First description of revertant mosaicism in familial platelet disorder with predisposition to acute myelogenous leukaemia: correlation with the clinical phenotype

by Ana C. Glembotsky, Cecilia P. Marin Oyarzún, Geraldine De Luca, Christophe Marzac, Nathalie Auger, Nora P. Goette, Rosana F. Marta, Hana Raslova, and Paula G. Heller

Haematologica 2020 [Epub ahead of print]

Citation: Ana C. Glembotsky, Cecilia P. Marin Oyarzún, Geraldine De Luca, Christophe Marzac, Nathalie Auger, Nora P. Goette, Rosana F. Marta, Hana Raslova, and Paula G. Heller. First description of revertant mosaicism in familial platelet disorder with predisposition to acute myelogenous leukaemia: correlation with the clinical phenotype. Haematologica. 2020; 105:xxx

Publisher's Disclaimer.
E-publishing ahead of print is increasingly important for the rapid dissemination of science. Haematologica is, therefore, E-publishing PDF files of an early version of manuscripts that have completed a regular peer review and have been accepted for publication. E-publishing of this PDF file has been approved by the authors. After having E-published Ahead of Print, manuscripts will then undergo technical and English editing, typesetting, proof correction and be presented for the authors' final approval; the final version of the manuscript will then appear in print on a regular issue of the journal. All legal disclaimers that apply to the journal also pertain to this production process.
First description of revertant mosaicism in familial platelet disorder with predisposition to acute myelogenous leukaemia: correlation with the clinical phenotype

Ana C. Glembotsky,1,2* Cecilia P. Marin Oyarzún,1,2,3* Geraldine De Luca, 1,2 Christophe Marzac,3,4 Nathalie Auger,5 Nora P. Goette,1 Rosana F. Marta,1,2 Hana Raslova,3** and Paula G. Heller 1,2**

1. Instituto de Investigaciones Médicas A. Lanari, Facultad de Medicina, Universidad de Buenos Aires, Buenos Aires, Argentina.
2. Departamento Hematología Investigación, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Universidad de Buenos Aires, Instituto de Investigaciones Médicas (IDIM), Buenos Aires, Argentina.
3. INSERM UMR 1170, Gustave Roussy, Université Paris-Saclay, Equipe Labellisée par la Ligue Nationale Contre le Cancer, Villejuif, France.
4. Department of Medical Biology and Pathology, Gustave Roussy Cancer Campus, Université Paris-Saclay, Villejuif, France.
5. Department of Tumor Genetics, Gustave Roussy Cancer Campus, Université Paris-Saclay, Villejuif, France.

* ACG and CPMO contributed equally to this work
** HR and PGH contributed equally to this work

Correspondence:
Paula G. Heller. Institute of Medical Research A. Lanari, School of Medicine, University of Buenos Aires; Department of Hematology Research, National Scientific and Technical Research Council (CONICET), University of Buenos Aires, Institute of Medical Research (IDIM). Combatientes de Malvinas 3150. Buenos Aires 1427. Argentina. Phone: 54 11 5287 3872. Email: paulaheller@hotmail.com

Running title: Revertant mosaicism in FPD/AML

Word count: 1746

Number of figures: 2

Number of Tables: 1
Keywords: FPD/AML, RUNX1, mosaicism, inherited platelet disorder, inherited myeloid malignancy

Familial platelet disorder with predisposition to acute myelogenous leukaemia (FPD/AML) is an autosomal dominant condition characterized by abnormal platelet number and function and 30-60% risk of haematologic malignancies, including myelodysplastic syndrome, acute myelogenous leukaemia and T-cell lymphoblastic leukaemia. It is caused by heterozygous germline mutations in the gene encoding the transcription factor RUNX1, which is essential in the emergence of definitive haematopoiesis and plays a key role in the lymphoid and megakaryocyte lineages. RUNX1 mutations predispose to leukaemia by inducing genomic instability which favours the acquisition of secondary somatic mutations. Thrombocytopenia is mild to moderate with normal-sized platelets and most patients display a platelet function defect with impaired platelet aggregation and dense-granule deficiency. However, the platelet phenotype is heterogeneous and even normal platelet count and function have been reported in rare carriers of RUNX1 mutations, highlighting that the diagnosis may be overlooked. Dysregulated expression of RUNX1-targets in platelets, including downregulation of the α2 subunit (GPIa) of collagen receptor α2β1 or persistent myosin 10 (MYH10) expression, have been proposed as screening tools to guide diagnosis, although it is at present unknown whether all FPD/AML patients harbour these defects. Therefore, molecular screening is still required to adequately identify RUNX1 mutation carriers.

