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Quantitative Immunophemotyping of Platelet Surface Glycoproteins among Iranian Patients with Bernard-Soulier Syndrome

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Abstract

Background: Bernard-Soulier syndrome is a rare inherited bleeding disease caused by quantitative or qualitative defect of GPIb/IX/V, a platelet complex that binds the Von Willebrand factor. The expression of GPIb-IX-V complex can be evaluated by flow cytometry and confirmed by the absence of ristocetin-induced platelet aggregation in platelet-rich plasma. The main aim of the present study was to classify Iranian Bernard-Soulier syndrome patients by a flow cytometric method, and to evaluate the correlation between platelet immunophenotype and clinical findings among patients.

Patients and Methods: The surface expression level of GPIb-IX-V on platelets was assessed in fifteen Bernard-Soulier syndrome patients, using a panel of antibodies using a quantitative flow cytometry method. The results of the physical examination, family history and clinical presentation were also recorded by a physician.

Result: The present study showed that all the patients suffer from a severe form of GPIb-IX-V complex deficiency. The study also found no correlation between the platelet surface glycoprotein expression and severity of bleeding among patients.

Conclusion: Severe quantitative defect is the most common subtype among Iranian patients with Bernard-Soulier syndrome. Platelet Immunophenotyping alone does not determine the severity of hemorrhage in patients with Bernard-Soulier syndrome.

Key words: Bernard Soulier Syndrome, GPIb-IX-V, flow cytometry, bleeding.

Introduction

Bernard-Soulier syndrome (BSS) is an inherited autosomal recessive platelet disorder, characterized by macrothrombocytopenia and prolonged bleeding time [1-6]. The most characteristic criteria of disease are severely reduced or absence of platelet aggregation in response to ristocetin 7,8. BSS platelets fail to express GPIb-IX-V complex on their surface.

In healthy individuals, resting blood platelets express approximately 25,000 to 30,000 copies of the GPIb-IX-V complex that, by binding to activated Von Willebrand factor, initiate hemostasis causing the rolling of platelets over the vascular surface. The receptor consists of four distinct subunits that are encoded by different genes [7]. The hitherto discovered molecular defects underlying BSS arise from missense, nonsense, or deletion mutations of the GPIba, GPIbb, or GPIX genes which produce unstable, truncated or dysfunctional glycoproteins. Despite the heterogeneous genetic abnormalities, BSS can be widely categorized in two groups. The first abnormality may be a biosynthetic defect affecting synthesis, processing, or expression of the GPIb-IX-V complex. The second is a qualitative

defect in which GPIba is expressed in a dysfunctional form that fails to bind ligand (Bolzano variant) [3,9-17]. Common clinical manifestations of BSS include easy bruising, nosebleeds, mucocutaneous bleeding, menorrhagia, and, occasionally, gastrointestinal bleeding. The severity of symptoms may vary considerably [10-14,18]. However, despite these typical characteristics, comparisons of the bleeding tendencies associated with BSS reveal considerable variation between individuals. Flow cytometric analysis of surface platelets glycoproteins is valuable for diagnosis of BSS: normal binding with CD41 and CD61 antibodies, but defective binding with CD42a, CD42b, CD42c, and CD42d antibodies suggest BSS [4,16,19,20]. In most cases GPIb-IX-V is not present on the surface of platelets; but in a few cases, up to 50% residual GPIb or GPIb-IX has been detected [2]. There are also BSS cases in which the platelet immunophenotype is normal. In the Bolzano variant, caused by a substitution of Val for Ala at position 156 of GPIba, the molecular change produces mutant complexes that appear on the cell surface essentially at normal levels but are unable to bind to Von Willebrand factor [13,16,21]. Flow cytometry also provides a simple and quick means to differentiate the homozygous and heterozygous states of BSS. Heterozygotes patients usually have intermediate amounts of the GP complex and mild thrombocytopenia with few giant platelets [13,22]. The objective of the present study was to determine the density of GPIb-IX-V among Iranian patients to classify them according the quantitative flow cytometric data and to correlate the severity of bleeding episode with the BSS platelet immunophenotype.

Patients and Methods

Antibodies and reagents

The purified monoclonal antibodies including; anti-CD41 (αIIb, catalog no. ab15021), anti-CD61 (β3, catalog no. ab33171), anti-CD42a (GPIX, cat no. ab23489), anti-CD42b (GPIbα, catalog no. Ab2578), anti-CD42d (GPV, catalog no. ab23773), goat anti-mouse secondary antibody (FITC conjugated, catalog no. F2653) was from Sigma (St, Louis, MO, USA). Mouse anti-rabbit macrophage RAM11 (purified isotype control, catalog no. M0633) was purchased from DAKO (Glostrup, Denmark). Calibration-beads (QuiFiKit, catalog no. K0078) were also obtained from DAKO.

