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The HR2 Haplotype of Factor V: Effects on Factor V Levels, Normalized Activated Protein C Sensitivity Ratios and the Risk of Venous Thrombosis

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Key words

HR2 haplotype, factor V, APC resistance, venous thrombosis

Summary

We studied the HR2 haplotype of the factor V gene in a case-control study for venous thrombosis including 474 patients with a first deep-vein thrombosis and 474 age- and sex-matched healthy controls (Leiden Thrombophilia Study, LETS). We investigated both the original His1299Arg (A4070G) polymorphism and the Met385Thr (T1328C) polymorphism. This latter polymorphism, located in exon 8 (heavy chain), is always present in the HR2 haplotype, but also occurs on its own in a His1299 (wt) background. The HR2 haplotype was not associated with an increased risk of venous thrombosis (OR = 1.2, 95% confidence interval: 0.8-2.0). We did not find an association between the HR2 haplotype and a reduced sensitivity for activated protein C (APC) in non-carriers of factor V Leiden (FVL). However, in compound heterozygous FVL/HR2 carriers the sensitivity for APC was reduced. The HR2 haplotype was also associated with reduced factor V antigen levels in both patients and controls. Sequence analysis of the promoter region of factor V in HR2 homozygotes did not reveal any sequence variations that could explain the reduced FV levels. Our results show that the HR2 haplotype is not associated with an increased risk of venous thrombosis or with a reduced sensitivity for APC in non-FVL carriers. However, the HR2 haplotype is associated with a reduced sensitivity for APC in carriers of FVL and with reduced factor V antigen levels.

Introduction

Human coagulation factor V (FV), which is synthesized in the liver and in megakaryocytes, circulates in plasma as a 330 kD single chain glycoprotein. The domain organization (A1-A2-B-A3-C1-C2) of FV is similar to that of factor VIII (FVIII) (1). By selective proteolytic cleavages the large B-domain is removed, yielding activated FV (FVa) which consists of a heavy chain (A1-A2) and a light chain (A3-C1-C2) that are noncovalently linked by a calcium ion [for a review see Rosing and Tans (2)]. The activated FV molecule acts as a cofactor to activated factor X (FXa) in the prothrombinase complex that proteolytically activates prothrombin to thrombin (3). FVa is inactivated by activated protein C (APC) by selected proteolytic cleavages in the heavy chain (4). This inactivation, with protein S as cofactor, is an important step in the anticoagulant pathway. Activated FVIII (FVIIIa) is also inactivated by APC and FV is thought to function as a cofactor, synergistic with protein S, in this reaction (5-7).

Activated protein C resistance, a poor anticoagulant response of plasma to APC, is almost always associated with the presence of a mutation in one of the APC cleavage sites (Arg506) of FV (8, 9). The activated FV variant (factor V Leiden, FVL) is inactivated more slowly than activated wildtype FV (10-13). APC resistance caused by the FVL mutation is a common and strong risk factor for venous thrombosis (14, 15). Recently, we reported that a reduced sensitivity for APC not due to FV Leiden is also associated with an increased risk of venous thrombosis (16).

The gene for human FV is localized on chromosome 1q23-24 and consists of 25 exons and 24 introns (17). The B-domain is fully encoded by the large exon 13. In 1996, the His1299Arg [A4070G, according to the cDNA sequence of Jenny et al. (1)] polymorphism in exon 13 was first described (18). In this study, the Arg1299 (R2) allele was reported to be more frequent in subjects with reduced FV activity levels. Subsequent studies which have investigated the HR2 haplotype have diverse results. Bernardi et al. reported that the R2 allele was associated with a reduced sensitivity for APC (19). An association with reduced FV levels was not found in this Italian study, nor an indication that the R2 allele is a risk factor for venous thrombosis. A French case-control study did show relationships between the HR2 haplotype and reduced FV levels and a reduced response to APC (20). Besides, this study showed that the R2 allele is associated with a 1.8-fold increased risk of venous thromboembolism. One family study showed that compound heterozygous FVL/HR2 carriers have a more reduced normalized APC-SR than FVL heterozygotes (21). The HR2 haplotype includes, in addition to the R2 allele, 7 other polymorphisms in exon 13 and one in exon 16. Four of these variations do not cause aminoacid substitutions and most of these polymorphisms have a much higher population frequency than the His1299Arg variation. The HR2 haplotype has an allele frequency of 8% in Italians and of 6% in the French study (19, 20).

