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IN FOCUS

Molecular defects in *ITGA2B* and ITGB3 genes in patients with Glanzmann thrombasthenia

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Summary. *Background*: Glanzmann thrombasthenia (GT) is an autosomal recessive inherited platelet function defect that is characterized by reduction in, or absence of, platelet aggregation in response to multiple physiologic agonists. The defect is caused by mutations in the genes encoding ITGA2B or ITGB3. This results in qualitative or quantitative abnormalities of the platelet receptor, αIIb-β3. *Objectives*: The aim of this study was to identify the mutations in GT patients and to correlate these with patient phenotype. *Subjects and methods*: A total of 45 unrelated patients with GT were enrolled in the present study to identify the causative molecular defects, and also to correlate their phenotype with their genotype. Platelet aggregation, flow cytometry, Western blotting, and mutation screening by conformation sensitive gel electrophoresis (CSGE) followed by sequencing were performed in all patients. Novel mutations were analyzed for penetrance in individual families. *Results*: A total of 22 novel mutations were identified in 45 unrelated GT patients. Mutations were identified in 36 of the 45 (80%) patients. Missense mutations were seen in most of the GT patients (59%). The remaining mutations were heterogeneous and were distributed throughout the length of the gene. Analysis of family members showed heterozygous mutations in all families. *Conclusions*: The severe type I GT was the most common subtype found in this study. Missense mutations were identified as the defects responsible for most GT patients. Carrier detection and genetic counseling in these families is a potentially effective alternative for decreasing the burden of severe type of GT.

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Introduction

Glanzmann thrombasthenia (GT) is a hereditary platelet function defect, characterized by normal platelet count, prolonged bleeding time, and abnormal clot retraction [1–3]. GT arises due to severe reduction in, or absence of, platelet aggregation in response to multiple physiologic agonists due to abnormalities in platelet glycoprotein αIIb and/or β3 [4–6]. GT occurs in high frequency in certain ethnic populations with an increased incidence of consanguinity, such as Indians, Iranians, Iraqi Jews, Palestinians, Jordanian Arabs and French gypsies [1,7–9]. GT is caused by mutations in the genes encoding ITGA2B or ITGB3 that result in qualitative or quantitative abnormalities in the platelet membrane proteins. For the detection of mutations affecting both genes, various screening methods including single stranded conformation polymorphism (SSCP), conformation sensitive gel electrophoresis (CSGE) and denaturing gradient gel electrophoresis (DGGE) are used [10]. Of these, CSGE is the most sensitive mutation screening technique. Identification of molecular defects not only helps in better understanding of the causal mechanism, but is also an important tool for carrier detection and prenatal diagnosis in GT [11]. It would also help in preimplantation genetics and in enrolling patients for gene therapy. In the current report we describe the mutation spectrum in Indian patients with GT.

Materials and methods

Study subjects

GT patients presenting at the hematology clinic of the All India Institute of Medical Sciences (AIIMS), New Delhi, were enrolled in this study. These patients were diagnosed on the basis of both clinical and hematological parameters. Informed consent from patients and their family members was obtained

as per guidelines of the institutional ethics committee. A total of 100 control subjects were also analyzed wherever required. The inclusion criteria were patients with history of muco-cutaneous bleeding, patients with absent or reduced platelet aggregation with agonists such as adenosine 5′-diphosphate (ADP), adrenaline (ADR), arachidonic acid (AA) and collagen, prolonged bleeding time (BT), absent or reduced clot retraction (CR), and normal platelet count ($> 150 \times 10^3\ \mu L^{-1}$), prothrombin time (PT) and activated partial thromboplastin time (aPTT). Patients with acquired bleeding, coagulation defects, thrombocytopenia, platelet function defects other than GT and patients on anti-platelet drugs were excluded from the study. Clinical history of bleeding, family history and history of blood/platelet transfusions were recorded at the time of enrollment.

Platelet receptor αIIb-β3 protein analysis

Platelet receptor αIIb-β3 protein was analyzed first by flow cytometry followed by Western blotting. Flow cytometry characterized the GT patients in to types I, II and III. Western blotting allowed detection of αIIb and β3 proteins in platelet lysates. Flow cytometry and Western blot were performed as described previously [12].

