Carrier Detection in Glanzmann Thrombasthenia

Comparison of Flow Cytometry and Western Blot With Respect to DNA Mutation

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Abstract

We studied 20 families with Glanzmann thrombasthenia, including 20 parents and 22 siblings, for carrier detection. Carrier detection was done by phenotypic analysis (flow cytometry and Western blot) and direct gene analysis (sequencing or restriction fragment length polymorphism). Reduced expression of the glycoprotein IIb/IIIa complex was found by flow cytometry in 17 (85%) of the parents and 12 (55%) of the siblings. Western blot showed a reduced or an abnormal band in 6 (30%) of the parents and 8 (36%) of the siblings. DNA analysis in family members showed all parents and 16 (73%) of siblings to be carriers. Both techniques, flow cytometry and Western blot, were evaluated with respect to DNA analysis as a "gold standard" method. The sensitivity of flow cytometry was higher (75%) than that of Western blot (39%). We concluded that flow cytometry can effectively be used for carrier detection in Glanzmann thrombasthenia.

Glanzmann thrombasthenia (GT) is an autosomal recessive inherited platelet function defect characterized by normal platelet count, prolonged bleeding time, and abnormal clot retraction.[1] GT is due to severe reduction in or absence of platelet aggregation in response to multiple physiologic agonists because of abnormalities of platelet glycoprotein (GP)IIb and/or GPIIIa. GT is caused by mutations in the genes encoding GPIIb or GPIIIa that result in qualitative or quantitative abnormalities of the platelet membrane proteins.[2,3] GPIIb and GPIIIa are the products of separate genes, and they form a heterodimer complex after synthesis in order to undergo final processing and transport to the platelet membrane. The types of mutations identified in both genes are missense mutations, nonsense mutations, deletions, and insertions leading to alternative splicing and/or premature termination of translation.[4] The clinical complications in GT include lifelong bleeding with easy bruising, epistaxis, menorrhagia, and gastrointestinal bleeding.[3] GT occurs in high frequency in certain ethnic populations with an increased incidence of consanguinity, such as Indians, Iranians, Iraqi Jews, Palestinian and Jordanian Arabs, and French Gypsies.[5-8]

Carrier detection and genetic counseling have become an important and integrated part of comprehensive care of patients with GT. In developing countries, owing to the higher cost and nonavailability of gene therapy, carrier detection followed by prenatal diagnosis is an effective alternative for decreasing the burden of the severe type of GT. Direct gene analysis is the most accurate method of carrier detection when the causative mutation is known in the affected patient. However, owing to its high cost and specialized technology required, there is a need to evaluate simpler and more economical methods for its detection. Flow cytometry and

Western blot are the potential candidates.[9,10] No studies have been reported on the role of these techniques for carrier detection in Indian patients with GT. The present study is the first such attempt in a large number of families. The present study was designed to evaluate carrier detection by flow cytometry and Western blot analysis with respect to DNA analysis as a "gold standard."

Materials and Methods

Study Subjects

Patients with a diagnosis of GT and their family members attending the hematology clinic of All India Institute of Medical Sciences, New Delhi, India, were the subjects of the study. GT was diagnosed on the basis of clinical and hematologic parameters. The patients' phenotypes and genotypes were studied to perform carrier studies in their families. Informed consent was obtained from the individuals as per guidelines of the institutional ethics committee. We also studied 50 healthy control subjects whenever required. The study was approved by the ethics committee.

Carrier Detection

Carrier detection in families (parents and siblings) of subjects with GT was done by direct mutation detection (sequencing or restriction fragment length polymorphism [RFLP]) and by phenotypic analysis (flow cytometry and Western blot).

Platelet Receptor Glycoprotein IIb/IIIa Complex Protein Analysis

Flow cytometry measured the expression of the glycoprotein IIb/IIIa complex (GPIIb-IIIa) on the surface of the platelets. Western blot allowed measuring the presence of GPIIb and GPIIIa protein in the platelet lysates.

Monoclonal Antibodies

Unconjugated and fluorescein isothiocyanate–conjugated monoclonal antibodies, CD41 and CD61 against human platelet GPIIb and GPIIIa receptors, respectively (DAKO, Glostrup, Denmark), were used.