Patients

Blood samples were obtained from 15 patients with BSS referred to the Imam Khomeini hospital, Tehran Iran, and Iranian Blood Transfusion Organization (IBTO) between 2008 and 2013. The patients should have not received any platelet containing blood product since one month before the blood sampling.

The BSS diagnosis was based on information extracted from the patients' records. These data included history of mucosal-dermal bleeding, prolonged bleeding time, thrombocytopenia and giant platelets, normal PT, PTT and clotting time and clot retraction test and no response of platelets to ristocetin in aggregometry test. We also defined a severe bleeding episode as an episode mandating blood transfusion and (or) hospitalization.

For each patient, bleeding severity and frequency was recorded, using information obtained from a copy of the questionnaire completed by a physician following an interview and examination of the patient. An informed consent form was filled by all patients prior to laboratory investigation, and sampling from children was performed with the permission of their parents. Fifteen control samples were collected from healthy volunteer individuals with no history of abnormal bleeding. We also included parents of the patients, in order to assess the phenotypic differences between normal individuals, carriers and the affected patients. The study was approved by the ethical review committee affiliated to the IBTO. This research was supported by the IBTO.

Flow cytometry

Platelet GPIb-IX-V density was determined in PRP using quantitative flow cytometry using QuiFiKit calibrator beads, (Dako-Denmark), according to the manufacturer's instructions. The kit includes a mixture of five calibration beads coated with increasing concentrations of mouse anti-human CD5 antibodies. Platelets were stained, using an indirect immunofluorescence protocol with the mouse IgG1 monoclonal antibodies including anti-CD41, anti-CD61, anti-CD42a, anti-CD42b, antiCD42c and, anti-CD42d. All monoclonal antibodies were used at saturating conditions, as determined in preliminary experiments. A negative isotypic control IgG1 was included in each series. The staining reagent was an anti-mouse IgG-fluorescein

isothiocyanate antibody. A calibration curve was constructed for each sample series, and a negative isotype control was run with each PRP sample. Ten thousand events were acquired on a Partec-PAS-III cytometer (Partec, Germany), and data were analyzed using FloMax software (Partec GmbH, Munster, Germany). The quantitative values of the glycoproteins were derived from the calibration curve after subtracting the negative isotype control value. Control samples were analyzed to make sure that the procedure was done correctly according to kit instruction.

Statistical methods

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

Results

Patients' characteristics

Fifteen patients with BSS including 6 males and 9 females were studied. The basic charactristics of patients is summarized in table 1. Mean age (±SD) was 19.0 ± 13.1 years (range: 2 to 48 years). Mean age at diagnosis had been 5.8 ± 6.09 years (range: 4 month to 22 years). Only two of the cases were diagnosed before the first year of their life. Consanguinity of first degree was present in all patients. Petechiae, epistaxis, and prolonged bleeding after trauma or surgery were the most common clinical symptoms. In five of the patients the clinical symptoms had decreased as the patient got older. Five of the patients had another relative who suffered from bleeding disorder. In seven of the patients bleeding symptoms correlated with the severity of thrombocytopenia. Six of the cases were female in reproductive age and one of them was giving birth to a child and none of others had a history of abortion. Ten of the patients had received platelet concentrations. The mean

Table 1: Basic characteristic of patients participating in the study.

Variable	number		percent	
Number of patients	**Male**	**Female**	**Male**	**Female**
	6	9	40	60
Platelet count More than 20000/µl	7		46.66	
Platelet count Less than 20000/µl	8		53.33	
Age	**Male**	**Female**		
	11-34	2-48		
Age at diagnosis	Above 10 year	Under 10 year	Above 10 year	Under 10 year
	8	7	53.33	46.66
Consanguineous marriage of parents	15		100	
Platelet product receiving patients	10		66.66	
Drug receiving patients	5		33.33	
Reaction to drug and platelet transfusion	1		6.6	
Epistaxis	12		80	
Gum bleeding	9		60	
Petechiae and purpura	1		6.6	
Hematoma	1		6.6	
Bleeding after surgery	1		6.6	
Bleeding episodes getting milder by aging	3		20	

