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Density of Platelet GPIIb-IIIa and Bleeding Severity in Iranian Patients with Glanzmann's Thrombasthenia

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Abstract

Background: Glanzmann's Thrombasthenia (GT) is a rare autosomal, recessive, bleeding syndrome. The main aim of this study was to investigate the relationship between symptoms, bleeding severity, and gender and subtypes of GT by platelet immunophenotyping.

Materials and Methods:Ninety five patients with Glanzmann's Thrombasthenia (GT) were assessed for the expression of GPIIb-IIIa on the platelet surface using flow cytometry, to determine the most common GT subtypes among Iranian patients. We also evaluated the severity of bleeding phenotype, and classified them as mild, moderate, or severe bleeders.

Results: On the basis of their platelet GPIIb-IIIa levels, 73 patients (77%) were classified as type I, 16 patients (17%) as type II, and 6 patients (6%) as type III. Historically, 15 of 95 patients had experienced minor bleeding, 32 reported clinically significant bleeding, and 48 patients had suffered severe bleeding. Thirty eight patients had needed packed red blood cell transfusion. However, no significant correlation was found between bleeding severity and subtypes of GT ($p > 0.05$).

Conclusion: Our study showed that there was no correlation between quantitative changes in the surface expression of platelet membrane glycoproteins, and the intensity and frequency of bleeding episodes in patients with GT.

Keywords:Phenotype, Thrombasthenia, Platelet glycoprotein GPIIb-IIIa complex, Flow cytometry.

Introduction

Glanzmann's Thrombasthenia (GT) is an autosomal recessive bleeding disorder, characterized by prolonged bleeding time, normal platelet count, and absence of platelet aggregation in response to all platelet agonists such as ADP, collagen, arachidonic acid, and thrombin except for ristocetin.[1] GT is caused by mutations in the genes encoding GPIIb(αIIb) or GPIIIa(β3) that result in qualitative or quantitative abnormalities of the platelet GPIIb-IIIa complex.[2,3] Both genes are located on chromosome 17q21–23 and various causative mutations have been identified.[4,5] Patients who are homozygous or compound heterozygote for GPIIb and/or GPIIIa pathologic mutations have symptoms of the disease, but heterozygous individuals (carriers) are asymptomatic.[6] The clinical complications of GT include lifelong bleeding with easy bruising, epistaxis, menorrhagia, and gastrointestinal bleeding.[7] Varying degrees of deficiency in platelet surface expression of GP αIIb/β3 may give rise to heterogeneity in severity of bleeding symptoms. Thus, patients with GT can be classified into three types depending on their integrin αIIb/β3 levels which may be markedly low (< 5%) in type I, reduced (10-20%) in type II, and near normal but dysfunctional in the variant type.[8] It has been demonstrated that the clinical phenotype of various diseases inherited in a classic

Mendelian fashion can be modulated by a series of factors, inherited as well as acquired. Moreover, a few reports suggest that while some patients bleed more than others, there is as yet no clear difference in phenotype between patients with different ITGA2B and ITGB3 defects.[9,10] To understand these issues we need to greatly increase the number of GT patients that are investigated. Epidemiological studies will also allow us to establish whether there is increased risk of a particular GT phenotype within different ethnic populations.[11] We have recorded a detailed bleeding history in the clinical assessment of patients referred to us for investigation. Proper evaluation of patients' bleeding symptoms is, therefore, the cornerstone of a questionnaire. Collected data should be interpreted to verify if bleeding history is not compatible with past history and modified scoring by sex and age. Therefore, the study of this relatively large group of Iranian patients with GT was designed to assess whether bleeding severity can be inferred solely from the platelet surface expression of IIb-IIIa, or whether other factors may also play a significant role.

Materials and Methods

Patients

In this study, we investigated 95 patients with GT referred to the Iranian Comprehensive Hemophilia Care Centre (ICHCC) between 2008 and 2010. Diagnosis was based upon a history of mucosal bleeding, easy bruising, and epistaxis since early childhood, as well as a prolonged bleeding time, abnormal clot retraction, and absent in-vitro platelet aggregation in response to ADP, epinephrine, and collagen, but relatively normal ristocetin-induced platelet aggregation. Control samples were collected from healthy donors with no history of abnormal bleeding. An informed consent form was obtained from all patients/their parents prior to testing and sampling. The study was approved by the ethical review committee affiliated to Tehran University of Medical Sciences (TUMS). This study was jointly supported by three centers: The Iranian Blood Transfusion Organization (IBTO), Tehran University of Medical Sciences (TUMS), and the Iranian Comprehensive Hemophilia Care Center (ICHCC).

