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ORIGINAL ARTICLE

Identification of Clinicopathological Spectrum, Platelet Glycoprotein IIb/IIIa complex and Platelet Antibodies in Egyptian Children with Glanzmann's Thrombasthenia

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Abstract Glanzmann's thrombasthenia (GT) is a rare genetic bleeding disorder. The aim of our study was to evaluate the clinicopathological spectrum of this syndrome and to study the platelet glycoprotein IIb/IIIa complex and platelet antibodies by flow cytometry in a cohort of children with GT in a tertiary care center in Upper Egypt. Forty children with GT were assessed for the expression of GPIIb-IIIa on the platelet surface and platelet antibodies by using flow cytometry, to determine the most common GT subtypes among Egyptian children. By analysis of platelet GP IIb-IIIa by flow cytometry the classification of patients with GT in our study was type I GT (47.5%), type II GT (32.5%) and type III GT (20%). In this study, we have delineated that type I is the most common type of GT in Upper Egypt. Our data suggested that there is a good correlation between quantitative changes in the surface expression of platelet membrane glycoproteins detected by flow cytometry and the clinical severity of bleeding. Therefore, classifying of severity of bleeding in patients with GT could possibly aid the pediatricians and hematologists in the implementation of ideal prophylactic measures.

Keywords Glanzmann's thrombasthenia · Platelet glycoprotein GPIIb-IIIa complex · Flow cytometry · Children

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Introduction

Glanzmann's thrombasthenia (GT) is a rare genetic bleeding disorder, characterized by prolonged bleeding time, normal platelet count, and absence of platelet aggregation in response to all platelet agonists, such as adenosine diphosphate (ADP), collagen, arachidonic acid, and thrombin, except for ristocetin. It is estimated that one in 1,000,000 individuals have GT, though the exact number is unknown (Nurden 2006; Solh et al. 2015; Toogeh et al. 2004). GT is an autosomal recessive disease caused by mutations in the genes encoding GPIIb/IIIa (ITG αIIbβ3) that result in qualitative or quantitative abnormalities of the platelet GPIIb-IIIa complex (Solh et al. 2015; Toogeh et al. 2004). Both genes are located on chromosome 17q21-23 and various causative mutations have been identified (Nurden 2006; Solh et al. 2015; Toogeh et al. 2004). On the basic of levels of GPIIb/IIIa as detected by CD61, GT has been subclassified into types I, II, and III. Patients with less than 5% of normal GPIIb/IIIa are classified as type I and 5–20% as type II. Type III variants usually have dysfunctional receptors with near-normal GPIIb/IIIa levels. Variability exists in the subtypes found in various ethnic groups (Kannan et al. 2009a).

Patients who are homozygous or compound heterozygous for GPIIb and/or GPIIIa pathologic mutations have symptoms of the disease, but heterozygous individuals (carriers) are asymptomatic. The clinical complications of GT include lifelong bleeding with easy bruising, epistaxis, menorrhagia, and gastrointestinal bleeding. Varying degrees of deficiency in the platelet surface expression of GP αIIb/β3 may give rise to heterogeneity in the severity of bleeding symptoms (Farsinejad et al. 2010; Nurden 2006). Nowadays, GT receives much recognition, as it was one of the first disorders to define GPIIb/IIIa as a platelet receptor

for adhesive molecules (such as von Willebrand factor and fibrinogen). The disease also served as a template for understanding processes of platelet aggregation as well as targets for therapeutic measures (Farsinejad et al. 2010). Since the inherited bleeding disorders are a big problem in Egypt with very limited studies in children and absent studies in Upper Egypt, the aims of our study were, first, to evaluate the clinicopathological spectrum of GT and to study the platelet glycoprotein IIb/IIIa complex and platelet antibodies by flow cytometry in a large cohort of children with GT in the largest tertiary care center in Upper Egypt. Second, to evaluate the severity of bleeding phenotype and its relation to the types of GT.

Patients and Methods

The study was approved by the Assiut University ethics committee and was conducted in accordance with the Declaration of Helsinki. An informed written consent in accordance with Assiut University Ethical Committee guidelines was taken from all guardians of patients and controls.

Study Setting

The study was conducted in the Pediatric Hematology Unit, Assiut University Children Hospital, which is the largest children's medical center in Upper Egypt.

