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Impact of Thrombogenic Mutations on Clinical Phenotypes of von Willebrand Disease

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Abstract: von Willebrand disease (VWD) is a most common inherited bleeding disorder. von Willebrand factor (VWF) exists as an extracellular adaptor molecule and generally involves in the hemostasis mechanism through binding with GP (Glycoprotein) Ib-IX-V platelet receptor. Clinical phenotype of bleeding disorders modulated to a decrease in bleeding symptoms by thrombogenic mutations. We made an attempt to investigate the impact of thrombogenic mutations/polymorphisms on the clinical phenotype of 114 different types of patients with VWD, and 120 healthy controls were screened for methylenetetrahydrofolate reductase (MTHFR) 677C/T, factor V (FV) Leiden (1691G/A), β_3 integrin (*HPA-I*) (Human platelets antigen-I) gene (1565T/C), and prothrombin 20210G/A mutations. Genotypic analysis was performed using polymerase chain reaction (PCR) and restriction fragment length polymorphism. Forty-five patients (39.5%) were found to be positive for at least one of the prothrombotic risk factors screened. Prothrombin 20210G/A was not found in any patient with VWD as well as healthy control. Eight patients with VWD were carrying the defective alleles of different thrombogenic markers, showing milder phenotypes than expected. A high prevalence was observed for MTHFR 677C/T (677C/C 73.6%, 677C/T 24.6%, 677T/T 1.8%) and PLA1/A2 (1565T/T 88.6%, 1565T/C 10.5%, 1565C/C 0.87%) polymorphism followed by FV Leiden (1691G/G 97.4%, 1691G/A 2.6%, 1691A/A 0.00%) in patients with VWD with allelic frequencies 11.4% (677T), 5% (1565C), and 1.3% (1691A). Hence, we concluded that thrombophilic markers were seen to be influencing the clinical phenotypes of patients with VWD.

Keywords: von Willebrand factor; clinical thrombophilia; bleeding; clinical phenotype; modulation; prevalence

Introduction

von Willebrand disease (VWD) is most frequent heterogeneous bleeding disorder with the prevalence in ≈1% of general population caused by mutation in von Willebrand factor (VWF) gene[1] located in short arm of chromosome 12 (12p13.3). von Willebrand factor is a complex multimeric glycoprotein, which acts as an extracellular adaptor molecule for platelet plug formation at the site of injury.[2] A modified clinical phenotype resulting from congenital bleeding disorders and concomitant thrombophilia has drawn the attention of hematologists in recent years.[3,4] Although thrombotic events occur rarely in patients with inherited bleeding disorders, the presence of single nucleotide polymorphisms predisposing to thrombophilia might modulate their clinical phenotypes.[5-8] The occurrence of thrombophilic mutations in patients with VWD has been reported by very few studies.[9,10] Hemostasis requires a delicate balance of coagulation and fibrinolysis. An important component of this system is the relationship of factor V (FV) with activated protein C (APC). Wild-type FV

