***RUNX1* Mutations Can Lead to Aberrant Expression of CD79a and PAX5 in Acute Myelogenous Leukemias: A Potential Diagnostic Pitfall**

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Established Facts

- Acute myelogenous leukemia (AML) with *RUNX1-RUNX1T1* fusion frequently aberrantly expresses the B cell antigens CD19, PAX5, and CD79a. This is due to net inactivation of the myeloid master regulator PU.1 by the chimeric *RUNX1-RUNX1T1* protein.
- AML with the *RUNX1* mutation is a provisional AML sub-entity in the WHO 2017 classification. Such mutations are commonly observed in mixed-lineage leukemia (MLL), and in *FLT3*-ITD and +13 and +21 AML.

Novel Insights

- *RUNX1* mutant AML can aberrantly express the B cell antigens CD79a and/or PAX5, similarly to AML with *RUNX1-RUNX1T1* fusion.
- CD79a and PAX5 expression combined with myeloperoxidase negativity or mere detectability in *RUNX1* mutant AML is a potential classificatory pitfall that may lead to misclassification of respective cases as acute lymphoblastic leukemia (ALL).
- The expression of B cell markers in *RUNX1* mutant AML may be linked to deregulation of the myeloid master regulator PU.1, similarly to what is known for AML with *RUNX1-RUNX1T1* fusion.

Keywords

RUNX1 · CD79a · PAX5 · Acute myelogenous leukemia · Acute lymphoblastic leukemia

Abstract

Background: *RUNX1* is a crucial transcription factor for hematological stem cells and well-known for its association with acute lymphoblastic leukemia (ALL) and acute myelogenous leukemia (AML). Besides the translocation t(8;21) that leads to the *RUNX1-RUNX1T1* fusion, somatic mutations of *RUNX1* have been discovered. **Methods:** Four bone marrow trephine biopsies of patients with CD79a-positive and/or PAX5-positive acute leukemias were investigated by immunohistochemistry (IHC), karyotyping, and next-generation sequencing-based genetic analysis. Data were then compared to a historical collective of AML (*n* = 42) and 42 cases of AML newly diagnosed at our institution between June

enous leukemia (AML). Besides the translocation t(8;21) that leads to the *RUNX1-RUNX1T1* fusion, somatic mutations of *RUNX1* have been discovered. **Methods:** Four bone marrow trephine biopsies of patients with CD79a-positive and/or PAX5-positive acute leukemias were investigated by immunohistochemistry (IHC), karyotyping, and next-generation sequencing-based genetic analysis. Data were then compared to a historical collective of AML (*n* = 42) and 42 cases of AML newly diagnosed at our institution between June

2017 and May 2018. **Results:** We report on 4 cases of acute leukemia with an equivocal immunophenotype showing expression of CD79a and/or PAX5, which led to a preliminary histopathologic classification as probable ALL/unclassifiable acute leukemia. All cases were positive for CD34 and TdT but negative for several myeloid markers on IHC. Mutational analysis revealed point mutations and indels of *RUNX1* and further mutations typical for AML such as *TET2*, *DNMT3A*, and *SRSF2*, and 2 cases had tetrasomy 13 characteristic of *RUNX1* mutant AML. **Conclusion:** Aberrant CD79a and/or PAX5 expression can be found in AML cases with *RUNX1* mutations even without the translocation t(8;21). Our series shows the expression of CD79a and PAX5 to be a potential pitfall in the classification of *RUNX1* mutant acute leukemia.

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Runt-related transcription factor 1 (*RUNX1*) (formerly known as acute myeloid leukemia 1 protein [AML1]) is crucial for hematopoietic stem cells [1] and plays an oncogenic role in both acute lymphoblastic leukemia (ALL) and acute myelogenous leukemia (AML) [2]. The best-studied aberration of *RUNX1* in AML is t(8;21)(q22;q22), generating the fusion protein RUNX1-RUNX1T1 (formerly described as AML1-ETO), resulting in a loss of function/repression of normal RUNX1 [3]. This translocation and t(12;21)(p13;q22) in ALL are considered to be initiating events in leukemogenesis [4]. AML with t(8;21) can aberrantly express B cell markers, especially CD19, CD79a, and PAX5 [5, 6].

