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

Familial platelet disorder with predisposition to acute myelogenous leukemia (FPD/AML) is an autosomal dominant condition characterized by abnormal platelet number and function and 30-60% risk of hematologic malignancies, including myelodysplastic syndrome, acute myelogenous leukemia and T-cell lymphoblastic leukemia.[1] It is caused by heterozygous germline mutations in the gene encoding the transcription factor RUNX1, which is essential in the emergence of definitive hematopoiesis and plays a key role in the lymphoid and megakaryocyte lineages.[1,2] *RUNX1* mutations predispose to leukemia by inducing genomic instability which favors the acquisition of secondary somatic mutations.[3] 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.[1] However, the platelet phenotype is heterogeneous[4] and even normal platelet count and function have been reported in rare carriers of *RUNX1* mutations,[5] highlighting that the diagnosis may be overlooked. Dysregulated expression of RUNX1-targets in platelets, including downregulation of the $\alpha 2$ subunit (GPIa) of collagen receptor $\alpha 2\beta 1$[6] or persistent myosin 10 (*MYH10*) expression,[7] have been proposed as screening tools to guide diagnosis, although it is at present unknown whether all FPD/AML patients harbor 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 *Online Supplementary Materials and Methods*. The girl, born to an FPD/AML patient, was referred for genetic testing at the age of 1 year. She had normal platelet counts and no bleeding. The pedigree includes five affected members (Figure 1A) with moderate thrombocytopenia and severe platelet dysfunction.[6,8,9] Three individuals developed myeloid malignancies, including AML,[3] chronic myelomonocytic leukemia (CMML)[8] and myelodysplastic syndrome (MDS) at the age of 43, 54 and 34 years, respectively. The familial *RUNX1* mutation involves one nucleotide deletion in a tandem of 5 C, 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).[8,9] Using ClinGen Myeloid Malignancy Variant Curation Expert Panel criteria recently developed for germline *RUNX1* variants,[10] this variant is classified as likely pathogenic (rules applied are detailed in the *Online 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.[11] 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 the age of 2 and 6 years. Matched samples of blood and buccal mucosa obtained at the age of 7 years 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 the age of 10 years, which revealed *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 impossible because of low numbers of circulating CD34$^+$ cells (*Online Supplementary Table S2*). Therefore, CD34$^+$ cells isolated from peripheral blood were amplified *in vitro* under myeloid conditions and revealed a VAF of 13%

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

	Platelets (x 10^9/L)	ISTH-BAT (score)	Platelet aggregation (%) AA	ADP	EPI	COL	δ-granules (RFI, P/C)	GPIa (RFI, P/C)	*MYH10* ($2^{\Delta\Delta Ct}$)	*RUNX1* (VAF)
Proband, age 1	180	0	nd	nd	nd	nd	nd	nd	nd	nd
Proband, age 2	176	0	96	48	65	10	nd	nd	nd	20
Proband, age 6	191	0	74	65	87	88	0.65	0.60	nd	17
Proband, age 7	169	0	nd	nd	nd	nd	nd	nd	nd	14
Proband, age 10	152	0	59	61	67	67	0.77	0.74	0.5	8
Proband, age 12	187	0	87	64	86	80	nd	nd	nd	5
Affected relatives	105	5	17	31	15	18	0.65	0.40	5.7	53
	(72-145)	(4-6)	(8-21)	(19-34)	(0-19)	(4-34)	(0.4-0.7)	(0.3-0.5)	(3-6)	(52-57)
Reference values	150-450	–	55-99	66-84	59-97	61-97	0.85-1.15	0.64-1.36	0.05-1.6	–

FPD/AML: familial platelet disorder with predisposition to acute myelogenous leukemia; BAT: 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. The age of the proband is given in years. Values in affected relatives (n=3 for surface GPIa, MYH10 expression and *RUNX1* 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 1D), confirming mosaicism at the hematopoietic stem cell level. A bone marrow biopsy was not performed. In order to quantify *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 the *RUNX1* VAF (20%-5%) (Table 1 and Figure 1E). In addition, the c.8560C>T (p.Arg2854Cys) single nucleotide variation in the *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 (*Online Supplementary Table S2*). Although moderate abnormalities in platelet aggregation, involving mainly ADP and