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serves as a substrate for APC and is inactivated after proteolytic cleavage at 3 different arginine residues, which is part of the normal coagulation regulatory mechanism. A common clinical manifestation, termed APC resistance, occurs when there is a single G to A point mutation (Arg506Gln) in the FV sequence, decreasing its susceptibility to APC cleavage. This single nucleotide polymorphism (SNP), termed FV Leiden,[11] has a prevalence of 2% of 5% in the United States and European populations, with wide variation among nationalities. Another common polymorphism that is seen in consonance with FV Leiden is the methylenetetrahydrofolate reductase (MTHFR) 677C/T. This polymorphism is seen in the *MTHFR* that codes for an enzyme methylenetetrahydrofolate reductase that converts 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate. This enzyme is important for metabolism of an active intermediate metabolite formed in the body known as homocysteine. Individuals who have a C to T substitution at base 677 of the *MTHFR* gene (Ala222Val) have lower enzyme activity and hence higher homocysteine levels, which predisposes them to a prothrombotic condition.[12,13] Apart from these 2 polymorphisms, 2 more polymorphisms that have been either not reported or rarely reported in the Indian population are the prothrombin 20210G/A and HPA-I (PLA1/A2) polymorphisms. Platelet aggregation results from platelet cross-linking by fibrinogen or VWF (or both) bound to the platelet-specific β_3 integrin (GPIIb-IIIa).[14] PLA1/A2 results from a Leu33Pro polymorphism in the extracellular portion of β_3.[15] If PLA2 variant affects platelet function, it likely does so by altering the interaction of $a_{IIb}\beta_3$ with ligands such as fibrinogen, VWF, or fibronectin.[16,17] The study demonstrated that Leu33 was responsible for the PLA1 epitope and Pro33 for the PLA2 epitope. The prevalence of the PLA1 and PLA2 alleles varies by ethnic background and geographical distribution. The common allele is PLA1 with reported frequencies of 0.84 to 0.89 in Whites, 0.92 in Blacks, and >0.99 in Asians. The PLA2 allele is not rare in certain populations, occurring with a frequency of 0.11 to 0.15 in Whites and 0.08 in Blacks. However, the PLA2 allele is nearly absent (<0.01) in rest of the Asians.[18]

To the best of our knowledge, no study has so far reported the prevalence and analyzed the clinical significance of inherited prothrombotic risk factors in Asian patients with VWD. In this study, the first of its kind from South Asia, we have investigated the prevalence of these prothrombotic polymorphisms in patients with VWD.

Patients and Methods

Participants

A total of 114 (52 male:62 female with mean age of 17.28 years) different types of unrelated patients with VWD and 120 healthy controls (78 male:42 female mean age of 23.1 years) were the study participants. There were 27, 68, and 19 patients with type 1, 2, and 3 VWD respectively. This study was granted ethical clearance by the hospital institutional review board (IRB). Clinical history and written informed consent was obtained from all the patients or patient's attendant participating in the study. Blood sample was collected from the patients with VWD as well as healthy controls, in 3.2% trisodium citrate (1:10), and centrifuged at 3500 rpm for 10 minutes. Platelet poor plasma (PPP) and peripheral blood leukocytes were stored at −70°C. DNA was extracted from peripheral blood leukocytes by phenol-chloroform method.[19]

Bleeding time (BT) in all patients was done by Ivy method.[20] Activated partial thromboplastin time (APTT) and prothrombin time (PT) tests were performed on automated coagulometer using patient PPP. Platelet aggregation was performed on platelet-rich plasma (PRP) with a standard count of 300×10^9/L (Counter-Sysmex America Inc, XT 1800i) using the standard concentration of agonists ristocetin, adenosine diphosphate (ADP), and adrenalin.[21] Mixing aggregation studies of patient PRP with normal pool plasma (NPP) was also performed to confirm the diagnosis of VWD. All aggregation responses were recorded on a Chrono-Log aggregometer (Havertown, Pennsylvania). Factor VIII (FVIII):C activity was assayed by manual clotting method by using FVIII-deficient plasma that had been standardized for the routine purpose in our laboratory.

VWF Assays

Plasma VWF antigen (VWF:Ag) levels were detected using an enzyme-linked immunosorbent assay kit (REEAD, Corgenix, 12061 Westminster, Colorado). Ristocetin cofactor activity (VWF:RCo) was assayed with formalin-fixed platelets.[22] Type 1 and type 2 patients with VWD were categorized on the basis of VWF:RCo and VWF:Ag ratios. A ratio less than 0.7 corresponded to a type 2 VWD and greater than 0.7 was considered type 1 VWD. When both

VWF:Ag and VWF:RCo values were less than 3 IU/dL, it was categorized as type 3 VWD.

The patients were also categorized into moderate and severe bleeders based on their bleeding tendencies. Moderate bleeders were defined as those who bled only after trauma or surgery with a history of prolonged bleeding (BT), epistaxis, and menorrhagia with low VWF:RCo value (<60 IU/dL) and normal VWF:Ag level. Severe bleeders were defined as those with prolonged BT, mucosal bleeding, epistaxis, and spontaneous or life-threatening hemorrhages, such as gastrointestinal bleeding. Severe bleeders also required repeated blood or platelet transfusions.

