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RUNX1 mutations in cytogenetically normal acute myeloid leukemia are associated with poor prognosis and up regulation of lymphoid genes

Philipp A. Greif[1,2], Nikola P. Konstandin[1,2], Klaus H. Metzeler[1], Tobias Herold[1], Zlatana Pasalic, Bianka Ksienzyk[1], Annika Dufour[1], Friederike Schneider[1], Stephanie Schneider[1], Purvi M. Kakadia[1,2,7], Jan Braess[3], Maria Cristina Sauerland[4], Wolfgang E. Berdel[5], Thomas Büchner[5], Bernhard J. Woermann[6], Wolfgang Hiddemann[1,2], Karsten Spiekermann[1,2], and Stefan K. Bohlander[1,2,7]

(1)Department of Internal Medicine 3, Ludwig-Maximilians-Universität (LMU) Munich, Munich, Germany; (2)Clinical Cooperative Group 'Leukemia', Helmholtz Zentrum München, German Research Center for Environmental Health, Munich, Germany; (3)Oncology and Hematology, St. John-of-God Hospital, Regensburg, Germany; (4)Institute of Biostatistics and Clinical Research; (5)Department of Medicine A-Hematology, Oncology and Pneumology, Universität Münster, Münster, Germany; (6)German Society of Hematology and Oncology, Berlin, Germany, and (7)Center for Human Genetics, Philipps Universität, Marburg, Germany

Correspondence
Stefan K. Bohlander, MD, Marchioninistr. 2581377 Munich, Germany.
Phone: international +49.089.7099410. Fax: international + +49.089.7099400.
E-mail: bohlander@helmholtz-muenchen.de/pgreif@med.uni-muenchen.de

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Abstract

Background. The *RUNX1* (*AML1*) gene is a frequent mutational target in myelodysplastic syndromes and acute myeloid leukemia. Previous studies suggested that *RUNX1* mutations may have pathological and prognostic implications.

Design and Methods. We have screened 93 cytogenetically normal acute myeloid leukemia patients for *RUNX1* mutations by capillary sequencing of genomic DNA. Mutation status was then correlated with clinical data and gene expression profiles.

Results. We identified 15 out of 93 cytogenetically normal acute myeloid leukemia patients with *RUNX1* mutations (16.1 %). 73 patients were enrolled in the AMLCG-99 trial and carried 10 *RUNX1* mutations (13.7%). Among these 73 patients *RUNX1* mutations were significantly associated with older age, male sex, absence of *NPM1* mutations and presence of *MLL*-partial tandem duplications. Moreover, *RUNX1* mutated patients had a lower complete remission rate (30% vs. 73% p=0.01) and shorter relapse-free survival (p=0.002; 3-year relapse-free survival 0% vs. 30.4%) and overall survival (p<0.001; 3-year overall survival 0% vs. 34.4%) than *RUNX1* wild type patients. *RUNX1* mutations remained associated with shorter overall survival in a multivariate model including the covariates age and the European Leukemia Net acute myeloid leukemia genetic classification. Patients with *RUNX1* mutations showed a unique gene expression pattern with differential expression of 85 genes. The most prominently upregulated genes in *RUNX1* mutated cytogenetically normal acute myeloid leukemia include lymphoid regulators like *HOPX* (HOP homeobox), *DNTT* (deoxynucleotidyltransferase, terminal) and *BLNK* (B cell linker) indicating lineage infidelity.

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Conclusions. Our findings firmly establish *RUNX1* mutations as marker of poor prognosis and provide insights into the pathogenesis of *RUNX1* mutation positive AML.