We studied the HR2 haplotype in a population-based case-control study on venous thrombosis (Leiden Thrombophilia Study, LETS). We screened for the His1299Arg polymorphism as well as for a novel variation, the Met385Thr polymorphism. This latter polymorphism, which was detected by sequencing of the FV gene of a FVL/HR2 compound heterozygote, is also part of the HR2 haplotype and is located in the

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heavy chain of the FV molecule. Furthermore we investigated the association between the HR2 haplotype and FV antigen levels and the sensitivity for APC. We also assessed the risk of venous thrombosis in compound heterozygous carriers of the R2 allele and the FVL allele.

Patients and Methods

Subjects

The Leiden Thrombophilia Study (LETS) is a population-based case-control study in three Dutch anticoagulation clinics. The study, of which the design has been described extensively elsewhere (22), includes 474 patients with a first episode of deep venous thrombosis and 474 age- and sex-matched healthy control subjects. Venepuncture took place at least 6 months after the thrombotic event. DNA analysis could be performed for 943 individuals (471 patients and 472 controls). For these individuals the ratio of male to female subjects was 1.3 for both patients and controls. The mean age was 47 years for both groups (range 16-70 for the patients and 16-73 for the controls).

Blood Collection and Laboratory Analysis

Blood was collected into tubes containing 0.106 mol/L trisodium citrate. Plasma was prepared by centrifugation for 10 min at 2000 g at room temperature and stored at –70° C. The sensitivity of the plasma activated partial thromboplastin time (APTT) to APC was measured as described before (22). Results were expressed as normalized APC sensitivity ratios (n-APC-SR). The APC sensitivity ratio is defined as the APTT in the presence of APC divided by the APTT in the absence of APC. The normalized APC-SR is calculated by dividing the APC-SR of the sample by the APC-SR of pooled normal plasma which is measured in the same run.

FV antigen (ag) was measured by a sandwich type enzyme-linked immunosorbent assay (ELISA) using two different monoclonal antibodies (V-6, V-9) against the light chain of FV (23). Briefly, wells coated with monoclonal antibody V-6 were incubated with diluted plasma sample. Monoclonal antibody V-9, conjugated to horseradish peroxidase, was used for the detection of immobilized FV. FVag levels were expressed in units per deciliter (U/dl). By definition 1 ml pooled normal plasma contains 1 unit.

FVIIIag levels were measured by a sandwich type ELISA with two different monoclonal antibodies directed against the light chain of FVIII (Kamphuisen et al., submitted). Briefly, wells coated with monoclonal antibody CLB Cag 117 were incubated with a diluted plasma sample. Monoclonal antibody CLB-Cag A conjugated to horseradish peroxidase was used for detection. Monoclonal anti-FVIII antibodies were kindly provided by Dr J. van Mourik (CLB, Sanguin Blood Supply Foundation, Amsterdam, The Netherlands).

FVIII coagulant activity (FVIII:C) was measured by a one-stage clotting assay as described before (24). Protein C activity and antithrombin activity were measured with Coamate (Chromogenix, Mölndal, Sweden) on an ACL-200 (Instrumentation Laboratory, Milan, Italy), factor II activity with a chromogenic method using S-2238 (Chromogenix) and Echis carinatus snake venom (Sigma Chemical Co, St Louis, USA) on an ACL-200 (25), and factor X antigen was measured by ELISA with a polyclonal antibody (DAKO, Denmark) (de Visser et al., in preparation). Factor VII was measured using Thromborel S reagent (Behringwerke AG, Warburg, Germany) and factor VII deficient plasma (Organon Teknica, Durham, USA) (26). Total protein S was measured by polyclonal ELISA (27) and free protein S was measured directly in plasma by ELISA using two monoclonal antibodies specific for free protein S (Asserachrom free protein S, Diagnostica Stago, Asnieres-sur-Seine, France) (28, 29). The fibrinogen concentration was determined according to method of Clauss using Dade® thrombin reagent (Baxter, Miami, USA) on an Electra 1000 (MLA, Pleasantville, USA) (26).

