Date: 14th Oct 2008

To

The Editor
Nature Medicine
75 Varick Street, 9th flr
New York NY 10013-1917
USA

Tel: (212) 726-9325; Fax: (212) 683-5751
E-mail:medicine@natureny.com

Dear Sir,

Further to your previous mail on 14th October regarding the submission, herewith I am submitting our manuscript **"Identification of 22 novel mutations in patients with Glanzmann's Thrombasthenia"** to your esteemed journal, Nature Medicine as an Original article. The work we submit here is on a rare disorder that included large number of patients which is of interest to clinicians and medical scientists. We analyzed both phenotype and genotype of the patients. Kindly convert the color figures into black and white. We request you to kindly acknowledge the receipt of the article. Please let me know if anything else is required.

Thanking you.

With regards,

Prof. (Dr). Renu Saxena, MD,
Director, Department of Hematology,
All India Institute of Medical Sciences,
IRCH Building 1st Floor, Ansari Nagar,
New Delhi-110 029. India.
Email: profrsaxena@gmail.com
Tel: +91-11-2659-4670
Fax: +91-11-2658 8663

Original research article

"Identification of 22 novel mutations in patients with Glanzmann's thrombasthenia"

Authors

[1,3]Meganathan Kannan PhD
Postdoctoral fellow

[1]Firdos Ahmad M Sc
PhD Student

[1]Birendra K Yadav M Sc
Research Scholar

[1]Abdul Samath Ethayathulla M Sc
PhD Student

[2]Rajive Kumar MD
Professor

[1]Ved P Choudhry MD
Professor

[1]Renu Saxena MD
Professor and Head

[1]Department of Hematology, [1]Department of Hematology All India Institute of Medical Sciences, [2]Laboratory Oncology, DR BRA IRCH, New Delhi, India, [3]Division of Hematology, U.S. Food and Drug Administration, NIH, Bethesda, MD 20892. USA

Running title: Identifying novel mutations in GT

Correspondence to:

Prof. (Dr). Renu Saxena, MD,
Director, Department of Hematology,
All India Institute of Medical Sciences,
IRCH Building 1st Floor, Ansari Nagar,
New Delhi-110 029. India.
Email: profrsaxena@gmail.com
Tel: +91-11-2659-4670
Fax: +91-11-2658 8663

Abstract:

Glanzmann's thrombasthenia (GT) is an autosomal recessive inherited platelet function defect that characterized by reduction in, or absence of, platelet aggregation in response to multiple physiologic agonists. GT is characterized by normal platelet count, prolonged bleeding time, and abnormal clot retraction. The defect is caused by mutations in the genes encoding GPIIb or GPIIIa that result in qualitative or quantitative abnormalities of the platelet membrane GPIIb/IIIa. GT occurs in high frequency in certain ethnic populations with an increased incidence of consanguinity, such as Indians, Iranians, Iraqi Jews, Palastinian and Jordanian Arabs and French gypsies. Forty-five unrelated patients of GT were enrolled in the study to identify the causative molecular defects and also to correlate the genotype with the phenotype. Molecular modeling was performed for the novel missense mutations. The current study identifies 22 novel mutations in these patients. Missense mutations were identified as the defects responsible for most of the GT patients (59%). Even though missense was common, the study concludes that the genetic defect is heterogeneous in nature and difficult to design a DNA marker for carrier detection in GT.

Key words: Glanzmann's thrombasthenia, Novel mutations, molecular genetics

Introduction:

Glanzmann's thrombasthenia (GT) is a hereditary platelet function defect, characterized by normal platelet count, prolonged bleeding time, and abnormal clot retraction. GT is due to severe reduction in, or absence of, platelet aggregation in response to multiple physiologic agonists because of abnormalities of platelet glycoprotein GPIIb and/or GPIIIa. The primary function of GPIIb/IIIa is to mediate platelet aggregation by acting as receptors for fibrinogen, fibrin, VWF, fibronectin and vitronectin, although fibrinogen is the predominant ligand in this function. Based on the presence of GPIIb/IIIa on platelets, GT is classified in to three types; Type I, II and III. The clinical complications in GT include lifelong bleeding with easy bruising, epistaxis, menorrhagia and gastrointestinal bleeding. GT occurs in high frequency in certain ethnic populations with an increased incidence of consanguinity, such as Indians, Iranians, Iraqi Jews, Palastinian and Jordanian Arabs and French gypsies.

