagulation factor synthesis. NQO1 is a cytosolic enzyme, which is expressed in the liver and converts vitamin K to hydroquinone vitamin K, the co-enzyme of gamma-glutamyl carboxylase (GGCX) (figure 2). Gamma-carboxyl modification enables proteins such as blood coagulation factors (i.e. protein C, S and Z, FII, FVII, FIX and FX), proteins involved in calcium homeostasis (osteocalcin and matrix Gla protein), cell growth (Gas6) and signal transduction (RPGP1 and RPGP2) to bind to calcium and thereby to be physiologically active [3]. Although factor V is not itself carboxylated, one can not exclude an indirect influence of NQO1. The precedent for this, is protein S that influences the level of tissue factor pathway inhibitor and the level of C4BP [4;5].

In the present study we investigated the association between haplotypes of *NQO1* and the levels of FV and FII and various other factors in GENES Family 8. To verify our findings we performed similar analyses in the control population of the Leiden Thrombophilia Study (LETS), a population based case-control study on venous thrombosis. Furthermore, we evaluated the risk of venous thrombosis for each *NQO1* haplotype in the LETS.

Figure 1: linkage results for FV and FII on chromosome 16

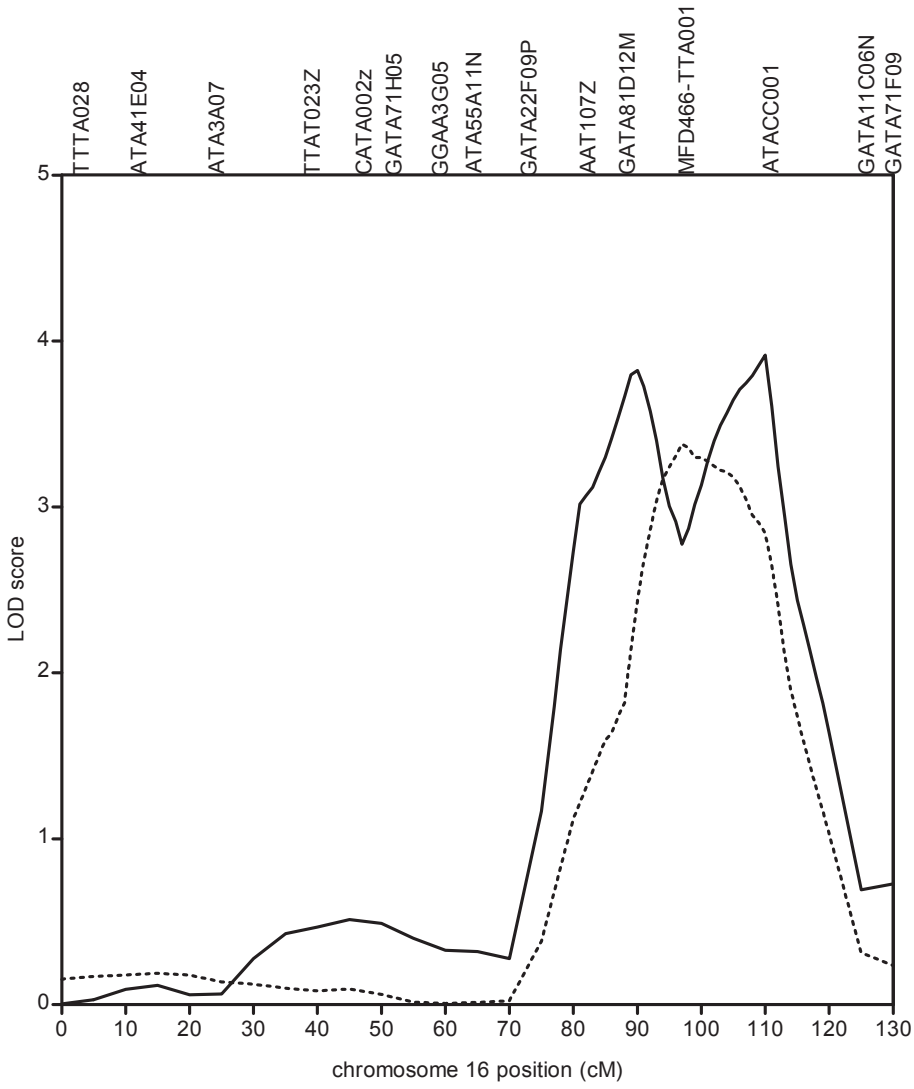
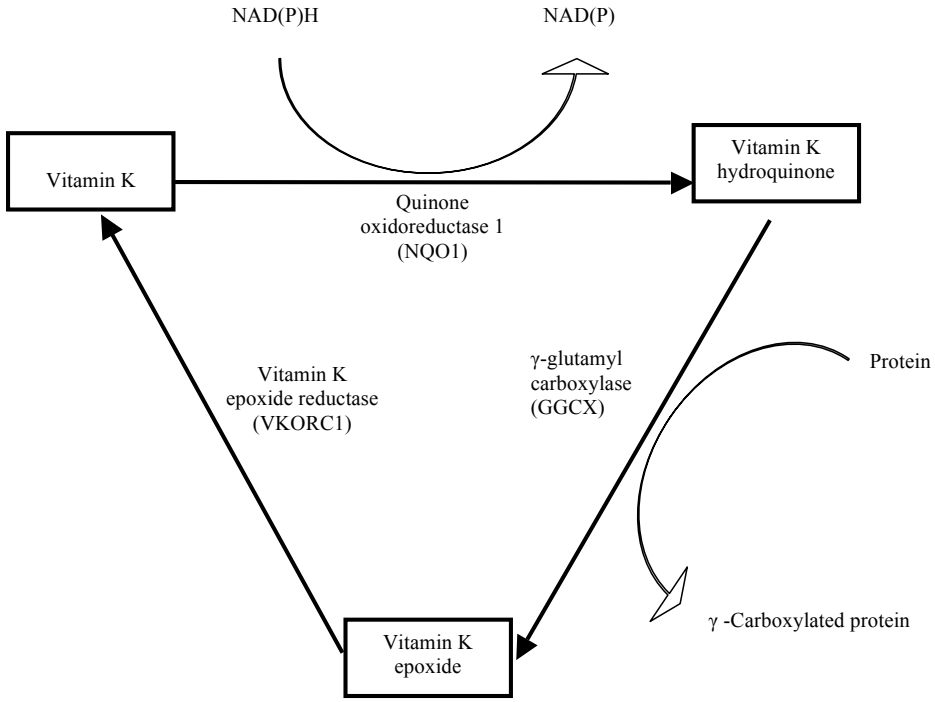


