Glanzmann thrombasthenia in Pakistan: molecular analysis and identification of novel mutations

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Accessibility
Glanzmann thrombasthenia in Pakistan: molecular analysis and identification of novel mutations

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Glanzmann thrombasthenia (GT) is an inherited genetic disorder affecting platelets, which is characterized by spontaneous mucocutaneous bleeding and abnormally prolonged bleeding in response to injury or trauma. The underlying defect is failure of platelet aggregation due to qualitative and/or quantitative deficiency of platelet integrin αIIbβ3 resulting from molecular genetic defects in either ITGA2B or ITGB3. Here, we examine a Pakistani cohort of 15 patients with clinical symptoms of GT who underwent laboratory and molecular genetic analysis. In patients with a broad range of disease severity and age of presentation, we identified pathogenic mutations in ITGA2B in 11 patients from 8 different families, including 2 novel homozygous mutations and 1 novel heterozygous mutation. Mutations in ITGB3 were identified in 4 patients from 3 families, two of which were novel homozygous truncating mutations. A molecular genetic diagnosis was established in 11 families with GT, including 5 novel mutations extending the spectrum of mutations in this disease within a region of the world where little is known about the incidence of GT. Mutational analysis is a key component of a complete diagnosis of GT and allows appropriate management and screening of other family members to be performed.

Conflict of interest

The authors declare no conflict of interest.

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Key words: bleeding disorder – consanguineous – integrin – ITGA2B – ITGB3 – Pakistan

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Glanzmann thrombasthenia (GT) is a rare mucocutaneous bleeding disorder with an autosomal recessive inheritance, first identified by Eduard Glanzmann in 1918 (1). Symptoms of spontaneous bleeding vary in frequency and intensity among GT patients (2). Frequently, petechiae, purpura and easy bruising are observable features at birth (3), allowing an early clinical diagnosis. Recurrent epistaxis, gingival bleeding and menorrhagia (in females of child-bearing age) are common symptoms while gastrointestinal bleeding, intracranial hemorrhage and hematuria have a lower incidental rate (2). In patients with GT, there is an increased bleeding risk after surgery and even dental procedures. Increased bleeding following accidents and trauma may also create major problems (4). Typically, the platelet count is normal (5) and platelet size remains normal in most patients, although cases with moderate thrombocytopenia and enlarged platelets have been reported (6). The combination of mucocutaneous bleeding, an absence of platelet aggregation and abnormal clot retraction associated with a normal platelet count and morphology, is diagnostic for GT (7).

Mutations in ITGA2B or ITGB3 may lead to GT type I or type II, where the expression of integrin αIIbβ3 at the platelet surface is reduced (respectively less than 5% and 20% of normal). In contrast, in variant form of GT there is a functional defect of the integrin αIIbβ3 complex (8). Both ITGA2B and ITGB3 are located on chromosome 17 and encode the platelet integrin αIIbβ3, previously known as glycoprotein Ib/IIa. Platelet aggregation is mediated by integrin αIIbβ3, an essential receptor for platelets. Normally, during platelet activation, the integrin αIIbβ3 binds fibrinogen, which leads to platelet aggregation (9). In GT, the process of thrombus formation fails and clot retraction is also affected (9).

In patients suspected of GT, a deficiency of integrin αIIbβ3 can be confirmed using flow cytometry or western blotting with monoclonal antibodies that recognize either the αIIb, or β3 subunits or the αIIbβ3 complex (2). Genetic analysis allows a definitive confirmation of diagnosis. Both ITGA2B and ITGB3 are polymorphic and are susceptible to germline mutations which occur more frequently in ITGA2B.

GT is a rare disease, occurring at a ratio of approximately 1:1,000,000, however it is more prevalent in certain ethnic groups (10). In this study, we analyze a cohort of 15 patients from Pakistan (from 11 different families) with GT in whom we have made a molecular genetic diagnosis. We found three novel mutations in ITGA2B and two novel mutations in ITGB3 extending the molecular diagnosis in this previously uncharacterized population.

**Materials and methods**

**Patients and families**

A total of 15 subjects with GT from 11 different families were investigated in the study. Informed consent was obtained from all patients and relatives according to a protocol approved by the National Institute of Blood Disease & Bone Marrow Transplantation, Karachi, Pakistan. Medical histories of patients were recorded in a questionnaire, which included age, gender, age at onset, consanguineous marriage between parents, a family history of bleeding, severity of bleeding and hemorrhagic clinical symptoms.

**Hematological and molecular analysis**

GT patients were screened based on platelet counts and morphology. Platelet aggregation tests were performed on a Helena AggRAM (Helena Laboratories, Beaumont, Texas, USA) using ristocetin, epinephrine, adenosine diphosphate (ADP) and collagen. Bleeding time (BT) measurements were performed and compared with reference values: 0–4 years, 4 ± 1 min; boys >4 years, 5 ± 1 min; girls >4 years, 5.5 ± 1 min. Genomic DNA was extracted from peripheral blood leukocytes isolated from whole blood obtained from patients and (where available) parents. Exon polymerase chain reaction (PCR) was used to amplify all coding regions of the ITGA2B and ITGB3 genes (using reference sequences NM_000419 and NM_000212, respectively). PCR products were purified and Sanger sequenced. Segregation using parental samples was undertaken when available. MutationTaster™ (http://www.mutationtaster.org/) and PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/index.shtml) were used to determine pathogenicity of novel mutations. The crystal structure of the complete ectodomain of integrin αIIbβ3 (PDB code 3FCS) (11, 12) was visualized using PyMOL (http://pymol.org/) to identify the position of the missense mutations identified in the present study.

