

Correspondence

Additional markers for genetic diagnosis of type 3 von Willebrand disease in Indian population

Sir,

von Willebrand disease (VWD) encompasses a wide spectrum of bleeding disorders with severity ranging from moderate bleeding tendency to severe life threatening haemorrhage, estimated to affect approximately 0.5 - 2 per cent of the population in the Western countries¹. Patients present with mucocutaneous bleeding symptoms, epistaxis, mucosal bleeding, prolonged bleeding from cuts, post-dental extraction bleeding, menorrhagia and gastrointestinal bleeding. These are classified as types 1, 2 and 3, depending on the qualitative and quantitative defects in von Willebrand factor (VWF) antigen. Diagnosis, genetic counselling, carrier and antenatal diagnosis play an important role in the comprehensive management of these cases.

Identification of mutations in von Willebrand factor gene (*VWF*) is hampered by the large size and the highly polymorphic nature of the gene, heterogeneity of mutations and a pseudogene corresponding to a large part of the gene². The spectrum of *VWF* mutations that cause VWD includes frame shift mutations resulting from small insertions or deletions, large deletions, missense mutations affecting single amino acid residues, splice-site mutations, duplications, nonsense mutations causing premature termination of translation and gene conversion due to recombination between the gene and pseudogene³. Even after sequencing the entire coding region, splicing region and the promoter regions of *VWF*, in a few cases the mutations are undetected^{4,5}. It has been reported that analysis of the essential regions of genomic DNA may not be adequate to identify the underlying molecular pathology in a proportion of type 3 VWD cases. Deep intronic mutations affecting mRNA splicing by creation of a cryptic splice site or introduction of additional in-frame exons are some of the plausible explanations for the disease manifestation,

which may be detected by mRNA analysis in at least a few cases⁶. Cost of DNA sequencing to screen 52 exons remains a major obstacle in implementing direct sequencing in the routine genetic diagnosis of VWD.

Restriction fragment length polymorphism (RFLP) technique has been the method of choice for offering carrier detection and antenatal diagnosis in VWD families, particularly in developing countries like India^{7,8}. The limitations of the technique are the requirement of all the family members and non-informativeness or homozygosity of the polymorphic markers. Heterozygosity of the polymorphisms varies in different populations and it is essential that this is analyzed prior to its application in the genetic diagnosis of the families.

A large number of RFLP markers have been identified for tracking the affected allele in *VWF*⁹⁻¹⁶. The most useful markers are the highly polymorphic tetranucleotide repeats *i.e.* variable number of tandem repeats (VNTR) of ATCT repeats in intron 40 of the *VWF*. These have been successfully used in prenatal diagnosis of VWD by different workers with different rates of heterozygosity. However, in a gene which is as complex as *VWF*, analysis of heterozygosity frequency of additional markers is always an added advantage to make the genetic diagnosis applicable to majority of the families.

In the present study, we analyzed the entire *VWF* coding region including intron-exon boundaries by DNA sequencing in 100 unrelated severe VWD patients. All consecutive severe type 3 VWD cases (VWF:Ag < 5 IU/dl and FVIII levels < 10 IU/dl)¹⁷ were included in the study. These patients attended the Comprehensive Haemophilia Care Center at the National Institute of Immunohaematology, Mumbai, India, between 2009 and 2013. All cases with acquired von Willebrand

syndrome were excluded from the study. The study was approved by the Institutional Ethics committee.

Genomic DNA was extracted using commercial kits (Invitrogen, USA). Fifty five sets of primers for 52 exons were designed in-house, using UCSC (<http://genome.ucsc.edu>), primer 3 output and the sequence from Ensembl¹⁸. The primers were obtained from Sigma Aldrich, USA. Primers were designed so as to include the entire exonic areas, promoter region, as well as exon-intron boundaries and minimum 50-100 bases upstream or downstream to exonic region. Oligo analysis was done for each set of primer to avoid looping or dimer formation. The primers for known Arginine hot spot region were adapted¹⁹. To amplify the specific gene without co-amplification of pseudogene, the primers were designed in such a way that those were specific to authentic *VWF* gene by 1-3

nucleotide mismatches at the region specific to gene and the PCR conditions were optimized for each exon to give authentic gene specific amplification. The DNA sequencing was performed in ABI Prism 3130 Genetic Analyzer (ABI, USA).