Blood sampling and preparation

Five milliliters of peripheral blood of patients from a single care center (ICHCC) and healthy blood donors (from IBTO) was collected by venipuncture into 3.8% tri-sodium citrate solution. Citrated blood samples were centrifuged at 1000 rpm for 10 minutes at room temperature, and platelet rich plasma (PRP) was removed into a separate tube. The PRP was then centrifuged once again at 2000 rpm for 10 minutes, in order to further concentrate platelets.

Antibodies and reagents

Purified monoclonal antibodies, including HIP8 (anti-αIIb, catalog no. ab15021) and PM6/13 (anti-β3, catalog no. ab33171) were obtained from Abcam (Cambridge, U.K.). Macrophage RAM11 (Mouse Anti-Rabbit, catalog no. M0633) was purchased from DAKO (Glostrup, Denmark) as a purified isotype control for indirect flow cytometry. The Goat anti-mouse secondary antibody (Anti-Mouse -FITC, catalog no. F2653) was taken from Sigma (St, Louis, MO, USA). Calibration-beads (QuiFiKit, catalog no.K0078) were obtained from DAKO (Glostrup, Denmark).

Quantitative measurement of platelet integrin IIb3

Aliquots of 100 µl from each PRP sample were incubated with saturating doses of unconjugated primary mouse monoclonal antibodies directed against GPIIb (CD41) and GPIIIa (CD61). For each individual sample, a negative control tube containing an irrelevant mouse monoclonal antibody (anti-Rabbit macrophage) of the same isotype and concentration was also mixed with platelets. After incubation at 4°C for 45 minutes, unbound antibodies were removed by washing with PBS-BSA-Azide (0.01 mol/L PBS, 0.1% BSA, 15 mmol/L NaN3, pH 7.4), followed by the addition of FITC conjugated goat anti-mouse Ig reagent (Dako). After 45 minutes, incubation in the dark at 4°C, stained platelets were washed twice with PBS-Azide (0.01 mol/L PBS, 15 mmol/L NaN3, pH 7.4). After that, platelet pellets were resuspended in 500 µL of 1% paraformaldehyde in PBS for 2 hours at room temperature. Finally, fixed platelets were washed once with and resuspended in 500 µL of PBS-Azide before analysis.[13] To quantify the absolute number of αIIb and β3 molecules expressed on the surface

of platelets, commercially available beads (QuiFiKit) were labeled in parallel with fluorescein-conjugated goat anti-mouse immunoglobulin according to the manufacturer's instructions.

Flow cytometry was performed using Partec-III cytometers (Partec) running FloMax (Partec GmbH, munster, Germany) acquisition and analysis software. Logarithmic amplification for the fluorescence parameter detecting FITC was selected and the window of analysis was established. Subsequently, the data from the "Set-Up" beads was acquired. Fluorescence analysis was confined to bead singlet clear of debris, as illustrated on a forward scatter versus side scatter dot plot. Afterward, without changing the gate region, the data from the Calibration Beads were collected to establish a calibration curve. The same settings with a different gate were then employed for cell analysis and 10,000 events were acquired for each sample. A histogram of the events found in the platelet gate was then displayed, and the MFI was recorded. The MFI for each population of the calibration beads was plotted on the abscissa and their corresponding number of monoclonal antibody molecules (as indicated in assay value insert) on the ordinate of double logarithmic paper. After creating the calibration curve, the MFI values of the samples and controls were interpolated on the calibration curve, and their corresponding molecule numbers (ABC) were read directly. The quantitative values (ABC) of the glycoproteins were calculated after subtraction of the corresponding negative isotypic control measurement.[13, 14]

Grading of bleeding severity

For each patient, bleeding severity was graded according to the information obtained from a copy of the questionnaire completed by a physician following an interview and examination of the patient, and modified for age and sex. For the purposes of this analysis, GT patients were clinically classified as mild, moderate, or severe bleeders, in accordance with the Glanzmann's Thrombasthenia Italian Team (GLATIT) protocol,[12] which was modified to include age and gender. Those who bled only after trauma or surgery or had minor symptoms, were classified as mild bleeders, those with history of spontaneous or life-threatening hemorrhages, such as gastro-intestinal bleeding, were classified as moderate bleeders and those who had repeated episodes requiring blood or platelet transfusions were classified as severe bleeders. The greatest benefit of using modified bleeding grading, however, lies in the possibilities of establishing likelihood ratio of GT for each level of bleeding grade for age and sex. This modified grading system has consequently been employed to classify our GT patients as grade 1 (mild), grade 2