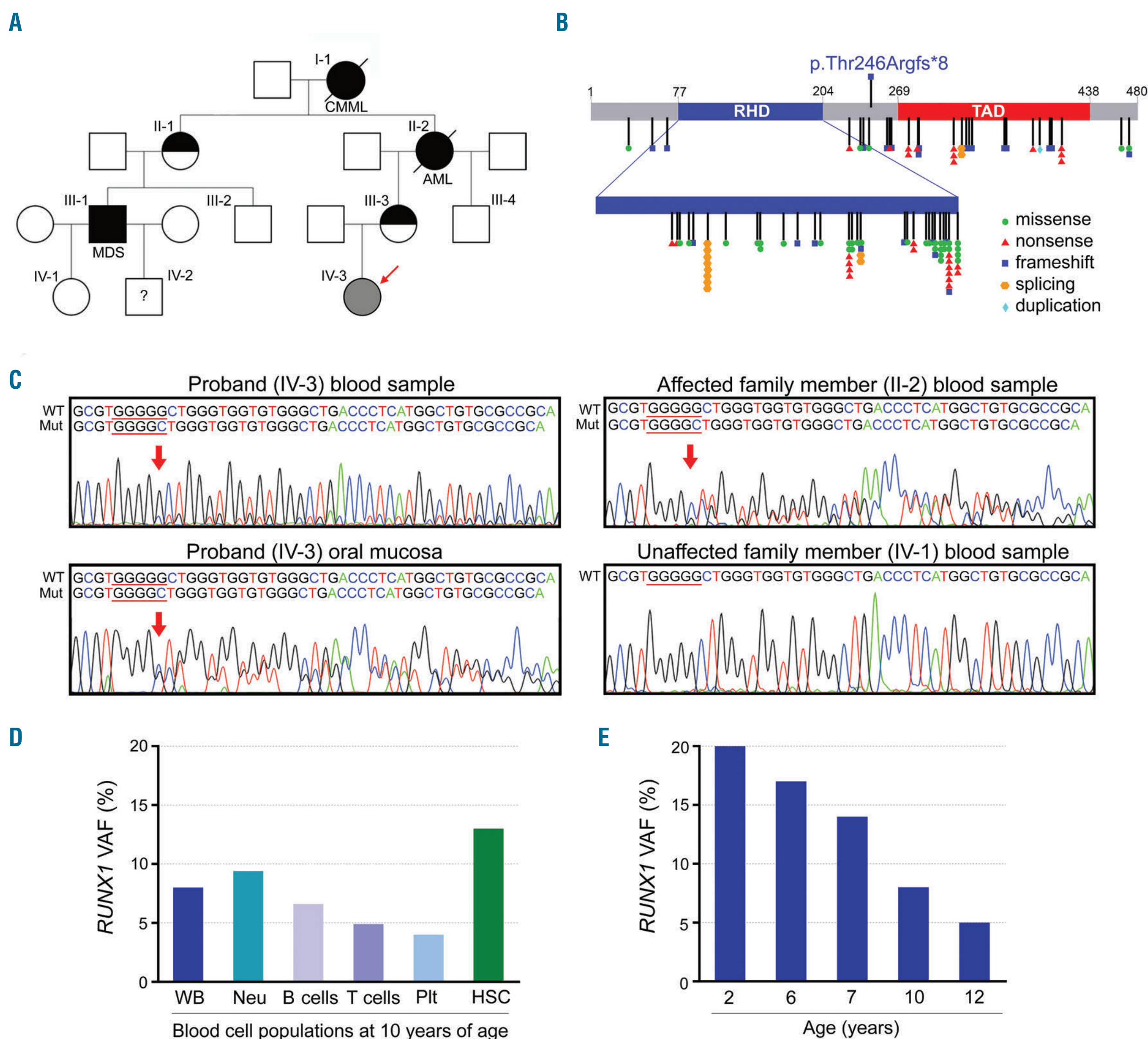

Figure 1. Molecular features of the proband and familial platelet disorder with predisposition to acute myelogenous leukemia (FPD/AML) pedigree. (A) FPD/AML pedigree. Half-filled symbols represent affected individuals presenting with platelet defects and filled black symbols represent patients with platelet defects who developed myeloid malignancies; empty symbols, individuals with a normal *RUNX1* genotype; the question mark depicts unknown mutation status. The proband is shown in gray and indicated by the arrow. CMML: chronic myelomonocytic leukemia; AML: acute myelogenous leukemia; 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 the *Online Supplementary References S7-23*), 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 (Plt) were purified from peripheral blood and CD34+ hematopoietic 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.

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 a moderate dense-granule deficiency and a mild decrease in GPIa at the age of 6 years, when the *RUNX1* VAF was 17%, whereas both parameters tended to reach normal levels at the age of 10 years, when the VAF was 8% (Table 1 and *Online Supplementary Figure S1*). Although no clear-cut relationship was evident between the *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 (*Online 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 *Online 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,[13] as highlighted by this report. Mosaicism can be found in a wide range of Mendelian genetic disorders

