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Biochemical and Molecular Genetic Analysis of a Patient with Glanzmann Thrombasthenia Revealed a *Novel* Likely Pathogenic *ITGA2B* Variant

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ABSTRACT

Glanzmann thrombasthenia (GT) is a bleeding disorder characterized by impaired platelet aggregation in response to most physiological agonists and caused by either a complete lack or dysfunction of the platelet integrin αIIbβ3 (glycoprotein IIb/IIIa). In this study we identified a novel homozygous non-synonymous variant (c.995A>T, p.Asp332Val) in exon 11 of *ITGA2B* leading to replacement of Aspartic Acid by Valine. According to the ACMG (American College of Medical Genetics and Genomics) variant classification guidelines, this variant is classified as likely pathogenic. This amino acid change affects the second Calcium-binding domain of αIIb integrin. The Index patient's platelet aggregation induced by all agonist except Ristocetin is absent, and the expression of the GPIIb/IIIa-complex is severely decreased.

Keywords: Glanzmann thrombasthenia, *ITGA2B*, Calcium-binding domain, Novel likely pathogenic variant

INTRODUCTION

Glanzmann thrombasthenia (GT) is the most common inherited platelet disorder characterized by a deficiency or functional defect of platelet integrin αIIbβ3. Most often GT is inherited autosomal recessively, a few patients with an autosomal dominant inheritance of GT have been identified. GT was first described by Glanzmann in 1918 as a platelet abnormality presenting with haemorrhage and thrombasthenia (weak) platelets. Characteristical for this disease is severely impaired or absent platelet aggregation in response to multiple physiological agonists. Platelets lacking integrin αIIbβ3 may adhere to the subendothelium after injury, however, platelet spreading on the exposed surface is defective as well as thrombus formation [1]. Patients suffer from a lifelong moderate to severe haemorrhagic syndrome which can manifest rapidly after birth. Bleeding symptoms comprise haematomas, petechiae, gastrointestinal and mucocutaneous bleeding (i.e., epistaxis). GT cannot be clinically distinguished from other platelet disorders; therefore, comprehensive diagnostic investigation is important to elucidate the underlying cause of the haemorrhagic diathesis [2-4].

The integrins are a family of type I transmembrane heterodimeric glycoprotein receptors. Integrin αIIbβ3 (also called glycoprotein (GP) IIb/IIIa complex) is an important, highly expressed platelet receptor which binds fibrinogen as well as Von Willebrand factor, vitronectin and fibronectin [5]. Both subunits of αIIbβ3, αIIb and β3, are encoded by separate genes less than 200 kb apart on chromosome 17q21. The gene coding for integrin αIIb subunit (*ITGA2B*) spans 17 kb and comprises 30 exons, whereas the gene that encodes the integrin β3 subunit (*ITGB3*) spans 59 kb and contains 15 exons. The polypeptide precursors of αIIb and β3 associate as a non-covalent heterodimer in the endoplasmic reticulum. Subsequently, single-chain pro-αIIb is cleaved into mature αIIb in the Golgi apparatus, comprising a heavy and light chain linked through a disulfide bridge [6]. Crystal structure analyses revealed that the head of the integrin contains a seven-bladed β-propeller

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structure from the α-subunit (comprising seven ~60-amino-acid N-terminal repeats) and a von Willebrand factor A-domain from the β-subunit (termed the β A-domain). The β A-domain is anchored to the upper face of the β-propeller, with an arginine residue in a 310 helical segment of the β A-domain (between β D and α5) linked to a hydrophobic 'cage' in the central shaft of the β-propeller. The remainder of the head composes an immunoglobulin module into which the β A-domain is inserted [7]. These two subunits associate in the membrane and in the cytoplasm with a low affinity [8]. In the inside-out signaling pathway, the cytoskeletal protein talin9 has been reported to be able to bind specifically to the cytoplasmic domain of β3 and in turn activate integrin αIIbβ3 [9]. The αIIb heavy chain includes four tandem domains, each containing a 12 amino acids stretch homologous to the calcium binding loops (EF-hand motif) of proteins such as calmodulin and parvalbumin [10]. The Calcium binding domain is part of the β-propeller domain at the N-terminus of the αIIb subunit and is of critical importance for αIIbβ3 biogenesis; at this location many mutations giving rise to type I and type II GT [11].