We report the finding of genetic mosaicism in a patient belonging to a well-characterized FPD/AML pedigree and describe the relationship between molecular and clinical features over a 12-year follow-up. Ethics Committee approval and written informed consent were obtained and methods are detailed in the Supplementary Material. The girl, born to an FPD/AML patient, was referred for genetic testing at age 1. She had normal platelet counts and no bleeding. The pedigree includes five affected members (Figure 1A) with moderate thrombocytopenia and severe platelet dysfunction. Three individuals developed myeloid malignancies, including AML, CMML and MDS at age 43, 54 and 34, respectively. The familial RUNX1 mutation involves one nucleotide deletion in a tandem of 5 C’s, generating a frameshift which results in p.Thr246Argfs*8 (previously named p.Thr219Argfs*8 according to RUNX1b isoform and reannotated as p.Thr246Argfs*8 using reference sequence NM_001754.5, isoform RUNX1c) (Figure 1B). Using ClinGen Myeloid Malignancy Variant Curation Expert Panel criteria recently developed for germline RUNX1 variants, this variant is classified as likely pathogenic (rules applied are detailed in Supplementary Table S1). Although germline RUNX1 mutations are frequently clustered in the runt homology domain, this variant is located in a C-terminal position (Figure 1B), potentially generating a truncated protein which lacks the transactivation domain and is predicted to act in a dominant-negative manner. Consistent with this possibility,
we have previously shown stable expression of the mutant transcript in platelets from affected family members from this pedigree, indicating this variant escapes nonsense-mediated decay mechanisms.8

Screening for the familial mutation by Sanger sequencing in blood DNA revealed a low, inconspicuous mutant trace, substantially lower than the 50% mutant/wild-type allelic ratio shown for heterozygous carriers (Figure 1C). A similar subtle but consistent pattern was shown in samples obtained at age 2 and 6. Matched samples of blood and buccal mucosa obtained at age 7 showed a balanced mutant/wild-type ratio in the oral swab, consistent with a heterozygous genotype (Figure 1C), and a low mutant trace in blood, suggesting somatic mosaicism. Mosaicism was confirmed by targeted next generation sequencing (NGS) performed at age 10, which revealed \textit{RUNX1} variant allele frequency (VAF) of 55% in buccal mucosa and 8% in blood. Analysis of purified blood cell fractions showed largely similar VAF in granulocyte, B- and T-cell DNA and platelet RNA (below 10%) (Figure 1D), indicating similar involvement of myeloid, lymphoid and megakaryocyte lineages. Measurement of VAF in CD34\(^+\) cells immediately ex vivo was not possible because of low numbers of circulating CD34\(^+\) cells (Supplementary Table S2). Therefore, CD34\(^+\) cells isolated from peripheral blood were amplified \textit{in vitro} under myeloid conditions and revealed a VAF of 13% (Figure 1D), confirming mosaicism at the haematopoietic stem cell level. A bone marrow biopsy was not performed. To quantify \textit{RUNX1} mutation over time and screen for acquired somatic variants, a 77-gene myeloid NGS panel was applied in DNA from sequential whole blood samples obtained at the age of 2, 6, 7, 10 and 12 years, revealing a progressive decline in \textit{RUNX1} VAF (20 to 5%) (Table 1 and Figure 1E). In addition, the c.8560C>T (p.Arg2854Cys) single nucleotide variation in the \textit{ATM} gene was found in blood, T-cells and mucosal tissue, indicating its germline origin. This variant is classified as of uncertain significance under ClinVar. It is reported in population databases at an allele frequency of 0.00018 (ExAC)/0.00019 (GnomAD) and at increased frequency in patients with breast cancer.12 PolyPhen, SIFT and Mutation Tester in-silico tools predict a deleterious effect.

Evaluation of the clinical phenotype revealed normal or low-normal platelet counts, with moderate fluctuations over time (Table 1) and normal platelet size (Supplementary Table S2). Although moderate abnormalities in platelet aggregation, involving mainly ADP and collagen, were noted at first evaluation, platelet function was largely normal at follow-up, with low-normal ADP-induced aggregation and preserved response to other agonists (Table 1). Regarding FPD/AML-related platelet features, although less pronounced than in her affected relatives,\(^6,9\) the proband displayed moderate dense-granule deficiency and mild decrease in GPIa at age 6, when \textit{RUNX1} VAF was 17%, whereas both parameters tended to reach normal levels at age 10, when the VAF was 8% (Table 1 and Supplementary Figure S1). Although no clear-cut
relationship was evident between RUNX1 VAF and platelet abnormalities, certain platelet features improved in parallel with the progressive decline in the mutant population (Table 1). Consistent with low GPIa, the GPIIa subunit was expressed at low-normal levels, whereas other glycoproteins were preserved (Supplementary Table S2). Regarding another FPD/AML biomarker, MYH10 levels were normal in the proband’s platelets, in contrast to her affected relatives, who displayed persistent MYH10 expression (Table 1 and Supplementary Figure S2), as described for other FPD/AML pedigrees.⁷