Study Design

The study was designed as a cross-sectional, case-control study, that is, clinical manifestations and laboratory investigations in children with GT were compared to healthy controls.

Study Subjects

Our study included 40 children with GT with mean age ± SD = 4.65 ± 3.91 (ranged: 1–10 years) referred to the Pediatric Hematology Unit, Assiut University Children Hospital from seven Governorates in Upper Egypt. Patients were diagnosed on the basis of clinical suspicion of the disease, positive history of mucosal bleeding, easy bruising, epistaxis and prolonged bleeding time, abnormal clot retraction, and absent in vitro platelet aggregation in response to ADP, epinephrine, and collagen, but normal ristocetin-induced platelet aggregation. Patients with other coagulation and platelet function disorders were excluded from this study. The study included 40 age and sex matched children with no history of abnormal bleeding as a control group.

Methods

In the clinical data, the points that we stressed upon were age, sex, sibling and family history, consanguineous marriage between parents, sites of bleeding, and the severity of bleeding. Bleeding assessment was recorded using a structured semi-quantitative questionnaire during an interview all guardians of patients. The questionnaire was designed to identify defects in primary hemostasis (mucocutaneous symptoms), secondary hemostasis (hemarthrosis, muscle bleeding), bleeding with surgery or tooth extraction. Those who bled only after trauma 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 severe bleeding episodes requiring blood or platelet transfusions were classified as severe bleeders (Farsinejad et al. 2010).

The assessments of the expression of GPIIb-IIIa on the platelet surface and platelet antibodies were done for all children with GT by using flow cytometry.

Flow Cytometric Analysis for Platelet Receptor GPIIb-IIIa

Five milliliters of peripheral blood of patients and controls were collected by venipuncture into 3.8% tri-sodium citrate solution. Citrated blood samples were centrifuged at 1000 rpm for 10 min 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 min, to further concentrate platelets. Flow cytometry measured the expression of the glycoprotein IIb/IIIa complex (GPIIb-IIIa) on the surface of the platelets. 100 µL of PRP were incubated with 10 µL fluorescein isothiocyanate (FITC) conjugated anti-CD41 and phycoerthren conjugated (PE) anti-CD61 against human platelet GPIIb and GPIIIa receptors, respectively, for 20 min at room temperature. Supernatant was discarded carefully, and the pellet washed twice with phosphate-buffered saline and resuspended in the same buffer that contained 1% paraformaldehyde. Flow cytometric analysis was done by FACSCalibur flow cytometry with Cell Quest software (BD Biosciences, USA). Anti-human IgG was used as an isotype-matched negative control for each sample. Forward and side scatter histogram was used to define the platelet population. Then, the expression of CD41 and CD61 were assessed in the platelet population (Fig. 1).

Flow Cytometric Detection of Platelet Antibodies

Peridinium-chlorophyll-protein conjugated anti-CD41 was used to identify the platelets population and

