Original Article

Comparative Frequency of Coagulation Factor II and Coagulation Factor V Alleles among New-Born and Senior Citizens

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Abstract

Resistance to activated protein C is one of the most common inherited disorders associated with hereditary thrombophilia. A missense mutation in the gene coding for coagulation factor V (CF V Leiden) and which renders this procoagulant factor resistant to inactivation by activated protein C results in an inherited risk for venous thrombosis. Recently, another mutation has been identified in the prothrombin gene (CF II G20210A) which was also associated with increased risk for venous thrombosis. In this study, we sought to establish the frequency of the two alleles in a random sample of Maltese newborn and compare these with the frequencies of the same alleles among senior citizens and patients with clinical thrombophilia.

The control population of 554 newborn samples processed for the same point mutations gave 13 (2.3%) who were CF V Leiden heterozygotes and 7 (2.7%) who were CF II G20210A heterozygotes. Neither homozygotes nor trans-heterozygotes (i.e. CF V Leden and CF II20210A heterozygotes) were observed.

The 348 senior citizens gave 9 (2.6%) CF V Leiden heterozygotes and 8 (2.4%) CF II G20210A heterozygotes. Neither homozygotes nor trans-heterozygotes (i.e. CF V Leden and CF II20210A heterozygotes) were observed. The 328 patients referred to the Laboratory of Molecular Genetics, University of Malta, with clinical thrombosis gave 23 (7.01%) CF V Leiden heterozygotes and 24 (7.31%) CF II G20210A heterozygous. One patient was found to be trans-heterozygous for the two mutations.

The data suggested that although CF V G1691A and CF II G20210A may increase risk for thrombophilia, they do not impact on the survival of the carriers, but the transheterozygosity may also confer increased risk. The high allele frequency may be best explained by positive natural selection.

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Introduction

The mutation in coagulation factor V (CF V) G1691A, known as factor V Leiden[1], and the recently described mutation in the prothrombin (CF II) gene G20210A[2] are the two most prevalent known causes of inherited thrombophilia. The

impaired neutralization of thrombin or a failure to control the generation of thrombin increases the risk of clinical thrombosis.[3] Since inherited hypercoagulability is a persistent, lifelong condition, whereas thrombosis occurs only episodically, one might argue that all thrombotic events must be precipitated by an acquired thrombogenic trigger. In many cases, the transient risk factor is clinically apparent (e.g., the postoperative state, immobilization, or the peripartum period). In others, in which thrombosis is considered to be idiopathic, the acquired trigger is probably a sub clinical thrombogenic stimulus. Thus, many cases of venous thrombosis may involve the convergence of one or more inherited prothrombotic mutations (leading to a lifelong state of hypercoagulability) and an acquired thrombogenic insult that precipitates the actual clinical event.[4] In patients with relatively low base-line degrees of hypercoagulability (e.g., those without any, or with a single mutation, such as CF V Leiden), a major, clinically clear stimulus would be required to trigger the thrombotic episode. In contrast, patients with higher base-line degrees of hypercoagulability (e.g., those with multiple mutations in the coagulation pathway) would require only relatively minor, often subclinical, acquired insults to precipitate thrombosis.

In haemostasis, CF V is activated by thrombin when the latter is produced by the action of CF Xa on prothrombin (CF II); CF Va then acts as a cofactor of CF Xa and together they associate with Ca^{2+} on the phospholipid surface of platelets or endothelia to form an active "*prothrombinase*" that converts additional prothrombin to thrombin.[5] CF Va catalyses the rate of CF Xa mediated prothrombin activation by a 1,000 fold.[6] CF Va is inactivated by activated protein C thus limiting thrombin formation from *prothrombinase*. However, the CF V G1691A mutant resists inactivation leading to excess thrombin generation and predisposition to thrombosis.[6] CF V Leiden is a common mutation in Caucasians, with a heterozygote frequency ranging from 2% to 15% in several populations.[7,8] However, it also shows important regional differences ranging from 2% in South Europe to 16% in parts of North Europe.[7] The risk of thrombosis is increased sevenfold in heterozygotes and 80-fold in homozygotes as compared with non carriers.[9] In Southeast Asia and Africa the frequency of the mutation is less than 1%.[7]

The CF II G20210A mutation has also been associated with increased prothrombin levels in the plasma.[2] The thrombosis risk is increased by two when plasma CF II levels exceed 115% of normal.[2,10] The frequency of the prothrombin variant in Caucasian populations is about 2%.[11] Within Europe the mutation frequency shows a marked increase from 1.7% in the North to 3% in the South.[11]

Both these mutations can be co-inherited (trans-heterozygotes) leading to much increased risk of thrombosis possibly manifesting in younger patients and having increased severity.

