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Haematologica 2020 [Epub ahead of print]

Citation: Serena Barozzi, Christian A. Di Buduo, Caterina Marconi, Valeria Bozzi, Marco Seri, Francesca Romano, Alessandra Balduini, and Alessandro Pecci. Pathogenetic and clinical study of a patient with thrombocytopenia due to the p.E527K gain-of-function variant of SRC. Haematologica. 2020; 105:xxx

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Pathogenetic and clinical study of a patient with thrombocytopenia due to the p.E527K gain-of-function variant of SRC

Serena Barozzi,¹* Christian A. Di Buduo,²,³* Caterina Marconi,⁴ Valeria Bozzi,¹ Marco Seri,⁴ Francesca Romano,⁵ Alessandra Balduini,²,³ and Alessandro Pecci.¹

¹Department of Internal Medicine, IRCCS Policlinico San Matteo Foundation and University of Pavia, Pavia, Italy.
²Department of Molecular Medicine, University of Pavia, Pavia, Italy.
³Laboratory of Biochemistry, Biotechnology and Advanced Diagnosis, IRCCS Policlinico San Matteo Foundation, Pavia, Italy.
⁴Department of Medical and Surgical Sciences, University of Bologna, Bologna, Italy.
⁵Postgraduate School of Pediatrics, University of Torino, Torino, Italy

*SB and CADB contributed equally as co-first authors.

Correspondence: Alessandro Pecci, MD, PhD, Department of Internal Medicine, IRCCS Policlinico San Matteo Foundation and University of Pavia, Piazzale Golgi, 2700 Pavia, Italy. Tel.: +39.0382.501385. Fax: +39.0382.526223. email: alessandro.pecci@unipv.it

Figure count: 3
Supplemental figures count: 4
Supplemental tables count: 4
The SRC gene is the first proto-oncogene discovered. Its product SRC is a nonreceptor protein tyrosine kinase that is the prototype, and a ubiquitously expressed member, of the SRC family kinases. SRC has been investigated for decades in mouse and in vitro models: these studies indicated that SRC signalling has a central role in many cellular functions and in oncogenesis. In platelets, SRC mediates signal activation pathways downstream different integrins and G protein-coupled receptors. However, much remains to be understood about SRC functions in human megakaryocytes and platelets.

Recently, the first germline mutation in SRC causing human disease has been reported in two families. The heterozygous c.1579G>A variant, causing the p.E527K substitution in SRC, was characterized as a gain-of-function mutation resulting in a constitutively active SRC due to the enhanced autophosphorylation of its Tyrosine-419. This mutation was associated with a complex syndromic phenotype characterized by thrombocytopenia variably associated with facial dysmorphism, severe osteoporosis, autism, intellectual disability, premature edentulism, and adult-onset myelofibrosis with splenomegaly. It has been suggested that thrombocytopenia derives from defective megakaryocyte maturation. Here we report the investigation of a new unrelated individual carrying the p.E527K variant that provides additional information on the clinical and pathogenetic features of the disorder and the role of SRC in human megakaryocytes. The study was approved by the Ethic Committee of Pavia and conducted according to the Declaration of Helsinki. Patient’s parents provided written informed consent.

Clinical and laboratory characterization. The proband was a 2-year-old male, born to healthy parents, referred for the investigation of congenital thrombocytopenia and excessive bleeding. Platelet count ranged from 55 to 100 x10⁹/L with no other cytopenias (Table S1). He presented at age 3 days with bilateral cephalohematoma with jaundice, and then had a lifelong history of easy bruising, petechiae, and occasional moderate epistaxis. Moreover, at age 3 months he was diagnosed with spontaneous left periventricular hemorrhage resulting in mild right hemiparesis. Physical examination showed no pathological findings, except for the hemiparesis. Intellectual development and learning were normal. Examination of peripheral blood smears revealed platelet anisocytosis and macrocytosis and a reduced α-granules content with about 30% of hypo- or agranular platelets (Figure S1,A-C). The α-granule defect was confirmed at ultrastructural analysis (Figure S1,D). Consistent with the α-granule deficiency, the platelet amount of thrombospondin-1 and von Willebrand factor was reduced (Figure S1,E-F), and the surface exposure of P-selectin was defective after stimulation with all tested agonists (Figure S1,G). Study of platelet aggregation showed a defective response to collagen, whereas response to ADP and TRAP was normal (Table S2). Platelet functional response to these agonists was also investigated as the induction of surface expression of the activated form of glycoprotein IIbIIIa: we found a significantly reduced response to collagen and a very mild
defect after stimulation with ADP and TRAP (Figure S1,H). Flow cytometry did not show any defects in the expression of the major platelet surface glycoproteins (Table S3).

