

Prognostic relevance of *RUNX1* mutations in T-cell acute lymphoblastic leukemia

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ABSTRACT

The runt-related transcription factor 1, *RUNX1*, is crucial in the development of myeloid and lymphoid cell lineages and has been reported to be mutated in myeloid malignancies in approximately 30% of cases. In this study, the mutational status of *RUNX1* was investigated in 128 acute lymphoblastic leukemia patients. We detected a mutation rate of 18.3% (13 of 71) in patients with T-cell acute lymphoblastic leukemia, 3.8% (2 of 52) in patients with B-cell acute lymphoblastic leukemia and no mutation (0 of 5) in patients with natural killer cell leukemia, respectively. In T-cell acute lymphoblastic leukemia patients, *RUNX1* mutations were significantly associated with higher age ($P=0.017$) and lower white blood cell count ($P=0.038$). Moreover, an inferior outcome was observed in the subgroup of early T-cell acute lymphoblastic leukemia patients carrying *RUNX1* mutations for overall survival

($P=0.043$). In conclusion, *RUNX1* mutations are an important novel biomarker for a comprehensive characterization of T-cell acute lymphoblastic leukemia with poor prognostic impact and have implications for use also in monitoring disease.

Key words: T-cell ALL, prognosis, *RUNX1* mutations.

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Introduction

Acute lymphoblastic leukemia (ALL) is characterized by an excessive accumulation of lymphoblasts and their progenitor cells.¹ ALL is the most frequent childhood cancer and accounts for approximately 25% of adult acute leukemias.¹ In approximately 80% of cases, ALL arises from B-cell lineage progenitor cells, whereas 20% are derived from T-cell lineage precursors.²

The diagnosis of ALL is based on immunophenotyping which allows lineage assignment as well as the identification of prognostically important disease subtypes.^{3,4} Furthermore, chromosomal aberrations have been shown to provide information of great prognostic relevance and are used to stratify patients according to different treatment regimens.¹ Today, virtually all patients with ALL can be classified according to specific genetic abnormalities.⁵ In addition, molecular analyses have shown that ALL subtypes harbor specific gene expression signatures, e.g. depending on the cell lineage or cytogenetic abnormalities,⁶ carry specific DNA copy number alterations,⁷ or molecular alterations such as mutations in single genes, e.g. *IKZF1*.^{5,8}

T-ALL, in particular, has been demonstrated to represent a heterogeneous disease with genetic subgroups that correspond to specific T-cell development stages, genomic alterations leading to deregulated expression of genes, e.g. *BAALC*, *ERG*, *TAL1* or *LMO2*, as well as harboring deletions or mutations of genes such as *PTEN*, *NOTCH1* or *FBXW7*.⁹

The runt-related transcription factor 1, *RUNX1*, located on chromosome 21q22, is essential in the development of all hematopoietic cell lineages, including lymphoid cells,⁹ and contains an 128-amino acid Runt domain (RUNT) and an 80-amino

acid transactivation domain (TAD).¹⁰ Through the RUNT domain, *RUNX1* is able to bind to a common DNA motif of its targets and this domain also mediates the heterodimerization with CBF β , which increases DNA binding activity to *RUNX* proteins.¹⁰ Furthermore, *RUNX1* both increases and inhibits transcriptional activity of target genes, depending on the cellular background and pathway.¹⁰ *RUNX1* has been reported to be mutated in AML (32%),¹¹ MDS (23%),¹² and CMML (37%),¹³ and is associated with a shorter overall and event-free survival in AML.^{11,14} Moreover, the gene is involved in a multitude of chromosomal translocations, e.g. the translocation t(12;21)(p13;q22) (*ETV6-RUNX1*) in B-cell ALL cases, in which the TAD domain but not the RUNT domain of *RUNX1* is retained in the fusion gene. In contrast, in the majority of other translocations involving *RUNX1*, e.g. t(8;21)(q22;q22) (*RUNX1-RUNX1T1*), the RUNT domain is retained in the fusion genes.¹⁵ Truncated *RUNX1* proteins act as dominant negative inhibitors of the wild-type protein by binding of DNA through the RUNT domain. Therefore, the wild-type protein loses its ability to regulate target genes.¹⁶