Molecular studies

For mutation analysis of *ITGA2B* and *ITGB3* genes, DNA extraction from whole blood, polymerase chain reaction (PCR), heteroduplex PCR followed by CSGE and DNA sequencing were performed. All 30 exons of *ITGA2B* and 15 exons of *ITGB3*, as well as their promoters, were analyzed by CSGE following heteroduplex PCR. In each gel, each patient's band pattern was compared with normal control band pattern. For novel missense mutations, Support Vector Machine (SVM) score was calculated using sequence homology and conservation to look for the deleterious effect of the mutations. Negative scores represent deleterious effects of the mutation. All mutation sites were examined for sequence conservation in four species (human, porcine, murine and canine). The nucleotide numbering was according to GeneBank accession numbers NM_000419.3 and NM_000212.2 for *ITGA2B* and *ITGB3* genes, respectively. The cDNA numbering was started with the A nucleotide of the ATG start codon as +1 in both *ITGA2B* and *ITGB3* genes. To examine the penetrance of the mutations within individual families, families of GT patients were analyzed either by sequencing the defective exon or by restriction fragment length polymorphism (RFLP) mapping. For RFLP, the PCR product was digested by the appropriate restriction endonucleases that cleaved at the particular mutation site.

Molecular modeling was performed for some of the novel missense mutations identified in both *ITGA2B* and *ITGB3* genes. A set of models for the potential MIDAS domain of ITGB3 and β propeller domain of ITGA2B were performed based on homology modeling to understand the potential influence of the mutations upon the protein. The sequence of the ITGA2B (PDB: 1jx5) and ITGB3 (PDB: 1txv) obtained was submitted to the SWISS-MODEL repository at ExPASY for homology modeling. The coordinates so obtained were viewed using the program PyMOL.

Results

Of the 45 GT patients, 25 were males and 20 were females, ranging from 5 months to 46 years of age. The clinical manifestations included epistaxis in 36 patients, gum bleeding in 31 patients, petechiae in 29 patients and ecchymoses in 21 patients. The major bleeding complications of hematuria and gastro-intestinal bleed were seen in three and eight patients, respectively. Other bleeding complications such as eye bleeding and bleeding during circumcision or tooth extraction were seen in six patients. Fourteen patients had prolonged bleeding after trauma; most of these 14 patients also had severe bleeding even from minor cuts. Of the 20 female GT patients, 12 were adults and 8 were premenarchal. Menorrhagia was seen in 10 out of 12 post-menarchal female patients (Fig. 1). Among the 45 GT patients, a blood or platelet transfusion was required in 36 patients, of whom 15 patients had multiple transfusions and 21 patients had transfusion once in their lifetime. Hemogram profiles of platelet count and platelet size were normal in all patients. The peripheral smear showed isolated, non-aggregated platelets under microscope. A clinical evaluation by BT, showed prolonged BT of more than 15 min in 39 patients and moderate BT in four patients. BT was not performed in two patients, as their ages were 5 months and 8 months. Absent or severely reduced clot retraction of 0% to 5%, was seen in 40 GT patients. Five patients showed moderate clot retraction (> 5%). These five GT patients exhibited mild clinical symptoms. All the GT patients had normal PT and APTT. Subsequent platelet function studies by platelet aggregation revealed either absent or reduced aggregation in response to ADP, ADR, AA and collagen and normal aggregation with ristocetin.

Analysis of αIIb and β3 receptor proteins

The αIIb-β3 expression as determined by flow cytometry in normal healthy individuals varied from 56.32% to 77.3%. In

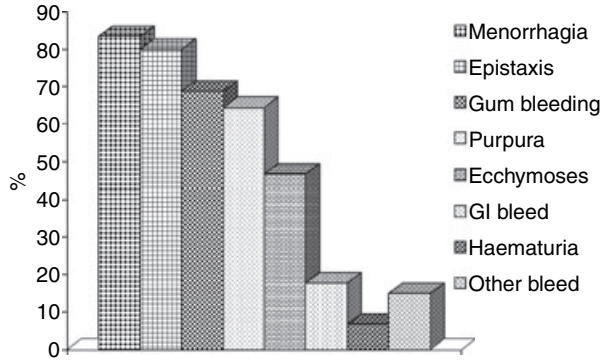

Fig. 1. Bar diagram showing clinical manifestations in patients with Glanzmann thrombasthenia ($n = 45$).

the patients with GT, the surface expression of αIIb-β3 was < 5% in 28 patients, between 5% and 20% in 7 patients and from 20% to near normal expression in 10 patients. Western blot analysis showed complete absence of αIIb protein in 24 patients. A trace amount of αIIb was seen in 16 GT patients; three patients showed reduced αIIb and two patients showed abnormal band pattern of αIIb when compared with normal healthy individuals. β3 was completely absent in 24 GT patients. A trace amount of β3 was seen in 14 GT patients; three patients showed reduced β3 and four patients were normal for β3. None of the patients had abnormally sized platelet β3.