This study describes the clinical and biochemical phenotype of a patient with GT caused by a novel homozygous likely pathogenic variant which results in an amino acid change located in the second Calcium-binding domain of αIIb integrin.

PATIENT, MATERIALS AND METHODS

The index patient is a 6 year old boy who came for counseling before a planned dental restoration. He and his family came originally from Iran. The parents are first cousins. He suffers from increased bleeding symptoms since early childhood, including petechiae on the entire skin and easy bruising **(Figure 1A)**. He reported prolonged epistaxis: 2-3 nose bleeding episodes during the year lasted as minor bleeding even for a couple of days. After minor injuries or blood drawing the bleeding symptoms persist for 2-3 h. His parents reported major bleeding and prolonged wound healing after circumcision as an infant. In Iran he successfully received repeated platelet concentrates after sustained bleeding episodes. After Glanzmann Thrombasthenia was diagnosed here, the index patient underwent tooth extraction (5 teeth) under treatment with activated recombinant factor VII (rFVIIa) without any major bleeding. He did not need any platelet concentrate.

The mother reported that she had minor bleeding symptoms as a child like epistaxis and easy bruising. She had no increased bleeding symptoms giving birth to her children. The father and the 4 year old brother are unaffected. One brother underwent an adenoidectomy without bleeding complications.

Platelet aggregometry analyses

Platelet-rich plasma (PRP) was prepared from citrated blood. Platelet aggregometry (APACT4) was performed using 2.0 and 10.0 µg/ml collagen (Takeda, Linz, Austria), 4.0 and 10.0 µmo/l/l adenosine diphosphate (ADP) (Sigma-Aldrich, St. Louis, MO, USA), 8.0 µmol/l epinephrine (Sanofi-Aventis, Frankfurt, Germany), 0.3 and 0.5 mg/ml arachidonic acid (MöLab GmBH, Langenfeld, Germany) and 1.2 mg/ml ristocetin (American Biochemical and Pharmaceutical LTD, Frankfurt, Germany).

Flow cytometry analyses

Flow cytometry analyses were performed according to Lahav et al. [12] using FACSCalibur (Becton Dickinson, Heidelberg, Germany). Aliquots of diluted PRP (5×10^7 to 5×10^7 platelets/ml) were fixed and stained with FITC-labelled monoclonal surface antibody against CD41 (fibrinogen receptor GPIIb/IIIa-complex), CD42a (von Willebrand factor (VWF) receptor GPIb/IX) and CD42b (VWF receptor GPIb) (Coulter, Immunotech, Marseille, France), respectively.

For VWF-binding analyses, diluted PRP (5×10^7 to 5×10^7 platelets/ml) was stimulated with different concentrations of ristocetin (0-1 mg/ml) and ADP (0-2 µmol/l) for 3 min at RT, respectively. Platelets were stained with FITC-labeled anti-VWF (Bio-Rad AbD Serotec, Puchheim, Germany) and Alexa Fluor 488-labelled anti-fibrinogen (Invitrogen, Waltham, MA, USA).

For secretion analyses, diluted PRP (5×10^7 platelets/ml) was stimulated with different concentrations of thrombin (0, 0.05, 0.1, 0.2, 0.5 and 1 U/ml; Siemens Healthineers, Marburg, Germany) in the presence of 1.25 mM fibrinolysis inhibiting factor Gly-Pro-Arg-Pro (Bachem, Bubendorf, Switzerland). Platelets were stained with monoclonal FITC-labelled anti-CD62 antibody (α-granule secretion) and anti-CD63 antibody (δ-granule secretion) (both Immunotech, Marseille, France).

