**RUNX1** mutations are frequent in de novo AML with non complex karyotype and confer an unfavorable prognosis

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ABSTRACT

Analyses of 164 RUNX1 mutations (RUNX1mut) in 147 of 449 patients (32.7%) with normal karyotype or non complex chromosomal imbalances were performed. RUNX1mut were most frequent in AML FAB M0 (65.2%) followed by M2 (32.4%) and M1 (30.2%). Considering cytogenetics, RUNX1mut were most frequent in cases with +13 (27/30, 90%) whereas frequencies were similar in other cytogenetic groups (26%-36%). The molecular genetic markers most frequently associated with RUNX1mut were MLL-PTD (19.7%), FLT3-ITD (16.3%), and NRAS mutations (9.5%). Patients with RUNX1mut had shorter overall and event free survival (OS, EFS) compared to RUNX1 wildtype cases (median: 378 days vs. not reached, p=0.003 and median: 285 vs. 450 days, p=0.003, respectively). In addition, it was shown that the adverse impact of RUNX1 was independent of the adverse impact of FLT3-ITD as well as of the high frequency of prognostically favorable NPM1mut and CEBPAmut in the RUNX1wt group. No effect of the type or localisation of the individual RUNX1 mutations was observed. Multivariate analysis revealed independent prognostic relevance for OS for RUNX1mut (p=0.029), FLT3-ITD (p=0.003), age (p<0.001) and WBC (p<0.002).
INTRODUCTION

Cytogenetic aberrations are the most important prognostic parameter in AML. During the last years it became clear that gene mutations add important information to the cytogenetic subtypes. AML with normal karyotype can be genetically further characterized by mutations in FLT3 in terms of internal tandem duplications (FLT3-ITD) or tyrosine kinase mutations (FLT3-TKD) as well as by partial tandem duplication in the MLL gene (MLL-PTD), NPM1 mutations, and CEBPA mutations. The prognostic impact of these mutations was established. NPM1 is regarded as favorable as long as not associated with FLT3-ITD. In addition, gene mutations can be associated with certain cytogenetic aberrations and impact on their primary prognostic significance. Thus, core binding factor leukemias (RUNX1-RUNX1T1 and CBFB-MYH11 positive AML) are associated with a favorable prognosis unless coinciding with KIT mutations which results in a very unfavorable prognosis.

A further candidate for prognostic impact in AML is RUNX1. The RUNX1 gene, located at chromosomal band 21q22, is crucial for the process of definite hematopoiesis and the generation of hematopoietic stem cells in the embryo as shown in a mouse model. It contains a “Runt homology domain” (RHD) which is responsible for heterodimerization with the core-binding factor β (CBFβ or PEBP2β) to form a transcription factor and for DNA binding. The second important part of the RUNX1 protein is the transactivation domain followed by a 5 amino acid sequence, VWRPY that is 100% conserved at the C-terminal end of the gene products. RUNX1 can act as activator or repressor of target gene expression depending upon the large number of interacting transcription factors, coactivators and corepressors. RUNX1 acts as a key regulator of hematopoiesis through the regulation of various hematopoietic genes, including growth factors (GM-CSF, MPO, IL3), surface receptors (TCRA, TCRB, M-CSF receptor, FLT3), signaling molecules (CDKN1A, BLK, BCL2), and transcription activators (STAT3, MYB). Thus, RUNX1-regulated target genes are essential for definite hematopoiesis of all lineages.
Three classes of acquired alterations of the *RUNX1* gene have been discovered in AML: intrageneic mutations, amplification and translocations. Intrageneic mutations have been described mainly for AML M0\(^{20,21}\), MDS and AML following MDS\(^{22}\) as well as for therapy-related MDS and AML\(^{23}\). In addition, *RUNX1* mutations have been associated with certain chromosome aberrations. In therapy-related MDS an association with monosomy 7 and rapid progression to AML has been shown\(^{23}\). Furthermore, *RUNX1* mutations have been shown to be frequently associated with trisomy 21\(^{20}\) and trisomy 13\(^{24}\). Overall *RUNX1* mutations have been described mainly in chemotherapy-related MDS, MDS of atomic bomb survivors or in de novo MDS\(^{23,25-27}\), and recently also in CMML\(^{28}\). Reports about *RUNX1* mutations in de novo AML are rare and mostly were focussed on the M0 subtype\(^{20,21}\).

The aim of the present study was to analyze the incidence and relevance of *RUNX1* mutations in de novo AML. As *RUNX1* mutations were found to be mutually exclusive of recurrent fusion genes and also of AML with complex aberrant karyotype\(^{24,29}\) we selected 449 patients with AML and normal karyotype or non complex chromosomal imbalances. 32.7% of this AML cohort was found to carry *RUNX1* mutations. As *RUNX1* mutations have been described not to be able *per se* to cause full blown leukemia\(^{30}\) we also focussed on the analysis of further genetic changes (*NPM1*, *FLT3*-ITD, *FLT3*-TKD, *CEBPA*, *NRAS*) and found that *MLL*-PTD is the most frequent additional aberration in *RUNX1* mutated AML followed by *FLT3*-ITD. A strong prognostically adverse impact of *RUNX1* mutations independent of other molecular mutations and other prognostically relevant factors was detected.
MATERIAL AND METHODS

Patients

All 449 patient samples were referred to the MLL Munich Leukemia Laboratory for diagnosis of AML between August 2005 and May 2009. AML was diagnosed according to the FAB- and WHO-classifications. To the best of our knowledge all patients had de novo AML without any preceding malignancy or MDS. Median age was 67.7 years (range: 18.3-90.1 years) (in detail see table 1). The patients received different treatment schedules and were in part included into controlled trials of German study groups (n=122; 27.2%). Clinical data were available from 316 patients. 36 cases did not receive standard chemotherapy including AraC and an anthracycline in a curative intend and were excluded from outcome analysis. Thus, 280 cases with clinical data and standard chemotherapy were subject to outcome analysis. Median follow up time of these patients was 642 days. In total 59 patients underwent allogeneic bone marrow transplantation (37 cases in first CR, 22 cases for salvage therapy). Prior to therapy all patients gave their informed consent for participation in the current evaluation after having been advised about the purpose and investigational nature of the study. The study design adhered to the tenets of the Declaration of Helsinki and was approved by the Munich Leukemia Laboratory institutional review board before its initiation.