(*ClinicalTrials.gov identifier NCT00266136*)

Introduction

The transcription factor *RUNX1* is the fusion partner of *RUNX1T1* (ETO) in the recurring t(8;21)(q22;q22) translocation present in 8-13% of adult *de novo* acute myeloid leukemia (AML) patients. (1) *RUNX1* is a key regulator of hematopoiesis and is involved in hematopoietic stem cell emergence and regulation. (2) The structure of the *RUNX1* protein is characterized by an N-terminal RUNT domain, which mediates DNA-binding as well as interaction with the core-binding-factor beta (CBFB), and a C-terminal transactivation domain. (3) Point mutations in *RUNX1* were initially described in AML secondary to myelodysplastic syndrome (MDS), radiation exposure or chemotherapy, at a frequency of 8 to 10%. (4) Later, analyses of cytogenetically heterogeneous AML cohorts found *RUNX1* mutations in 6-33% of patients. (5-7) The mutational spectrum includes N-terminal missense mutations, affecting mostly the RUNT domain, and C-terminal truncating mutations, deleting the transactivation domain. Both missense and truncating mutations were reported not only to cause a loss of normal *RUNX1* function, but also to act in a dominant negative fashion on the transactivation capacity of wild-type *RUNX1*. (3) In minimally differentiated AML (AML M0) with *RUNX1* mutations, deregulation of lymphoid genes was observed, indicating linage infidelity. (8) Mutations in *RUNX1* are associated with poor prognosis in AML (5-7) in cytogenetically heterogeneous patient cohorts. Therefore, we studied the prognostic implications of *RUNX1* mutations in a cohort homogeneous both with

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regard to cytogenetics (only cytogenetically normal AML; CN-AML) and treatment (all patients treated on the AMLCG-1999 trial). To learn about the biology of *RUNX1* mutated AML, we analyzed differential gene expression of *RUNX1* mutated versus *RUNX1* wild type CN-AML.

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Design and Methods

Patients

Ninety-three adult patients with CN-AML with available material and gene expression data were analyzed for *RUNX1* mutations. Seventy-three were enrolled in the multicenter AMLCG-1999 trial of the German AML Cooperative Group, and an additional 20 CN-AML patients were not treated on trial and could thus not be evaluated for outcome, but were studied for *RUNX1* mutation status and gene expression profiles. Diagnostics were performed centrally at the Laboratory for Leukemia Diagnostics, University of Munich, and included standard cytomorphology, cytogenetics, fluorescence *in situ* hybridization and testing fo*r FLT3*-internal tandem duplications (ITD), *MLL*-partial tandem duplications (PTD), and *NPM1, CEBPA*, *NRAS*, *KIT*, *IDH1* (R132) and *IDH2* (R140 and R172) mutations. The diagnosis of CN-AML was based on the analysis of at least 20 metaphases in >90% of patients, and on the analysis of at least 10 metaphases in the remaining patients. All patients received intensive cytarabine-based double-induction and consolidation chemotherapy. (9) The AMLCG-1999 trial is registered at ClinicalTrials.gov (NCT00266136) and approved by the local institutional review boards of all participating centers. Informed consent was obtained from all patients in accordance with the Declaration of Helsinki.

Mutation screening

The entire open reading frame of *RUNX1* (NM_001754.4) was analyzed from genomic DNA using PCR amplification with exon-spanning primers and bidirectional DNA sequencing on an ABI 3100 Avant instrument. Primer sequences are listed in *Online Supplementary Table S1*.

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Microarray Analyses

Pretreatment bone marrow samples were analyzed using Affymetrix HG-U133 A/B oligonucleotide microarrays (Affymetrix, Santa Clara, CA). Details regarding sample preparation, hybridization and image acquisition have been described previously. (10, 11) For combining individual oligonucleotide probes to probe sets and for annotating these probe sets to genes, we used custom chip definition files (CDFs) based on the GeneAnnot database (available online at *http://www.xlab.unimo.it/GA_CDF/*).(12) In contrast to standard Affymetrix annotations, in these CDFs each gene is represented by one single probe set comprising only probes that exclusively match to the gene of interest. This approach reduces the multiple testing burden by decreasing the total number of probe sets, and potentially increases the specificity of the analyses by eliminating cross-hybridizing probes. Data normalization was performed using the variance stabilizing normalization algorithm (13) and expression values were calculated by the median polish method.