Genotypic Analysis

DNA amplification for FV Leiden, prothrombin 20210G/A, MTHFR 677C/T, and PLA1/A2 was performed separately as individual polymerase chain reaction (PCR). Polymerase chain reaction was performed on PCR mixture containing total volume of 25 μL that contained 200 ng DNA, 0.2 mmol/L of each of the deoxynucleotide triphosphates; 25 pmol of each oligonucleotide primer, each forward and reverse, reaction buffer (giving a final concentration of 10 mmol/L Tris-HCl, 50 mmol/L KCL, 1.5 mmol/L $MgCl_2$, and 0.05% gelatin); and 1 U Taq polymerase. The PCR conditions were common for all the above 4 PCRs. The conditions were 95°C for 5 minutes followed by 30 cycles at 95°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute followed by an extension step at 72°C for 10 minutes. Oligos sequence used for different polymorphism are as follows: FV Leiden (sense 5′-CATGAGAGAACATC GCCTCTG-3′, antisense 5′-ACCTAACATGTTC-TAGCCAGAAG-3′), MTHFR (sense 5′-TGAAG GAGAAGGTGTCTGCGGGA-3′, antisense 5′-AG GACGGTGCGGTGAGAGTG-3′), HPA-I (sense 5′-TTCTGAT TGCTGGACTTCTCTT-3′, antisense 5′-TCTCTCCCCACGGCAAAGAGT-3′), and prothrombin (sense 5′-TCTAGAAACAGTTGCCTGGC-3′, anti-sense 5′-ATAGCACTGGGAGCA TTGAAGC-3′).

Restriction Digestion

MTHFR 677C/T. Hinf1 digestion was done in a total volume of 10 μL containing 5 μL of PCR product, *HinfI* enzyme (5 U; MBI Fermentas Inc, Suit A Hanover, Maryland), and 1 μL of the restriction buffer. The digestion reaction was kept at 37°C overnight. The digestion product was run on a 2% agarose gel for 1 hour at 150 and visualized in UV light.

FV Leiden 1691G/A. MnlI digestion was done in a total volume of 20 μL containing 15 μL of PCR product, 10 U of *Mnl1* enzyme (MBI Fermentas Inc), and 2 μL of the *Mnl1* restriction buffer. The digestion reaction was kept at 37°C overnight and the digested product was run on 7% polyacrylamide gel electrophoresis (PAGE) for about 2 hours at 200 V and visualized in UV light.

Prothrombin 20210G/A. HindIII digestion was done in a total volume of 10 μL containing 5 μL of PCR product, 5 U of *HindIII* enzyme (MBI Fermentas Inc), and 1 μL of the *HindIII* restriction buffer. The digestion reaction was kept at 37°C overnight. The digestion product was run on a 2% agarose gel for 1 hour at 150 V and visualized in UV light.

β3 *integrin (PLA1/A2). MspI* digestion was done in a total volume of 20 μL containing 17 μL of PCR product, 10 U (0.5 μL) of *MspI* enzyme (New England Biolabs Inc, Ipswich, Massachusetts), and 2 μL of the *MspI* restriction buffer. The digestion reaction was kept at 37°C overnight and the digested product was run on a 2% agarose gel for 2 hours at 200 V and visualized in UV light.

Results

A total of 114 patients with VWD and 120 controls were included in this study. Of 114 cases with VWD, 27 and 68 patients were diagnosed for type 1 and type 2 VWD, respectively. Total of 19 cases were diagnosed as type 3 VWD with reduced or absent VWF:Ag and VWF:RCo activity. Several cases of VWD showed severe bleeding manifestations with low VWF:Ag or VWF:RCo. Forty-five patients (39.5%) were found positive for at least one of the studied thrombotic factor (Table 1). The genotypic and allelic frequencies of all thrombophilic mutations have been described in Figure 1A and B.

