Dominant inheritance of a novel integrin $\beta_3$ mutation associated with a hereditary macrothrombocytopenia and platelet dysfunction in two Italian families

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ABSTRACT

Background
Defects of integrin $\alpha_{IIb}\beta_3$ are typical of Glanzmann’s thrombasthenia, an inherited autosomal recessive bleeding disorder characterized by the failure of platelets to aggregate in response to all physiological agonists, but with no abnormalities in the number or size of platelets. Although large heterogeneity has been described for Glanzmann’s thrombasthenia, no family has so far been described as having an autosomal dominant form of this disease.

Design and Methods
We describe two Italian families with moderate thrombocytopenia with large platelets, defective platelet function and moderate/severe mucocutaneous bleeding, transmitted as an autosomal dominant trait and associated with a novel integrin $\beta_3$-gene ($ITGB3$) mutation.

Results
The characteristics of our families are moderate macrothrombocytopenia and defective platelet function associated with a mild reduction of surface $\alpha_{IIb}\beta_3$, impaired platelet aggregation to physiological agonists but not to ristocetin, normal clot retraction, reduced fibrinogen binding and expression of activated $\alpha_{IIb}\beta_3$ upon stimulation, normal platelet adhesion to immobilized fibrinogen but reduced platelet spreading and tyrosine phosphorylation, indicating defective $\alpha_{IIb}\beta_3$-mediated outside-in signaling. Molecular analysis revealed a novel mutation of $ITGB3$ that determines an in-frame deletion producing the loss of amino acids 647-686 of the $\beta$TD ectodomain of integrin $\beta_3$. Haplotype analysis indicated that the two families inherited the mutation from a common ancestral chromosome.

Conclusions
This novel autosomal dominant macrothrombocytopenia associated with platelet dysfunction raises interesting questions about the role of integrin $\beta_3$, and its $\beta$TD domain, in platelet formation and function.

Key words: glycoprotein IIb/IIIa, megakaryocytes, outside-in signaling, thrombocytopenia.
**Introduction**

Glanzmann’s thrombasthenia (GT) is a bleeding disorder caused by mutations of the ITGA2B or ITGB3 genes encoding for integrins αIIb and β3, respectively. Platelets do not aggregate in response to any physiological agonist, but they do not show abnormalities in number or size.

GT is an autosomal recessive disease and is, therefore, phenotypically expressed in homozygotes or compound heterozygotes, given that 50% of normal αβ3 is sufficient to guarantee unimpaired platelet function.1,2 However, one GT-like patient with a partial deficiency of αβ3, a life-long bleeding history and alterations in platelet size was previously reported.3 More recently, a missense mutation affecting only one allele of ITGB3 was described in a family with macrothrombocytopenia.4 Thus, depending on the site and the extent of the αβ3 mutation, defective platelet function or impaired thrombocytopenia may occur. Indeed, a role for αβ3 in platelet release from megakaryocytes has been shown.5

We report here two unrelated Italian families with an autosomal dominant form of macrothrombocytopenia showing impaired platelet function associated with a previously undescribed ITGB3 mutation and a partial integrin β3 defect.

**Patients**

The proband, a 35-year-old woman from southern Italy, had life-long moderate/severe bleeding, mainly mucocutaneous. She and some members of her family have been studied on several occasions over a 4-year period; on each occasion one or more controls, members of the research and hospital staff, were simultaneously studied. No abnormalities in blood clotting were detected but her template bleeding time was more than 20 min (normal 5.2±1.0). Electronic platelet counts (Genius S, Sec, Italy) were 26-79 ×109/L (by optical microscopy: 69-98×109/L); mean platelet volume was 14.2 fl (normal: 10.7±0.9 fl), with 84% normal-sized (controls: 0.2±0.4%) platelets on peripheral blood smears.3 Bone marrow showed mild megakaryocytic hyperplasia with all stages of maturation. Clinical and laboratory investigations of 36 members of the proband’s family identified 17 subjects with thrombocytopenia transmitted as an autosomal dominant trait (Online Supplementary Figure S1).

