A gene-centric analysis of activated partial thromboplastin time and activated protein C resistance using the HumanCVD focused genotyping array

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Abstract

Activated partial thromboplastin time (aPTT) is an important routine measure of intrinsic blood coagulation. Addition of activated protein C (APC) to the aPTT test to produce a ratio, provides one measure of APC resistance. The associations of some genetic mutations (eg factor V Leiden) with these measures are established, but associations of other genetic variations remain to be established. The objective of this work was to test for association between genetic variants and blood coagulation using a high density genotyping array. Genetic association with aPTT and APC resistance was analysed using a focused genotyping array that tests approximately 50,000 single nucleotide polymorphisms (SNPs) in nearly 2000 cardiovascular candidate genes, including coagulation pathway genes. Analyses were conducted on 2544 European origin women from the British Women's Heart and Health Study. We confirm associations with aPTT at the *F12*/*GRK6* and *KNG1*/*HRG* loci, and identify novel SNPs at the *ABO* locus and novel locus *KLKB1*/*F11*. In addition, we confirm association between APC resistance and factor V Leiden mutation, and identify novel SNP associations with APC resistance in the *HRG* and *F5*/*SLC19A2* regions. In conclusion, variation at several genetic loci influences intrinsic blood coagulation as measured by both aPTT and APC resistance.

Keywords

aPTT; coagulation; genotype; SNP

Introduction

Blood coagulation is an important process in preventing blood loss from damaged vessels, but can also be responsible for thrombosis leading to ischaemic heart disease, stroke or

venous thromboembolism[1]. An informative measure of efficacy of the intrinsic coagulation pathway is activated partial thromboplastin time (aPTT), measured as time taken for a clot to form in plasma in the absence of tissue factor following introduction of an activator (eg silica). An abnormally short aPTT can indicate a hypercoaguable state in acute coronary syndromes[2], and is associated with increased risk of venous thrombosis[3–5], whilst abnormally long aPTT may also indicate thrombotic risk in the case of the lupus anticoagulant[6]. Addition of activated protein C (APC), which deactivates factors Va and VIIIa, and calculation of an APC resistance provides one measure of APC resistance [7], including effects of factor V Leiden mutation as its major determinant[8,9]. There is evidence to suggest aPTT is highly heritable[10], thus meriting investigation of its genetic basis, but to date the only high-density genetic association study of aPTT was a recently reported genome-wide association study (GWAS) of aPTT in 1477 subjects from the Lothian Birth Cohorts, which identified three novel loci associated with aPTT, namely: coagulation factor XII (*F12*), kininogen 1 (*KNG1*), and histidine-rich glycoprotein (*HRG*)[11]. The IL3581Thr variant in KNG1 has since been found to associate with risk of venous thrombosis as well as aPTT[12]. No genome-wide association studies has been reported for APC resistance.

Materials and Methods

Subjects were from the British Women's Heart and Health Study (BWHHS), a prospective cohort study of heart disease in British women. Baseline recruitment was 1999-2001 (age 60-79 years), with blood samples for DNA, APC ratio and aPTT measurement taken from consenting individuals. Protocols and consents were approved by relevant research ethics committees[13]. aPTT measurements were available on 2962 women (mean age 68.8 years, SD 5.5), and APC resistance on 2953 women (mean age 68.8 years, SD 5.5). Data are not available where either insufficient blood was available to assay, consent was not given, or assays failed in the laboratory.

DNA was extracted from whole blood using a salting-out procedure[14]. Genotyping was successfully performed on 3445 of 3838 available samples using the Illumina HumanCVD Beadchip[15]. Principal components analysis was used to check self-reported ancestry, with 32 individuals excluded to avoid stratification issues, leaving 3413 samples for analysis. aPTT and APC resistance were assayed in an automated coagulometer (MDA-180, Organon Teknika, Cambridge, UK) using reagents and standards from the manufacturer as previously described[16]. APC ratio was assayed without factor V deficient plasma. Citrated plasma samples were stored at $-80°C$ for a maximum of 12 months prior to assay. Genotype and phenotype data were available on 2618 women for aPTT (mean age 68.9 years, SD 5.5) and 2610 women for APC resistance (mean age 68.9 years, SD 5.5).

