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Familial platelet disorder due to germline exonic deletions in RUNX1: a diagnostic challenge with distinct alterations of the transcript isoform equilibrium

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ABSTRACT
Germline pathogenic variants in RUNX1 are associated with familial platelet disorder with predisposition to myeloid malignancies (FPD/MM) with intragenic deletions in RUNX1 accounting for almost 7% of all reported variants. We present two new pedigrees with FPD/MM carrying two different germline RUNX1 intragenic deletions. The aforementioned deletions encompass exons 1–2 and 9–10 respectively, with the exon 9–10 deletion being previously unreported. RNA sequencing of patients carrying the exon 9–10 deletion revealed a fusion with LINC00160 resulting in a change in the 3’ sequence of RUNX1. Expression analysis of the transcript isoform demonstrated altered RUNX1a/b/c ratios in carriers from both families compared to controls. Our data provide evidence on the impact of intragenic RUNX1 deletions on transcript isoform expression and highlight the importance of routinely performing copy number variant analysis in patients with suspected MM with germline predisposition.

INTRODUCTION
Familial platelet disorder with predisposition to myeloid malignancies (FPD/MM, OMIM 601399) is a rare autosomal-dominant inherited disorder caused by germline heterozygous pathogenic variants in the Runt-related transcription factor 1 (RUNX1) gene [1–4]. FPD/MM is associated with various grades of thrombocytopenia, while almost 40% of all cases develop a hematological malignancy, primarily acute myeloid leukemia (AML), or myelodysplastic syndrome (MDS) [1]. Interestingly, pathogenic variants within the RUNX1 gene are somatically acquired in up to 10–15% of sporadic MDS [5]. In AML, somatic pathogenic variants in RUNX1 are reported in 4–16% of cases and define a novel provisional AML entity associated with worse prognosis for de novo AML in the absence of recurrent genetic abnormalities [2,6].

The RUNX1 gene acts as a transcription factor involved in the regulation of hematopoiesis [7]. The gene has three main transcript isoforms, regulated by two promoters and alternative splicing (Figure 1(A)). In intron 1, an enhancer has been identified [8,9]. RUNX1a is the shortest form and its expression is, together with RUNX1b, regulated by a proximal promoter. RUNX1a is the only isoform that expresses exon 8. RUNX1c is regulated through a distal promoter and is the longest isoform, consisting of exons 1–7, 9, and 10. The RUNX1 protein contains a DNA-binding domain, the RUNT domain, and a transactivating domain (Figure 1(B)). In addition to binding DNA, the RUNT domain binds co-transcription factors [7].
Pathogenic germline variants in RUNX1 were first reported in 1999 in six pedigrees, one of which was an intragenic deletion of exons 4–5, while the remaining were either nonsense (n = 3) or missense mutations (n = 2) located in the RUNT domain [10]. To date, large deletions in RUNX1 have been reported in 38 of 153 cases with FPD/MM included in the Human Gene Mutation Database (HGMD Professional 2021.3), with 21 of them (55%) referring to deletions including the whole RUNX1 gene [11]. The same number of gross deletions is reported in the RUNX1 database (RUNX1db) where the reported number of pathogenic variants is 164 [11]. Intragenic deletions accounted in the past for only a minor proportion of the germline gross deletions in the RUNX1 gene, mainly due to methodological issues that did not allow for their detection. Recently however, the number of cases carrying intragenic deletions has increased dramatically [3,11–16], reaching today up to 6.5% of all described RUNX1-related FPD/MM, according to the RUNX1db [17]. Interestingly, little is known regarding the alterations resulting from such deletions on the transcriptome level. Here, we report two new pedigrees with FPD/MM carrying germline intragenic deletions in RUNX1 and we investigate the effect of their deletions on exon boundary and transcript isoform expression.

