Original Article

Co-inheritance of Factor V Leiden in Cases with Inherited Hemophilia A in North India

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Abstract

Background: Considerable variation has been noted in the age at onset and bleeding pattern in Cases with Hemophilia-A (CWH) of severe type. Mechanisms seem multifactorial with co-inheritance of Factor V Leiden mutation (FVL) with severe Hemophilia A (HA) being one of them. There is evidence that carrier ship of FVL mutation in CWH might confer an evolutionary selective advantage leading to a milder clinical phenotype. The authors hypothesized that FVL mutation may be more prevalent in CWH. The present study was undertaken to determine the prevalence of FVL mutation in CWH and evaluate the phenotypic effect of this co-inheritance on clinical severity of hemophilia.

Methods: A total of 100 CWH were recruited for this study. Detailed clinical history regarding the age at onset, type, frequency and site of bleeding was taken. Coagulation work up including Factor VIII (FVIII) assay and von willebrand factor antigen (VWFAg) were done. FVL mutation and intron 22 inversion mutation were studied.

Result: Fifty six CWH had FVIII <1% categorizing them into severe category. FVL mutation was present in 3 cases. All the three cases had FVIII levels <1% with Intron 22 inversion, were heterozygous for FVL and were clinically severe. Out of these three patients, two were siblings and the third sibling who is also a severe hemophilia patient, did not carry FVL mutation.

Conclusion: The results suggest that prevalence of FVL mutation in CWH is similar to that in non-hemophilic population and this mutation has no definite influence on the clinical severity in CWH.

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Introduction

Hemophilia A (HA), an X-linked recessive bleeding disorder, affects approximately 1 in 5,000 males and is caused by deficiency of coagulation factor VIII (FVIII) resulting from mutations in FVIII gene including Inversion 1, Inversion 22 and small mutations in any of the 26 exonic regions. Based on measurable FVIII activity, Hemophilia is classified as severe (<1%), moderate (1-5%) and mild (6-35%). Symptoms usually correlate well with the residual FVIII activity. Clinical phenotype of cases with Hemophilia (CWH) with FVIII <1% consists of spontaneous joint and muscle bleeds. Typically, the onset of bleeding in such patients is early, coinciding with increasing physical activity. However, there is clear evidence that there is considerable variability in the bleeding pattern and age of onset of hemorrhage among patients with severe hemophilia.[1] The mechanism of this phenotypic heterogeneity is not well understood, although it seems multifactorial and involve acquired or genetic factors [2,3] including co-inheritance of some prothrombotic genetic mutations, impaired fibrinolysis, and elevated endogenous thrombin generation.[4-6] Of these, prothrombotic genetic mutations have drawn much attention especially Factor V Leiden mutation (FVL) and Factor II mutation which are the two most frequent genetic risk factors for venous thrombosis. There are several reports available on severe hemophiliacs which suggest that co-inheritance of prothrombotic mutations was related to a milder clinical phenotype. [7-9] However few reports have advocated that co-inheritance of HA with FVL did not seem to influence the severity of bleeding events. [10,11]

The prevalence of FVL in general population world over varies from 0-15% being almost absent in Africa, South East Asia and Middle East and highest in Greeks.[12] Its prevalence in various parts of India has been reported to be between 1-4.1%.[13-15] Factor II mutations, however, have not been reported either in controls or patients in India.[13,16]

There is mounting evidence that carrier ship of FVL mutation might confer an evolutionary selective advantage. [17] The authors hypothesized that FVL mutation may be more prevalent in CWH. Prevalence of FVL mutation in CWH from India has not been reported earlier. Although there are few studies on the effect of co-inheritance of thrombophilia in hemophilia cases from India, the results are discrepant. [7,18-20] The present study was therefore undertaken to determine the prevalence of FVL mutation in CWH in North India and its effect on modulating the clinical phenotype.

Materials and Methods

One hundred CWH were recruited through the Hemophilia Federation of India, Lucknow and Varanasi Chapter representing the North Indian population. Peripheral venous blood (5ml) was collected in 0.5M Ethylene diamine tetraacetic acid and 3.2% Sodium citrate anticoagulant (1:9). Consent for entry into the study was taken from all cases/families on approved format. Institutional Ethical approval was obtained.

Clinical assessment: History regarding type of bleeding, frequency of bleeding episodes, age of onset, bleeding into joints/ joint deformity was taken and accordingly cases were categorized into mild-moderate and severe HA as per the defined criteria. [21,22]

Factor VIII bioassay: Factor VIII bioassay was performed by the standard method of one stage assay for FVIII based on Activated Partial Thromboplastin Time on fully automated coagulation analyser (STA Compact-Diagnostica Stago, France) using APTT reagent, FVIII deficient plasma, Unicalibrator and Calcium chloride 0.025M (All from Diagnostica Stago, France). Depending on the factor levels, cases were categorized into mild (6-35%), moderate (1-5%) and severe (<1%).