**Design and Methods**

**Platelet morphology**

Blood films stained with May-Grünwald Giemsa were examined using an immersion oil objective with a Laborox 12 microscope (Leitz, Germany). For electron microscopy, washed platelets were prepared from blood collected in EDTA, and fixed for 4 h at 4°C, using cacodylate buffer containing 4% wt/vol of glutaraldehyde. The samples were then washed, and kept in cacodylate buffer for 4 h; the buffer was then replaced with 1% osmic acid and the samples were pelleted by centrifugation at 10,000×g for 30 sec. Ultrathin sections of the platelet pellets were stained with uranyl acetate and lead citrate and observed with a Philips Electron Optics EM208 transmission electron microscope at 80 kv.

**Platelet function**

Platelet aggregation induced by collagen (1.2-4 μg/mL), adenosine diphosphate (ADP 2-4 μM), epinephrine (10-100 μM), UA (1-2 μM), arachidonic acid (0.6-4 mM), A23187 (4-40 μM), PFAA (1-50 nM) and PAF (0.1-10 μM), was studied with an four-channel aggregometer (APACT4, Helena Biosciences, UK), using either platelet rich-plasma or washed platelets.9

Clot retraction was assessed using non-anticoagulated whole blood added to pre-warmed glass tubes (45×12 mm) and placed in a water bath at 37°C for 24 h; tubes were then inspected for clot retraction and results expressed as percentage of the original volume.8 The PFA-100® (Dade Behring, Marburg, Germany) was employed using both collagen/ADP (C/ADP) and collagen/epinephrine (C/EPI) cartridges in citrate whole blood samples; normal ranges were established using samples from 40 healthy volunteers.10

Platelet adhesion to a collagen-coated surface (collagen from equine tendon, 30 μg/cm2) under flow conditions was studied in a parallel plate perfusion chamber, at a wall shear rate of 3000s⁻¹.

**Flow cytometry**

The expression of platelet membrane glycoproteins (GP) was investigated by flow cytometry with the following monoclonal antibodies: P2 and SZ22 for integrin αIIb (CD41); SAP, SZ21 and AP3 for integrin β3 (CD61); A2A9/6 and AP2 for integrin αIIbβ3; SZ2 for GPIIbα (CD42b); SZ1 for GPIX (CD42a); FA6-152 for GPLI (CD42c); ALB6 for CD9; AMF7 for αIIb (CD51, αV-chain); 5.6E for PECAM-1 (CD31), and 11E4B7-6 for glycoporphin A. An isotypic antibody was used as a negative control. All antibodies were fluorescein isothiocyanate (FITC)-conjugated, except for AP2 and AP3 which were unconjugated and their binding was determined using a secondary FITC-conjugated anti-mouse IgG. Mean fluorescence intensity and the percentage of positive cells were measured and normalized to those of GPIIbα, in order to take into account the large volume of platelets.14

The activation of platelets, induced either by ADP (10 μM) or thrombin receptor activating peptide (TRAP-6, 20 μM) in whole blood, was assessed by measuring the expression of P-selectin on their surface.15 Moreover, the binding of PAC-1 (recognizing activated αIIbβ3) or of a FITC-conjugated monoclonal antibody anti-fibrinogen, was studied.16

All samples were analyzed in an EPICS XL-MCL flow cytometer (Coulter Corporation, Miami, FL, USA), equipped with an argon laser operating at 488 nm.

**Western blotting**

Washed platelets from the proband and control or Chinese hamster ovary cells expressing αIIbβ3 (kindly provided by Prof. H. Deckmyn, University of Kortrijk, Belgium) were lysed and 20 μg of proteins for each sample were
analyzed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) (8% gel) for 4 h and 50 min at 20 mA. Separated proteins were transferred onto a nitrocellulose membrane at 100 V for 90 min. Membranes were saturated with 5% fat-free dried milk in T-TBS and incubated overnight with an anti-integrin β mouse monoclonal antibody (Calbiochem, USA) and then incubated with horseradish peroxidase-conjugated anti-mouse IgG for 2 h at room temperature. Immunoreactive bands were detected using peroxidase-conjugated IgG antibody and chemiluminescence detection.