Materials and methods

Patients and samples

The patients included in the study belong to the 150 families that have been referred to our department (Department of Clinical Genetics, Uppsala University Hospital) for genetic counseling and investigation due to suspected predisposition for myeloid malignancies (MM) during the last 3 years. DNA was collected from peripheral blood, cultured fibroblasts, buccal swabs, or bone marrow specimens. For RNA-based exon boundary expression and transcript isoform ratio analyses, mononuclear cells from blood were used. Depending on the indication, these 150 families were either investigated with targeted panels or whole exome sequencing (WES) including analysis for the detection of deletions and duplications (del/dup analysis) with arrays or Multiplex Ligation-dependent Probe Amplification (MLPA). The methodologies used in the analysis of the pedigrees included in this report are described in detail below. All samples were collected at Uppsala University hospital in accordance with the Declaration of Helsinki following informed consent from the patients. The study was approved by the ethical board at Gothenburg University (Dnr: 2019-05635).

Multiplex Ligation-dependent Probe Amplification

Multiplex Ligation-dependent Probe Amplification for the detection of deletions or duplications was performed using the MLPA kit Familial MDS-AML P437-A1 (MRC-Holland, Amsterdam, The Netherlands). The kit includes probes for GATA2 (exons 1–8), TERC (exon 1), TERT (exons 1–3, 5–8, 10–16), CEBPA (exon 1), and RUNX1 (exons 1–10). Details of probe locations are available upon request.

SNP-array analysis

SNP-array analysis was performed with a CytoScan HD Array Kit and reagent kit bundle (Thermo Fisher, Waltham, MA), a CytoScan HD Chip (Thermo Fisher, Waltham, MA) and a Titanium DNA Amplification kit (Takara Bio Inc., Kusatsu, Japan). The chip was scanned using a GeneChip Scanner 3000 7G (Thermo Fisher, Waltham, MA). Analysis of copy number aberrations was performed with the Chromosome Analysis Suite (Thermo Fisher, Waltham, MA).

Next-generation sequencing analysis

Next-generation sequencing (NGS) analysis of genomic samples for somatic pathogenic variants was performed using the TruSight myeloid panel (Illumina, San Diego, CA) library kit followed by sequencing on a MiSeq instrument using reagent kit version 3 (Illumina, San Diego, CA). Variants were called using Ingenuity Variant analysis and QIAGEN Clinical Insight (QCI) Interpret (QIAGEN, Hilden, Germany). Details of the genes and exons included in the panel are listed in...
**Supplementary Table 1.** Details regarding filtering of variants are available upon request.

Targeted sequencing of RNA for the evaluation of *RUNX1* gene fusions was performed using the Archer™ FusionPlex™ Heme Panel v2 for the Illumina Platform. Library preparation was carried out according to the manufacturer’s instructions (ArcherDX, Boulder, CO) and 200 ng total RNA was used as input material. Libraries were sequenced by pooling four samples, at a concentration of 18 pM, using sequencing kit version 2 on a MiSeq instrument (Illumina, San Diego, CA). *RUNX1* fusions were assessed using Archer analysis software version 6.0.3.2 (ArcherDX, Boulder, CO).