Molecular genetic analysis

Sanger sequencing was performed for the coding region and splice sites of *ITGA2B* (NM_000419.4) and *ITGB3* (NM_000212.2) as described previously [13] and aligned using CodonCode® software. Variants were evaluated using mutation analyzing software Alamut®, *in silico* pathogenicity prediction and occurrence in Glanzmann database (Sinai central), NCBI ClinVar and HGMD® [Access 11/2018].

RESULTS

The Index patient showed normal values for platelet count (279 Gg/l), von Willebrand factor parameters (VWF:Ag, VWF:Collagen-binding activity, VWF:CBA/VWF:Ag-ratio and VWF-multimeric analysis), factor VIII- and factor XIII-activity. Platelet aggregometry analyses revealed absent aggregation with all agonists except for Ristocetin. Maximal agglutination after stimulation with Ristocetin was 76% followed by desaggregation **(Figure 1B)**. Flow cytometry analyses indicated severely decreased expression of the

GPIIb/IIIa-complex (CD41) **(Figure 1C)** and impaired thrombin-and ADP induced fibrinogen-binding **(Figure 1D)**, respectively. Normal values were measured for expression of CD42a (GPIb/IX-complex), CD42b (GP Ib), CD 63 (δ-granule secretion) and CD62 (α-granule secretion). The VWF-binding after stimulation with Ristocetin was normal.

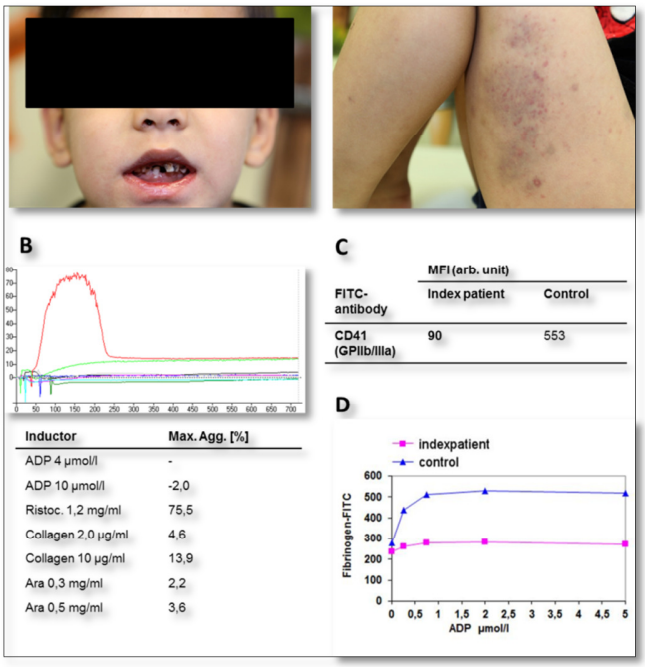

Figure 1. A: Clinical phenotype of the index patient with remaining blood at nostrils, dental status before intervention and pronounced hematomas at the left tight. **B:** Platelet aggregometry analyses: absent aggregation after stimulation with all agonists except with Ristocetin (Red colour; Max. agglutination of 76% followed by early desaggregation). **C:** Severely decreased expression of the GPIIb/IIIa-complex (CD41) compared to healthy control (MFI, mean fluorescence intensity). **D:** Impaired ADP induced fibrinogen-binding in flow cytometry analyses.

Molecular genetic analysis of the index patient revealed a novel homozygous variant (c.995A>T, p.Asp332Val) in exon 11 of *ITGA2B*. The highly conserved (up to *C. elegans*) Aspartic Acid at position p.332 (301 in mature protein) is exchanged by the physiochemical different Valine (Grantham dist.:152 [0-215]). The variant is neither listed in population databases (dbSNP, gnomAD, ESP) nor in disease databases (Sinai central, HGMD and ClinVar) [Access 11/2018]. *In silico* pathogenicity prediction was concordant pathogenic using SIFT, Polyphen2 and MutPred).