The results of all the above mentioned measurements, except for FVag, FVIIIag and FXag have been reported previously (22, 24, 26, 30).

High molecular weight DNA was isolated from leukocytes and stored at 4° C. A list of the primers used for sequencing of all exons of FV in the FVL/HR2 heterozygote and a description of the sequencing procedure have been reported elsewhere (31). The detection of three polymorphisms (A4070G, T1328C and A6755G; numbering according to Jenny et al. (1)) in the FV gene was performed by polymerase chain reaction (PCR) followed by restriction enzyme digestion. The PCR mixture consisted of 50 ng of both oligonucleotides, 200 μM of each dNTP, 67 mM Tris-HCl pH 8.8, 6.7 mM $MgCl_2$, 10 mM β-mercaptoethanol, 6.7 μM EDTA, 16.6 mM $(NH_4)_2SO_4$, 0.5 mg/ml BSA, 0.2 units AmpliTaq polymerase (Perkin-Elmer) and 10% DMSO (only for the A4070G and A6755G polymorphisms) in a total volume of 10 μl. The reactions were performed in a T3 Thermocycler (Biometra, Göttingen, Germany). The PCR conditions were as follows: 4 min initial denaturation at 94° C, followed by 33 cycles of 1 min at 94° C, 1 min at 65° C and 90 sec at 72° C. A final extension was performed at 72° C for 4 min. For detection of the A4070G (= His1299Arg) polymorphism a 828 bp fragment was amplified with primer A (5'-CATGAAGTCTGGCAGACAGTC-3') and primer B (5'-TATCTGGCTGAGATCCGGGAG-3'). The T1328C (= Met385Thr) polymorphism was determined after amplification of a 153 bp fragment with primer C (5'-CACCAAACATACAGTGAATCCCAGTA-3') and primer D (5'-AATAACCAGGTACTCCATAATATTTTAC-3'). The underlined nucleotide in primer C corresponds to a mismatch with the gene sequence and was introduced to create a RsaI restriction site (in the presence of the 1328C allele) for detection of the polymorphism. For detection of the Asp2194Gly (= A6755G) polymorphism a 440 bp fragment was amplified with primers E (5'-GTGTTCTATGTGTTCTTTGATATCCTCATT-3') and F (5'-GGGTTTTTGAATGTTCAATTCTAGTAGATA-3'). PCR products were digested by incubation with either RsaI (New England Biolabs, A4070G and T1328C polymorphisms) or with EcoRV (New England Biolabs, A6755G polymorphism) overnight at 37° C. The restriction fragments (A4070G polymorphism: 828 bp for the 4070A allele, and 381 and 447 bp for the 4070G allele; T1328C polymorphism: 142 and 11 bp for the 1328T allele, and 117, 25 and 11 bp for the 1328C allele; A6755G polymorphism: 390, 29 and 21 bp for the 6755A allele, and 419 and 21 bp for the 6755G allele) were separated in 2% agarose gels and visualized after ethidium bromide staining. The determination of the FVL mutation in the LETS samples has been described previously (9). Almost 2 kb of the upstream region of the FV gene (bases -1 to -1933, according to GenBank sequence U83346) was amplified by PCR with primers G (5'-TCAGTAGGCTAGGTGTTCTAGGAC-3') and H (5'-GCTTCCTTTCCTGCTCCCGC-3') with the Expand Long Template PCR System (Boehringer Mannheim). Sequencing of this PCR fragment was performed with the ABI Prism® BigDye terminator cycle sequencing ready reaction kit (Perkin-Elmer Applied Biosystems) according to manufacturer's protocol. Sequences of the used primers can be asked for. The reactions were run on an ABI Prism 310 (Perkin-Elmer Applied Biosystems).

