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Relationship between factor VIII genetic pattern and presence of mild hemophilia, severity of bleeding

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We aimed to assess mild hemophilia and relationship between blood parameters and genetic pattern and bleeding in patients with mild hemophilia. This cross-sectional descriptive-analytic study was performed on 50-member family was screened for people with mild hemophilia. To detect gene mutations in patients, peripheral blood was taken DNA was extracted to assess DNA variant of factor VIII by the PCR method and finally DNA sequences was determined using the ALFexpress auto read sequencing. The rate and severity of bleeding was evaluated using a special questionnaire. Out of 50 subjects studied, 32 (64.0%) including 16 men and 16 women were carriers for hemophilia, while 18 (36.0%, 13 men and 5 women) were normal for the disease. The mean level of factor VIII was significantly lower in those suffering mild hemophilia as compared to normal people (23.69 ± 4.30 versus 73.39 ± 3.42, $p < 0.001$). Similarly, the mean PTT was also higher in mild hemophilia group than in healthy group (34.00 ± 1.69 versus 26.95 ± 0.60, $p < 0.001$). Most cases with mild hemophilia had no bleeding or suffering mild bleeding. There was no significant correlation between severity of bleeding and the level of factor VIII in mild hemophilia. The gene analysis could confirm the genotype of hemophilia in those patients who were homozygous for factor VIII gene. Lowering factor VIII with overexpression of its related gene is dominant in patients with mild hemophilia; however, it may not be associated with the severity of bleeding in such patients.

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Introduction

Hemophilia is an X-linked heritable hemorrhagic disorder due to reduced coagulation factors (Srivastava A. et al. 2013). In this disease, bleeding occurs in bruises, gastrointestinal tract, joints, and even genital tract with various intensities. Hemophilia A is a result of a reduction in factor VIII, which is seen in one case per 5000 births in a single case and accounts for 80% of cases of hemophilia (Peters R. 2018, Lippi G. 2007). The tendency to bleeding in hemophilia type-A carriers is already known and some of them, even with a level of about 50%, also develop abnormal hemorrhage (Paroskie A. et al. 2015). Individuals who are definite hemophilia A carriers include women with hemophilic, women with a hemophilic child and positive family history of hemophilia A, and women with more than one hemophilic child (Allan JN. Et al. 2014, Miesbach W. et al 2010). If the coagulation factor is less than normal, it strongly suggests the carrier state, however normal values of these factors do not rule out it. The importance of measuring the amount of coagulation factors is to determine the risk of bleeding during or after delivery as well as within or after surgical and dental interventions (Fijnvandraat K. et al 2012, Bhoi D. 2013). In the case of asymptomatic patients with a family history of bleeding, even if the laboratory phenotype (factor VIII) is known, it can be used to predict bleeding (Mannucci PM. 2012). Therefore, doctors dealing with these people should have sufficient information about their condition. Because these people should be considered to take some preventive measures or avoid some of the measures that put them at risk. Individuals with mild hemophilia A do not have spontaneous bleeding episodes; however, without pre- and postoperative treatment, abnormal bleeding occurs with surgery or tooth extractions; the frequency of bleeding episodes varies widely, typically from once a year to once every ten years (Powell JS.2009, Bhopale GM.2003). Individuals with mild hemophilia A are often not diagnosed until later in life. Approximately 30% of heterozygous females have clotting activity below 40% and are at risk for bleeding (even if the affected family member is mildly affected) (Konkle BA. Et al. 2018). Because mild hemophilia without spontaneous bleeding may remain undiagnosed for a lifetime, continuous screening of coagulation factors with the approach to familial linkage in such patients is necessary (Biswas A. et al. 2014). The present study aimed to epidemiologically assess mild hemophilia A in a large Iranian family and the relationship between blood parameters and genetic pattern and bleeding in patients with mild hemophilia.

Method

Study population

This cross-sectional descriptive-analytic study was performed on the relatives of a hemophilic woman who were referred to Shafa Hospital in Ahvaz in 2017. Due to financial and administrative constraints, only a 50-member family was screened for people with mild hemophilia (Figure 1).