It has to be stressed that the analyzed cohort was selected: Patients were enrolled with normal karyotype (NK) or non-complex chromosomal imbalances (i.e. trisomies, monosomies or deletions). Furthermore, patients with clinical follow-up data available were preferentially analyzed. The patient characteristics at diagnosis of AML are depicted in table 1.

Molecular analysis

Isolation of mononucleated bone marrow (n=404) or peripheral blood (n=45) cells, mRNA extraction, and random primed cDNA synthesis was performed as described previously. The entire coding region of the RUNX1 isoform AML1b (GenBank entry D43968) was amplified from cDNA using four separate polymerase chain reactions (PCR) and analyzed by
denaturing high performance liquid chromatography (DHPLC) (WAVE system, Transgenomic Inc., Omaha, USA). Depending on the kind and position of the different mutations the sensitivity of this assay varies but is at least 10% and can reach up to 1% of all cells. All fragments that revealed an aberrant dissociation pattern by DHPLC analysis were subsequently analyzed by Sanger sequencing. Similarly, the CEBPA gene was first amplified in four PCR fragments and subsequently analyzed by DHPLC. Analyses for FLT3-length mutation (FLT3-LM)=FLT3 internal tandem duplication (FLT3-ITD), NPM1 mutations, KITD816 mutations, NRAS mutations, JAK2V617F, and FLT3-TKD mutations also were described previously.

Cytomorphology, Cytogenetics, Immunophenotyping

Cytomorphologic assessment were done according to the criteria defined in the FAB and the WHO classification. Cytogenetic studies were performed after short-term culture. Karyotypes, analyzed after G-banding, were described according to the International System for Human Cytogenetic Nomenclature. Immunophenotyping was performed as described previously. All analyses were performed in the MLL Munich Leukemia Laboratory.

Statistical analysis

Survival curves were calculated for overall survival (OS) and event free survival (EFS) according to Kaplan-Meier and compared using the two sided log rank test. OS was the time from diagnosis to death or last follow-up. EFS was defined as time from diagnosis to treatment failure, relapse, death, or last follow-up. Relapse was defined according to Cheson et al. Cox regression analysis was performed for OS and EFS with different analyzed parameters (age, sex, WBC, blast count, CD34 positivity, cytogenetics, FLT3 status) as covariates. Parameters which were significant in univariate analyses were included into multivariate analyses. Dichotomous variables were compared between different groups using the χ²-test and continuous variables by Student’s T-test. Spearman rank correlation was used to analyze correlations between continuous parameters. For all analyses, results were
significant at a level of p<0.05 at both sides. SPSS (version 14.0.1) software (SPSS, Chicago, IL) was used for statistical analysis.

RESULTS

Characterization and distribution of mutations

In total 164 \textit{RUNX1} mutations were detected in 147 of these 449 selected patients (32.7%). Two mutations were reproducibly detected by DHPLC but were not further characterizable by sequencing due to small clone size (10-20%). Thus the further characterization refers to 162 mutations. Of the 147 mutated patients 101 (68.7%) revealed a mutation pattern indicating a low allelic mutation burden (mutation/wildtype ratio of 1 or lower) regarded as “heterozygous”, in 29 patients (17.9%) no \textit{RUNX1} wildtype (wt) was detectable and these cases were subsequently indicated as “LOH cases” (loss of heterozygousity), and 14 (8.6%) had two different heterozygous mutations. Three cases (1.9%) had one mutation with an LOH pattern and had a second mutation with a heterozygous pattern at a region different from the region bearing the LOH type mutation. According to the mutation load in these three latter cases one case with a 50% and 100% load obviously has a subclone with two mutations in one allele, whereas the other two cases with a 25% and a 75% load, each, most probably had two clones with a different \textit{RUNX1} mutation each. Due to methodological reasons in all patients with obvious heterozygousity (low allele burden) cells with LOH at the \textit{RUNX1} locus diluted by unmutated cells can not be excluded.

The mutations consisted of 60 (37.0%) missense, 18 (11.1%) nonsense, 72 (44.4 %) frameshift, 9 (5.6%) in frame insertion/deletion mutations as well as one exon 5 and two exon 7 skipping mutations (1.9%). The mutations are distributed throughout the gene as indicated in figure 1. Mutations are numbered according to Ensemble cDNA sequence ENSG00000159216 transcript RUNX1-001(ENST00000344691).

According to the position of the mutation within the gene 98 (60.5%) of all mutations were in the RUNT homology domain (RHD), 24 (14.8%) in the transactivation domain (TAD), 26
(16.0%) in between these two domains and 13 (8.0%) were found 3´ of the TAD. A single frame shift mutation was detected 5´ of the RHD. In the RHD all different kind of mutations were detected, whereas 3´ of the RHD missense mutations were very rare and totally absent in the TAD. Patients with an LOH pattern most frequently revealed mutations in the RHD (29/32; 90.6%) whereas the mutations of heterozygous cases are scattered throughout the gene. Frameshifts are greatly underrepresented in mutations with LOH pattern (3/32; 9.4%) compared to heterozygous mutations (69/118; 58.5%, p<0.001).

In additional 8 cases the L29S amino acid exchange was observed which has been described to be a functional neutral polymorphism. These cases were not regarded to be mutated. Other non-functional polymorphisms that have been described previously, S21syn, G42R, H58N, and I87syn were not observed in our cohort.

A familial history of thrombocytopenia or familial platelet disorders were not known in any of the 147 RUNX1 mutated cases. Remission samples were available in 60 patients (data not shown) and did not reveal a RUNX1 mutation at remission, thus a germline mutation could be excluded in all cases tested in parallel.