Statistical Analysis

Odds ratios (OR) were calculated in the standard unmatched fashion. Ninety-five percent confidence intervals (95% CI) were constructed according to Woolf (32). The OR is used as an estimate of the relative risk, which indicates the risk of developing venous thrombosis in a category of exposure (e.g., HR2 carriers) relative to the reference category (e.g., HR2 wildtype). An OR of 1 indicates no effect on risk, while an OR above 1 indicates an increase in risk.

Results

The Met385Thr Polymorphism

In the population-based case-control study LETS, one heterozygous FVL carrier was present with a normalized APC-SR of 0.44. In our laboratory this ratio is within the range of homozygous FVL carriers (n-APC-SR <0.45) (33). Because of this discrepancy all exons of the FV gene were sequenced in this individual. Besides the FVL mutation and some previously described polymorphisms, the A4070G (His1299Arg) variation, which is part of the HR2 haplotype, was detected in heterozygous form (19). Previous investigations had already shown that the FVL allele is not present in the HR2 haplotype (34-37).

Furthermore one novel variation (T1328C, Met385Thr) in exon 8 was detected. This latter variation was not present in 8 homozygous FVL carriers (Guasch et al., unpublished results), so the Thr385 allele is not part of the FVL haplotype. Screening of a panel of 90 normal individuals for the His1299Arg and Met385Thr variations revealed 9 individuals who were heterozygous for both variations. Two individuals were homozygous for His1299 and heterozygous for Met385Thr. So, the Thr385 allele is always present in the HR2 haplotype, but can also occcur on its own in a His1299 (wildtype, R1) background. Previous studies (19, 20) have reported an association between reduced normalized APC-SR and the presence of the HR2 haplotype, but no plausible explanation for this reduction has been found yet. The Met385Thr polymorphism is located in the heavy chain of FV, which is directly involved in the generation of thrombin and the inactivation of FVa by APC. Therefore we hypothesized that this variation might be responsible for the reduced normalized APC-SR. Because the two variations His1299Arg and Met385Thr are not in absolute linkage disequilibrium, we looked at both polymorphisms in LETS.

Table 1 Frequencies of the His1299Arg and Met385Thr polymorphisms

		His1299Arg		
	Met385Thr	++	+-	--
Patients (n=471)	++	2	0	0
	+-	0	46	8
	--	0	0	415
Controls (n=472)	++	0	0	0
	+-	0	39	9
	--	0	0	424

Table 2 Co-inheritance of factor V Leiden and HR2 haplotype and the risk of venous thrombosis

FVL	His1299Arg	Patients (n=461)	Controls (n=472)	OR	95% CI
–	–	340	420	1 *	
+	–	75	13	7 1	3 9 – 13 1
–	+	37	38	1 2	0 7 – 1 9
+	+	9	1	11 1	1 4 – 88

* Reference category

Subjects homozygous for factor V Leiden (8 patients) or homozygous for the R2 allele (2 patients) are not included in this table

Table 3 Mean normalized APC sensitivity ratio according to factor V Leiden and the factor V His1299Arg polymorphism

		Patients		Controls	
FVL	His1299Arg	n	n-APC-SR (95% CI)	n	n-APC-SR (95% CI)
++	--	8	0 43 (0 42 - 0 44)	0	
+-	--	65	0 57 (0 56 - 0 58)	13	0 57 (0 56 - 0 59)
	+-	8	0 51 (0 48 - 0 54)	1	0 52
--	--	303	0 96 (0 95 0 97)	415	1 02 (1 01 - 1 04)
	+-	32	0 95 (0 92 - 0 99)	38	1 03 (0 98 - 1 08)
	++	2	0 92, 0 93	0	

Patients using oral anticoagulants or with a lupus anticoagulant were excluded for this analysis