GT is caused by mutations in the genes encoding GPIIb or GPIIIa that result in qualitative or quantitative abnormalities of the platelet membrane proteins[1,2]. GPIIb and IIIa 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 different types of mutations identified in both the genes are missense mutations, nonsense mutations, deletions and insertions either leading to alternative splicing and/or premature termination of translation. For the detection of mutations affecting GPIIb and GPIIIa genes, a number of screening methods like single stranded conformation polymorphism (SSCP), conformation sensitive gel electrophoresis (CSGE), denaturing gradient gel electrophoresis (DGGE) is being used[3]. Of these, CSGE is the sensitive

mutation screening technique. The novel mutations identified are generally analyzed further by expression studies or molecular modeling to evaluate the effect of these mutations on GT phenotype. 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. It would also help in pre-implantation genetics and in enrolling patients for gene therapy. Carrier detection and genetic counseling have become an important and integrated part of comprehensive care of most of the bleeding disorders. In the current report we are describing the mutation spectrum in patients with GT.

Materials and Methods:

***Study Subjects*:** Glanzmann's thrombasthenia patients presenting at the hematology clinic of All India Institute of Medical Sciences (AIIMS), New Delhi, India were the subjects of the study. These patients were diagnosed on the basis of clinical and hematological parameters. Informed consent from patients 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 ADP, ADR, AA and collagen, prolonged bleeding time (BT), absent or reduced clot retraction (CR), normal platelet count (>1.5 lac/mm^3), 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 was recorded at the time of enrollment.

Platelet receptor GPIIb/IIIa protein analysis: Whole blood was collected in 2% EDTA from patients and from normal healthy controls. Platelet receptor GPIIb/IIIa protein was analyzed first by flow cytometry followed by western blot technique. Flow cytometry characterized the GT patients in to types I, II and III. Western blot allowed measuring the presence of GPIIb and GPIIIa protein in the platelet lysates. For flow cytometry platelets were incubated with FITC conjugated monoclonal antibodies CD41, CD61 and negative control antibody (DAKO, Produktionsvej 42, Denmark), for 20 minutes at RT. Non platelet particles were removed by lysis solution. Samples were processed within an hour in flow cytometry (Beckman Coulter, Switzerland). Normal control was run along with the patients' samples. For western blot, platelet lysate was prepared using lysis solution (3% Sodium dodecyl sulphate (SDS) and 6mM N-Ethyl maleimide) and equal amount of protein (5 μg) was separated on SDS PAGE under reducing and non reducing conditions for GPIIb and GPIIIa respectively. Membrane was incubated with CD41 (anti-GPIIb) and CD61 (anti-GPIIIa) antibodies, followed by chemiluminescence detection, which is considered to be a higher sensitive method of protein detection. α-Tubulin antibody was used in each blot as an internal control.

Molecular studies: For mutation analysis of GPIIb and GPIIIa genes, DNA extraction from whole blood, polymerase chain reaction (PCR), heteroduplex PCR followed by Conformation sensitive gel electrophoresis (CSGE) and sequencing were performed. All the 30 exons of GPIIb gene and 15 exons of GPIIIa gene along with the promotor were analyzed by CSGE following heteroduplex PCR. In each gel a normal control was

loaded and patient's' band pattern was always compared with normal band pattern. For novel missense mutations, SVM score was calculated using sequence homology and conservation to look for the deleterious effect of the mutations. Negative score represents deleterious effect of the mutation. All the mutations were looked for sequence conservation in 4 species namely Human, pig, murine and canine.

Molecular modeling for novel mutations identified: Molecular modeling was done for some of the novel missense mutations identified in both GPIIb and GPIIIa gene. A set of models for the potential MIDAS domain of GPIIIa and β propeller domain of GPIIb were performed based on homology modeling. The sequence of the GPIIb (PDB: 1jx5) and GPIIIa (PDB: 1txv) obtained was submitted to the SWISS-MODEL repository[4] at ExPASY for homology modeling. The coordinates so obtained were viewed using the program PyMOL.

Results:

Forty- five GT patients included 25 males and 20 females of age ranging from 5 months to 46 years. The clinical manifestations included epistaxis in 36 patients, Gum bleeding in 31 patients, petechiae in 29 patients and Ecchymotic spots in 21 patients. The major bleeding complications of hematuria and gastro-intestinal bleed were seen in 3 patients and 8 patients respectively. Other bleeding complications like eye bleed, bleeding during circumcision and tooth extraction was seen in 6 patients. 14 patients had prolonged bleeding after trauma; most of these 14 patients had severe bleeding even from minor cuts. Of 20 female GT patients, 12 were adults and 8 were too small to attain menarche. Menorrhagia was seen in 10 out of 12 female patients who attained the menarche (Fig 1). Among the 45 GT patients, a blood or platelet transfusion was

required in 36 patients; of whom 15 patients had multiple transfusions (more than once) and 21 patients had transfusion once in their life time.

