The HR2 Haplotype of Coagulation Factor V: Biochemistry, Epidemiology, and Clinical Relevance

Der HR2-Haplotyp des Gerinnungsfaktors V: Biochemie, Epidemiologie und klinische Bedeutung

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Summary: The HR2 haplotype of factor V represents an apparently very ancient group of tightly linked mutations throughout the factor V gene. It has been associated with increased activated protein C resistance, reduced factor V levels, and an increased risk of venous thromboembolism. However, reports are not consistent. Recent studies about the functional activity of factor V HR2 demonstrated that factor V HR2 is inactivated by activated protein C at the same rate as normal factor V and that in the prothrombinase complex it acts as well as factor V. On the other hand, it is more prothrombotic because of an increased ratio of factor V1 to factor V2, with factor V1 being the more procoagulant isoform of circulating factor V, with reduced affinity to negatively charged phospholipids. It is possible that discrepancies in the results of epidemiological studies linking factor V HR2 to thrombosis are due to several factors, e.g. differences in end-points, characteristics of patients and controls, and methods of measurement of resistance to activated protein C. Then again, factor V HR2 might be a mild risk factor on its own, while it might increase thrombotic risk if in combination with other risk factors (such as factor V Leiden) as preliminary studies suggest. Another intriguing possibility is that the association of reduced factor V levels and factor V HR2 might also be prothrombotic, since the combination would presumably lead to a globally reduced cofactor activity of factor V to factor VIII inactivation by activated protein C. Finally, several aspects of the relationship of HR2 and venous thromboembolism have not been explored until now, such as the association of this haplotype with thrombosis in unusual sites or in combination with acquired risk factors.

Keywords: factor V HR2; venous thrombosis; arterial thrombosis; APC resistance; factor V deficiency.

Zusammenfassung: Der HR2-Haplotyp des Faktor V repräsentiert eine vermutlich sehr alte Gruppe von fest gekoppelten Mutationen im ganzen Faktor V-Gen. Er wurde mit einer erhöhten Aktiviertes Protein C

(APC)-Resistenz, niedrigen Faktor V-Spiegeln und einem erhöhten Risiko für venöse Thromboembolien in Verbindung gebracht. Diesbezügliche Berichte sind jedoch nicht einheitlich. Neuere Arbeiten in bezug auf die funktionelle Aktivität von Faktor V HR2 haben gezeigt, dass der Faktor V HR2 durch aktiviertes Protein C genauso rasch wie normaler Faktor V inaktiviert wird und Faktor V HR2 im Prothrombinasekomplex die Cofaktorfunktion genauso gut wie Faktor V erfüllt. Andererseits zeichnet er sich aber durch eine erhöhte Ratio von Faktor V1 zu Faktor V2 aus. Damit ist Faktor V HR2 thrombogener als Faktor V, denn Faktor V1 ist eine mehr prokoagulatorische Isoform des zirkulierenden Faktor V mit herabgesetzter Affinität zu negativ geladenen Phospholipiden. Epidemiologische Studien, die den Zusammenhang von Faktor V HR2 und Thrombose zum Gegenstand hatten, führten möglicherweise deshalb zu unterschiedlichen Ergebnissen, weil unterschiedliche Endpunkte, ungleich charakterisierte Patienten bzw. Kontrollpersonen und unterschiedliche Methoden zur Messung der APC-Resistenz zugrundegelegt wurden. Der Faktor V HR2 könnte alleine ein recht milder Risikofaktor sein, das Thrombooserisiko aber, wie erste Studien zeigen, vor allem dann erhöhen, wenn es in Verbindung mit anderen Risiken (z. B. Faktor V Leiden) auftritt. Möglicherweise wirkt der Faktor V HR2 zusammen mit niedrigen Faktor V-Spiegeln prokoagulatorisch. Diese Kombination könnte nämlich zu einer insgesamt reduzierten Cofaktor-Aktivität des Faktor V bei der Inaktivierung des Faktor VIII durch APC führen. Letztlich sind einige Seiten der Beziehung von HR2 und venöser Thromboembolie noch nicht erforscht, so z.B. der Zusammenhang dieses Haplotyps mit Thrombosen an seltenen Stellen oder mit erworbenen Risikofaktoren.

Schlüsselwörter: Faktor V HR2; Venöse Thrombose; Arterielle Thrombose; APC-Resistenz; Faktor V-Mangel.