HR2 Haplotype and the Risk of Venous Thrombosis

All subjects were screened for both the His1299Arg (A4070G) polymorphism in the B-domain and the Met385Thr (T1328C) polymorphism in the heavy chain of FV (Table 1). It was found that 2 of the 471 patients were homozygous for the R2 allele and 46 were heterozygous (allele frequency 5.3%). Thirty-nine of the 472 controls were heterozygous for the R2 allele and none of the controls was homozygous for the R2 allele (allele frequency 4.1%). The odds ratio (OR), calculated as a measure of the relative risk of venous thrombosis, for subjects carrying the R2 allele (in heterozygous or homozygous form) was 1.2 (95% CI: 0.8-2.0) compared to homozygous R1 (wildtype) carriers. In addition to all carriers of the R2 allele, seventeen homozygous carriers of the R1 allele (8 patients and 9 controls) were heterozygous for the Thr385 allele. The allele frequency for the Thr385 allele was 6.2% and 5.1% for patients and controls, respectively. The Thr385 allele was also not associated with an increased risk of venous thrombosis (OR = 1.2, 95% CI: 0.8-1.8).

Compound Heterozygous HR2/Factor V Leiden Carriership and Risk of Venous Thrombosis

We investigated whether co-inheritance of the R2 allele influenced the risk of venous thrombosis in heterozygous carriers of FVL (Table 2). Homozygous FVL or HR2 carriers were not included in this analysis because the FVL allele and the R2 allele are not present in the same haplotype (37). The OR for heterozygous carriers of FVL who are not carrying the R2 allele was 7.1 (95% CI: 3.9-13) compared to wildtype carriers (no HR2 and no FVL). The OR for compound heterozygous FVL/HR2 carriers was slightly higher (OR = 11, 95% CI: 1.4-88), but the confidence intervals largely overlapped.

HR2 Haplotype and Normalized APC Sensitivity Ratio

We investigated the relationship between the HR2 haplotype and the sensitivity for APC, which was measured in undiluted plasma by an APTT-based assay with Cephotest® as activator. The association between genotype and normalized APC-SR is shown in Table 3. All FVL homozygotes carried the R1 allele, because this allele is part of the FVL haplotype. Compound heterozygous carriers for FVL/R2 have reduced normalized APC-SRs compared to FVL/R1 carriers. In non-FVL carriers no difference in APC sensitivity was found between carriers of the R1 allele and carriers of the R2 allele in both patient and control groups. None of the 17 subjects who were only heterozygous for Met385Thr did carry the FVL mutation. For both patients and controls, no significant difference in mean normalized APC-SR was found between this group and non-FVL carriers who were homozygous for R1/Met385.

	Patients		Controls	
His1299Arg	n	Mean FVag (95% CI)	n	Mean FVag (95% CI)
- -	423	136 (133 - 140)	433	134 (131 - 137)
+ -	46	117 (107 - 128)	39	107 (97 - 117)
+ +	2	79, 105	0	-

Table 4 Mean factor V antigen levels (U/dl) according to the factor V His1299Arg polymorphism

HR2 Haplotype and Factor V Antigen Levels

The association between the HR2 haplotype and FVag levels was investigated. The results are shown in Table 4. Mean FVag levels were 134 U/dl and 132 U/dl for patients and controls, respectively (23). The R2 allele was associated with reduced FVag levels in both patients and controls. To assess whether the reduction in FVag levels was specific and not due to a difference in liver function, mean levels of other coagulation factors were calculated for the different genotype subgroups. For the investigated coagulation factors (FVIIIag, FVIII:C, antithrombin, fibrinogen, factor II, factor VII, factor X, protein C, protein S [free and total]) no differences in mean levels were found between homozygous R1 carriers and heterozygous R1R2 carriers. To establish whether the reduction in FV levels was caused by the Thr385 variation we compared the mean FV levels of subjects homozygous for R1 and Met385 (N = 839, mean FVag = 135 U/dl, 95% CI: 133-137) and subjects homozygous for R1 but heterozygous for Met385Thr (N = 17, mean FVag = 124 U/dl, 95% CI: 108-141). For this analysis patients and controls were taken together because of the small size of the group of subjects heterozygous for the Met385Thr variant. Again, no difference in mean FV levels was found. So, the established reduction in FV levels in carriers of the HR2 haplotype is probably not due to the Thr385 allele.