This is, to our knowledge, the first description of genetic mosaicism in FPD/AML. The finding of a subtle mutant trace in blood by careful analysis of Sanger chromatograms, coupled with a heterozygous pattern in mucosa, suggested mosaicism, which was confirmed by NGS. Low-level mosaicism is often overlooked in clinical practice and can be missed or regarded as background noise in Sanger-based sequencing, masking clinical diagnosis, whereas NGS has emerged as a powerful tool in this setting,¹⁵ as highlighted by this report. Mosaicism can be found in a wide range of Mendelian genetic disorders and results from diverse molecular mechanisms.¹⁴ Reversion mosaicism is a special class of mosaicism in which cells undergo a somatic genetic event that counteracts the effects of a disease-causing germline mutation, followed by selective advantage of the rescued clone.¹⁴,¹⁵ This phenomenon may involve correction of the mutation to the wild-type genotype or the occurrence of compensatory second-site mutations which offset the primary variant.¹⁴,¹⁵ It has been described in diverse genetic disorders; including haematological diseases, such as Wiskott-Aldrich syndrome, Fanconi anaemia and several immunodeficiencies.¹⁴,¹⁵ In this patient, mosaicism involved the haematopoietic stem cell and mature blood cells, indicating genetic reversion in the haematopoietic system. In rescued cells, the constitutional RUNX1 mutation was reverted to the wild-type sequence. To determine the mechanism underlying this reversion, a single nucleotide polymorphism (SNP) array was performed in peripheral blood CD34⁺ cells revealing uniparental disomy and thus copy-neutral loss of heterozygosity of chromosome 21 that led to duplication of the paternal wild-type RUNX1 allele with restoration of two wild-types copies of the gene (Figure 2A, B). As RUNX1 is essential for definitive haematopoiesis,¹ it may be envisioned that RUNX1-corrected haematopoietic stem cells might have experienced growth advantage versus RUNX1-mutant cells, leading to positive selection and expansion of the revertant clone. The proposed sequence of events is depicted in Figure 2C. Interestingly, the mutant population decreased progressively over 12-year follow-up, as shown by VAF decline, indicating persistent selective advantage of the revertant clone over time. The presence of mosaicism in blood (mesodermal origin) and its absence in buccal mucosa (ectodermal origin) indicates that the genetic rescue event occurred after gastrulation, although it is uncertain whether it took place at the level of the haematopoietic stem cell or earlier during ontogeny.
The finding of a constitutional ATM variant is intriguing. Although its pathogenic potential remains uncertain, in-silico analysis supports a deleterious effect. Considering that ATM plays an essential role in DNA repair, it might be hypothesized that the ATM variant could have increased the likelihood of reversion of the RUNX1 mutation, although this possibility remains a matter of speculation. In this regard, it is noteworthy to highlight the relatively high frequency of somatic rescue events in genome instability syndromes, such as Fanconi or Bloom syndromes. More widespread use of high-throughput technologies might determine whether somatic rescue events occur more frequently than currently appreciated in FPD/AML, which might contribute to variability in phenotypic presentation.

Although revertant mosaicism is frequently associated with a less severe clinical course, as described for Wiskott-Aldrich syndrome and Fanconi anaemia, it may also have detrimental effects. In this regard, a worse outcome has been described for certain inherited bone marrow failure syndromes, such as myelodysplasia and leukaemia syndrome with monosomy 7 (MLSM7) caused by germline mutations in SAMD9 or SAMDL9, in which particular reversion mechanisms (e.g. monosomy 7 leading to removal of the pathogenic allele) may determine progression to myeloid malignancies. In the patient described in the present report, the clinical presentation was substantially attenuated compared to her FPD/AML affected family members. Although certain FPD/AML-related features, such as dense-granule deficiency and low GPIa expression, were present in this patient, especially when the VAF was higher, the platelet phenotype was mild and variable, hampering clinical diagnosis, which relied on molecular analysis. On a similar line, the patient had an uneventful course without leukaemic transformation. Although follow-up is short, it is tempting to speculate that the risk of leukaemia might be attenuated in this case. However, the remaining pool of RUNX1-mutant cells, while comprising a low proportion of haematopoietic cells, are still susceptible to acquire additional mutations which may promote malignant transformation. On this basis, careful follow-up, including bone marrow biopsy, is warranted, especially considering that dominant-negative mutations, as the one found in this pedigree, are associated with higher risk of leukaemic transformation.

In conclusion, this report illustrates the challenges of establishing a conclusive diagnosis in mosaic disorders presenting with an attenuated clinical phenotype and highlights the usefulness of new genetic technologies for adequate detection of these conditions. Revertant mosaicism might be more frequent than previously recognized and long-lived, rapidly replicating haematopoietic cells may be particularly susceptible to suffer somatic rescue events. In this setting, FPD/AML may now be added to the growing list of haematologic genetic disorders associated with revertant mosaicism.
Acknowledgements

This study was supported by grants from the Fondation Nelia et Amadeo Barletta (to Paula Heller, 2017), the Ligue Nationale Contre le Cancer (équipe labellisée 2019 to H. Raslova) and the cooperation program between France and Argentina, Ecos-Sud-Ministerio de Ciencia, Tecnología e Innovación Productiva (MINCyT) (to Hana Raslova and Paula Heller, 2016). Cecilia Marin Oyarzún was supported by a postdoctoral fellowship from the Fondation Nelia et Amadeo Barletta and from the Fondation de la Recherche Médicale.