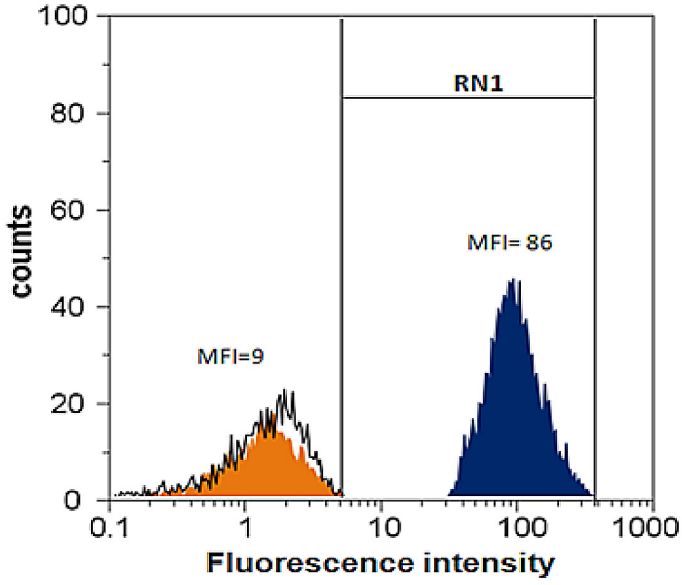

Figure1. Representative flow cytometric analysis of GPIIIa (CD61) subunit on the platelet surface. The orange histogram shows absence of the GPIIIa in a patient with GT type-I, while the blue histogram exhibits normal expression of the GPIIIa in a healthy donor. Background staining with FITC conjugated mouse IgG is shown in the open histogram. For the sake of clarity, the original plots have been redrawn.

(moderate), grade 3 (severe).

Statistical methods

SPSS/PC 15.0 version software was used (SPSS Inc., Chicago, Illinois). Results are presented as mean±SD and range. The relationships between immunophenotyping and bleeding grade were assessed by the non-parametric test of Pearson chi-square. P values <0.05 were considered significant.

Results

Quantitative measurement of platelet integrin IIb3

The results of our study revealed different patterns of GPIIb/IIIa expression among the patients compared with normal controls. Variable results were also obtained for patients classed as type 1. Fifty eight patients presented the classical profile for GT, characterized by absence or extremely reduced levels (<5%) of both GPIIb (CD41) and GPIIIa(CD61), as compared with normal platelets, while 15 patients were characterized by a heterogeneous expression pattern, as follows: 10 patients showed lack of expression for CD41 and diminished levels (<5%) of CD61; five patients exhibited severe reduction (<5%) of CD41 and weak expression (10-20% of controls) of CD61 (data not shown). Sixteen patients were classified as type II, because their GPIIb/IIIa levels varied between 7.1% and 16.3%, and six patients were diagnosed as type III (variant type), because they exhibited a normal or near-normal amount of GPIIb/IIIa complexes (data not shown), corresponding to approximately 80-100% of control levels. (Figure 1)

Grading of bleeding severity

We analyzed the clinical records of a cohort of 95 GT patients, who had already been interviewed by a physician, considering age, sex and their recorded files of bleeding severity (BS). The most frequent clinically significant bleeding symptoms in the cohort were menorrhagia (57% of menstruating females), epistaxis (36%), and gum bleeding (35%). The prevalence of each bleeding symptom is shown in tables 1 and 2. Based on the severity of bleeding history and score according to Italian study, 15 of 95 patients (15%) had minor bleeding (grade 1), 32 (33%) had a clinically significant bleeding (grade 2), and 48 (50%) had severe bleeding (grade 3). (Table 3)

Clinical and Demographic characteristics of patients

Ninety five patients with GT including 43 males and 52 females were studied. Mean age (±SD) was 19.90±12.01 years (range: 1 to 45 years). Consanguinity of first degree was present in all except six patients. Mean age at diagnosis had been 2.8 years (±4.6); 53% of patients were diagnosed before the age of 1 year, 23% between 1 and 3, 16% between 3 and 10, and only 8% subsequently. Petechiae, epistaxis, and prolonged bleeding after trauma were the most common symptoms at the time of admission, and were present in 32%, 31%, and 22% of patients, respectively. Bleeding severity in relation to the different GT subtypes is shown in table 2. There was significant correlation (Pearson Chi-square test) between sex, and age group and menorrhagia (p=0.000). Platelet concentrate had been administered to 67 patients; rFVIIa (Novoseven) to 25, and packed red cells to 38. Overall, 48 patients had experienced adverse reactions to blood products.

Discussion

Based on the historical severity of bleeding, the clinical phenotypic classification assigned to our GT

Table 1. The most prominent bleeding symptoms in GT patients.