Analysis for Platelet Receptor GPIIb-IIIa by Flow Cytometry

Whole blood was collected in 2% EDTA from the family members and from healthy control subjects. Platelets were counted and adjusted to 1.5 million cells per 100 μL with phosphate-buffered saline (PBS). Cells were incubated with fluorescein isothiocyanate–conjugated monoclonal antibodies CD41 and CD61 and a negative control antibody for 20 minutes at room temperature. To remove nonplatelet particles, 1 mL of lysis solution was added to each tube, mixed, and kept in the dark for 30 minutes. Tubes were then centrifuged at 1,500*g* for 10 minutes. Supernatant was discarded carefully, and the pellet washed twice with PBS-EDTA and resuspended in the same buffer that contained 1% paraformaldehyde. Samples were processed within an hour in flow cytometry (Beckman Coulter, Nyon, Switzerland).[11,12] Each time, a normal control sample was run along with the patients' samples. Gating was done to include the platelet particles and leave debris and WBCs outside the gate.

Analysis for Platelet Glycoprotein IIb and IIIa by Western Blot

For this part of the study, 10 mL of blood was collected in 2% EDTA from family members and healthy control subjects. Platelet-rich plasma was prepared by centrifuging at 150*g* to 200*g* for 15 minutes. The platelet-rich plasma was then washed twice with wash buffer (0.01 mol/L of tris(hydroxymethyl)aminomethane hydrochloride, 0.15 mol/L of sodium chloride, and 10 mmol/L of EDTA), and the platelet pellet was resuspended in the same wash buffer. An equal volume of lysis solution (3% sodium dodecyl sulfate and 6 mmol/L of *N*-ethyl maleimide) was added and heated in a boiling water bath for 3 minutes. The platelet lysates then were frozen immediately at –70°C.

Before the Western blot, total protein in the platelet lysate was quantified and an equal amount of protein (5 μg) was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis under reducing and nonreducing conditions for GPIIb and GPIIIa, respectively. A Ponceau-S stain of membrane revealed complete transfer of total protein from the gel onto the membrane before further membrane processing in all the cases. The membrane was incubated with CD41 (anti-GPIIb) and CD61 (anti-GPIIIa) antibodies, followed by chemiluminescence detection, which is considered a more sensitive method of protein detection. α-Tubulin antibody was used in each blot as an internal control.[13,14]

Molecular Studies

To find the mutation status of *GPIIb* and *GPIIIa* genes in family members, DNA extraction from whole blood, polymerase chain reaction, and direct sequencing were performed. In 1 family, RFLP was performed to look for heterozygote status in the family members.[15]

Statistical Evaluation

Sensitivity, specificity, positive predictive value, negative predictive value, and confidence interval of the flow cytometric and Western blot results for diagnosis and to detect carriers of GT were calculated with respect to DNA analysis as the gold standard. The statistical analysis was

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done by using CATmaker statistical software (version 1.1, Center for Evidence Based Medicine, John Radcliffe Hospital, Oxford, England).

Results

Carrier detection by phenotypic analysis (flow cytometry/Western blot) and direct gene analysis (sequencing or RFLP) was carried out in 20 parents (20 fathers and 20 mothers) and 22 siblings. Of the 20 families (parents), 13 included patients with type I disease (0%-5% of GPIIb-GPIIIa on platelets), 3 included patients with type II disease (>5%-20% of GPIIb-GPIIIa on platelets), and 4 included patients with type III disease (>20% of GPIIb-GPIIIa on platelets). Of the 22 siblings studied, 13 were siblings of patients with type I disease, 3 of patients with type II disease, and 6 of patients with type III disease.

Carrier Detection by Flow Cytometry

A total of 17 families showed reduced expression of GPIIb-IIIa, of whom 11 were families of patients with type I GT, 2 of patients with type II, and 4 of patients with type III **Figure 1**. A total of 12 siblings showed reduced expression of GPIIb-IIIa **Table 1**.