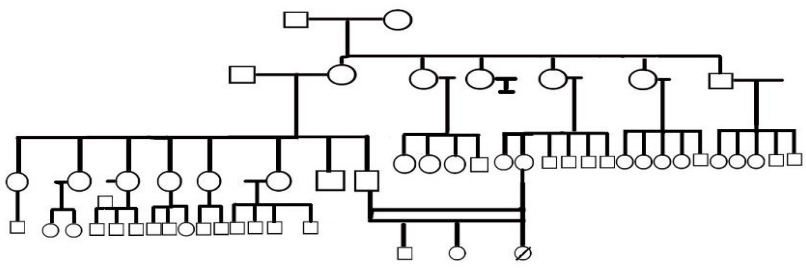

Figure 1: The pedigree of the family with mild hemophilia

Criteria for entering into the study were to confirm the mild hemophilia based on patient biography and genetic testing. Exit criteria included dissatisfaction or non-cooperation of hemophilic patients for the study, or receiving coagulation factors 10 days before the study. After receiving the informed consent of the patients and explaining how the study was performed and possible unwanted side effects to the patients, cell blood count, coagulation biomarkers, ferritin, and factor VIII tests were planned for the selected patients.

Gene assessment

To detect gene mutations in patients, peripheral blood was taken and salting out technique and protein kinase by using a DNA kit (Yekta Tajhiz Azma Co. Iran) was used for DNA extraction. The DNA samples were kept in the freezer at temperatures of -20°C until the tests were carried out. The spectrophotometric method will be used to evaluate the quality and quantity of DNA. Also, to ensure that the genome is healthy and not to have broken fragment, the genome of each extraction is electrophoresed by agarose gel. Suitable primers for proliferation of exons in the F8 gene were designed and ordered using the Polymerase Chain Reaction (PCR) using Primer3plus and MFE Primer online software. To perform PCR, Taq DNA Polymerase, primers, deoxynucleotide triphosphates (dNTPs) and magnesium ions (Mg^{2+}) were used with sterile buffer and distilled water, and the final volume of the PCR mix was considered to be 20µl. The PCR program consisted of 35 cycles of 95ºC for 60 seconds, 60ºC for one minute and finally 72ºC for 45 seconds. Then, PCR products will be examined by agarose gel electrophoresis and if the band was visible, DNA sequences were determined using the ALFexpress autoread sequencing kit, and the resulting sequences were then adapted using different software such as blast and Gene Runner and the mutations were finally identified. After obtaining the blood and genetic data of patients, its association with the amount of bleeding from the questionnaire of each patient will be measured. First, exons with a high frequency of reported mutations are sequenced, which consists of 8 exons of 24 exons.

Assessment of bleeding rate

The rate of bleeding in patients was evaluated using a special questionnaire and the relationship between the laboratory parameters and the rate of bleeding in the patients was evaluated. Considering that one of the goals of this study was to investigate the association between bleeding severity and factor VIII, therefore, the types of bleeding including nasal bleeding, skin bleeding, bleeding from small scars, oral bleeding, gastrointestinal bleeding, hematuria, hemorrhage after tooth extraction, postoperative hemorrhage, severe bleeding after surgery or trauma, menstrual bleeding, postpartum hemorrhage, spontaneous hemorrhage, intra-articular bleeding, and central nervous system hemorrhage were evaluated using a special questionnaire (appendix). Also, using the scoring system defined in this questionnaire, the scores were as follows: no bleeding (score 0), mild bleeding (score: 1), moderate bleeding (score 2), moderate to severe bleeding (score 3) and severe bleeding (score 4).

Statistical analysis

The results were presented as mean ± standard deviation (SD) for quantitative variables and were summarized by absolute frequencies and percentages for categorical variables. Normality of data was analyzed using the Kolmogorov-Smirnoff test. Categorical variables were compared using chi-square test or Fisher's exact test when more than 20% of cells with expected count of less than 5 were observed. Quantitative variables were also compared with t test or Mann U test. For the statistical analysis, the statistical software SPSS version 16.0 for windows (SPSS Inc., Chicago, IL) was used. P values of 0.05 or less were considered statistically significant.