Laboratory parameters including platelet aggregation

Hemogram profile of platelet count and platelet size showed normal in all the patients. The peripheral smear showed isolated, non aggregated platelets under microscope. A clinical evaluation by bleeding time (BT), showed prolonged BT of more than 15' in 39 patients and moderate BT in 4 patients. BT was not done 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. 5 patients showed moderate clot retraction (>5%) and these patients were clinically mild. All the GT patients had normal PT and APTT. Subsequent platelet function study by platelet aggregation revealed absent or reduced aggregation with ADP, ADR, AA and collagen and normal aggregation with ristocetin.

Analysis of GPIIb and GPIIIa receptor proteins

The surface expression of platelet membrane glycoprotein GPIIb/IIIa was analyzed by flow cytometry. The GPIIb/IIIa expression by flow cytometry in normal healthy individuals varied from 56.32% to 77.3%. In the patients with GT, the surface expression of GPIIb/IIIa was less than 5% in 28 patients; from 5% to 20% in 7 patients and from 20% to near normal expression in 10 patients. These patients were classified as types I, II and III respectively (Table 1). The bar diagram shows the comparison of αIIbβ3 expression in different types of GT in India with west (Fig 2).

Western blot showed complete absence of GPIIb protein in 24 patients (Fig 3). A trace amount including mild amount of GPIIb was seen in 16 GT patients; three patients showed reduced GPIIb and two patients showed abnormal band pattern of GPIIb when

compare with normal healthy individuals. GPIIIa was completely absent, no traces, in 24 GT patients. A trace amount including mild amount of GPIIIa was seen in 14 GT patients; three patients showed reduced GPIIIa and 4 patients were normal for GPIIIa. None of the patients had abnormal fragment of GPIIIa.

Genotypic analysis of GPIIb and GPIIIa genes

All the 45 GT patients were subjected to mutation screening by CSGE. 4 out of 45 GT patients did not show band shift in any of the exons of the GPIIb and GPIIIa gene. DNA sequencing revealed nucleotide changes (either mutation or polymorphism), when compared to the GenBank 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 (Fig 4a-c). Of these, 22 patients (48.8%) showed defect in GPIIb gene and 14 patients (31.1%) showed defect in GPIIIa gene. In 9 out of 45 (20%) GT patients, no gene alterations were identified. Thirty one different mutations have been identified in 36 patients, of which 22 mutations were novel. Of the 31 different mutations, 17 were identified in GPIIb gene and 14 were identified in GPIIIa gene. The mutation nomenclature was according to published reports[5,6].

Mutations in GPIIb gene

Seventeen different mutations (a total of 29 GPIIb mutations, including compound heterozygous and homozygous) were identified in 22 GT patients. Of 29 GPIIb mutations, 16 were missense, 4 were deletions, 5 were insertions and 4 were splice site mutations. Six different missense mutations were identified in 16 patients. Of these, 3 mutations were previously reported. SVM score was calculated using sequence homology and conservation. Negative score represents deleterious effect of the mutation.

All the mutations were conserved in 4 species (Human, pig, murine and canine) (Table 2). Of the 7 insertions/deletions found in GPIIb gene of GT patients, 3 were deletions, 3 were insertions and one was duplication. Table 3 represents GT patients with insertions and deletions. Of these 7 insertions/deletions, 6 were novel mutations and only one insertion was previously reported[7]. Four splice site variations have been identified in GPIIb gene, of which two were previously reported (Table 4).

Mutations in GPIIIa gene

Fourteen different mutations (a total of 15 GPIIIa mutations, including compound heterozygous and homozygous) were identified in 14 unrelated GT patients. Of 15 GPIIIa mutations, 10 were missense, 4 were deletions and 1 was indel (insertion-deletion) mutation. Ten different missense mutations were identified in 10 patients, of these 7 were novel and 3 were previously reported. Of the 4 deletions/insertions found in GPIIIa gene of GT patients, 3 were deletions and one was indel mutation (insertion-deletion). None of these mutations (Deletions and insertions) were previously reported.

Genotype and phenotype correlation:

Of all the mutations identified missense were the most common cause of GT (59%). The missense mutations were distributed among the various types of GT, as follows; Type I (58%), type II (57.1%) and variant type (66.6%). Of the 8 deletions identified, 6 (75%) resulted in type I GT and 2 (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 i.e. type I GT. Splice site mutations were equally represented in both type I and variant type GT (Table 5).

Novel mutations: Of the 31 different mutations, 17 were identified in GPIIb gene and 14 were identified in GPIIIa gene. A total of 22 mutations were not described earlier in mutation database and hence were novel. Of these 22 novel mutations, 10 were missense mutations, 10 were deletions/insertions and 2 were splice site mutations. Table 6 shows the novel mutations obtained in this current study.

