**Introduction**

Cytogenetic aberrations are the most important prognostic parameter in acute myeloid leukemia (AML). During the past years it became clear that gene mutations add important information to the cytogenetic subtypes. AML with normal karyotype (NK) can be genetically further characterized by mutations in the FLT3 gene 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 effect of these mutations was established. NPM1 is regarded as favorable as long as it is not associated with FLT3-ITD. In addition, gene mutations can be associated with certain cytogenetic aberrations and can affect 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 effect 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) that is responsible for heterodimerization with the core-binding factor β (or PEBP2B) 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, WRVPY, 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 on 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 those coding for growth factors (GM-CSF, MPO, IL3, surface receptors (TCRA, TCRC, 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: intragenic mutations, amplification and translocations. Intragenic mutations have been described mainly for AML M0, myelodysplastic syndrome (MDS) and AML after MDS as well as for therapy-related MDS and AML. 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. Furthermore, RUNX1 mutations have been shown to be frequently associated with trisomy 21 and trisomy 13. Overall, RUNX1 mutations have been described mainly in chemotherapy-related MDS, MDS of atomic bomb survivors, or in de novo MDS, and recently also in chronic myelomonocytic leukemia. Reports about RUNX1 mutations in de novo AML are rare and mostly were focused on the M0 subtype.

The aim of the present study was to analyze the incidence and relevance of RUNX1 mutations in de novo AML. Because RUNX1 mutations were found to be mutually exclusive of recurrent fusion genes and also of AML with complex aberrant karyotype, we selected 449 patients with AML and NK or noncomplex chromosomal imbalances. A portion (32.7%) of this...
AML cohort was found to carry RUNX1 mutations. Because RUNX1 mutations have been described not to be able per se to cause full-blown leukemia,\textsuperscript{30} we also focused 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 effect of RUNX1 mutations independent of other molecular mutations and other prognostically relevant factors was detected.

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 French-American-British (FAB) and World Health Organization (WHO) classifications.\textsuperscript{31,32} 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; for details 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.

Thirty-six cases did not receive standard chemotherapy, including cytosine arabinoside and an anthracycline in a curative intent 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 (BMT; 37 cases in first complete remission, 22 cases for salvage therapy). Before 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 NK or noncomplex chromosomal imbalances (ie, 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.\textsuperscript{2} The entire coding region of the RUNX1 isoform AML1b (GenBank entry D43968) was amplified from cDNA with the use of 4 separate polymerase chain reactions (PCRs) and analyzed by denaturing high-performance liquid chromatography (DHPLC; WAVE system; Transgenomic Inc). Depending on the kind and position of the mutation, the use of 4 separate polymerase chain reactions (PCRs) and analyzed by denaturing high-performance liquid chromatography (DHPLC; WAVE system; Transgenomic Inc) was subject to outcome analysis. Median follow-up time of these patients was 642 days. In total 59 patients underwent allogeneic bone marrow transplantation (BMT; 37 cases in first complete remission, 22 cases for salvage therapy). Before 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.

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Cytomorphology, cytogenetics, immunophenotyping

Cytomorphologic assessments were done according to the criteria defined in the FAB and the WHO classifications.\textsuperscript{31,32} Cytogenetic studies were performed after short-term culture. Karyotypes, analyzed after G-banding, were described according to the International System for Human Cytogenetic Nomenclature.\textsuperscript{36} Immunophenotyping was performed as described previously.\textsuperscript{37} 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 with the use of the 2-sided log-rank test. OS was the time from diagnosis to death or last follow-up. EFS was defined as the time from diagnosis to treatment failure, relapse, death, or last follow-up. Relapse was defined according to Cheson et al.\textsuperscript{38} Cox regression analysis was performed for OS and EFS with different analyzed parameters (age, sex, white blood cell [WBC] count, blast count, CD34 positivity, cytogenetics, FLT3 status) as covariates. Parameters that were significant in univariate analyses were included into multivariate analyses. Dichotomous variables were compared between different groups with the use of the chi-square test and continuous variables by Student t test. Spearman rank correlation was used to analyze correlations between continuous parameters. For all analyses, results were significant at a level of P < .05 at both sides.