Promoter Polymorphisms

We wondered whether the reduced FV levels that were found in carriers of the HR2 haplotype were caused by a polymorphism in the promoter region of FV in linkage disequilibrium with the R2 polymorphism. Therefore we sequenced 1933 basepairs of the promoter region of FV of the two HR2 homozygotes and one wildtype control. No sequence variations were identified between the three sequenced individuals. The sequences we found were identical to the PAC sequence submitted by Bird (GenBank accession number Z99572).

His1254Arg Polymorphism

During screening of the His1299Arg polymorphism, one aberrant *Rsa*I restriction pattern was detected, suggesting the presence of the His1254Arg (A3935G) polymorphism in the FV gene which was previously described by Lunghi et al. (38). The presence of this variation in heterozygous form was confirmed by sequence analysis. Like the R2 polymorphism, the His1254Arg polymorphism is located in a highly repeated area of exon 13 with 31 tandem repeats of 27 bp. It is interesting that the two polymorphisms are located in exactly the same position (20th nucleotide) of two similar repeats (His1254Arg in the 11th repeat and His1299Arg in the 16th repeat). The female control carrying this variant had a normalized APC-SR of 0.78 and a FVag level of 117 U/dl, which is relatively low for the LETS population. She did not carry the R2 allele or the Thr385 allele.

Asp2194Gly Polymorphism

Recently, a missense polymorphism (A6755G) in exon 25 of the FV gene was reported, predicting an aminoacid substitution (Asp2194Gly) in the C2 domain of the FV molecule (39). This polymorphism was found to be tightly linked to the R2 allele. We screened all carriers of the R2 allele for this polymorphism and found that 2 of the 85 heterozygous carriers did not carry this novel variation. Two homozygous R2 carriers did carry the Gly2194 allele in homozygous form. Furthermore this variation in the light chain was not detected in 150 homozygous His1299 carriers. We had not detected this variation by sequence analysis of all FV exons of the compound heterozygous FVL/HR2 subject. Re-analysis revealed the presence of the Asp2194Gly polymorphism in heterozygous form. Our results confirm the tight linkage of this variation to the R2 allele.

Discussion

The HR2 haplotype of the FV gene was not found to be associated with an increased risk in our population-based case-control study for venous thrombosis (LETS). These findings correspond with the results of Bernardi and Luddington (19, 40). Our results differ from the results of a recent French study that did find an increased risk of venous thrombosis associated with the HR2 haplotype (20). The study population of this French study was not exactly defined, but allele frequencies of FVL and the prothrombin G20210A variant in this French study were not different from the frequencies in our study (15, 20, 41).

Faioni et al. recently showed in a family study that co-inheritance of the R2 allele resulted in an increased risk of venous thromboembolism in FVL carriers (42). Our data do not support this observation, but cannot exclude it either. The OR for compound heterozygous FVL/HR2 carriers was slightly higher (OR = 11, 95% CI: 1.4-88) than the OR for heterozygous FVL carriers (OR = 7.1, 95% CI: 3.9-13), but the confidence intervals largely overlapped. Because the OR for compound heterozygotes depends on only one control, these data suffer from statistical uncertainty.

In addition to the His1299Arg polymorphism we studied the Met385Thr polymorphism in the heavy chain of FV. This variation, that had not been previously reported, is part of the HR2 haplotype and was detected by sequencing of a FVL heterozygote with an APC resistant phenotype similar to a homozygous FVL carrier. In our large population sample the Thr385 allele was always present in the HR2 haplotype, but it also occurred with the R1 allele. Just like the R2 allele, the Thr385 allele was not associated with an increased risk of venous thrombosis.

Recently, a missense polymorphism (A6755G) in exon 25 of the FV gene was reported, predicting an aminoacid substitution (Asp2194Gly) in the C2 domain of the FV molecule (39). We confirmed the tight linkage of this variation to the R2 allele by screening of all R2 carriers and 150 homozygous R1 carriers. It was reported that in carriers of the Gly2194 allele the ratio of the two isoforms of FV (FV1 and FV2) is shifted in favor of FV1, the isoform which has the highest overall procoagulant activity (39, 43, 44).