In a recent study investigating 39 patients with chronic myeloid leukemia in blast crisis (BC-CML) with either myeloid or lymphoid features, 3 out of 10 patients with a lymphoid BC-CML harbored a *RUNX1* mutation indicating a potential role of *RUNX1* alterations in lymphatic malignancies which has not yet been discussed.¹⁷ Here, we analyzed the *RUNX1* mutation status in a cohort of 128 adult patients harboring T-ALL, B-ALL, or natural killer (NK) cell leukemia to further study the impact of *RUNX1* alterations in *de novo* acute lymphoblastic leukemias.

The online version of this article has a Supplementary Appendix.

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Design and Methods

Peripheral blood or bone marrow mononuclear cells were collected between October 2005 and December 2010 from the purified fraction of mononuclear cells after Ficoll density centrifugation from 128 thoroughly characterized patients with T-ALL (n=71), B-ALL (n=52), or natural killer (NK) cell leukemia (n=5). T-ALL cases were differentiated by immunophenotyping into early T-ALL (n=30), cortical T-ALL (n=30), and mature T-ALL (n=3). A distinction according to pre- and pro-subtypes is given in the *Online Supplementary Spreadsheet 1* (data not available for 8 cases). The expression intensity of T-cell markers, clinical, pathological and cytogenetic data for these patients are also available (*Online Supplementary Spreadsheet 1*). Study design adhered to the tenets of the Declaration of Helsinki and was approved before initiation by the institutional review board.

Multiparameter flow cytometry (MFC) was performed by applying five-color stainings and isotype controls with monoclonal antibodies conjugated with the fluorochromes FITC, PE, ECD, PC-5, and PC-7. Samples were processed as previously described.¹⁸ MFC analyses were performed using FC500 and Navios flow cytometers (Beckman Coulter, Miami, FL, USA). List mode files were analyzed using CXP Software version 2.0 and Kaluza version 1.0 (Beckman Coulter). Diagnoses were assigned according to EGIL and WHO classifications.^{1,3}

For molecular analyses, a sensitive next-generation amplicon deep-sequencing assay (NGS) was applied, using the small volume Titanium amplicon chemistry (454 Life Sciences, Branford, CT, USA). A sequencing library was prepared interrogating the complete coding region of *RUNX1*, split into 7 distinct PCR amplicons (transcript identifier: ENST00000344691). Data on primer sequences, molecular barcodes used and the PCR amplification protocol are available in the *Online Supplementary Tables S1, S2, and S3*. NGS data were analyzed using the GS Variant Analyzer Software 2.5.3 (454 Life Sciences) and Sequence Pilot version 3.4.2 (JSI Medical Systems, Kippenheim, Germany). All variants reported were independently validated using Sanger sequencing or a separate setup of NGS PCR, emPCR and sequencing run. Statistical

analyses were performed using SPSS version 14.0.1 (SPSS Inc., Chicago, IL, USA). Reported *P* values are two-sided and not corrected for multiple testing.

Results and Discussion

RUNX1 was successfully analyzed in all cases, i.e. in total 896 PCR amplicons were generated for the subsequent characterization by next-generation deep-sequencing. A median of 776 reads per amplicon and patient (range 217-1,654) were obtained thus yielding sufficient coverage for mutation detection with high sensitivity (<5%). Overall, 17 mutations were detected in 15 patients. In the cohort of B-cell ALL, 2 of 52 cases were found to be *RUNX1* mutated, both of them exclusively detected in the subgroup of patients harboring a *BCR-ABL1* translocation (n=2 of 22 *BCR-ABL* mutated B-cell ALL). In T-ALLs, 15 distinct mutations were observed in 13 of 71 cases (18.3%). Interestingly, 8 cases were harboring an early T-ALL (8 of 30, 26.6%) and only 2 cases a cortical T-ALL (2 of 30, 6.6%); subgroup data of 3 *RUNX1* mutated cases were not available (Figure 1A).