**Adhesion and spreading assays**

Washed platelets resuspended in Tyrode's buffer (200×10^9/L) were incubated for 30 or 60 min on 100 μg/mL fibrinogen-coated dishes under static conditions, as previously described. The protein content of adherent platelets was determined with the Bradford assay, and results expressed as a percentage of the total added platelets. Platelet lysates were used for the detection of tyrosine phosphorylation by immunoblotting. Aliquots from total platelets or adherent platelets, containing the same amount of proteins, were added to an equal volume of SDS-sample buffer, heated at 95°C for 5 min and then subjected to SDS-PAGE on 7.5% acrylamide gels. Proteins were transferred to nitrocellulose and tested with an antibody against phosphotyrosine. After extensive washing, blots were revealed by chemiluminescence with an ECL kit and the bands corresponding to pp125FAK were quantified by using Quantscan software (Biosoft, Cambridge, UK) and expressed as arbitrary units.

For platelet spreading, washed platelets (40×10^9/L) were layered onto human fibrinogen-coated (100 μg/mL) glass coverslips. Adherent platelets were fixed, permeabilized and stained with FTC-conjugated phalloidin. Slides were then analyzed using a fluorescence microscope (Zeiss Axioplan fluorescence microscope) with a 100x objective lens, controlled by a Spot-2 cooled camera (Diagnostic Instruments) and digital images were acquired. Ten different fields were analyzed for each specimen. For scanning electron microscopy analysis, adherent platelets were fixed with 2.5% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.3, containing 2% sucrose. Fixed plates were washed, and then processed for dehydration and gold-labeling, and scanning electron microscopy images were captured using a Philips XL30 scanning electron microscope.

For all the assays the proband’s samples were compared with those of ten normal subjects.

**Results**

On light microscopy platelets were large but did not show any other visible alterations. No leukocyte inclusions were observed.

On transmission electron microscopy platelets were larger than normal (average diameter: 3.5±0.5 μm vs. 2.6±0.7 μm of controls, p<0.005) and revealed an increased number of α and α granules/platelet, compatible with the larger platelet size (8 granules/platelet: 1.56±0.6 vs. 1.13±0.9, p<0.05; α granules/platelet: 10.9±5 vs. 6.1±2.7, p<0.005) (Figure 1). In the proband, platelet aggregation induced by ADP and epinephrine was severely impaired while that induced by collagen was reduced. Response to ristocetin, PMA or A23187 was normal; arachidonic acid-induced aggregation was absent (Figure 2). Spontaneous aggregation was always

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**Figure 1. Platelet morphology as determined by optical microscopy and transmission electron microscopy.** (A) Light microscopy (under oil immersion, 1000X) of a blood smear of control and proband’s platelets: platelets are indicated by arrowheads. (B) Transmission electron microscopy of control and proband’s platelets (original magnification 13000X).
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platelets (39% at 30 min. vs. normal of 71.4±5.4%), unlike that of a simultaneously studied 
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Platelet adhesion to collagen at high shear rate was impaired (44±0.9% vs. 22±2.6% of surface covered by 
platelets). Clot retraction was normal (77.5% vs. normal of 71.4±5.4%), unlike that of a simultaneously studied 

A genome-wide search mapped the involved gene to chromosome 17q in a candidate region of approximately 
18 Mb between microsatellites D17S798 and D17S1319 (Online Supplementary Figure S1). Marker D17S950 gave a 
maximum multi-point location score of 8.4. Among possible candidates, ITGB3 and ITGA2B were analyzed in 
two affected individuals and a heterozygous G>C conversion (c.2134+1G>C) in the donor splice site of intron 
13 of ITGB3 was identified (Figure 5). Mutational analysis in 36 family members showed that only the affected 
subjects were heterozygous for the mutation while this was absent in 150 unrelated control subjects. The effect 
of the splice site mutation was determined by reverse transcriptase-polymerase chain reaction (PCR) and 
sequencing analysis showing an in-frame deletion of 120 bp of exon 13, with loss of 40 residues of the extracellular 
βTD subdomain of integrin β3, from aspartic acid 647 to glutamic acid 686 of the mature polypeptide 
(p.D647_E686del) (Figure 5).