Results

Characterization and distribution of mutations

In total 164 RUNX1 mutations were detected in 147 of these 449 selected patients (32.7%). Two mutations were reproducibly detected by DHPLC but were not further characterize by sequencing because of small clone size (10%-20%). Thus, the further characterization refers to 162 mutations. Of the 147 patients with mutation 101 (68.7%) showed a mutation pattern that indicated a low allelic mutation burden (mutation/wild-type [wt] ratio of < 1) regarded as “heterozygous,” in 29 patients (17.9%) no RUNX1 wt was detectable, and these cases were subsequently indicated as “LOH cases” (loss of heterozygosity), and 14 (8.6%) had 2 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 3 latter cases 1 case with a 50% and 100% load obviously has a subclone with 2 mutations in one allele, whereas the other 2 cases with a 25% and a 75% load, each, most probably had 2 clones with a different RUNX1 mutation each. Because of methodologic reasons in all patients with obvious heterozygosity (low allele burden) cells with LOH at the RUNX1 locus diluted by unmutated cells cannot 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 1 exon 5- and 2 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 ENST00000344691 transcript RUNX1-001. According to the position of the mutation within the gene 98 (60.5%) of all mutations were in the RHD, 24 (14.8%) in the transactivation domain (TAD), 26 (16.0%) in between these 2 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 showed mutations in the RHD (29 of 32; 90.6%) whereas the mutations of heterozygous cases are scattered throughout the gene. Frameshifts are greatly underrepresented in mutations with...
LOH pattern (3 of 32; 9.4%) compared with heterozygous mutations (69 of 118; 58.5%; P < .001).

In an additional 8 cases the L29S amino acid exchange was observed which has been described to be a functional neutral polymorphism.21 These cases were not regarded to be mutated. Other nonfunctional polymorphisms that have been described previously, S21syn, G42R, H58N, and I87syn30 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 show 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 males and 33.8% in females; NS). The median age in the RUNX1-mutated cohort was higher (70.5 years) compared with the RUNX1 wt cohort (67.1 years) (P = .002). Accordingly, the frequency of RUNX1 mutations was higher in patients > 60 years (35.8% vs 26.3%; P = .050). Bone marrow blasts were higher in the RUNX1-mutated cases (60.0% vs 52.8%; P = .016), and also CD34 positivity as measured by immunophenotyping was higher in the RUNX1-mutated cases (54.5% vs 32.8% of cells; P < .001) compared with the RUNX1 wt cohort. In contrast, median platelet count was lower in the RUNX1-mutated compared with the RUNX1 wt cases (56 × 10^9/L vs 71 × 10^9/L; P = .027). No differences with respect to WBC count 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 of 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 of 96; 30.2% and 46 of 142; 32.4%, respectively), followed by myelomonocytic AML or AML M4 (8 of 40; 20%). Few RUNX1 mutations were detected in acute erythroid leukemia or AML M6 (1 of 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 NK and noncomplex chromosomal imbalances were included (Table 1). The highest frequency of RUNX1 mutations with 90.0% (27 of 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 into one group they also showed an overall RUNX1 mutation frequency of 22.8% (5 of 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 because only 2 cases and 1 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 wt groups. FLT3-TKD mutations were detected more frequently (P = .125), NRAS codon 12, 13, and 61 mutations slightly less frequently (P = .104), and JAK2V617F mutations were never detected in the RUNX1-mutated cohort (P = .032; Table 2; supplemental Table 1; supplemental Figure 1, available on the Blood Web site; see the Supplemental Materials link at the top of the online article).

In total, in 60 of the 147 RUNX1-mutated cases (40.8%) ≥ 1 additional mutation was identified. In 17 cases even 2 and in 1 case 3 different additional mutations were detected (total of 79 additional mutations). The MLL-PTD was found to be the most frequent mutation detected in RUNX1-mutated AML (n = 29; 36.7% of additional mutations), followed by FLT3-ITD (n = 24; 30.4%), WO/NRAS (n = 14; 17.7%), and FLT3-TKD (n = 9; 11.4%); in addition 2 CEBPA as well as one NPM1 mutation were detected.
(supplemental Table 2). A detailed association analysis of mutations in addition to RUNX1 mutations within a single cytogenetic subgroups is given in Figure 2 and in the supplement (supplemental Figure 1A-D; supplemental Tables 1-2).