	Patients			
Spontaneous bleeding symptoms	Type-I (n=73)	Type-II (n=16)	Type-III (n=6)	All (n=95)
Epistaxis	26	5	2	33
Gum bleeding	19	3	4	26
Menorrhagia (31 cases >11yr)	23	3	1	27
Petechiae	27	18	39	84

patients was: mild, moderate, and severe. In the present study, the severe phenotype was seen in 50%, followed by moderate and mild, seen in 33% and 15%, respectively.

Glanzmann's Thrombasthenia is definitively diagnosed by tests that determine if there is a deficiency of αIIb or β3 (GPIIb or GPIIIa) proteins. These tests use techniques involving specific monoclonal antibodies and flow cytometry.[15-17] Although some GT patients have severe deficiency of GPIIb/IIIa, they may have less severe symptoms and hence be classified as mild or moderate bleeders without any correlation to genotype.[6,8,12,13] The main goal of the present study was to determine the immunophenotypic characteristics of GT in Iranian patients, and their correlation to bleeding severity. Our study did not reveal any correlation between quantitative changes in the surface expression of platelet membrane glycoproteins and the intensity of bleeding manifestations, as some with negligible bleeding symptoms had virtually no detectable GPIIb/IIIa, while others who had 10%-15% of the normal level of functional platelet GPIIb/IIIa experienced severe hemorrhage. Sites of bleeding were similar to other reports. Lack of relationship between bleeding severity and GT subtypes is entirely consistent with previous reports (Nurden, Bellucci).[9,10,11] These data suggest that the severity of bleeding in affected individuals cannot readily be predicted from currently available laboratory findings. Indeed, some patients have never had serious bleeding, and most have been healthy and free of bleeding complications throughout their adult lives. Our study also shows that the frequency of consanguinity among Iranian patients with GT enrolled in the present study was about 94%. Therefore, although Glanzmann's thrombasthenia is usually considered to be a rare disorder, it can be almost as frequent as hemophilia and von Willebrand disease in regions where consanguinity is common.10 Because intra-familial marriage is common in Iran, some inherited disorders have a higher prevalence, and this study confirms previous observations of the more frequent occurrence of GT in Iran.[17,18]

Finally, the severity of bleeding in thrombasthenia, in contrast to coagulation disorders such as hemophilia where FVIII levels are a relatively useful measure of clinical phenotype, does not correlate with the severity of the platelet GP IIb-IIIa abnormality. However, the importance of platelet GP IIb-IIIa for normal hemostasis is clear: patients with homozygous thrombasthenia can have serious hemorrhage, while heterozygous subjects with half the normal levels of GP IIb-IIIa have no clinically significant bleeding. Therefore, quantification of bleeding severity in GT could potentially help the clinician in the adoption of optimal prophylactic measures, and in a research context, the BS may allow an improved comparison of bleeding symptoms between patient subgroups, in order to identify valid predictive determinants of bleeding.

Conclusion

In conclusion, according to our data, there is no discernible correlation between bleeding severity and immunophenotypically determined GT subgroups, and it may therefore be that other important genetic factors have a role in fostering or preventing bleeding among these patients. For instance, the coinheritance of certain defects in thrombotic factors such as genes leading to deficiency of protein C, protein S, antithrombin, factor V Leiden, MTHFR, and PG 20210 A may predispose some GT patients to a milder bleeding

Table 2. Distribution of the bleeding scores in patients with different subtypes of GT.

GT subtypes	Bleeding Score			
	1	2	3	total
Type-I	8	26	39	73
Type-II	5	5	6	16
Type-III	2	1	3	6
Total	15	32	48	95

Table3. Type, age, gender and severity of disease.

Glanzmann (95)	≤10 y (29)	Male (18)	Type I (16)	Mild = 3
				Moderate = 9
				Severe = 4
			Type II (1)	Mild = 0
				Moderate = 1
				Severe = 0
			Type III (1)	Mild = 0
				Moderate = 0
				Severe = 1
		Female (11)	Type I (9)	Mild = 3
				Moderate = 3
				Severe = 3
			Type II (1)	Mild = 0
				Moderate = 1
				Severe = 0
			Type III (1)	Mild = 0
				Moderate = 0
				Severe = 1
	11-20 (19)	Male (7)	Type I (6)	Mild = 0
				Moderate = 1
				Severe = 5
			Type II (1)	Mild = 0
				Moderate = 0
				Severe = 1
			Type III (0)	Mild = 0
				Moderate = 0
				Severe = 0S
		Female (12)	Type I (11)	Mild = 0
				Moderate = 5
				Severe = 6
			Type II (1)	Mild = 0
				Moderate = 0
				Severe = 1
			Type III (0)	Mild = 0
				Moderate = 0
				Severe = 0
	>20 (47)	Male (18)	Type I (8)	Mild = 1
				Moderate = 2
				Severe = 5
			Type II (8)	Mild = 5
				Moderate = 0
				Severe = 3
			Type III (2)	Mild = 0
				Moderate = 0
				Severe = 2
		Female (29)	Type I (23)	Mild = 1
				Moderate = 6
				Severe = 16
			Type II (4)	Mild = 0
				Moderate = 3
				Severe = 1
			Type III (2)	Mild = 1
				Moderate = 0
				Severe = 1