**Results**

**Clinical and laboratory findings**

A total of 15 GT patients from 11 consanguineous Pakistan families were evaluated in this study (Table 1). The age of the patients ranged from 1 to 16 years with an age of disease onset ranging from 7 days to 4 years (mean age of onset of 2 years). Fifty percentage of patients had a positive family history of a bleeding disorder. Common clinical symptoms included recurrent epistaxis and gingival bleeding (73%), and easy bruising (67%) (Table 1). Gastrointestinal hemorrhage (four cases), petechiae and purpura (two cases) and hemathrosis (one case) were rare. BT was prolonged in all of our patients and was often over 10 min, while activated partial thromboplastin time (APTT) and prothrombin time (PT) remained within normal range in 14 (93%) and 13 (87%) patients, respectively. Platelet aggregation assays for all cases measured were defective when using ADP, adrenaline, collagen and arachidonic acid. These findings are typical of GT, as platelet aggregation tends to be defective with all agonists. The response to ristocetin was maintained in 13 of 14 patients tested and platelet count was within normal limits for all patients except one (Table 1).

**Genetic findings**

We identified 7 different mutations in ITGA2B in 11 patients (from 8 families). Three of these
Table 1. Clinical and laboratory findings in the GT cohort

| Patient ID | A | B | C | D | E | F | G | H | I | J | K | L | M | N | O |
|------------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Family number | 1 | 2 | 3 | 3 | 4 | 5 | 6 | 7 | 8 | 8 | 8 | 9 | 9 | 10 | 11 |
| Age (years), sex | 6, M | 16, M | 12, M | 10, M | 1, F | 16, F | 1, F | 8, F | 3, F | 5, F | 5, M | 10, F | 15, F | 7, M | 7, F |
| Age at onset | 4.5 y | 5 d | 2 y | 1 y | 3 m | 12 y | 6 m | 6 m | 3 m | 6 m | 7 d | 4 y | 4 y | 1 y | 1 y |

Clinical symptoms

- Petechiae and purpura
- Easy bruising
- Recurrent epistaxis
- Gingival bleeding
- Gastrointestinal bleeding
- Intracranial hemorrhage
- Hematuria
- Hemorrhage after dental
- Hemorrhage after trauma
- Ear bleeding
- Hemarthrosis
- Hematemesis
- Hematoma

Bleeding time (min) (for NR see Methods)

| Patient ID | A | B | C | D | E | F | G | H | I | J | K | L | M | N | O |
|------------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Platelet aggregation assay | ADP | X | X | N/A | X | X | X | X | X | X | X | X | X | X | X |
| ADP | Adrenaline | X | X | X | N/A | X | X | X | X | X | X | X | X | X | X |
| Collagen | X | X | X | N/A | X | X | X | X | X | X | X | X | X | X | X |
| Arachidonic acid | X | X | N/A | N/A | X | X | X | X | X | X | X | X | X | X | X |
| Ristocetin | N | N | N | N/A | N | N | N | N | N | N | N | N | N | N | N |

Hematological evaluation

| Patient ID | A | B | C | D | E | F | G | H | I | J | K | L | M | N | O |
|------------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Platelet count (x 10⁹/l) (NR 150–450) | 371 | 205 | 156 | 150 | 212 | 235 | 407 | 312 | 212 | 129 | 272 | 225 | 199 | 250 | 301 |
| Red blood cell count (x 10⁶/ml) | 3.9 | 3.7 | 3.4 | 3.7 | 3.2 | 2.5 | 4.12 | 3.93 | 3.69 | 3.1 | 3.57 | 3.2 | 3.5 | 2.6 | 3.8 |
| Family history of bleeding | + | + | N/A | + | + | + | + | + | + | + |

+, present; −, absent; ADP, adenosine diphosphate; d, day; F, female; GT, Glanzmann thrombasthenia; m, month; M, male; min, minutes; N, normal; N/A, not available; NR, Normal range; s, second; X, defective; y, year. Abnormal results are shown in bold.

mutations in this gene (p.N145K, p.S477Gfs*111 and p.N536Kfs*29) were novel (Table 2) and were not found on database searches including 1000 Genomes (http://www.1000genomes.org/), Exome Variant Server (http://evs.gs.washington.edu/EVS/) and the GT database (http://sinaicentral.mssm.edu/intranet/research/glanzmann/menu). One patient (A, Family 1) had compound heterozygous mutations which included the novel missense change p.N145K together with a nonsense mutation, p.C177* previously reported by Jallu et al. (in its homozygous state) in a GT patient from China (13). Unfortunately, parental samples were unavailable to confirm segregation, but given the negative family history of GT in this family, it is possible that one of these mutations may have occurred de novo. The remaining patients had homozygous mutations, in keeping with known consanguinity. Mutations included p.L214R, which has been previously reported and investigated in terms of its pathogenicity (14). The nonsense mutation p.Y411* found in two siblings of Family 3 and is predicted to affect the integrin beta subunit (Fig. 1, Table 2). All mutations in ITGB3 were homozygous and two mutations were novel nonsense mutations (p.C258* and p.Q694*) not found in database searches. The L143W mutation in ITGB3 was found in two patients, and has been previously described in a patient also from Pakistan (17) and in two patients from India (18).