To investigate the genetic cause of the thrombocytopenia, we performed whole exome sequencing of the proband. The analysis identified the c.1579G>A variant in the SRC gene (p.E527K substitution in SRC) in heterozygosis. No other potentially pathogenetic variants were found in the other genes known to be responsible for inherited thrombocytopenia. Sanger sequencing showed that the proband’s parents, who had normal blood cell counts, did not carry the variant, which was therefore considered as de novo.

The clinical picture remained stable until age 3.5 years, when the proband began to present unexplained, moderate hyporegenerative anemia (Table S1). A bone marrow (BM) biopsy was therefore performed. Examination concluded for a hypercellular BM with moderate trilineage dysplasia, no excess blasts, and significantly increased reticulin (fibrosis grade 2 according to Thiele). Megakaryocytes (Mks) were numerous, irregularly distributed in intertrabecular areas, and represented at all stages of maturation with dysmegakaryopoiesis and some micromegakaryocytes. No signs of Mk emperipolesis were present. Subsequently, anemia progressively worsened and required periodic red blood cell transfusions; we observed also a progressive decrease in platelet count and a mild splenomegaly (Table S1). The search for a donor for hematopoietic stem cell transplantation was therefore started.

Pathogenetic studies. We first checked that the SRC p.E527K variant actually results in constitutive increased activation of SRC. Consistent with previous findings, SRC was in a hyperactivated state in patient’s resting platelets (Figure S2). We also found increased activation of the tyrosine kinase FAK, a substrate of SRC involved in several mechanisms, such as cell adhesion and cytoskeleton reorganization (Figure S2).

The experiments reported from now on were carried out on two different occasions, when the patient was 2.5- and 3.0-year-old, and the results were compared with those obtained in three healthy controls. Given the suggested role of SRC in cell adhesion, we tested the ability of patient platelets to interact with different proteins of the extracellular matrix (ECM). Mutant platelets showed a significantly increased adhesion and spreading on fibrinogen, type I collagen, and von Willebrand Factor (Figure S3), suggesting that SRC hyperactivation induces a generalized enhancement of platelet adhesion to the ECM.

We then differentiated in vitro Mks from peripheral blood progenitors of the patient according to a standard protocol. At the end of the culture, the maturation profile of patient Mks was similar to that of controls (Figure 1,A–C). Differently from previous findings, proplatelet formation (PPF) of mutant Mks in
suspension liquid cultures was comparable to controls (Figure 1, D-E). However, when Mks were let adhere to fibrinogen or type I collagen, two components of the BM ECM that regulate platelet formation, mutant Mks exhibited a markedly increased adhesion and spreading, often with aberrant morphology (Figure 2 and S4). This prominent adhesion phenotype was associated with increased number and density of podosomes, i.e. the actin-based focal adhesion structures that mediate Mk contact with ECM proteins (Figure 3, A-C). Figure 3A shows that the active form of SRC, recognized by an antibody specific to phospho-Tyrosine-419, was closely associated with these adhesion structures, supporting the role of the constitutively active SRC in their formation. Importantly, in adhesion to fibrinogen, an ECM substrate that promotes PPF,\(^8,9\) the increased spreading of patient Mks was associated with a significantly reduced extension of typical proplatelets (Figure 2, A-B). PPF of mutant Mks adhering to ECM proteins had not been investigated in previous studies.\(^3,4\) 

Finally, Mk migration on ECM proteins toward a gradient of stromal cell-derived factor 1 (SDF1) is an essential mechanism for platelet production, as it allows Mks to reach the BM vascular structures in order to release proplatelets into the circulation.\(^12\) Using a modified transwell assay, we found that patient Mks presented a significantly impaired SDF1-driven migration both in adhesion to fibrinogen and type I collagen (Figure 3, D-E).

**Discussion.** The study of this patient contributes to elucidate the pathogenesis of SRC-related thrombocytopenia and the role of SRC in human Mks. We provide evidence that thrombocytopenia derives from an altered interaction of Mks with the ECM components. In fact, mutant Mks presented normal PPF when cultured free from the engagement with ECM proteins; however, when plated on fibrinogen, they showed an abnormally increased adhesion that associated with impaired PPF and SDF1-driven migration. We conclude that a physiologically modulated SRC signalling in Mks is crucial for adhesion and migration on ECM components. In fact, pharmacological inhibition of SRC in mouse Mks induced decreased adhesion/spreading and defective migration;\(^7\) moreover, reduced activation of SRC in Mks of patients with thrombocytopenia due to PTPRJ loss-of-function variants also associated with defective migration.\(^13\) On the other hand, here we showed that SRC hyperactivation in human Mks causes increased adhesion/spreading, which also results in impaired SDF1-driven migration. The latter is probably due to altered turnover of focal adhesion structures, which is essential for migration.\(^11\) Since actin cytoskeleton reorganization after Mk interaction with the ECM is crucial for proplatelet extension,\(^14\) an altered cytoskeletal rearrangement upon Mks adhesion to fibrinogen, due to SRC constitutive activation, likely underlies the impaired PPF.