FVL mutation detection: DNA was extracted using column based Kit (Invitrogen, Germany) from peripheral blood leucocytes as per manufacturer's instruction and stored at -80^{o}C. Extracted DNA was tested for FVL mutation using polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP). In brief, a 220 bp segment of Factor V gene was amplified using specific primers (5' TGC CCA GTG CTT AAC AAG ACC A 3') and (5' CTT GAA GGA AAT GCC CCA TTA 3') as previously described. [10] The PCR product (15ul) was digested with 3ul of Mnl 1 restriction enzyme (Thermoscientific) and Buffer G at 37 ºC for 16 hours and subjected to 30% polyacrylamide gel electrophoresis. This region of exon 10 of Factor V gene contains two restriction sites for Mnl1 at nucleotides 1637 and 1694.The gel was stained with ethidium bromide and viewed with a gel documentation system. The digested amplicon of normal Factor V gene (allele 1691G), gives three bands of size 116, 67 and 37bp. When allele 1691A was present, the cleavage site for Mnl1 at nucleotide 1694 was involved and fragments of 67 and 153bp were obtained.

Intron 22 inversion: Intron 22 inversion was performed by Inverse PCR protocol described by Rossetti et al [23].

Other coagulation tests like Factor IX assay, von Willebrand Factor Antigen assay (VWFAg), mixing studies for inhibitor screen were performed as and when indicated.

Result

A total of 100 CWH were included in this prospective study with an age range of 1-48 years. Based on the FVIII assay, 9 cases were categorized into mild, 35 into moderate and 56 into severe HA. There was family history suggestive of hemophilia in 68 cases and no history in 32 cases. Intron 22 inversion was detected in 33 cases out of which 9 (25.7%) were moderate and 24 (52%) severe HA. FVL mutation was detected in 3 cases (3% of CWH) all of whom had FVIII <1% and carried Intron 22 inversion. All three were heterozygous for FVL mutation (Table 1). Further analysis of clinical details of the three cases revealed that all of them had first symptomatic bleeding by the age of first year with spontaneous

Table 1: showing the distribution of cases with Hemophilia A into various categories of severity, age range, family history, Intron 22 inversion mutation and factor V Leiden mutation (n=100)

Category (Factor VIII %)	Number of cases	Age range (years)	Positive family history (%)	Intron 22 inversion (%)	Factor V Leiden mutation	Zygosity
Mild (6-35)	9	7-33	4 (44)	0 (0)	0	-
Moderate (1-5)	35	4-48	9 (25)	9 (25.7)	0	-
Severity (<1)	56	1-47	19 (34)	24 (52)	3	heterozygous

Table 2: showing Factor VIII levels and clinical severity profile of cases with Factor V Leiden mutation (n=3)

Case number	Age (years)	Category/Factor VIII %	Age at onset of bleeding	Frequency of bleeding/ year	Site of bleeding
330P	24	Severe/<1	1 year	4-5	Joint, epistaxis
107P2	31	Severe/<1	Within 1st year	5-9	Joints, muscle
107P3	28	Severe/<1	Within 1st year	4-7	Joints, muscle

bleeding episodes (4-9/year) commonly into joints suggesting a clinically severe phenotype (Table 2). All three patients were screened for inhibitors by mixing studies and found to be negative. Appropriate tests to exclude deficiency of Factor IX and von Willebrand Antigen were performed. An interesting observation noted was that patients 107p2 and 107p3 were siblings with their third sibling 107p1 not carrying the mutated FVL gene (Figure 1). This patient also had a bleeding history suggestive of severe HA with first symptomatic bleeding before first year of life, nature of bleeding being spontaneous.

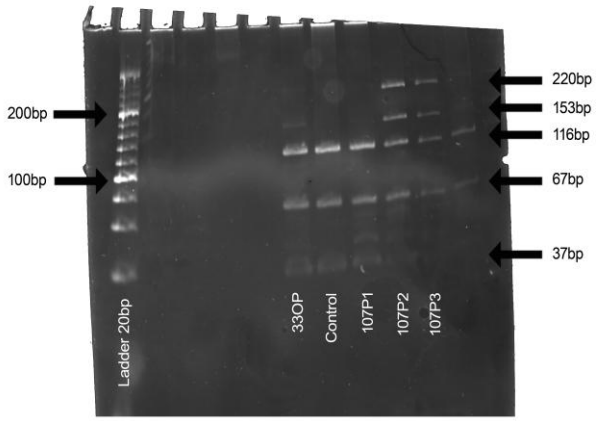

Figure 1: The digestion of PCR products of exon 10 of the Factor V gene by Mnl 1restriction enzyme. Lane 1: 20bp ladder. Lane 6, 9 and 10 show three patients with the mutated Factor V. Lane 7 shows a normal control and Lane 8 shows case 107P1.