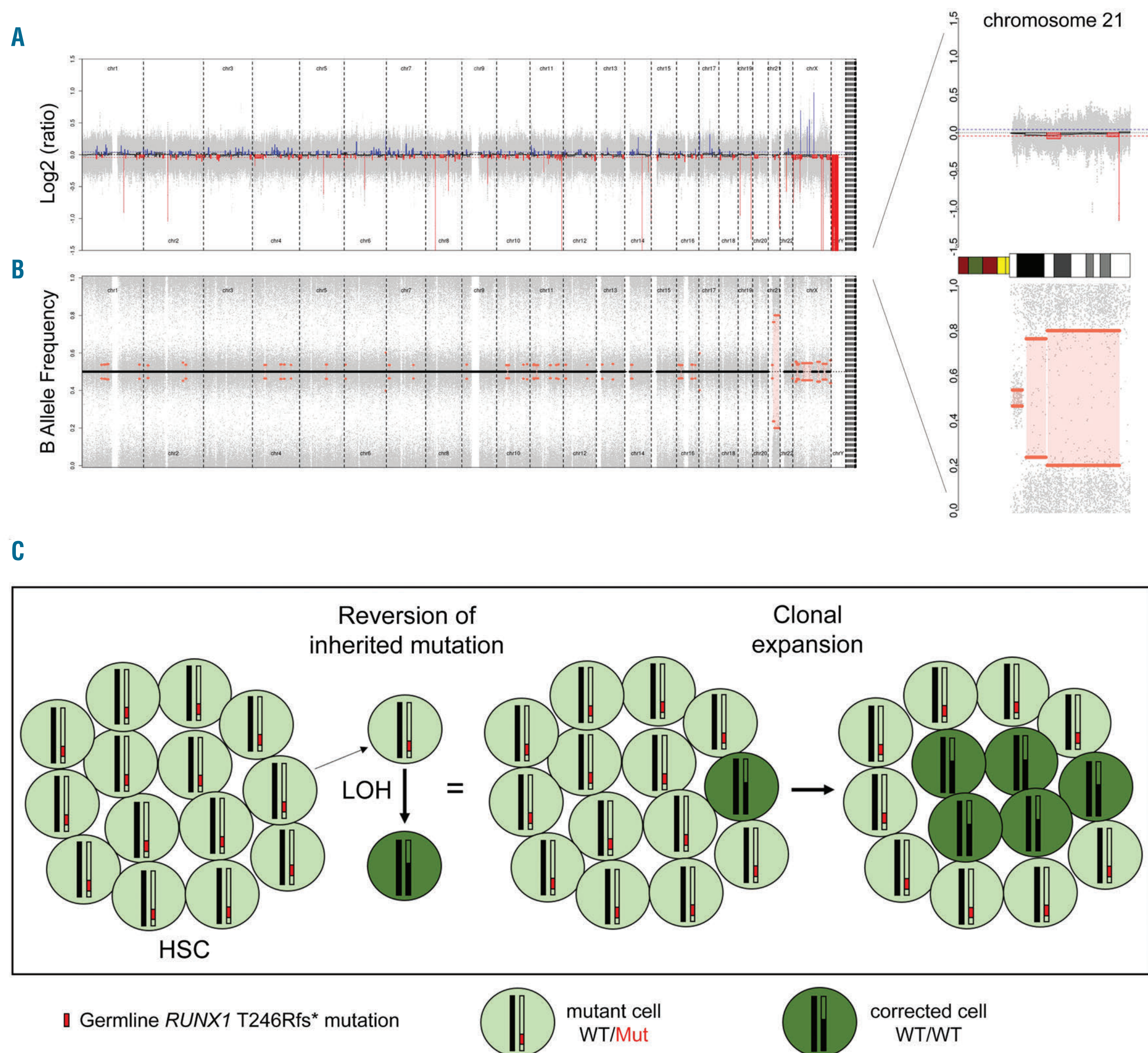

Figure 2. Loss of heterozygosity. (A-B) SNP array profile. (A) Log2ratio profile showing the absence of copy number variation. (B) Biallelic frequency profile showing an unbalanced ratio of chromosome 21. The right panel shows an enlarged view of chromosome 21 profile. (C). Proposed model of mosaicism development in familial platelet disorder with predisposition to acute myelogenous leukemia (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 the duplication of the paternal wild-type (WT) *RUNX1* allele with restoration of two WT copies of the gene (depicted in black in corrected cells) and resulting in a mixed population of mutant and corrected cells. Reverted hematopoietic stem cells (HSC) underwent selective advantage and clonal expansion, whereas cells harboring the mutant allele decreased progressively over time.

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 a selective advantage of the rescued clone.[14,15] This phenomenon may involve the 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 hematological diseases, such as Wiskott-Aldrich syndrome, Fanconi anemia and several immunodeficiencies.[14,15] In this patient, mosaicism involved the hematopoietic stem cell and mature blood cells, indicating genetic reversion in the hematopoietic 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 hematopoiesis,[1] it may be envisioned that RUNX1-corrected hematopoietic stem cells might have experienced growth advantage *versus* RUNX1-mutant cells, leading to the 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 the 12-year follow-up, as shown by the 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 hematopoietic 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.[15] 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 anemia,[15] 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 leukemia 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.[16] 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 leukemic transformation. Although the follow-up is short, it is tempting to speculate that the risk of leukemia might be attenuated in this case. However, the remaining pool of RUNX1-mutant cells, while comprising a low proportion of hematopoietic 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 a higher risk of leukemic transformation.[1]

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 hematopoietic cells may be particularly susceptible to suffer somatic rescue events. In this setting, FPD/AML may now be added to the growing list of hematologic genetic disorders associated with revertant mosaicism.

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