Figure 2: vitamin K cycle: NQO1 converts vitamin K to hydroquinone vitamin K, the co-enzyme of gamma-glutamyl carboxylase (GGCX). After gamma carboxylation vitamin K transforms to vitamin K epoxide which then by vitamin K epoxide reductase complex subunit 1 (VKORC1), the rate limiting enzyme in the vitamin K cycle, is reduced to vitamin K



Subjects and methods

Family 8 and the GENES study

Family 8 is one of the thrombophilic families included in a larger study called the GENES study. GENES is described in a previous publication [2]. In short, 43 families which were ascertained through probands with venous thromboembolism (VTE) and a strong family history of VTE participated in this study. Family history of VTE was defined as at least one first degree or two second degree family members affected by VTE. As the purpose of GENES was to identify new genetic risk factors for VTE, none of the probands should have any of the known thrombophilic defects: factor V Leiden (FVL), prothrombin G20210A or deficiencies of antithrombin, protein C and protein S. The diagnosis of VTE was established based on a standardized questionnaire or on documented medical records. Blood and DNA samples were available for the participants of GENES. The GENES study was approved by the Central Committee on Research Involving Human Subjects (CCMO) and all subjects provided an informed consent.

Leiden Thrombophilia Study

Details of the Leiden Thrombophilia Study (LETS) have been published previously [6]. Briefly, 474 patients younger than 70 years of age with a first deep vein thrombosis were recruited from anticoagulation clinics in Leiden, Amsterdam and Rotterdam (the Netherlands) between January 1988 and December 1992. None of the participants had overt malignancy. As controls, partners or friends of the patients who did not have venous thrombosis were included. Levels of FII [7], FV [8], FVII [9], FIX [10], FVIII [11], protein C and protein S [12] have been determined before and were expressed in U/dl.