Authorship contributions

A.C.G. and C.P.M.O. designed and performed experiments, analysed data, contributed to the manuscript draft and prepared figures; G.DL performed experiments and prepared figures; N.P.G., R.F.M, C.M and N.A performed experiments; P.G.H. provided patient samples and clinical follow-up; P.G.H. and H.R. designed and supervised the work and wrote the paper. All authors discussed results and contributed to manuscript editing and final approval.

Declaration of interest

The authors declare no conflicts of interest.

References


Table 1. Platelet features in the proband and affected FPD/AML relatives.

<table>
<thead>
<tr>
<th>Platelets (x 10^9/L)</th>
<th>ISTH-BAT (score)</th>
<th>Platelet aggregation (%)</th>
<th>δ-granules (RFI, P/C)</th>
<th>GPIa (RFI, P/C)</th>
<th>MYH10 (ΔΔCt)</th>
<th>RUNXI (VAF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proband, age 1</td>
<td>180</td>
<td>0</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Proband, age 2</td>
<td>176</td>
<td>0</td>
<td>96</td>
<td>48</td>
<td>65</td>
<td>10</td>
</tr>
<tr>
<td>Proband, age 6</td>
<td>191</td>
<td>0</td>
<td>74</td>
<td>65</td>
<td>87</td>
<td>88</td>
</tr>
<tr>
<td>Proband, age 7</td>
<td>169</td>
<td>0</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Proband, age 10</td>
<td>152</td>
<td>0</td>
<td>59</td>
<td>61</td>
<td>67</td>
<td>67</td>
</tr>
<tr>
<td>Proband, age 12</td>
<td>187</td>
<td>0</td>
<td>87</td>
<td>64</td>
<td>86</td>
<td>80</td>
</tr>
<tr>
<td>Affected relatives</td>
<td>105 (72-145)</td>
<td>5</td>
<td>17</td>
<td>31</td>
<td>15</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>(4.6)</td>
<td>(8.21)</td>
<td>(19.34)</td>
<td>(0.19)</td>
<td>(4.34)</td>
<td>(0.4-0.7)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(0.3-0.5)</td>
<td>(3.6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(52-57)</td>
</tr>
<tr>
<td>Reference values</td>
<td>150-450</td>
<td>-</td>
<td>55.99</td>
<td>66.84</td>
<td>59.97</td>
<td>61.97</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.85-1.15</td>
<td>0.64-1.36</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.05-1.6</td>
<td>-</td>
</tr>
</tbody>
</table>

BAT means bleeding assessment tool; AA, arachidonic acid; EPI, epinephrine; COL, collagen; RFI, relative fluorescence intensity; P/C, patient/control ratio; VAF, variant allele frequency; nd, not done.

Values in affected relatives (n=3 for surface GPIa, MYH10 expression and RUNXI VAF; n=4 for all other parameters) are given as median and range. Data on dense granule content and GPIa expression in affected relatives has been previously published.6,9

Normal reference values for all tested parameters are provided at the bottom of the table.
Figure legends

**Figure 1.** Molecular features of the proband and FPD/AML pedigree. A. FPD/AML pedigree. Half-filled symbols represent affected individuals presenting with platelet defects and filled black symbols represent patients with platelet defects who developed myeloid malignancies; empty symbols, individuals with normal RUNX1 genotype; the question mark depicts unknown mutation status. The proband is shown in gray and indicated by the arrow. CMML, chronic myelomonocytic leukaemia; AML, acute myelogenous leukaemia; MDS, myelodysplastic syndrome. B. Schema of the RUNX1 protein showing the position of the familial **RUNX1** mutation (p.Thr246Argfs*8), which is located between the runt homology domain (RHD) and the transactivation domain (TAD). Also shown is a summary of reported germline **RUNX1** variants described in other FPD/AML pedigrees\(^1\) (see also Supplemental references 7-23), annotated according to reference sequence NM_001754.5. Intragenic and whole gene deletions are not depicted. C. DNA sequencing chromatograms obtained by Sanger method. Reverse traces from blood and buccal mucosa samples from the proband (IV-3) and blood samples from an affected (II-2) and unaffected (IV-1) family members are shown. Arrows indicate the single base deletion which generates a frameshift. D. **RUNX1** variant allele frequency (VAF) in whole blood (WB) and blood cell populations obtained from the proband at the age of 10 years. Neutrophils (Neu), B- and T-cells and platelets (Plts) were purified from peripheral blood and CD34+ haematopoietic progenitors (HSC) were separated from peripheral blood mononuclear cells and cultured *in vitro* in the presence of a cytokine cocktail. VAF was measured by next generation sequencing (NGS). E. **RUNX1** VAF measured by NGS in sequential samples of whole blood DNA obtained at the age of 2, 6, 7, 10 and 12 years.