Genotypic analysis of ITGA2B and ITGB3 genes

All the 45 GT patients were subjected to mutation screening by CSGE. Four out of 45 GT patients showed no band shift in any of the exons of the *ITGA2B* or *ITGB3* genes. DNA sequencing revealed nucleotide changes (either mutation or polymorphism), when compared with the Gene Bank and published sequences, in all 41 patients in whom at least one band shift was observed by CSGE. Mutations were identified in 36 of 45 (80%) unrelated GT patients. Of these, 22 patients (48.8%) showed defects in *ITGA2B* and 14 patients (31.1%) showed defects in *ITGB3*. In 9 out of 45 (20%) GT patients, no gene alterations were identified. In total, 31 different mutations were identified from 36 patients, of which 22 mutations were novel. Of the 31 different mutations, 17 were identified in *ITGA2B* and 14 were identified in *ITGB3*. The mutation nomenclature used was according to previously published reports [13].

Mutations in ITGA2B

Seventeen different mutations (a total of 29 αIIb mutations, including compound heterozygous and homozygous) were identified in 22 GT patients. Of the 29 *ITGA2B* mutations, 16 were missense, 4 were deletions, 5 were insertions and 4 were splice site mutations (Tables 1–3). Six different missense mutations were identified in 16 patients. Of these, three

Table 1 Missense mutations found in GT patients

Patient ID	Gene	Exon	Nucleotide change	Codon change	Amino acid change	SVM score	Conservation
*GT1, 18, 34, 54, 69	αIIb	Promoter	g.951G > A	Promoter	–	–	–
*GT1, 18, 54, 58, 69	αIIb	Exon 12	c.1028T > C	C**T**G > C**C**G	Leu343Pro	−2.0	HPMC
GT12, 26, 68	αIIb	Exon 6	c.641T > C	C**T**T > C**C**T	Leu214Pro	−1.71	HPMC
*GT19	αIIb	Exon 10	c.937G > A	**G**CA > **A**CA	Ala313Thr	−0.97	HPMC
GT45	αIIb	Exon 12	c.1073G > A	C**G**T > C**A**T	Arg358His	−1.5	HPMC
GT37	αIIb	Exon 13	c.1234G > A	**G**GG > **A**GG	Gly412Arg	−1.63	HPMC
*GT39	β3	Exon 2	c.92G > A	T**G**T > T**A**T	Cys31Tyr	−2.83	HPMC
GT9	β3	Exon 3	c.356G > A	C**G**G > C**A**G	Arg119Gln	−2.33	HPMC
GT2	β3	Exon 5	c.752G > A	C**G**A > C**A**A	Arg242Gln	−0.76	HPMC
*GT24	β3	Exon 7	c.953T > C	T**T**G > T**C**G	Leu318Ser	−1.94	HPMC
*GT28	β3	Exon 7	c.1031A > G	T**A**T > T**G**T	Tyr344Cys	−3.13	HPMC
*GT72	β3	Exon 10	c.1641C > G	T**G**C > T**G****G**	Cys547Trp	−3.25	HPMC
*GT3	β3	Exon 15	c.2315T > C	C**T**G > C**C**G	Leu772Pro	−0.12	HPMC
*GT53	β3	Exon 4	c.415G > C	**G**AC > **C**AC	Asp139His	−3.06	HPMC
GT53	β3	Exon 4	c.422A > G	T**A**C > T**G**C	Tyr141Cys	−3.4	HPMC
*GT10	β3	Exon 1	c.59T > C	C**T**G > C**C**G	Leu20Pro	–	HPMC

*Novel missense mutations. HPMC, human, pig, murine and canine; SVM score, Support Vector Machine score.

