Clinical and pathogenic features of *ETV6*-related thrombocytopenia with predisposition to acute lymphoblastic leukemia

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SUPPLEMENTARY INFORMATION OF THE MANUSCRIPT ENTITLED "CLINICAL AND PATHOGENETIC FEATURES OF *ETV6*-RELATED THROMBOCYTOPENIA WITH PREDISPOSITION TO CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA", by Melazzini et al.

SUPPLEMENTARY METHODS

Mutation screening and RT-PCR

For Sanger sequencing PCR was carried out in 35 µl of total reaction volume with 25 ng of genomic DNA, 10 µM of each primer, and Kapa 2G Fast Hot Start ReadyMix 2X (KapaBiosystems, Cape Town, South Africa). PCR products were sequenced using the ABI PRISM BigDye v3.1 Terminator Cycle Sequencing Ready Reaction Kit and ABI PRISM 3130xl sequencer (Applied Biosystems, Foster City, CA).
Whole exome sequencing (WES) was performed on DNA samples using the solid-phase NimbleGen SeqCap EZ Exome 44Mb array (Nimblegen Inc., Madison, WI) and sequenced as 91/100 bp paired-end reads on Illumina HiSeq2000 platform (Illumina Inc., San Diego, CA). Variants passing quality filters were annotated using ANNOVAR[1] against NCBI RefGene (http://www.ncbi.nlm.nih.gov).
cDNA was synthesized with the Go Script Reverse Transcription System kit (Promega, Madison, WI, USA). Amplification reactions were performed using the following pairs of primers: F (5'-ACCAGGAGAACAACCACCAG-3') in exon 5 and R (5'-AAGTGTCCCTGCCATTTCTG-3') in exon 8 using DNA polymerase KAPA2G Fast HS Ready Mix (Kapa Biosystems, Wilmington, MA, USA). PCR products were sequenced as indicated above.

Bioinformatic tools and analysis of the structures

Missense variants were evaluated using prediction programs, such as PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/), Mutation Taster (http://www.mutationtaster.org/) Mutation Assessor (http://mutationassessor.org/), and SIFT (http://sift.jcvi.org). The only available structure of an ETV6 construct containing residues C338 to L442 (numbered in the file C8 and L112 using the construct numbering) was obtained from the PDB database. The structure was displayed by the graphic program MOLMOL and analyzed by the DSSP program to assess water exposure.

Measurement of platelet diameters

Platelet diameters were measured on May-Grünwald-Giemsa-stained blood smears by software-assisted image analysis (Axiovision 4.5, Carl Zeiss, Gottingen, Germany). On average, 200 platelets were evaluated in

each subject, and the maximum diameter of each platelet was recorded. A previous examination of a wide series of patients with IT showed that the percentage of large platelets can be reliably estimated without the support of image analysis, by visually comparing the diameters of platelets with those of erythrocytes and calculating the percentage of platelets larger than half an erythrocyte.[2] Thus, the percentage of large platelets was calculated also by this empirical method on MGG-stained blood smear by a blinded operator, as reported.[2]

Flow cytometry study of platelet surface glycoproteins

The following fluorescein isothiocyanate-conjugated (FITC) monoclonal antibodies (moAbs) from Immunotech (Marseille, France) were used: SZ21 that recognizes GPIIIa (CD61), P2 recognizing GPIIb in the intact complex with GPIIIa (CD41), SZ1 recognizing GPIX when correctly complexed with GPIbα and SZ2 against GPIbα (CD42b).

Platelet aggregation

Platelet aggregation was investigated according to the densitometric method of Born in response to the following agonists: collagen (4 and 20 µg/mL; Mascia Brunelli, Milan, Italy), adenosine diphosphate (ADP, 5 and 20mM) and ristocetin (1.5 mg/mL), both from Sigma-Aldrich (St Louis, MO).

Flow cytometry study of platelet activation

Platelet activation in response to ADP or TRAP (Tocris Bioscience, Bristol, UK) was investigated by flow cytometry.[3] Samples of patients and controls (unaffected relatives of *ETV6*-RT patients and age-matched healthy volunteers) were processed in parallel. Aliquots of whole blood were incubated with moAbs and either TRAP 25 µM, ADP 1 µM, or vehicle HEPES buffer for 10 minutes at 37°C and fixed with paraformaldehyde. The following moAbs were used: PAC1, which specifically binds to the activated conformation of GPIIb-IIIa (Becton Dickinson, San Josè, CA); CLB-Thromb/6 against P-selectin (Immunotech); SZ2 against GPIbα; P2 against GPIIb-IIIa. Platelets were gated by GPIIb-IIIa expression. Platelet activation was expressed as the ratio between mean fluorescence intensity (MFI) measured after stimulation with each agonist and MFI measured after incubation with the buffer alone.