Correlation to clinical features

The distribution of RUNX1 mutations according to sex was equal (31.9% in male and 33.8% in females, n.s.). The median age in the RUNX1 mutated cohort was higher (70.5 years) compared to the RUNX1 wildtype cohort (67.1 years) (p=0.002). Accordingly, the frequency of RUNX1 mutations was higher in patients above age 60 (35.8% vs. 26.3%, p=0.050). Bone marrow blasts were higher in the RUNX1 mutated cases (60.0 vs. 52.8%; p=0.016) and also CD34 positivity as measured by immunophenotyping was higher in the RUNX1 mutated cases (54.5 vs. 32.8% of cells; p<0.001) compared to the RUNX1 wildtype cohort. In contrast, median platelet counts were lower in the RUNX1 mutated compared to the RUNX1 wildtype cases (56 x10^9/L vs. 71 x10^9/L, p=0.027). No differences with respect to white blood cell count (WBC) were observed (table 1).
Distribution of RUNX1 mutations according to WHO/FAB subtypes

Highest frequency of RUNX1 mutations was found in AML with minimal differentiation or AML M0 (30/46; 65.2%). We also detected a high proportion of mutations in AML without maturation or AML M1 and in AML with maturation or AML M2 (29/96; 30.2% and 46/142; 32.4%, respectively), followed by myelomonocytic AML or AML M4 (8/40; 20%). Few RUNX1 mutations were detected in acute erythroid leukemia or AML M6 (1/17; 5.9%) and none in acute monoblastic or monocytic leukemia or AML M5 or in acute megakaryoblastic leukemia or AML M7.

Distribution of RUNX1 mutations according to cytogenetics

The cohort was selected according to cytogenetics. Only patients with normal karyotype (NK) and non complex chromosomal imbalances were included (table 1). The highest frequency of RUNX1 mutations with 90.0% (27/30 cases) was detected in the group with trisomy 13. The frequencies in the groups with NK and single chromosomal losses or gains (-7/del(7q), +8, +11, and +21) were similar ranging from 29% to 36%, respectively (table 1). Only few cases with chromosomal deletions mainly del(5q), del(9q) and del(20q) were analyzed (total: n=22), however, if combined to one group they also revealed an overall RUNX1 mutation frequency of 22.8% (5/22 cases) (table 1).

Cooperating mutations

A further aim of this study was the identification of cooperating mutations in RUNX1 mutated AML. The results of this analysis are summarized in table 2. In the total cohort CEBPA and NPM1 mutations were almost exclusive of RUNX1 mutations as only two and one case, respectively, were detected that had a RUNX1 mutation in addition. MLL-PTD and FLT3-ITD are equally distributed between the RUNX1 mutated and the RUNX1 wildtype group. FLT3-TKD mutations were detected more frequently (p=0.125), NRAS codon 12, 13 and 61 mutations slightly less frequently (p=0.104) and JAK2V617F mutations were never
detected in the \textit{RUNX1} mutated cohort (p=0.032; table 2, supplementary table 1, supplementary figure 1).

In total, in 60 of the 147 \textit{RUNX1} mutated cases (40.8\%) at least one additional mutation was identified. In 17 cases even two and in one case three different additional mutations were detected (total of 79 additional mutations). The \textit{MLL}-PTD was found to be the most frequent mutation detected in \textit{RUNX1} mutated AML (n=29; 36.7\% of additional mutations), followed by \textit{FLT3}-ITD (n=24; 30.4\%), \textit{NRAS} (n=14; 17.7\%) and \textit{FLT3}-TKD (n=9; 11.4\%); in addition 2 CEBPA as well as one NPM1 mutation were detected (supplementary table 2). A detailed association analysis of mutations in addition to \textit{RUNX1} mutations within single cytogenetic subgroups is given in figure 2 and in the supplement (supplementary figure 1a-d, supplementary table 1 and 2).

**Stability of the \textit{RUNX1} mutations**

For 10 patients mutation status and cytogenetics were available at diagnosis and at relapse of AML. One case relapsed with a \textit{RUNX1} mutation different from that at diagnosis (p.V103F and 47,XY,+13 at diagnosis and p.N112_Y113insP and NK at relapse) and was subsequently regarded as secondary AML. \textit{RUNX1} was stable with the same \textit{RUNX1} mutation status at diagnosis and relapse in 9 cases. In contrast, karyotype was stable in only 5 of these cases (NK: n=2, +13: n=1; +21: n=1; del(7q): n=1). One case had a shift from NK to del(12p), one from NK to t(2;12)(q31;p13), one from +8 to +14, and one from +13 to NK. The case with t(2;12)(q31;p13) lost the \textit{MLL}-PTD and \textit{FLT3}-ITD at relapse. The case with del(7q) gained an \textit{FLT3}-ITD and the case with +21 lost the \textit{NRAS} mutation at relapse. These data show genetic heterogeneity between diagnosis and relapse with respect to cytogenetic and molecular markers. Solely the \textit{RUNX1} mutation was stably retained. Even the \textit{RUNX1} status (6 heterozygously mutated, 2 with loss of wildtype, and one with two different mutations) was identical in the diagnostic and relapsed samples. These data indicate that \textit{RUNX1} is the underlying initial or at least the first detectable genetic event in these cases.
Prognostic relevance of RUNX1 mutations

Clinical data for the evaluation of prognosis parameters were available from 316 cases (110 RUNX1mut and 206 RUNX1wt). 36 cases were excluded from this analysis as they did not receive standard chemotherapy including AraC and an anthracycline in a curative intent. Finally in the analysis for prognostic parameters 280 patients (97 RUNX1mut and 183 RUNX1wt) were included. The age of these patients ranged from 18.3 to 87.0 years (median, 68.3 years) for RUNX1 mutated cases and from 20.4 to 88.1 years (median, 65.4 years) for unmutated cases.