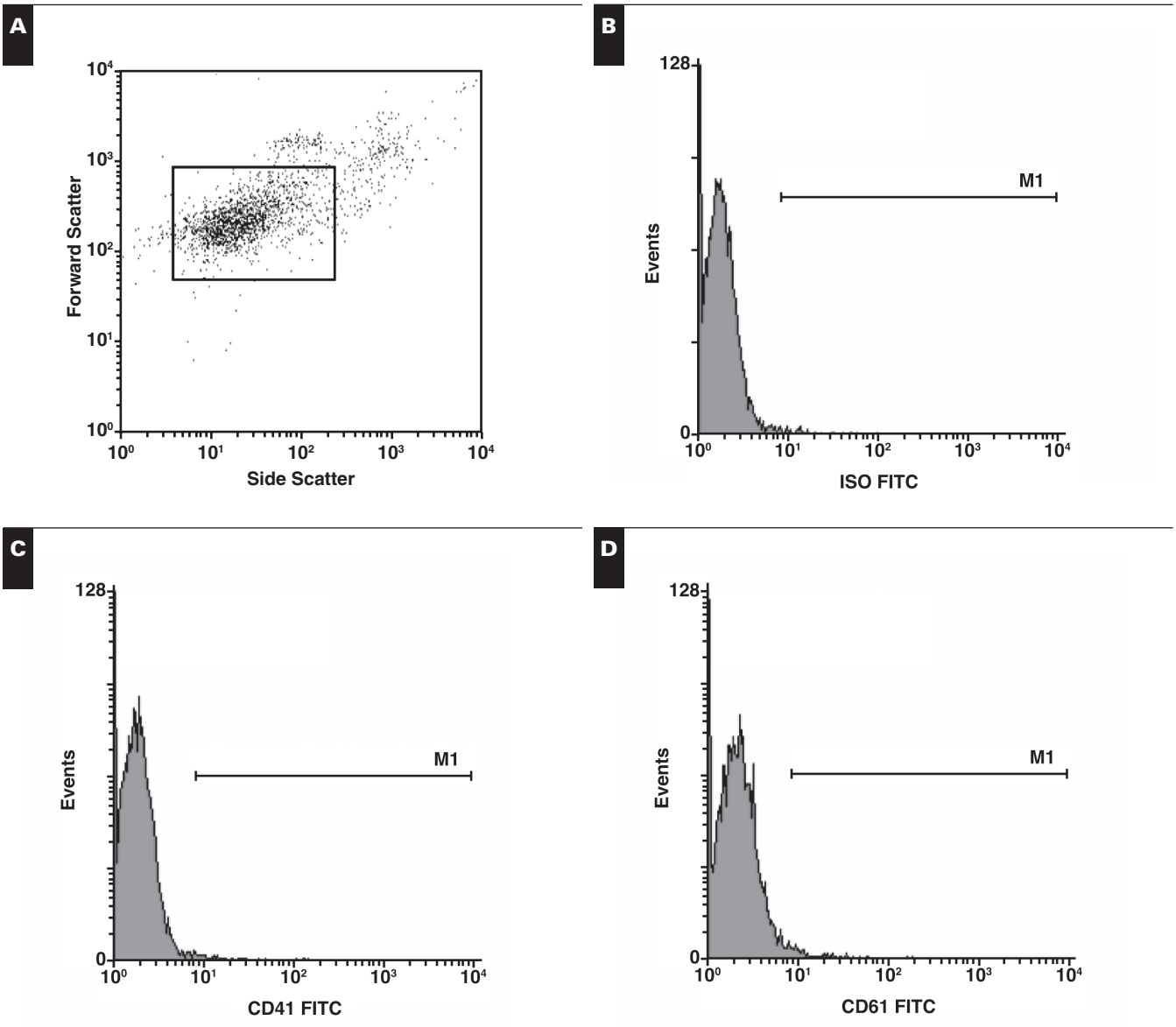

Figure 1 Examples of histograms showing absence of the glycoprotein IIb/IIIa complex. **A**, Dot plot along with gating. **B**, Histogram for negative control. **C**, Histogram for CD41 FITC. **D**, Histogram for CD61 FITC. FITC, fluorescein isothiocyanate.