Analysis of genetic association was performed using linear regression without covariables (adjustment for age had little effect; whilst clotting phenotypes are age dependent this cohort are all post-menopausal and within the relatively narrow age-range 60-79 years.) using PLINK[17]. SNPs out of Hardy-Weinberg equilibrium ($p < 0.0001$) were excluded, as were any with a minor allele frequency below 0.1%, leaving 36,529 SNPs for analysis. Both traits were natural log transformed, outliers greater than 2.5 standard deviations from the mean were removed (on the basis that extreme values may represent either technical errors or biological abnormalities unrelated to common polymorphic variants which are the focus of our analyses), and warfarin users excluded, leaving 2510 participants with non-missing data for aPTT (arithmetic mean 30.06 seconds, SD 1.103 seconds) and 2500 with non-missing data for APC resistance (arithmetic mean 2.924, SD 1.134). Exclusion of women on hormone replacement therapy (HRT, shown to associate with these measures[16]) was evaluated, but did not substantially change the results. A stringent (given non-independence of many SNPs) Bonferroni correction for 36,529 tests gives a threshold of $p = 1.37 \times 10^{-6}$ as

equivalent to a single-test p = 0.05. Variable selection was performed in R using Akaike Information Criterion (AIC)[18] with the stepAIC function of the "MASS" library.

Results

Results of the HumanCVD BeadArray-wide association analysis with aPTT and APC resistance are presented in table 1, with results of variable selection presented in table 2. The SNPs most significantly associated with aPTT are in or near the *F12* gene on chromosome 5. The top SNP rs2545801 is a non-coding SNP upstream of *F12*, p = 1.39×10^{-59}), with an (antilogged) per-allele effect of 1.05 seconds on aPTT (95% CI 1.04-1.06). Other gene regions showing association include the *HRG/KNG1* region on chromosome 3 (top hit SNP rs710446, p = 2.68×10^{-19}), the ABO blood group (*ABO*) locus on chromosome 9 (rs657152, p = 2.45×10^{-11}) and the kallikrein B (*KLKB1*) region on chromosome 4 (rs4253304, p = 1.67×10^{-7}). Variable selection identified multiple statistically independent signals at each locus except *KLKB1* (table 2).

Table 1 also presents genetic association results for APC resistance. The most strongly associated SNP with APC resistance is the factor V Leiden mutation (rs6025, p = 4.2×10^{-104}) in the factor V (*F5*) gene on chromosome 1. The other region associated with APC resistance is the *HRG* region on chromosome 3 (top SNP rs16860992, p = 2.29×10^{-15}).

Variable selection (table 2) suggests that all association with APC resistance in the *F5* and solute carrier family 19 member 2 (*SLC19A2*) region on chromosome 1 is attributable to the functional factor V Leiden mutation, with no evidence of statistically independent effects for other SNPs. In the *HRG* region on chromosome 3 there are three potentially independent SNPs.

Discussion

Whilst the HumanCVD array is a candidate gene array, the coagulation pathway is well represented, with SNPs in the genes for the majority of intrinsic and extrinsic pathway proteins (table 3). We confirmed previous reports[11] of effects in *F12* (our "top hit" rs2545801 is the best HumanCVD tag of rs2731672, HapMap r^2 = 0.935 [19]), *KNG1* ("top hit" rs710446) and *HRG* (rs9898, significantly associated, but not our top hit at this locus). We also found positive associations with aPTT at the G protein-coupled receptor kinase 6 (*GRK6*) gene, genomically adjacent to the *F12* gene, although low LD between the "top hit" SNPs at each locus suggests that these are marking independent effects (even if both the effects are actually in the *F12* gene). GRK6 (G protein-coupled receptor kinase 6) deactivates G protein-coupled receptors, and thus may potentially also have a biological effect in the clotting mechanism. The results of variable selection suggest that there may be more than one causal site at each of the three main loci (excluding *KLKB1*).

We also found significant associations between aPTT and SNPs at the *ABO* and *KLKB1* loci. Blood group O versus non-O becomes associated with lower levels of factor VIII and von Willebrand factor (vWF) during childhood[20] and continues into adulthood [21]. Assuming this relationship is causal, and given that aPTT is prolonged with both severe von Willebrand Disease (vWF deficiency in type 1 and 3) and Hemophilia A (factor VIII deficiency), we hypothesize that *ABO* genotype could associate with aPTT through alteration of levels of vWF or factor VIII. There is also a previous report describing association of ABO OO genotype with aPTT using a combined linkage and association approach[22]. Our highest *ABO* locus association is with rs657152, which is in high linkage disequilibrium (LD, r^2=0.98)[23] with rs8176719 (the O/non-O variant), and thus rs657152 closely marks the association of O blood group with clotting. SNP rs657152 is also in high

LD (r^2=0.93) with the myocardial infarction risk SNP reported by Reilly *et al*[24], and hence likely to tag the functional mechanism of that risk. *KLKB1* encodes plasma kallikrein B (Fletcher factor) 1, a glycoprotein which is involved in the intrinsic coagulation pathway[25], and also neighbours the *F11* locus, encoding the factor XI protein, an important factor in the intrinsic coagulation pathway.