Linkage analysis in Family 8

Using the Sequential Oligogenic Linkage Analysis Routines (SOLAR) program probability of identity by descent and variance components linkage analysis in Family 8 members were previously performed for several intermediate phenotypes such as prothrombin F1+2, thrombin generation time, endogenous thrombin potential, clot lysis time, activated protein C sensitivity ratio, prothrombin time, activated partial thromboplastin time, activity of FII, FVII, FVIII, FIX, FXI and

antithrombin, and antigen concentrations of total and free protein S, protein C, FV and tissue factor pathway inhibitor [2]. Genotyping was conducted by the NHLBI Mammalian Genotyping Service at the Marshfield Medical Foundation (Marshfield, WI, USA, Weber and Broman, 2001) using the 10 cM spaced short tandem repeat polymorphism screening set 16 [2]. In the current study, linkage analyses for FV and FII levels were performed adjusted for *NQO1* haplotypes to investigate whether these haplotypes influenced the linkage signals on chr 16.

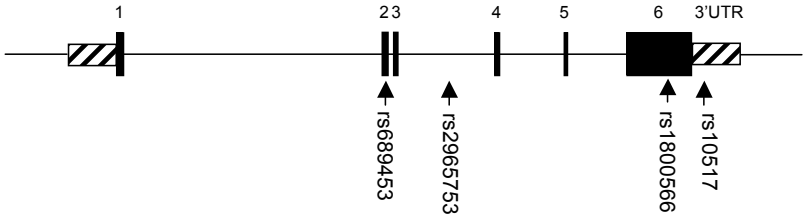
SNP genotyping in Family 8 and LETS

We searched for haplotype tagging (ht) SNPs in *NQO1* in the European Hapmap population (CEU) using the Genome Variation Server (http://gvs.gs.washington.edu/GVS). A htSNP is a polymorphism whose minor allele is specific to one haplotype (H). Four htSNPs (rs689453, rs10517, rs1800566 and rs2965753) were identified, which together tag five haplotypes (figure 3). We used TaqMan® SNP genotyping assays to determine the htSNPs. Fluorescent allele-specific oligonucleotide probes (Applied Biosystems, CA, USA) were used for PCR amplification and fluorescence endpoint reading for allelic discrimination was done on an ABI 7900 HT (Applied Biosystems).

Statistical analysis

We used linear regression analysis in Family 8 as well as in the LETS control population to investigate the association between the haplotypes of *NQO1* and vitamin K-dependent (protein C and S, FII, FVII and FIX) and independent (FV and FVIII) coagulation factor levels. The regression coefficient (β) represents the mean difference of the levels related to each haplotype copy. Furthermore, to investigate whether carrying a certain haplotype increases the risk of deep venous thrombosis, we computed the odds ratios (OR) and 95% confidence intervals (CI), as an estimate of relative risk of thrombosis for subjects heterozygous or homozygous for each *NQO1* haplotype compared with non-carriers (reference group). The latter analyses were also performed in the LETS.

Figure 3: Five haplotypes of *NQO1* and their frequencies in Family 8 and controls of the Leiden Thrombophilia Study



	Frequency (%)					
	LETS	Family 8				
H1	9	8	A	A	C	C
H2	62	55	G	A	C	C
H3	2	1	G	A	C	T
H4	17	18	G	A	T	C
H5	11	18	G	G	C	T

Results

Family 8 characteristics

Family 8 comprises 218 individuals in 5 generations. Plasma and DNA samples are available for 161 members. The mean (range) age of the members is 46 (15-87) years and 80 (50%) of them are men. Four members experienced thrombosis of whom two had a recurrent event. The linkage signals for FV and FII levels were located at chromosomal regions 16q23 (location maximum LOD score: 110 cM; LOD-1 support interval: 98-114 cM) and 16q22 (location 97 cM; LOD-1 support interval: 89-113 cM), respectively (figure 1).