Discussion

Resistance to anticoagulant effect of Activated Protein C (APC) due to Factor V Leiden mutation is the most frequent inherited risk factor for venous thromboembolism. [17] Factor V gene is located on chromosome 1q21-25. [24] Factor V is a cofactor for Factor Xa in the Prothrombinase complex which results in thrombin generation in the coagulation pathway. Activated Factor V (FVa) is inactivated by APC by proteolysis first at R506, followed by cleavage at R306 and R679 that results in loss of co-factor activity. Factor V Leiden mutation is characterized by substitution of guanine to adenine at nucleotide 1691 (G to A) of factor V gene which results in replacement of Arginine by Glutamine at position 506. Cleavage by APC at this Arginine site is required for efficient inactivation of activated factor V. FVa from patients lacking R506 (FVL) is still inactivated after cleavage at R306 and R679, however rate of inactivation is much slower than normal. Hence FVL retains cofactor activity and continues to promote thrombin generation for an extended period of time. Heterozygous carriers of Factor V Leiden have an approximately 7-fold greater risk of thrombosis than normal, whereas homozygous individuals have 80-fold greater risk. [24]

After the first evidence in 1996 by Nichols and colleagues, that severe hemophiliacs carrying FVL had less bleeding severity than those with identical FVIII gene mutations but no FVL, a couple of groups have demonstrated the protective effect of this mutation, with fewer bleeding episodes, [7,19,25-28] less factor concentrate utilization, [7,18,25,27] lower age of first symptomatic bleeding, [8] and hemophilic arthropathy. [26,27] However these results were not uniformly consistent. Contradictory to these results, there are other reports that fail to demonstrate a correlation between clinical course of

severe HA and prothrombotic mutations. Studies by Arbini et al., [29] Ahmed et al., [20] and Beltran-Miranda [30] have reported that none of their cases of severe Hemophilia with milder phenotype carried FVL. On the similar lines, other studies have reported that presence of FVL mutation in severe HA patients did not appear to have any protective effect. [4,10,11,31,32] In the present study, coinheritance of FVL mutation did not attenuate the frequency of bleeding and the first symptomatic bleeding appeared by first year of age suggesting a severe phenotype. In contrast to this, Escuriola and colleagues showed that the first symptomatic bleeding in 6 FVL carriers occurred later in life than in non-carriers (1.6years vs 0.9 years). [8]

A very interesting aspect of our study is the study of a HA family wherein we were able to study three brothers of the same family. All three had FVIII <1%, Intron 22 inversion and a clinically severe disease. However FVL mutation was present in two of them. This is in contrast to a family study conducted by Ghosh et al., a patient heterozygous for FVL had a milder phenotype, while his maternal uncle carrying the same FVIII molecular defect, had severe clinical manifestations. [19]

Such variable results suggest that hemophilia severity may be modulated by multiple factors including undetected additional mutation in FVIII gene, coinheritance of mutations in other coagulation factors (eg FXI, Type 2N VWD) or another prothrombotic mutation (Prothrombin gene G20210A, Protein C, Protein S, Antithrombin III etc). Our patients were not screened for these mutations however their role in the three CWH with FVL in the present study seems unlikely. Intron 22 inversion mutation leads to a truncated protein with hardly any functional FVIII activity hence there is no role of any additional mutation. Coinheritance of FXI and Type 2N VWD with FVL is highly unlikely and an extremely rare phenomenon not reported earlier. One limitation of the study is that because the supply of Factor VIII concentrate is still not streamlined in our country in most of the cities, we could not use the Hemophilia Severity Score [11] as a tool for assigning the severity of disease.

The current study shows prevalence of FVL in CWH to be 3% which is similar to the prevalence reported from various parts of India in normal healthy individuals (in the range of 1-4.1%). With few exceptions, as in Greek hemophiliacs the overall prevalence of FVL is not increased in hemophiliacs, but it is similar to that of non-hemophilic populations belonging to the same geographical area. [17, 33] Presence of FVL in two out of three brothers with clinically severe HA strongly suggest that this mutation does not attenuate the bleeding severity in CWH.

Conclusion

Prevalence of Factor V Leiden mutation in hemophilia cases is similar to that in non-hemophilic population implying that this mutation does not offer any evolutionary advantage to CWH. This mutation has no definite influence on the clinical severity in CWH. Multicenter large scale studies conducted on genotypically homogenous groups of severe HA aimed at studying the range of factors (discussed above) will help to clarify the role of FVL as well as other factors involved in modulation of severity in HA.

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Competing Interests

None declared.

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