We also present results for genetic associations with APC resistance. The HumanCVD array directly assays the factor V Leiden mutation (rs6025), which is known to influence the APC resistance[26]. This mutation shows the strongest genetic association with APC resistance in our dataset ($p = 4.2 \times 10^{-104}$). Although our other association signals in the *F5* gene are with SNPs in little LD with rs6025 (eg $r^2 = 0.104$), the magnitude of signal with rs6025 and results of variable selection (table 2) suggest these SNPs are simply showing a "bystander" effect. The other locus containing SNPs associating with APC resistance is *HRG* (same SNPs as with aPTT, and showing consistent direction of effect on both tests). HRG (histidine-rich glycoprotein) has a complex role in coagulation, with both anticoagulant and antifibrinolytic properties reported [27,28]. In our data we observe concordant effects of *HRG* genotype on both aPTT (clotting speed) and APC resistance (response to inhibition). Whilst there is a SNP in *SLC19A2* (the gene for solute carrier family 19 (thiamine transporter), member 2) this is physically close to the *F5* gene on chromosome 1, so may simply tag functional variation in *F5*. Although variable selection excludes the *SLC19A2* SNP (table 2), the LD between all our top hits in *F5* (including rs6025) and the *SLC19A2* SNP is very low ($r^2 < 0.006$), suggesting this may either mark an independent effect in the *F5* gene, or a biological relevance of thiamine transport in coagulation.

With the exception of the factor V Leiden mutation (rs6025), already known to influence APC resistance[26], the majority of these results represent relatively small genetic effects on aPTT and APC resistance. They therefore have very limited *predictive* value (especially as individual variants), but instead offer additional insight into the functional pathways underlying blood coagulation.

Our study has three principal limitations: (i) The HumanCVD array is not "genome-wide". Although even genome-wide arrays do not capture all genetic variation they offer a relatively unbiased representation of the genome. Table 3 illustrates the extent to which this candidate gene array represents coagulation system genes. (ii) The population we have analysed is female, of European ancestry, and represents a fairly narrow age-range (post-menopausal, 60-79 years). The results may therefore not be generalisable to other ancestries, males or younger people. Further studies are needed to examine the associations of newly identified genotypes with risk of venous and arterial thrombosis. (iii) These phenotypes (in particular APC resistance) are infrequently measured on a cohort scale, and we were unable to identify a suitable replication cohort with both these measures and appropriate genotyping data. Appropriate caution should therefore be applied in interpreting results close to our significance threshold. Our replication of published aPTT GWAS results[11] and the very strong statistical evidence (most of our reported p-values several orders of magnitude below the nominal HumanCVD significance threshold of 1×10^{-6}) support the reliability of these findings.

In conclusion, we have both confirmed previous reports that *F12/GRK6*, *KNG1* and *HRG* are associated with aPTT[11] and identified new SNPs at *ABO* and new genomic locus *KLKB1* associated with aPTT. We also present the first high-density genetic association analysis of APC resistance, and identify signals in the *F5* and *HRG* genomic regions. Our findings suggest that *KLKB1* and *HRG* may play potentially important roles in blood coagulation.

Acknowledgments

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Table 1

Associations between SNPs and either aPTT or APC resistance.