Results

A 50-member family including 29 males (58.0%) and 21 females (42.0%) were assessed, the mean age of participants was 22.17 ± 15.12 years ranged 2 to 57 years. The mean age of female individuals was significantly higher than male people (28.40 ±

16.37 years versus 17.50 ± 12.58 years, p < 0.001). Of 50 subjects studied, 32 (64.0%) including 16 men and 16 women were carriers for hemophilia, while 18 (36.0%, 13 men and 5 women) were normal for the disease. The mean level of factor VIII was significantly lower in those suffering mild hemophilia as compared to normal people (23.69 ± 4.30 versus 73.39 ± 3.42, p < 0.001). Similarly, the mean PTT was also higher in mild hemophilia group than in healthy group (34.00 ± 1.69 versus 26.95 ± 0.60, p < 0.001). There was no difference in the mean level of other serum biomarkers such as serum ferritin, hemoglobin, RBC, hematocrit, MCV and MCH levels across the two groups with and without mild hemophilia (Table 1).

Table 1: Blood indexes in healthy subjects and those with mild hemophilia

Index	Mild hemophilia group	Healthy group	Total
Ferritin	**20.23 ± 5.12**	**24.82 ± 6.74**	**23.11 ± 4.61**
F VIII	**23.69 ± 4.30***	**73.39 ± 3.42**	**54.93 ± 4.89**
WBC	**7377 ± 662.2**	**7589 ± 343.1**	**7503 ± 331.5**
RBC	**4.753 ± 0.12**	**4.584 ± 0.07**	**4.653 ± 0.06**
Hb	**14.10 ± 0.47**	**13.95 ± 0.39**	**14.01 ± 0.29**
HCT	**39.41 ± 0.97**	**38.45 ± 0.86**	**38.84 ± 0.64**
MCV	**83.08 ± 1.52**	**83.81 ± 1.15**	**83.52 ± 0.91**
MCH	**29.71 ± 0.75**	**30.39 ± 0.58**	**30.11 ± 0.46**
MCHC	**35.71 ± 0.37**	**36.22 ± 0.30**	**36.01 ± 0.23**
PLT	**241.3 ± 19.37**	**254.9 ± 16.52**	**249.4 ± 12.43**
RWDsd	**44.28 ± 0.65**	**44.02 ± 0.56**	**44.12 ± 0.42**
RWDsv	**13.75 ± 0.40**	**13.74 ± 0.28**	**13.75 ± 0.23**
PWD	**14.04 ± 0.80**	**12.89 ± 0.36**	**29.57 ± 0.98**
MPV	**10.76 ± 0.31**	**10.27 ± 0.18**	**10.47 ± 0.17**
PTT	**34.00 ± 1.89***	**26.95 ± 0.60**	**29.57 ± 0.98**
Neutrophil	**49.62 ± 5.14**	**44.13 ± 3.52**	**44.98 ± 3.21**
Lymphocyte	**38.18 ± 3.05**	**42.09 ± 2.20**	**40.50 ± 1.80**

In the present study, there was a significant adverse relationship between factor VIII and PTT in patients with mild hemophilia. Furthermore, there was no significant association of factor VIII with ferritin and hemoglobin in such patients (Table 2).

Table 2: relationship between factor VIII and blood indices in healthy and mild hemophilia patients

Index	Mild hemophilia group	Healthy group	Total
Ferritin	**r= 0.230** **P=0.448**	**r= 0.950** **P=0.674**	**r= 0.136** **P=0.433**
F VIII	**r=-0.286** **p=0.342**	**r=-0.046** **p=0.850**	**r=-0.040** **p=0.824**
RBC	**r=-0.594** **p=0.320**	**r=-0.160** **p=0.512**	**r=-0.177** **p=0.467**
Hb	**r=-0.418** **p=0.154**	**r=-0.416** **p=0.075**	**r=-0.252** **p=0.164**
HCT	**r=-0.369** **p=0.213**	**r=-0.343** **p=0.149**	**r=-0.293** **p=0.104**
MCV	**r=0.306** **p=0.307**	**r=-0.405** **p=0.084**	**r=0.017** **p=0.926**
MCH	**r=0.037**	**r=-0.494**	**r=-0.019**

