A A386G biallelic GPIbα gene mutation with anomalous behavior: a new mechanism suggested for Bernard-Soulier syndrome pathogenesis

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Citation: Vettore S, Tezza F, Malara A, Vianello F, Pecci A, Scandellari R, Floris M, Balduini A, and Fabris F. A A386G biallelic GPIbα gene mutation with anomalous behavior: a new mechanism suggested for Bernard-Soulier syndrome pathogenesis. Haematologica 2011;96(12):1878-1882. doi:10.3324/haematol.2010.039008

Design and Methods

Antibodies

We used different monoclonal antibodies (mAbs) for studying surface platelet glycoproteins. AntiCD41a-FITC clone SZ22 (BD Beckton Dickinson, San José, CA, USA) recognizes the complex GPIIb/IIIa. AntiCD42a-FITC clone GRP-P (AbD Serotec, Oxford, UK) binds to GPIX. Three different mAbs were used for the N-terminal region of GPIbα (antiCD42b). Clone SZ2 (FITC-conjugated, Immunotech, Marseilles, France) identifies a narrow epitope of GPIbα (Tyr276-Glu282). This antibody is conformation-sensitive for the diagnosis of Bernard-Soulier syndrome, type Bolzano.[1] AntiCD42b clone LJ-Ib1 (kindly provided by Dr. L. De Marco and Dr. ZM Ruggeri) recognizes an epitope located between the residues His1-Arg293 of the GPIbα chain.[2] The antiCD42b clone LJIb10 mAb (kindly provided by Dr L De Marco and Dr. ZM Ruggeri) was used for the WB assay after 7.5% or 12.5% SDS-page gel electrophoresis.[2] The other antibodies used in WB analysis were anti-CD42a (GPIX) clones BL-H6 and SZ1[3] (Santa Cruz Biotechnology Inc., CA, USA) anti-CD42c (GPIbβ) clone Gi27 (Enzo Lifescience, Farmingdale, NY, USA).

Megakaryocytes and proplatelet formation evaluation

The extent of PPF was calculated as the percentage of proplatelet-bearing CD41$^+$ cells *versus* total CD41$^+$ cells. Immunofluorescence analysis was always performed as described in detail elsewhere.[4] The following primary antibodies were used: goat polyclonal anti-CD41, rabbit polyclonal anti-CD61 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-GPIbα, clone AK2 (Sanquin, Amsterdam, Netherlands). After washing with PBS, cells were incubated with the appropriate secondary antibody conjugated with either Alexa Fluor 594 or Alexa Fluor 488 (Invitrogen, Milan, Italy). Nuclei were counterstained with Hoechst 33258.

RNA extraction

RNA was extracted from cultured MK with the RNeasy Kit (Qiagen, Germany) and treated with RNAse-free DNAse (Ambion/Applied Biosystem, Foster City, CA, USA) according to the manufacturer's instructions. Retrotranscription was performed using M-MLV retrotranscriptase (Promega Corp., Madison, WI, USA) with oligo-dT primers. cDNA was amplified in a 12.5 μL reaction using the following 15 μM primers: 8F: 5'-CTGCTCTTCCTTCGAGGTTC-3' and 5R: 5'-CACAGGCTCTTCTCTCAAGG-3' and a commercial PCR Master Mix (Promega Corp., Madison, WI, USA). The amplicons were revealed on a 2% agarose gel stained with ethidium bromide. Housekeeping gene reaction controls (FcγIIA receptor cDNA) were set up for each experiment.

References

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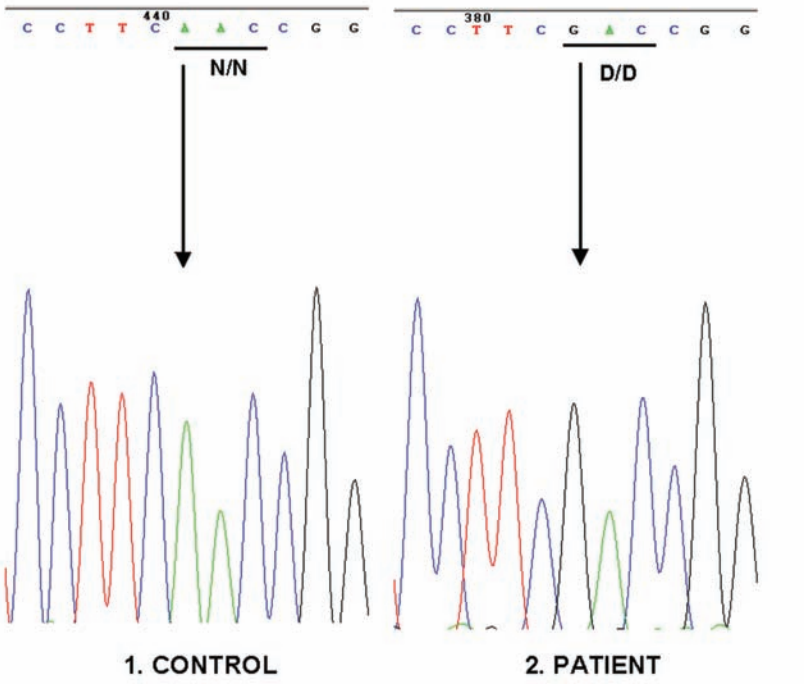
Online Supplementary Table S1. Matrices and thresholds used in computational analysis.