Table 2 Deletions and insertions found in GT patients

Patient ID	Gene	Exon	Nature of deletion/insertion	Codon position	A new stop codon at	Comments
*GT62	αIIb	Exon 4	c.559delG	187	223	Frame shift
*GT32, 67	αIIb	Exon 19	c.1919_1920delTG	640	659	Frame shift
*GT38	αIIb	Exon 23	c.2338delG	780	909	Frame shift
*GT70	αIIb	Exon 26	c.2674_2675insGA	892	910	Frame shift
GT18	αIIb	Exon 28	c.2915_2916insC	972	1035	Frame shift
*GT51	αIIb	Exon 30	c.3117_3118insTGGAG	End	Stop codon abortion	Frame shift
*GT20, 70	αIIb	Exon 14	c.1424_1427dupAGGT	476	661	Frame shift
*GT30	β3	Exon 5	c.674delA	225	282	Frame shift
*GT11	β3	Exon 14	c.2217delC	740	AA change	Frame shift
*GT31, 41	β3	Exon 6	c.887_901delACGGGCA GTGTCATG	Deletion of amino acids from 296 to 300		
*GT59	β3	Exon 2	c.155_156delGCinsTT	52	–	Indel

Table 3 Patients with splice site mutations and the mutation type

Patient ID	Gene	Exon/Intron	Nucleotide change	Genotype	Mutation type
GT23	αIIb	c.800−2A > T	A > T	Heterozygous	Missense
*GT23	αIIb	c.1210 + 4A > G	A > G	Heterozygous	Missense
GT15	αIIb	c.1753−1G > A	G > A	Homozygous	Missense
*GT14	αIIb	c.188 + 8delG	delG	Heterozygous	Deletion

*Novel mutations identified in the current study

mutations were previously reported. SVM score was calculated using sequence homology and conservation. All the mutation sites were conserved amongst human, porcine, murine and canine genes. Of the seven insertions/deletions found in *ITGA2B* of GT patients, three were deletions, three were insertions and one was a duplication. Of these seven insertions/deletions, six were novel mutations and only one insertion was previously reported. Four splice site variations have been identified in *ITGA2B*, of which two have been reported previously.

Mutations in ITGB3

Fourteen different mutations (a total of 15 *ITGB3* mutations, including compound heterozygous and homozygous) were identified in 14 unrelated GT patients (Tables 1 and 2). Of the 15 *ITGB3* mutations, 10 were missense, 4 were deletions and 1 was an insertion/deletion mutation. Ten different missense mutations were identified in 10 patients; of these, seven were novel and three were previously reported. Of the four deletions/insertions found in ITGB3 gene of GT patients, three were deletions and one was an insertion/deletion mutation. None of these mutations were previously reported.

Novel mutations

Of the 31 different mutations, 17 were identified in *ITGA2B* and 14 were identified in *ITGB3*. A total of 22 mutations were not previously described and hence were novel. Of these 22 novel mutations, 10 were missense mutations, 10 were deletions/insertions and 2 were splice site mutations. Molecular modeling of a novel missense mutation in ITGA2B (Ala313Thr) and four novel missense mutations (Leu318Ser, Tyr344Cys, Asp139His, and Cys547Trp) in ITGB3 showed that these mutations could potentially adversely affect the functional domains of these proteins (Figs 2 and 3).

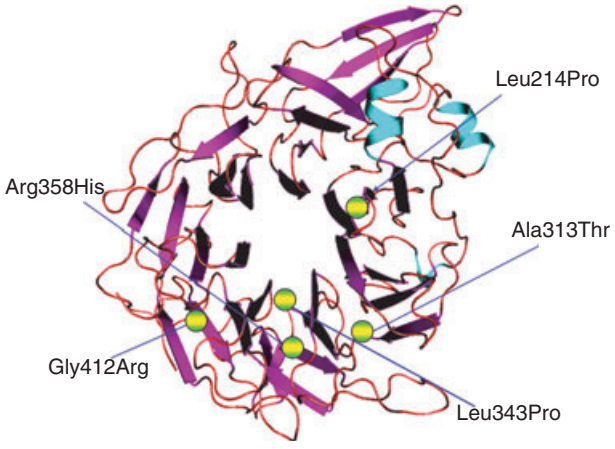

Fig. 2. Molecular modeling showing location of some of the missense mutations in the beta propeller domain of the αIIb protein.