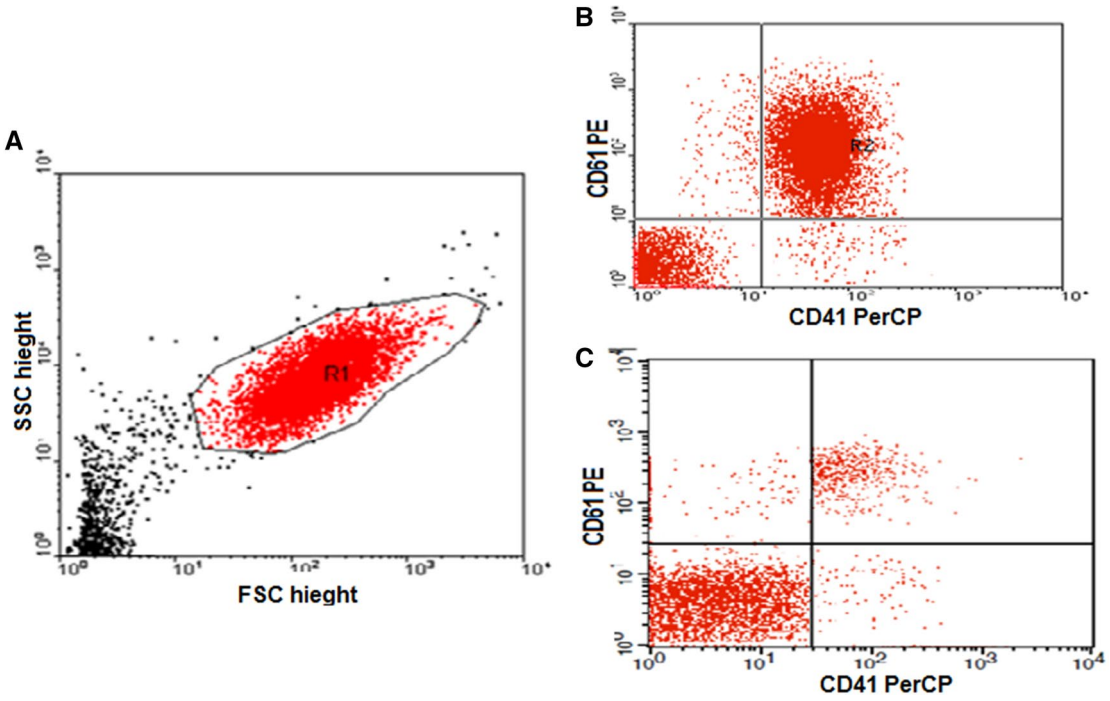

Fig. 1 Flow cytometric detection of platelet glycoproteins. **a** Forward and side scatter histogram was used to define the platelet population (R1). The percentages of CD41 and CD61 were determined in the platelet population of a GT patient (**c**) compared to a normal control (**b**)

FITC-conjugated anti-human IgG was used to detect platelet antibodies, both were purchased from Becton Dickinson Biosciences, USA. In a test tube, 20 µl of patient's or control's serum were incubated with 20 µl of platelet concentrate of normal person for 30 min at room temperature. Then, 10 µl of FITC conjugated anti-human IgG and PerCP-conjugated anti-CD41 were added. The samples were incubated for 30 min at room temperature to be ready for analysis by flow cytometry. Negative control (serum sample of the donor of that platelet concentrate) was treated as a patient sample. Sample analysis was done using FACSCaliber (Becton Dickinson, USA) flow cytometry. Identification of platelets (R1) according to their forward scatter and side scatter characteristic. Then CD41 was used to confirm platelet gate. Then the expression of IgG in $CD41^+$ population was detected. The level of IgG was detected as a percentage of antibody positive cells and as the geometric mean of fluorescence intensity (MFI). The positivity was defined as fluorescence higher than that of the negative control as shown in Fig. 2.

Statistical Analysis

Statistical analyses were performed using the Statistical Package for Social Sciences, version 16.0 (SPSS Inc., Chicago, IL, USA). Data are expressed as mean ± standard error of mean (SEM) for continuous variables and percentages for categorical variables. Mann-Whitney test was used to analyze continuous variables, whereas Chi-square test was used to analyze categorical variables. The p value is considered statistically significant when less than 0.05. Spearman correlation co-efficient was used to assess the correlation among different studied parameters.

Results

Table 1 shows some demographic characteristics of children with GT. The mean age of the patients was 4.65 ± 3.91 years with a mean onset of the disease of 1.50 ± 1.42 years. Male: female ratio was 1:1.1. Consanguineous marriage was present in 65% of parents of the patients. Parents of 26 GT children were first or second cousins. Positive family history of bleeding was noted in 20 (50%) patients and no history of bleeding was found in 20 (50%) patients. All clinical manifestations of bleeding of GT patients were summarized in Table 1. Table 2 shows the classification of GT and bleeding severity in all studied patients. 57.5% of our patients were severe bleeders, most of them were belonged to type I GT with GP IIb-IIIa levels <5%. Our results show a significant correlation between bleeding severity and types of GT ($p = 0.002$). Table 3 shows the mean levels of GPIIb (CD41) and GPIIIa (CD61) in patients and healthy children. CD41 was significantly lower