Patients with no mutations in GPIIb and GPIIIa genes

In 9 out of 45 (20%) GT patients, no causative gene alterations were identified in either GPIIb gene or GPIIIa gene. Of these, 4 patients were born to consanguineous parents. A total of 5 patients had family history of bleeding; that included death episode, due to prolonged bleeding in all four families and bleeding complication in sibling in one family. The clinical manifestations, Hemogram profile and protein analysis is mentioned in the table 8. Based on flow cytometry, 5 patients were classified as type I, 3 were type III and one was type II. Western blot analysis revealed complete absent of both GPIIb and GPIIIa in 6 patients. In remaining 3 patients, one had trace amount of GPIIb and reduced amount of GPIIIa; another had mild amount of GPIIIa with no trace of GPIIb, the third patient had abnormal GPIIb and normal GPIII protein. DNA analysis showed polymorphisms in 5 out of 9 patients in either GPIIb or GPIIIa genes. However, 4 patients did not show any sequence variation.

Molecular modeling for novel mutations

Molecular modeling was done for some of the novel missense mutations identified in both GPIIb and GPIIIa gene. Modeling showed the exact region of amino acid change on proteins caused by mutations (Fig 5-6). Molecular modeling of a novel missense mutation of GPIIb gene (Ala313Thr) and four novel missense mutations

(Leu318Ser, Tyr344Cys, Asp139His, and Cys547Trp) of GPIIIa gene revealed the mechanism by which the protein becomes non- functional. Table 7 describes the novel missense mutations and its effect as explained by molecular modeling.

Discussion:

The clinical manifestations in GT observed in these patients were purpura, ecchymotic spots, epistaxis, Gum bleeding, gastrointestinal hemorrhage, hematuria, menorrhagia, and other bleeding like eye bleed. Of these, epistaxis was most commonly seen (80%) followed by gum bleeding (68.8%). Platelet GPIIb/IIIa analysis by flow cytometry revealed 62.2%, 15.5% and 22.2% of GT to have types I, II and III respectively. Various reports support the findings of current study, where type I was the common type of GT. In a study on 64 GT patients from Paris, 50 were type I (78%) followed by type II and type III seen in 14% and 8% respectively (George et al 1990). Previous report on subtypes of GT revealed type I to be the commonest subtype in North Indians[8]. Western blot analysis for platelet GPIIb and GPIIIa, in the current study, revealed no detectable GPIIb or GPIIIa in 53.3% of the patients. Very trace amount including mild presence of GPIIb and GPIIIa were seen in 35.5% and 31.1% respectively. About 6.6% patients had reduced GPIIb and GPIIIa. Abnormal fragment of GPIIb was seen in two patients (4.4%) who showed mutation in GPIIb gene. These two patients along with two additional GT patients had normal GPIIIa protein by western blot. Type I patients had either absent or severely reduced both GPIIb and GPIIIa protein. A severely reduced equal amount of pro-GPIIb and mature GPIIb was seen in 2 of type I patients. A published report revealed that residual amounts of GPIIb and GPIIIa could usually be detected even in type I patients[9,10]. It has also been mentioned that in type I patients, the

amount of GPIIIa was up to 10% of normal, whereas the amount of GPIIb was less than 3% and in some cases the GPIIb is often not detected. In the present study, absent GPIIb was seen in 2 of the 7 type II patients and absent GPIIIa was seen in 4 patients. Rest of the type II patients had either trace or mild protein. Of the 10 cases of type III, only 2 patients were absent for both GPIIb and GPIIIa. The normal amount of GPIIIa in 4 patients of type III revealed the presence of dysfunctional receptors. Literature reports that in some of the variant GT, though the number of GPIIb/IIIa is normal, they are functionally abnormal as shown by the absence of platelet aggregation.

Sequencing revealed mutations in 36 of 45 (80%) unrelated GT patients. Of these, 48.8% patients had GPIIb gene defect and 31.1% patients had GPIIIa gene defect. In 9 out of 45 (20%) GT patients, no gene alterations were identified. These 9 patients though their mutation status were not identified, their hematological tests including platelet aggregation and flow cytometry revealed that they are definite GT.

Defects in GPIIb gene

Seventeen different mutations (a total of 29 GPIIb mutations, including compound heterozygous and homozygous) were identified in 22 unrelated GT patients. Of the total of 29 GPIIb mutations identified, 55.1% were missense mutations, 13.8% were splice site mutations and deletions each. 10.3% and 6.9% of GPIIb 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.

