Frequency of type 2N von Willebrand disease among patients with mild or moderate factor VIII deficiency in an 18-month period in Iran

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Background - Type 2N von Willebrand disease (VWD) increases the clearance rate of factor VIII (FVIII) in patient's plasma, leading to clinical symptoms and laboratory findings similar to those of mild or moderate hemophilia A. Differentiating between these conditions is crucial for genetic counseling and treatment. This research aims to use genetic analysis to distinguish type 2N VWD from mild or moderate FVIII deficiency.

Materials and methods - During 18 months, suspected type 2N patients with mild or moderate FVIII deficiency, were selected from those referred to the Iranian Blood Transfusion Organization (IBTO) coagulation laboratory. Exons 18, 19, and 20 of the *VWF* gene were sequenced using Sanger's method. Subsequently, sequenced data were analyzed using NCBI's BLAST database and Chromas Pro.

Results - In the current study of 35 participants with mild or moderate FVIII deficiency, 5 were found to have type 2N VWD mutations. The mutations detected were p.Arg816Gln (No.=3) and p.Thr789Pro (No.=2), which are common in Iran. Three patients were misdiagnosed with hemophilia A at a young age, while 2 were not diagnosed until later in life.

Discussion - The current study discovered that 14.2% of the participants carried known type 2N VWD mutations, a higher prevalence compared to previous studies using a similar approach. Additionally, the study indicated that the limited availability of accurate diagnostic tests for type 2N VWD in our country could lead to a high probability of misdiagnosing or underdiagnosing patients with this condition.

Keyword: *von Willebrand factor, von Willebrand disease, hemophilia A, factor VIII.*

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INTRODUCTION

With a worldwide estimated incidence rate of 0.1 to 1%, von Willebrand disease (VWD) is known to be the most common inherited bleeding disorder[1-3]. It is caused by either qualitative or quantitative abnormalities of the von Willebrand factor (VWF)[4]. Based on the type of deficiency, VWD is classified into 3 types. Types 1 and 3 involve relative and absolute deficiencies in VWF antigen levels, respectively. Type 2 VWD is caused by mutations in the *VWF* gene that result in qualitative abnormalities of VWF[1,5]. Studies estimate that 30% to

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40% of affected patients have qualitative defects within VWF that can be classified as types 2A, 2B, 2M, and 2N[6]. Among these variants, type 2N VWD is an autosomal recessive disorder, which was first described in a patient from Normandy, France, who exhibited a normal bleeding time with a low FVIII activity level[7-9]. Type 2N VWD is defined by compromised binding of FVIII to VWF as a result of changes in the D'D3 region of VWF, which can lead to the increased clearance rate of FVIII in plasma[8]. Thus, affected individuals have reduced FVIII levels ranging from 1 to 40 U/dL[10,11]. Due to the pathophysiology of this disorder, clinical manifestations of the patients, including epistaxis, mucocutaneous bleeding, or surgery-related bleeding, and their primary laboratory findings resemble those of patients with mild or moderate hemophilia A[12-14]. It is crucial to differentiate these conditions, as their pattern of inheritance and genetic counseling are different from each other. Moreover, differentiation between type 2N VWD and hemophilia A is essential for affected patients to receive the appropriate treatment[1,11,14]. Administration of DDAVP, the treatment of choice in type 1 VWD, typically elevates FVIII:C levels in type 2N VWD patients but fails to improve the binding capacity of VWD to FVIII, leading to poor therapeutic responses in most cases[3]. However, several studies indicate that in type 2N VWD patients with the R854Q mutation, administration of DDAVP shows almost optimal results[15]. Due to the elevated clearance rate of FVIII in type 2N patients, the infusion of recombinant FVIII alone yields short-term benefits, which is an ineffective treatment for affected individuals[3]. The potential for adverse clinical consequences of misdiagnosis, such as mistreatment or ineffective treatment with DDAVP or recombinant FVIII concentrate, underscores the urgency of accurate differentiation between type 2N VWD and hemophilia A[16]. In order to correctly diagnose type 2N and differentiate it from hemophilia A, the International Society of Thrombosis and Hemostasis (ISTH) recommends a diagnostic panel consisting of a specialized binding assay named VWF:FVIIIB and specific molecular studies[1]. The VWF:FVIIIB assay is an ELISA-based method first introduced in 1989[9]. This method allows laboratories to determine the binding capacity of an individual's VWF to FVIII[17,18]. In type 2N VWD patients, this binding capacity is significantly reduced, while it remains within normal values in individuals with hemophilia A[5,8]. As a result, this assay serves as a valuable tool in differentiating between these two conditions[4]. However, the VWF:FVIIIB assay is complex and necessitates specialized laboratories[14,19]. Additionally, recent guidelines state that the VWF:FVIIIB assay does not provide as much information as molecular studies for genetic counseling[1]. Consequently, targeted genetic testing has become more widely utilized for diagnosing type 2N VWD patients[3]. This method identifies and analyzes specific causative mutations associated with type 2N VWD, which can provide valuable information for differentiating type 2N VWD from hemophilia A[20]. Moreover, it can determine the genotype of an identified mutation, which can be used to discriminate homozygous patients from heterozygous carriers or individuals with compound heterozygous mutations[4]. Nowadays, the emergence of new advanced and state-of-the-art sequencing techniques, such as next-generation sequencing (NGS), alongside other well-established methods like Sanger's sequencing, has revolutionized molecular studies. These advancements have not only led to streamlined processes but have also significantly reduced turnaround times while concurrently enhancing the reliability and accuracy of the results obtained[3]. However, the ISTH has stated that there is not enough evidence to support the use of genetic studies or the VWF: FVIIIB assay for suspected patients. Therefore, it is recommended that both tests be utilized during the diagnostic process, emphasizing the importance of comprehensive testing[1].