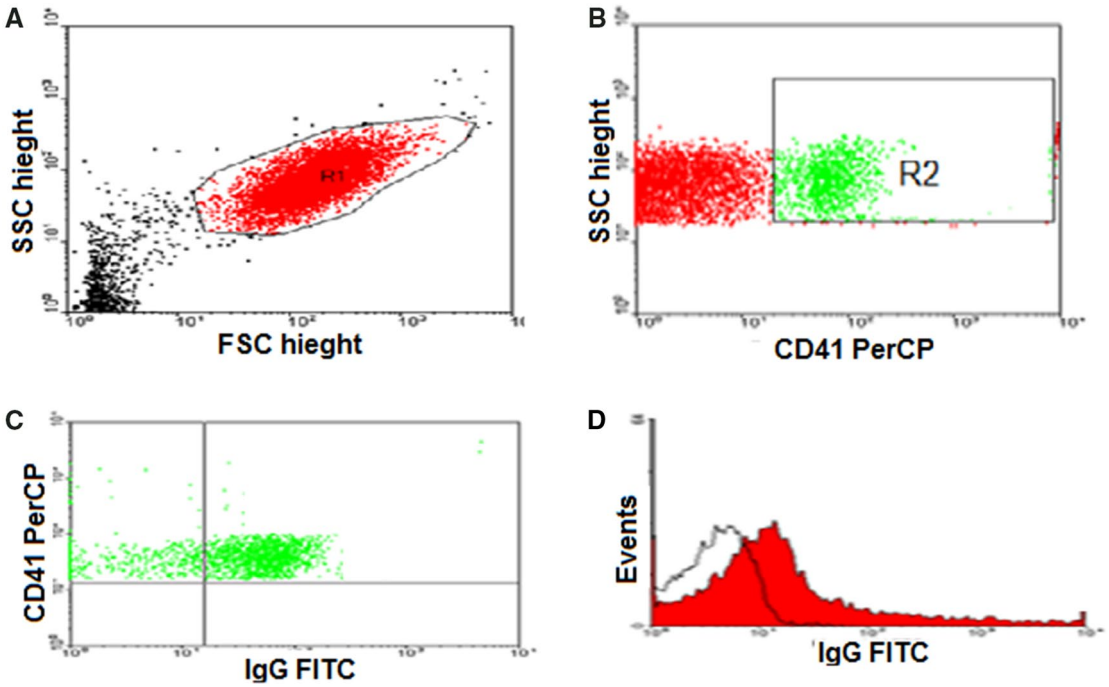

Fig. 2 Flow cytometric detection of platelet antibodies in Glanzmann thrombasthenia. **a** Forward and side scatter histogram was used to define the platelet population (*R1*). **b** CD41 and side scatter histogram were used to confirm platelet population. **c, d** Then, the expression of IgG in $CD41^+$ population (*R2*) was detected. The level of IgG was detected as percentage of antibody positive cells and as geometric MFI. The positivity was defined as fluorescence (*red histogram*) higher than that of the isotype control (*open histogram*)

in GT children than healthy control. In addition, CD61 was significantly lower in patients when compared to controls. Figure 2 and Table 3 show the expression of IgG in $CD41^+$ population and MFI of platelet antibodies (IgG) in GT children. The frequency of IgG platelet antibodies was significantly higher in patients when compared to controls. The MFI of platelet antibodies (IgG) in GT patients was significantly higher in patients as compared to controls. Figure 3 and Table 4 show the correlations between the percentages of CD41 and CD61 with the percentages of IgG platelet antibodies and MFI of platelet antibodies (IgG) in GT patients. The percentage of CD41 had significant negative correlations between the percentage of platelet antibodies ($r = -0.601$, $p < 0.001$) and IgG MFI ($r = -0.586$, $p \leq 0.001$). Furthermore, the percentage of CD61 had significantly negative correlations between the percentage of platelet antibodies ($r = -0.629$, $p < 0.001$) and IgG MFI ($r = -0.597$, $p \leq 0.001$).

Discussion

Bleeding disorders are rare diseases that are both complicated and expensive to manage. Consanguinity increases the risk of occurrence of rare bleeding disorders, which is more common in the Middle East especially Egypt. In Upper Egypt consanguineous marriage is common with high prevalence of autosomal recessive disorders (Elsayh et al. 2016; Fahim et al. 2013). GT can be found in individuals across the globe, but is more common in ethnic groups that display higher incidences of consanguineous marriages such as Iraqi Jews, Northern Iranians, Southern Indians, Jordanians, Saudi Arabians and Arabs living in Israel (Farsinejad et al. 2010; Haghighi et al. 2016; Kannan et al. 2009a). In this study, 65% of parents of the patients were first or second cousins. However, there is a widespread variation in the consanguinity reported in the previous studies. It ranged from 14% (Ali et al. 2008), 93.6% (Farsinejad et al. 2010), 94% (Farsinejad et al. 2011) up to100% (Haghighi et al. 2016) in GT patients.