Missense mutations: The missense mutations of the GPIIb gene may lead to a variety of phenotypes from mild to severe. Of the 6 different missense mutations identified in 16

patients, 3 were novel mutations and 3 mutations were reported previously in the database/ literature. The three novel mutations identified in this study are Ala313Thr, Leu343Pro and g.951G>A (promoter region). Ala313Thr change resulted in type I GT phenotype. Molecular modeling revealed that Ala313 is presented within the blade W5 of beta propeller domain. Residues 294 to 314, of beta propeller domain, are potential for ligand binding sites. Because of the amino acid change, there was a hydrogen bond formation between the carboxyl groups of Gly, Leu and Thr. Leu343Pro resulted in type I GT in 4 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 important for the ligand binding.

Five patients carried missense mutations, which have been reported earlier in the database as causative changes. Three published missense mutations (Leu214Pro, Arg358His and Gly412Arg) were seen in 5 GT patients. Leu214Pro was seen in 3 unrelated GT patients and all of them were type III. Earlier report suggested that Leu214Pro mutation disrupts the structural conformation and the ligand binding properties of the heterodimeric complex. In the present study, all the three patients resulted in type III GT phenotype. Arg358His showed trace amount of GPIIb and GPIIIa by western blot, which supports the earlier published study. Gly412Arg was seen in one patient who was type I GT. This mutation is associated with the creation of a stop codon on the other GPIIb allele, thus responsible for the lack of GPIIb/IIIa on the platelet surface[11]. The flow cytometry analysis revealed that the patient was GT type I since he had severely reduced expression GPIIb/IIIa on the platelets.

Deletions and Insertions: Of the 7 insertions/deletions found in GPIIb gene of GT patients, 3 were deletions, 3 were insertions and one was duplication. Of these 7 insertions/deletions, 6 were novel mutations and only one insertion was previously reported. Three different deletions were seen in 4 unrelated GT patients all caused GT type I phenotype. None of them were reported earlier. All the three deletions found were resulted in premature truncated GPIIb protein. Because of the truncated protein, these patients had complete absence of GPIIb protein by western blot analysis and flow cytometry analysis revealed the expression of severely reduced GPIIb/IIIa on the patient's platelets. Clinically these patients were severe and required blood transfusion. One of these patients had severe menorrhagia.

Three insertions were seen in three patients of which two were novel and one was reported earlier. The insertions identified in the current study were c.2674_2675insGA, c.2915_2916insC and c.3117_3118insTGGAG. An insertion c.2674_2675insGA was seen in exon 26 in one patient. The truncated protein resulted in GT type I phenotype and clinically the patient was very severe. The patient had multiple transfusions to stop bleed from nose. The same duplication was seen in another patient who was homozygous for this mutation. The patient was type I GT and had severe gum bleeding and nose bleeding. Clinically the patient was severe and was required blood transfusion during prolonged bleeding from nose. An insertion, c.3117_3118insTGGAG, was seen in one patient who was type I GT and is resulted in frame shift and thus abortion of normal stop codon at the end of the GPIIb protein. The patient had gum bleeding since birth and later developed clinical complication of menorrhagia. A heterozygous insertion c.2915_2916insC was seen in one patient who was type I GT. There is a report

mentioning that InsC results in GT phenotype by affecting transmembrane domain and cytoplasmic tail (Hayashi et al 2005). In addition to this mutation, this patient carried two missense mutations in GPIIb gene. One was c.1028T>C and another was g.951G>A in the promoter region. Clinically the patient was very severe and required blood transfusion during severe menorrhagia. Apart from this, she also had frequent gum bleeding and epistaxis. It may be concluded that the triple heterozygosity was responsible for severe form of type I GT phenotype in this patient.

Splice site mutations: Of the 4 different splice site mutations identified, 2 were novel mutations and 2 were reported previously in the literature. A heterozygous missense variation c.800-2A>T was resulted in aberrant splicing in the GPIIb gene in one patient. In addition to this mutation, the patient had another heterozygous splice site c.1210 (c.1210+4A>G). The patient had severe menorrhagia and she had gum bleeding and epistaxis since childhood. The compound heterozygous splice site mutation presumed to contribute this severe type I GT phenotype. A patient with c.188+8delG mutation, had menorrhagia. A homozygous splice site mutation c.1753-1G>A was identified in type III GT. This mutation was causative as no other gene alterations were identified in this patient. It was reported in the literature that the patient with the heterozygous splice site mutation (c.1753-1G>A) was clinically moderate[12]. Similarly, in the current study the patient with the same mutation but in homozygous state was clinically mild. Western blot and flow cytometry revealed mild expression of GPIIb/IIIa on the platelets. The Thrombogenic polymorphism was looked for in this patient, interestingly, he carried a homozygous HPA-1 polymorphism. The presence of homozygous polymorphism along with this mutation in this patient is of special interest.

