Dysregulation of oncogenic factors by GFI1B p32: investigation of a novel *GFI1B* germline mutation

Michela Faleschini,[1] Nicole Papa,[1] Marie-Christine Morel-Kopp,[2] Caterina Marconi,[3] Tania Giangregorio,[1] Federica Melazzini,[4] Valeria Bozzi,[4] Marco Seri,[3] Patrizia Noris,[4] Alessandro Pecci,[4] Anna Savoia[1,5] and Roberta Bottega[1]

[1]Institute for Maternal and Child Health – IRCCS Burlo Garofolo, Trieste, Italy; [2]Department of Hematology and Transfusion Medicine, Royal North Shore Hospital and Northern Blood Research Center, Kolling Institute, University of Sydney, Sydney, New South Wales, Australia; [3]Department of Medical and Surgical Sciences, University of Bologna, Bologna, Italy; [4]Biotechnology Research Laboratories, IRCCS Policlinico San Matteo Foundation, Pavia, Italy and [5]Department of Medical Sciences, University of Trieste, Trieste, Italy

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Correspondence: *ANNA SAVOIA* - anna.savoia@burlo.trieste.it

Supplementary Information

Supplementary Methods

Family study and clinical features

The propositus was a 65-year-old female referred for chronic thrombocytopenia discovered at the age of 31 when diagnosed with breast cancer. The degree of thrombocytopenia was mild to moderate, with platelet count always fluctuating between 50 and 100 $x10^9$/L. She reported a lifelong history of bleeding tendency, including easy bruising, bleeding from minor wounds, occasional gum bleeding and menorrhagia. She underwent three surgical procedures: quadrantectomy at age 32, bilateral annessiectomy at age 49, and radical mastectomy at age 55 without any bleeding complications. She had three pregnancies, resulting in one early abortion and two vaginal deliveries. The first delivery was complicated with post-partum hemorrhage requiring red blood cells transfusion, whereas the second was carried out without any major bleeding complications.

The proband's oldest daughter (III-1) was found to have mild thrombocytopenia at the age of 14 when in hospital for appendectomy, which was not complicated by excessive bleeding. Thrombocytopenia was persistently mild, ranging from 124 to 135 $x10^9$/L. She never presented significant bleeding symptoms and did not report any hemorrhagic complication during two caesarian deliveries. The proband's second daughter (III-2) had a platelet count always around the lower limit of normal (150 - 170 $x10^9$/L). She has never reported bleeding symptoms. She had three vaginal deliveries and underwent surgery for carpal tunnel syndrome without any complications.

The proband's sister (II-3) also presented with platelet counts at the lower limit of normal (145-155 $x10^9$/L) and no spontaneous bleeding tendency. She had 5 pregnancies, resulting in two miscarriages and three vaginal deliveries without bleeding complications. At age of 55, she received a diagnosis of ovarian cancer that relapsed twice despite surgery and chemotherapy. One of the two surgical procedures for cancer removal was complicated by major hemorrhage requiring red blood cells transfusion, which led us to assign a bleeding score of 3. However, since such a complication can occur also in individuals who are not at risk for hemorrhages due to platelet or coagulation defects, the WHO score of individual II-3 should not regarded as expression of bleeding diathesis.

Of note, during the genetic counselling, the proband (II-2) reported to carry, as well as her sister (II-3), a pathogenic mutation in the BRCA1 gene that was not transmitted to her daughters.

The proband's brother (II-4), who never had any bleeding symptoms, was incidentally found to have mild thrombocytopenia (120 $x10^9$/L) at the age of 55. Platelet count decreased over the last years together with worsening of liver function. A diagnosis of liver cirrhosis was then made. Finally, another proband's sister (II-5) had no thrombocytopenia or bleeding tendency.

Except for the proband and her sister, the other family members (II-4, II-5, III-1, and III-2) did not develop malignancies except for the proband's mother (I-2), who, at advance age, was reported to be affected by

myelodysplastic syndrome that was later responsible for her death. She also had chronic thrombocytopenia and suffered from menorrhagia, though she had eight pregnancies resulting in two abortions and six vaginal deliveries, all without bleeding complications. No clinical information was available for I-1, as well as the proband's younger sister and brother, who were not included in this study.

The institutional review board of the IRCCS "Policlinico San Matteo Foundation" of Pavia approved the study. All subjects provided written informed consent for the study, which was conducted in accordance with the Declaration of Helsinki.