Stability of the RUNX1 mutations

For 10 patients mutation status and cytogenetics were available at diagnosis and at relapse of AML. One case relapsed with a 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 mutation different from that at diagnosis (p.V103F and 47,XY,del(7q), n (%) 24 (5.3) 7 (29.2) 17 (70.8) NS

Table 2. Distribution of other mutations

<table>
<thead>
<tr>
<th>Mutated total, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Available, n</td>
</tr>
<tr>
<td>437</td>
</tr>
<tr>
<td>58 (12.9)</td>
</tr>
<tr>
<td>449</td>
</tr>
<tr>
<td>77 (17.1)</td>
</tr>
<tr>
<td>449</td>
</tr>
<tr>
<td>73 (16.3)</td>
</tr>
<tr>
<td>446</td>
</tr>
<tr>
<td>18 (4.0)</td>
</tr>
<tr>
<td>446</td>
</tr>
<tr>
<td>60 (13.5)</td>
</tr>
<tr>
<td>377</td>
</tr>
<tr>
<td>9 (2.4)</td>
</tr>
</tbody>
</table>

Table 1. Patients’ characteristics at diagnosis of de novo AML

<table>
<thead>
<tr>
<th>Sex</th>
<th>RUNX1mutated (n = 147; 32.7%)</th>
<th>RUNX1wt (n = 302; 67.3%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female, n (%)</td>
<td>201</td>
<td>68 (33.8)</td>
<td>133 (66.2)</td>
</tr>
<tr>
<td>Male, n (%)</td>
<td>248</td>
<td>79 (31.9)</td>
<td>169 (68.1)</td>
</tr>
<tr>
<td>Median age, y (range)</td>
<td>67.7 (18.3-90.1)</td>
<td>70.5 (18.3-90.1)</td>
<td>67.1 (20.4-88.1)</td>
</tr>
<tr>
<td>WBC count, x10^9/L (median, range)</td>
<td>8.2 (0.14-365)</td>
<td>9.6 (0.4-211)</td>
<td>7.15 (0.14-365)</td>
</tr>
<tr>
<td>Platelet count, x10^9/L (median, range)</td>
<td>64 (6-1116)</td>
<td>56 (10-301)</td>
<td>71 (6-1116)</td>
</tr>
<tr>
<td>FAB subtype*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AML M0</td>
<td>46</td>
<td>30 (65.2)</td>
<td>16 (34.8) &lt; .001</td>
</tr>
<tr>
<td>AML M1</td>
<td>96</td>
<td>29 (30.2)</td>
<td>67 (69.8) NS</td>
</tr>
<tr>
<td>AML M2</td>
<td>142</td>
<td>46 (32.4)</td>
<td>96 (67.6) NS</td>
</tr>
<tr>
<td>AML M4</td>
<td>40</td>
<td>8 (20.0)</td>
<td>32 (80.0) NS</td>
</tr>
<tr>
<td>AML M5</td>
<td>10</td>
<td>0</td>
<td>10 (100.0) .026</td>
</tr>
<tr>
<td>AML M6</td>
<td>17</td>
<td>1 (5.9)</td>
<td>16 (94.1) .017</td>
</tr>
<tr>
<td>AML M7</td>
<td>1</td>
<td>0</td>
<td>1 (100.0) NS</td>
</tr>
<tr>
<td>Cytogenetics available</td>
<td>262 (58.7)</td>
<td>69 (26.3)</td>
<td>193 (73.7) .001</td>
</tr>
<tr>
<td>Normal karyotype, n (%)</td>
<td>43 (9.6)</td>
<td>13 (30.2)</td>
<td>30 (69.8) NS</td>
</tr>
<tr>
<td>Trisomy 8, n (%)</td>
<td>13 (2.9)</td>
<td>4 (30.8)</td>
<td>9 (69.2) NS</td>
</tr>
<tr>
<td>Trisomy 11, n (%)</td>
<td>30 (6.7)</td>
<td>27 (90.0)</td>
<td>3 (10.0) &lt; .001</td>
</tr>
<tr>
<td>Trisomy 13, n (%)</td>
<td>14 (3.1)</td>
<td>5 (35.7)</td>
<td>9 (64.3) NS</td>
</tr>
<tr>
<td>Other trisomies, n (%)</td>
<td>9 (2.0)</td>
<td>3 (33.3)</td>
<td>6 (66.7) NS</td>
</tr>
<tr>
<td>del(7q), n (%)</td>
<td>24 (5.3)</td>
<td>7 (29.2)</td>
<td>17 (70.8) NS</td>
</tr>
<tr>
<td>del(5q), n (%)</td>
<td>5 (1.1)</td>
<td>1 (20.0)</td>
<td>4 (80.0) NS</td>
</tr>
<tr>
<td>del(13q), n (%)</td>
<td>8 (1.8)</td>
<td>0</td>
<td>8 (100.0) .046</td>
</tr>
<tr>
<td>del(20q), n (%)</td>
<td>3 (0.7)</td>
<td>2 (66.7)</td>
<td>1 (33.3) NS</td>
</tr>
<tr>
<td>Other deletions, n (%)</td>
<td>6 (1.3)</td>
<td>2 (33.3)</td>
<td>4 (66.7) NS</td>
</tr>
<tr>
<td>Others or combinations of 2 of above aberrations, n (%)</td>
<td>32 (7.1)</td>
<td>14 (43.7)</td>
<td>18 (56.3) NS</td>
</tr>
</tbody>
</table>