Defects in GPIIIa gene

Fourteen different mutations (a total of 15 GPIIIa mutations, including compound heterozygous and homozygous) were identified in 14 unrelated GT patients. Of the 15 GPIIIa mutations, 66.6% were missense and 26.6% were deletions. 6.6% of the mutations were Indel (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.

Missense mutations: The missense mutations of the GPIIIa gene may lead to a variety of phenotypes from mild to severe. Of the 10 different missense mutations identified in 11 patients, 7 were novel mutations and 3 mutations were reported previously in the database/ literature. The 7 novel missense mutations identified in this study are Cys31Tyr, Leu318Ser, Tyr344Cys, Cys547Trp, Leu772Pro, Asp139His and Leu20Pro. Leu318Ser missense mutation resulted in type I GT phenotype. Molecular modeling revealed that Leu318 is presented in the β3 A domain. Because of the mutation, the patricianly charged Serine at 318 reduced the hudrophobic nature of the protein. The patient was clinically mild and showed reduced expression of GPIIb and GPIIIa by western blot. Molecular modeling revealed that Tyr344 was presented in the β3 A domain. Residues 324 to 366 are cysteine rich region and moreover, this region is important for GPIIb/IIIa heterodimer inter-subunit surface interactions. The patient with this mutation had a clinical complication of nose bleed as well as eye bleed. The loss of intersubunit interaction might have role in this kind of clinical bleeding and resulted in GT type I phenotype in this patient. Molecular modeling for Cys547 revealed its presence in the EGF-3 (epidermal growth factor) domain. There is a significant contact

between calf-1 of αIIb and EGF-3 of β_3. Hence, in the current study, it is presumed that the mutation Cys547Trp gives rise to conformational changes that are responsible for retention of the aberrant GPIIb/IIIa complex in the ER. Leu772Pro mutation resulted in type III GT. The conserved NPLY sequence represents a potential beta-turn motif in the β_3 cytoplasmic tail and has been suggested to mediate the interaction of beta(3) integrins with talin[13]. The study demonstrated that the NPLY beta-turn motif regulates post-ligand binding functions of alpha(IIb)beta(3) in a manner independent of talin interaction. Thus variation in the sequence allowed the synthesis of protein but affected the post ligand binding function of GPIIb/IIIa. In the current study, the patient had the clinical complication of menorrhagia and required blood transfusion to stop bleeding. The Leu772Pro at cytoplasmic domain of β_3 resulted in alteration in the ligand binding and raised the type III GT phenotype. Cys31Tyr novel missense mutation was identified in a patient who had severe menorrhagia and flow cytometry revealed the patient was type I GT phenotype. It is presumed that the mutation Cys31Tyr in the PSI domain might be responsible for type I GT phenotype. Leu20Pro novel missense mutation resulted in type I GT in a patient who had gum bleeding. Though the flow cytometry data revealed that the patient was type I GT, he was clinically mild. Asp139His mutation was seen as a heterozygous state in a patient with type I GT phenotype. Molecular modeling revealed that the amino acid Asp139 was presented in the βA domain. Since the mutation was located in the beta-sheet of the protein, it might have deleterious effect on the protein conformation.

The 3 previously reported missense mutations identified in this study are Tyr141Cys, Arg119Gln and Arg242Gln. Tyr141Cys mutation was previously reported by

Wilcox et al in 2000 and was seen as heterozygous state in a patient who also had a novel heterozygous change Asp139His[14]. In the current study, the patient with compound heterozygous mutation was type I GT and had severe nose bleeding and gum bleeding. He needed blood transfusion to stop bleed during prolonged bleeding from nose. The novel Asp139His together with reported Tyr141Cys presumed to contribute the sever phenotype in this patient. Arg119Gln mutation was seen in a type II GT patient. The homozygous Arg119Gln mutation was located within the hybrid domain at the interface with the PSI domain. In the current study, the patient with Arg119Gln mutation had severe menorrhagia and needed blood transfusion to stop bleed. Thus the mutation predicted to cause miscoding of protein and result in severe GT phenotype. Another reported mutation c.752G>A resulted in amino acid change Arg242Gln[15]. The amino acid change was reported to affect calcium binding subunit association that affected intracellular trafficking and surface expression. In the present study, the Arg242Gln mutation was seen in a type III GT patient. The patient was 8 month old child who had gum bleeding and nose bleeding. Though the patient had reduced expression of GPIIb/IIIa on the platelets, he was clinically very severe and required blood transfusion to stop bleed. It is postulated that the mutation Arg242Gln in the calcium binding regions affected the function of the protein.