Differentially expressed probe sets were identified by comparing *RUNX1*-mut and *RUNX1*-wild type (wt) patients, using a permutation-based algorithm to adjust for multiple testing. (14) Genes were called significant if their adjusted *q* value was <0.05 and the fold change between the two groups was >1.5 or <0.66. (15) Microarray analyses were performed using the R software package, version 2.13.0. (16)

To identify functionally related sets of genes which are deregulated in *RUNX1*-mut CN-AML, we performed gene set enrichment analysis (GSEA). Gene sets were obtained from the curated ‘canonical pathways’ (c2:cp) collection of the Molecular Signatures Database (MSigDB version 3.0.; *http://www.broadinstitute.org/gsea/msigdb/*).(17)

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Only gene sets comprising between 15 and 200 individual genes (654 of the 880 total gene sets) were included in the analysis. Gene sets were considered significant at a false discovery rate (FDR; adjusted for gene set size and multiple testing) of $q<0.10$

Statistical analysis

Fisher's exact test was used to compare categorical clinical variables of the *RUNX1*-mut and *RUNX1*-wt cohorts. For the continuous variables we used the Mann-Whitney U-Test. The clinical endpoints complete remission (CR), non-responder AML, relapse free survival (RFS) and overall survival (OS) were defined as reported previously. (11, 18) In brief, patients with more than 5% residual bone marrow blasts after induction treatment were judged to be non-responders. Relapse-free survival (RFS) was defined as time from the date of complete remission (CR) until relapse or death, regardless of cause. Overall survival (OS) was defined as time from study entry until death from any cause. Patients alive without an event were censored at the time of their last follow-up.

The prognostic impact of *RUNX1* mutations was first evaluated according to the Kaplan-Meier method and the log-rank test. To adjust for important clinical and molecular prognostic variables, we derived a multivariate Cox model for OS with age as continuous parameter (10-year difference), ELN genetic group and *RUNX1* mutational status as covariates.

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Results

Patient characteristics and clinical outcome

In a cohort of 93 adult CN-AML patients, 15 (16.1%) were found to carry *RUNX1* mutations (Figure 1 A, Table 1). Four patients carried several RUNX1 mutations. Among the 73 patients enrolled in the AMLCG-1999 trial, 10 (13.7%) had *RUNX1* mutations. Clinical and molecular patient characteristics are listed in Table 2. *RUNX1* mutations were associated with older age (p=0.001), male sex (p=0.005), higher LDH levels (p=0.003) and a trend towards a lower WBC (p=0.08) when compared with *RUNX1*-wt patients.

No patient with mutated *RUNX1* carried a concurrent *NPM1* mutation, while the frequency of *NPM1* mutations in the *RUNX1*-wt group was 66.7% (p<0.001). The *MLL*-PTD was more frequent among the *RUNX1*-mutated than among RUNX1-wt patients (p=0.02). There was no significant association of *RUNX1* mutations with *FLT3*-ITD or *CEBPA*, *NRAS*, *KIT*, *IDH1* (R132), *IDH2* (R140/R172) (Figure 1 B).

Only 3/10 (30%) *RUNX1*-mutated patients achieved a CR after intensive induction treatment, whereas the CR rate in the control group was 46/63 (73%; p=0.01). Four of ten (44%) *RUNX1* mutated patients were primarily refractory to induction treatment, whereas this rate was only 11.1% in the control group (p=0.04).

Log rank test identified *RUNX1* mutations as a significant strong negative predictor of RFS (p=0.002) and OS (p<0.001). Three-year RFS and OS rates for *RUNX1* mutated patients were 0%, whereas they were 30.4% (RFS) and 34.4% (OS) for patients with wild type *RUNX1*.

Kaplan-Meier estimates were calculated to display the negative prognostic influence of *RUNX1* mutations on OS in all 73 study patients and the ELN intermediate I and elderly AML patient subgroups (Figure 2).

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In a multivariate model for OS, including age (10-year difference), the ELN genetic groups and *RUNX1* mutational status all covariates were significant parameters (Table 3).