In more detail, 17 different *RUNX1* mutations were observed in 15 patients (Figure 1B): 8 missense alterations, one nonsense mutation, 7 frame shift alterations, and one in-frame insertion. Two of the 15 affected patients concomitantly harbored two distinct *RUNX1* mutations. In both cases, these were located on two separate amplicons thus not allowing the discrimination between a mono- or biallelic state. As shown in Figure 1B, the *RUNX1* mutations were generally distributed across several exons, but exclusively clustered in the RUNT (amino acid 50-177, 13 of 17 mutations) and TAD domain (amino acid 291-371, 4 of 17 mutations). The double-mutated cases were harboring a mutation affecting each of the two domains (Figure 1B).

As assessed by the percentage of single sequencing reads carrying the alteration, the median burden of *RUNX1* mutations was 42% (range 6-53%) per patient. Information on

Table 1. *RUNX1* mutations and functional consequences.

Sample N.	Entity	Exon	Mutation	Consequence	% Reads
1	T-ALL	4	343_351delinsACCCC	115_117delinsThrPro	50
2	T-ALL	4	340T>C	Ser114Pro	6
3	T-ALL	5	508G>T	Val170Leu	48
3	T-ALL	8	1009_1022delATCGGCAT	Ile337ValfsX231	11
4	T-ALL	5	502_503insAT	Ile168AsnfsX17	17
4	T-ALL	8	1108delC	Gln370LysfsX197	22
5	T-ALL	5	520C>T	Arg174X	45
6	T-ALL	8	996_997insTGGGGC	Pro332_Val333insTrpGly	20
7	T-ALL	3	215G>A	Cys72Tyr	53
8	T-ALL	8	992dupC	Pro332AlafsX241	39
9	T-ALL	4	350T>C	Leu117Pro	41
10	T-ALL	3	235T>C	Trp79Arg	47
11	T-ALL	4	412G>A	Gly138Ser	42
12	T-ALL	4	416_417insTTCAACG	Gly141ThrfsX47	45
13	T-ALL	4	398A>T	Asp133Val	47
72	B-ALL	4	351_360delGAGAAATGCT	Arg118ProfsX4	34
73	B-ALL	5	521G>A	Arg174Gln	45

blast count and clonality as detected by FISH or cytogenetics are given in the *Online Supplementary Spreadsheet 1*. Moreover, for one patient carrying the Ser114Pro mutation (case n. 2), a paired DNA sample from the remission and relapse state was available. At remission, the Ser114Pro mutation was undetectable, thus confirming the somatic nature as disease-specific alteration at diagnosis. In the relapse sample the mutation had emerged again with a clonality of 10%.

Here, we not only demonstrated for the first time the very high incidence of *RUNX1* mutations in T-ALL, but also addressed the clinical impact of *RUNX1* mutations detected in great detail. We observed that *RUNX1* mutations in T-ALL were associated with a higher age (mean±SD 42±18 vs. 56±18 years; $P=0.017$), and lower white blood cell count (mean±SD 82±96 10⁹/L vs. 38±45×10⁹/L cell count; $P=0.038$), but not with platelet count, hemoglobin level, gender or karyotype (Table 2). Furthermore, a trend for a higher incidence of *RUNX1* mutations in the subgroup of early T-ALL was detected ($P=0.038$).

Table 2. Clinical associations.