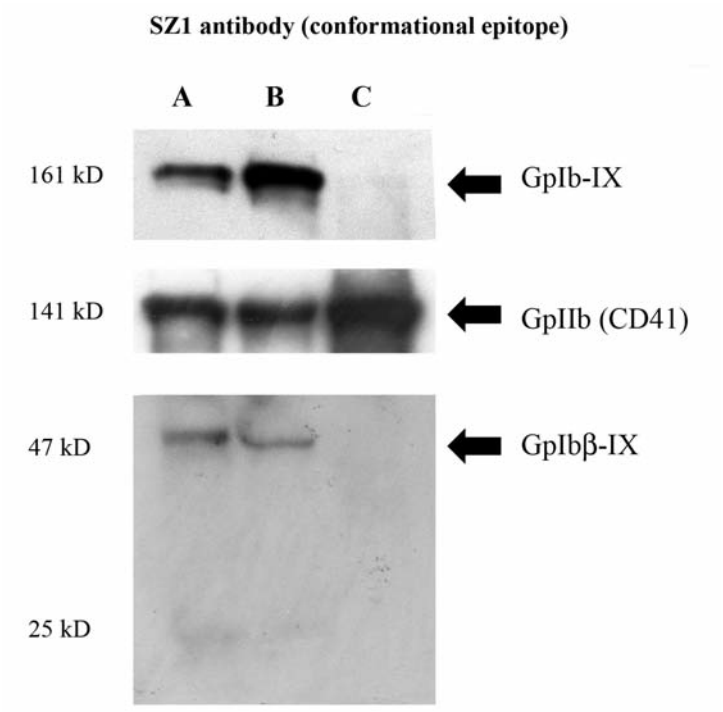
Number of motifs: 5		
#1:SF2/ASF	SF2/ASF round 3 winner	1.956 active
#2:SF2/ASF (IgM-BRCA1)	Smith06-HMG-matrix	1.867 active
#3:SC35	SC35 round 3 winner	2.383 active
#4:SRp40	SRp40 round 3 winner	2.670 active
#5:SRp55	SRp55 round 3 winner	2.676 active

Online Supplementary Table S2. MK maturation profiles. Megakaryocyte maturation profiles. After 14 days of culture, cells from the patient's peripheral blood were cytospun, in parallel with control samples, onto glass coverslips and stained with antibodies against CD41. The table shows the percentages of CD41+ cells with an MK morphology and their distribution over the different stages of maturity.

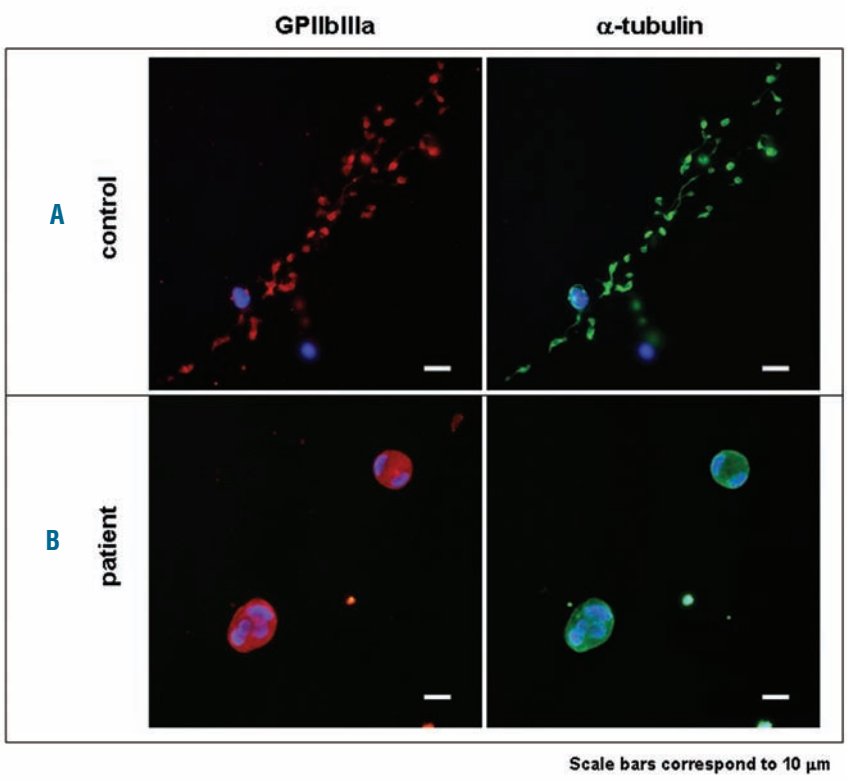
Samples (MKs)	CD41+ (%)	Stage I (%)	Stage II (%)	Stage III (%)	Stage IV (%)
Control peripheral blood	7	58.5	32.2	5.3	4
Patient's peripheral blood	6.8	51.8	37	6.2	5



Online Supplementary Figure S1. Sequencing results. Panel 1: healthy subject with wild-type sequence. Panel 2: the patient shows a biallelic substitution at the first base of the codon encoding the N110 residue of GPIbα (c.386A>G), theoretically leading to an N to D amino acid change in the protein sequence.



Online Supplementary Figure S2. WB analysis of partial GPIb/IX complex. The SZ1 antibody recognizes a conformational epitope generated on GpIX as a consequence of interaction with Gplbβ. A band corresponding to the full Gplb/GplX complex (161 kD) is visible in healthy control (A) and in N41H heterozygous substitution (B). Only traces of the complex are present in the patient (C). The mAb also recognizes another band corresponding to the partial Gplbβ/GplX complex (47 kD), which is absent in the patient with c.386A>G substitution



Online Supplementary Figure S3. Defective formation of proplatelets in c.386A>G substitution. The figure shows representative immunofluorescence images of CD41+ extensions (proplatelets, red) from control (A) and patient (B) derived-MKs. MKs were also stained with an anti alpha-tubulin antibody to reveal proplatelet structure (green). Nuclei were counterstained with Hoechst 33288 (blue). Scale bar=10 μm.