The analysis of data herein showed that most of our patients with GT had severe bleeding episodes (57.5%). In line with our results (Haghighi et al. 2016) reported 50% of their patients with GT had a history of severe bleeding. In addition, Farsinejad et al. (2011) reported 48% of their patients with GT (59/123) were severe bleeders. Although the severity of bleeding may differ from patient to another, GT is considered a severe bleeding syndrome as at least 75% of patients require blood and/or platelet transfusions at least once in their life (Di Minno et al. 2015).

Our series with GT showed a significant correlation between bleeding severity and types of GT ($p = 0.002$).

Table 1 Baseline characteristics of GT patients and controls

Clinical data	Patients (40)	Controls (40)	*p* value
Age (years)			
Range	1–10	1–10	NS
Mean ± SD	4.65 ± 3.91	5.1 ± 2.80	NS
Male/female ratio	19/21	19/21	NS
Age at onset (years)			
Range	0.5–4	–	–
mean ± SD	1.50 ± 1.42	–	–
Platelets (10^9/L)	318.34 ± 14.43	297.45 ± 13.88	NS
WBCs (10^9/L)	6.3 ± 0.3	7.52 ± 0.4	NS
Hemoglobin gm/dl	11.29 ± 0.3	12.51 ± 0.41	NS
Petechiae and purpura *N* (%)	27 (67.5)	–	–
Recurrent epistaxis *N* (%)	29 (72.5)	–	–
Gingival bleeding *N* (%)	17 (42.5)	–	–
Gastrointestinal bleeding *N* (%)	13 (32.5)	–	–
Hematuria *N* (%)	8 (20)	–	–
Hemorrhage after trauma *N* (%)	19 (47.75)	–	–
Family history of bleeding *N* (%)	10 (25)	–	–
Consanguineous marriage *N* (%)	26 (65)	25 (62.50)	NS

Data represented as means ± SEM, $p \leq 0.05$ is significant

WBCs white blood cells, *NS* non-significant

Table 2 The classification of GT and bleeding severity in all studied patients

GT patients	Type 1 <5%	Type 2 (15–20%)	Type 3 >20%
Number (%)	19 (47.5)	13 (32.5)	8 (20)
Mild bleeding *N* (%)	1 (5.2)	2 (15.4)	6 (75)
Moderate bleeding *N* (%)	4 (21.1)	3 (23)	1 (12.5)
Severe bleeding *N* (%)	14 (73.7)	8 (61.6)	1 (12.5)

Table 3 Platelet glycoprotein and platelet antibodies of GT patients and controls

	Patients (40)	Control (40)	*p* value
$CD41^+$ %	16.25 ± 2.72	85.6 ± 2.35	<0.001
$CD61^+$ %	14.61 ± 1.9	69.15 ± 3.22	<0.001
IgG %	48.31 ± 2.1	10.53 ± 1.6	<0.001
MFI of IgG	143.24 ± 5.69	34.65 ± 2.73	<0.001

Data represented as means ± SEM

MFI mean fluorescent intensity; $p \leq 0.05$ is significant

Most of patients with severe bleeding were from type I GT with severe deficiency of GP IIb-IIIa levels (<5%). Therefore, our study supported the positive correlation between quantitative changes in the surface expression of platelet membrane glycoproteins and the clinical severity of bleeding. Contrary to our results, previous researches (Farsinejad et al. 2010, 2011; Nelson et al. 2006) have failed to demonstrate any consistent relationship between the severity of bleeding and the type of GT. These researches suggested that the severity of bleeding in affected individuals cannot readily be predicted from currently available laboratory findings. The difference between our results and the previous reports might be due to epigenetic, environmental factors, and to ethnic differences. In addition, previous studies have shown that pro-thrombotic factors may play a role in the phenotype of GT, e.g., factor V Leiden, the PG 20210 A, and MTHFR mutations (George et al. 1990; Kannan et al. 2009b).

By analysis of platelet GP IIb-IIIa by flow cytometry the classification of patients with GT in our study was type I GT (47.5%), type II GT (32.5%) and type III GT (20%). Previous studies (Di Minno et al. 2015; Farsinejad et al. 2010, 2011; Kannan et al. 2009a) reported that type I GT was the most common type of GT in their series.