In this study we report the comparative frequencies of the CF V Leiden and CF II G20210A alleles in a number of random newborn, a number of senior citizens and patients referred for investigation of clinical thrombotic episodes. The data showed that although there was no significant difference in frequencies between the newborn and the senior citizens, thus excluding effects on survival, a much higher frequency was found among the patients with thrombosis.

Materials and Methods

The study was approved by the Research Ethics Review Board of the Faculty of Medicine of the University of Malta. The blood samples were collected by venipuncture in EDTA vacutainers (Becton Dickinson Vacutainer Systems, UK) anonymised and stored at a temperature of -20∞C in a locked freezer. Blood was collected from random senior citizens (348) referred through the Haematology Department from the outpatients clinic at St. Luke's Hospital, Gwardamangia, Malta. Umbilical cord blood samples (554) were collected daily from the maternity ward at St. Luke's Hospital and patients (328) were referred for investigation in connection with clinical thrombosis.

Genetic Analysis

DNA extraction was performed using the Nucleon!" BACC1 Kit (Amersham Life Science). DNA analysis for the 1691 G to A substitution in the CF V gene was conducted by restriction endonuclease digestion of a polymerase chain reaction (PCR) product as described by Bertina *et al* (1994; Figure 1). Restriction endonuclease digestion of the CF V PCR product with Mnl 1 resulted in three fragments of 22bp, 37bp and 116bp (Figure 1). In the presence of the A at 1691(CF V Leiden), one of the recognition sites is blocked and the enzyme cuts only once resulting in two fragments of 22bp and 153bp. Analysis of the 20210 G to A substitution in the 3'-untranslated region of the prothrombin gene was conducted by restriction endonuclease digestion of the corresponding PCR product as described by Poort *et al* (1996; Figure 2). The CF II PCR was carried out using a mutagenised reverse primer 5'-ATA GCA CTG GGA GCA TTG A<u>A</u>G C-3' where the underlined adenosine

base is absent from the wild type sequence. The primer together with the G20210A point mutation of CF II, created a HindIII[2] recognition site. The undigested CF II PCR product was 345bp long. In the presence of the G20210A point mutation, the CF II PCR product had one HindIII recognition site resulting into two fragments, one of 322bp and one of 23bp in length (Figure 2).
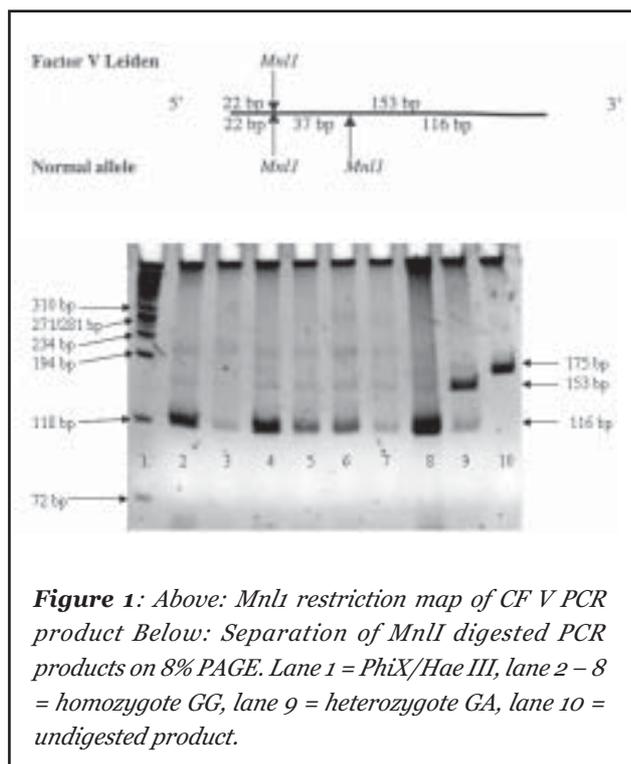
Statistical Analysis

The data were analysed statistically by the Hardy-Weinberg Equilibrium, Fisher Exact test, Chi Square Contingency test and Odds Ratio.

Results

All data are summarised in Table I

Coagulation Factor V

CF V restriction endonuclease digestion yielded 13 GA heterozygotes (2.3%) and no abnormal homozygotes among the newborn population and 9 GA heterozygotes (2.6%) and no abnormal homozygotes among the senior citizen population. No significant difference was observed between these two populations. CF V restriction endonuclease digestion among the referred patients yielded 23 GA heterozygotes (7.0%) and no abnormal homozygotes. The referred population frequencies were found to be significantly different when compared to the frequencies found in the random newborn and the senior citizens (Table I).