The HR2 haplotype of factor V consists of several mutations linked with different strength throughout the factor V gene. It is associated with functional modifications of factor V and possibly with clinical effects. In order to gain insight into this ancient haplotype and its clinical relevance, it is first of all necessary to re-

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Figure 1 Schematic representation of factor V and factor Va, with the potential glycosylation site at Asp 2181 (C2-domain).

view some of the current knowledge about the properties and functions of coagulation factor V. I shall then proceed to describe the genetic composition and functional properties of HR2 and the results of several clinical studies. I have to forewarn the readers that this topic is still "in construction", that is to say that all conclusions are provisional, as an Italian poet wrote [1], and subject to change. Similar to other polymorphisms and as mentioned in another setting [2], answers to clinical queries are lagging behind functional biochemical data because of the small size of most epidemiological studies and because of the heterogeneous end-points. Still, there are some indications that HR2 might be clinically relevant, and thus, I believe it is worthwhile, even at this stage of our knowledge, to keep it in mind when evaluating the proneness of a patient to inherited thrombophilia.

Factor V

Factor V is a single-chain plasma glycoprotein, also stored in platelets, synthesised mainly in the hepatocytes (reviewed extensively in [3]). After synthesis, it is extensively modified by glycosylation, sulphuration and phosphorylation [3]. N-glycosylation, which occurs predominantly in the B domain, is relevant to secretion and function. Particularly relevant to our topic is N-glycosylation in position Asn 2181 in the C2 domain. In fact, factor V circulates as two isoforms, which differ by glycosylation [4]: Factor V₁ is glycosylated in position Asn 2181, while factor V₂ is not. This apparently small difference lowers the affinity of factor Va₁ for negatively charged phospholipid membranes 45-fold compared to factor Va₂ [5]. Consequently, since factor Va is inactivated by activated protein C (APC) mainly in the presence of phos-

phatidylserine, factor Va₁ "lasts" longer and thus is 7-fold more thrombogenic than factor Va₂ [6].

Factor V is composed by a heavy chain (A1 and A2 domains), an activation peptide (B domain) and a light chain (A3 domain, C1 and C2 domains, Fig. 1). In order to function, factor V must be converted to its active form, factor Va. This, *in vivo*, is thought to be accomplished by limited proteolysis, by Xa and thrombin through multistep mechanisms (Fig. 1; [7]). Upon activation of factor V, the B domain is released in two fragments of 71 kd and 150 kd [7]. Such a large activation peptide, compared to those present in several other coagulation proteins containing only a few amino acids, poses the question of the role of the activation peptide in factor V. Several studies have concluded that the B domain might function as a spacer between the heavy and light chain in order to prevent constitutive FV activity [8]. Furthermore, sequences within the B domain are necessary for proper factor V activation by thrombin [9]. All these observations indicate that the B domain has an important role in factor V.

Physiologically, factor Va participates in the procoagulant mechanism as a non-enzymatic cofactor to factor Xa in prothrombin activation in the prothrombinase complex. This complex consists of factor Xa and factor Va, negatively charged phospholipids, prothrombin, and Ca²⁺ ions [3, 10, 11]. The structural requirements for the interaction of factor Va with the different components of the prothrombinase complex are partially resolved. The factor Va light chain participates in binding to phospholipid membranes and to APC, while protein S and prothrombin bind to the heavy chain [3]. Factor Xa binding occurs to both chains [3]. Calcium ions, besides favouring the inter-

action of Gla-containing proteins with negatively charged membranes, stabilise factor Va in the prothrombinase complex [3].

Factor Va is inactivated by APC in the presence of its cofactor, protein S [12]. Inactivation proceeds stepwise by sequential cleavage at Arg 506, 306, and 679. A common mutation at Arg 506 (G1619A, Arg506Gln, factor V Leiden) is an example of genetic variations in coagulation factors that lead to a pro-thrombotic rather than pro-haemorrhagic condition (gain-of-function mutation) [13]. Also factor VIIIa, the cofactor of the tenase complex and a glycoprotein in part homologous to factor V, is inactivated by limited proteolysis by APC, though this does not seem to be the only inactivation mechanism. In the case of factor VIIIa inactivation, APC is aided by factor V, which functions as a cofactor [14]. The carboxy-terminal portion of the B domain of factor V is probably the region relevant for this cofactorial activity [15]. Thus, a coagulation protein, which was previously considered only in its pro-coagulant capacity, is in fact also an anticoagulant. This knowledge sustains studies aimed at clarifying the procoagulant-prothrombotic role of mutations and polymorphisms in factor V.