**Figure 2.** Loss of heterozygosity. A, B. SNP array profile. A. Log2ratio profile showing absence of copy number variation. B. Biallelic frequency profile showing an unbalanced ratio of chromosome 21. The right panel shows an enlarged view of chromosome 21 profile. C. Proposed model of mosaicism development in FPD/AML. The germline **RUNX1** mutation (Mut) (red dot), which was inherited from the mother, underwent somatic reversion by means of uniparental disomy and thus copy-neutral loss of heterozygosity (LOH), leading to duplication of the paternal wild-type (WT) **RUNX1** allele with restoration of two wild-types copies of the gene (depicted in black in corrected cells) and resulting in a mixed population of mutant and corrected cells. Reverted haematopoietic stem cells (HSC) underwent selective advantage and clonal expansion, whereas cells harbouring the mutant allele decreased progressively over time.
Reversion of inherited mutation

Clonal expansion

Germline RUNX1 T246Rfs* mutation

mutant cell WT/Mut

corrected cell WT/WT
Supplementary Methods

Biological samples

Samples were obtained from the proband and family members who presented with platelet defects without or prior to malignant transformation. DNA was obtained from whole blood, oral swabs and purified blood cell populations. Briefly, mononuclear cells were isolated using a Ficoll-Hypaque gradient followed by magnetic cell separation of B- and T- cells using CD19 and CD3 microbeads (Miltenyi Biotec, Germany), respectively. Granulocytes were recovered from the bottom layer and subjected to dextran sedimentation and red cell hypotonic lysis. Haematopoietic progenitors (CD34+) were isolated from mononuclear cells by immunomagnetic enrichment (Miltenyi, Biotech), stained with CD34-PE antibody (Beckton Dickinson) and sorted by the Influx flow cytometer (Beckton Dickinson). They were cultured in serum-free medium in the presence of a cocktail of human recombinant cytokines containing EPO (1 U/mL) (Amgen Thousand Oaks, CA), TPO (20 ng/mL) (Krin, Japan), SCF (25 ng/mL) (Biovitrum AB, Sweden), IL-3 (10 ng/mL) (MiltenyiBiotec), FLT3-L (10 ng/mL), G-CSF (20 ng/mL) and IL-6 (100 U/mL) for 8 days, pelleted and stored at -80°C. DNA was extracted using standard procedures. DNA concentrations and OD260/280 ratio were measured with the NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA) and DNA integrity was assessed by electrophoresis.

To obtain platelet RNA, platelet-rich plasma (PRP) was prepared by centrifugation at 200g for 10 min at room temperature, supplemented with 0.042M indomethacin, subjected to an additional centrifugation step at 200 g during 8 min to remove leukocytes and red cells, filtered through a leukocyte reduction filter (Purecell PL, Pall Biomedical Products Co, NY, USA) and red blood cells were lysed with 8.6 g/L ammonium chloride solution. After this procedure, the leukocyte:platelet ratio was <1:10^6. Total RNA was prepared from 1-3x10^9 platelets using glycogen as a carrier and 1 µg RNA was reverse transcribed using SuperScript (Life Technologies, Carlsbad, CA, USA).

Sanger sequencing

Amplification of a fragment of the RUNX1 gene spanning the previously characterized familial mutation (exon 6) and a region of ATM encompassing the mutation found by NGS (exon 58) was done by PCR. To exclude allelic drop-out yielding random non-amplification of RUNX1 mutant allele, a previously sequenced DNA fragment bearing no polymorphisms was sequenced using a different pair of primers, as detailed below. The amplified fragments were purified using the ADN PuriPrep GP kit (Inbio Highway, Argentina), followed by Sanger bidirectional sequencing using
the respective forward (F) or reverse (R) primer for fluorescent labelling and analysis on an ABI 3100/3730XL or ABI PRISM 310 Analyzer (Applied Biosystems, Foster City, CA, USA).

Next generation sequencing (NGS)

2 methods were used:

1) The mutated RUNX1 region was amplified by PCR using primers listed below. PCR products were end-repaired, extended with an 'A' base on the 3'end, ligated with indexed paired-end adaptors (NEXTflex, Bioo Scientific) using the Bravo Platform (Agilent) and amplified by PCR for 4 cycles. Amplicon libraries were sequenced in an IlluminaMiSeq flow cell using the onboard paired-end sequencing (2x250 bp reads) (Illumina, San Diego, CA).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>RUNX1 DNA 1</td>
<td>GGCAATTCTCTAGGATCATATGTT</td>
<td>CAGAGATCACTGTATTTGC</td>
</tr>
<tr>
<td>RUNX1 DNA 2</td>
<td>AAAGGGGGCCATATTCTTCTTCTGTA</td>
<td>CCTTGTGGGATCTGTGTTAC</td>
</tr>
<tr>
<td>RUNX1 DNA 3</td>
<td>CTGATCTTCCCTCCCTCCCTCC</td>
<td>AGGTGTTGCTTGGTTCG</td>
</tr>
<tr>
<td>RUNX1 DNA 4*</td>
<td>CTGATCTTCCCTCCCTCCCTCC</td>
<td>ATCTGACTCTGAGGCTTAC</td>
</tr>
<tr>
<td>RUNX1 cDNA*</td>
<td>GAAGACATCGGAGCAACTA</td>
<td>GAAGGCAATGGGATCCCAAGTTA</td>
</tr>
<tr>
<td>ATM</td>
<td>GAAGTCTTCGAGGATTTGCC</td>
<td>GCCAAACAAACACAGTCC</td>
</tr>
</tbody>
</table>

Primer sequences used for Sanger sequencing and targeted NGS.