**Figure 2.** Representative platelet aggregation tracings, in response to different agonists in platelet-rich plasma from the 
proband (P) and controls (C). Agonist concentrations are indicated in the figure.

**Figure 3.** Flow cytometry of surface glycoproteins on platelets from the 
proband (black histograms) and controls (white histograms). The expression of CD9, αvβ3, GPIb/IX/V, PECAM-1 or GPIV on the 
platelet surface was normal, or slightly increased. In contrast, expression of GPIIbα (integrin β3) and the complex GPIIIa/IIa (αIIbβ3) 
was reduced. The gray curve is a non-specific signal.
The 120 bp deletion was confirmed in platelet β3 mRNA by reverse transcriptase-PCR of purified platelet cDNA using primers flanking the deletion (data not shown).

Moreover, the expression level of normal β3 mRNA was quantified by real-time PCR using primers overlapping the deleted region: expression of the normal allele was reduced by approximately 50% in the proband (Online Supplementary Figure S2).

The mutated β3 was expressed in the proband’s platelets. Western blotting of control platelet lysate (Figure 6) showed a 90 kDa band, consistent with wild type β3, while platelet lysate from the proband showed normal β3 and a low molecular weight band product of mutated β3, of an expected molecular weight of approximately 85 kDa (Figure 6).

In order to define whether other patients with thrombocytopenia carried the c.2134+1G>C mutation, we selected from our database of patients with inherited forms of thrombocytopenia of unknown origin six patients with platelet surface expression of αGP<lower>β3 lower than 50% of control: in one of them we found the same mutation. This was a 34-year old woman from southern Italy, with moderate/severe bleeding since birth, electronic platelet counts of 48-95x10⁹/L (by optical microscopy: 52-95x10⁹/L), mean platelet volume 14.5fL, and 78% of normal-sized, 21% large and 1% giant platelets on blood smears. This patient’s 9-year old daughter had similar clinical and laboratory features. Platelet membrane glycoproteins and platelet aggregation showed the same alterations as the proband of the first family. Although no consanguinity was reported, microsatellites within the candidate region revealed that the affected members from the two families shared the same haplotype (Figure 7), indicating that the mutation occurred as a single event on an ancestral chromosome and that other cases with the same mutation are likely to be found in the Italian population.

**Figure 4.** (A) Adhesion to immobilized human fibrinogen. Washed platelets were allowed to attach to wells coated with 100 µg/mL fibrinogen for 30 and 60 min. After washing out non-adherent platelets, the protein content of adherent platelets was determined with the Bradford assay, and results were expressed as the percentage of total added platelets. The percentages of adherent platelets were similar in the proband (black columns) and in controls (white columns). (B) Spreading and protein phosphorylation upon adhesion on immobilized fibrinogen. Washed platelets were layered onto fibrinogen-coated plates for 30 min. After washing away unbonded platelets, adherent platelets were examined by fluorescence microscopy (B1) or by scanning electron microscopy (B2) and digital images (x100) were acquired. (C) Platelet spreading expressed as mean platelet area/pixel². The proband’s platelets show an approximately 50% reduction of spreading (n=10, p<0.05 vs. controls). (D) Protein phosphorylation. Densitometric analysis of pp125FAK, showing a significant reduction in the proband’s samples as compared to normal controls (p<0.05 vs. controls). For all the assays the proband’s samples were compared to those of five normal subjects.

**Figure 5.** Identification of the c.2134+1G>C mutation and its effect on the splicing of the ITGB3 gene. (A) Sequencing analysis of exon 13 and its flanking region from a control and from the proband, showing the nucleotide substitution G>C in the first position of intron 13 (lower case); (B) Reverse transcriptase-PCR of RNA extracted from peripheral blood of three controls (ctrl) and of patient IV-L2 (proband) (Online Supplementary Figure S1): the proband presents an additional fragment of 486 bp corresponding to an in frame deletion of 120 bp of exon 13, as detected by sequence analysis; M, marker 100 bp (Biolabs); (C) Schematic representation of the alternative splicing of the ITGB3 gene due to the c.2134+1G>C mutation leading to a deletion of 40 amino acids (p.D647_E686del).
Discussion