	p=0.903	**p=0.031**	**p=0.917**
MCHC	**r=-0.428** **p=0.144**	**r=-0.522** **p=0.021**	**r=-0.078** **p=0.668**
PLT	**r=0.103** **p=0.735**	**r=0.210** **p=0.387**	**r=0.167** **p=0.359**
RWDsd	**r=-0.172** **p=0.573**	**r=-0.015** **p=0.951**	**r=-0.088** **p=0.631**
RWDsv	**r=-0.449** **p=0.123**	**r=0.289** **p=0.229**	**r=-0.035** **p=0.848**
PWD	**r=-0.356** **p=0.232**	**r=0.076** **p=0.756**	**r=-0.303** **p=0.090**
MPV	**r=-0.303** **p=0.313**	**r=-0.046** **p=0.850**	**r=-0.307** **p=0.086**
PTT	**r=-0.682** **p=0.010**	**r=-0.455** **p=0.033**	**r=-0.733** **p=0.065**
Neutrophil	**r=0.126** **p=0.679**	**r=0.132** **p=0.600**	**r=-0.158** **p=0.386**
Lymphocyte	**r=-0.202** **p=0.500**	**r=-0.202** **p=0.956**	**r=0.121** **p=0.507**

In this study, the severity of bleeding was studied at different points. The results showed that most cases with mild hemophilia had no bleeding or suffering mild bleeding (Table 3).

Table 3: Evaluation of bleeding severity in patients with mild hemophilia

Type	None	Mild	Moderate	Moderate to severe	Severe
Nasal	**10 (76.92)**	**0 (0.0)**	**0 (0.0)**	**2 (15.38)**	**1 (7.7)**
Skin	**10 (76.92)**	**0 (0.0)**	**0 (0.0)**	**2 (15.38)**	**1 (7.7)**
Small wound	**0 (0.0)**	**0 (0.0)**	**0 (0.0)**	**0 (0.0)**	**0 (0.0)**
Mouth	**10 (76.92)**	**0 (0.0)**	**0 (0.0)**	**2 (15.38)**	**1 (7.7)**
gastrointestinal	**10 (76.92)**	**1 (7.7)**	**2 (15.38)**	**0 (0.0)**	**0 (0.0)**
Hematuria	**12 (92.30)**	**0 (0.0)**	**1 (7.7)**	**0 (0.0)**	**0 (0.0)**
Dental extract	**6 (46.15)**	**2 (15.38)**	**2 (15.38)**	**2 (15.38)**	**1 (7.7)**
Surgery or trauma	**8 (61.54)**	**2 (15.38)**	**0 (0.0)**	**0 (0.0)**	**3 (23.08)**
Intramuscular	**11 (84.62)**	**0 (0.0)**	**2 (15.38)**	**0 (0.0)**	**0 (0.0)**
Articular	**11 (84.62)**	**0 (0.0)**	**0 (0.0)**	**0 (0.0)**	**2 (15.38)**
Nervous system	**0 (0.0)**	**0 (0.0)**	**0 (0.0)**	**0 (0.0)**	**0 (0.0)**
Others	**5 (30.77)**	**6 (46.15)**	**3 (23.08)**	**0 (0.0)**	**0 (0.0)**

There was no significant correlation between severity of bleeding and the level of factor VIII in mild hemophilia (Table 4). The gene analysis could confirm the genotype of hemophilia in those patients who were homozygous for F8 gene.