* primers used for targeted NGS

2) NGS gene panel

A panel of 77 genes designed for the diagnosis of myeloid malignancies used for NGS includes:

ABL1 (Chr9; NM_007313; exons 4-9), ADGRV1(chr5; NM_032119; exons 1-90), ANKRD26 (Chr10; NM_014915; exons 1 and 5'UTR), APC (Chr5; NM_001127510; exons 1-15), ASXL1 (Chr20; NM_015338; exons 11-12), ASXL2 (Chr2; NM_018263; exons 11-12), ATG2B (Chr14; 20 polymorphisms), ATM (Chr11; NM_000051; exons 2-63), ATRX (ChrX; NM_000489; exons 1-35), BCOR (ChrX; NM_001123385; exons 3-16), BCORL1 (ChrX; NM_001184772; exons 1-13), BRAF (Chr7; NM_004333; exons 15), CALR (Chr19; NM_004343; exons 9), CBL (Chr11; NM_005188; exons 4, 8-9,12,16), CEBPA (Chr19; NM_001287424; exons 1), CHEK2 (Chr22; NM_001005735; exons 3-16), CREBBP (Chr16; NM_004380; exons 1-31), CSF3R (Chr1; NM_156039; exons 14 et 17), CUX1 (Chr7; NM_001202543; exons 1-24), DDX41 (Chr5; NM_016222; exons 1-17), DDX54 (chr12; NM_001111322; exons 1-20), DHX29 (chr5; NM_019030; exons 1-27), DIS3 (chr13; NM_014953; exons 1-21), DNMT3A (Chr2;
NM_022552; exons 2-23), EED (Chr11; NM_001308007; exons 1-13), EP300 (Chr22; NM_001429; exons 1-31), EPOR (Chr19; NM_000121; exon 8), ERBB4 (Chr2; NM_005235; exons 1-28), ETV6 (Chr12; NM_018638; exon 3), ETV6 (Chr12; NM_001987; exons 1-8), EZH2 (Chr7; NM_004456; exons 2-20), FLI3 (Chr13; NM_004119; exons 8-21), GATA1 (ChrX; NM_002049; exons 2-4), GATA2 (Chr3; NM_032638; exons 4-6; c.1017_462-703), HRAS (Chr11; NM_005343; exons 2-3 and Codon 117 in exon 4), IDH1 (Chr2; NM_005896; Codons 130-134 in exon 4), IDH2 (Chr15; NM_002168; exon 4), JAK2 (Chr9; NM_004972; exons 3-25; 46/1 Haplotypes; c.*122G>A), KDM6A (ChrX; NM_001291415; exons 1-30), KMT2A (Chr11; 6 polymorphisms), KIT (Chr4; NM_000222; exons 2, 8-17), KRAS (Chr12; NM_033360; exons 2 and 4; Codons 57-64 in exon 3), MPL (Chr1; NM_005373; exons 1-12), MYC (Chr8; NM_002467; exon 2), NF1 (Chr17; NM_001042492; exons 1-58), NFE2 (chr12; NM_001261461; exons 3-4), NPM1 (chr5; NM_002520; exon 11), NRAS (Chr1; NM_002524; exons 2-3), PHF6 (ChrX; NM_001015877; exons 2-10), PPM1D (Chr17; NM_003620; exon 6), PRPF40B (Chr12; NM_001031698; exons 1-26), PRPF8 (Chr17; NM_006445; exons 2-43), PTPN11 (Chr12; NM_002834; exons 3-4; 8; 11-13), RAD21 (Chr8; NM_006265; exons 2-14), RUNX1 (Chr21; NM_001001890; exons 3-9), SETBP1 (Chr18; NM_015559; exon 4), SETD2 (Chr3; NM_014159; exons 5-9), SF1 (Chr11; NM_004630; exons 1-13), SF3A1 (Chr22; NM_005877; exons 1-16), SF3B1 (Chr2; NM_012433; exons 13-16), SH2B3 (chr12; NM_005475; exons 2-8), SIMC1 (Chr5; NM_001308195; exons 2-10), SMCL1A (ChrX; NM_6306; exons 1-25), SMC3 (Chr10; NM_5445; exons 1-29), SRP72 (Chr4; NM_006947; exons 1-19), SRSF2 (Chr17; NM_001195427; exon 1), STAG2 (ChrX; NM_001042749; exons 3-35), SUZ12 (Chr17; NM_015355; exons 1-16), TERC (Chr3; NR_001566), TERT (Chr5; NM_198253; exons 1-16), TET2 (Chr4; NM_001127208; exons 3-11), THPO (Chr3; NM_001289998; 5'UTR), TP53 (Chr17; NM_001126112; exons 2-11; NM_001126113_130exon 10; NM_001126114_134exon 10), U2AF1 (Chr21; NM_001025203; Codon 34 in exon 2 and Codon 157 in exon 6), U2AF2 (Chr19; NM_007279; exons 1-12), WT1 (Chr11; NM_0024426; exons 1-4; 7-9), ZRSR2 (ChrX; NM_005089; exons 1-11).