**Transcript isoform expression assay**

For the isoform expression assay, cDNA synthesis was carried out using 0.5–1 μg RNA and SuperScript IV VILO Master Mix according to the manufacturer’s instructions (Invitrogen, ThermoFisher Scientific, Waltham, MA). TaqMan® Gene Expression Assays were used with primer–probe mix with probes spanning exons 2–3, Hs01021966_m1, exons 6–7, Hs01021970_m1, exons 7–9, Hs01021971_m1, exons 7–8, Hs04186042_m1, and exons 9–10, Hs0231079_m1 (Invitrogen, ThermoFisher Scientific, Waltham, MA). GUS was used as a reference gene, primer probe sequence: forward primer: GAAAATATGTGGTGGAGAGCTCATT, reverse primer: CCAGCAGCTCTCGTGGTAGCTTCA, and probe: CCAGCAGCTCTCGTGGTAGCTTCA. Real-time PCR was performed using a TaqMan Universal Master Mix (Applied Biosystems, ThermoFisher Scientific, Waltham, MA) and an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, ThermoFisher Scientific, Waltham, MA) according to the manufacturer’s instructions. Patient data were compared to data generated using blood samples from two healthy donors (one male and one female between 40 and 50 years old), and all samples were run in triplicate on the same plate. Assuming equal efficiency of the TaqMan® Gene Expression Assays, the expression of each exon boundary was calculated using the delta Ct-method with GUS as a reference gene using the formula $2^{-\Delta\Delta Ct\text{ exon boundary}-\Delta Ct\text{ GUS}}$ [18]. Real-time PCR data using the primer–probe mix spanning exons 6–7 was considered as the total *RUNX1a/b/c* expression since this exon junction is present in all *RUNX1* isoforms and is not deleted in any of the pedigrees. The expression of all investigated exon boundaries was calculated by dividing the relative expression of each exon boundary with the relative expression of total *RUNX1a/b/c* to normalize for differences in total *RUNX1a/b/c* expression between tested samples. Expression of the *RUNX1* isoforms *RUNX1a* and *RUNX1c* was extrapolated from real-time PCR data using the primer–probe mix spanning exons 7–8 and exons 2–3, respectively. For the *RUNX1b* transcript isoform, no unique exon-usage compared to the other isoforms is present. Thus, in order to calculate the expression of *RUNX1b*, the relative expression of the primer–probe mix spanning exons 7–8 (*RUNX1a*) and exons 2–3 (*RUNX1c*) was subtracted from the relative expression of the primer probe mix spanning exons 6–7 (total *RUNX1a/b/c*). The ratio of the transcript isoforms was calculated by dividing each isoform expression with the combined expression of the other two isoforms.

**Results**

**Pedigree A**

In pedigree A (Figure 2(A)), index patient V:1 was first referred for genetic investigation/counseling at the age of 6 due to thrombocytopenia (100 × 10^9/L) presenting at the age of 3 when she was diagnosed with immune thrombocytopenia (ITP). IV:3 was also diagnosed with thrombocytopenia from birth and at the age of 4 she developed AML, for which she underwent allogeneic hematopoietic stem cell transplantation (allo-HSCT) with IV:1 as the donor. III:5 was diagnosed with MDS at the age of 46 and underwent allo-HSCT with III:1 as the donor. Unfortunately, 6 years after the transplantation both III:1 and III:5 died due to MM (data regarding the actual nature of these myeloid neoplasms are not available). The chimerism analysis at the time of the development of the MM in III:5 showed only alleles from III:1, indicating a donor-derived neoplasia. At the first referral (2013) for V:1, sequencing of *RUNX1* and ANKRD26 was performed with no pathogenic variants detected. Five years later, V:2 at the age of 3, also showed signs of thrombocytopenia/thrombocyte defect in the form of easy bruising. NGS using a myeloid gene panel (TruSight myeloid panel from Illumina, San Diego, CA, details provided in Supplementary Table 1) including 54 genes (among them *RUNX1* and *ETV6*) was performed on V:1, but no pathogenic variant was found. Further analysis of *RUNX1* with MLPA (familial MDS-AML panel, details provided in section ‘Materials and methods’) on peripheral blood and cultured fibroblasts from V:1 and III:5, respectively, revealed an intragenic deletion of exons 9 and 10 (Figure 3(A)). Analysis of a buccal swab from IV:3 showed a borderline result for *RUNX1* deletion of exons 9 and 10 (Supplementary Figure 1A). Contamination of blood cells in the buccal swab was suspected and a skin biopsy was performed confirming the deletion in
cultured fibroblasts from IV:3 (Supplemental Figure 1B). MLPA analysis of peripheral blood from V:2, also confirmed the deletion of exons 9 and 10. In addition, single nucleotide polymorphism (SNP) array analysis was performed on V:1 demonstrating a 24 kb deletion (arr[GRCh37] 21q22.12(36147494_36171830)x1) encompassing RUNX1 exons 9 and 10 but also part of the non-coding RNA, LOC100506385 (LINC01426, LincRNA uc002yug.2) on the opposite strand (Figure 3(B)).