Besides t(8;21), different types of *RUNX1* somatic mutations have been more recently discovered in myeloid malignancies [2]. They all result in a loss of function of RUNX1 and mainly affect the Runt homology domain, which is responsible for the DNA-binding and transcriptional activity of RUNX1 [7]. In contrast to AML with t(8;21), these cases are associated with a worse prognosis, referred to by the WHO as a provisional entity “AML with mutated *RUNX1*” [8]. The negative impact of *RUNX1* mutations has also been very recently confirmed in AML in children [9]. Contrary to t(8;21)(q22;q22), *RUNX1* mutations are rarely discovered as initiating mutations in AML, and are therefore considered secondary events driving the progression of the disease [10]. They are also rare in clonal cytopenia of undetermined significance (CCUS) and overt myelodysplastic syndromes and have not yet been observed in clonal hematopoiesis of indeterminate potential (CHIP) [4]. *RUNX1* germline mutations are associated with “familial platelet disorder with predisposition to AML” (FPD/AML), and implicate a 44% life-

time risk of developing myelodysplastic syndrome or AML [11], but they usually also affect domains other than the Runt homology domain.

We report on a case series of 4 patients with CD79a-positive and/or PAX5-positive, difficult-to-classify AML, with mutated *RUNX1* (Table 1). Cases were investigated by immunohistochemistry (IHC), karyotyping, and next-generation sequencing (NGS)-based panel sequencing, and then compared to a historical collective of AML ($n = 42$) stained for the B cell markers CD19, CD20, CD79a, and PAX5 [12] as well as 42 AML cases newly diagnosed in routine practice at our institution between June 2017 and April 2018.

These 4 cases with an equivocal immunophenotype, sharing negativity for myeloperoxidase (MPO; 3/4 cases determined on IHC), CD19, and CD20, but expressing CD79a (clone SP18) and/or PAX5 (clone SP34), were preliminary histopathologically classified as probable ALL or unclassifiable acute leukemia, most probably AML or mixed-phenotype acute leukemia (MPAL). All were positive for CD34 and TdT, all (but one) were negative for the additional myeloid marker CD11c, and all were negative for CD14 and CD117 on IHC (Fig. 1). On flow cytometry (FCM), the blasts of all cases were positive for the myeloid markers CD33, CD13 ($\pm$), CD117 ($\pm$ and dim), and CD15 (2/4 cases), and negative for CD19, CD20, CD10, CD22, and cCD79a. FCM thus favored AML (Table 1).

NGS analysis was performed on bone marrow DNA, isolated using QIAasympy (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. Patient NGS libraries were prepared using the AML community panel from ThermoFisher (Waltham, MA, USA) containing 19 genes frequently mutated in AML (www.ampliseq.com), sequenced using the Ion S5 platform, and analyzed on the Ion Reporter AML pipeline (ThermoFisher). The average coverage per sample was >3,000. Sensitivity for calling a mutation was set at 3%. Mutational analysis revealed deleterious (frameshift and non-sense) or expected deleterious double or single mutations of *RUNX1*, i.e., G165fs and E86fs (with a variant allelic frequency [VAF] of 46 and 48%, respectively), R166* (VAF of 14%), R166Q and D160E (VAF of 15 and 18%, respectively), and W106C (VAF of 86%), with a pathogenic score of at least 0.86. The VAF of mutant *RUNX1* in the last 3 patients strongly argued against germline mutations. While bearing *RUNX1* mutations with a VAF close to 50%, the first patient presented with AML at the age of 84 years and had no history of hematological illness, particularly not of an inherited bleeding disorder,