*FAB subtypes (n = 352), RUNX1mutated (n = 114), RUNX1wt (n = 238).

Stability of the RUNX1 mutations

For 10 patients mutation status and cytogenetics were available at diagnosis and at relapse of AML. One case relapsed with a 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. RUNX1 was stable with the same 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(3q), 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 MLL-PTD and FLT3-ITD at relapse. The case with del(7q) gained an FLT3-ITD, and the case with +21 lost the NRS mutation at relapse. These data show genetic heterogeneity between diagnosis and relapse with respect to cytogenetic and molecular markers. Solely the RUNX1 mutation was stably maintained. Even the RUNX1 status (6 heterozygously mutated, 2 with loss of wild-type, and 1 with 2 different mutations) was identical in the diagnostic and relapsed samples. These data indicate that 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 prognostic parameters were available from 316 cases (110 RUNX1mut and 206 RUNX1wt). Thirty-six cases were excluded from this analysis because they did not receive standard chemotherapy, including cytosine arabinoside 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 unmutilated cases.

In total 59 patients underwent allogeneic BMT (37 cases in first complete remission, 22 cases for salvage therapy). The number of patients who underwent allogeneic BMT were equally distributed from 316 cases (110 RUNX1mut and 206 RUNX1wt).
(17 of 97; 17.5%) patients. In addition it was found that the survival was not significantly influenced by transplantation ($P = .637$; supplemental Figure 3). Therefore, in all analyses the patients were not censored at time of BMT.

In the total cohort with $RUNX1$mut ($n = 97$) OS and EFS were significantly worse compared with the cohort with $RUNX1$wt ($n = 183$) (median, 378 days vs not reached, $P = .003$; median, 285 vs 450 days, $P = .003$, respectively) (Figure 3A). The results were similar if only the cases with NK that were included for analysis of OS and EFS with $RUNX1$mut ($n = 45$; median, 361 days vs not reached; $P = .050$) and $RUNX1$wt ($n = 111$; median, 285 vs 504 days; $P = .038$) (Figure 3B).