**Pedigree B**

In pedigree B (Figure 2(B)), the index patient was III:1. She presented with thrombocytopenia (80–100 × 10^9/L) during childhood, while her sister (III:2) was diagnosed with MDS at the age of 16 and underwent allo-HSCT with an unrelated donor. According to their family history II:2 and I:1 also had low platelets. III:1 and I:1 were initially analyzed with NGS using the previously described myeloid panel. III:1 did not show any pathogenic variants, while I:1 carried a pathogenic variant in the BCO2 gene (NM_017745.5:c.3645delG, p.Thr1216fsX44) in 64% of the sequences using DNA from a peripheral blood sample. In addition, the MLPA panel for familial MDS/AML was performed using peripheral blood and cultured fibroblasts from I:1 and II:2 as well as III:1, respectively, demonstrating a deletion of RUNX1 exon 1 and 2 (Figure 3(C)).

**RUNX1 transcript isoform analysis**

To evaluate the phenotypic effect of the two different deletions seen in pedigree A and B on exon expression and RUNX1 isoforms, expression analyses with real-time PCR and primer–probe mixes spanning exon junctions 2–3, 6–7, 7–8, 7–9, and 9–10 were performed (Figure 4(A)). RNA from blood samples from a RUNX1 deletion-carrier from each pedigree (pedigree A V:1 and pedigree B I:1) was analyzed with the inclusion of healthy blood donors as controls. The relative expression of exons to total RUNX1 expression was calculated. In addition, the ratios of RUNX1a/b/c transcript isoforms were compared between patients and control samples. For the patient from pedigree A with exon 9–10 deletion, a reduced relative expression of exon boundaries 7–9 and 9–10 was detected (Figure 4(D,E)), as expected. Interestingly, higher levels of exon 7–8 boundary expression, specific for the RUNX1a isoform, were seen compared to controls (Figure 4(C)). Consistent with this, when comparing the RUNX1a transcript isoform ratios, the pedigree A patient sample showed a higher ratio of RUNX1a transcript isoform compared both to the controls and the pedigree B sample (Figure 4(F)). For the pedigree B
Figure 3. Exonic \textit{RUNX1} deletions in pedigrees A and B. (A) MLPA Familial MDS-AML analysis showed a heterozygous deletion of probes for exons 9 and 10, with peak height ratios of approximately 0.5. Red squares: target probes with deletion. Green squares: target probes without deletion. Blue squares: control region probes. Gray squares: mutation-specific probes and Y chromosome probe. (B) CytoScan HD array analysis verified the MLPA result and detected a 24 kb deletion of GRCh37:21q22.12 (36147494–36171830) encompassing exons 9 and 10 of \textit{RUNX1} and part of the \textit{LINCO1426}-gene. (C) MLPA Familial MDS-AML analysis showed a heterozygous deletion of probes for exons 1 and 2, demonstrated with peak height ratio of approximately 0.5. Red squares: target probes with deletion. Green squares: target probes with no deletion. Blue squares: control region probes. Gray squares: mutation-specific probes and Y chromosome probe. (D) CytoScan HD array analysis verified the MLPA result and showed a 221 kb deletion of GRCh37:21q22.12 (36289047–36510008) encompassing exons 1 and 2 of \textit{RUNX1}. The bars represent the weighted Log2 ratio. Note that the SNP arrays have no markers in a specific region around exon 1, resulting in a gap and causing the software to translate the weighted Log2 ratio close to 0, as seen here.
A lower relative expression of exons 2–3 was seen compared to controls and pedigree A, consistent with the exon 1–2 deletion present in this family (Figure 4(B)). A reduced RUNX1c transcript isoform ratio was also seen compared to controls and pedigree A (Figure 4(H)). In addition, a trend toward a higher expression of exon boundaries 7–9 and 9–10 was seen in the sample from pedigree B.
and the RUNX1b transcript isoform ratio was higher compared both to the controls and the pedigree A sample (Figure 4(D–E,G)).