Trait	SNP	Chr[1]	Position[2]	Gene symbol[3]	Tested allele	N	Ln Effect[4] (95% CI)	Effect in secs[5] (95% CI)	p-value[6]
aPTT	rs2545801	5	176773945	*FI2*	A	2498	0.05 (0.04,0.06)	1.05 (1.04,1.06)	1.39×10^{-59}
aPTT	rs1801020	5	176769138	*FI2*	A	2505	0.05 (0.05,0.06)	1.05 (1.05,1.06)	1.55×10^{-59}
aPTT	rs17876032	5	176763233	*FI2*	G	2507	0.04 (0.03,0.04)	1.04 (1.03,1.04)	1.40×10^{-37}
aPTT	rs710446	3	187942621	*KNG1*	G	2501	−0.03 (−0.03,−0.02)	0.97 (0.97,0.98)	2.68×10^{-19}
aPTT	rs2228243	3	187877807	*HRG*	G	2503	0.03 (0.02,0.04)	1.03 (1.02,1.04)	1.35×10^{-17}
aPTT	rs16860992	3	187876732	*HRG*	C	2503	0.03 (0.02,0.04)	1.03 (1.02,1.04)	4.37×10^{-17}
aPTT	rs5030062	3	187936874	*KNG1*	C	2506	−0.02 (−0.03,−0.02)	0.98 (0.97,0.98)	3.55×10^{-15}
aPTT	rs1621816	3	187921867	*KNG1*	G	2500	0.02 (0.02,0.03)	1.02 (1.02,1.03)	2.33×10^{-14}
aPTT	rs5030028	3	187928448	*KNG1*	A	2502	0.03 (0.02,0.03)	1.03 (1.02,1.03)	3.78×10^{-14}
aPTT	rs5030023	3	187927338	*KNG1*	A	2508	0.03 (0.02,0.03)	1.03 (1.02,1.03)	4.16×10^{-14}
aPTT	rs3856930	3	187941016	*KNG1*	A	2506	−0.02 (−0.03,−0.02)	0.98 (0.97,0.98)	6.90×10^{-14}
aPTT	rs1624230	3	187921629	*KNG1*	A	2499	0.02 (0.02,0.03)	1.02 (1.02,1.03)	9.47×10^{-14}
aPTT	rs657152	9	135129086	*ABO*	A	2507	−0.02 (−0.03,−0.01)	0.98 (0.97,0.99)	2.45×10^{-11}
aPTT	rs266723	3	187929741	*KNG1*	C	2501	0.02 (0.01,0.02)	1.02 (1.01,1.02)	3.31×10^{-10}
aPTT	rs7447593	5	176756743	*FI2*	C	2504	−0.02 (−0.02,−0.01)	0.98 (0.98,0.99)	1.81×10^{-09}
aPTT	rs7381103	5	176770918	*FI2*	G	2510	0.05 (0.03,0.07)	1.05 (1.03,1.07)	4.35×10^{-09}
aPTT	rs651007	9	135143696	*ABO*	A	2502	−0.02 (−0.03,−0.01)	0.98 (0.97,0.99)	5.97×10^{-09}

Trait	SNP	Chr[1]	Position[2]	Gene symbol[3]	Tested allele	N	Ln Effect[4] (95% CI)	Effect in secs[5] (95% CI)	p-value[6]
aPTT	rs1042445	3	187878130	HRG	A	2508	−0.02 (−0.03,−0.01)	0.98 (0.97,0.99)	2.27×10^{-08}
aPTT	rs1624569	3	187932763	KNG1	G	2499	0.02 (0.01,0.02)	1.02 (1.01,1.02)	5.15×10^{-08}
aPTT	rs2062632	3	187943875	KNG1	G	2503	0.02 (0.01,0.02)	1.02 (1.01,1.02)	1.04×10^{-07}
aPTT	rs2287694	5	176792899	GRK6	G	2508	0.02 (0.02,0.03)	1.02 (1.02,1.03)	1.39×10^{-07}
aPTT	rs4253304	4	187410565	KLKB1	C	2498	−0.01 (−0.02,−0.01)	0.99 (0.98,0.99)	1.67×10^{-07}
aPTT	rs13177732	5	176789531	GRK6	C	2500	−0.02 (−0.02,−0.01)	0.98 (0.98,0.99)	2.56×10^{-07}
aPTT	rs9898	3	187873321	HRG	A	2505	0.02 (0.01,0.02)	1.02 (1.01,1.02)	2.61×10^{-07}
aPTT	rs2304595	4	187409274	KLKB1	A	2507	−0.01 (−0.02,−0.01)	0.99 (0.98,0.99)	3.60×10^{-07}
aPTT	rs5030091	3	187943571	KNG1	G	2507	0.01 (0.01,0.02)	1.01 (1.01,1.02)	7.29×10^{-07}
APC-R	rs6025	1	1.68E+08	F5	A	2500	−0.28 (−0.31,−0.26)	0.76 (0.73,0.77)	4.2×10^{-104}
APC-R	rs6682179	1	1.68E+08	F5	A	2499	−0.08 (−0.1,−0.07)	0.92 (0.9,1.07)	1.43×10^{-28}
APC-R	rs6427196	1	1.68E+08	F5	C	2500	−0.08 (−0.1,−0.07)	0.92 (0.9,1.07)	2.11×10^{-28}
APC-R	rs6009	1	1.68E+08	F5	A	2500	−0.08 (−0.1,−0.07)	0.92 (0.9,1.07)	1.24×10^{-27}
APC-R	rs16860992	3	1.88E+08	HRG	C	2492	0.04 (0.03,0.04)	1.04 (1.03,1.04)	2.29×10^{-15}
APC-R	rs2228243	3	1.88E+08	HRG	G	2492	0.03 (0.03,0.04)	1.03 (1.03,1.04)	9.55×10^{-15}
APC-R	rs9898	3	1.88E+08	HRG	A	2496	0.02 (0.02,0.03)	1.02 (1.02,1.03)	5.26×10^{-11}
APC-R	rs2038024	1	1.68E+08	SLC19A2	C	2496	−0.03 (−0.04,−0.02)	0.97 (0.96,0.98)	1.75×10^{-09}