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Table 1
Carrier Detection in Glanzmann Thrombasthenia Based on Glycoprotein IIb/IIIa Complex Expression by Flow Cytometry*

Disease Type	Absent or Reduced Expression (0%-56%)	Normal Expression (>56%)	Total
Parents (n = 20)			
I	11 (65)	2 (67)	13
II	2 (12)	1 (33)	3
III	4 (23)	0 (0)	4
Total	17 (85)	3 (15)	20
Siblings (n = 22)			
I	7 (58)	6 (60)	13
II	1 (8)	2 (20)	3
III	4 (33)	2 (20)	6
Total	12 (55)	10 (45)	22

Type I, 0%-5% of GPIIb-GPIIIa on platelets; type II, >5%-20% of GPIIb-GPIIIa on platelets; type III, >20% of GPIIb-GPIIIa on platelets.
* Data are given as number (percentage).

Carrier Detection by Western Blot

An abnormal protein fragment or absent or reduced GPIIb or GPIIIa protein was considered to be defective. A defect in GPIIb alone was seen in a total of 5 families, of whom 4 were families of patients with type I GT and 1 was a family of a patient with type II. A defect in the GPIIb and GPIIIa proteins was seen in 1 family of a patient with type III. Of the 22 siblings, a defect in GPIIb alone was seen in 6, of whom 5 were siblings of patients with type I disease and 1 was a sibling of a patient with type II disease. A dual GPIIb-IIIa defect was seen in 2 siblings **Table 2**.

Carrier Detection by Direct Mutation Analysis

Heterozygous mutations were seen in all parents. Of the siblings, 16 carried at least 1 defective allele and, hence, were carriers. Of these, 2 siblings carried a homozygous mutation, and the aggregation and other protein analysis revealed that the mutations were GT **Table 3**.

Evaluation of Techniques to Detect Carriers of GT

A total of 62 individuals (fathers, mothers, and siblings) were available for carrier detection by all 3 techniques, flow cytometry, Western blot, and DNA studies **Table 4**. DNA-based mutation detection was considered the gold standard method. Sensitivity, specificity, positive predictive value, negative predictive value, and the confidence interval for flow cytometric and Western blot results for diagnosis and to detect carriers of GT were calculated with respect to DNA analysis **Table 5**.

Discussion

Because GT results in severe bleeding and the management of GT involves repeated transfusions of platelets in severe episodes, a treatment of limited availability in developing countries, it becomes important to control the disease. This can be carried out by appropriate genetic counseling and prenatal screening.[16,17] Carriers can be identified by protein analysis using flow cytometry and Western blot or by direct gene analysis. Carrier detection by protein analysis is an immediate method of diagnosis and the method of choice in many developing countries. In the present study, carrier status was detected by flow cytometry in 17 parents (85%) and 12 (55%) siblings.

Carrier detection originally demonstrated by biochemical and immunologic methods for total or surface expression of GPIIb-IIIa found significant reduction of total and surface expression of GPIIb and/or GPIIIa.[9] Flow cytometry that distinguished heterozygote carriers by relative fluorescence intensity was used to detect potential carrier status in GT from

Table 2
Carrier Detection in Glanzmann Thrombasthenia Based on Absent or Reduced or an Abnormal Fragment of GPIIb and/or GPIIIa Protein by Western Blot*

Type of Disease in Families	GPIIb Defect Alone	GPIIIa Defect Alone	Dual GPIIb-IIIa Defect	Normal
Parents (n = 20)				
I (n = 13 families)	4 (80)	0 (0)	0 (0)	9 (64)
II (n = 3 families)	1 (20)	0 (0)	0 (0)	2 (14)
III (n = 4 families)	0 (0)	0 (0)	1 (100)	3 (21)
Total	5 (25)	0 (0)	1 (5)	14 (70)
Siblings (n = 22)				
I (n = 13 siblings)	5 (83)	0 (0)	1 (50)	7 (50)
II (n = 3 siblings)	1 (17)	0 (0)	0 (0)	2 (14)
III (n = 6 siblings)	0 (0)	0 (0)	1 (50)	5 (36)
Total	6 (27)	0 (0)	2 (9)	14 (64)

GP, glycoprotein; type I, 0%-5% of GPIIb-GPIIIa on platelets; type II, >5%-20% of GPIIb-GPIIIa on platelets; type III, >20% of GPIIb-GPIIIa on platelets.
* Data are given as number (percentage).