The macrothrombocytopenia with platelet dysfunction we report here represents a novel clinical entity because it does not meet the criteria for the diagnosis of any of the known forms.3,11,26

The p.D647_E686del mutation we found produces the loss of 40 amino acids of a region of integrin β₃ located in the carboxyterminal tail of the ectodomain called βTD.27 Little is known about the function of this domain. Although random mutagenesis of βTD has produced mainly gain-of-function mutants, with a permanently activated αIIbβ₃,28 the loss of amino acids 657-692, which partially overlaps the deletion in our families, was shown to prevent intracellular αIIbβ₃ complex formation.29 We have shown the concomitant presence of both the normal and a mutated β₃ protein in platelet lysates of the proband; thus, a loss-of-function hypothesis, with a dominant negative effect resulting from the p.D647_E686del mutation, can be made. The β₃ mutation in our family leads to impaired αIIbβ₃-mediated outside-in signaling. The patient’s platelets adhere to fibrinogen but fail to spread on it and tyrosine phosphorylation is impaired too, suggesting that the signal transduction pathways activated by the αIIbβ₃ ligation and clustering are altered in our patient.30

In addition to platelet dysfunction our patient also had macrothrombocytopenia. It has been reported that the interaction of fibrinogen with αIIbβ₃ is capable of stimulating proplatelet development in megakaryocytes and that the integrin is necessary for proplatelet formation.6

Recently, an ITGB3 mutation associated with autosomal dominant macrothrombocytopenia was described, and the β₃ dysfunction was shown to cause defective megakaryopoiesis;3 in accordance with a role of αIIbβ₃ in platelet formation and release.31 Differently from our cases, however, this mutation resulted in a significant constitutive, partial activation of αIIbβ₃ and was not associated with bleeding. Moreover, unlike GT patients, our families manifest additional features, such as macrothrombocytopenia, defective response to arachidonic acid but a normal clot retraction. It has previously been reported that some variant αIIbβ₃ molecules retain clot retraction capacity, even if aggregation is prevented.31

We cannot exclude that the platelet defects in our families are associated with an unidentified mutant allele in linkage disequilibrium with c.2134+1G>C; however, all candidate genes of known autosomal dominant thrombocytopenias were normal in our families (data not shown), and it is unlikely that two independent mutation events causing the same disease occurred in two different families and in the same relatively small region.

In conclusion, we have reported here a novel autosomal dominant macrothrombocytopenia with platelet dysfunction associated with a previously undescribed ITGB3 mutation. Although the full comprehension of the pathogenic mechanisms caused by p.D647_E686del

Figure 6. Western blot of integrin β₃. Control platelet lysate (lane 1) showed a band at the same level of the band of extract of Chinese hamster ovary cells expressing normal αIIbβ₃ (lane 2). The proband’s platelet lysate showed two distinct bands (lane 3): a band corresponding to normal integrin β₃ (~90 kDa) and a lower band of the molecular weight expected for the mutated protein (~85 kDa).

Figure 7. Haplotypes of the ITGB3 gene. (A) Panel of the pedigree used for linkage analysis; alleles of microsatellites indicated in bold are the same as those reported in Online Supplementary Figure S1. (B) Mother and daughter of the second family; alleles in italics are not informative. The largest haplotype potentially shared by affected individuals is boxed. Asterisks indicate microsatellites of the ABI PRISM Linkage Mapping set 2.5 (see Materials and Methods). nd, allele not determined.
requires further investigations, this novel hereditary thrombocytopoiesis raises interesting questions about the β3 domains involved in thrombocytopoiesis and outside-in signaling.

**Authorship and Disclosures**

PG contributed patients to the study, designed and supervised the research and wrote the paper; EF, SG, TC, AMM, and GG collected patients' data, carried out experimental studies and analyzed data; LC performed RT-PCR and real-time PCR of platelet mRNA; CLB contributed patients to the study; PN carried out experimental studies; FDB, AS, PD'A and AD'E performed sequencing and molecular analysis and analyzed data; CLB and AS revised the final manuscript.

The authors report no potential conflicts of interest.

**References**