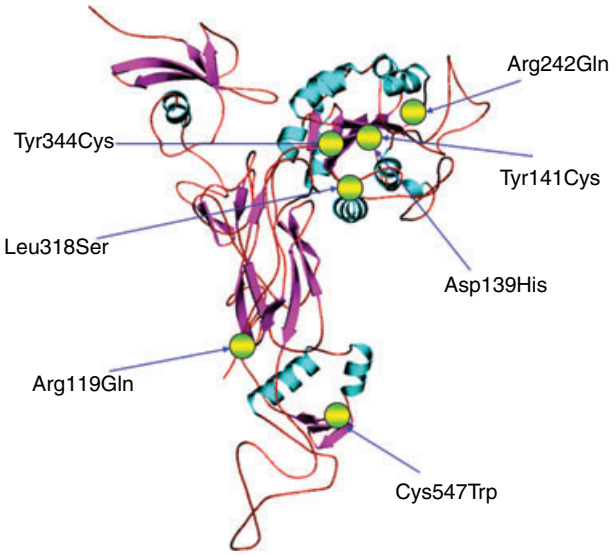

Fig. 3. Molecular modeling showing location of some of the missense mutations in the different domains of the β3 protein.

Penetrance of mutations within the individual families

Sixteen different mutations, including 11 novel mutations, were analyzed for penetrance into the individual family members (Table 4). Direct mutation analysis by sequencing for particular mutation of the defective exon was done in the patients' family members. Figure 4(a) shows the heterozygous pattern of the Leu343Pro mutation by direct sequencing of defective exon 12 in a family member. In a few families, RFLP mapping was done by identifying restriction enzyme cleavage sites that overlapped the mutation site. A restriction enzyme, TspRI, was used against c.1028T > C (exon 12 of GPIIb) for carrier detection in four families (GT1, GT18, GT54 and GT58) (Fig. 4b). A total of 46 family individuals were available for this analysis. Heterozygous mutation was seen in all the families. Of these, most of the parents were heterozygous for a single

Table 4 Penetrance of the mutations within the individual families

Mutation	Gene/exon	Patient ID	Parents		Siblings		
			F	M	B1	B2	S1
g.951G > A	αIIb of promoter	GT1	+/−	+/−	+/−		+/−
		GT18	−/−	+/−	−/−		
		GT54	+/−	+/−			+/−
Leu343Pro	αIIb of exon 12	GT1	+/−	+/−	+/−		+/−
		GT18	−/−	+/−	−/−		
		GT54	+/−	+/−			+/−
Ala313Thr	αIIb of exon 10	GT19	+/−	+/−	+/+	+/−	
Arg242Gln	β3 of exon 5	GT2	+/−	+/−			+/−
Tyr344Cys	β3 of exon 7	GT28	+/−	+/−			
Leu772Pro	β3 of exon 15	GT3	+/−	+/−	−/−	+/−	−/−
Asp139His	β3 of exon 4	GT53	−/−	+/−			−/−
Tyr141Cys	β3 of exon 4	GT53	+/−	−/−			−/−
c.1919_1920delTG	αIIb of exon 19	GT32	+/−	−/−			
c.2915_2916insC	αIIb of exon 28	GT18	+/−	−/−	−/−		
c.1424_1427dupAGGT	αIIb of exon 14	GT20	+/−	+/−	+/+	+/−	
c.674delA	β3 of exon 5	GT30	+/−	+/−			
c.155_156delGCinsTT	β3 of exon 2	GT59	+/−	+/−	+/+	+/−	
c.800−2A > T	αIIb splice site	GT23	+/−	−/−	+/−	+/−	−/−
c.1210 + 4A > G	αIIb splice site	GT23	−/−	+/−	−/−	−/−	−/−
c.1753−1G > A	αIIb splice site	GT15	+/−	+/−			

+/−, heterozygous; +/+, homozygous; −/−, normal. F, father; M, mother; B1, brother 1; B2, brother 2; S1, sister 1. Empty box represents not applicable.