In control population from same geographic and ethnic background, the results were noncomparable from patients as none of the control carried defective genotype for thrombophilia except one who was found positive for heterozygous genotype for FV

Table 1. Thrombogenic Polymorphisms in Patients With VWD

Clinical phenotype[a]	Patients with VWD (n = 114)	HPA-I (PLA1/A2) T1565C Leu33Pro	FV Leiden G1691A Arg 506Gly	MTHFR C677T Ala222Val
Type 1	27	1	0	9
Type 2	68	8	3	17
Type 3	19	4	0	4

NOTES: Ag = antigen; FV = factor V; MTHFR = methylenetetrahydrofolate reductase; RCo = Ristocetin cofactor; VWD = von Willebrand disease; VWF = von Willebrand factor.
[a] On the basis of ratio of VWF:RCo to VWF:Ag.

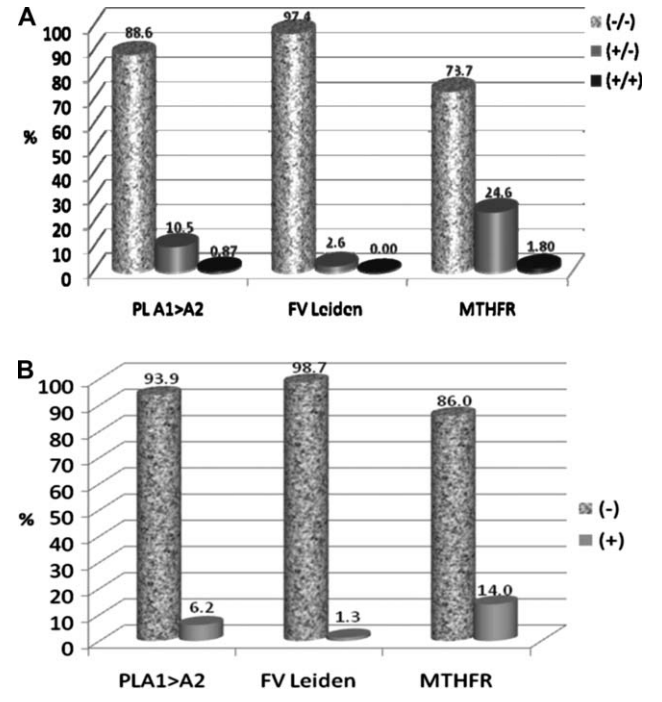

Figure 1. A, Genotypic frequencies of different thrombophilic markers in patients with VWD cases. B, Allelic frequencies of different thrombophilic markers in patients with VWD. FV indicates factor V; MTHFR = methylenetetrahydrofolate reductase; VWF = von Willebrand factor.