In total 59 patients underwent allogeneic bone marrow transplantation (37 cases in first CR, 22 cases for salvage therapy). The number of patients that underwent allogeneic bone marrow transplantation were equally distributed between the RUNX1wt (42/183; 23.0%) and the RUNX1mut patients (17/97; 17.5%). In addition it was found that the survival was not significantly influenced by transplantation (p=0.637, supplementary figure 3). Therefore in all analyses the patients were not censored at time of BMT.

In the total cohort with RUNX1mut (n=97) OS and EFS were significantly worse compared to the cohort with RUNX1wt (n=183) (median: 378 days vs. not reached, p=0.003 and median: 285 vs. 450 days, p=0.003, respectively) (figure 3a). The results were similar if only the cases with NK where included for analysis of OS and EFS with RUNX1mut (n=45) and RUNX1wt (n=111) (median: 361 days vs. not reached, p=0.050 and median: 285 vs. 504 days, p=0.038, respectively) (figure 3b).

A detailed analysis of RUNX1mut versus RUNX1wt in the total cohort, in the NK subcohort as well as in the cytogenetically intermediate cohort as defined by MRC criteria according to age ≤ 60 and > 60 years is shown in supplementary figure 4. The impact of RUNX1 mutations could be shown for EFS in patient ≤60 years in the total cohort (median: 299 vs. 595 days, p=0.022), in the NK cohort (median: 232 vs. 723 days, p=0.011), as well as in the cytogenetically intermediate cohort (median: 299 vs. 731 days, p=0.007; supplemental figures 4a to 4c). The respective differences in EFS in patients > 60 years were significant only for the intermediate cohort (median: 280 vs. 387 days, p=0.022; supplemental
Furthermore, in the cytogenetically intermediate cohort in patients > 60 years OS was significantly worse in RUNX1mut versus RUNX1wt cases (median: 300 vs. 595 days, p=0.022).

**Prognostic relevance of MLL-PTD and FLT3-ITD in addition to RUNX1 mutations**

As MLL-PTD and FLT3-ITD were found to be the most frequent mutations in addition to RUNX1 mutations a possible prognostic effect was analyzed. The first analysis was performed in the total cohort. For each comparison four different groups were defined 1) RUNX1wt/MLL-PTD- (n=154), 2) RUNX1mut/MLL-PTD- (n=118), 3) RUNX1mut/MLL-PTD+ (n=19), 4) RUNX1wt/MLL-PTD+ (n=26) and 1) RUNX1wt/FLT3-ITD- (n=150), 2) RUNX1mut/FLT3-ITD- (n=82), 3) RUNX1mut/FLT3-ITD+ (n=15), 4) RUNX1wt/FLT3-ITD+ (n=30). Subsequently the same analysis was restricted to cases with a normal karyotype only: 1) RUNX1wt/MLL-PTD- (n=93), 2) RUNX1mut/MLL-PTD- (n=33), 3) RUNX1mut/MLL-PTD+ (n=12), 4) RUNX1wt/MLL-PTD+ (n=17) and 1) RUNX1wt/FLT3-ITD- (n=89), 2) RUNX1mut/FLT3-ITD- (n=36), 3) RUNX1mut/FLT3-ITD+ (n=9), 4) RUNX1wt/FLT3-ITD+ (n=21). Although FLT3-ITD and MLL-PTD are both unfavorable markers they both do not confer an additional unfavorable impact on RUNX1 (see supplementary material, supplementary figure 2).

**Impact of different RUNX1 and NPM1 mutation status**

Only one case was RUNX1/NPM1 double mutated and thus these two markers are almost exclusive. We performed a survival analysis of the three remaining constellations: RUNX1wt/NPM1wt (n=136), RUNX1mut/NPM1wt (n=96), and RUNX1wt/NPM1mut (n=47) to exclude that the prognostic adverse impact of RUNX1mut compared to RUNX1wt is due to the positive impact of NPM1 in the RUNX1 unmutated group. As shown in figure 4a RUNX1mut/NPM1wt was worse compared to RUNX1wt/NPM1wt and RUNX1wt/NPM1mut (OS: median: 361 days vs. n.r., p=0.002 and 361 vs. 645 days, p=0.087, respectively and EFS: 285 vs. 387 days, p=0.012 and 285 vs. 616 days, p=0.009, respectively). There was a
similar trend when the same analysis was restricted to the NK group only with (OS: 361 days vs. n.r., \( p=0.029 \) and 361 vs. 733 days, n.s., respectively) and (EFS: median: 285 days vs. 420, \( p=0.091 \) and 285 vs. 675 days, \( p=0.062 \), respectively) (figure 4b).

**Analysis of RUNX1 mutation status in different molecular genetic risk groups**

In a further analysis the prognostic influence of \( \text{RUNX1} \) mutations was evaluated in intermediate (\( \text{NPM1}^- \), \( \text{CEBPA}^- \), \( \text{FLT3}^- \), and \( \text{MLL-PTD} \) unmutated) and adverse (\( \text{FLT3-ITD} \) and/or \( \text{MLL-PTD} \) mutated) risk groups (only cases with intermediate cytogenetics were included, grouping was performed regardless of the presence of \( \text{NRAS} \) and \( \text{JAK2} \) mutations). As there was only one case each with coincidence of \( \text{RUNX1} \) and \( \text{NPM1} \) or \( \text{CEBPA} \) this kind of analysis was not done for the good risk group. A highly adverse impact of \( \text{RUNX1}\text{mut} \) on OS and EFS compared to \( \text{RUNX1}\text{wt} \) could be shown in this analysis (median: 348 days vs. not reached, \( p=0.001 \), and 285 vs. 480 days, \( p=0.011 \), respectively) (figure 5a). In the high risk group (\( \text{FLT3-ITD} \) and/or \( \text{MLL-PTD}^+ \)) no further impact of the \( \text{RUNX1} \) mutations could be shown (OS: median: n.r. vs. 645 days, n.s., and EFS: median: 232 vs. 252, n.s., respectively) (figure 5b).