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Table 3
Carrier Detection in Glanzmann Thrombasthenia by Direct Mutation Analysis*

Disease Type	Carriers	Normal	Total
Parents (n = 20)			
I	13 (65)	0 (0)	13
II	3 (15)	0 (0)	3
III	4 (20)	0 (0)	4
Total	20 (100)	0 (0)	20
Siblings (n = 22)			
I	9 (56)	4 (67)	13
II	3 (19)	0 (0)	3
III	4 (25)	2 (33)	6
Total	16 (73)	6 (27)	22

Type I, 0%-5% of GPIIb-GPIIIa on platelets; type II, >5%-20% of GPIIb-GPIIIa on platelets; type III, >20% of GPIIb-GPIIIa on platelets.
* Data are given as number (percentage).

Table 4
Comparison of Flow Cytometry and Western Blot With DNA Mutation in Cases of Glanzmann Thrombasthenia*

	DNA (n = 62)	
	Mutation	Normal
Flow cytometry		
Reduced GPIIb-IIIa	38 (61)	5 (8)
Normal GPIIb-IIIa	13 (21)	6 (10)
Western blot		
Reduced GPIIb and IIIa	20 (32)	0 (0)
Normal GPIIb and IIIa	31 (50)	11 (18)
Flow cytometry and Western blot		
Reduced GPIIb-IIIa	45 (73)	5 (8)
Normal GPIIb-IIIa	6 (10)	6 (10)

GPIIb-IIIa, glycoprotein IIb/IIIa complex.
* Data are given as number (percentage).

Table 5
Statistical Evaluation of Flow Cytometry and Western Blot for the Diagnosis of Glanzmann Thrombasthenia Carriers*

	Flow Cytometry	Western Blot	Flow Cytometry and Western Blot
Sensitivity (%)	75 (63-86)	39 (26-53)	88 (79-97)
Specificity (%)	55 (25-84)	100 (100-100)	55 (25-84)
Positive predictive value (%)	88 (79-98)	100 (100-100)	90 (82-98)
Negative predictive value (%)	32 (11-52)	26 (13-39)	50 (22-78)

* The values in parentheses are 95% confidence intervals.

healthy people.[10] In the present study, flow cytometry was found to be the more sensitive technique (75%) compared with Western blot (39%) for carrier detection. The sensitivity was increased to 88% when flow cytometry and Western blot detection were combined. The positive predictive value was 90% for the protein analysis. Thus, it is suggested that protein analysis may be effectively used to detect carriers, especially where DNA techniques are not available. To the best of our knowledge, this is the first study in India to attempt to detect carrier status in a large number of family members. A study from Oman showed that healthy subjects had GPIIb-IIIa expression occasionally overlapping with that in GT carriers; in such cases, the mean platelet volume was used as a correction factor for the detection of carrier status. This study indicated that it is possible to confidently and rapidly predict the carrier status by flow cytometry.[18]

Although protein analysis for carrier detection has its own advantages, such as rapid carrier detection, DNA-based analysis is considered to be an accurate and a reliable means of carrier detection of GT. Carrier detection by direct gene analysis is possible if the patient's mutation is known.[15] Heterozygous mutation was seen in all families (100%). Of 22 siblings, 16 (73%) were found to carry at least 1 defective allele and, hence, were carriers. Of these 16 siblings, 9 were siblings of patients with type I disease, 3 of patients with type II disease, and 4 of patients with type III disease. These findings show that the genotype assessment constitutes a more accurate method of carrier detection. When circumstances permit, the genetic diagnosis of GT should be done based on the direct identification of the causative mutation in the *GPIIb* or *GPIIIa* gene.

The present study reveals that carrier detection by flow cytometry is more sensitive than by Western blot and, hence, can be used for carrier detection in GT in developing countries.

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