A detailed analysis of $RUNX1$mut versus $RUNX1$wt in the total cohort, in the NK subcohort as well as in the cytogenetically intermediate cohort as defined by criteria from the Medical Research Council1 according to age $\leq 60$ and $> 60$ years is shown in supplemental Figure 4. The effect of $RUNX1$ mutations could be shown for EFS in patients $\leq 60$ years in the total cohort (median, 299 vs 595 days; $P = .022$), in the NK cohort (median, 232 vs 723 days; $P = .011$), as well as in the cytogenetically intermediate
cohort (median, 299 vs 731 days; \( P = .007 \); supplemental Figure 4A-C). The respective differences in EFS in patients > 60 years were significant only for the intermediate cohort (median, 280 vs 387 days; \( P = .022 \); supplemental Figure 4C). Furthermore, in the cytogenetically intermediate cohort in patients > 60 years OS was significantly worse in \( RUNX1 \) mutation than in \( RUNX1 \) wt cases (median, 300 vs 595 days; \( P = .022 \)).

Prognostic relevance of \( MLL-PTD \) and \( FLT3-ITD \) in addition to \( RUNX1 \) mutations

Because \( MLL-PTD \) 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 4 different groups were defined (1) \( RUNX1 \) wt/\( MLL-PTD \)− (\( n = 154 \)), (2) \( RUNX1 \) mut/\( MLL-PTD \)− (\( n = 118 \)), (3) \( RUNX1 \) mut/\( MLL-PTD \)− (\( n = 19 \)), and (4) \( RUNX1 \) wt/\( MLL-PTD \)− (\( n = 26 \)) and (1) \( RUNX1 \) wt/\( FLT3-ITD \)− (\( n = 150 \)), (2) \( RUNX1 \) mut/\( FLT3-ITD \)− (\( n = 82 \)), (3) \( RUNX1 \) mut/\( FLT3-ITD \)− (\( n = 15 \)), and (4) \( RUNX1 \) wt/\( FLT3-ITD \)− (\( n = 30 \)). Subsequently, the same analysis was restricted to cases with a NK only: (1) \( RUNX1 \) wt/\( MLL-PTD \)− (\( n = 93 \)), (2) \( RUNX1 \) mut/\( MLL-PTD \)− (\( n = 33 \)), (3) \( RUNX1 \) mut/\( MLL-PTD \)− (\( n = 12 \)), (4) \( RUNX1 \) wt/\( MLL-PTD \)− (\( n = 17 \)) and (1) \( RUNX1 \) wt/\( FLT3-ITD \)− (\( n = 89 \)), (2) \( RUNX1 \) mut/\( FLT3-ITD \)− (\( n = 36 \)), (3) \( RUNX1 \) mut/\( FLT3-ITD \)− (\( n = 9 \)), and (4) \( RUNX1 \) wt/\( FLT3-ITD \)− (\( n = 21 \)).

Although \( FLT3-ITD \) and \( MLL-PTD \) are both unfavorable markers, they both do not confer an additional unfavorable effect on \( RUNX1 \) (see supplemental material; supplemental Figure 2).

Effect of different \( RUNX1 \) and \( NPM1 \) mutation status

Only one case was \( RUNX1/NPM1 \) double mutated; thus, these 2 markers are almost exclusive. We performed a survival analysis of the 3 remaining constellations: \( RUNX1 \) wt/\( NPM1 \) wt (\( n = 136 \), \( RUNX1 \) mut/\( NPM1 \) wt (\( n = 96 \), and \( RUNX1 \) wt/\( NPM1 \) mut (\( n = 47 \)) to exclude that the prognostic adverse effect of \( RUNX1 \) mut compared with \( RUNX1 \) wt is because of the positive effect of \( NPM1 \) in the \( RUNX1 \) unmutated group. As shown in Figure 4A \( RUNX1 \) mut/\( NPM1 \) wt was worse compared with \( RUNX1 \) wt/\( NPM1 \) wt and \( RUNX1 \) wt/\( NPM1 \) mut (OS: median, 361 days vs not reached, \( P = .002 \) and 361 vs 645 days, \( P = .087 \); EFS: 285 vs 387 days, \( P = .012 \) and 285 vs 616 days, \( P = .009 \)). There was a similar trend when the same analysis was restricted to the NK group only with OS (361 days vs not reached, \( P = .029 \) and 361 vs 733 days, NS, respectively) and EFS (median, 285 vs 420, \( P = .091 \) and 285 vs 675 days, \( P = .062 \), respectively) (Figure 4B).