Concerning the clinical aspects, this case demonstrates that the disorder deriving from SRC mutation can present as isolated, non-syndromic thrombocytopenia. Of note, the only feature common to all the
The other inherited thrombocytopenias that can present with this picture are thrombocytopenia due to GFI1B mutations and gray platelet syndrome due to NBEAL2 variants, although in the latter the α-granule defect is usually more pronounced. Therefore, these disorders should be considered in the differential diagnosis. A defective aggregation or activation response to collagen, albeit of varying degrees, was observed in most reported patients: this finding too could be considered a feature of the disorder. Similar to other cases, our patient presented a bleeding tendency more severe than expected based on the platelet count: the α-granule deficiency and the impaired response to collagen could explain the excessive bleeding. Finally, the moderate thrombocytopenia observed at diagnosis evolved during childhood toward a picture characterized by transfusion-dependent anemia, severe thrombocytopenia, and trilineage BM dysplasia with significant fibrosis. The disorder can therefore be rapidly progressive, underlining the importance of recognizing SRC-related thrombocytopenia among the other forms of inherited thrombocytopenia. In conclusion, the investigation of this patient provides novel information on the pathogenetic and clinical features of SRC-related thrombocytopenia and on the functions of SRC in human Mks.
FUNDING

This study has been supported by the Telethon Foundation (grant no. GGP17106, to AP); the IRCCS Policlinico San Matteo Foundation (to AP); the European Union (H2020-Project ID 767309, to AB); the Italian Ministry of Health (GR-2018-12367235, to CADB).

ACKNOWLEDGMENTS

The authors would like to thank the patient and his family, and Dr. Emanuela Boveri, Dept. of Human Pathology, IRCCS Policlinico San Matteo Foundation, for helpful discussion on bone marrow histological findings.

CONFLICT OF INTERESTS DISCLOSURE

The authors declare no competing financial interests.
REFERENCES


FIGURE LEGENDS

**Figure 1. Analysis of maturation and of proplatelet formation in suspension liquid cultures of megakaryocytes of the investigated patient.** Megakaryocytes (Mks) were differentiated from peripheral blood progenitors through 14-day culture. Samples of the patient (PT) were processed in parallel with those of three healthy controls (HCs). **(A-C): Analysis of Mk maturation. (A):** At the end of the culture, the proportion of mature megakaryocytes was measured by flow cytometry, as the percentage of CD41-positive cells co-expressing the CD42b antigen. **(B,C):** Mks were also cytopun onto slides and stained with an anti-β1-tubulin antibody (red). Hoechst (blue) was used for counterstaining nuclei (C). Mks were then classified into maturation stages I to IV by morphological analysis according to standard criteria (B).

Overall, the maturation profile of the patient’s Mks was not different from that of healthy controls. Scale bars, 10 µm. **(D-E):** Analysis of proplatelet formation in suspension liquid cultures, which measures the intrinsic ability of Mks to form proplatelets, i.e. free from the engagement with proteins of the extracellular matrix. **(D):** Representative examples of proplatelet formation of Mks of the patient and controls (phase-contrast microscopy). Scale bars, 10 µm. **(E):** Proplatelet formation was quantified as the percentage of cells displaying at least one proplatelet with respect to the total number of cells. Overall, the rate of proplatelet formation and the morphology of proplatelets in suspension were similar in patient and controls. The data are expressed as means ± SD.

**Figure 2. Analysis of proplatelet formation and spreading in adhesion to fibrinogen of megakaryocytes of the investigated patient.** Megakaryocytes (Mks) were incubated on fibrinogen-coated coverslips for 16 hours at 37°C and 5% CO₂, fixed and stained with an anti-β1-tubulin antibody (red). Hoechst (blue) was used for counterstaining nuclei. **(A-D):** Mks of the patient exhibited a markedly increased spreading, often with aberrant morphology, associated with defective extension of typical proplatelets. **(A) (ii-iv):** Representative examples of patient’s Mks. **(i-ii):** proplatelets-forming Mks of healthy controls processed in parallel are shown for comparison. Scale bars, 60 µm. **(B):** Proplatelet formation was quantified using fluorescence microscopy as the proportion of Mks displaying at least one proplatelet with respect to the total number of Mks. **(C,D):** Spreading was measured through image analysis as the average area covered by each Mk (C), and as the percentage of spread Mks with respect to the total number of Mks (D). The data reported with the histograms are expressed as means ± SD. *** $p < 0.001$, and * $p < 0.05$ by two-tailed Student t test.