The aim of the current study was to identify type 2N VWD patients among individuals with mild to moderate FVIII deficiency referred to the coagulation laboratory of the Iranian Blood Transfusion Organization (IBTO) over an 18-month period using genetic analysis as a definite diagnostic method.

MATERIAL AND METHODS

Study sample

This cross-sectional study enrolled patients with mild or moderate FVIII deficiency referred to the special coagulation laboratory of the IBTO during an 18-month period from September 2021 to March 2023, based on the inclusion and exclusion criteria presented in **Table I**.

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Table I - *Inclusion and exclusion criteria*

Inclusion criteria	Exclusion criteria
FVIII:C levels between 50% to 1%	History of blood transfusion or receiving FVIII concentrate within 7 days
FVIII:C/VWF:Ag ratios below 1	Existence of conditions leading to multiple coagulation factor depletion or acquired FVIII deficiency
-	Familial history indicating mild or moderate hemophilia A

FVIII:C factor VIII activity; FVIII: factor VIII; VWF:Ag: von Willebrand factor antigen.

The study was approved by the High Institute for Education and Research in Transfusion Medicine Ethics Committee (IR.TMI.REC.1401.028). All participants signed and provided informed consent.

Sample size calculation

To determine the minimum sample size required for this study, the pwr package of R software was utilized (R Foundation, Vienna, Austria). Given the rarity of type 2N VWD among the population, a high effect size (h=0.8) with a significance level of 0.05 and a power of 80% was considered for a two-sided test. Based on these parameters, a minimum of 13 participants were necessary for this study (**Figure 1**).

Genetic analysis

In this research, genomic DNA was extracted from peripheral blood leukocytes using a commercial DNA extraction kit (Qiagen N. V., Hilden, Germany) based on the spin-column method. Afterward, exons 18, 19, and 20 of the *VWF* gene, the central mutation regions associated with type 2N VWD, were amplified using polymerase chain reaction(PCR) with specific primers[21-24]. Subsequently, PCR products were sequenced using Sanger's sequencing technique, and the generated SEQ files for each patient were analyzed using UGENE (Unipro, Novosibirsk, Russia) and Chromas (Technelysium Pty Ltd., Brisbane, Australia) bioinformatic software.

RESULTS

Over a period of 18 months, 1,092 patients with bleeding disorders were referred to IBTO's special coagulation laboratory to assess FVIII activity levels. Among them, 971 individuals had above FVIII:C levels above 50%, while 48 cases were patients with severe hemophilia A with FVIII activity levels below 1%. The remaining 73 individuals with FVIII:C levels ranging from 1 to 50% were described as patients with mild or moderate FVIII deficiency. This group was selected for further evaluations that revealed 19 patients with FVIII:C/VWF:Ag ratios above 1, 10 individuals with multifactorial deficiencies, 8 patients who had received FVIII concentrates 7 days prior to the study, and 1 patient with a family history indicating hemophilia A that was confirmed by clinical and laboratory findings. All these cases, totaling 38 individuals, were excluded. After applying the inclusion and exclusion criteria, the study enrolled 35 patients (70% men) with a mean age of 18±14.5 (range 1-62) years from 33 unrelated families.