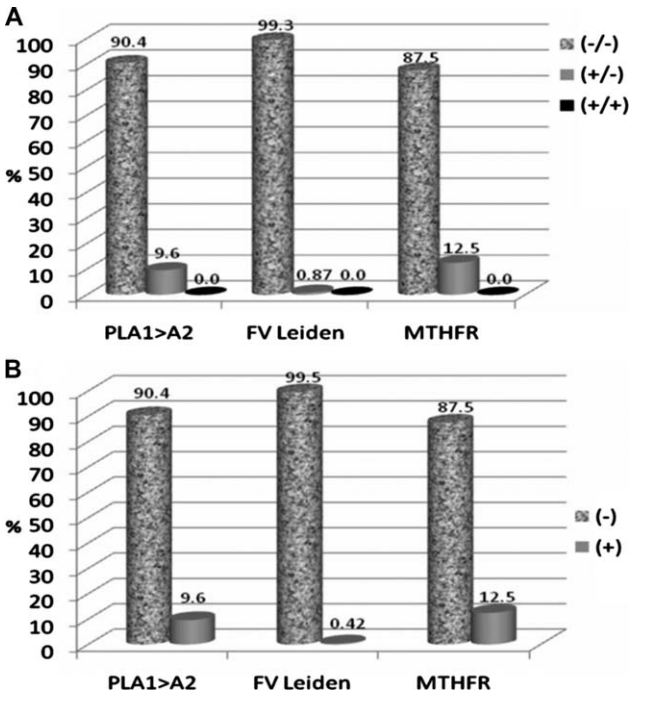

Figure 2. A, Genotypic frequencies of different thrombophilic markers in healthy controls. B, Allelic frequencies of different thrombophilic markers in healthy controls. FV indicates factor V; MTHFR = methylenetetrahydrofolate reductase; VWF = von Willebrand factor.

Leiden. Allelic frequencies from control population are shown in Figure 2A and B.

Factor V Leiden mutation was detected in 2.6% (3/114) of patients with mutated allelic frequency of 1.3% and prothrombin was not found in any patient with VWD as well as healthy controls. PLA1/A2 polymorphism of β_3 integrin was found in 11.4% (13 of 114) of patients. Of these 13 patients, 1 patient carried the homozygous variant form (PLA2/A2), whereas the remaining 12 patients were heterozygous (PLA1/A2) for this polymorphism. Methylenetetrahydrofolate reductase polymorphism was found in 26.31% (30 of 114) of the patients. Of 30 patients with VWD, 2 cases including 1 type 1 and type 2 each carried the homozygous MTHFR 677T genotype and rest 28 were heterozygous for the MTHFR 677C/T genotype. Prothrombin 20210G/A mutation was not detected in any patient with VWD and healthy controls. It has been observed that VWD cases with heterozygous MTHFR 677C/T and PLA1/A2 alleles independently showed no modulating effect. Heterozygous alleles of FV Leiden were seen to be modulating factor for clinical phenotype of VWD toward mild bleeding. One of male patient with type 1 VWD was carrying homozygous genotype

Table 2. Correlation of Thrombogenic Mutation and Clinical Phenotype in 8 Different Types of Patients With Clinically Modulated VWD

						Clinical presentation			Genotype		
S No	Age/sex	VWD type	Bleeding complication	VWF:RCo (IU/dL)	VWF:Ag (IU/dL)	EMS	EPT	GB	β_3 integrin (HPA-I)	FV Leiden	MTHFR
1	5/M	Type 1	Mild	32	32	√			1565T/T	1691G/G	677T/T
2	8/M	Type 2	Mild	43	100	√			1565C/C	1691G/A	677C/C
3	9/F	Type 2	Mild	34	110	√			1565T/T	1691G/G	677T/T
4	33/M	Type 2	Mild	29	70		√		1565T/T	1691G/A	677C/C
5[a]	14/M	Type 2	Mild	42	140		√		1565T/C	1691G/G	677C/C
6	13/F	Type 2	Mild	35	75		√		1565T/C	1691G/A	677C/C
7[a]	27/F	Type 2	Mild	25	102			√	1565T/C	1691G/G	677C/C
8	5/M	Type 3	Mild	0.5	2	√			1565T/C	1691G/G	677C/T

NOTES: Ag = antigen; EMS = ecchymosis; EPT = epistaxis; FV = factor V; GB = gum bleeding; MTHFR = methylenetetrahydrofolate reductase; RCo = Ristocetin cofactor; VWF = von Willebrand factor; VWF = von Willebrand factor.
[a] Modulated cases were carrying only β_3 integrin (HPA-I) heterozygous alleles.