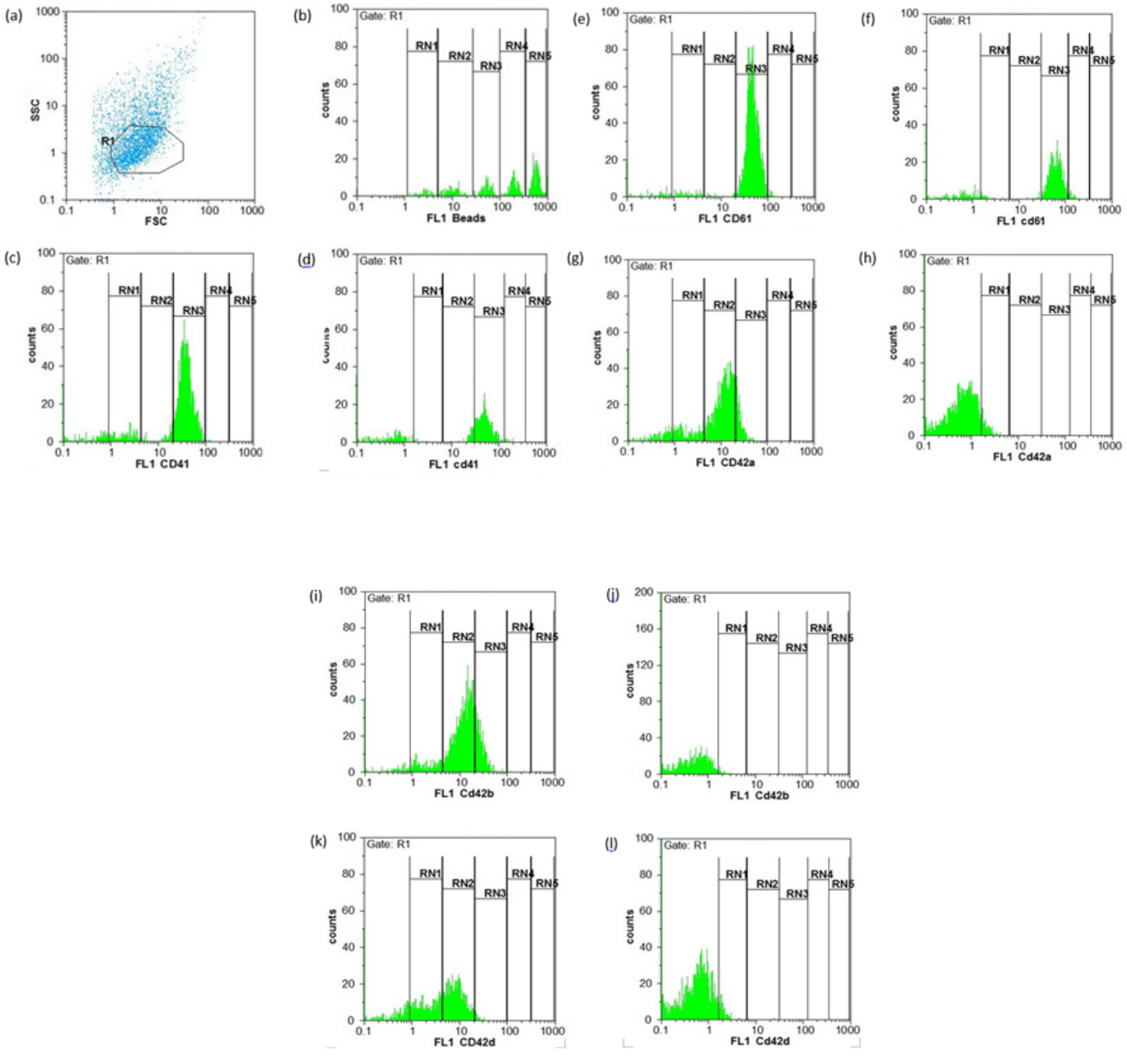

Figure 1: Flow cytometric histograms of gating region (a) calibration beads (b) normal platelet CD41 (c), BSS platelet CD41(d), normal platelet CD61 (e), BSS platelet CD61 (f), normal platelet CD42a (g), BSS platelet CD42a (h), normal platelet CD42b (i), BSS platelet CD42b (j), normal platelet CD42d (k), BSS platelet CD42d (l).

platelet count was 33307.69 ± 25509.42 (range: 65000 - 4000 per µl).

Quantitative flow cytometry

In direct qualitative method none of the patients expressed CD42a, Cd42b and CD42d on their platelets surface (Figure 1). In all samples CD61 was analyzed to confirm the identity of platelet population and correct gating which was positive in all patients. In quantitative analysis, all the patients showed extreme decrease in surface expression of complex subunits (compared to normal platelets) and in thirteen cases antigen density was less than 500 per platelet (less than 1%). In all patients the decreased level of evaluated antigens (Cd42a,Cd42b,Cd42d) was similar on platelets surfaces. None of patients revealed significant alteration of CD41 and CD61 expression, but in four patients they were expressed slightly above the normal range (80000-90000). This finding should be interpreted with caution because other factors including the absence of GpIb-IX-V complex which may allow the antibody to access more GpIIb-IIIa binding sites on platelet surface must be considered.