Blood cell studies

Complete blood cell counts for all subjects were obtained from the same automated cell counter (Cell-Dyn 3700 from Abbott, using the impedance channel of the instrument). Mean Platelet Diameter (MPD) was measured by software-assisted image analysis on May-Grünwald-Giemsa stained blood films, as previously described.[1] Surface expression of platelet glycoproteins (GPs) was investigated by flow cytometry as reported;[2] results were expressed as the percentages of mean fluorescence intensity patient/control run in parallel. Platelet aggregation was measured in platelet rich plasma by the densitometric method of Born, as reported.[2] Platelets were stimulated with collagen (20 and 4μg/ml) (Mascia Brunelli, Milan, Italy), adenosine diphosphate (ADP, 20 and 5 μM, Sigma-Aldrich, St. Louis, MO, USA), ristocetin (1.5 mg/ml, Sigma-Aldrich), epinephrine 10 μM (Mascia Brunelli), arachidonic acid 1 μM (Sigma Aldrich) and TRAP 25 μM (Sigma-Aldrich). The extent of platelet aggregation was measured 5 minutes after the addition of the stimulating agents. Adenosine triphosphate (ATP) platelet release was measured by lumiaggregometry after stimulation with collagen 20 μg/ml and ADP 20 μM.

Expression vectors and dual-luciferase reporter assay

Wild-type (WT) and mutant (c.648+5G>A) *GFI1B* were amplified from patient's platelet cDNA and cloned into the tagged (myc) expression vector pcDNA3.1(Invitrogen, Carlsbad, CA, USA). The GFI1B, GFI, MEIS1 and CD34 promoters were generated by PCR from normal human DNA, cloned in the pGEM vector (Promega, Madison, WI, USA) and inserted into a reporter firefly luciferase vector (pGL4/luc2, Promega).

Plasmids were transiently co-transfected in Meg01 as previously described.[3] Transfection efficiency was monitored by co-transfection of the Renilla luciferase vector (LucR) acting as internal control and an empty vector was used to clean the background noise due to the possible baseline activation of the promoters.

Cell lysates were prepared 48h post-transfection and assayed for luciferase activity using the Dual Luciferase reporter assay system kit (Promega) according to the manufacturer's instructions. All transfection experiments were performed three times in triplicate (N=9), results were expressed as a ratio of firefly to renilla (LucF/LucR) and normalized to the activity of the empty vector.

Immunofluorescence assay

HeLa cells were seeded on chamber slides and transiently transfected with myc-tagged wild-type or mutant GFI1B plasmids. After 16 hours cells were fixed with 4% paraformaldehyde for 20 minutes and then permeabilized with 0.1% Triton X-100. GFI1B was detected using a primary antibody against c-myc (9E10, Santa Cruz Biotechnology) followed by a secondary FITC-labelled anti-mouse antibody (F0479, DakoCytomation – Agilent Technologies, Santa Clara, CA, USA), while nuclei were stained with Propidium Iodide (Sigma Aldrich). Images were obtained with a Nikon C1si confocal microscope, using a 60X Plan Apo objectives. Images were processed for brightness and contrast regulation using ImageJ 1.45 (NIH, Bethesda, USA) as previously described.[4]

Peripheral blood smears were fixed with methanol/acetone 1:1 at 4°C and double-labelled with a goat antibody against CD41 (Santa Cruz Biotechnology) and the AC136 mouse monoclonal antibody against CD34 (Miltenyi Biotec, Bergisch Gladbach, Germany). The appropriate chicken Alexa Fluor 594- or Fluor 488-conjugated anti-goat or anti-mouse were used as secondary antibodies (Invitrogen). General staining procedures have been already described.[5] Blood smear of the proband was stained and analyzed in parallel with blood smears of three healthy volunteers. Images were acquired through an Axioscope 2 Plus microscope (Carl Zeiss, Gottingen, Germany) equipped with a AxioCam MRc5 camera (Carl Zeiss).