Analysis of \( RUNX1 \) mutation status in different molecular genetic risk groups

In a further analysis the prognostic influence of \( RUNX1 \) mutations was evaluated in intermediate (\( NPM1 \)-, \( CEBPA \)-, \( FLT3 \)-, and \( MLL-PTD \) unmutated) and adverse (\( FLT3-ITD \) and/or \( MLL-PTD \) mutated) risk groups (only cases with intermediate cytogenetics were included, grouping was performed regardless of the presence of \( NRAS \) and \( JAK2 \) mutations). Because there was only one case each with coincidence of \( RUNX1 \) and \( NPM1 \) or \( CEBPA \), this kind of analysis was not done for the good-risk group. A highly adverse effect of \( RUNX1 \) mut on OS and EFS compared with \( RUNX1 \) wt could be shown in this analysis (median, 348 days vs not reached, \( P = .001 \); and 285 vs 480 days, \( P = .011 \), respectively) (Figure 5A). In the high-risk group (\( FLT3-ITD \) and/or \( MLL-PTD \)−) no further effect of the \( RUNX1 \) mutations could be shown (OS: median, no response vs 645 days, NS; and EFS: median, 232 vs 252, NS, respectively) (Figure 5B).
Influence of RUNX1 mutation characteristics on outcome

To analyze for possible differences of certain mutation types, first heterozygously RUNX1-mutated AML cases (n = 101) were analyzed in comparison to cases with LOH (n = 29), and cases with 2 different mutations (n = 17; including the 3 cases with LOH and 2 different mutations which are separately displayed in Figure 1). There were no significant difference in OS and EFS between these 3 groups. A detailed analysis of the position of the mutations did not show any prognostic relevance either (see supplemental 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:

A Intermediate Cytogenetic Risk Group without Molecular Mutations

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

Figure 4. OS and EFS taking RUNX1 and NPM1 into account. (A) Analysis of the total cohort; (B) analysis restricted to NK cases. Only P values < .05 are indicated.

Figure 5. Kaplan-Meier plots of adverse effect (OS and EFS) of RUNX1 mutations. (A) 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- or MLL-PTD-positive AML; n.r. indicates not reached.
age, sex, WBC count, FAB subtype, and CD34 positivity. As summarized in supplemental Table 3 an unfavorable effect on OS was shown for higher age \((P < .001)\), higher WBC count \((P = .008)\), and higher FLT3-ITD/FLT3wt ratio \((P = .031)\). Surprisingly, females were found to have a better OS \((P = .012)\) than were males. An unfavorable effect on EFS was shown for higher age \((P < .001)\), male sex \((P = .031)\), higher WBC count \((P = .019)\), and a favorable effect on EFS was shown for NPM1 mutations \((P = .049)\).

Subsequently all parameters that were significantly associated with prognosis in the univariate Cox regression analysis where further analyzed in a multivariate analysis. As shown in supplemental Table 4, RUNX1 mutation positivity \((P = .029)\), a higher FLT3-ITD/wt ratio \((P = .003)\), high age \((P < .001)\), and high WBC count \((P = .002)\) 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**

Because RUNX1 mutations were shown to be mutually exclusive of recurrent reciprocal translocations and complex aberrant karyotype,20,29 we selected our cohort according to NK karyotype (NK AML) and those with noncomplex chromosomal imbalances. We detected a considerable frequency of nearly one-third of these AML cases that carried a RUNX1 mutation. This data are 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 compared with previous studies.20,22,23,29 This may be caused by our selection for NK and noncomplex 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 al29 (median, 52.0 years) and that of Gaidzik et al40 (range, 16-60 years). In addition, as described previously, 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 RUNX1 mutations in the present cohort because Tang et al29 reported a significantly higher age of patients with RUNX1 mutations compared with patients without (median, 62.0 vs 48.0 years). Taken together, the present data should be reproduced in unsellected cohorts of AML before generalizing the findings on the frequency of RUNX1 mutations for AML.