**Influence of RUNX1 mutation characteristics on outcome**

To analyze for possible differences of certain mutation types, first heterozygously \( \text{RUNX1} \) mutated AML (\( n=101 \)) were analyzed in comparison to cases with LOH (\( n=29 \)) and those with two different mutations (\( n=17 \)) (including the 3 cases with LOH and two different mutations which are separately displayed in figure 1). There were no significant difference in OS and EFS between these three groups. A detailed analysis of the position of the mutations did not show any prognostic relevance either (see supplementary material).
Prognostic influence of age, karyotype and morphology on prognosis

In addition to other molecular mutations and mutation type the following prognostic factors were analyzed in a univariate analysis: age, gender, white blood cell count (WBC), FAB subtype, CD34 positivity. As summarized in supplementary table 3 an unfavorable impact on OS was shown for higher age (p<0.001), higher WBC (p=0.008), and higher FLT3-ITD/FLT3wt ratio (p=0.031). Surprisingly, females were found to have a better OS (p=0.012) compared to males. An unfavorable impact on EFS was shown for higher age (p<0.001), male gender (p=0.031), higher WBC (p=0.019), and a favorable impact on EFS was shown for NPM1 mutations (p=0.049).

Subsequently all parameters that were significantly associated with prognosis in the univariate Cox regression analysis where further analysed in a multivariate analysis. As shown in supplementary table 4, RUNX1 mutation positivity (p=0.029), a higher FLT3-ITD/wt ratio (p=0.003), high age (p<0.001), and high WBC (p=0.002) came out to be independent adverse prognostic parameters for OS. The proportional hazards assumption was met for all parameters entered into the multivariate analysis.

DISCUSSION

As RUNX1 mutations were shown to be mutually exclusive of recurrent reciprocal translocations and complex aberrant karyotype, we selected our cohort according to normal karyotype (NK AML) and those with non complex chromosomal imbalances. We detected a considerable frequency of nearly one third of these AML that carried a RUNX1 mutation. This data is supportive of RUNX1 mutations belonging to the most frequent molecular aberrations in de novo AML. However, it has to be considered that our analysis for RUNX1 mutations was restricted to cytogenetically defined subsets of AML.

Our study suggests a higher overall frequency of RUNX1 mutations as compared to previous studies. This may be caused by our selection for normal karyotype and non complex chromosomal imbalanced cases. An additional reason of different mutation frequencies not only of RUNX1 but also of NPM1 most obviously are different age structures...
in the different studies. The age in the present cohort was clearly higher (median 67.7 years) than in the study of Tang et al (median 52.0 years)\textsuperscript{29} and that of Gaidzik (range 16-60 years)\textsuperscript{40}. In addition, as described previously, \textit{NPM1} mutations occur at higher frequencies in younger patients which is in line with the differences between the present cohort and the above mentioned cohorts. These differences in age also have to be considered the cause for a higher frequency of \textit{RUNX1} mutations in the present cohort since Tang et al. reported a significantly higher age of patients with \textit{RUNX1} mutations as compared to those without (median 62.0 vs. 48.0 years). Taken together, the present data should be reproduced in unselected cohorts of AML before generalizing the findings on the frequency of \textit{RUNX1} mutations for AML.

Although previous data also showed that \textit{RUNX1} is most frequently mutated in AML of the FAB M0 subtype\textsuperscript{20,21}, and in those with a trisomy 13\textsuperscript{24} we detected an even higher rate in our cohort. This can be explained by the same reasons as mentioned above. Furthermore, the high frequency of 65.2\% \textit{RUNX1} mutations in M0 in our cohort is in part the result of an additional selection bias, as complex aberrant karyotypes, which are frequent in M0 were not included in this study\textsuperscript{41}.

We were able to confirm the higher age of the \textit{RUNX1} mutated patients compared to the \textit{RUNX1} wildtype cases as recently published\textsuperscript{29}. However, the high male prevalence (18.4\% vs. 6.4\%) as reported in the same study could was not confirmed in our cohort (31.9\% vs. 33.8\%) possibly due to differences in ethnical background. The higher frequency of the CD34 positivity in the \textit{RUNX1} mutated blast cells might reflect the association with immature phenotypes.

Until now the position and kind of \textit{RUNX1} mutations were largely discussed. A recent paper\textsuperscript{42} defined 4 subtypes with respect to position and function (N-terminal in-frame mutations: Ni-type, C-terminal truncated mutations: Ct-type, truncating mutations in N-terminal region: Nt-type, and so-called “chimera-like mutations” which are frameshift mutations in C-terminal region resulting in the formation of bigger proteins than wild-type AML1: Cc-type). Although we detected very similar frequencies of the different mutation
types than previous studies. In contrast to these studies we observed no associations to certain additional mutations or differences of WBC or outcome between these mutation types. This again may be a difference between MDS/s-AML and de novo AML, but it may also rely on differences in additional genetic events (see below). In our study the only different finding was age being higher in Ni (68.7 years) compared to Nt (64.6 years; p=0.006) and also higher compared to Ct (64.8 years; p=0.005).

Similar to the findings by Kuo in CMML we found that missense mutations of RUNX1 preferentially (91.7%) located in the RHD. On the other hand, frameshift or nonsense mutations were distributed throughout the entire RUNX1 gene in both N-terminal and C-terminal parts. However, patients with an LOH pattern most frequently revealed mutations in the RHD (90.6%) and also in these patients frameshift mutations are greatly underrepresented in compared to heterozygous cases (9.4% vs. 58.5%, p<0.001). Although biallelic mutations were previously observed predominantly in AML M0 and in myeloid malignancies with acquired trisomy 21 and trisomy 13, there was a random distribution in our cohort. All the controversial findings with respect to mutation types may be a matter of small patient cohorts in most of the studies. To our knowledge our study contains the largest cohort of mutated patients a fact that may normalize for those differences.

From other entities we know that certain cytogenetic aberrations or molecular mutations cooperate frequently, e.g. t(15;17) with FLT3-ITD, or core binding factor leukemias with KIT. Previous reports have shown that RUNX1 mutations are frequently associated with activating mutations e.g. in FLT3 and NRAS. Especially the RAS pathway has been described to be the most frequent second hit in RUNX1 mutated MDS and secondary AML. In the present study we found MLL-PTD to be the most frequent mutation in addition to RUNX1. This might be a particular attribute for de novo AML.