Supplementary Results

Figure S1

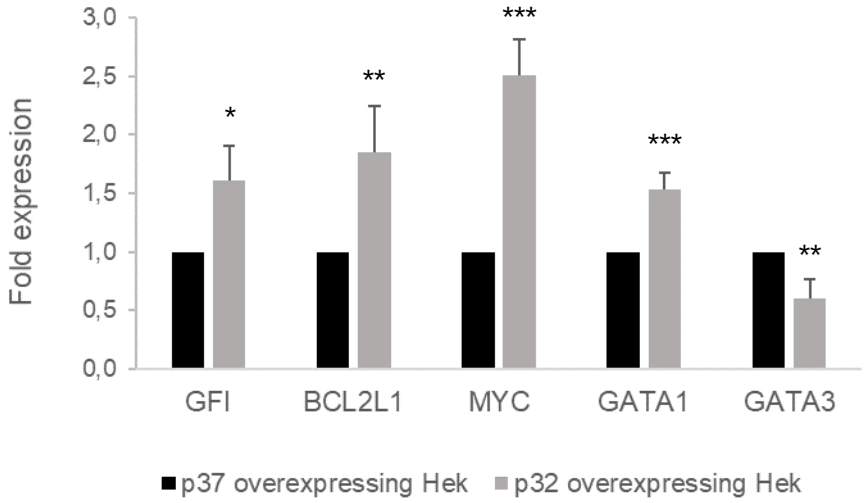

Figure S1. **Quantitative PCR (qPCR) of GFI1B transcriptional target genes in HEK cells.** The expression level of *GFI, BCL2L1, TGFBR3, MYC, GATA1* and *GATA*3, the genes up-regulated in patients blood cells was evaluated in HEK cells overexpressing p37 or p32. TGFBR3 was not detected at valuable Ct and therefore was not included in the graph. Error bars represent the standard deviation of three independent experiments. Statistical analysis was performed using T-test.

Supplementary Tables

Table S1

***GFI1B*: NM_004188.6**	
Primer	**Sequence: 5'→ 3'**
Genomic primers for Sanger sequencing	
4F	TCCCCTCTCTCACATCCAAC
4R	ACCCCTCCCAATCTGTGTC
Cloning primers	
F_kpn_myc	TAAGGTACCATGGAACAAAAACTCATCTCAGAAGAGGATCTGATGCCACGCTCCTTCCTG
R_not	TATGCGGCCGCCTCACTTGAGATTGTGCTGG
cDNA primers for RT	
GFI1b_F cDNA	TACCACTGTGTGAAGTGCAAC
GFI1b_R cDNA	CACCTGCTCATCCACTCAGAC

Table S2

cDNA primers used for qPCR			
Gene	**GenBank**	**Primer Forward (5'-3')**	**Primer Reverse (5'-3')**
GFI1b_p37	NM_004188.6	TACCACTGTGTGAAGTGCAAC	CTGCGCTCCTGGGAGTG
GFI1b_p32		CAACCTATGGCCACAGCTAC	GAAGCTGCGCTCCTTGTTG
GFI1b_p39		TGAAGTGCAACAAGGGGAT	GTCTGAGTGGATGAGCAGGTG
GFI1b_p34		ACGTCCACTCCCAGGGGATC	GTCTGAGTGGATGAGCAGGTG
GFI1	NM_005263	ACCCCTGTCAGTACTGTGGC	GAAGGGTTTCCAGAGGAAGG
BCL2L1	NM_138578	GTAAACTGGGGTCGCATTGT	GGCTGGGATACTTTTGTGGA
TGFB3	NM_003239.2	AAGAAATCCATAAATTCGACATGATC	AAGGTTTTCCGCTTCAATGTG
MYC	NM_002467	AATGAAAAGGCCCCCAAGGTAGTTATCC	GAAGAGGACTTGTTGCGGAAACGAC
GATA1	NM_002049	CCAAGCTTCGTGGAACTCTC	ATTGTCAGTAAACGGGCAGG
GATA2	NM_032638.4	GTCACTGACGGAGAGCATGA	CTCGTTCCTGTTCAGAAGGC
GATA3	NM_002051.2	CTCATTAAGCCCAAGCGAAG	CCAGACCAGAAACCGAAAAA
TUBB1	NM_030773.3	GGATCATGAATTCCTTCAGC	CACCTAGTGTCCTTGACCAT

Supplementary Bibliography

1. Noris P, Biino G, Pecci A, et al. Platelet diameters in inherited thrombocytopenias: analysis of 376 patients with all known disorders. Blood. 2014;124(6):e4-e10.

2. Noris P, Guidetti GF, Conti V, et al. Autosomal dominant thrombocytopenias with reduced expression of glycoprotein Ia. Thromb Haemost. 2006;95(3):483-489.

3. Stevenson WS, Morel-Kopp MC, Chen Q, et al. GFI1B mutation causes a bleeding disorder with abnormal platelet function. J Thromb Haemost. 2013;11(11):2039-2047.

4. Faleschini M, Melazzini F, Marconi C, et al. ACTN1 mutations lead to a benign form of platelet macrocytosis not always associated with thrombocytopenia. Br J Haematol. 2018;183(2):276-288.

5. Balduini CL, Pecci A, Loffredo G, et al. Effects of the R216Q mutation of GATA-1 on erythropoiesis and megakaryocytopoiesis. Thromb Haemost. 2004;91(1):129-140.