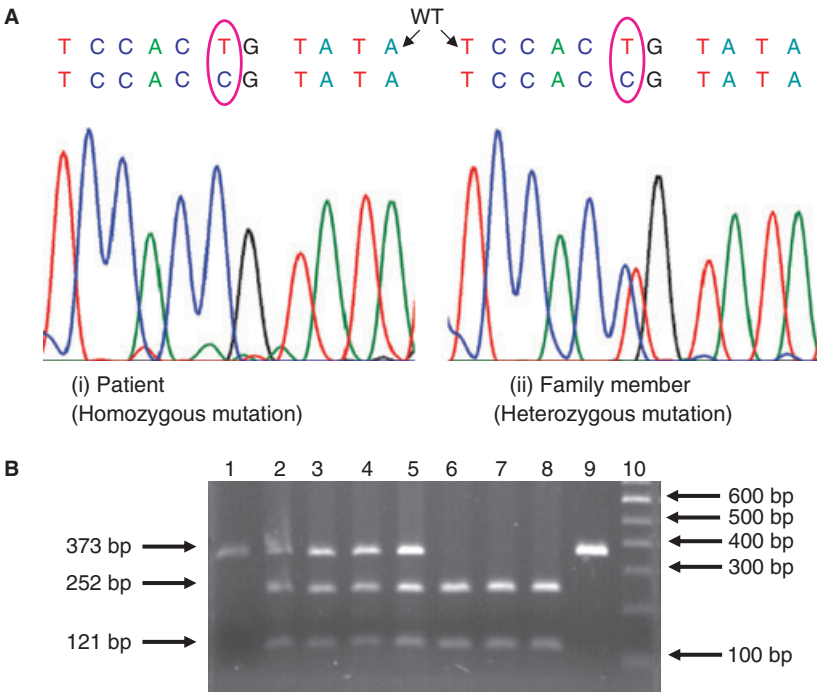

Fig. 4. Analysis of Leu343Pro mutation in family individuals. (A) Direct sequencing of defective exon 12 showing heterozygous pattern in a family member. (B) The same mutation was confirmed by RFLP in family members of a GT patient. Lane 1: Patient, Lane 2: Father, Lane 3: Mother, Lane 4: Brother, Lane 5: Sister, Lane 6-8: Normal, Lane 9: Amplified undigested PCR product and Lane 10: Molecular weight marker (DNA ladder).

mutation, whereas the parents of patients that were compound heterozygous were each heterozygous for two different mutations. Of the 18 siblings, 15 carried at least one defective allele.

Genotype and phenotype correlation

Of all the mutations identified in this study, missense mutations were the most common cause of GT (59%). The missense

Table 5 Genotype and phenotype correlation

Mutation type	Mutation frequency or distribution			
	GT Type I (n = 28)	GT Type II (n = 7)	GT Variant (n = 10)	Total GT patients (n = 45)
Missense mutation	18 (58%)	4 (57.1%)	4 (66.6%)	26 (59%)
Deletions	6 (19.3%)	2 (28.5%)	0	8 (18.1%)
Insertions	3 (9.6%)	0	0	3 (6.8%)
Duplications	2 (6.4%)	0	0	2 (4.5%)
Indel mutations	0	1 (14.2%)	0	1 (2.2%)
Splice site mutations	2 (6.4%)	0	2 (33.3%)	4 (9%)
Total mutations	31	7	6	44

mutations were distributed among the various types of GT; type I (58%), type II (57.1%) and variant type (66.6%). Of the eight deletions identified, six (75%) resulted in type I GT and two (25%) resulted in type II GT, whereas none of the deletions were seen in variant type (non-severe type of GT). Insertions and duplications were seen only in severe type of GT (type I). Splice site mutations were equally represented in both type I and variant type GT (Table 5).

Discussion

The clinical manifestations of GT observed in the patients enrolled in our study were purpura, ecchymoses, epistaxis, gum bleeding, gastrointestinal hemorrhage, hematuria, menorrhagia, and certain other types including eye bleeding. Of these, epistaxis was most commonly seen (80%) followed by gum bleeding (68.8%). Platelet αIIb-β3 analysis by flow cytometry revealed that 62.2%, 15.5% and 22.2% of these patients had types I, II and III GT, respectively. Previously, we reported type I GT to be the most common subtype in the North Indian population [9]. Western blot analysis revealed no detectable αIIb or β3 in 53.3% of the patients. Trace amounts of αIIb and β3 were seen in 35.5% and 31.1% of patients, respectively. Abnormal sized αIIb was seen in two patients (4.4%) with mutations in *ITGA2B*.

Sequencing revealed mutations in 36 out of 45 (80%) unrelated GT patients. Of these, 48.8% of patients had *ITGA2B* defects and 31.1% of patients had *ITGB3* defects. In 9 out of 45 (20%) GT patients, no gene alterations were identified. Although no mutations in *ITGA2B* or *ITGB3* were identified in these nine patients, their hematological tests (including platelet aggregation and flow cytometry) confirmed their diagnosis of GT.