The high coincidence of RUNX1 and MLL-PTD suggest that these two mutations cooperate to cause leukemia. According to the Gilliland model RUNX1 are typical type 2 mutations responsible for a differentiation stop. MLL has many functions like methyltransferase and acetylase activity as well as DNA binding by zinc fingers and leucine
zippers\textsuperscript{48,49}. However, \textit{MLL}-PTD does not seem to carry out a typical type 1 mutation function responsible for proliferation. As in 17 of the 147 \textit{RUNX1} mutated cases (11.6\%) at least two further mutations were detected in addition to \textit{RUNX1} a more than 2-hit hypothesis may be discussed at least for the \textit{RUNX1} mutated AML.

Cytogenetic and molecular genetic data were available in paired samples from diagnosis and relapse in ten cases and showed stability of \textit{RUNX1} in 9/10 samples in contrast to instability of other markers like \textit{FLT3}, \textit{NRAS} and cytogenetic aberrations. One additional patient developed secondary AML with a different type of \textit{RUNX1} mutation as compared to primary AML. These data suggest that \textit{RUNX1} is the initiating event or at least the earliest detectable event, so far, in \textit{RUNX1} mutated AML. In analogy to \textit{NPM1} and CEBPA\textsuperscript{32,50}, \textit{RUNX1} mutated AML may therefore be suggested to be a specific AML entity. This is further supported by the clinical data as the effect of \textit{RUNX1} on outcome is impressive. For OS and EFS a highly significant unfavorable effect of \textit{RUNX1} could be shown in the total group, in NK, and also if other molecular aberrations were taken into account. Very recently, an unfavorable outcome of \textit{RUNX1} mutated de novo AML also has been shown\textsuperscript{29}. Our study supports these data. Furthermore, data on the correlation of \textit{RUNX1} mutations to other molecular mutations showed that FLT3-ITD\textsuperscript{2} and the \textit{MLL}-PTD\textsuperscript{5} did not confer an additional unfavorable impact on the \textit{RUNX1} mut status and vice versa.

In conclusion, these data clearly show that \textit{RUNX1} is frequently mutated in de novo AML with normal karyotype or non complex chromosomal imbalances. It can be detected especially in cases that lack other aberrations as defined by the WHO classification and that represent certain biological subgroups (e.g. \textit{PML-RARA}, \textit{RUNX1-RUNX1T1}, \textit{CBFB-MYH11}) or provisional entities like \textit{NPM1} or CEBPA mutated AML. As \textit{RUNX1} mutations have a strong adverse prognostic effect in AML with NK or non complex chromosomal imbalances especially in those that do not carry CEBPA, NPM1, FLT3-ITD or MLL-PTD it is strongly suggested to be implemented into the diagnostic workup of AML. Furthermore, it is suggested as a new candidate molecular marker along with NPM1/FLT3-ITD and CEBPA mutations, to stratify patients for treatment. However, in the current study patient were
treated very heterogeneously, thus the optimum therapy for patients with \textit{RUNX1} mutations should be defined in randomized therapeutic trials. This also may have implications for follow up studies and therapy monitoring.

\textbf{Acknowledgment}

We thank all the coworkers in our laboratory for their excellent technical assistance as well as all physicians for referring sample material to our center.

\textbf{Author contributions}

SS was the principal investigator of this study, analyzed the data and wrote the manuscript. FD, JS and NW did molecular analysis of the \textit{RUNX1} mutations. CH was responsible for chromosome analysis. WK was responsible for immunophenotyping and was involved in the statistical analysis. TH was responsible for cytomorphologic analysis and was involved in the collection of clinical data. TA collected clinical data and helped in the preparation of the figures. All authors read and contributed to the final version of the manuscript.

\textbf{Disclosures}

SS, WK, CH, and TH in part own the Munich Leukemia Laboratory (MLL). FD, JS, NW, and TA are employed by the Munich Leukemia Laboratory (MLL).
References


23. Christiansen DH, Andersen MK, Pedersen-Bjergaard J. Mutations of AML1 are common in therapy-related myelodysplasia following therapy with alkylating agents and are significantly associated with deletion or loss of chromosome arm 7q and with subsequent leukemic transformation. Blood 2004;104(5):1474-1481.