Platelet adhesion and spreading

Glass coverslips were coated with type I collagen, von Willebrand Factor, or fibrinogen, and blocked with bovine serum albumin (BSA), as reported.[4] Washed platelets were prepared [5] and aliquots of 1 x 10^8 platelets in 2.5 mL HEPES with 1 mg/mL BSA, 5.5 mM glucose and 2 mM $MgCl_2$ were incubated on coated coverslips for 45 minutes at 37°C. After washing, specimens were fixed and stained with Alexa Fluor 488-conjugated phalloidin (Life Technologies, Carlsbad, CA) as reported.[4] Samples of patients and controls were processed simultaneously. At least 10 random microscopic fields at 63x magnification were acquired per each specimen for image analysis, which was performed by the Axiovision 4.6 software (Carl Zeiss, Oberkochen, Germany). The following parameters were assessed: number of adherent platelets, percentage of spread platelets and average platelet area.

Differentiation of human megakaryocytes and morphological analysis

$CD45^+$ cells from peripheral blood samples were separated by immunomagnetic bead selection (Miltenyi Biotech, Bologna, Italy), cultured in Stem Span medium (StemCell Technologies, Canada) and analyzed using an Olympus BX51 microscope (Olympus, Deutschland GmbH, Hamburg, Germany) as previously described.[6,7] Mk diameters were measured using the LCmicro software by Olympus Soft Imaging Solutions GmbH. Samples of patients and controls were processed in parallel.

Megakaryocyte flow cytometry

To analyze the percentage of fully differentiated Mks at the end of the culture, 200 x 10^3 cells were recovered, washed with PBS, and double-stained with the FITC-conjugated antibody HIP8 against GPIIb (eBioscience, Milan, Italy) and the PE-conjugated antibody HIP1 against GPIbα (Abcam, Cambridge, UK). Cells were analyzed using a Navios flow cytometer (Beckman Coulter). The analysis of DNA content was performed as described.[8] A minimum of 10,000 Mks were acquired. Ploidy was analyzed upon gating $GPIIb^+$ events.

Evaluation of proplatelet formation by *in vitro* differentiated megakaryocytes

Proplatelet yields were evaluated both in suspension and in adhesion on fibrinogen at the end of the culture (14th day), as previously described.[6,9] Proplatelets on fibrinogen were stained with rabbit anti-β1-tubulin primary antibody (kindly provided by Prof. J. Italiano Jr.) and Alexa Fluor-conjugated anti-rabbit antibody (Life Technologies). The size of proplatelet tips was measured by image analysis as previously described.[6]

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Supplementary Table S1. Prediction of the effect of the two missense variants identified in families C, D, and E on protein function by different tools.