Seventeen different mutations were identified in 22 unrelated GT patients. Of these, 55.1% were missense mutations, 13.8% were splice site mutations or deletions; 10.3% and 6.9% of *ITGA2B* mutations were insertions and duplications, respectively. Missense mutations were seen in all the three types of GT, whereas deletions, insertions and duplications were seen only in type I GT patients. Fourteen different mutations were identified in 14 unrelated GT patients. Of these, 66.6% were missense and 26.6% were deletions; 6.6% of the mutations were insertion/deletion mutations. Missense mutations were seen in all the three types of GT, whereas deletions and splice site mutations were seen only in types I and II, and not in type III.

Structural modeling of αIIb Ala313Thr revealed that Ala313 is presented within the W5 blade of the beta propeller domain. It is possible that the mutation creates a hydrogen bond between the carboxyl groups of adjacent Glu, Leu and Thr. Moreover, residues 294–314 of the beta propeller domain are potential ligand binding sites. Hence, the mutation may potentially affect the ligand binding to the receptor, resulting in type I GT.

Structural modeling of β3 Leu318Ser revealed that Leu318 is located in the A domain of β3. The Leu318Ser substitution very likely reduces the hydrophobic nature of the protein. Clinically, this patient exhibited mild symptoms and showed reduced expression of both αIIb and β3, as determined by Western blotting. Molecular modeling for Tyr344Cys revealed that Tyr344 was also located in the β3 A domain. Residues 324–366 represent a cysteine-rich region and moreover this region is important for αIIb-β3 heterodimer inter-subunit surface interactions. The patient carrying this mutation presented with nosebleeds, as well as eye bleeding. The loss of intersubunit interaction may influence this kind of clinical bleeding and in turn resulted in type I GT phenotype in this patient. Molecular modeling for Cys547 revealed its presence in the epidermal growth factor (EGF) 3 domain. There is extensive contact between calf-1 of αIIb and EGF-3 of β3. Hence, in the current study, it is presumed that the mutation Cys547Trp gives rise to conformational changes that result in retention of the aberrant αIIb-β3 complex in the endoplasmic reticulum.

Genotype and phenotype correlations

Of all the mutations identified in the current study, missense mutations were the most common cause of GT (59%). The missense mutations were distributed among the various types of GT; type I (58%), type II (57.1%) and variant type (66.6%). In the literature, the majority of mutations responsible for GT were reported to be missense mutations (50%). The current study revealed that the missense mutations were more common (59%) in Indian patients with GT than in Western populations (50%). Of the eight deletions identified in the current study, six (75%) resulted in type I, and two (25%) resulted in type II phenotype, whereas none of the deletions were seen in variant type (non-severe type of GT). Published reports have stated

that deletion mutations responsible for type I GT arise due to premature truncation of αIIb or β3, or through single base substitutions that result in amino acid changes in areas critical for normal subunit stability and processing [14–16]. In the current study, insertions and duplications were seen only in type I GT, suggesting that these genotype variations cause severe phenotype of GT. Insertion mutations invariably result in premature or non-functional αIIb protein. None of the type III patients had deletions or insertions. Splice site mutations were equally represented in between type I (severe) and variant type GT (non-severe).

A novel missense mutation, Ala313Thr in ITGA2B resulted in type I GT phenotype. Molecular modeling revealed that Ala313 is presented within the blade W5 of beta propeller domain. Residues 294–314, of beta propeller domain, are potential ligand binding sites. The amino acid change may result in hydrogen bond formation between the carboxyl groups of Gly, Leu and Thr that possibly affected the binding of the ligand to the receptor. Another novel mutation, Leu343Pro, resulted in type I GT in four patients and type II GT in one patient. Molecular modeling confirmed that the amino acid was located in the alpha loop of the beta propeller domain that is also important for the ligand binding.

Three published missense mutations (Leu214Pro, Arg358His and Gly412Arg) were seen in five GT patients. Leu214Pro was seen in three unrelated GT patients and all of them were type III GT. An earlier report suggested that Leu214Pro mutation disrupts the structural conformation and the ligand binding properties of the heterodimeric complex. In the present study, all three patients exhibited a type III GT phenotype. Arg358His showed trace amounts of αIIb and β3 by Western blotting and supports the earlier published study. Gly412Arg was seen in one patient with type I GT. This mutation is associated with the creation of a stop codon on the other αIIb allele that may be responsible for the lack of αIIb-β3 on the platelet surface. The flow cytometry analysis revealed that the patient was GT type I because he had severely reduced expression αIIb-β3 on the platelets.