Genetic studies on exons 18, 19, and 20 of the *VWF* gene revealed that 5 patients harbored known causative mutations associated with type 2N VWD. These mutations were p.Thr789Pro (No.=2) and p.Arg816Gln (No.=3) located on exons 18 and 19, respectively (**Table II**). Both mutations are among Iran's most commonly reported mutations associated with type 2N VWD[25]. The clinical history of all identified patients indicated a mean bleeding score of 7 based on the ISTH-BAT scoring system,

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>pwr: :pwr.p.test(h=0.8,sig. level = 0.05,power=0.8,
+                  alternative = 'two.sided')

     proportion power calculation for binominal distribution (arcsine
   transformation)

              h = 0.8
              n = 12.26384
      sig. level = 0.05
          power = 0.8
    alternative = two.sided
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Figure 1 - The input code and the output of R software to calculate the minimum sample size

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Table II - *Results of the genetic analysis of patients with confirmed type 2N VWD associated mutations*

Patient No.	VWF domain	Gene region	Nucleotide change	Amino-acid change	Variant type	Genotype
1	D'	Exon 19	c.2449C>T	p.Arg816Gln	Missense	Homozygous
2	D'	Exon 18	c.2365A>C	p.Thr789Pro	Missense	Homozygous
3	D'	Exon 19	c.2449C>T	p.Arg816Gln	Missense	Homozygous
4	D'	Exon 18	c.2365A>C	p.Thr789Pro	Missense	Heterozygous
5	D'	Exon 19	c.2449C>T	p.Arg816Gln	Missense	Homozygous

VWD: von Willebrand disease; VWF: von Willebrand factor.

Table III - *Demographic data and results of coagulation testing of identified patients with type 2N VWD*

Patient No.	Gender	Age	BAT score	Primary laboratory findings			
				VWF:Ag	VWF:Rco	FVIII:C	FVIII:C/VWF:Ag ratio
1	Male	18	7	57.0	48.0	8.0	0.14
2	Male	20	12	44.0	42.0	12.0	0.27
3	Male	33	7	50.0	32.0	20.0	0.40
4	Female	62	4	41.0	20.0	37.0	0.90
5	Male	18	5	75.0	76.0	17.6	0.23

VWD: von Willebrand disease; BAT: bleeding assessment tool; VWF:Ag: von Willebrand factor antigen; VWF:Rco: von Willebrand factor ristocetin cofactor; FVIII:C: factor VIII activity.

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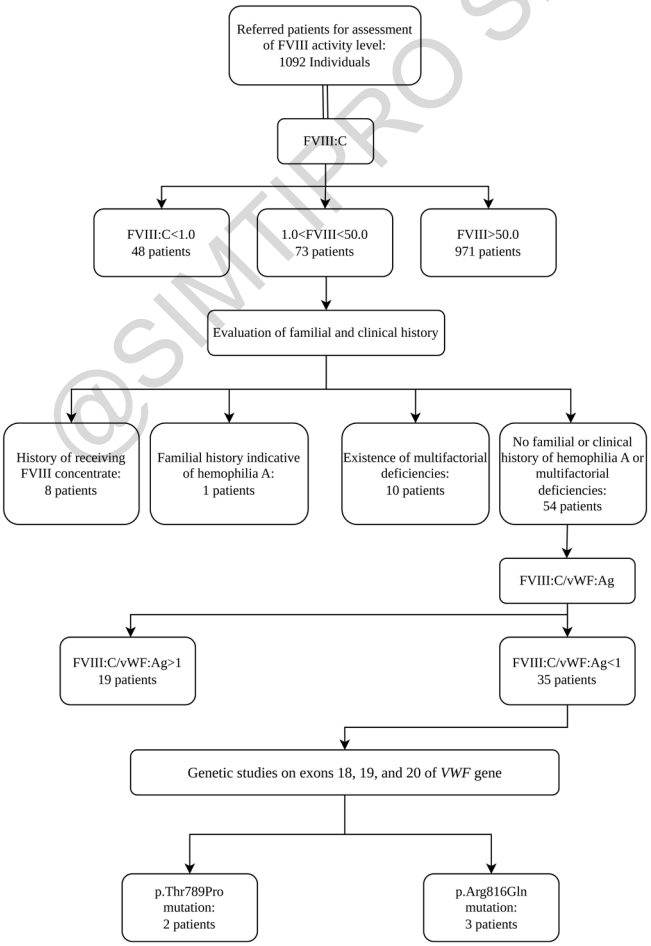