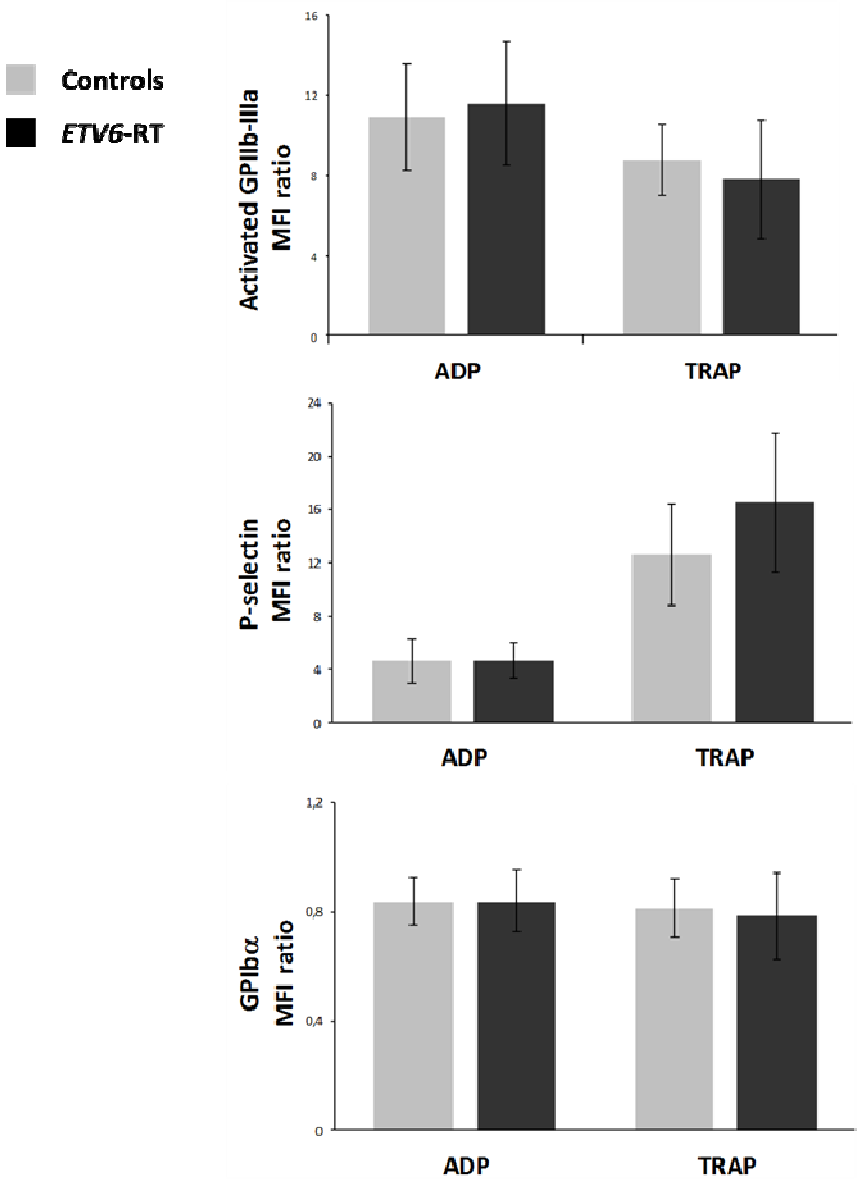
	Mutation Taster	**Mutation Assessor**	**Polyphen**
p.R369W	Disease-causing	Medium	Probably damaging
p.W380R	Disease-causing	High	Probably damaging

Supplementary Table S2. Summary of the available platelet counts in the investigated patients.

Family/ Individual	No. of measurements[1]	Age - range[2], y	Platelets, $x10^9$/L - range (median)
A/I-1	3	48-57	83-125 (110)
A/II-1	7	3-20	41-70 (61)
A/II-2 [3]	7	9-27	62-97 (84)
B/I-2 [3]	21	17-43	70-119 (94)
B/II-1	29	0-15	62-105 (71)
B/II-21	25	2-18	31-85 (55)
C/I-1	2	38-48	84-112 (98)
C/II-1	4	3-13	55-87 (79)
D/I-1	2	47-53	110-137 (123)
D/II-1	4	1-7	97-113 (103)
E/I-1	1	37	105
E/I-3[4]	3	7-32	55-71 (63)
E/I-4	3	43-45	93-109 (98)
E/II-1[5]	3	4-6	48-72 (60)
E/II-3	15	0-13	75-114 (99)
F/I-2	6	30-49	77-115 (107)
F/II-1	5	5-12	57-95 (65)
F/II-2 [3]	5	5-17	59-70 (64)
G/I-2	5	20-51	85-110 (95)
G/II-1	5	3-28	80-105 (99)

Notes: [1] Number of the available measurements of automated platelet count (including last evaluation). [2] Range of the age when the available measurements have been performed. [3] Platelet counts measured during chemotherapy for ALL were not included. [4] Platelet counts measured during chemotherapy for ALL and after HSCT were not included. 5 Platelet count measured after the diagnosis of polycythemia vera were not included.

SUPPLEMENTARY FIGURE 1



Supplementary Figure 1. Flow cytometry analysis of platelet activation in response to ADP or TRAP in *ETV6*-RT patients. The expression of activated GPIIb-IIIa (GPIIb-IIIa, PAC1 antibody binding), P selectin and GPIbα was measured after the addition of TRAP 25 µM, ADP 1 µM or the vehicle buffer alone. Data are expressed as the ratio between mean fluorescence intensity (MFI) measured after stimulation with each agonist and MFI measured after incubation with buffer alone. No significant differences were observed between 11 *ETV6*-RT patients and 20 healthy subjects analyzed in parallel (two tailed Student's t test).