Three different deletions were seen in four unrelated GT patients and all caused GT type I phenotype. None of these had been reported previously. All three deletions found resulted in premature truncated αIIb protein. Flow cytometry analysis revealed the expression of severely reduced αIIb-β3 on the patients' platelets. Clinically, all of these patients had severe GT and required blood transfusion. One of these patients also had severe menorrhagia.

An insertion, c.2674_2675insGA, was seen in one patient with type I GT, which was clinically very severe. This patient required multiple transfusions to stop nose bleeding. The same duplication insertion was seen in another type I GT patient who was homozygous for this mutation. He had severe gum and nose bleeding and also required blood transfusion. An insertion, c.3117_3118insTGGAG, was seen in one type I GT and resulted in a frame shift that disrupted the normal stop codon at the end of the αIIb protein. This patient also had gum bleeding since birth and later developed clinical complications associated with menorrhagia.

The missense mutations of the *ITGB3* gene lead to a variety of phenotypes from mild to severe. Of the seven novel missense mutations of *ITGB3*, Leu318Ser substitution resulted in type I GT phenotype. Molecular modeling revealed that Leu318 is presented in the A domain of β3. The Leu318Ser substitution very possibly reduced the hydrophobicity in this region of the protein. The patient was clinically mild and showed reduced expression of αIIb-β3. Molecular modeling of Tyr344Cys revealed that Tyr344 was present in the A domain of β3. Residues 324–366 correspond to the cysteine-rich region and are important for αIIb-β3 heterodimer inter-subunit surface interactions. The patient with this mutation had a clinical complication of both nosebleeds and eye bleeding. The loss of intersubunit interaction might have a role in this kind of clinical bleeding, and so result in the type I GT phenotype in this patient. Molecular modeling for Cys547Trp revealed that Cys547 is located in the EGF-3 domain. There is a significant contact between the calf-1 of αIIb and EGF-3 of β3. Hence, it is possible that the mutation Cys547Trp gives rise to conformational changes that are responsible for retention of the aberrant αIIb-β3 complex in the endoplasmic reticulum.

A novel deletion c.674delA was seen in one type I GT patient. The patient had severe gum bleeding and epistaxis and required blood transfusion to stop the bleeding. It is possible that the novel deletion resulted in severe GT phenotype. A 15 bp deletion c.887_901delACGGGCAGTGTCATG was identified in two patients and resulted in deletion of five amino acids DGQCH from codon 296 to 300. The deletion was presented in the A domain of β3. Both patients presented with type II GT and both had similar clinical complications of gum bleeding and epistaxis.

Unexpectedly, no gene alterations were identified in either the coding or exon-flanking regions of either *ITGA2B* or *ITGB3* in 9 out of 45 (20%) patients from our cohort. In two previous studies no mutations were detected in 20% of patients diagnosed with GT [17]. It is clear therefore from these and our studies that *ITGA2B* or *ITGB3* mutations may not be present in about 20% of GT patients. Such patients with no detected causative mutations may carry defects in regulatory elements that adversely affect the transcription of these genes [18]. Alternatively, abnormalities in mechanisms that are responsible for post-translational modifications and trafficking of integrin subunits may also potentially account for some cases of GT. A recent study on Indian GT patients, reported that no causative mutation in either the αIIb or β3 gene was identified in 27% of patients [19]. The strategy used to detect mutations in this current study was based on CSGE, the most sensitive screening method for detection of mutations. We carefully set up the CSGE approach and validated this with known mutations, making it highly unlikely that any nucleotide substitutions were missed in the 20% of the GT patients. Despite published literature reporting that CSGE does not generate a significant number of false positive or false negative results [20], we

repeated the CSGE twice in these patients and still found no sequence alterations.

In conclusion, our present study identifies the causal mutations, including 22 novel mutations, in 80% of GT patients in our cohort. Missense mutations were identified as the defects responsible for most of the GT patients (59%). Of the two genes involved, *ITGA2B* is found to be defective in most of the patients, which gives the possibility of targeting this gene first in new cases of GT. Because the type I GT is the most common subtype found in this study, carrier detection and genetic counseling of these families may be an effective alternative for decreasing the burden of severe type I GT.

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Disclosure of Conflict of Interests

All authors declare that they have no conflict of interest.

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