677T/T for MTHFR showed mild ecchymosis but had low VWF:Ag level (32 IU/dL). Similarly, a female patient with type 2 VWD was carrying the homozygous genotype 677T/T for MTHFR and modulated to mild clinical phenotype and showed only ecchymosis but had low VWF:RCo 4 (34 IU/dL). Three patients with type 2 VWD were carrying heterozygous 1691G/A FV Leiden alleles. Two of these 3 FV Leiden-positive cases coinherited a homozygous (PLA2/A2) and a heterozygous (PLA1/A2) alleles and presented clinically mild symptoms; one of them showed only ecchymotic spots on skin with low VWF:RCo (43 IU/dL) and another one showed mild epistaxis with 35 IU/dL of low VWF:RCo. Patient carrying heterozygous allele for FV Leiden was clinically mild and presented with mild epistaxis. Two patients with type 2 VWD were presenting mild bleeding (VWF:Ag 140 and 102 IU/dL, VWF:RCo 42 and 25 IU/dL), while both of them were carrying heterozygous genotype for PLA1/A2 polymorphism. One patient with type 3 VWD carrying MTHFR 677C/T heterozygous genotype together with PLA1/A2 heterozygous presented with mild ecchymosis but had very low VWF:Ag (2 IU/dL) level and VWF:RCo (0.5 IU/dL). Detail genotypic and clinical phenotype of the patients with VWD is given in Table 2.

Discussion

Thrombotic event is rare in patients with VWD, but it has also been reported concomitantly in congenital bleeding disorder.[23] von Willebrand disease is a most frequent bleeding disorder among general population. A few studies have so far reported the correlation between the presence of thrombophilic markers and phenotypic heterogeneity of VWD.[7,24] Literatures have stated that arterial occlusion is higher in patients with hemophilia in comparison to patients with VWD. A critical review of arterial and venous thrombosis in patients with VWD presented the 55 cases of myocardial infarction (MI) in patients with hemophilia A and B,[25] while only 11 cases of VWD with arterial thrombosis were reported. In patients with VWD, there is also possibility of thrombotic thrombocytopenic purpura (TTP) in presence of large VWF multimers due to a congenital or acquired deficiency of a VWF-cleaving metalloprotease ADAMTS-13. Generally, patients with TTP present with low platelet count but our patients showed normal counts. A high plasma level of VWF has also been associated with a slightly increased risk of arterial thrombosis.[26] Studies suggested that replacement therapy of VWF/FVIII also leads to thrombotic manifestation due to posttransfusional high level of VWF and FVIII. A large retrospective study in VWD patients carried out by a group for thrombosis and found no positive case.[3] This result supports the hypothesis that elevated FVIII/VWF levels are usually maintained to represent a prothrombotic condition.[4] It is revealed that some of the mutations detected are well-recognized prothrombotic risk factors (ie, FV Leiden, prothrombin 20210G/A), while for other mutations (eg, MTHFR 677C/T mutation), the association with an increased prothrombotic risk factor is still controversial.[24]

Different genotypic patterns of inheritance were seen to influence the clinical phenotype of VWD in

our study. Heterozygous variant of FV Leiden, homozygous variant of PLA2/A2 as well as the compound heterozygosity of MTHFR 677C/T with PLA1/A2 polymorphisms were seen to have a probable impact on modulation of clinical phenotype. Eight (7.0%) of 114 patients with VWD (1 type 1; 6 type 2; 1 type 3) showed phenotypic variability owing to the presence of thrombophilic mutation. Prothrombin 20210G/A was seen to be completely absent in our study population. Methylenetetrahydrofolate reductase 677C/T and PLA1/A2 variants were seen at much higher prevalence than FV Leiden. However, looking at the bigger picture, the allelic frequencies in the healthy controls as well as the VWD population showed that the prevalence of thrombogenic markers in Asian Indians is lower than that in the Caucasian population. This is in accordance with many of the previously determined studies.[24,27,28] Of 8, 6 were carrying variant of thrombogenic alleles and other 2 carried only heterozygous PLA1/A2 genotype variant. The ameliorating factors for these 2 patients might be other thrombotic factors such as defects in protein C, protein S, antithrombin genes, homocysteine, APC resistance, or presence of HPA-$2^{a/b}$ polymorphism of GpIbα—those have not been studied in present investigation. It is thus concluded that thrombogenic mutations/polymorphisms in Asian Indian VWD population have their probable impact on modulation of clinical phenotypes that leads to mild bleeding. The results of the current study would be of clinical interest.

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