Table 4: Relationship between bleeding severity and factor VIII in patients with mild hemophilia

Type	Bleeding score	Correlation with factor VIII
Nasal	**1.00 ± 1.60**	**r= -0.049** **P=0.884**
Skin	**1.00 ± 1.60**	**r= -0.210** **P=0.534**

Small wound	**0.0±0.0**	**-**
Mouth	**0.87 ±1.64**	**r= -0.267 P=0.426**
gastrointestinal	**0.37 ± 0.74**	**r= -0.291 P=0.384**
Hematuria	**025 ± 0.90**	**r= -0.101 P=0.767**
Dental extract	**1.25±1.58**	**r= -0.204 P=0.545**
Surgery or trauma	**1.13 ± 1.81**	**r= -0.387 P=0.239**
Intramuscular	**0.25 ± 0.71**	**r= -0.195 P=0.565**
Articular	**0.50 ± 1.41**	**r= -0.258 P=0.442**
Nervous system	**0.00±0.00**	**-**
Others	**0.87 ± 0.64**	**r= -0.811 P=0.002**
Total	**0.62 ± 1.22**	**r= -0.768 P=0.061**

Discussion

The present study led to an overall prevalence of 26% for mild hemophilia that was shown to be 16% in the study by Poon et al among Chinese population (Poon MC. 2008), 32% in the study by Geraghty et al among American nation (Walker I. et al. ,1995), 34% in IORIO et al study in Italy (Iorio A. et al. 2008), and even 50% in the study by Walker et al among Canadian population (Walker I. 1995). It seems that the reasons for these differences can be the difference in the size of samples studied, economic and cultural conditions, and differences in the amount of family marriage. In countries with higher per capita income, the prevalence of mild hemophilia was found to be 34%, while in countries with lower incomes it was estimated to be 16%. A study in Sweden has shown that the incidence of mild hemophilia has risen from 35% to 54% over the past 20 years, which the authors attribute to increased consciousness of people on the different aspects of disease. These studies show that economic and cultural factors can help in more accurate identification of people with mild hemophilia and thus provide more accurate results (Jensson O. et al. 1994).

Our study could show lower level of factor VIII as well as higher PTT value in the patients with mild hemophilia as compared to normal condition. Also, the association between the two pointed parameters was also significant. Previous studies have shown that there is a negative correlation between factor VIII and PTT; however, some people with mild hemophilia may have a normal PTT. Therefore, in these cases, the diagnostic test for mild hemophilia is only based on factor VIII. Moreover, the level of factor VIII may increase in inflammation and bleeding processes and this increase may cause a false negative for the presence or absence of mild hemophilia, thus when measuring factor VIII, attention should be paid to the inflammatory and infectious aspects related to serum factor VIII. In addition, thromboplastin time is affected by liver function, vitamin K deficiency and vitamin K analogues, and it is important to check these points when investigating the relationship with factor VIII.

There was no significant correlation between severity of bleeding and level of factor VIII. Also, the results showed that there was no significant relationship between factor VIII and the bleeding types in men. Furthermore, among women with mild hemophilia, there was a significant correlation

between Factor VIII level and mouth bleeding and menstruation. In patients with mild hemophilia, excessive hemorrhage usually occurs after minor trauma, dental or surgical procedures, and this is in contrast to severe hemophilia, in which a person without impulse is also bleeding. As shown by Castaman et al (Castaman G. et al. 2016), mild hemophilia A was defined as an X-recessive hemorrhagic disorder with a factor VIII in the range of 5 to 40 units per deciliter. In the study, a mild hemophilia was detected later than severe or moderate hemophilia. In that study, researchers concluded that when bleeding with normal or sub-normal levels of factor 8 is not justifiable, chromogenic assessments for the diagnosis of mild hemophilia A seem logical.

Conclusion

Hemorrhagic symptoms usually correlate with the plasma levels of factor VIII and include a wide range of hemorrhagic events, including spontaneous bleeding in the brain to ecchymosis. In developing countries, due to limited homeostasis diagnostic facilities and a low number of experts in this field, there appears to be a significant number of unknown patients with hemophilia. Sometimes people with incomplete diagnosis are exposed to hemorrhagic symptoms. Performing a complete physical examination, collecting medical records beyond suspicious and family and collecting demographic information is important in diagnosis. To diagnose, a proper analysis of the hemostasis should be performed. Hemostasis laboratories should pay close attention to the characteristics of the screening assemblies used to evaluate patients to cover the range of cases of hemophilia suspected and mild hemophilic cases.

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