**Figure 3. Analysis of podosome formation and of SDF1-driven migration of megakaryocytes of the investigated patient.** **(A-C):** Megakaryocytes (Mks) were let adhere to fibrinogen or type I collagen, fixed, and double-stained for polymerized actin (phalloidin, green) and the active form of SRC (antibody
selectively recognizing SRC phosphorylated at Tyrosine-419, red). Scale bars, 10 µm. (A): Patient’s Mks exhibited a markedly increased number and density of podosomes that were recognized as actin-based focal adhesion structures (green); moreover, patient’s Mks presented a strong positivity for the active form of SRC, which was highly and selectively concentrated in podosomes (red). The inserts on the right represent a merge of the images taken in the green and red signals, which demonstrates that the active form of SRC localizes in correspondence of podosomes recognized by phalloidin staining (yellow signal). (B,C): Podosomes formed on fibrinogen (FBG) and type I collagen (COLL) were quantified by image analysis of phalloidin-labelled Mks, as the number of podosomes per each Mk and the number of podosome per Mk area unit. (D-E): SDF1-driven migration of Mks was investigated using a modified transwell assay. Transwell systems having a polycarbonate membrane with an 8-µm pore size were coated with fibrinogen (FBG) or type I collagen (COLL). Aliquots of 4 x10^3 Mks were seeded in the upper chamber of the transwell insert, whereas the lower chamber was filled with medium containing 100 ng/mL SDF1. After an incubation of 16 hour at 37°C and 5% CO_2, cells that migrated to the lower face of the membrane were fixed and labeled with an anti-β1-tubulin antibody (red) and counted using fluorescence microscopy. (D): Representative images of microscopic fields of migrated cells. Scale bars, 60 µm. (E): Mk migration was quantified as the number of migrated Mks per field by analyzing the entire polycarbonate membrane area. For each experiment, the assays were performed in triplicate wells for each condition. Data are expressed as means ± SD. **** p<0.0001 and ** p<0.01 by two-tailed Student t test.
Figure A: Comparison of podosomes in healthy control and patient samples. Images show green and red fluorescence, with merged images on the right.

Figure B: Bar graphs comparing the number of podosomes per Mk for FBG and COLL in healthy controls (HCs) and patients (PT). The graphs show statistical significance with asterisks.

Figure C: Graphs showing the number of podosomes per Mk area for FBG and COLL in HCs and PT. The graphs indicate statistical significance with asterisks.

Figure D: Microscopy images of Fibrinogen and Collagen. Healthy controls and patients are compared.

Figure E: Graphs showing the number of MkS per field for FBG and COLL in HCs and PT. The graphs highlight statistical significance with asterisks.
SUPPLEMENTAL DATA of the paper entitled “PATHOGENETIC AND CLINICAL STUDY OF A PATIENT WITH THROMBOCYTOPENIA DUE TO THE p.E527K GAIN-OF-FUNCTION VARIANT OF SRC”