<i>RUNX1</i>	Mutated (n=13)	Wild-type (n=58)	P
Age	55.6	42.0	0.017
White blood cell count (×10 ⁹ /L)	38.1	82.4	0.038
Hemoglobin (g/dL)	10.7	11.4	0.51
Platelets (×10 ⁹ /L)	112	139.8	0.65
Gender (female:male)	6:7	15:43	0.18
Karyotype (normal:aberrant)	3:10	20:36	0.52

Moreover, the most interesting observation from a clinical point of view was the negative impact of *RUNX1* mutations on the overall survival in T-ALL. First, when taking the complete cohort into account, we observed a generally poor survival for *RUNX1* mutated cases (alive at two years: 44.4% mutated vs. 66.6% wild-type patients; $P=0.011$; *Online Supplementary Figure S1*). Secondly, we confirmed that the cortical immunophenotype had a positive effect on the overall survival of T-ALL patients (including the 63 cases with the known subtype; alive at two years 82.1% vs. 42.8%, $P=0.002$; *Online Supplementary Figure S2*).¹⁹ A multivariable analysis further demonstrated the cortical T-ALL subtype to be an independent prognostic parameter ($P=0.016$) and in *RUNX1* mutations indicated a strong trend for an independent prognostic impact ($P=0.062$). Since in the cortical T-ALL patients only 6.6% (2 of 30) were carrying *RUNX1* mutations and no mutation was observed in the 3 cases with the mature subtype, we then restricted the analysis of the prognostic impact of *RUNX1* mutations to early T-ALL. In this subtype, 26.6% (8 of 30) of patients carried a mutation in *RUNX1*. Again, mutated *RUNX1* conferred a poor prognosis (alive at two years 28.6% mutated vs. 46.0% wild-type patients, $P=0.043$; Figure 1C).

RUNX1 mutations have so far been described in myeloid malignancies such as AML and MDS.¹¹⁻¹³ In our T-ALL cohort, the location of mutations was observed to be similar in comparison to AML and MDS, as well as in families carrying *RUNX1* germline mutations predisposing to platelet disorders and other myeloid malignancies.²⁰ Generally, with respect to its involvement in hematopoietic processes, *RUNX1* has been shown to play a fundamental part in differentiation and that its role would not be limited to myeloid differentiation only.²¹ As reported by Wong *et al.*,

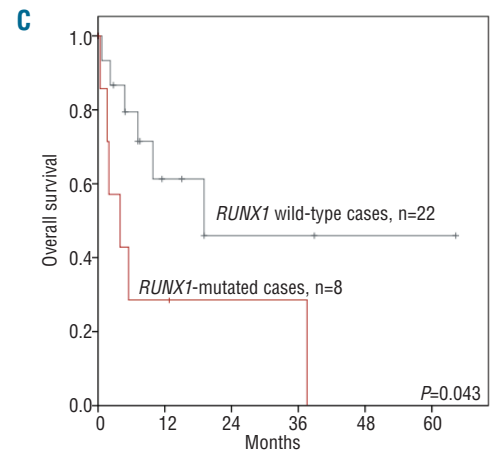
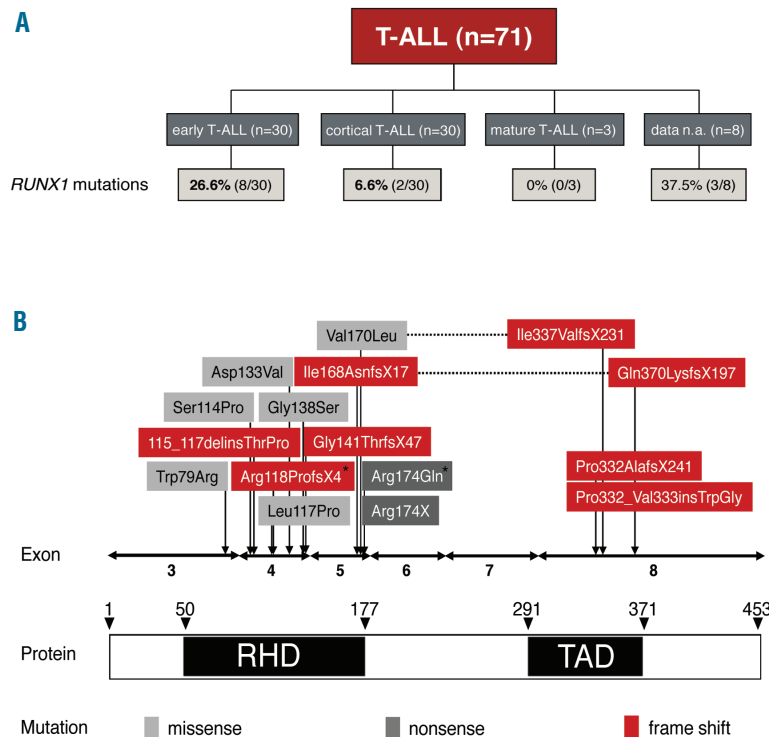


