World Journal of Medical Sciences 2 (2): 128-131, 2007
ISSN 1817-3055

Molecular Biology of von Willebrand Disease (vWD): A Study among Unrelated Pakistani Patients

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Abstract: Von Willebrand Disease (vWD) is an autosomal dominant hereditary blood coagulation disorder that is highly variable in expression. Although the frequency of vWD is low in Pakistan but since the disease has similar manifestations as that of hemophilia A, some of the vWD patients are misdiagnosed. The molecular basis of vWD in Pakistan is also unknown. The main objective of this study is to identify the mutation that produces vWD in Pakistani patients and compare the ethnic differences. For this purpose the blood samples were collected from 11 unrelated vWD patients from southern Pakistan. DNA was extracted by using standard techniques. The DNA for only 15 exons was amplified. The reason for the selection of these exons was that most of mutations in these exons have been reported in South East Asian population. Sequencing results showed two point mutations were present within a same exon 28 in one vWD Sindhi patient, at codon 1229 and 1231 with changes GTT→GGT and AAC→ACC which results in the Asn→Thr and Val→Gly substitution respectively. These mutations have been previously reported in two separate individuals of different ethnic backgrounds. Four known SNPs in eight vWD patients were observed in exon 18, 20, 42. These SNPs are already been documented in other world populations. These results of mutational analysis on vWD patients are significant for the reason that this is the first study that has been carried out in Pakistan and it has provided some insight to molecular pathology of vWD in Pakistan which can be useful in conducting further studies. Also it can be helpful in the molecular diagnosis of vWD.

Key words: Coagulation Disorders · von Willebrand Disease · Molecular Biology · Pakistan

INTRODUCTION

Von Willebrand disease (vWD) is a hereditary autosomal dominant bleeding disorder which results from quantitative or qualitative defects of Von Willebrand factor (vWF) that serves as carrier for factor VIII and adhesive link between platelets and the injured blood vessel wall. It is named after the Finnish physician, Erik Von Willebrand, who first described the condition in 1926 in 24 of 66 members of a family from the Alands islands [1]. th sexes were affected and the bleeding time was prolonged despite normal platelet counts and normal clotting reaction. vWD affects approximately 125 persons per million population, with severe disease affecting approximately 0.5-5 persons per million population [2]. vWF is a large multimeric glycoprotein that circulates in blood plasma at concentrations of approximately 10 mg/mL. In response to numerous stimuli, vWF is released from storage granules in platelets and endothelial cells. It performs 2 major roles in hemostasis. First, it mediates the adhesion of platelets to sites of vascular injury. Second, it binds and stabilizes the procoagulant protein factor VIII (FVIII). vWF exists as a series of multimers, varying in molecular weight between 0.5 (dimer) and 20 million kD (multimer). The building block of multimers is a dimer, held together by disulfide nds located near the C-terminal end of each subunit [3].

There are 3 main subtypes, classically characterized by excessive mucocutaneous bleeding, a positive family history and abnormal von Willebrand factor (VWF) laratory studies. Type 1 VWD is a partial deficiency of qualitatively normal VWF, type 2 VWD is caused by functionally abnormal VWF and type 3 VWD represents a virtual absence of the VWF protein [4].

The spectrum of clinical severity is broad, ranging from a severe disorder beginning in early life with

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recurring life threatening to a very mild disorder in which abnormal bleeding does not occur except during trauma or surgery. Early bruising is common and pitechia or purpura may be noted. Mucous membrane bleeding including epistaxes, upper GI bleeding and menorrhagia may be prominent. The diagnosis is established by obtaining a appropriate family history on demonstration of a prolonged bleeding time, defective ristocetin-induced platelet aggregation in the presence of normal aggregation to other agent and reduced FVIII R:Ag and FVIIIC activities. A delayed marked but sustained increase in FVIIIC activity after infusion of cryoprecipitate confirms the diagnosis and forms the basis of treatment [5].

In Pakistan no molecular study on vWD has been conducted and only clinical data is available for some scattered patients. This study was therefore, planned to:

- Characterize the molecular basis of vWD in Pakistan
- Compare the ethnic differences
- Evaluate the usefulness of the mutational analysis in pre and post-natal diagnosis.