NQO1 haplotype tagging SNPs

Four htSNPs identified five haplotypes of *NQO1* (figure 3). Haplotypes could not be assigned to three members of Family 8 and 14 controls and 15 patients of the LETS because of missing genotypes due to technical failure in SNP genotyping. Haplotypes and their frequencies in Family 8 and in LETS controls are shown in figure 3. Haplotype 5 (H5) was tagged by two SNPs and H2 consisted of the common alleles of all htSNPs.

Association of NQO1 haplotypes with coagulation factor levels in Family 8 and LETS controls

The regression coefficients (β) for the association of each *NQO1* haplotype with coagulation factor levels are summarized in table 1. Members of Family 8 who inherited H4 had lower FV levels than H4 non-carriers while H4 had no effect on FV levels in the LETS controls. Each H4 copy was associated with 11.8 U/dl decrease in FV level (95% CI: -21.4, -2.3). No other haplotypes showed significant association with FV level, neither in Family 8 nor in LETS controls. In the LETS controls, H4 was consistently associated with lower levels of vitamin K-dependent coagulation factors. The strongest association of H4 was with the levels of factor II (95% CI: -5.2, -0.1) and total protein S (95% CI: -7.6, -1.0). We did not observe similar pattern in the Family 8.

Association of NQO1 haplotypes with thrombosis risk

Table 2 shows the thrombosis risks for homozygous and heterozygous carriers of each *NQO1* haplotype as compared with non-carriers of that particular haplotype in the LETS. H1 carriers (homozygotes and heterozygotes) had a 30% lower risk of thrombosis (95% CI: 0.5-1.1) than non-carriers of H1. Contrarily, H4 carriers (homozygotes and heterozygotes) had a 1.3 times higher thrombosis risk than non-carriers (95% CI: 0.96-1.7). Other haplotypes of *NQO1* were not associated with thrombosis risk.

Linkage analysis with adjustment for NQO1 haplotypes

Adjusting the linkage analyses of FV and FII levels for *NQO1* haplotypes did not reduce the linkage signal on chr16 noticeably. The LOD score after adjustment was reduced mildly.

Table 1: Association of *NQO1* haplotypes with levels of coagulation factors in Family 8 and in controls of the Leiden Thrombophilia Study

NQO1 haplotype	FII		FVII		FIX		FV		FVIII		PC		tPS	
	LETS	Family	LETS	Family	LETS	Family	LETS	Family	LETS	Family	LETS	Family	LETS	Family
H1	0.5	-2.9	0.4	1.6	-4.3	-1.9	-1.8	1.8	-5.8	-0.8	-1.0	-0.0	1.1	-6.7
H2	0.7	2.7	2.2	-1.3	7.4	1.1	-1.2	2.1	-1.6	3.8	1.2	-0.02	1.9	1.9
H3	0.2	2.9	-3.9	18.7	29.8	8.5	-4.6	16.5	-28.4	13.6	-1.1	4.1	2.5	-12.7
H4	**-2.6**	-3.7	-2.6	2.1	-15.8	0.9	2.7	**-11.8**	6.1	-3.6	-0.7	4.4	**-4.3**	-1.1
H5	1.7	0.05	-1.1	-3.2	3.9	-2.8	1.0	4.5	2.1	-4.5	-0.7	-4.6	0.7	2.9

Regression coefficients β are shown. The direction of the regression coefficient represents the effect of each extra copy of the haplotype (i.e. a positive regression coefficient means that the haplotype increases phenotype mean).
The bold typed ciphers are statistically significant.