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Supplemental Methods .............................................................. page 12
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Figure S1. Morphology, content in α-granules secretory proteins, and functional response of platelets of the investigated patient. (A): Peripheral blood smears, May-Grünwald-Giemsa (MGG) staining. Representative examples of platelets of the patient, which presented anisocytosis, macrocytosis with large platelets, and reduced α-granules content with about 30% of hypo- or agranular platelets. Scale bars, 5 µm. (B): Mean platelet diameter (MPD) was measured on MGG-stained blood smears through software-assisted image analysis according to a previously reported method \(^1\). The data obtained in the patient (PT) on three different occasions were compared with those previously measured in a cohort of 55 consecutive healthy controls (HCs) \(^1\). Data are presented as means ± SD. (C): Platelet diameter distribution width (PDDW) was calculated with the same method \(^1\) as a measure of platelet anisocytosis. (D): Transmission electron microscopy analysis of patient’s platelets. Representative example of platelets presenting a markedly reduced content in α-granules. Scale bar, 1 µm. (E-F): Immunoblotting analysis of patient’s platelet content in thrombospondin 1 (TSP-1) and von Willebrand factor (vWF). Lysates were obtained from washed resting platelets of the patient (PT) and healthy controls (HCs). β-actin was used as loading control. (E): Representative image of immunoblotting experiments. (F): Densitometric analysis of the bands obtained with two separate experiments (means ± SD). Patient’s samples were processed in parallel with those of 4 healthy controls. TSP-1 and vWF levels were expressed as the TSP-1/β-actin and vWF/β-actin ratio, respectively. (G-H): Flow cytometry investigation of activation response of patient’s platelets to stimulation with adenosine diphosphate (ADP), thrombin receptor activating peptide (TRAP), and collagen. Platelet surface expression of P-selectin and of the activated form of GPIIbIIIa (PAC-1 antibody binding), was measured after incubation with ADP (5 µM), TRAP (25 µM), and collagen (COLL) (4 µg/mL), or vehicle alone (HEPES buffer). Platelet activation is expressed as the ratio between mean fluorescence intensity (MFI) measured after stimulation with each agonist and MFI measured after incubation with the buffer alone (resting platelets, plts). The values obtained in the patient were compared with those of 4 healthy controls processed in parallel. Data are expressed as means ± SD. *** p <0.001, ** p<0.01, and * p<0.05 by two-tailed Student t test.
Figure S2. Activation status of SRC and FAK in platelets of the investigated patient. Lysates were obtained from washed resting platelets of the patient (PT) and healthy controls (HCs). Immunoblotting was performed with an antibody recognizing SRC phosphorylated at tyrosine 419 (SRC activation tyrosine, Y419) and an antibody recognizing SRC phosphorylated at tyrosine 530 (SRC inhibitory tyrosine, Y530). Membranes were also incubated with an antibody against the activated form of the tyrosine kinase FAK (phosphorylated at tyrosine 397, Y397). β-actin was used as loading control. (A): Representative images of the immunoblotting experiments. (B): Densitometric analysis of SRC phosphorylation. SRC activation status is expressed as the SRC phospho-Y419/phospho-Y530 ratio normalized to β-actin. (C): Densitometric analysis of FAK activation expressed as the FAK phospho-Y397/β-actin ratio. Data are expressed as means ± SD. ** p<0.01, and * p<0.05 by two-tailed Student t test.
Figure S3. Adhesion and spreading of platelets of the investigated patient on extracellular matrix proteins. Aliquots of 1 x10^8 washed platelets were incubated for 45 minutes at 37°C on glass coverslips previously coated with fibrinogen, type I collagen, or von Willebrand factor. Specimens were then fixed and stained for polymerized actin (phalloidin, green). Adhesion and spreading were investigated by fluorescence microscopy and software-assisted image analysis. Samples of the patient (PT) were processed in parallel with those of 4 healthy controls (HCs). (A): Representative example of platelet adhesion and spreading on fibrinogen. Scale bars, 10 µm. (B): Platelet adhesion on each extracellular matrix protein was quantified as the number of adherent platelets (plts) per microscopic field. Platelet spreading was measured as the percentage of spread platelets with respect to the number of total adherent platelets, and as the average value of the area covered by each platelet (plt) (µm^2). The data reported with the histograms are expressed as means ± SD. **** p <0.0001, and ** p<0.01, by two-tailed Student t test.
Figure S4. Spreading of megakaryocytes of the investigated patient on type I collagen. Megakaryocytes (Mks) of the patient (PT) and 3 healthy controls (HCs) were incubated on type I collagen-coated coverslips for 16 hours at 37°C and 5% CO₂, fixed and stained with phalloidin. (A-B): Spreading was measured through image analysis as the percentage of spread Mks with respect to the total number of Mks (A), and the mean area covered by each Mk (μm²) (B). The data reported with the histograms are expressed represent as means ± SD. *** p < 0.001, and * p < 0.05 by two-tailed Student t test.
**SUPPLEMENTAL TABLES**

**Table S1.** Main parameters of the blood cell count of the investigated patient carrying the p.E527K mutation of SRC.

<table>
<thead>
<tr>
<th></th>
<th>Age 2 yrs (at diagnosis)</th>
<th>Age 3.5 yrs</th>
<th>Age 4.5 yrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hgb (gr/dL)</td>
<td>12.4</td>
<td>9.6</td>
<td>7.3</td>
</tr>
<tr>
<td>MCV (fl)</td>
<td>77.6</td>
<td>78.3</td>
<td>79.1</td>
</tr>
<tr>
<td>WBC (x10^9/L)</td>
<td>7.3</td>
<td>5.7</td>
<td>5.8</td>
</tr>
<tr>
<td>Neu (x10^9/L)</td>
<td>2.8</td>
<td>2.9</td>
<td>2.7</td>
</tr>
<tr>
<td>Platelets (x10^9/L)</td>
<td>71</td>
<td>50</td>
<td>19</td>
</tr>
<tr>
<td>MPV (fl)^1</td>
<td>10.9</td>
<td>9.1</td>
<td>nr</td>
</tr>
</tbody>
</table>

**Note:** ^1 mean platelet volume evaluated using an automated blood cell counter, normal range 6.4 - 8.0 fl.

**Abbreviations:** yrs, years; Hgb, hemoglobin; Neu, neutrophils; nr, not reported by the automated counter.
Table S2. Results of the study of *in vitro* platelet aggregation in the investigated patient, maximal extent (percentage).

<table>
<thead>
<tr>
<th></th>
<th>ADP, 5 µM</th>
<th>TRAP, 25 µM</th>
<th>Collagen, 4 mg/mL</th>
<th>Collagen, 20 mg/mL</th>
<th>Ristocetin, 1.5 mg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>81</td>
<td>79</td>
<td>10</td>
<td>60</td>
<td>100</td>
</tr>
</tbody>
</table>

**Note:** Normal ranges: ADP, 43-86%; TRAP, 70-100%; collagen, 66-90%; ristocetin, 67-90%.
Table S3. Results of flow cytometry investigation of surface expression of the major platelet glycoproteins (GP) in the investigated patient.