Deletions: A total of 3 deletions were seen in 4 unrelated GT patients. None of these deletions were previously reported. A novel deletion c.674delA was seen in one patient who was found to be type I phenotype by flow cytometry. The patient had severe gum bleeding and epistaxis and blood transfusion was required to stop the bleed. It is presumed that the novel deletion if single base pair resulted in severe GT phenotype. A

c.2217delC was found in one patient that resulted in type I GT phenotype. Though the flow cytometry revealed the patient to be type I, he was clinically mild and had gum bleeding and nose bleeding. Western blot revealed the presence of equal amount of trace amount of pro GPIIb and matured GPIIb. A 15bp deletion c.887_901delACGGGCAGTGTCATG was identified in two patients and is resulted in deletion of 5 amino acids DGQCH from codon 296 to 300. The deletion was presented in the β3A domain. Both the patients resulted in type II GT phenotype and both of them had similar clinical complications of gum bleeding and epistaxis. One indel mutation c.155_156delGCinsTT was identified in one patient that resulted in type II GT. The patient was clinically severe and was required blood transfusion.

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, as follows; Type I (58%), type II (57.1%) and Variant type (66.6%). In the literature, majority of the missense mutations (50%) were reported to be responsible for GT (Mitchell et al 2003). The current study revealed that the missense mutations in Indian patients with GT were more common (59%) when compare to that of west (50%). Of the 8 deletions identified in the current study, 6 (75%) resulted in type I GT phenotype and 2 (25%) resulted in Type II GT phenotype whereas none of the deletions were seen in Variant type (non severe type of GT). Published reports stated that deletion mutations that raised Type I GT resulted in premature truncation of GPIIb or GPIIIa; moreover single base deletions that resulted in single amino acid changes in areas critical for normal subunit stability and processing[16-18]. In the current study, insertions

and duplications were seen only in type I GT, suggesting that these genotype variations causes severe phenotype of GT. Maximums of insertion mutations resulted in premature or non- functional αIIb protein. None of the type III patients had deletions or insertions. Splice site mutations were equally represented in both type I GT (severe) and variant type GT (non- severe).

Unexpectedly, 9 out of 45 (20%) from our cohort, no gene alterations could be identified in the coding as well as the exon-flanking region in either GPIIb gene or GPIIIa gene. Previous two studies could not find any mutation in 20% of the diagnosed GT patients[19,11]. It is evident from the above studies that GPIIb or GPIIIa mutation may not be present in about 20% of the patients with GT. GT patients with no possible causative mutations were may be due to defect in a regulatory element affecting the transcription of these two genes[20]. On the other hand, abnormalities in mechanisms that are responsible for post-translational modifications and trafficking of integrin subunits may account for some cases of GT. A recent study on Indian GT patients, reported that no causative mutation in either GPIIb or GPIIIa gene was identified in 27% of the patients[21].

In conclusion the present study identifies the causal mutations that included 22 novel mutations 80% of the patients. Missense mutations were identified as the defects responsible for most of the GT patients (59%). The remaining mutations were heterogeneous and were distributed throughout the length of the gene. Even though missense was common, the study concludes that the genetic defect is heterogeneous in nature and difficult to design a DNA marker for carrier detection in GT.

Acknowledgments:

We express our sincere gratitude to Dr. Barry S. Coller, Head, Laboratory of Blood and Vascular Biology, The Rockefeller University, New York for his great help to complete this study. We also wish to acknowledge our technical staffs Mr. S. Dutta, Mr. Suresh, Mr Sha alam and Mr. Chander for their support.