Both aPTT and APC resistance ("traits") are presented in this table.

[1] Chromosome ("Chr").

[2] Chromosomal position.

[3] Mapped gene.

[4] Logged effect sizes (as tested by regression).

[5] Anti-logged effect sizes (for direct interpretation), where effect is change in trait per tested allele.

[6] P-value indicates strength of evidence against the null hypothesis as tested by linear regression of trait on number of tested alleles.

Table 2

Variable selection results.

Genomic region[1]	Location[2]	Phenotype	SNPs	Estimate	Individual p-value[3]	Overall p-value[4]
F12 & GRK6	5q33-35	aPTT	rs2545801	−0.029507	0.041	$<2.2\times10^{-16}$
			rs1801020	−0.022793	0.1303	
HRG & KNG1	3q27	aPTT	rs1042445	0.010413	0.00704	$<2.2\times10^{-16}$
			rs2062632	0.007951	0.02904	
			rs2228243	0.018532	2.34×10^{-05}	
			rs710446	−0.021965	2.96×10^{-12}	
			rs9898	−0.014528	6.23×10^{-05}	
KLKB1	4q35	aPTT	rs4253304	0.015523	6.15×10^{-08}	6.15×10^{-08}
ABO	9q34	aPTT	rs657152	0.015269	0.000263	1.269×10^{-10}
			rs651007	0.006843	0.154113	
F5 & SLC19A2	1q23	APC resistance	rs6025	0.28882	$<2\times10^{-16}$	$<2.2\times10^{-16}$
HRG	3q27	APC resistance	rs16860992	−0.116523	0.043113	2.2×10^{-16}
			rs2228243	−0.088207	0.125584	
			rs9898	−0.014962	0.000618	

For each SNP in a genomic region the estimate gives an indication of the independent effect of that SNP on the trait. Akaike Information Criterion was used to identify SNPs with a significant independent contribution to the phenotype of interest in a step-wise multiple regression of significantly associated SNPs in each genomic region.

[1] Genomic regions are represented by gene names.

[2] Location is cytogenetic location.

[3] The individual p-value gives an indication of the statistical independence of a SNP from others included in the model.

[4] The overall p-value indicates the strength of the combined evidence against the null hypothesis when all SNPs are included.

Table 3

Representation of coagulation factor genes on the HumanCVD array.

Coagulation Factor	Gene symbol	Number of SNPs
Fibrinogen	*FGA*	23
Fibrinogen	*FGB*	28
Fibrinogen	*FGG*	20
Prothrombin	*F2*	36
Tissue Factor	*F3*	38
Factor V	*F5*	126
Factor VII	*F7*	34
Factor VIII	*F8*	19
Factor IX	*F9*	18
Factor X	*F10*	20
Factor XI	*F11*	20
Factor XII	*F12*	19
Factor XIII	*F13A1*	133
Factor XIII	*F13B*	9
Von Willebrand Factor	*VWF*	128
Prekallikrein	*KLKB1*	21
Fibronectin	*FN*	0
Antithrombin III	*SERPINC1*	9
Heparin cofactor II	*SERPIND1*	5
Protein C	*PROC*	8
Protein S	*PROS1*	8
Protein Z	*PROZ*	6
Protein Z-related protease inhibitor (ZPI)	*SERPINA10*	11
Plasminogen	*PLG*	35
Alpha 2-antiplasmin	*SERPINF2*	7
Tissue Plasminogen Activator	*PLAT*	19
Plasminogen Activator	*PLAU*	6
Plasminogen Activator Inhibitor-1	*SERPINE1*	33
Plasminogen Activator Inhibitor-2	*SERPINB2*	16

For each of the main proteins in the coagulation cascade the corresponding gene(s) is/are shown, along with the number of SNPs included in the HumanCVD array for that gene.