Table 1: Patients' characteristics at diagnosis of de novo AML

<table>
<thead>
<tr>
<th></th>
<th>Total cohort: n=449</th>
<th>RUNX1mutated n=147 (32.7%)</th>
<th>RUNX1wildtype n=302 (67.3%)</th>
<th>p=</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>201</td>
<td>68 (33.8%)</td>
<td>133 (66.2%)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Male</td>
<td>248</td>
<td>79 (31.9%)</td>
<td>169 (68.1%)</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>Median: 67.7</td>
<td>Median: 70.5</td>
<td>Median: 67.1</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>Range: 18.3-90.1</td>
<td>Range: 18.3-90.1</td>
<td>Range: 20.4-88.1</td>
<td></td>
</tr>
<tr>
<td>WBC (x10^9/L)</td>
<td>Median: 8.2</td>
<td>Median: 9.6</td>
<td>Median: 7.15</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>Range: 0.14-365</td>
<td>Range: 0.4-211</td>
<td>Range: 0.14-365</td>
<td></td>
</tr>
<tr>
<td>Platelets (x10^9/L)</td>
<td>Median: 64</td>
<td>Median: 56</td>
<td>Median: 71</td>
<td>0.027</td>
</tr>
<tr>
<td>FAB-Subtype available (n=352)</td>
<td>RUNX1mutated (n=114)</td>
<td>RUNX1wildtype (n=238)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AML M0</td>
<td>46</td>
<td>30 (65.2%)</td>
<td>16 (34.8%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>AML M1</td>
<td>96</td>
<td>29 (30.2%)</td>
<td>67 (69.8%)</td>
<td>n.s.</td>
</tr>
<tr>
<td>AML M2</td>
<td>142</td>
<td>46 (32.4%)</td>
<td>96 (67.6%)</td>
<td>n.s.</td>
</tr>
<tr>
<td>AML M4</td>
<td>40</td>
<td>8 (20.0%)</td>
<td>32 (80.0%)</td>
<td>n.s.</td>
</tr>
<tr>
<td>AML M5</td>
<td>10</td>
<td>0</td>
<td>10 (100.0%)</td>
<td>0.026</td>
</tr>
<tr>
<td>AML M6</td>
<td>17</td>
<td>1 (5.9%)</td>
<td>16 (94.1%)</td>
<td>0.017</td>
</tr>
<tr>
<td>AML M7</td>
<td>1</td>
<td>0</td>
<td>1 (100.0%)</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>total</td>
<td>RUNX1mutated</td>
<td>RUNX1wildtype</td>
<td></td>
</tr>
<tr>
<td>Cytogenetics available</td>
<td>449</td>
<td>147</td>
<td>302</td>
<td></td>
</tr>
<tr>
<td>Normal karyotype</td>
<td>262 (58.7%)</td>
<td>69 (26.3%)</td>
<td>193 (73.7%)</td>
<td>0.001</td>
</tr>
<tr>
<td>Trisomy 8</td>
<td>43 (9.6%)</td>
<td>13 (30.2%)</td>
<td>30 (69.8%)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Trisomy 11</td>
<td>13 (2.9%)</td>
<td>4 (30.8%)</td>
<td>9 (69.2%)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Trisomy 13</td>
<td>30 (6.7%)</td>
<td>27 (90.0%)</td>
<td>3 (10.0%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Trisomy 21</td>
<td>14 (3.1%)</td>
<td>5 (35.7%)</td>
<td>9 (64.3%)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Other Trisomies</td>
<td>9 (2.0%)</td>
<td>3 (33.3%)</td>
<td>6 (66.7%)</td>
<td>n.s.</td>
</tr>
<tr>
<td>-7 / del(7q)</td>
<td>24 (5.3%)</td>
<td>7 (29.2%)</td>
<td>17 (70.8%)</td>
<td>n.s.</td>
</tr>
<tr>
<td>del(5q)</td>
<td>5 (1.1%)</td>
<td>1 (20.0%)</td>
<td>4 (80.0%)</td>
<td>n.s.</td>
</tr>
<tr>
<td>del(9q)</td>
<td>8 (1.8%)</td>
<td>0</td>
<td>8 (100.0%)</td>
<td>0.046</td>
</tr>
<tr>
<td>del(20q)</td>
<td>3 (0.7%)</td>
<td>2 (66.7%)</td>
<td>1 (33.3%)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Other deletions</td>
<td>6 (1.3%)</td>
<td>2 (33.3%)</td>
<td>4 (66.7%)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Others or combinations of two of the above aberrations</td>
<td>32 (7.1%)</td>
<td>14 (43.7%)</td>
<td>18 (56.3%)</td>
<td>n.s.</td>
</tr>
</tbody>
</table>
**Table 2:** Distribution of other mutations

<table>
<thead>
<tr>
<th>Marker</th>
<th>Available</th>
<th>Mutated total</th>
<th>Mutated in RUNX1mut</th>
<th>Mutated in RUNX1wt</th>
<th>p=</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CEBPA</strong></td>
<td>437</td>
<td>41 (9.4%)</td>
<td>2/147 (1.4%)</td>
<td>39/290 (13.4%)</td>
<td>↓&lt;0.001</td>
</tr>
<tr>
<td><strong>NPM1</strong></td>
<td>449</td>
<td>58 (12.9%)</td>
<td>1/147 (0.7%)</td>
<td>57/302 (18.9%)</td>
<td>↓&lt;0.001</td>
</tr>
<tr>
<td><strong>MLL-PTD</strong></td>
<td>449</td>
<td>77 (17.1%)</td>
<td>29/147 (19.7%)</td>
<td>48/302 (15.9%)</td>
<td>0.351</td>
</tr>
<tr>
<td><strong>FLT3-ITD</strong></td>
<td>449</td>
<td>73 (16.3%)</td>
<td>24/147 (16.3%)</td>
<td>49/302 (16.2%)</td>
<td>1.000</td>
</tr>
<tr>
<td><strong>FLT3-TKD</strong></td>
<td>446</td>
<td>18 (4.0%)</td>
<td>9/145 (6.2%)</td>
<td>9/301 (3.0%)</td>
<td>0.125</td>
</tr>
<tr>
<td><strong>NRAS</strong></td>
<td>446</td>
<td>60 (13.5%)</td>
<td>14/147 (9.5%)</td>
<td>46/299 (15.4%)</td>
<td>0.104</td>
</tr>
<tr>
<td><strong>JAK2</strong></td>
<td>377</td>
<td>9 (2.4%)</td>
<td>0/127</td>
<td>9/250 (3.6%)</td>
<td>↓0.032</td>
</tr>
</tbody>
</table>

Arrows indicate whether the respective marker occurs more or less frequent in RUNX1 mutated AML, respectively.
FIGURES

Figure 1: Position, characterization and frequencies of different RUNX1 mutations. The position of all analyzed exons with numbering of the exons and indication of the numbers of amino acids (AA) is demonstrated at the top. Below a scheme with the position of the functional domains RHD (runx homology domain) and TAD (transactivation domain). Below the scheme the positions of the mutations are depicted in four different rows differentiated according to the presence in 1) heterozygous cases (mutation/wildtype ratio of 1 or lower), only 99/101 of these heterozygous cases are depicted, because two were below detection limit of Sanger sequencing. 2) cases with an LOH pattern (mutation/wildtype ratio of >1), 3) cases with two different heterozygous mutations and 4) cases with one LOH type and one heterozygous mutation. Different colors indicate whether the mutations are of missense (black), nonsense (red), frameshift (green), insdel (insertion/deletion) (blue), or of the ExScip (exon skipping) (yellow) type. The table below summarizes the results with respect to frequency and position of the different RUNX1 mutations. # As two of the total 164 were not further characterized by sequencing due to mutation load of <20%, only 162 mutations are depicted.