The results of our study revealed a significantly higher frequency of IgG platelet antibodies in patients with GT when compared to controls ($p \leq 0.001$). Initial studies of platelet antibody formation against αIIb/β3 in GT were largely performed on isolated cases and mainly concerned antibody characterization (Fiore et al. 2012). Poon et al. (2004) reported the status of platelet antibodies in 54 non genotyped patients with GT. They found 30% were positive against αIIb/β3. Santoro et al. (2010) investigated 17 GT patients for αIIb/β3 antibodies. Twenty-five percent of these patients had antiplatelet antibodies. More recently, Fiore et al. (2012) reported that 81.25% of their patients with GT (13/16) developed platelet antibodies. Though, additionally studies are needed to clarify the importance of antibody development in GT patients, the previous studies suggested that early transfusion therapy in children might increase the hazard of antibody formation, and, if so, it should be taken into account by the physicians (Fiore et al. 2012).

Most patients in our cohort presented with mucocutaneous bleeding such as epistaxis (72.5%), skin bleeds (67.5%) and gingival bleeding (42.5%). However, no patients had menorrhagia as all females in our study were ≤10 years. Our finding is supported by other studies as well (Di Minno et al. 2015; Farsinejad et al. 2010, 2011; Kannan et al. 2009a).

The age at onset and diagnosis of our cohort is ranged 0.5–4 years with mean ± SD = 1.50 ± 1.42. In previous reports, the age at diagnosis varied in different studies,

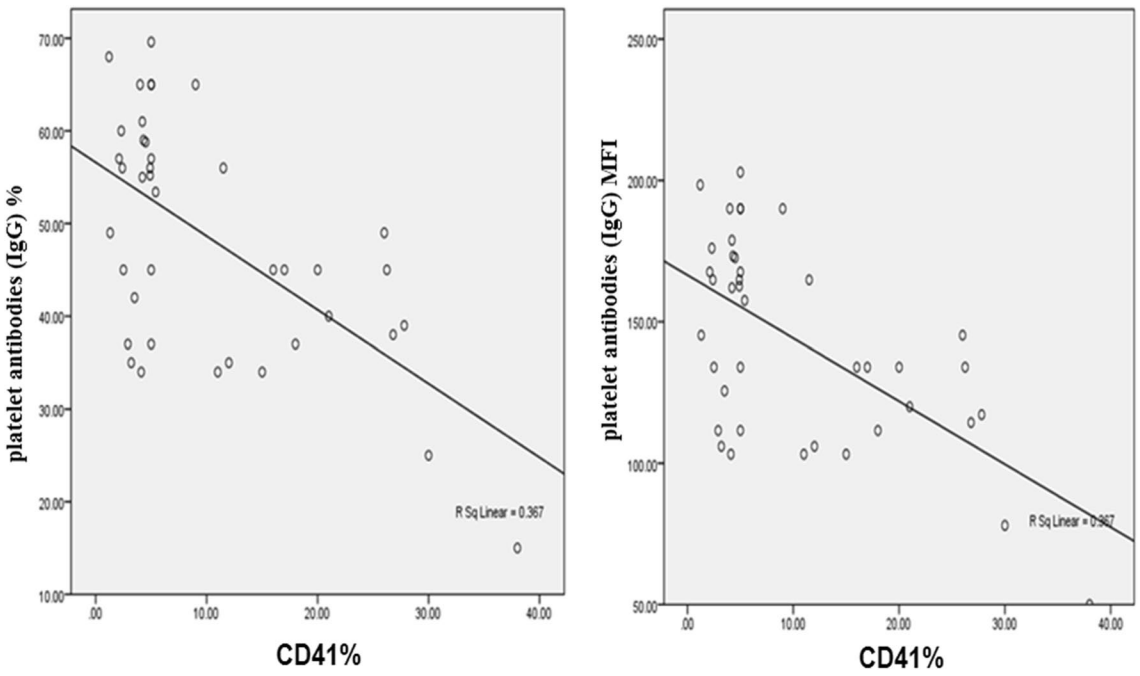

Fig. 3 Correlation between platelet glycoprotein (CD41) with the percentage and MFI of platelet antibodies (IgG) in GT patients

Table 4 Correlation between platelet glycoprotein and platelet antibodies of GT patients

		IgG %	IgG MFI
CD41%	*r*	–0. 601	–0.586
	p value	<0.001	<0.001
CD61%	*r*	–0.629	–0. 597
	p value	<0.001	<0.001

MFI: Mean fluorescent intensity; $p \leq 0.05$ is significant

however, most cases are diagnosed at an early age 0.5–5 years. Several researches have shown that GT is a disease of children and young adults with the majority of patients being less than 20 years (Badhe et al. 2006; Farsinejad et al. 2010, 2011; George et al. 1990).