Figure 2 - Identification algorithm of type 2N VWD patients with molecular studies
VWD: von Willebrand disease; FVIII: factor VIII; FVIII:C: factor VIII activity; vWF:Ag: von Willebrand factor antigen; VWF: von Willebrand factor.

with mucocutaneous and post-surgery bleeding being the most common clinical symptoms. In addition, primary laboratory findings of these individuals showed FVIII:C, VWF:Ag, and FVIII:C/VWF:Ag values of 18.8±10.9 percent, 49.4±16.8 IU/dL, and 0.30±0.13, respectively. Moreover, VWF:RCo levels for these patients were 43.6±20.9 IU/dL (**Table III**). Furthermore, their clinical history indicated that 3 patients were misdiagnosed with hemophilia A at young ages, while the remaining 2 cases were not diagnosed until the ages of 33 and 62 (**Figure 2**).

DISCUSSION

The challenges in diagnosing type 2N VWD are significant and often underappreciated. The condition's similar clinical presentations and laboratory results to mild or moderate hemophilia A can be a major hurdle[3]. Diagnosing type 2N VWD requires a comprehensive assessment of the patient's symptoms and familial history of bleeding disorders, along with a series of specialized coagulation tests[1]. This study aimed to use targeted molecular studies as a definitive diagnostic method to identify and differentiate type 2N VWD from mild or moderate hemophilia A, despite its limited utilization in Iran.

The current study revealed that during 18 months, 1092 patients with bleeding disorders were referred to IBTO's coagulation laboratory for measurement of their FVIII levels. Considering the rarity of type 2N VWD, 35 individuals with mild or moderate FVIII deficiency were chosen for further evaluation after applying the inclusion and exclusion criteria. Since IBTO's coagulation laboratory serves as a major referral center for patients with bleeding disorders in Iran, the identification of this number of cases within 18 months may suggest a referral bias. Results of targeted molecular studies revealed that, among all participants, 5 individuals (14.2%) had known causative mutations associated with type 2N VWD. This finding is slightly higher than the previous estimates, as Meyer *et al.* estimated in 2010 that only 10% of individuals with low levels of FVIII activity and FVIII:C/VWF:Ag ratios below 1 are type 2N VWD patients[26]. The incidence of consanguineous marriages is higher in Iran than in developed countries. This could be a contributing factor to the slightly higher prevalence of type 2N VWD patients in our study. It should be noted that among all identified patients, 3 individuals (60%) had parents with consanguineous marriages. Similarly, Seidizadeh *et al.* conducted a 10-month study with 128 participants in Iran. Their results showed that 23 patients were diagnosed with VWD, with 5 of them having type 2N VWD, and 3 of these 5 had parents with consanguineous marriages[14]. Similar results were obtained in another study in India, a developing country with approximately 70% incidence of consanguinity. During a 5-year study, 796 participants with bleeding diathesis were evaluated and 9 type 2N patients were identified[27]. These comparisons emphasize the impact of consanguineous marriages on the occurrence of congenital bleeding disorders, which can be considered one of the main reasons for the high frequency of type 2N patients in our studied population during 18 months.