References

1. George JN, Caen JP, Nurden AT. 1990. Glanzmann's thrombasthenia: the spectrum of clinical disease. Blood. 75(7):1383-95.
2. French DL. 1998. The molecular genetics of Glanzmann's thrombasthenia. Platelets. 9; 5- 20.
3. Kannan M, Ahmad F, Kumar R, Choudhry VP, Saxena R. 2006. Use of CSGE, TspRI- RFLP and Western Blot in Carrier Detection in an Indian Family with Type I Glanzmann Thrombasthenia. Blood. 108 (11) 3975a.
4. Schwede T, Kopp J, Guex N, Peitsch MC. SWISS-MODEL: An automated protein homology-modeling server. Nucleic Acids Res. 2003 Jul 1;31(13):3381-5.
5. den Dunnen JT, Antonarakis SE. 2000. Mutation nomenclature extensions and suggestions to describe complex mutations: a discussion. Hum Mutat. 15(1):7-12. Erratum in: Hum Mutat 2002 Nov;20(5):403.
6. Beutler E, McKusick VA, Motulsky AG, Scriver CR, Hutchinson F. 1996. Mutation nomenclature: nicknames, systematic names, and unique identifiers. Hum Mutat. 8(3):203-6.
7. Hayashi T, Tanaka S, Hori Y, Terada C, Ueda Y, Tani Y. 2005. Insertion of a C in the exon 28 of integrin alphaIIb gene leading to a frameshift mutation is responsible for Glanzmann thrombasthenia in a Japanese case. J Thromb Haemost. 3(3):489-96.
8. Kannan M, Ahmed RP, Jain P, Kumar R, Choudhry VP, Saxena R. 2003. Type I Glanzmann thrombasthenia: most common subtypes in North Indians. Am J Hematol. 74(2):139-41.
9. Nurden AT, Didry D, Kieffer N, McEver RP. 1985. Residual amounts of glycoproteins IIb and IIIa may be present in the platelets of most patients with Glanzmann's thrombasthenia. Blood. 65(4):1021-4.
10. Coller BS, Seligsohn U, Little PA. 1987. Type I Glanzmann thrombasthenia patients from the Iraqi-Jewish and Arab populations in Israel can be differentiated by platelet glycoprotein IIIa immunoblot analysis. Blood. 69(6):1696-703.

11. Vinciguerra C, Bordet JC, Beaune G, Grenier C, Dechavanne M, Negrier C. Description of 10 new mutations in platelet glycoprotein IIb (alphaIIb) and glycoprotein IIIa (beta3) genes. Platelets. 2001 Dec;12(8):486-95.
12. Tadokoro S, Tomiyama Y, Honda S, Arai M, Yamamoto N, Shiraga M, Kosugi S, Kanakura Y, Kurata Y, Matsuzawa Y. A Gln747-->Pro substitution in the IIb subunit is responsible for a moderate IIbbeta3 deficiency in Glanzmann thrombasthenia. Blood. 1998 Oct 15;92(8):2750-8.
13. Patil S, Jedsadayanmata A, Wencel-Drake JD, Wang W, Knezevic I, Lam SC. 1999. Identification of a talin-binding site in the integrin beta(3) subunit distinct from the NPLY regulatory motif of post-ligand binding functions. The talin n-terminal head domain interacts with the membrane-proximal region of the beta(3) cytoplasmic tail. J Biol Chem. 274(40):28575-83.
14. Wilcox DA, Olsen JC, Ishizawa L, Bray PF, French DL, Steeber DA, Bell WR, Griffith M, White GC 2nd. Megakaryocyte-targeted synthesis of the integrin beta(3)-subunit results in the phenotypic correction of Glanzmann thrombasthenia. Blood. 2000 Jun 15;95(12):3645-51.
15. Newman PJ, Weyerbusch-Bottum S, Visentin GP, Gidwitz S, White GC II. 1993a. Type II Glanzmann Thrombasthenia due to a destabilizing amino acid substitution in platelet membrane glycoprotein IIIa. Thromb Haemost 69:1017.
16. Basani RB, French DL, Vilaire G, Brown DL, Chen F, Coller BS, Derrick JM,Gartner TK, Bennett JS, Poncz M. 2000. A naturally occurring mutation near the amino terminus of alphaIIb defines a new region involved in ligand binding to alphaIIbbeta3. Blood. 95(1):180-8.
17. French DL, Coller BS. 1997. Hematologically important mutations: Glanzmann thrombasthenia. Blood Cells Mol Dis. 23(1):39-51.
18. Peyruchaud O, Nurden AT, Milet S, Macchi L, Pannochia A, Bray PF, Kieffer N, Bourre F. 1998. R to Q amino acid substitution in the GFFKR sequence of the cytoplasmic domain of the integrin IIb subunit in a patient with a Glanzmann's thrombasthenia-like syndrome. Blood. 92(11):4178-87.
19. D'Andrea G, Colaizzo D, Vecchione G, Grandone E, Di Minno G, Margaglione M; GLAnzmann's Thrombasthenia Italian Team (GLATIT). 2002. Glanzmann's

thrombasthenia: identification of 19 new mutations in 30 patients.Thromb Haemost. 87(6):1034-42.

20. Bray PF, Rosa JP, Lingappa VR, Kan YW, McEver RP, Shuman MA. 1986. Biogenesis of the platelet receptor for fibrinogen: evidence for separate precursors for glycoproteins IIb and IIIa. Proc Natl Acad Sci U S A. 83(5):1480-4.
21. Nelson EJ, Nair SC, Peretz H, Coller BS, Seligsohn U, Chandy M, Srivastava A. 2006. Diversity of Glanzmann thrombasthenia in southern India: 10 novel mutations identified among 15 unrelated patients. J Thromb Haemost. 4(8):1730-7.