The nucleotide and amino acid numbering of each polymorphism was determined by reference to the published *VWF* sequence²⁰. Sequence variants have been checked manually by identifying the base position on the cDNA sequence. Previously reported single nucleotide polymorphisms were checked in the Ensembl database¹⁸.

Several intronic and exonic single nucleotide variations (SNVs) were found with different rates of heterozygosity (Table). Most of the variations were intronic and a few silent synonymous changes in exonic

Table. Single nucleotide variations (SNVs) identified in Indian VWD patients in *VWF*

SNP Ref. no.	Nucleotide change	Amino acid no.	Intron/ Exon no.	Alleles	Heterozygosity (%)	Restriction enzyme digestion
rs216902	5844C>T	C1948	Exon 35	C/T	43	Mse I
rs1800383	4414G>C	<i>p.D1472H</i>	Exon 28	G/C	32	
rs1800387	954T>A	<i>p.N318K</i>	Exon 8	T/A	11.4	Msl I
rs1800379	1548T>C	<i>p.Y516Y</i>	Exon 14	C/T	27	
rs35365059	1626G>A	<i>p.A542A</i>	Exon 14	A/G	12	
rs216321	2555A>G	<i>p.RL852Q</i>	Exon 20	G/A	10	Nla IV
rs36105228	2282-122_2282-121invGT	-	In 18	GT/AC	8	-
rs216293	2282-42C>A	-	In 18	A/C	8	-
rs216312	3675-75A>G	-	In 28	A/G	7	-
rs1063856	2365A>G	<i>p.T789A</i>	Exon 18	A/G	8	Rsa I
rs1063857	2385T>C	<i>p.Y795Y</i>	Exon 18	T/C	8	-
rs1800375	1173A>T	<i>p.T391T</i>	Exon 11	T/A	6	Bsr I
rs1800378	1451A>G	<i>p.H484R</i>	Exon 13	A/G	4	Rsa I
rs3819540	7288-19	-	In 43	C/T	7	-
rs3819539	7288-21	-	In 43	C/T	7	-
novel	8439+207	-	3 untranslated region	C/T	6	-
rs177702	6799-14C>T	-	In 39	T/C	4	-
rs2270239	7549-59	-	In 45	C/A	4	-
13/83-85*	1591delTCT	-	In 16	del TCT	4	-

*mancuso nucleotide numbering
SNP, single nucleotide polymorphism

region. The c.5844C>T SNV (rs216902), *i.e.* in the splice site of exon 35 was found to be heterozygous in 43 per cent of our patients. The heterozygosity rates of polymorphic markers *p.D1472H*, *p.N318K*, *p.T789A*, *p.R852R*, rs177702 C/T, rs216312 A/G, *p.T391T* and *p.H484R* polymorphisms were found to be 32, 11.4, 8, 10, 4, 7, 6 and 4 per cent, respectively. The two polymorphisms *i.e.* *p.T789A* (rs1063856) and *p.Y795Y* (rs1063857) were found to be in strong linkage disequilibrium with each other ($r^2=1.0$), each showing 8 per cent heterozygosity, which also showed moderate degree of linkage disequilibrium with c.2282-42C>A (rs216293) ($r^2=0.656$) (<http://www.broadinstitute.org/mpg/snap/ldsearchpw.php>). Other polymorphisms which showed significant degree of linkage disequilibrium were c.7288-21C>T(rs3819539) and c.7288-19C>T(rs3819540)($r^2=0.867$). *p.N318K* has been reported both as mutation and polymorphism; however, the present analysis showed high heterozygosity (11.4%). The novel variant c.8439+207 was checked for its effect on the splicing mechanism by using ALAMUT software²¹ and was found to be benign.

Due to the complexity of the gene and heterogeneity of mutations, RFLP analysis using intragenic polymorphisms has been an important diagnostic tool to track the affected allele in the family members of severe VWD patients. Rapid automated methods to simultaneously analyze multiple markers by multiplex fluorescent PCR followed by capillary electrophoresis have also been reported to make the diagnosis more cost-effective and less time consuming²². Our study shows the utility of additional polymorphic markers with high heterozygosity frequencies for genetic diagnosis in type 3 VWD.

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Conflicts of Interest: None.

Priyanka Kasatkar, Kanjaksha Ghosh & Shrimati Shetty*

National Institute of Immunohaematology (ICMR),
KEM Hospital, Parel,
Mumbai 400 012, Maharashtra, India

*For correspondence:
shrimatishetty@yahoo.com

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