The HR2 haplotype

Initially, a mutation in exon 13 (B domain) of the factor V gene, A4070G, predicting the amino acid change His1299Arg, was detected by sequencing [16]. This mutation creates a restriction site for RsaI and was thus termed the R2-polymorphism. It is frequent in the general population of many European countries and is present also in Turks, Indians, and Somali (see Tab. 1 for estimates). A highly conserved haplotype surrounds the R2 polymorphism as shown by several studies. Thus R2 is probably very ancient, dating back to times preceding the migration of man out of Africa, and is probably derived from a single mutational event.

Figure 2 shows the composition of the HR2 haplotype. Linkage disequilibrium analysis showed that the haplotype is highly conserved at the 3' end and between exons 8 and 13, while consensus decreases at

the 5' end. Three transitions, among which is R2, are invariantly present in this haplotype and characterise it, while the others can be present also in other factor V alleles (Fig. 2; [3, 16–18]).

Since several gene variations in the HR2 haplotype predict amino acid changes, including some in the B domain, it is reasonable to suppose that the HR2 factor V could have altered function compared to the wild type factor V. This has fostered extensive biochemical studies of HR2 factor V aiming at detecting its functional characteristics.

Biochemical aspects of factor V HR2

Activation and cofactor activity in prothrombinase

In vitro, extensive biochemical studies on the kinetics of activation of HR2 factor V and on the cofactor activity of HR2 factor Va in the prothrombinase complex allow us to conclude that these two functions overlap those of wild type factor V [19]. Similarly, HR2 factor V is inactivated by APC at the same rate as normal Va, and is equally susceptible to the enhancing effect of protein S [19].

The V₁/V₂ ratio

Factor V exists in plasma as two isoforms. The ratio between factor V₂ and factor V₁ in plasma in normal individuals was measured by a functional assay that exploits the increased prothrombinase activity of Va₂ at limiting concentrations of Va, Xa and phosphatidylserine compared to Va₁ [20]. The Va₂/Va₁ ratio was estimated at 2.8 (mean value) compared to 1.4 in homozygous carriers of the HR2 factor V [20]. Ion-exchange chromatography had previously yielded the same results, i.e. showed that factor VHR2 is enriched in V₁, the more procoagulant isoform of factor V [21]. According to the research group who reported these results, the most likely structural determinant of this altered ratio is one of the amino acid substitutions predicted by the HR2 haplotype, namely Gly2194Asp [21]. The reasons are related to its absolute linkage to R2, to the dramatic amino acid change (by bulk and by charge) and to its location in the linear sequence, 13 amino acids carboxy-terminal of the N-glycosylated Asn 2181 residue. However, subsequent crystal analysis of the mutant allowed to establish that Asp 2194 and Asn 2181 are spatially opposite, so that changes in glycosylation efficiency determined by the mutation is an unlikely explanation [3].

Resistance to activated protein C

Since the first report that HR2 is enriched in patients with APC resistance, either carriers or non-carriers of factor V Leiden, most clinical studies have measured APC resistance in their cases and controls. There exists no consensus concerning this topic, although most papers suggest an interaction between HR2 and factor V Leiden in enhancing APC resistance [17, 18, 22, 23]. Recently, Kostka et al. [24] and Hoekema et al. [20] reported that when APC resistance is measured in

Table 1 Prevalence of HR2 in different countries and ethnic groups (modified from ref [3])

Population	Sample size	Allelic frequency of R2	Authors [Ref. Number]
Italians	98	0.046	Bernardi et al. [17]
French	394	0.058	Alhenc-Gelas et al. [22]
Dutch	474	0.041	De Visser et al. [18]
English	469	0.055	Luddington et al. [23]
Germans	84	0.095	Kostka et al. [24]
Turks	144	0.048	Akar et al. [28]
Indians	39	0.102	Bernardi et al. [17]
Somali	40	0.087	Bernardi et al. [17]

Figure 2 The HR2 haplotype. The three transitions that are invariably part of the haplotype are highlighted in grey. The R2 polymorphism is indicated by an arrow. The HR2 haplotype is in bold. Exon lengths are not proportional (modified from reference 3).

HR2 carriers by a method which is sensitive to the APC cofactor activity of factor V, lower ratios could be shown compared to non-carriers. This would suggest that HR2 factor V loses its cofactor activity towards APC in the degradation of factor VIIIa. Discrepancies in the literature regarding the effect of HR2 on APC resistance could then be due both to patient selection and to the methodology employed to measure APC-resistance.

Stability in plasma and association with reduced factor V levels

In most HR2 studies, factor V levels have been measured as well. The majority of them showed lower factor V levels in carriers than in non carriers of the HR2 allele, though differences are rather small [18, 20, 22, 23]. All these studies selected patients because of thrombosis and HR2 carriership. The first-mentioned study selected patients because of factor V levels and then verified the prevalence of HR2 [16].