<table>
<thead>
<tr>
<th>Glycoprotein</th>
<th>GPIbα (clone SZ2)</th>
<th>GPIbα (clone MB45)</th>
<th>GPIbIX (clone SZ1)</th>
<th>GPIIbIIIa (clone P2)</th>
<th>GPIIIa (clone VIPL2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>181</td>
<td>174</td>
<td>186</td>
<td>105</td>
<td>111</td>
</tr>
</tbody>
</table>

**Note:** data are expressed as the percentage of mean fluorescence intensity compared to two healthy controls processed in parallel.
Table S4. Summary of the clinical features of the individuals carrying the p.E527K germline variant of SRC described so far.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient ID¹</td>
<td>13</td>
<td>31</td>
<td>35</td>
</tr>
<tr>
<td>Age at diagnosis (yrs)</td>
<td>55</td>
<td>31</td>
<td>26</td>
</tr>
<tr>
<td>Thrombocytopenia [platelet count, x10⁹/L]</td>
<td>yes [33]</td>
<td>yes [124-188]</td>
<td>yes [55-88]</td>
</tr>
<tr>
<td>Bleeding symptoms</td>
<td>yes</td>
<td>nr</td>
<td>yes</td>
</tr>
<tr>
<td>Platelet morphology</td>
<td>Anisocytosis, macrocytosis, α-granule deficiency</td>
<td>Anisocytosis, macrocytosis, α-granule deficiency</td>
<td>Anisocytosis, macrocytosis, α-granule deficiency</td>
</tr>
<tr>
<td>Platelet function</td>
<td>nd</td>
<td>Delayed platelet aggregation after stimulation with collagen</td>
<td>nd</td>
</tr>
<tr>
<td>Myelofibrosis</td>
<td>yes, diagnosed at age 22 yrs</td>
<td>no</td>
<td>yes, diagnosed at age 35 yrs</td>
</tr>
<tr>
<td>Osteoporosis</td>
<td>yes</td>
<td>nr</td>
<td>yes</td>
</tr>
<tr>
<td>Facial dysmorphism</td>
<td>no</td>
<td>nr</td>
<td>yes</td>
</tr>
<tr>
<td>Premature edentulism</td>
<td>no</td>
<td>nr</td>
<td>yes</td>
</tr>
<tr>
<td>Other phenotypes</td>
<td>none²</td>
<td>none²</td>
<td>none²</td>
</tr>
</tbody>
</table>

Notes: ¹ Patient identification in the original report. ² Intellectual disability and behavior abnormalities were noted in some patients of this family, but these neurological manifestations were not investigated in details. ³ Abbreviations: n. = number. nd = not determined. nr = not reported. yrs = years
SUPPLEMENTARY METHODS

Analysis of platelet size on peripheral blood smears

Platelet diameters were measured by software-assisted image analysis of May-Grünwald-Giemsa-stained peripheral blood smears, according to a previously published protocol. Mean platelet diameter and platelet diameter distribution width (the 97.5th to 2.5th percentiles difference of platelet diameters measured in each sample) were calculated as reported. Image analysis was carried out through the Axiovision 4.6 software (Carl Zeiss, Oberkochen, Germany). Analysis of patient’s samples was performed on the slides prepared on three different occasions. Data are expressed as means ± SD.

Platelet aggregation

Platelet aggregation was studied according to the densitometric method of Born as previously reported. Collagen was purchased from Mascia Brunelli (Milan, Italy). Adenosine diphosphate (ADP) and ristocetin were from Sigma-Aldrich (St Louis, MO). Thrombin receptor activating peptide (TRAP) was purchased from Tocris (Bristol, UK).

Flow cytometry of platelet glycoproteins expression

Aliquots of whole blood were incubated for 30 minutes in the dark at room temperature with the following FITC-conjugated monoclonal antibodies (moAbs) from Immunotech (Marseille, France): P2 against GPIIib in the intact complex with GPIIia (CD41); SZ2 against GPIbα (CD42b); MB45 against GPIbα (CD42b); SZ1 recognizing GPIX (CD42a) when correctly complexed with GPIbα; mouse IgG1 isotype control. FITC-conjugated VIPL2 against glycoprotein GPIIIa (CD61) was from Immunostep (Salamanca, Spain). PE-conjugated P2 against CD41 (Immunotech) was used to gate platelets. Platelets were analyzed using a FC-500 flow cytometer (Beckman Coulter, Brea, CA, USA). Data obtained in the patient were expressed as the percentage of mean fluorescence intensity compared to two healthy controls processed in parallel.