For pedigree A, no obvious difference was seen for the RUNX1b or RUNX1c transcript isoform ratio compared to the controls. This indicates that these isoforms are present at similar levels despite the exon 9–10 deletion; however, they are likely partly truncated due to the reduced expression of exon 9 and 10. To investigate this further, targeted RNA sequencing was performed. For the patient from pedigree A, RNA sequencing data revealed the expression of a gene fusion between RUNX1 exon 7 and a non-coding RNA LINC00160, with 3537 reads spanning the breakpoint (Figure 5). The fusion resulted in a predicted frameshift from RUNX1 amino acid p.269 and a premature stop codon after 27 amino acids.

**Discussion**

MM with germline predisposition are a group of rare diseases, recently recognized as a novel dedicated entity in the latest World Health Organization (WHO) classification of MM and acute leukemia [2,13]. Among them, FPD/MM due to germline pathogenic variants in the RUNX1 gene are probably one of the best characterized. Identification of MM with germline predisposition is of crucial clinical significance not only for the patient, as it affects decisions related to donor choice or conditioning regimen, but also for the patient's family, as is the case for all tumor predisposition syndromes [19,20]. This can be challenging in the clinical setting as there is no consensus regarding the criteria defining which patients should be tested for these conditions or the diagnostic algorithm that should be followed [21].

We report two novel pedigrees with FPD/MM where the underlying germline genomic aberration is a deletion of RUNX1 encompassing either exons 1–2 or 9–10. Although the particular deletions detailed here have not previously been reported in the literature, RUNX1 exon 1–2 deletions have been described in previous studies (albeit with different breakpoints than in this study), with a total number of nine probands, which makes this region the second most common hotspot for pathogenic germline variants in RUNX1 (for comparison of breakpoints see Supplementary Table 2) [13–15,17,22–24]. Interestingly, these two families represent one-third of the six families with RUNX1-related FPD/MM that have been diagnosed at our department over the past three years, when a special screening program focusing on MM with germline predisposition was established. Based on all reported pedigrees so far, no higher penetrance for hematological malignancies among FPD/MM families due to RUNX1 exonic deletions is observed. Nevertheless, we do notice a rather strong trend for anticipation. Moreover, in our pedigrees, all carriers had thrombocytopenia, contrasting a recent report of FPD/MM due to deletion of exons 1–2 with different breakpoints than ours, where not all carriers exhibited low platelet count [15]. Lastly, it should be mentioned that the patients with FPD/MM who underwent allo-HSCT with an unrelated donor or a related non-carrier donor are in complete hematological remission, suggesting that timely allo-HSCT offers a curative option for these patients.

Intragenic deletions in RUNX1 have probably been underdiagnosed for years. Recently however, the number of reported cases has increased significantly and today they account for 45% of all reported germline gross deletions in RUNX1 [3,11,12,13,14,15,16,25]. Of note, similar copy number variants (CNVs) have been reported in other conditions related to germline predisposition to MM such as GATA2-related syndrome and ETV6-related thrombocytopenia [26,27]. Deletions in RUNX1 have been also reported in studies characterizing the clonal population in patients with MM, although no germline material was tested in order to provide proof of somatic origin [28].

In everyday clinical practice, most laboratories perform diagnostics using Sanger sequencing or NGS,
with workflows that are mainly developed for the detection of single nucleotide variants (SNVs) or insertions/deletions of a small number of nucleotides. Such methods are usually limited by the size of the reads for the detection of larger CNVs. Even with the advent of WES, the detection of larger CNVs demands specific bioinformatics approaches. Therefore, the investigation of CNVs, at least at the exon level, has been based mainly on the performance of MLPA. Nowadays, microarrays with increased exon coverage hold promise for the identification of such CNVs. The high reporting rate of intragenic deletions the last 2–3 years indicates that the majority of clinical hematopoietic laboratories complement sequencing analysis with methods that allow for the detection of such aberrations.

Another caveat of routine diagnostics is the analysis of patients with MDS or AML using gene panels designed for the detection of somatic variants. A negative result with such a gene panel should by no means be considered ‘safe’ for the exclusion of germline conditions as many regions with enrichment for germline variants are not well covered. With that being said, the investigation of patients with previously identified germline variants that predispose to MM using targeted somatic panels may be useful in detecting clonal hematopoiesis which usually precedes the development of MM, at least in a fraction of cases [20].