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

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

Until now the position and kind of RUNX1 mutations were largely discussed. A recent paper42 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 wt AML1, Ct-type). Although we detected very similar frequencies of the different mutation types compared with previous studies,28,42 in contrast to these studies we observed no associations to certain additional mutations or differences of WBC count or outcome between these mutation types. This again may be a difference between MDSs/s-AML and de novo AML, but it may also rely on differences in additional genetic events (see supplemental data). In our study the only different finding was age being higher in Ni (68.7 years) compared with Nt (64.6 years; \(P = .006\)) and also higher compared with Ct (64.8 years; \(P = .005\)).

Similar to the findings by Kuo et al28 in chronic myelomonocytic leukemia, we found that missense mutations of RUNX1 preferentially (91.7%) located in the RHD. However, 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 with heterozygous cases (9.4% vs 58.5%; \(P < .001\)). Although biallelic mutations were previously observed predominantly in AML M0 and in myeloid malignancies with acquired trisomy 21 and trisomy 13,24,30,43,44 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, for example, t(15;17) with FLT3-ITD, or core-binding factor leukemias with KIT.2,15,45 Previous reports have shown that RUNX1 mutations are frequently associated with activating mutations, for example, in FLT3 and NRAS.23 Especially the RAS pathway has been described to be the most frequent second hit in RUNX1-mutated MDS and secondary AML.42,46 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 suggests that these 2 mutations cooperate to cause leukemia. According to the Gilliland model RUNX1 is a typical type 2 mutation responsible for a differentiation stop.47 MLL has many functions such as methyltransferase and acetylase activity as well as DNA binding by zinc fingers and leucine zippers.13,48 However, MLL-PTD does not seem to carry out a typical type 1 mutation function responsible for proliferation. Because in 17 of the 147 RUNX1 mutated cases (11.6%) ≥ 2 further mutations were detected in addition to RUNX1, a > 2-hit hypothesis may be discussed at least for the RUNX1-mutated AML.

Cytogenetic and molecular genetic data were available in paired samples from diagnosis and relapse in 10 cases and showed stability of RUNX1 in 9 of 10 samples in contrast to instability of
other markers such as FLT3, NRAS, and cytogenetic aberrations. One additional patient developed secondary AML with a different type of RUNX1 mutation compared with primary AML. These data suggest that RUNX1 is the initiating event or at least the earliest detectable event, so far, in RUNX1-mutated AML. In analogy to NPM1 and CEBPA, RUNX1-mutated AML may therefore be suggested to be a specific AML entity. This is further supported by the clinical data because the effect of RUNX1 on outcome is impressive. For OS and EFS a highly significant unfavorable effect of 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 RUNX1-mutated de novo AML also has been shown.29 Our study supports these data. Furthermore, data on the correlation of RUNX1 mutations to other molecular mutations showed that FLT3-ITD5 did not confer an additional unfavorable effect on the RUNX1mut status and vice versa.

In conclusion, these data clearly show that RUNX1 is frequently mutated in de novo AML with NK or noncomplex chromosomal imbalances. It can be detected especially in cases that lack other aberrations as defined by the WHO classification and that represent certain biologic subgroups (eg, PML-RARA, RUNX1-RUNX1T1, CBFB-MYH11) or provisional entities such as NPM1- or CEBPA-mutated AML. Because RUNX1 mutations have a strong adverse prognostic effect in AML with NK or noncomplex 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 patients were treated very heterogeneously; thus, the optimum therapy for patients with RUNX1 mutations should be defined in randomized therapeutic trials. This also may have implications for follow-up studies and therapy monitoring.

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Authorship

Contribution: S.S. was the principal investigator of this study, analyzed the data, and wrote the manuscript; F.D., J.S., and N.W. did molecular analysis of the RUNX1 mutations; C.H. was responsible for chromosome analysis; W.K. was responsible for immunophenotyping and was involved in the statistical analysis; T.H. was responsible for cytomorphologic analysis and was involved in the collection of clinical data; and T.A. collected clinical data and helped in the preparation of the figures. All authors read and contributed to the final version of the manuscript.

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RUNX1 mutations are frequent in de novo AML with noncomplex karyotype and confer an unfavorable prognosis

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