Flow cytometry investigation of platelet activation

Platelet activation in response to different agonists was investigated by flow cytometry as reported. Briefly, aliquots of whole blood were incubated with moAbs and ADP 5 µM (Sigma-Aldrich), TRAP 25 µM (Tocris), collagen 4 µg/mL (Mascia Brunelli), or vehicle HEPES buffer alone, for 10 minutes at 37°C, and fixed with paraformaldehyde. The following moAbs were used: FITC-conjugated PAC-1, which specifically binds
to the activated conformation of GPIIbIIIa (Becton Dickinson, San José, CA, USA); FITC-conjugated CLB-Thromb/6 against P-selectin (Immunotech); PE-conjugated P2 against CD41 (Immunotech). Platelets were gated by CD41 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. Data obtained in patient’s samples with two separate set of experiments were compared with those of 4 healthy controls processed in parallel.

**Immunoblotting assays**

General procedures of immunoblotting analysis of lysates of resting platelets have been previously described in detail. Briefly, whole platelet lysates were prepared and dissociated under reducing conditions, loaded on the gradient AnyKd gels, and transferred to nitrocellulose (Biorad, Hercules, CA, USA). Membranes were probed with the following antibodies: mouse A6.1 against Thrombospondin-1 (Abcam, Cambridge, UK); rabbit polyclonal against von Willebrand Factor (Dako, Santa clara, CA, USA); rabbit D49G4 against Phospho-SRC Tyr419 (Cell Signaling Technology, Massachusetts, USA); rabbit polyclonal against Phospho-SRC Tyr530 (Cell Signaling); rabbit D20B1 against Phospho-FAK Tyr397 (Cell Signaling); mouse AC-15 against β-actin (Sigma-Aldrich). The appropriate HRP-conjugated secondary antibodies (Dako) were used for detection. Protein bands were visualized by an enhanced chemiluminescence method (ECL, GE Healthcare, Waukesha, WI, USA). Patient’s samples were collected on two different occasions and processed in parallel with those of 5 healthy controls. Densitometric analysis was performed by Image J software (https://imagej.nih.gov/ij/).

**Transmission electron microscopy analysis of platelets**

Transmission electron microscopy analysis of platelet ultrastructure was performed according to previously reported standard methods.

**Whole exome and Sanger sequencing**

Whole exome sequencing on gDNA samples of the proband and raw data analysis were performed as previously described in details, using the hg19 assembly as reference. Candidate variants where further selected among those having frequency less than 1:1000 in the gnomAD database. Candidate variants confirmation and segregation analysis were carried out by Sanger sequencing with BigDye Terminator v1.1 Cycle Sequencing Kit (ThermoFisher Scientific, Waltham, MA, USA) following manufacturer’s instructions. Primer sequences are available upon request.
Platelet adhesion and spreading

Platelet adhesion and spreading were investigated according to a previously reported protocol. Glass coverslips were coated with fibrinogen (Sigma-Aldrich), type I collagen purified from bovine tendon, as reported, or von Willebrand Factor (Merck KGaA, Darmstadt, Germany), and blocked with bovine serum albumin (BSA), as reported. Aliquots of $1 \times 10^8$ washed platelets suspended 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 with buffer, specimens were fixed with paraformaldehyde 4% and stained with Alexa Fluor 488-conjugated phalloidin (Life Technologies, Carlsbad, CA). At least 10 random microscopic fields at 63x magnification were acquired per each specimen to the purpose of image analysis, which was carried out through the Axiovision 4.6 software (Carl Zeiss). Platelet adhesion on each extracellular matrix protein was quantified as the number of adherent platelets per field. Platelet spreading was measured as the percentage of spread platelets with respect to the number of total adherent platelets, and as the average value of the area covered by each platelet. Patient’s samples were collected and processed on two different occasions and compared with those obtained in 4 healthy controls processed in parallel.

Analysis of megakaryocyte maturation

CD45$^+$ cells from peripheral blood were separated by immunomagnetic bead selection (Miltenyi Biotech, Bologna, Italy) and cultured for 14 days according to a previously reported protocol. Aliquots of $50 \times 10^3$ cells were collected at the end of the culture, washed in PBS, and double-stained with the FITC-conjugated moAb HIP8 against CD41 (eBioscience, ThermoFisher Scientific) and the PE-conjugated moAb HIP1 against CD42b (Abcam). Cells were analyzed using a Navios flow cytometer (Beckman Coulter). A minimum of 10,000 events was acquired. Off-line data analysis was performed using the Beckman Coulter Navios software package. The proportion of mature megakaryocytes was measured as the percentage of CD41-positive cells co-expressing the CD42b antigen, as reported. Cells were also cytospun onto glass slides, fixed with paraformaldehyde and stained with a rabbit anti-β1-tubulin antibody (kindly provided by Prof. J. Italiano Jr.). An Alexa Fluor 594-conjugated goat anti-rabbit antibody (Life Technologies) was used as secondary antibody. Hoechst (Life Technologies) was added for counterstaining nuclei. Megakaryocytes were then classified into maturation stages I to IV by morphological analysis according to standard criteria. Patient’s samples were collected and processed on two different occasions and compared with those obtained in three healthy controls processed in parallel.