Figure 1: *Above: Mnl1 restriction map of CF V PCR product Below: Separation of MnlI digested PCR products on 8% PAGE. Lane 1 = PhiX/Hae III, lane 2 – 8 = homozygote GG, lane 9 = heterozygote GA, lane 10 = undigested product.*
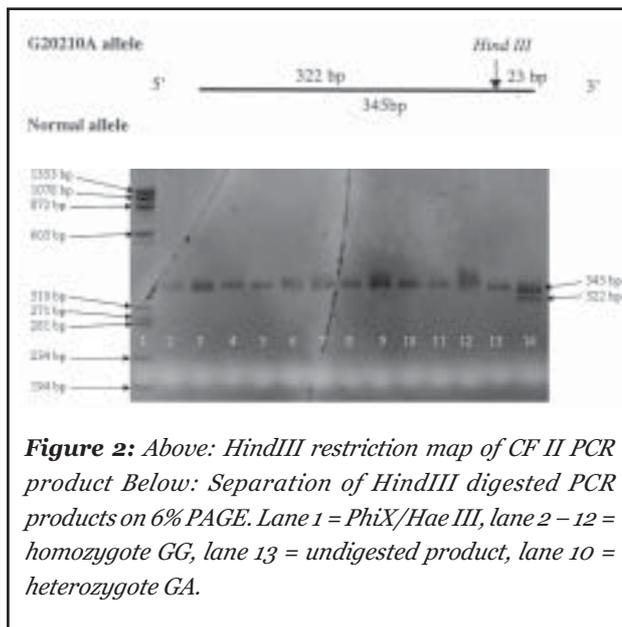
Coagulation Factor II

CF II restriction endonuclease digestion yielded 7 GA heterozygotes (2.7%) and no abnormal homozygotes among the newborn population and 8 GA heterozygotes (2.4%) and no abnormal homozygotes among the senior citizens. No significant difference was observed between these two populations. CF II restriction endonuclease digestion among the referred patients yielded 24 GA heterozygotes (7.3%) and no abnormal homozygotes. The referred population frequencies were found to be significantly different when compared to the frequencies found in the senior citizen population (Table I). One trans-heterozygote was also found among them.

Discussion

We have shown here that, somewhat contrary to expectations, the allele frequency of the CF II G20210A and the CF V G1691A, although high were practically the same among the random newborn and the senior citizens in our study. Although both alleles increase the risk of thrombosis, as shown by the finding of even higher frequencies (0.035 and 0.037) among the patients with a variety of clinical thrombosis and the studies of others[9-11], the relatively high frequency should imply a positive element of natural selection, although founder effects and genetic drift cannot be formally excluded. The high frequency of haemolytic anaemia due to haemogobinopathy and G6PD deficiency among populations from geographic areas endemic for malaria[12] or, possibly other intra-erythrocytic parasites such as Leishmania, and, of Cystic Fibrosis with respect to diarrhoeal virus[13] are good examples of positive natural selection. One may assume that in the

Figure 2: *Above: HindIII restriction map of CF II PCR product Below: Separation of HindIII digested PCR products on 6% PAGE. Lane 1 = PhiX/Hae III, lane 2 – 12 = homozygote GG, lane 13 = undigested product, lane 10 = heterozygote GA.*

Table 1: *Genotype distribution and allele frequencies of CF V Leiden and CF II G20210A in Senior Citizens, Control Cords and Referred Samples. The results of the statistical analysis are given for every mutation. Bold result means statistical significance.*

MUTATION											
	SENIOR CITIZENS			**CONTROL CORDS**[A]			**REFERRED SAMPLES**[B]			
	Genotype		Allele Freq.	Genotype		Allele Freq.	Genotype		Allele Freq.	
FACTOR V LEIDEN	N = 348	%	G=0.987	N = 554	%	G=0.988	N = 328	%	G=0.965	
	GG 339	97.4		GG 541	97.7		GG 305	93.0		
	GA 9	2.6	A=0.013	GA 13	2.3	A=0.012	GA 23	7.01	A=0.035	
	AA 0	0		AA 0	0		AA 0	0		
	Senior Citizens versus			Fisher Exact 2-Tail : p-value = 0.82 Chi Square = 0.0516 OR = 0.9 (95% C.I. from 0.38 to 2.14)			Fisher Exact 2-Tail : p-value = *0.01* Chi-square = *7.33* (significant to 0.01 level) OR = *2.84* (95% C.I. from 1.29 to 6.23)			
FACTOR II G20210A	N = 335	%	G=0.988	N = 260	%	G=0.987	N = 328	%	G=0.963	
	GG 327	97.6		GG 253	97.3		GG 304	92.7		
	GA 8	2.4	A=0.012	GA 7	2.7	A=0.013	GA 24	7.31	A=0.037	
	AA 0	0		AA 0	0		AA 0	0		
	Senior Citizens versus			Fisher Exact 2-Tail : p-value = 0.79 Chi Square = 0.0551 OR = *1.13* (95% C.I. from 0.4 to 3.16)			Fisher Exact 2-Tail : p-value = *0.003* Chi-square = *8.76* (significant to 0.01 level) OR = *3.227* (95% C.I. from 1.43 to 7.29)			