phenotype. In contrast, presence of synergistic action of the GPIIb and IIIa gene defects is important.

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References

1. Phillips DR, Charo IF, Scarborough RM. GPIIb-IIIa: the responsive integrin. Cell; 1991. 65: 359-62.

2. George JN, Caen JP, Nurden AT. Glanzmann's thrombasthenia: the spectrum of clinical disease. Blood. 1990; 75: 1383–95.

3. French DL. The molecular genetics of Glanzmann's thrombasthenia. Platelets. 1998; 9: 5–20.

4. French DL, Coller BS. Hematologically important mutations: Glanzmann thrombasthenia. Blood Cells Mol Dis. 1997; 23: 39–51.

5. D' Andrea G, Colaizzo D, Vecchione G, Grandone E, Di Minno G, Maraglione M, Glanzmann's Thrombasthenia Italian Team (GLATIT). Glanzmann's thrombasthenia: Identification of 19 new mutations in 30 patients. Thromb Haemost. 2002; 87: 1034–42.

6. Srivastava A, Usher S, Everette J, Nelson R, Jayandharan G, Ramachandran V, et al. Prenatal diagnosis of Glanzmann thrombasthenia. Natl Med J India ; 2003; 16: 207-8.

7. George JN, Caen JP, Nurden AT. Glanzmann's thrombasthenia: the spectrum of clinical disease. Blood. 1990; 75: 1383-95.

8. Kannan M, Ahmad F, Yadav B, Anand M, Jain P, Kumar R, et al. Glanzmann's thrombasthenia in North Indians: Sub classification and carrier detection by flow cytometry. Platelets. 2009; 20: 12-5.

9. Bellucci S, Caen J. Molecular basis of Glanzmann's Thrombasthenia and current strategies in treatment. Blood Rev. 2002; 16: 193–202.

10. James N, Caen J, Nurden A. Glanzmann's Thrombasthenia: The Spectrum of Clinical Disease. Blood. 1990; 75: 1383-95.

11. Nurden A. Glanzmann thrombasthenia: the need for epidemiological studies. J Thromb Haemost. 2009; 7: 1875–7.

12. D'Andrea G, Maraglione M, Glanzmann's Thrombasthenia Italian Team (GLATIT). Glanzmann's thrombasthenia: modulation of clinical phenotype by alpha2C807T gene polymorphism. Haematologica. 2003; 88:1378–82.

13. Kannan M, Ahmad F, Yadav BK, Anand M, Jain P, Kumar R, et al. Glanzmann's thrombasthenia in North Indians: sub classification and carrier detection by flow cytometry. Platelets. 2009; 20: 12-5.

14. Papin J, Loiseau S, Kayaba M, Dombrowicz D, Woerly G. Human eosinophils and human high affinity IgE receptor transgenic mouse eosinophils express low levels of high affinity IgE receptor, but release IL-10 upon receptor activation. J Immunol. 2001; 167: 995-1003.

15. Kathryn E, Webert J, Cook S, Sigouin M, Heddle I. The risk of bleeding in thrombocytopenic patients with acute myeloid leukemia. Haematologica. 2006; 91: 1530-7.

16. Donald M, Donahoe L, France J, Andrea J, Heels-Ansdell D, Zytaruk N, et al. Bleeding during critical illness: A retrospective cohort study using a new measurement tool. Clin Invest Med. 2007; 30: 93-102.

17. Bussel J, Cheng G, Saleh M, Psaila B, Kovaleva L, Meddeb B, et al. Eltrombopag for the Treatment of Chronic Idiopathic Thrombocytopenic Purpura. N Engl J Med. 2007; 357: 2237-47.

18. Toogeh G, Sharifian R, Lak M, Safaee R, Artoni A, Peyvandi F. Presentation and pattern of symptoms in 382 patients with Glanzmann thrombasthenia in Iran. Am J Hematol. 2004; 77: 198-9.