Identification of genes differentially expressed between *RUNX1*-mutated and *RUNX1*-wild type cases

To gain insights into the biology of *RUNX1*-mutated *CN-AML*, we derived RUNX1 mutation-associated gene expression signatures. Of note, *RUNX1* mutations were found exclusively in *NPM1*-wt CN-AML patients, while over 60% of *RUNX1*-wild type patients carried *NPM1* mutations which themselves are associated with a strong gene expression signature. (19) To avoid confounding our analyses through the impact of *NPM1* mutations, we only analyzed *NPM1*-wild type patients. Comparing 15 *RUNX1*-mutated/*NPM1*-wild type and 26 *RUNX1*-wt/*NPM1*-wt patients, we identified a set of 85 differentially expressed genes (Figure 3 and *Online Supplementary Table S2)*. Sixty-nine genes showed higher expression in the *RUNX1*-mutated cases, while 16 genes were down-regulated. The most prominently upregulated genes in *RUNX1* mutated CN-AML include lymphoid regulators like *HOPX* (HOP homeobox), *DNTT* (deoxynucleotidyltransferase, terminal) and *BLNK* (B cell linker) indicating lineage infidelity.

To investigate whether specific functional pathways are overrepresented among the genes deregulated in *RUNX1*-mut CN-AML, we performed GSEA. We found 71 gene sets to be significantly enriched in *RUNX1*-mut patients, while 51 gene sets were enriched in *RUNX1*-wt patients (*Online Supplementary Table S3*). Gene sets upregulated in *RUNX1*-mutated patients included signaling pathways highly expressed in lymphoid cells, such as the B-cell receptor (BCR) signaling pathway and the toll-like receptor

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4 (TLR4) and NOTCH1 pathways (*Online Supplementary Figure S1 A-C*). Conversely, pathways related to DNA synthesis, DNA repair and DNA damage response pathways were downregulated in *RUNX1*-mutated AML (*Online Supplementary Figure S1 D-F*).

Discussion

In our analysis of a homogenous and uniformly treated cohort of CN-AML patients enrolled in the AMLCG-99 trial, we found *RUNX1* mutations in 13.7% of patients. This frequency is similar to the one reported by Tang et al (6) who found mutations in 13.9% of CN-AML, and higher than the frequency reported by Gaidzik et al (3.9%) (5) who only studied patients below the age of 60 years. We confirmed that *RUNX1* mutations are more frequent in elderly, male patients and that *RUNX1* mutations are associated with some established genetic markers like *MLL*-PTD (positively) and *NPM1* mutations (negatively). (5-7) In our cohort, *NPM1* and *RUNX1* mutations were mutually exclusive.

Our analyses revealed *RUNX1* mutations as a highly significant predictor of inferior outcomes, including a lower CR rate, shorter RFS and shorter OS. A high proportion of patients with *RUNX1* mutations did not respond to intensive induction treatment, and only 3 of 10 (30%) achieved a CR. Even these three responders all died within 9.5 months (two in relapse; one in CR). *RUNX1* mutations were a significant covariate in a multivariate model for OS including age (≥60), the ELN genetic classification and *RUNX1* mutational status. These findings are consistent with the study by Tang and colleagues, (6) who reported that *RUNX1* mutations associate with shorter RFS and OS in homogenously treated CN-AML patients. In contrast, Gaidzik and colleagues previously reported a negative prognostic impact of *RUNX1* mutations in a cytogenetically heterogeneous cohort, but found no significant impact on RFS or OS within the CN-AML subset. (5) Of note, their study only included

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younger patients, suggesting that the negative impact of mutated *RUNX1* might be age-related. Our findings strongly support that CN-AML patients with *RUNX1* mutations do not benefit from standard treatment. Screening for *RUNX1* mutations might thus identify candidates for alternative treatment approaches. In summary, *RUNX1* mutational status might be considered for inclusion in a revised version of the European Leukemia Net (ELN) AML risk classification particularly for older patients.