MATERIALS AND METHOD

5ml of venous blood samples from 11vWD patients from southern Pakistan were taken for DNA in a EDTA vacutainer tube and the samples were stored at -20 C. The clinical data was collected from the files of the patients and were recorded on a Performa designed for this purpose. 11 patients had family history. Nasal bleeding, bruises, gum bleeding were most common prevalent and common symptoms of the disease. Mostly PT, APTT, BT, CT values were available for nearly all the patients. DNA from blood samples was extracted using Phenol Chloroform Method. The quantity of the extracted DNA in the TE solution was estimated by spectrophotometery yield gel electrophoresis. Out of the 52 Exons, 15 Exons (Exon 3, Exon 5, Exon 6, Exon 7, Exon 9, Exon 10, Exon 16, Exon 18, Exon 19, Exon 20, Exon 27, Exon 28, Exon 42, Exon 43, Exon 45) were selected considering there high mutation rate. In order to design the primers various primer choices were examined for 15 Exons, using the computer program Primer 3. 20 sets of primers were chosen and were synthesized in Primer designing laratory of CEMB. The primers were mostly 20 nucleotide G+C rich sequence. Sizes of amplified products range from 218pb to 475 bp. Sizes of products to be amplified range from 218 pb to 475 pb. These primers were then utilized to amplify selected portion of DNA using PCR amplification technique of each amplified exon [6]. Amplification was carried out in BIO-RAD I-Cycler and 1-2 ul of genomic DNA was used as template. Quantity and quality of amplification was checked on agarose gel using ethidium bromide staining. All fragments exhibiting aberrant banding pattern were then subjected to cycle sequencing using Big Dye termination technology. Sequencing of the amplified DNA products was done by di-deoxy termination method on ABI 3100 genetic analyzer capillary electophoresis (Applied Biosystems).

RESULTS

Sequencing data was analyzed by using software Chromas2. The sequence was also blasted against normal sequence by using BLAST 2 sequences on NCBI web. BLAST (Basic Local Alignment Search Tool) a search program designed to explore all the available databases against a query protein or DNA sequence. It utilizes the BLAST algorithm for pairwise DNA-DNA or protein-protein sequence comparison. The resulting alignments

Table 1: Mutations Detected in a vWD Patient

Patient's Name	Age	Exon#	Nucleotide Change	A.A Change	Codon#	Comments
M.S (Karachi)	----	E 28	A→C	Asn→Thr	1231	(Eikenboom *et al*., 1994)
	----	E 28	T→G	Val→Gly	1229	(Eikenboom *et al*., 1994)

Table 2: SNPs in vWD Patients

Sr.#	Patient's Name	Age	Exon#	Nucleotide Change	Amino acid Change	Codon#	Comments
1	M.Sa (Karachi)	34	18	SNP→A/G	Thr→Ala	789	Reperted
			18	SNP→T/C	Tyr→Tyr	795	Reperted
			42	SNP→T/C	Thr→Thr	2413	Reperted
2	S (Karachi)	24	20	SNP→A/G	Gln→Arg	874	Reperted
3	A (Karachi)	25		SNP→A/G	Gln→Arg	874	Reperted
4	N (Karachi)	20		SNP→A/G	Gln→Arg	874	Reperted
5	R (Karachi)	17		SNP→A/G	Gln→Arg	874	Reperted
6	AM (Karachi)	----	20	SNP→A/G	Gln→Arg	874	Reperted
		----	42	SNP→T/C	Thr→Thr	2413	Reperted
7	Sh (Karachi)	17	42	SNP→T/C	Thr→Thr	2413	Reperted
8	M.Sh (Karachi)	----	42	SNP→T/C	Thr→Thr	2413	Reperted

are presented in graphic form. Any change in the DNA sequence was confirmed by sequencing of th sense and antisense strands for all the patients. Sequencing results showed that out of 11 patients, one patient had 2 point mutations shown in Table 1 and 4 SNPs were detected in 8 samples of patient shown (Table 2).

The mutations were reconfirmed by repeating the sample with th forward and reverse primers.