If one concurs with the conclusion that factor V levels are lower in HR2 carriers, the subsequent step is to establish why. Stability of factor V HR2 in plasma is apparently normal as measured by decay curves over time in plasma compared to normal factor V [17]. As mentioned earlier, inactivation by APC is similar to wild-type factor V. A possible alternative explanation of the lower levels could be a polymorphism in the promoter region, in close linkage disequilibrium with HR2. de Visser et al. looked at the promoter of factor V HR2 and did not detect mutations [18]. Another possibility is just chance. Since both factor V deficiency and HR2 are common in the general population, the probability of finding them in association is relatively high. By doing some rough conservative estimates, one might expect approximately 5 in 1000 individuals to carry both defects. Finally, the association of low factor V levels and HR2, similarly to what happens for pseudohomozygous APC resistance as suggested by one study [25], could be more thrombogenic than HR2 alone. Thus, one might expect to find the association more commonly in thrombophilic patients. The pro-

thrombotic potential of this association may be due to the greatly reduced cofactor activity for factor VIIIa inactivation by APC. Despite the fact that no formal evidence exists so far for this occurrence, it is a hypothesis worth testing on a large scale.

Table 2 summarizes the functional characteristics of HR2 factor V.

Clinical studies on thrombotic risk

In consideration of the fact that biochemical data show that HR2 is prothrombotic in its nature, and since epidemiological data report it to be very frequent, one might expect that HR2 would be definitely associated with an increased risk of venous thrombosis. However, reports from the literature are very disappointing in this regard. An overview of the available data will illustrate this point.

Association with an increased risk of venous thromboembolism

A number of studies that deal with HR2 have attempted to estimate an association between this allele and thrombotic risk. Table 3 reports these results schematically. No final conclusion can be drawn, possibly due to heterogeneity of the studies both in terms of patient selection (type of thrombosis, referral criteria), control selection (from the general population, blood donors), and in terms of numbers of patients analysed. As can be seen from the Table, the most striking association is

Table 2 Functional characteristics of HR2 factor V

- Increased ratio between factor V1, the more procoagulant isoform, and factor V2
- Decreased cofactor activity to activated protein C in the degradation of factor VIIIa
- Decreased plasma levels of factor V (not consistent)
- Increased resistance to activated protein C (not consistent)

that of the synergic effect with factor V Leiden. Though these results were derived from a very large study, the actual numbers of patients who carried both defects was small [26]. Results derived from the LETS study, a large population-based survey, were inconclusive in this regard, too [18].

Association with an increased risk of arterial thrombosis

Results concerning the association of HR2 with arterial thrombosis are even more difficult to interpret. So far, only three studies have dealt with this issue. One analysed the frequency of HR2 in patients with coronary artery disease (any type of coronary event) and concluded that HR2 carriership is a risk factor for coronary artery disease (OR=2.0, 95 % CI= 1.4–2.9) [29]. The other dealt with cerebral infarction in children and found an OR for carriership of 3.9, 95 % CI 1.2–12.3 [30]. Finally, *Doggen* et al. in the SMILE study, a large case-control study of 560 males with a first myocardial infarction before the age of 70, reported that there was no association between HR2 and myocardial infarction (OR 0.9, 95 % CI 0.6–1.4) [31].

Comments

At the end of the story, the question always is, and understandably so, what should one do with HR2?

Health policies in most countries in which thrombophilia is an issue are ever more constrained in terms of money. Should one include HR2 in thrombophilia screening, among the many other tests? The answer is not an easy one. First of all, there is some doubt as to the usefulness of any thrombophilia screening to start with [32]. A thrombophilia test, to be considered useful, should have a number of characteristics: it should reasonably predict a thrombotic event or be convincingly associated with an increased risk of venous thromboembolism. It should be easy to perform and relatively inexpensive. It should be linked to thrombophilia by understanding of its potential causative effect. Even if all these conditions held true, one should still evaluate the benefit of testing. Would knowledge of carriership of HR2 (or any other risk factor, for that matter) change the clinical course of thrombophilia? I do not wish to enter the more general debate on thrombophilia testing. I believe so far we do not have the necessary information to answer the basic question: does testing for thrombophilia change the clinical course of thrombophilia (for example, by modifying type of treatment, duration of anticoagulant therapy, prophylaxis of risk situations or counselling for pregnancy or oral contraceptive use etc.). I will just attempt an answer circumscribed to HR2, for which some of the conditions described above are met. HR2 is easily screened for (due to linkage within the haplo-