A) *Based on genetic analysis of random Maltese cord samples [24] pooled together with recent cord analysis of 285 cord in CF V Leiden and 126 cords in CF II.*

B) *Based on genetic analysis of patient samples that are referred to the Laboratory of Molecular Genetics for thrombosis analysis*

C) *P- Value is greater than 0.05, the null hypothesis is accepted i.e. there is no significant difference between the frequencies in Senior Citizens and the other group under study.*

D) *For significant difference at the 0.05 level, chi-square should be greater than or equal to 3.84. The null hypothesis is accepted, i.e. the frequencies do not differ.*

E) *An OR > 1 indicates that there might be an association between the genotype and the phenotype or medical disorder.*

migration of humans out of Africa into Northern Europe either the CF II G20210A or the CF V G1691A carrier could have survived traumatic or obstetric bleeding better than their wild type travellers.   Recently, it has been shown that the CF V Leiden heterozygotes preferentially survived severe sepsis and a similar observation was made on a mouse model of endotoxaemia.[14] Thus, natural selection appears a more satisfactory and patho-physiologically sound explanation than drift or founder effects. Based on our prior observation on the population distribution of CF VII frameworks[15, 16], we had anticipated a lower frequency of one or both thrombogenic alleles among the older subjects assuming that fatal thrombosis would have decreased the number of surviving heterozygotes, homozygotes and trans-heterozygotes.

The modern view of haemostasis places the CV VII-tissue factor complex in a critical step for initiation and amplification of thrombosis.   We[15, 16] and others[17-21] showed that the production of CF VIIa is genetically regulated due to two alternate forms of the CF VII gene with one being more active than the other. The allele frequency of the slower variant, CF VII Framework II in Shinawi et al[16] is found more frequently among the older sector of the population. Presumably, they may have been protected from the "hypercoaguable" state by the slow variant. In this study, however, the results showed no significant difference between the control newborn population and the senior citizens population, implying that the frequencies of both the CF V Leiden and the CF II G20210A alleles did not change with age and thus did not reflect the occurrence of fatal outcomes.

Similarly the frequency of the HFE allele of hereditary haemochromatosis has been found higher among the elderly in Sicily compared to younger subjects.[22-24] Clearly, there must be many other alleles of many other genes whose enrichment among senior citizens compared to neonates, or vice versa, provides an efficient tool for the discovery of "patho-physiologically meaningful sequence variation" in man.

Thus, unlike CF VII frameworks, the CF II G20210A and CF V G1691A alleles did not appear to increase the risk of fatal thrombosis.   Alternatively the current level of healthcare in Europe compensates for the additional risk.   The interplay between alpha Thalassaemia and Hb S homozygotes (sickle cell disease) provides grounds for comparison.[25,26]   A concurrent alpha thalassaemia modifies the developmental haematology of sickle cell disease with alpha plus thalassaemia homozygotes having a much milder anaemia.   A higher frequency of alpha Thalassaemia can be found among older patients with sickle cell disease from less resourced countries where health care is poor. We could not show any difference among the patients from North America with appropriate health care.[26]

There is a reciprocal North-South gradient in the relative frequency of the CF II G20210A and CFV G1691A alleles. It appears that because of the higher risk of the trans-heterozygotes and homozygotes the higher frequency of one allele could keep the other down. Geographic gradients have also been seen in beta-Thalassaemia with the b+ (Beta positive) IVS-1, 6C thalassaemia being commoner in the Western Mediterranean and the b+ (Beta positive) IVS-I , 110A thalassaemia being commoner in the Eastern Mediterranean[27]. In Cystic Fibrosis the CFTR; DF508 mutation frequency is increased in the North and declines in the South except in Malta and the Basque region of Spain.[28] The geographic epidemiology of alleles may well reflect the outcome of evolutionary pressures on human migration, which resulted in contemporary genomes.

These data strongly confirm the pathophysiologic risk of thrombosis due to hypercoagulability among CF V G1619A, CF II G20210A heterozygotes and trans-heterozygotes. However, the two mutations do not appear to influence mortality from the clinical events.   In future studies it would be useful to conduct prospective clinical follow up among CF V G1619A, and CF II G20210A carriers conditioned on their CF VII genotype and the response to therapeutic procedures.

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