Family genotyping revealed that the consanguine parents and the brother of the index patient are heterozygous carriers of this variant **(Figure 2)**.

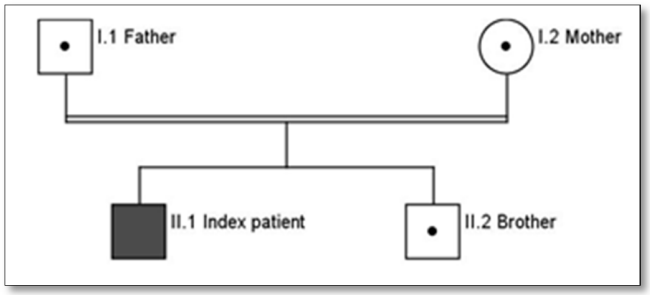

Figure 2. Pedigree; black indicates homozygous carrier and therefore clinically affected index patient of the novel likely pathogenic variant c.995A>T. Consanguine parents and one brother are heterozygous carrier (symbols with dot inside). They are clinically unaffected or mildly affected (mother).

DISCUSSION AND CONCLUSION

In this study we describe the severe bleeding phenotype of a young boy carrying a novel likely pathogenic (Class4) non-synonymous variant in the *ITGA2B*-gene. Wild type Aspartic Acid (Asp; D) at position p.301 in the mature protein (p.332 with signal peptide) is replaced by Valine. This change affects the second Calcium-binding domain of αIIb integrin, which is a sequence of 9 amino acids: p. 297-DVNGDGRHD-305 in mature protein (UniProtKB P08514 Calcium binding domain 2: p. 328-336 with signal peptide). Several groups investigated the effect of variants in the calcium-binding domains and showed how important these regions are to facilitate proper αIIbβ3 biogenesis or calcium-binding [14,15]. Gidwitz et al. used site-directed mutations to substitute Ala or Gln for wildtype Asp or Asn at several positions in the calcium-binding domain, including an Asp301Ala (D301A) mutant. This position is identical with the altered amino acid position in αIIb of our index patient (D301V in mature protein). αIIb mutants were transiently co-expressed with β3 and cell-surface expression was examined by immunoprecipitation of biotinylated cell-surface proteins with antibodies to αIIb. Their results demonstrated that the conformation of the second calcium-binding loop is critical for maturation and cell-surface expression of αIIbβ3, but not for the formation of the αIIbβ3 heterodimer. Each of the point mutations that they induced to residues in the second calcium-binding domain blocked expression of the heterodimer on the cell surface, but none of them blocked interaction with β3 [14]. Nelson et al. [16] described three missense mutations (G128S, S287L and G357S in mature protein) within αIIb β-propeller, located on the upper face of the propeller in the area involved in interaction with β3. They showed that all three pro-αIIb mutants were synthesized, stable and able to form a complex with β3 and also the mutations had no effect on pro-αIIbβ3 complex formations. However, the mutations compromised the receptor maturation and surface expression. The authors proposed that the major limiting step in αIIbβ3 biogenesis in the mutations is not complex formation, but transport from

the ER to the Golgi for carbohydrate modification, pro-αIIb cleavage and transport to the cell surface [16]. In another publication, Shen et al. [17] characterized the P126H missense mutation within the αIIb β-propeller and showed that the mutation blocked the pro-αIIbβ3 transport from the ER to Golgi and the surface expression, but not the complex formation [17].

Biochemical and molecular genetic analysis of our patient revealed the diagnosis of Glanzmann Thrombasthenia and identified most likely the disease causing mutation. Therefore, successful treatment in case of bleeding symptoms or surgery could be successfully performed. Identifying the disease causing mutation in GT enables to understand the biochemical and molecular genetic mechanisms responsible for the bleeding phenotype.

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