In addition, we demonstrate that *RUNX1* mutated CN-AML shows a distinct gene expression pattern characterized by differential expression of 85 genes. 26 out of these 85 differentially expressed genes were previously reported to be deregulated in *RUNX1* mutated AML M0 (minimally differentiated AML according to the FAB classification), indicating that the expression of these genes is very likely to be influenced by *RUNX1* mutations. (8) These 26 genes include the T cell markers *DNTT* and *BLNK,* suggesting that mutations in the early hematopoietic stem cell regulator *RUNX1* may disturb differentiation resulting in lineage infidelity in early progenitor cells. Since AML M0 is cytogenetically diverse, the study by Silva and colleagues is limited by the influence of multiple cytogenetic aberrations including complex karyotype and trisomy 8. (8, 20) Furthermore, Silva and colleagues limited their mutation screening to the RUNT-domain of *RUNX1*, which likely resulted in an underestimation of the *RUNX1* mutation burden. In contrast, our study of *RUNX1* mutations in CN-AML is not biased by the impact of cytogenetic aberrations on gene expression and an underestimation of *RUNX1* mutations (the complete coding sequence of *RUNX1* gene was sequenced). Gaidzik et al also studied the association between *RUNX1* mutational status and gene expression in a large cohort of AML patients including various cytogenetic subgroups. (21) However, that cohort included only 5 CN-AML patients with *RUNX1* mutations and several different

microarray platforms were used in their study, thus limiting the comparability with the study presented here.

High expression levels of several genes we found upregulated in *RUNX1*-mutated CN-AML, namely *DNTT*, *SETBP1*, *BAALC* and *PTK2*, were previously shown to be associated with adverse prognosis in AML. (22-25)

In summary, we provide further evidence for the unfavourable impact of *RUNX1* mutations on clinical outcomes in a cytogenetically homogenous and uniformly treated cohort of AML patients. Compared to previous reports on *RUNX1* mutation-related gene expression signatures who were based on cytogenetically diverse patient cohorts, (5, 8) our study specifically focused on CN-AML. Our findings reveal the unique biology of *RUNX1* mutation positive AML and may provide the basis for the development of novel diagnostic tools and therapies. Importantly, our findings that *RUNX1* mutations in elderly CN-AML patients are associated with a dismal prognosis should aid in defining this group of patients as potentially benefitting from alternative treatment strategies.

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Authorship and Disclosures

P.A.G. and S.K.B. conceived and designed the research. N.P.K. and B.K., performed laboratory work. P.A.G., N.P.K., Z.P., K.H.M and T.H. analyzed data. A.D., P.M.K., F.S., S.S., J.B. and K.S. characterized patient samples. J.B., K.S., M.C.S., W.E.B., T.B., B.J.W. and W.H. coordinated the AMLCG clinical trial. P.A.G., K.H.M, T.H. and S.K.B. wrote the manuscript. The authors declare no conflict of interest.

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Table legends

Table 1. Molecular details of RUNX1 mutations in 94 CN-AML patients. 15 CN-AML patients carried *RUNX1* mutations. 4 out of 15 patients had several *RUNX1* mutations. Sequence variations are indicated on the cDNA and protein level with reference to the longest isoform of *RUNX1* (NM_001754.4). UPN: Unique Patient Number.

Table 2. Patient characteristics. Correlation of clinical characteristics and RUNX1 mutation status is indicated for 73 patients enrolled in the AMLCG-99.

Table 3. Multivariate analysis. HR: Hazard ratio; CI: Confidence interval; Multivariate Cox regression model with covariates *RUNX1* mutational status, age (10-year difference) and the European LeukemiaNet AML risk classification (ELN).

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Table 1. Molecular details of RUNX1 mutations in 94 CN-AML patients.