Proplatelet formation assay
Proplatelet formation was investigated at the end of the megakaryocyte culture both in suspension liquid cultures and in adhesion to fibrinogen according to a previously described protocol. Proplatelet formation in suspension was quantified by phase-contrast microscopy at day 14 as the percentage of cells presenting at least one proplatelet with respect to the total number of cells. For the analysis in adhesion to fibrinogen, glass coverslips were coated with 100 µg/ml fibrinogen (Merck-Millipore) and aliquots of $1 \times 10^5$ megakaryocytes were allowed to adhere at 37°C and 5% CO$_2$ for 16 hours. Samples were then fixed with paraformaldehyde and stained with the anti-β1-tubulin antibody as reported above. Hoechst was used for counterstaining nuclei. Proplatelet forming megakaryocytes were identified by fluorescence microscopy as the β1-tubulin$^+$ cells displaying at least one proplatelet with respect to the total number of β1-tubulin$^+$ cells. At least 50 fields per sample were analyzed using an Olympus IX53 microscope (Olympus Deutschland GmbH, Hamburg, Germany). Samples of the patient were collected on two different occasions and processed in parallel with those of three healthy controls.

**Analysis of megakaryocyte spreading**

Megakaryocyte spreading was assessed on fibrinogen and type I collagen as previously reported. Briefly, megakaryocytes were allowed to adhere for 16 hours on fibrinogen-coated coverslips, as reported above, or on coverslips previously coated with 25 µg/ml type I collagen. Cells were then fixed and stained with the anti-β1-tubulin antibody (see above) or with Alexa Fluor 488-conjugated phalloidin (Life Technologies) (see below). Spreading was quantified through image analysis as the percentage of spread megakaryocytes with respect to the total number of megakaryocytes, and as the average area covered by each megakaryocyte. At least 20 random microscopic fields at 20x magnification were acquired per each specimen to the purpose of image analysis, and at least 500 Mks per each sample were analyzed. Image analysis was performed through the Axiovision 4.6 software (Carl Zeiss). Data obtained in the patient’s samples with two separate set of experiments were compared with those of three healthy controls processed in parallel.

**Analysis of megakaryocyte podosomes**

Aliquots of $1 \times 10^5$ megakaryocytes were allowed to adhere on fibrinogen or type I collagen as reported above, fixed, and double-stained with Alexa Fluor 488-conjugated phalloidin (Life Technologies) and the rabbit D49G4 against Phospho-SRC Tyr419 (Cell Signaling Technology), which specifically recognizes the active form of SRC phosphorylated at the activation Tyrosine 419. An Alexa Fluor 594-conjugated goat anti-rabbit antibody (Life Technologies) was used as secondary antibody. Hoechst was added for counterstaining nuclei. Podosome were recognized in the green channel as polymerized actin-based focal structures.
Podosome number and density were quantified by image analysis as the number of podosomes for each megakaryocyte and the number of podosomes per unit of megakaryocyte area. The Axiovision 4.6 software (Carl Zeiss) was used for image analysis. At least 20 megakaryocytes for each specimen were analyzed. Samples of the patient were processed in parallel with those of three healthy controls.

**Analysis of megakaryocyte SDF1-driven migration**

SDF1-driven migration of megakaryocytes on fibrinogen and type I collagen was investigated using the Transwell migration chamber system (Merck-Millipore, Milan, Italy). Briefly, 96-well plates with polycarbonate inserts having 0.3 cm²/well membrane area with 8 μm pore size were coated with 100 μg/ml fibrinogen (Merck-Millipore) or 25 μg/ml type I collagen. Aliquots of 4 x10⁵ megakaryocytes suspended in 100 μL of Stem Span medium (Stem Cell Technologies) were seeded in the upper chambers, while the lower chambers were filled with 150 μL of medium with 100 ng/ml SDF1 (PeproTech, London, UK). After incubation for 16 hours at 37°C and 5% CO₂, cells remaining on the upper face of the filters were removed by a cotton wool swab. Inserts were then washed with buffer and cells on the underside of the membrane were fixed and stained for β1-tubulin as reported above. Membranes were cut out with a scalpel, mounted onto glass slides, and the number of migrated cells was counted by analyzing the entire membrane area using a Olympus BX51 microscope. The assays were performed in triplicate wells for each condition. Data obtained in the patient’s samples with two separate set of experiments were compared with those of three healthy controls processed in parallel.
REFERENCES TO SUPPLEMENTAL DATA