In conclusion, in this study, we have delineated that type I is the most common type of GT in Upper Egypt. Our data suggested there is a good correlation between quantitative changes in the surface expression of platelet membrane glycoproteins detected by flow cytometry and the clinical severity of bleeding. Therefore, classifying of severity of bleeding in patients with GT could possibly aid the pediatricians and hematologists in the implementation of ideal prophylactic measures. According to these results we recommend that flow cytometry is to be used as an important tool in the screening and typing of GT patients.

Compliance with ethical standards

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Conflict of interest All authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

References

Ali N, Moiz B, Shaikh U et al (2008) Diagnostic tool for Glanzmann thrombasthenia clinicopathologic spectrum. J Coll Physicians Surg Pak 18:91–94

Badhe BA, Jayanthi S, Dutta T (2006) Clinical spectrum of Glanzmann thrombasthenia. Indian J Pathol Microbiol 43:297–302

Di Minno G, Zotz RB, d'Oiron R et al (2015) The international, prospective Glanzmann thrombasthenia registry: treatment modalities and outcomes of non-surgical bleeding episodes in patients with Glanzmann thrombasthenia. Haematologica 100:1031–1037

Elsayh KI, Mohammed WS, Zahran AM et al (2016) Leukocytes apoptosis and adipocytokines in children with beta thalassemia major. Clin Exp Med 16:345–350

Fahim FM, Saad K, Askar EA et al (2013) Growth parameters and vitamin D status in children with thalassemia major in Upper Egypt. Int J Hematol Oncol Stem Cell Res 7:10–14

Farsinejad A, Abolghasemi H, Kazemi A et al (2010) Density of platelet GPIIb-IIIa and bleeding severity in iranian patients with Glanzmann's thrombasthenia. IJBC 3:115–121

Farsinejad A, Abolghasemi H, Kazemi A et al (2011) classification of Iranian patients with Glanzmann's thrombasthenia using a flow cytometric method. Platelets 22:321–327

Fiore M, Firah N, Pillois X et al (2012) Natural history of platelet antibody formation against αIIbβ3 in a French cohort of Glanzmann thrombasthenia patients. Haemophilia 18:e201–e209

George JN, Caen JP, Nurden AT (1990) Glanzmann's thrombasthenia: The spectrum of clinical disease. Blood 75:1383–1395

Haghighi A, Borhany M, Ghazi A et al (2016) Glanzmann thrombasthenia in Pakistan: molecular analysis and identification of novel mutations. Clin Genet 89:187–192

Kannan M, Ahmad F, Yadav BK et al (2009a) Glanzmann's thrombasthenia in North Indians: sub classification and carrier detection by flow cytometry. Platelets 20:12–15

Kannan M, Yadav BK, Ahmad F et al (2009b) Modulation of clinical phenotype of Glanzmann's thrombasthenia by thrombogenic mutations. Clin Chim Acta 403:156–158

Nelson EJ, Nair SC, Peretz H et al (2006) Diversity of Glanzmann's thrombasthenia in southern India: 10 novel mutations identified among 15 unrelated patients. J Thromb Haemost 4:1730–1737

Nurden AT (2006) Glanzmann thrombasthenia. Orphanet J Rare Dis 1:10

Poon MC, D'Oiron R, Von Depka M et al (2004) Prophylactic and therapeutic recombinant factor VIIa administration to patients with Glanzmann's thrombasthenia: results of an international survey. J Thromb Haemost 2:1096–1103

Santoro C, Rago A, Biondo F et al (2010) Prevalence of allo-immunization anti-HLA and anti-integrin alphaIIbbeta3 in Glanzmann thromboasthenia patients. Haemophilia 16:805–812

Solh T, Botsford A, Solh M (2015) Glanzmann's thrombasthenia: pathogenesis, diagnosis, and current and emerging treatment options. J Blood Med 6:219–227

Toogeh G, Sharifian R, Lak M et al (2004) Presentation and pattern of symptoms in 382 patients with Glanzmann thrombasthenia in Iran. Am J Hematol 77:198–199