Libraries were obtained from 200 ng of genomic DNA using HaloPlex Target Enrichment System (Agilent technologies) and sequencing was performed using a MiSeq sequencing (Illumina, San Diego, CA), according to the manufacturer’s protocols. Results were analysed after alignment of the reads using two dedicated pipelines, SOPHiA DDM® (Sophia Genetics) and an in-house software GRIO-Dx®. For all samples, an average depth exceeding 200X for > 90% of the target regions was required. All pathogenic variants were manually checked using Integrative Genomics Viewer software. The disease-causing potential of variants was checked by using online prediction programs which included Polyphen2, MutationTaster and SIFT.

Cytoscan high density (HD) SNP array
Chromosome analysis was performed using CytoScan HD array/Oncoscan (Affymetrix) according to the manufacturer's recommendations. The data from microarray scans were extracted by Chromosome Analysis Suite software (ChAS; v3.1, Affymetrix) and analysed as previously reported.1

Platelet features

Platelet studies were performed as previously described.2,3 Briefly, platelet aggregation was evaluated in PRP by light transmission aggregometry (LTA) using a lumiaggregometer (Chrono Log Corp., Havertown, PA, USA) after stimulation with 1 mM arachidonic acid, 4 μg/mL collagen, 2 μM ADP, and 1 μM epinephrine (Biopool, Bray, Ireland) and expressed as maximal light transmission percentage. To study dense (δ)-granule content, PRP was adjusted to 100 x 10⁹/L platelets, labelled with 2mM mepacrine (Sigma-Aldrich Inc.) for 30 minutes at room temperature, analysed by flow cytometry and a ratio between mean fluorescence intensity (MFI) with and without mepacrine was calculated and expressed as relative fluorescence intensity (RFI). To assess platelet surface expression of integrin α2 (GPIa) and β1 (GPIIa) subunits of collagen receptor α2β1, PRP was adjusted to 10 x 10⁹/L platelets, incubated with fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody against GPIa (CD49b) and phycoerythrin (PE)-conjugated antibody against GPIIa (CD29) (BD Biosciences, San José, CA, USA) or the corresponding isotypic controls, analysed in a flow cytometer and expressed as a ratio between the antibody and the isotypic control (RFI). The platelet population was identified by labelling with CD41-PE or CD61-FITC, respectively. Assays were run in duplicate. To assess levels of other glycoproteins (GP), platelets were labelled with FITC or PE-conjugated antibodies against GPVI, GPIIb (CD41a), GPIIIa (CD61), GPIb (CD42b) and GPIX (CD42a) (BD Biosciences). For all assays, a healthy subject was studied simultaneously. Mean platelet diameter (MPD) was determined by measuring the largest diameter of 100 platelets in May–Grünwald-Giemsa-stained blood smears by using the VideoTesT-Master (Morphology) image analysis software (St Petersburg, USSR). Reference values for platelet studies were established by the mean ± two standard deviations values of healthy subjects (n=6-20).

MYH10 expression levels in platelets by qPCR

Platelet RNA was prepared from the proband, three affected family members (II-1, III-1 and III-3) and 5 healthy subjects. Platelet MYH10 expression was measured in triplicate by qPCR (primer sequences, forward 5’-TGCATGCTTCAAGATCGTGAG-3’; reverse 5’-AGCAACATGGGCAAGGTACTG-3’) relative to GAPDH using SYBR® Green (Life Technologies, Grand Island, NY, USA) in an iCycler (Bio Rad Life Science, Hercules, CA, USA).
The identity of the PCR products was assessed by melting analysis and electrophoresis. Serial cDNA dilutions were assessed to ensure similar amplification efficiency of the target and control genes.

**Circulating CD34+ cells**

Whole blood was incubated with fluorescein isothiocyanate (FITC)-conjugated anti-CD45 monoclonal antibody and phycoerythrin (PE)-conjugated anti-CD34 monoclonal antibody (BD Biosciences) or the corresponding isotype control. FACS Lysing Solution (BD Biosciences) was then added to lyse the red blood cells. After washing, cells were fixed with 1% paraformaldehyde, analysed by flow cytometry and the percentage of CD34+ cells was calculated as described.4

**Statistical analysis**

Comparison between patients and controls was performed by Mann-Whitney test. \( P \) values <0.05 were considered significant.
Supplementary Tables

Table S1. Classification of the germline variant using ClinGen Myeloid Malignancy Variant Curation Expert Panel criteria for germline RUNX1 variants

<table>
<thead>
<tr>
<th>RUNX1 variant</th>
<th>ClinGen classification</th>
<th>Rules applied</th>
</tr>
</thead>
<tbody>
<tr>
<td>c.735delC</td>
<td>Likely Pathogenic</td>
<td>Absent from controls (PM2), null variant in a gene where LOF is a known mechanism of disease (PVS1_strong)*, 1 proband meeting at least one of RUNX1 phenotypic criteria (PS4_supporting), segregation in 5 affected family members (PP1_moderate).</td>
</tr>
</tbody>
</table>

* PVS1_strong criteria was applied because the variant was previously shown to escape nonsense-mediated decay\(^5\) but the altered region is critical to protein function, as specified in Luo et al.\(^6\)

RefSeq NM_001754 (RUNX1 isoform c) was used.