Table 1. Characteristics of 4 cases

	Patient #1	Patient #2	Patient #3	Patient #4
Age at presentation	84	63	48	74
Sex	male	male	female	male
FAB AML classification	M0	M0	M1	M0
Chemotherapy regimen	1 cycle of azacitidine	induction therapy (idarubicin and cytarabine)	HOVON 132 (lenalidomide arm)	azacitidine
HCT	none	allogenic	allogenic	none
Current status	died of progressive disease and infectious complications 3 months after initial diagnosis	complete remission 2 months after HCT	complete remission 1 year after HCT	2nd cycle of azacitidine
<i>Immunohistochemistry</i>				
Positive markers	CD34, CD79a , CD123±, PAX5 , TdT	CD34, CD79a , LMO2, TdT	CD11c dim, CD34, CD79a , CD117 dim, CD123±, CD303±, TdT	CD11c, CD34, CD79a , CD123±, MPO±, PAX5 , TdT
Negative markers	CD3, CD19, CD20, CD303, MPO	CD3, CD11c, CD14, CD20, CD68, CD123, CD303, MPO, PAX5	CD3, CD14, CD20, CD61, CD68, MPO, PAX5	CD3, CD20, CD117, CD303
<i>Flow cytometry</i>				
Positive markers	CD4, CD7±, CD9±, CD13, CD15, CD25, CD33, CD34, CD38, CD117±, CD123, cTdT, HLA-DR	CD7, CD11b±, CD33, CD34, CD38, CD71±, CD117±, CD123, cTdT±	CD4 dim, CD13, CD15±, CD34, CD71 dim, CD117, CD123±, HLA-DR, cMPO (13%), cTdT	CD7±, CD13, CD34, CD38, CD71, CD117, CD123, cTdT, HLA-DR
Negative markers	CD3, CD9, CD10, CD11b, CD14, CD16, CD19, CD21, CD35, CD36, CD41, CD42a, CD56, CD58, CD64, CD79a, CD105, cMPO, CD203, CD300e	CD1a, CD2, cCD3, CD3, CD4, CD5, CD8, CD9, CD10, CD13, CD14, CD15, CD16, CD19, CD22, CD25, CD35, CD36, CD41, CD42a, CD42b, CD56, CD61, CD64, cCD79a, CD105, CD203c, CD300e, HLA-DR, cMPO, NG2	CD2, cCD3, sCD3, CD7, CD10, CD14, CD16, CD19, CD33, CD35, CD36, CD64, cCD79a, CD79b, CD56, CD300e, lactoferrin, lysozyme	CD3, CD4, CD10, CD11b, CD14, CD15, CD16, CD19, CD20, CD21, CD22, CD33, CD35, CD36, CD56, CD64, CD79a, CD105, CD203, CD300e, cMPO
<i>Genetic analysis</i>				
NGS panel analysis	<i>RUNX1</i> (insCAGGGGCT, G165fs; insA, E80fs) <i>SRSF2</i> (G/A, P95L) <i>BCR</i> (3' splice site, G/A)	<i>RUNX1</i> (G/A, R166*) <i>DNMT3A</i> (C/T, R882H) <i>TET2</i> (delC, H717fs)	<i>RUNX1</i> (C/T, R166Q; G/C, D160E) <i>DNMT3A</i> (C/T, W860X) <i>NRAS</i> (C/T, D160E)	<i>RUNX1</i> (G/T, W106C) <i>NRAS</i> (A/C, Q61H) <i>PTPN11</i> (T/C, S502P) <i>SRSF2</i> (C/A, P95H)
Cytogenetics	48,XY,+13,+13	46,XY	46,XX,del(11)(p11.2p15)	48,XY,+13,+13

Bold type denotes B cell markers aberrantly expressed on AML blasts. AML, acute myelogenous leukemia; MPO, myeloperoxidase; FAB, French-American-British; HCT, hematopoietic cell transplantation; NGS, next-generation sequencing.