cDNA (NM_001754.4)	Protein (NP_001745.2)	Exon	UPN	AMLCG-99
c.167T>C	p.(Leu56Ser)	4	1	Included
c.319C>T	p.(Arg107Cys)	4	2	-
c.329A>G	p.(Lys110Arg)	4	3	-
c.387_388insTATTG	p.(Val130Tyrfs*5)	5	4	-
c.485G>A	p.(Arg162Lys)	5	5	Included
c.493G>T	p.(Gly165Cys)	5	6	-
c.524T>C	p.(Leu175Pro)	6	7	Included
c.592G>A	p.(Asp198Asn)	6	3	-
c.593A>T	p.(Asp198Val)	6	8	-
c.601C>T	p.(Arg201*)	6	9	Included+
c.602G>A	p.(Arg201Gln)	6	8	Included
c.611_612insTGTCCCACAGGGAAAAGCTTCAC TCTGACCATCACTGTCTTCACAAACCCACCGC AAGTCGCCACCTACCACAGAGCCATCAAAAT	p.(Arg205Valfs*9)	6	8	-
c.620_621insACTTTACTTCCG	p.(Arg207_Gln208insLeuTyrPheArg)	7	3	-
c.861C>G	p.(Tyr287*)	8	10	Included
c.881delC	p.(Pro294Leufs*17)	8	11	Included
c.958C>T	p.(Arg320*)	8	4	-
c.965C>G	p.(Ser322*)	8	9	-
c.1003_1015dupCAGTTCCCCGCGC	p.(Leu339Profs*265)	9	12	Included
c.1243dup	p.(Gln415Profs*185)	9	13	Included
c.1243dup	p.(Gln415Profs*185)	9	14	-
c.1347_1348insGCTTCCTTCCTCCTAG	p.(Ser450Alafs*155)	9	15	Included

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Table 2. Patient characteristics.

Variable	***RUNX1* wt**	***RUNX1* mut.**	**P-value**
No. of patients	63	10	
Median age, years (range)	54 (27-83)	73 (54-78)	**0.001**
Male sex, no. (%)	26 (41.3)	9 (90)	**0.005**
White-cell count, G/l, median(range)	39.5 (0.1-486.0)	11.70 (1.8-105.3)	0.08
Hemoglobin, g/dl, median(range)	9.7 (5.5-14.2)	8.4 (4.9-9.3)	0.76
Platelet count, G/l ,median(range)	52.0 (0.02-268.0)	37.0 (18.0-111.0)	0.52
LDH (U/l), median(range)	694 (181-2814)	328 (186-784)	**0.003**
Bone marrow blasts, %, median(range)	80 (20-100)	85 (20-95)	0.82
Performance Status (ECOG) ≥ 2 (%)	21 (35)	5 (50)	0.48
de novo AML (%)	57 (93.4)	8 (80)	0.2
FAB			
M0, no. (%)	1 (1.6)	1 (10)	0.26
M1, no. (%)	11 (18)	6 (60)	**0.01**
M2, no. (%)	20 (32.8)	2 (20)	0.71
M4, no. (%)	19 (31.1)	1 (10)	0.26
M5, no. (%)	9 (14.8)	0 (0)	0.34
M6, no. (%)	1 (1.6)	0 (0)	1
NPM1 mut., no. (%)	42 (66.7)	0 (0)	**<.001**
FLT3-ITD, no. (%)	31 (49.2)	3 (30)	0.32
FLT3-TKD	5 (7.9)	2 (20)	0.24
Monoallelic CEBPA mut., no. (%)	3 (4.8)	2 (20)	0.14
Biallelic CEBPA mut., no. (%)	6 (9.5)	0 (0)	0.59
MLL-PTD, no. (%)	2 (3.2)	3 (30)	**0.02**
NRAS mut., no. (%)	9 (14.3)	1 (10)	1
KIT mut., no. (%)	1 (1.6)	0 (0)	1
IDH1 R132 mut., no. (%)	5 (7.9)	1 (10)	1
IDH2 R140 mut., no. (%)	11 (17.5)	1 (10)	1
IDH2 R172 mut., no. (%)	0	0	
ELN classification			
Favorable (ELN I), no. (%)	27 (42.9)	2 (20)	0.3
Complete remission, no. (%)	46 (73)	3 (30)	**0.01**
Non-responder AML, no. (%)	7 (11.5)	4 (40)	**0.04**

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Table 3. Multivariate analysis.

	Overall survival[1]	
Variable	**HR (95% CI)**	**p-value**
RUNX1 mutation	2.51 (1.1-5.8)	**0.03**
Age[2]	1.24 (1.01-1.52)	**0.04**
ELN	4.35 (2.19-8.63)	**<0.001**

[1] N=71/73 (97.3%); 2 observations missing due to missing follow up data

[2] The hazard ratio refers to a 10-year difference in age.