Table S2. Haematologic features in the proband and affected family members.

<table>
<thead>
<tr>
<th></th>
<th>CD34+ (%)</th>
<th>Haemoglobin (g/dL)</th>
<th>WBC (x 10(^9)/L)</th>
<th>MPD (µm)</th>
<th>MPV (fL)</th>
<th>GPIIa (β1) RFI (P/C)</th>
<th>GPVI RFI (P/C)</th>
<th>GPIb RFI (P/C)</th>
<th>GPIIIa RFI (P/C)</th>
<th>GPX RFI (P/C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proband, age 1</td>
<td>nd</td>
<td>12.2</td>
<td>13.1</td>
<td>nd</td>
<td>8.6</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Proband, age 2</td>
<td>nd</td>
<td>12.7</td>
<td>6.5</td>
<td>2.3</td>
<td>9.3</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Proband, age 6</td>
<td>nd</td>
<td>13.9</td>
<td>8.4</td>
<td>2.6</td>
<td>9.3</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Proband, age 7</td>
<td>nd</td>
<td>13.6</td>
<td>6.0</td>
<td>2.3</td>
<td>8.7</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Proband, age 10</td>
<td>0.02</td>
<td>14.7</td>
<td>5.3</td>
<td>2.4</td>
<td>9.0</td>
<td>0.78</td>
<td>0.80</td>
<td>1.01</td>
<td>0.84</td>
<td>0.86</td>
</tr>
<tr>
<td>Proband, age 12</td>
<td>nd</td>
<td>12.0</td>
<td>4.6</td>
<td>2.5</td>
<td>9.4</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Affected relatives</td>
<td>0.05</td>
<td>13.3</td>
<td>6.5</td>
<td>3.0</td>
<td>9.9</td>
<td>0.65</td>
<td>0.80</td>
<td>0.79</td>
<td>1.02</td>
<td>1.51</td>
</tr>
<tr>
<td>(0.05-0.06)</td>
<td>(12.5-13.8)</td>
<td>(5.9-6.9)</td>
<td>(2.5-3.1)</td>
<td>(9.6-10.2)</td>
<td>(0.4-0.7)</td>
<td>(0.8-0.9)</td>
<td>(0.8-1.0)</td>
<td>(0.9-1.0)</td>
<td>(1.4-1.6)</td>
<td>(1.3-1.8)</td>
</tr>
<tr>
<td>Reference values</td>
<td>0.004</td>
<td>12-16</td>
<td>4-10</td>
<td>2.3-3.1</td>
<td>8.9-10.5</td>
<td>0.81-1.19</td>
<td>0.73-1.27</td>
<td>0.78-1.22</td>
<td>0.78-1.22</td>
<td>0.65-1.35</td>
</tr>
</tbody>
</table>

WBC means white blood cells, MPD; mean platelet diameter; MPV; mean platelet volume; GP, glycoprotein; RFI; relative fluorescence intensity; P/C, patient/control ratio.

Values in affected relatives (n= 2 for circulating CD34+ cells; n=3 for platelet GP; n=4 for all other parameters) are given as median and range. Data on platelet GPs in affected relatives was previously published.\(^3\)

Normal reference values for all tested parameters are provided at the bottom of the table.
Supplementary Figures

**Figure S1.** FPD/AML-related platelet features. Dense granule content in the proband at (A) age 6 and (B) age 10. Platelets were incubated with mepacrine, analysed by flow cytometry and a ratio between mean fluorescence intensity of platelets incubated with and without mepacrine was calculated and expressed as relative fluorescence intensity (RFI). A control sample was run simultaneously with the patient at both time points. Colour histograms represent platelets incubated with mepacrine and gray histograms, those without mepacrine. Surface expression of GPIa at (C) age 6 and (D) age 10. Platelets were incubated with FITC-conjugated CD49b or the corresponding isotype control, analysed by flow cytometry and a ratio between mean fluorescence intensity of CD49b and the isotype control was expressed as relative fluorescence intensity (RFI). A control sample was run simultaneously with the patient at both time points. Colour histograms represent platelets stained with CD49b and gray histograms, isotype controls.
**Figure S2.** MYH10 gene expression in platelets. Real-time PCR analysis of MYH10 (myosin 10) relative to GAPDH in platelets from the proband, affected family members (n=3) and healthy subjects (n=5). *P <0.05, Mann-Whitney test.

**Supplementary References**