Table 3 Results of clinical studies reporting on the association between HR2 factor V and venous thromboembolism

Author, year [reference number]	Type of patients and number	Type of controls and number	OR for HR2 carriership
<i>Bernardi</i> et al., 1997 [17]	With venous thromboembolism, consecutively referred –100	Population based 98	n.d.
<i>Alhenc-Gelas</i> et al., 1999 [22]	Unselected with venous thromboembolism 205	Healthy controls from health center 394	1.8 (95 % CI 1.1–2.8)
<i>Faioni</i> et al., 1999 [26]	Family study 810 family members 23 with HR2 and fV Leiden	238 without HR2, with fV Leiden	10.9 (95 % CI 2.9–40.6)*
<i>Le Cam-Duchez</i> et al., 1999 [27]	Family study 370 members	Controls from health care center 394	0.05 vs 0.06#
<i>De Visser</i> et al., 2000 [18]	Patients with a first deep-vein thrombosis 474 (LETS study)	Population based 474	1.2 (95 % CI 0.8–2.0)
<i>Luddington</i> et al., 2000 [23]	Consecutive patients with history of venous thrombosis 581	Blood donors 469	1.21 (95 % CI 0.84–1.74)
<i>Akar</i> et al., 2000 [27]	Patients with venous thromboembolic disease 148	Unrelated healthy individuals 82	0.8 (95 % CI 0.16–0.94)§

*OR for carriership of HR2 and factor V Leiden compared to factor V Leiden alone

#allelic frequency of HR2 in cases compared to controls

§after exclusion of factor V Leiden carriers

n.d. not determined

type, one can test just for R2, by enzyme restriction or ARMS) and relatively inexpensive. We know enough of its functional characteristics to understand its pro-thrombotic potential. The problem is that we do not have convincing clinical data to associate it without doubt with an increased thrombotic risk. At best, it seems to be a mild risk factor for venous thromboembolism. In the frame of the modern concepts of thrombophilia, it might be more relevant, due also to its high frequency and widespread occurrence, in association with other established risk factors. The more promising association seems to be with factor V Leiden. The sum of two dysfunctional factor V alleles, both of which are pro-thrombotic, is in fact expected to lead to a more marked thrombotic clinical phenotype. For the same reason, the association of HR2 with low factor V levels might lead to thrombophilia, although even less data are available. Other up to now unknown associations might be relevant and explain the inconsistency of the results of the clinical studies. Moreover, there is some recent indication that risk factors for thrombophilia might differentially interact with local factors to localise thrombosis in some sites rather than others. So far, this has not been explored for HR2 and could open a different direction of prospective investigations. At present, however, without solid epidemiological information and authoritative guidelines, clinicians can rely only on their perception of the problem, and ultimately on cost-benefit considerations within the health policies of their individual countries or institutions.

Relating HR2 to arterial thrombosis is even more complex, and not only because of the limited number of subjects tested in the two positive studies mentioned above, or the wide, ill-defined end-points. The main problem is that arterial thrombosis results from the interaction of multiple acquired and inherited risk factors on the background of atherogenesis. Thus, it is highly unlikely that a strong inherited risk factor for arterial thrombosis, the final event of a long multi-step process, could be identified. It is more likely that risk factors would be identified for each step of the pathological process and for each system involved in the atherogenesis and thrombogenesis processes. A large number of weak risk factors have been identified so far, which are often contradictory when subsequently tested in larger studies or the plausibility of which is ignored. Possibly, a different approach to identify risk factors for arterial thrombosis among coagulation proteins should be designed.

Perspectives

As for other putative risk factors for venous thromboembolism or for arterial thrombosis, for HR2 a need for some more epidemiological data is also felt. The interaction with other defects of factor V seems to be most promising. Due to the widespread occurrence of HR2 factor V and its similar prevalence in all countries, a multicenter effort in designing well-defined

studies should be affordable. In the end, such an approach aiming at getting convincing answers relative to the clinical impact of HR2 carriership, might be more cost-effective than screening all (or even some) thrombophilic patients for HR2. On the other hand, most likely the time has come to revise our approach to thrombophilia screening in general and to the search for new thrombophilic defects in particular. Whatever new directions these novel approaches might take, I believe they necessarily should include multicentre and multidisciplinary co-operation.

Acknowledgements

The author is particularly indebted to *Elisabetta Castoldi* who did a lot of the work on HR2 and whose PhD thesis was a generous source for this review.

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