A novel variant Glanzmann thrombasthenia due to co-inheritance of a loss- and a gain-of-function mutation of *ITGB3*: evidence of a dominant effect of gain-of-function mutations

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SUPPORTING INFORMATION

METHODS

Blood samples were taken from healthy volunteers and patients after written informed consent in accordance with the Declaration of Helsinki; the study was approved by the ethical committee of the University of Perugia.

Measurement of platelet diameter and platelet function tests

Platelet diameter and ultrastructure were assessed by electron microscopy, platelet volume was assessed by evaluation of blood smears as described (**7**). Platelet function was assessed by light transmission aggregometry on PRP (**6**) or by impedance aggregometry. $\alpha_{IIb}\beta_3$ expression was assessed by flow cytometry with the CD41-FITC clone P2 mAb (Immunotech, Beckman Coulter, Milan, Italy) (**6**), $\alpha_{IIb}\beta_3$ activation was assessed by flow cytometry with the PAC-1 FITC mAb (BD Biosciences, Milan, Italy) upon platelet activation with ADP 10 μM or TRAP-6 20 μM. $\alpha_V\beta_3$ expression was assessed by flow cytometry with the CD51-FITC clone P2 mAb (Immunotech, Beckman Coulter, Milan, Italy).

Variant analysis

DNA was extracted from whole blood, all exons of *ITGA2B* and *ITGB3* were amplified by PCR and analyzed by Sanger sequencing as described (**8**). After identification of the two *ITGB3* variants we excluded their presence in 50 healthy controls and in the ExAC and 1000 Genomes control databases.

Megakaryocyte differentiation from CD34+ stem cells

CD34+ cells were separated from peripheral blood of patients and healthy controls and were induced to differentiate into megakaryocytes, as previously described (**5**). Proplatelet formation, proplatelet tips number and diameter were assessed by fluorescence microscopy after the incubation with an anti-β_1-tubulin antibody (a kind gift of Professor J.E. Italiano, Harvard Medical School, Boston, MA, USA) to stain microtubules, TRITC-phalloidin (Life Technologies Italia) to stain actin and Hoechst to stain nuclei, as previously described (**4, 5**).

CHO cells culture and transfection

CHO cells (from European Collection of Cell Cultures, Salisbury, UK) were grown in IMDM supplemented with 10% FCS, 2mM L-Glutamine, 100U/ml penicillin and 100μg/ml streptomycin.

The cDNA constructs pcDNA3-N331Sβ_3 and pcDNA3-R786Wβ_3 were generated by site-directed

mutagenesis starting from the pcDNA3/β_3 vector (**9**), and transfected in CHO cells stably expressing the α_{IIb} subunit using Turbofect (Fermentas, Glen Burnier, MD, USA) (**4**).

$\alpha_{IIb}\beta_3$ expression, activation and internalization

$\alpha_{IIb}\beta_3$ expression was assessed by flow cytometry by incubation of CHO cells for 30 minutes with the CD41-FITC clone P2 mAb (Immunotech, Beckman Coulter, Milan, Italy).

$\alpha_{IIb}\beta_3$ activation was assessed by flow cytometry after stimulation with 20μM ADP (platelets) or 10mM DTT (CHO cells) and incubation with the PAC-1 mAb, recognizing activated $\alpha_{IIb}\beta_3$ (**4, 6**).

To assess internalization of $\alpha_{IIb}\beta_3$ CHO cells were incubated for 60 minutes at 37°C with the CD41-FITC clone P2 mAb and then treated with 10mM DTT or PBS for 20 minutes at 37°C, and analyzed by flow cytometry. After the acquisition of the surface mean fluorescence intensity a saturating concentration of an anti-fluorescein rabbit polyclonal IgG (Molecular Probes) was added to quench the fluorescence of surface-bound antibodies, and samples were immediately re-analyzed for residual CD41-FITC fluorescence. The percentage of internalized $\alpha_{IIb}\beta_3$ was calculated as described (**4**).

Actin polymerization

Wild type or mutant $\alpha_{IIb}\beta_3$-expressing CHO cells were stimulated with DTT (10mM) for 15 min at 37°C, fixed with 4% PFA, permeabilized with 0.01% Triton-X, stained with FITC-conjugated Phalloidin (Molecular Probes, Life Technologies, Monza, Italy) and analyzed by flow cytometry (**4**).

Adhesion assay

CHO cells ($3x10^6$/ml) were resuspended in IMDM, washed platelets ($50x10^6$/ml) were resuspended with Tyrode and layered onto glass coverslips coated with 100 μg/ml of human fibrinogen (American Diagnostica, Stamford CT, USA). May-Grunwald-Giemsa staining was performed and samples were analyzed by optical microscopy.

Spreading was assessed at 15 minutes (15 and 60 minutes for platelets) and the mean percentage of the total surface covered was calculated by using the ImageJ software (NIH, USA) (**4, 6**).

FAK phosphorylation

CHO cells were resuspended in IMDM and plated for 1 h at 37° C in 6-well plates pre-coated with 100 μg/ml of purified human fibrinogen or with 1% BSA (suspension cells). Washed platelets were resuspended in Tyrode. Cells were then washed twice with PBS and lysed with lysis buffer.

Lysates were recovered, clarified and 20 μg of proteins were analyzed by SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were probed with a rabbit anti-phospho-FAK MoAb (Cell Signalling Technology, Danvers, MA) and immunoreactive bands were detected using a peroxidase-conjugated anti-rabbit IgG antibody by chemiluminescence and measured by densitometric analysis using the ImageJ software (NIH, USA) (**4**).

Statistical analysis

Data are expressed as means±standard deviation. Unpaired t-test or the two-way ANOVA with the Bonferroni post-test were applied, where appropriate, using GraphPad Prism version5.00 (GraphPad Software, San Diego, California, USA). Differences were considered significant when $p<0.05$.