Our observation that the HR2 haplotype is associated with reduced FV levels corresponds with the results of all previous studies on the HR2 haplotype (18, 20, 21), except the study of Bernardi et al. (19). In this Italian study FV levels were only measured in a small sample of HR2 carriers and not in non-carriers. Our findings that the HR2 haplotype is associated with reduced FV levels but not with an increased risk of venous thrombosis agrees with the recent finding that reduced FV levels are not a risk factor for venous thrombosis (23). To assess whether the reduction in FV levels was due to the Thr385 allele we compared mean FV levels of subjects homozygous for R1 and Met385 and subjects homozygous for R1 but heterozygous for Met385Thr. No difference was found, so we showed that the Thr385 variation in the heavy chain is probably not responsible for the reduced FV levels in the HR2 haplotype. Another possible explanation for the reduced FV levels is that the His1299Arg polymorphism is in linkage disequilibrium with a polymorphism in the promoter region of the FV gene that may cause a reduced expression of the FV gene. To investigate this we sequenced a large upstream region (1933 bp) of two homozygous HR2 carriers and one wildtype control, but no sequence variations were detected. So, the mechanism by which the genotype is associated with reduced FV levels is not clear yet. The most simple explanation is that the His1299Arg variant itself is responsible for the reduction in FV levels. The substitution of histidine to arginine at amino acid 1299, which is located in a highly repeated region in exon 13 of the FV gene, will give a repeat with a unique sequence and could be responsible for reduced FV levels through the production of a less stable protein or interference with intracellular trafficking. One indication for this explanation is that the heterozygous carrier of the His1254Arg polymorphism, which is located in homologous position in the repeat as the His1299Arg mutation, also has a relatively reduced FV level (117 U/dl).

In our study population the HR2 haplotype was not associated with a reduced sensitivity for APC in non-FVL carriers. However, in compound heterozygous FVL/HR2 carriers, the normalized APC-SR was reduced compared to heterozygous FVL/R1 carriers. This observation corresponds with the results of the family study of Castaman et al. (21). It also corresponds with the results of previous reported plasma experiments in which a mixture of homozygous HR2 plasma and homozygous FVL plasma led to a reduced normalized APC-SR compared to FVL heterozygous plasma (19). Our finding that the HR2 haplotype in non-FVL carriers is not related with a reduced sensitivity for APC can be shared with a recent French study in which the APC ratio, measured with an APC resistance assay with undiluted plasma, in patients carrying the R2 allele was not different from patients not carrying the allele (20). In the control group of this latter study only a small difference in APC ratio, measured with a modified FV specific assay, was found. In the original study of Bernardi, who was the first to point to an association between the HR2 haplotype and a reduced sensitivity for APC, this relation was only proven in a small study population (19). The use of different APC resistance tests can also be the cause of the discrepancies between the above mentioned studies. The measured APC response depends on the type of clotting test and the particular reagent that is used. Besides, the influence of other factors (e.g. FVIII levels), apart from FVL, that determine the APC response may differ between assays (16, 45, 46). The observation that the effect of the HR2 haplotype on the normalized APC-SR is only found in FVL carriers can be explained by the fact that the APC resistance assay reflects, among other things, the ratio between the concentrations of the wildtype FV molecule and the FVL molecule in plasma (9). The R2 allele is associated with reduced FV levels and as a result the relative concentration of the FVL molecule increases, leading to a more reduced normalized APC-SR. More gravely, this effect is observed in compound heterozygous carriers of FVL and a quantitative FV deficiency (47-50). These so-called pseudo-homozygous FVL carriers have reduced normalized APC-SRs within the range of homozygous FVL carriers, due to the decrease in the relative concentration of the normal FV molecule.

We conclude that the HR2 haplotype is associated with reduced FVag levels and with a reduced sensitivity for APC in FVL carriers. It is not associated with a reduced sensitivity for APC in non-FVL carriers or with an increased risk of venous thrombosis. The mechanism which underlies the reduction of FV levels has to be further investigated.

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