DISCUSSION

Von Willebrand disease (VWD) is an autosomal inherited congenital bleeding disorder which either is due to deficiency or dysfunction of von Willebrand factor (VWF), a large multimeric glycoprotein. VWF has two functions, first it attaches to sub endothelial collagen and to platelets, promoting formation of a platelet plug at the site of injury of small blood vessels and second it binds and transports Factor VIII (FVIII) [7].

Many molecular defects that results in vWD have been described. To date 316 mutations have been reported in vWF gene in different ethnic groups of world population [8]. These defects include point mutations, insertions or deletion of some nucleotides. The majority of abnormalities are missense mutations that are associated with the functionally abnormal vWD types, such as types IIA and type IIB and the variants with defective factor VIII binding. In severe type III vWD gene deletions, non-sense mutations and defective mRNA expression have been described as the cause of quantitative deficiency of vWF.

The size of the samples of this study was small as vWD has very low prevalence in Pakistan and sometimes it is misdiagnosed as Hemophilia A. Blood samples from 11 patients were collected from Karachi, a cosmopolitan city with mix up of individuals of different ethnic backgrounds. 11 v WD patients included in this study are: 3 Pathans, 2 Sindhis, 1 Memon, 1 Brohi, 2 Mahajirs, 1 Sheikh and 1 Behari indicating an ethnic diversity. In this study 15 exons were selected because of high rate of mutations that has been documented in South East Asian populations. [8].

The two point mutations were identified in a Sindhi patient in exon 28 of vWF gene. The first was at codon number 1229 (GTT→GGT) that results in the Asn→Thr amino acid substitution. The second was at codon number 1231 (AAC→ACC) resulting into the Val→Gly amino acid substitution.

Exon 28 of the vWF gene encodes for functional domains A1 and A2. The mutations that have been detected in this study are in these domains and they code for amino acid 1212-1491 [3]. These domains are involved in the binding of vWF to GPIb which is platelet specific glycoprotein. In first mutation Asparagines is replaced by Threonine whereas in 2nd mutation Valine is replaced by Glycine. th of these substituted amino acids (Thr and Gly) are non polar with different R groups. These R groups can play important role in the binding of vWF to GPIb which is platelet specific glycoprotein. Changes in these R groups can result in abnormal interaction of vWF with platelets. Nevertheless, it is still not clear how these domains interact to bind to the platelet receptor and what role intervening or adjacent sequences play in this interaction [9]. The two point mutations described ave have been reported previously in two different vWD patients with different ethnic background [10]. Interestingly in our study, these two point mutations are present in a single Sindhi patient which is not unusual as such examples are already present in literature [11]. The clinical consequences of this double mutation can be easily inferred, as the composite effect of double mutation can be in the form of severe phenotypic expression of the disease. This is substantiated by the fact that this patient has very low Hb, excessive nasal and gum bleeding and bleeding into the soft tissues. Moreover the disease is also present in other members of family showing severe manifestation.

Out of 11 in 8 patients 4 SNPs were detected in exon 18, exon 20 and exon 42. They account for 72% of the patients. These nucleotide changes are seemed to be common to different in all ethnic groups of Pakistan (Table 2). All the SNPs have previously been reported in other ethnic groups [12]. In 3 patients we could not detect any mutation because these mutations may be present in other coding and non-coding regions of vWD gene.

Some vWd patients had more then one SNP. In one patient 3 SNPs were found. 2 were in exon 18. The first SNP A/G was at codon number 789 with changes ACC→GCC which results in the Thr→Ala amino acid substitution. The second SNP T/C was at codon number 795 with changes TAT→TAC which resulted into the same amino acid Tyr. 3rd SNP T/C was found in exon 42 at codon number 2413 with changes ACT→ACC which resulted into the same amino acid Thr. This 3rd SNP T/C was also present in patients 3 other patients with different ethnic backgrounds. The 4th SNP A/G was detected in 4 patients in exon 20 at codon number 874 with changes AGG→GGG which resulted into Arg→Gly amino acid substitution. This 4th SNP accounts for 45% of population

The main objective of this study was to characterize the molecular basis of vWD in Pakistani population as such studies have not been conducted before. The results of this study which are preliminary in nature are significant as these have provided us an insight into the molecular pathology of vWD in Pakistan. Further these results can form the basis of future studies and consequently can be helpful in the molecular diagnosis of the vWD.

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