and G165fs was near to the well-known R166 mutational hot-spot in AML, all of which argued against germline mutations of *RUNX1*. Indeed, there are no germline variants reported at codon 165 or 166 that would be compatible with the mutations observed in this study. In FPD/AML, >40 different *RUNX1* mutations are described and show varied distribution at many different sites [13], but they are expected to occur with a VAF near to 50 or 100%. Additional prototypic mutations of AML such as *DNMT3A*, *NRAS*, and *SRSF2* in 2 patients, and *PTPN11* and *TET2* in 1 patient each, respectively, were detected (Table 1); these mutations as well as the tetrasomy 13 of cases 1 and 4 being prototypic for *RUNX1*-mutated AML

subcohorts of larger studies [14]. All cases were devoid of t(8;21). In our previously studied collective of 42 AML cases [12], only 2 cases showed an expression of CD19 and PAX5, both of them harboring t(8;21). Neither CD79a-positive nor PAX5-positive cases were detected in the 42 *RUNX1* mutation- and t(8;21)-negative newly diagnosed AML cases in our routine practice between June 2017 and May 2018.

Expression of CD19, CD79a, and PAX5 in AML with t(8;21) is well documented in the literature [5, 6]. With the case series presented here, we show that aberrant CD79a and PAX5 expression can also be recurrently found in AML with *RUNX1* mutations in the absence of

Fig. 1. **a** Morphology showing a diffuse infiltrate of blastoid cells resembling lymphoid rather than myeloid blasts. HE, $\times 200$. The blastoid cells show expression of CD34 (**b**, $\times 200$), CD79a (**c**, $\times 200$; clone SP18), and focally PAX5 (**d**, $\times 360$; clone SP34).

t(8;21). The reason why CD79a was not detected by FCM could have been the use of a different clone (HM57), which binds to an epitope different from what the clone SP18 used for IHC binds (SP18: epitope at the N-terminal extracellular region of CD79a; HM57: epitope within the cytoplasmic domain of CD79a).

Importantly, translocations and mutations lead to a loss of function of RUNX1, thus postulating a protective, antileukemogenic effect of functional RUNX1. The leukemogenic effect of RUNX1 alterations expands beyond its role as a transcription factor; RUNX1 alterations also play a role in DNA damage repair and cell cycle control (review [4]). Cell culture experiments have shown that the RUNX1-RUNX1T1 fusion protein interferes with DNA repair, mainly by depressing genes related to base-excision repair and double-strand break repair (mainly homologous recombination). Mutant, and thus inactivated, RUNX1 has negative effects on the Fanconi anemia (FA)/Breast Cancer (BRCA) susceptibility pathway.

RUNX1 mutations may also foster the survival of mutated hematopoietic stem cells by reducing p53 protein levels and activity as mutant RUNX1 can no longer act as a co-factor of p53.

In t(8;21)-positive AML, the mechanism of PAX5 (and, consecutively, CD79a) expression has already been uncovered. Ray et al. [15] demonstrated, in cell line models, that PAX5 is not a direct target of the chimeric RUNX1-RUNX1T1 but is activated by aberrant mitogen-activated protein kinase (MAPK) signaling. This is a result of the chimeric protein's action, leading to a dissociation of the Polycomb complex. As some, but not all, patients with t(8;21) have *CKIT* mutations, which can explain the activation of MAPK signaling as well, another putative mode of action has been assumed by the induction or repression of various miRNAs by mutant/altered RUNX1 [16]. Finally, chimeric RUNX1-RUNX1T1 proteins inactivate the myeloid master regulator transcription factor PU.1, disturbing the cellular identity program

and leading to differentiation stop, which may contribute to indel (B cell) lineage marker expression [17].

The aberrant B cell marker expression observed by us in MPO-negative or merely positive RUNX1 mutant AML is an important potential pitfall for the differential diagnosis of ALL, showing the significance of including FCM and genetic data in the integrative diagnosis of leukemias. FCM could render the diagnosis of AML versus ALL with greater accuracy, by relying on myelogenous markers

such as CD33, which are only rarely part of the routine IHC panel of bone marrow biopsies because of weaker expression and a lack of blast specificity. Our observation suggests that, in addition to AML cases with t(8;21) leading to RUNX1-inactivating *RUNX1-RUNX1T1* fusions, deleterious point mutations of *RUNX1* can, analogously to the former, promote the PAX5-mediated activation of B cell marker expression in neoplastic myeloid blasts. It is important for diagnosticians to be aware of this.

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