Discussion

GT is an autosomal recessively inherited platelet function disorder that appears to be more common in areas with high consanguinity rate. Consanguineous unions are commonly seen among Pakistanis, as in other ethnic
| Patient ID | A | B | C | D | E | F | G | H | I | J | K | L | M | N | O |
|------------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Family number | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
| Gene | ITGA2B | ITGA2B | ITGA2B | ITGA2B | ITGA2B | ITGA2B | ITGA2B | ITGA2B | ITGA2B | ITGA2B | ITGA2B | ITGA2B | ITGB3 | ITGB3 | ITGB3 |
| Homozygous/ Heterozygous | Homo | Homo | Homo | Homo | Homo | Homo | Homo | Homo | Homo | Homo | Homo | Homo | Homo | Homo | Homo |
| Nucleotide change | c.435C>A | c.641T>G | c.1265C>T | c.1265C>T | c.1460insAGGT | p.N145K | p.L214R | p.Y411C | p.Y411C | p.N536Kfs*29 | p.R551Q | p.R551Q | p.R551Q | p.R551Q | p.L143W |
| Amino acid change | p.N145K/p.C177* | p.L214R | p.Y411C/p.Y411C | p.Y411C/p.Y411C | p.N536Kfs*29 | p.R551Q | p.R551Q | p.R551Q | p.R551Q | p.R551Q | p.R551Q | p.R551Q | p.R551Q | p.R551Q | p.L143W |
| Parental consanguinity | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Segregation | N/A | N/A | + | + | + | + | N/A | N/A | + | + | + | + | + | + | N/A |
| Novel/reference | Novel/Jallu et al. (13) | N/A | Michel et al. (15) | + | + | + | Novel/Jallu et al. (13) | N/A | + | + | + | + | + | + | N/A |
| MutationTaster (score) | (0.999) | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A |
| PolyPhen2 (score) | (1.000) | (1.000) | (1.000) | (1.000) | (1.000) | (1.000) | (1.000) | (1.000) | (1.000) | (1.000) | (1.000) | (1.000) | (1.000) | (1.000) | (1.000) |
| Location of mutation within protein domains | Within beta propeller repeat | Within beta propeller repeat | Within beta propeller repeat | Within Thigh domain | Within Thigh domain | Within Thigh domain | Within Thigh domain | Within Thigh domain | Within Thigh domain | Within Thigh domain | Within Thigh domain | Within Thigh domain | Within integrin beta subunit | Within integrin beta subunit | Within integrin beta subunit |

GT, Glanzmann thrombasthenia; N/A, not available.

*Novel nucleotide changes, amino acid changes, and scores using MutationTaster and PolyPhen2 are shown in bold.
defect that was recently modeled in GT may affect platelet production, such as the R560B3 defect that was recently modeled in vivo (22).

We identified a wide spectrum of mutations within ITGA2B and ITGB3. Several families identified here had novel ‘private’ mutations, emphasizing a need to sequence the entire coding regions of both genes. The establishment of infrastructures to allow such molecular genetic diagnostics in remote regions of the world, where consanguinity rates are high, would be a real advance.

Integrins have two subunits, alpha and beta and form an obligate heterodimer (Fig. 1). In mammals, there are 18 different alpha and 8 different beta subunits. Integrin subunits penetrate the plasma membrane and have short (40–70 amino acid) cytoplasmic domains (23). The integrin αIIbβ3 complex is formed from a calcium-dependent association of glycoprotein IIb and IIIb. The crystal structure of integrin αIIbβ3 has been described (11, 12). Mapping of known mutations to the functional domains of the integrin αIIbβ3 complex shows that a broad range of mutations exists and that genotype/phenotype correlations are difficult within this complex molecular structure.

Conclusion

GT occurs in high frequency in certain ethnic populations with an increased incidence of consanguinity. GT is caused by mutations in the genes encoding ITGA2B or ITGB3 that result in qualitative or quantitative abnormalities in the platelet membrane proteins leading to bleeding disorders. A precise molecular diagnosis in patients with suspected GT allows an increased understanding of the disease pathogenesis, and is important for screening of at risk family members and establishing a prenatal diagnosis. Here, we have successfully identified pathogenic mutations in either ITGA2B or ITGB3 in 15 patients with GT from a previously uncharacterized ethnic group and country, where little is known about the incidence and molecular genetics of GT.

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