Regarding primary laboratory findings, results of our study revealed that VWF antigen and activity levels of the 5 identified individuals were 49.4±16.8 IU/dL and 43.6±20.9 IU/dL, respectively. These levels were slightly below the lower limit of the normal diagnostic range, possibly due to conformational changes in the D'D3 region of VWF. Studies indicate that these changes can lead to defects in intracellular packaging, secretion, or increased binding of VWF to clearance receptors, resulting in subnormal VWF levels in affected individuals[28,29]. Regardless, these findings did not provide valuable data to distinguish between type 2N VWD and mild or moderate hemophilia A. However, the FVIII activity levels of the patients were 18.8±10.9 percent with an FVIII:C/VWF:Ag ratio of 0.30±0.13, which was below normal diagnostic limits. Thus, these parameters can be used to identify suspected type 2N VWD cases for further evaluation[1,30]. Similar to our findings, Casonato *et al.* demonstrated that FVIII activity level and FVIII:C/VWF:Ag ratio decreases significantly in homozygous type 2N VWD patients, while FVIII:C/VWF:Ag ratio remains within the normal diagnostic range in compound heterozygous type 2N patients with qualitative VWF defects. Therefore, the hemostatic results of the first group of patients can be used to identify suspected type 2N VWD cases. In contrast, the decrease in FVIII activity level of the second group of patients may be attributed to the decrease in VWF: Ag levels[31]. Overall, a definitive diagnosis of type 2N patients based on clinical manifestations and primary laboratory results can be challenging, leading to potential misdiagnosis of affected individuals. In this regard,

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Favaloro *et al.* conducted a study that pointed out that 91% of requests for further evaluations and correct typing of type 2N VWD failed, resulting in the misdiagnosis of patients[32].

The current study's targeted genetic testing revealed that, among the identified patients, 3 individuals had the p.Arg816Gln mutation, while the other 2 had the p.Thr789Pro mutation. These results are consistent with a 13-year study by Rasoulzadegan *et al.* in Iran, which found that among 24 type 2N VWD patients, 6 cases had the p.Thr789Pro mutation, and 1 patient had the p.Arg816Gln mutation[25]. Therefore, all identified mutations in our study were among the known causative mutations associated with type 2N VWD in Iran. However, none of these mutations were among the most common type 2N VWD mutations. According to several studies, p.Arg854Gln, p.Arg816Trp, and p.Thr791Met are considered the most prevalent type 2N VWD mutations worldwide[33]. Additionally, a study by Liang *et al.* in China failed to identify patients with the p.Arg854Gln mutation, the most commonly found causative type 2N VWD mutation among Caucasians[34]. The presence of a globally rare but locally common mutation may have arisen from a founder effect, where a population is established by a few individuals carrying specific genetic variants. These genetic variants are often preserved and amplified in communities with limited gene flow caused by geographical barriers, cultural practices, or marriage patterns[2]. Thus, the molecular profile of type 2N patients varies based on their geographical regions. This underscores the importance of identifying the most common mutations in a population, revealing its regional mutational landscape to diagnose affected individuals accurately.

Limitations

Out of 35 patients with mild or moderate FVIII deficiency, 5 individuals were identified with type 2N VWD, while the cause of reduced FVIII activity levels in the remaining participants remained unknown. Therefore, further genetic analysis of exons 17, 21 through 25, and 27 of the *VWF* gene as other possible mutation sites is required. Moreover, detection of large gene deletions associated with type 2N VWD requires multiplex ligation-dependent probe amplification, a method that was not utilized in this study[35]. Additionally, molecular analysis of *F8* gene for identification of pathogenic variants can confirm the diagnosis of hemophilia A.

In this study, the VWF:FVIIIB assay was not conducted due to limited access to the test in Iran. Consequently, the diagnosis of type 2N VWD patients relied solely on molecular studies. Therefore, this study suggests that future research should incorporate both the VWF:FVIIIB assay and complete molecular analysis to reduce the risk of underdiagnosing affected individuals.

CONCLUSIONS

In contrast to several prior studies utilizing a similar approach, which found that only 10% of patients with reduced FVIII:C levels and FVIII:C/VWF:Ag ratios below 1 could be classified as type 2N VWD cases, the current study conducted in Iran revealed that 14.2% of the participants carried known type 2N VWD mutations. This slightly higher prevalence is likely due to the increased frequency of consanguineous marriages in the region. Moreover, the study uncovered that out of 5 affected cases, 3 patients had been misdiagnosed and mistreated, while 2 individuals had been overlooked for diagnosis. It seems that the limited availability of precise diagnostic assays for type 2N VWD in Iran may contribute to a heightened risk of misdiagnosis or underdiagnosis among patients with this condition. Therefore, the authors recommend the further establishment of a comprehensive diagnostic panel for type 2N VWD, including both the VWF:FVIIIB assay and molecular studies, to be implemented in referral coagulation laboratories throughout Iran.

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The Authors declare no conflicts of interests.

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