Figure 1. (A) Distribution of *RUNX1* mutations between the three subgroups of T-ALL. **(B)** Distribution of *RUNX1* mutations in ALL. Location of the 17 mutations in *RUNX1* according to the functional domains as detected in 15 patients. Vertical arrows indicate the location of the mutations; corresponding concurrent mutation pairs of the same patient are connected by horizontal lines. Two mutations marked with an asterisk indicate the 2 B-ALL cases. **(C)** Kaplan-Meier overall survival estimates including 30 early T-ALL cases. Data are shown for overall survival of T-ALL patients separated into two groups of *RUNX1*-mutated (n=8) and *RUNX1* wild-type patients (n=22; alive at two years 28.6% vs. 46.0%).

RUNX1 has been demonstrated to be involved in lymphoid differentiation, which is particularly true for T-cell differentiation.⁹ Moreover, expression of *RUNX1* is observed in naïve CD4-positive T cells and *RUNX1* proteins repress the expression of *GATA3* and *IL4*, thereby influencing their further development into Th2 T-helper cells. Our data reporting on 18.3% *RUNX1* mutations in T-ALL leading to a disrupted *RUNX1* function clearly supports a rationale for a pathogenetic role of *RUNX1* alterations in T-ALL. Finally, in B-cell ALL, *RUNX1* mutations were exclusively detected in *BCR-ABL1* positive cases, which are known to frequently co-express myeloid markers such as CD13 and CD33.²⁰ This may further point to a potential correlation of *RUNX1* mutations that can be found not only in myeloid neoplasms but in lymphatic leukemias also demonstrating some myeloid features.

Moreover, it has been proposed that mutated *RUNX1* represent a clinically useful biomarker to indicate disease progression from MDS to s-AML,²² as well as a characteristic to monitor minimal residual disease (MRD) during AML treatment.²³ While clonal rearrangements of T-cell receptor (TCR) genes also enable MRD assessment; these analyses are time-consuming and expensive.²⁴ As such, in addition to the significant relevance of *RUNX1* mutations with respect to survival, monitoring of MRD in T-ALL can also be accomplished by investigating *RUNX1* alterations in combination with next-generation deep-sequencing and generating thousands of reads for a patient-specific mutation.

In conclusion, here we have demonstrated for the first time the fundamental importance of *RUNX1* mutation assessment in hematologic diseases, not only in myeloid but also in lymphoid malignancies. We detected *RUNX1* mutations in 13 of 71 (18.3%) patients with T-ALL and thereby provide a better molecular understanding of underlying disease mechanisms. The high incidence of *RUNX1* mutations in early T-ALL may indicate an important role in the early steps of hematopoietic development. However, testing in larger independent patient cohorts is required. Moreover, *RUNX1* mutations were significantly associated with higher age and lower white blood cell count and characterized a novel as yet undefined poor prognosis risk group, i.e. in the subtype of early T-ALL. Therefore, *RUNX1* mutation screening is proposed for future clinical studies of T-ALL and may thus allow for an even more individualized diagnosis and treatment strategy to be adopted.

Authorship and Disclosures

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