Interestingly, RNA expression data revealed that the two distinct deletions in the families resulted in phenotypic effects altering the isoform expression levels of RUNX1. In pedigree A, an increased ratio of RUNX1a was seen compared to the controls and pedigree B (Figure 4(F)). Studies have demonstrated an increased RUNX1a isoform expression in patients with MM while RUNX1a-transduced mice developed leukemia [29,30]. Furthermore, increased RUNX1a isoform expression has been shown to enhance self-renewal activity and hematopoietic stem/progenitor cell expansion in mice (both ex vivo and in vivo) [31]. As the RUNX1a isoform has higher affinity for DNA-binding compared to RUNX1b and lacks the C-terminal regulatory domains, this isoform may act as a dominant-negative regulator of the other two isoforms [32].

Different types of pathogenic RUNX1 variants have been described: (i) mutations that truncate the RUNX1 protein’s N-terminal to or within the DNA-binding domain thereby inactivating the protein, (ii) mutations that disrupt DNA-binding thereby acting as weakly dominant negative mutations, and (iii) dominant negative mutations that remove the C-terminal transactivation domain, allowing mutated RUNX1 to bind DNA and presumably block transactivation by full length RUNX1 proteins [33–35].

In pedigree A, our RNA sequencing result shows that the RUNX1b/c isoforms are expressed, but that they form a RUNX1 transcript fused with a non-coding RNA, LINC00160 (Figure 5), resulting in a frameshift and an early stop codon and thereby an mRNA with an altered sequence, lacking exons 9 and 10. Looking at the isoform ratios of the pedigree A patient, no obvious differences were seen between the RUNX1b/c isoform ratios compared to controls. This may reflect the notion that no nonsense mediated decay of the fusion transcript occurs. If translated, this gene fusion would result in a protein lacking the transcription-activating domain, thus acting in a dominant negative manner by presumably blocking the binding of full length RUNX1. This closely resembles what is commonly seen in somatic frameshift mutations in exons 6–10 of RUNX1 in MM and is most likely mechanistically similar [5].

For pedigree B, a lower ratio of the RUNX1c isoform was observed when compared to all other samples (Figure 4(H)). This was expected, since the RUNX1c isoform is regulated through the distal promoter which is deleted in this case. Interestingly, we observed a higher ratio of RUNX1b isoform (Figure 4(G)), which could be attributed to the decreased RUNX1c ratio. Nevertheless, a trend toward an increased expression of exon boundaries 7–9 and 9–10 was also observed (Figure 4(D,E)). Draper et al. demonstrated that absence of RUNX1c in knock-in adult mice with RUNX1b expression solely causes defective megakaryopoiesis and thrombocytopenia and suggests that RUNX1b and RUNX1c isoforms have distinct and specific roles in adult megakaryopoiesis despite a high degree of structural similarity [36]. Thus, changes in RUNX1b and RUNX1c levels may be causative of the impaired megakaryopoiesis seen in the carriers in pedigree B. It is important to note that in the isoform ratio analyses, only one family member from each pedigree has been studied and the isoform ratios may be affected by differences in age and blood status. Therefore, our results should be considered with caution. For example, one of the control samples showed a significantly lower RUNX1a ratio compared to all other investigated samples (Figure 4(F)). However, differences in total RUNX1 expression have been taken into consideration in the analyses and only blood samples from non-transplanted individuals with no diagnosed neoplasia were used.

In conclusion, we report two new pedigrees with germline exonic deletions in RUNX1. Our analyses
demonstrated that the deletions resulted in changes in RUNX1 exon boundary and transcript isoform expression in deletion-carriers compared to controls. Acknowledging the rather low number of cases with FPD/MM diagnosed at our department to date, we would like to highlight the need to adjust diagnostic approaches in order not to miss RUNX1 exonic deletions, which appear to be more prevalent than previously thought.

**Disclosure statement**

The authors declare no conflicts of interest.

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