Considering the bleeding severity, statistical analysis showed no relation between bleeding severity and expression of GpIb-IX-V. Our findings show that despite dramatic reduction of GpIb-IX-V complex on platelets surfaces, clinical symptoms and bleeding episode among the patients were quite different. For example, one of BSS patients did not develop any symptoms until child delivery, while others were referred to a physician with signs of bleeding in the neonatal period.

Discussion

Although the prevalence of BSS is estimated to be less than one in a million in the world but its incidence is higher than expected in countries with high rate of consanguineous marriages. That is the reason why Iran is among countries with a high number of patients with rare inherited platelet surface glycoprotein disorders such as Glanzmann's thrombasthenia and BSS [23-26]. The evaluation of clinical symptoms, laboratory findings, and immunophenotyping of these patients can provide a better understanding of pathophysiology, diagnosis and management of the platelet related disorders. The purpose of this study was to classify the patients with Bernard-Ssoulier syndrome based on platelet immunophenotyping and also to evaluate the relationship between the platelet surface GP Ib/IX/V expression levels and the severity of bleeding.

In this study we evaluated a relatively large cohort of 15 Bernard-Soulier syndrome patients. History of our patients indicated that in eleven of them diagnosis was made in the first decade of their life, probably reflecting the improvement and expertise available for detecting this rare disorder in Iran. Sex distribution among our patients showed none statistically significant higher prevalence among females. Other studies have showed almost equal prevalence between genders and some others have reported more male subjects [6,14,22]. The higher prevalence of female patients in our study is possibly due to the susceptibility of females for menstrual bleeding. Parental consanguinity was found among all of our patients due to familial marriage which is very common in Iran [23-26]. Clinical symptoms were similar to previous studies with regard to sites of bleeding and the need for transfusion of blood products [2-7].

Demographic data showed that affected individuals were from all geographic parts of Iran indicating the wide distribution of the syndrome in the country. According to previous studies, there are at least two distinct BSS immunophenotypes. One of them is a quantitative defect with absence or severe reduction of platelets surface GPIb-IX-V (Type I) and the other is a qualitative defect (variant type) in which a dysfunctional GPIb-IX-V complex is present on the platelet surface at slightly reduced or completely normal levels [27, 28]. Our investigation showed that all of our patients suffered from complete absence or severe reduction of GPIb-IX-V complex on their platelets.

The results of the present study suggest that the majority of Iranian patients with Bernard-Soulier syndrome are classified as quantitative defects. However, we must keep in mind that most patients were distributed in different geographical locations and their family proximity was few. If more patients were participating in this study it would probably be possible to find variant forms as well. However, there is some evidence which may validate the results of this research, including molecular studies on patients with BSS. To date, about 50 different genetic lesions associated to

the BSS have been identified. Defects are due to mutations in GPIbA, GPIbB and GP9 [7,27]. The genetic defects can be separated into two major categories including mutations that lead to an unstable complex with strongly decreased or absent surface expression (quantitative defects) and rare gene alterations which give rise to normal or slightly decreased expression of a dysfunctional receptor (qualitative defects) [3,9,27]. Therefore it is logical that immunophenotypes with severe reduction of platelets surface GPIb-IX-V are the most common form of platelet immunophenotype among patients with BSS [13-26].

This study also showed that the bleeding severity does not associate with the density of the GPIb/IX/V receptor in the surface of platelet membrane. We observed patients with no detectable GPIb-IX-V on their platelets who had no signs of severe bleeding while some patients with the same immunophenotype had the worst bleeding. It can be concluded that although genetic defects can cause GPIb/IX/V function or expression abnormality, bleeding diathesis is quite variable in patients who have the same mutations. However other molecular differences and acquired conditions affecting vessel wall, platelet function and/or quantity and coagulation factors may influence bleeding severity among patients. Of note, bleeding diathesis also varied considerably among patients without any apparent association with their platelet count. In particular, some patients with mild thrombocytopenia, had a bleeding tendency that affected their quality of life, requiring platelet transfusions or clinical measures (grade 3 or 4), whereas some patients with severe thrombocytopenia had a mild bleeding tendency (grade 2) which did not necessitate any particular interventions.

Conclusion The vast majority of the Iranian patients with Bernard-Soulier syndrome can be classified as patients with severe quantitative defect of platelet surface GPIb-IX-V. Platelet immunophenotyping must be performed in families at risk for BSS to improve the detection of patients and carriers. We should be aware that flow cytometry is not sensitive enough to determine low quantities of antigen so further studies using Western blot analysis are required before excluding any correlation between phenotype and glycoprotein expression on platelet membrane.

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