Figure 2: Diagrams with frequencies of cooperating mutations in RUNX1wt compared to RUNX1 mutated cases in the NK group (a) and the +13 group (b). The inner circles demonstrate all cases with a certain karyotype that are analyzed for RUNX1 mutations. In dark grey the percentages of RUNX1wt and in red the percentages of RUNX1 mutated are indicated in the inner circle. The segments in the periphery indicate the percentages of cases with other mutations for RUNX1wt and
$RUNX1$mut subcohorts, respectively. As some cases have more than one additional mutation there are overlapping segments in the periphery. In some cases not all markers were analyzed as indicated by *. Further details are given in supplementary table 1 and supplementary figure 1.

**Figure 3**: Kaplan Meier plots of $RUNX1$ mutated compared to $RUNX1$wt AML showing overall (OS) and event free survival (EFS) in the total cohort (a) and restricted to the normal karyotype group (b). n.r.: not reached

**Figure 4**: OS and EFS taking $RUNX1$ and $NPM1$ into account. In a) analysis of the total cohort. In b) analysis restricted to normal karyotype cases. Only p-values below 0.05 are indicated.

**Figure 5**: Kaplan Meier plot showing a) adverse effect (OS and EFS) of $RUNX1$ mutations in patients with intermediate cytogenetic risk without mutations in $NPM1$, $CEBPA$, $FLT3-ITD$, or $MLL$-$PTD$. b) no effect of $RUNX1$ mutations in patients with high risk $FLT3-ITD$ and/or $MLL$-$PTD$ positive AML. n.r.: not reached
Figure 1

De novo AML (n=147)

Type of mutations in cases:

1) 1 heterozygous (n=99)

2) 1 with LOH pattern (n=29)

3) 2 heterozygous (n=14)

4) 1 heterozygous + 1 with LOH* (n=3)

<table>
<thead>
<tr>
<th></th>
<th>total</th>
<th>RHD</th>
<th>TAD</th>
</tr>
</thead>
<tbody>
<tr>
<td>missense</td>
<td>60</td>
<td>55</td>
<td>4</td>
</tr>
<tr>
<td>nonsense</td>
<td>18</td>
<td>13</td>
<td>3</td>
</tr>
<tr>
<td>frameshift</td>
<td>72</td>
<td>1</td>
<td>25</td>
</tr>
<tr>
<td>insdel</td>
<td>9</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>ExScip</td>
<td>3</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>total</td>
<td>162*</td>
<td>98</td>
<td>26</td>
</tr>
</tbody>
</table>
Figure 2a

NK

FLT3-TKD 0.5%
JAK2 0.6%
NRAS 5.9%
CEBPA 6.8%
FLT3-TKD 2.1%

FLT3-ITD 17.0%

MLL-PTD 29.0%

RUNX1 mut 26.3% (n=69)
RUNX1 wt 73.7% (n=193)

NPM1 21.2%
NRAS 6.7%
FLT3-TKD 0.5%
JAK2 1.2%
CEBPA 1.4%
NRAS 5.8%
CEBPA 5.8%
Figure 2b

Trisomy 13

- FLT3-ITD: 33.3% (7.4% in red)
- FLT3-TKD: 7.4%
- MLL-PTD: 11.1%
- NRAS: 3.7%
- RUNX1 wt: 10% (n=3)
- RUNX1 mut: 90% (n=27)
Figures 3a and 3b illustrate the survival analysis of two cohorts: the overall cohort and the NK cell cohort.

### Total Cohort

- **RUNX1wt** (n=183; median: n.r.)
- **RUNX1mut** (n=97; median: 378 days)

**p** = 0.003

### NK Cohort

- **RUNX1wt** (n=111; median: n.r.)
- **RUNX1mut** (n=45; median: 361 days)

**p** = 0.050

- **RUNX1wt** (n=111; median: 504 days)
- **RUNX1mut** (n=45; median: 285 days)

**p** = 0.038

The figures depict Kaplan-Meier survival curves for each cohort, showing the overall survival (left) and event-free survival (right) over time.
Figure 4a  Total cohort

RUNX1wt/NPM1wt (n=136)  median: n.r.  p=0.002
RUNX1wt/NPM1mut (n=47)  median: 616 days
RUNX1mut/NPM1wt (n=96)  median: 361 days  p=0.012

RUNX1wt/NPM1wt (n=136)  median: 387 days
RUNX1wt/NPM1mut (n=47)  median: 645 days
RUNX1mut/NPM1wt (n=96)  median: 285 days

Figure 4b  NK cohort

RUNX1wt/NPM1wt (n=75)  median: n.r.  p=0.029
RUNX1wt/NPM1mut (n=36)  median: 733 days
RUNX1mut/NPM1wt (n=45)  median: 361 days

RUNX1wt/NPM1wt (n=136)  median: 420 days
RUNX1wt/NPM1mut (n=47)  median: 675 days
RUNX1mut/NPM1wt (n=45)  median: 285 days
**Figure 5a**

Intermediate Cytogenetic Risk Group without Molecular Mutations

- RUNX1 wt (n=81; median: n.r.)
- RUNX1 mut (n=67; median: 348 days)

p=0.001

**Figure 5b**

FLT3-ITD+ and/or MLL-PTD+ group

- RUNX1 wt (n=50; median: 645 days)
- RUNX1 mut (n=28; median: n.r.)

p=0.830

- RUNX1 wt (n=50; median: 252 days)
- RUNX1 mut (n=28; median: 232 days)

p=0.470
RUNX1 mutations are frequent in de novo AML with non complex karyotype and confer an unfavourable prognosis

Susanne Schnittger, Frank Dicker, Wolfgang Kern, Nicole Wendland, Jana Sundermann, Tamara Alpermann, Claudia Haferlach and Torsten Haferlach