Table 2: The risk of thrombosis for *NQO1* haplotypes in the Leiden Thrombophilia Study

Haplotype	Patients (%) N=459	Controls (%) N=460	OR	95% CI
H1 (rs689453)				
HxHx	399 (87)	382 (83)	1*	
H1Hx	55 (12)	76 (16)	0.7	0.5-1.0
H1H1	5 (1)	2 (0.4)	2.4	0.5-12.4
H1Hx/ H1H1	60 (13)	78 (17)	0.7	0.5-1.1
Frequency H1	7	9		
H2 (all common)				
HxHx	61 (13)	68 (15)	1	
H2Hx	239 (52)	216 (47)	1.2	0.8-1.8
H2H2	159 (35)	176 (38)	1.0	0.7-1.5
H2Hx/ H2H2	398 (87)	392 (85)	1.1	0.8-1.6
Frequency H2	61	62		
H3 (rs10517)				
HxHx	447 (97)	447 (97)	1	
H3Hx	12 (3)	12 (3)	1.0	0.4-2.3
H3H3	-	1 (0.2)	0.9	0.4-2.0
H3Hx/ H3H3	12 (3)	13 (3)		
Frequency H3	1	2		
H4 (rs1800566)				
HxHx	293 (64)	318 (69)	1	
H4Hx	155 (34)	126 (27)	1.3	1.0-1.8
H4H4	11 (2)	16 (4)	0.7	0.3-1.6
H4Hx/ H4H4	166 (36)	142 (31)	1.3	1.0-1.7
Frequency H4	19	17		
H5 (rs10517 and rs2965753)				
HxHx	361 (79)	368 (80)	1	
H5Hx	89 (19)	84 (18)	1.1	0.8-1.5
H5H5	9 (2)	8 (2)	1.1	0.4-3.0
H5Hx/ H5H5	98 (21)	92 (20)	1.1	0.8-1.5
Frequency H5	12	11		

* Reference category; Hx: all haplotypes but the one given.

Discussion

We studied the effect of *NQO1* haplotypes on the levels of several coagulation factors in a large family and in a population based case-control study on venous thrombosis, the Leiden Thrombophilia Study. We observed that H4 carriers had lower levels of FV in the family but were unable to confirm this difference in LETS controls. In the LETS controls, H4 appears to be associated with lower levels of vitamin K-dependent coagulation factors. It is worth mentioning that coagulation factor levels can not easily be compared between Family 8 and LETS because assays were performed in different laboratories at different time points and sometimes other assays were used (e.g. for FV levels).

The effect of *NQO1* polymorphisms on the levels of coagulation factors is poorly studied and the results remain controversial. Rs1800566, which tags the haplotype 4, results in a proline to serine substitution. This amino acid change was reported to cause rapid degradation of the enzyme [13] and thereby lower or undetectable enzymatic activity in heterozygous and homozygous carriers respectively [14]. This postulates that H4 carriers have lower levels of vitamin K-dependent coagulation factors. Similar to our observation in the LETS, a significant correlation between rs1437135 in *NQO1* (a SNP in complete linkage disequilibrium with rs1800566) and the levels of protein C, protein S and FII but not FVII, FIX and FX is reported in a genome-wide linkage study of Spanish families (Genetic Analysis of Idiopathic Thrombophilia (GAIT)) [15]. However, no relation was evident between the levels of protein C and S and rs1800566 (the htSNP of H4) in a study in the Japanese population [16]. Unfortunately, the Spanish authors did not consider FV levels in their analysis, probably because vitamin K is not deemed essential for FV biosynthesis. In both genome-wide linkage studies (GAIT and Family 8), adjusting for *NQO1* polymorphisms did not attenuate the linkage signal suggesting the presence of other genetic variation on chr 16 that is responsible for the linkage peaks. Since identity by descent probabilities are already quite accurate in Family 8, the possibility to narrow down the linkage signal by fine mapping is limited. We assessed the risk of venous thrombosis in the LETS for each *NQO1* haplotype. Heterozygous carriers of haplotype 4 were at almost significantly 30% higher risk of venous thrombosis as compared with

non-carriers. However, as the effect was not dose-dependent we presume it would lose its significance by enlarging the sample size.

In conclusion, we observed that haplotype 4 carriers of *NQO1* gene have lower levels of vitamin K-dependent coagulation factors especially lower FII and total protein S, in the control population of the Leiden Thrombophilia Study. None of the haplotypes seem to affect the risk of venous thrombosis. One *NQO1* haplotype was associated with FV levels in Family 8. However, linkage analyses adjusted for *NQO1* haplotypes showed that variations in *NQO1* could not explain the linkage peaks on chromosome 16 for the levels of FV and FII.

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