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Figure legends

Figure 1. (A) Overview of mutations in RUNX1. Linear structure of the RUNX1 protein (NP_001745.2) includes the N-terminal RUNT domain and the C-terminal transcriptional activation (TAD) domain. Amino acids (aa) changes resulting from mutations found in our cohort of CN-AML patients are detailed. The graph was generated using the software DOG 2.0. (26) **(B) Distribution of mutations in *RUNX1* and 8 additional genes in 93 CN-AML patients.** Distribution of additional mutations among patients with RUNX1 mutations (n=15) or RUNX1-wild type (n=78). 73 CN-AML patients were enrolled in the AMLCG-99 clinical trial (left panel). Another 20 CN-AML patients were not homogenously treated (right panel). Genes analyzed for mutations are indicated on the left side.

Figure 2. Influence of *RUNX1* mutations on clinical outcome. Kaplan-Meier estimates for intensively treated CN-AML patients with or without RUNX1 mutations. The censored patient in the *RUNX1* mutated group experienced a relapse and was then lost to follow up. (A) Median overall survival in *RUNX1* mutated patients is 75 days compared to 442 days for patients with *RUNX1* wild-type status. (B) For ELN Intermediate I patients (CN-AML with wild-type CEBPA and wild-type NPM1 and/or FLT3-ITD) median overall survival in *RUNX1* mutated patients was 75 days compared to 293 days for patients without this mutation. (C) In elderly AML patients (≥ 60 years) the median OS for RUNX1 mutated patients was 86 days and 432 days for patients with wild-type status.

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Figure 3. Heatmap of genes differentially expressed between *RUNX1*-mutated and *RUNX1*-wild type patients. Each column represents one of 41 CN-AML patients, grouped according to RUNX1 mutation status, and each row represents one of 85 genes that were differentially expressed. Yellow indicates high and blue indicates low gene expression.

Figure 1 A. Overview of mutations in RUNX1.
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RUNX1 (NP_001745.2)

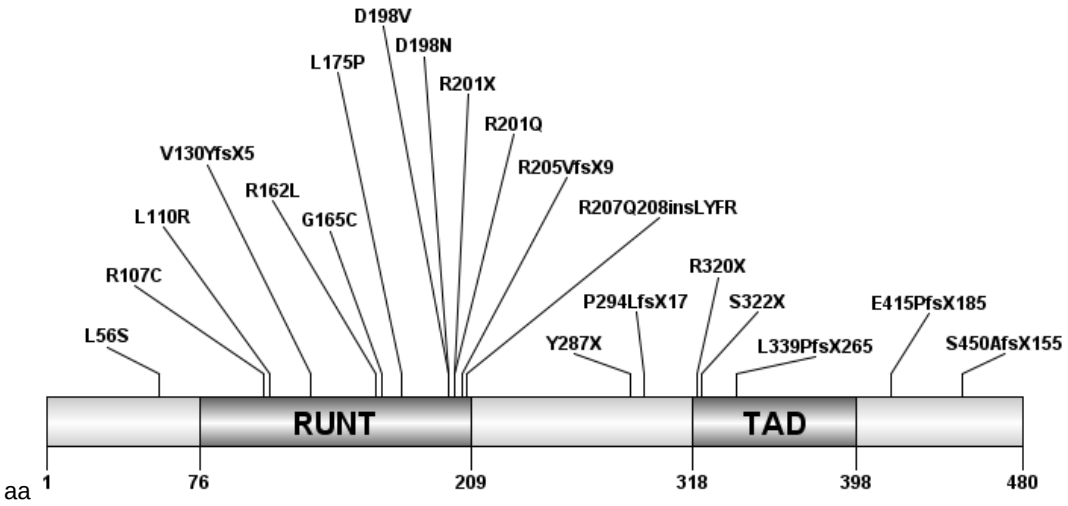

Figure 1B. Distribution of mutations in *RUNX 1* and 8 additional genes in 93 CN-AML patients.

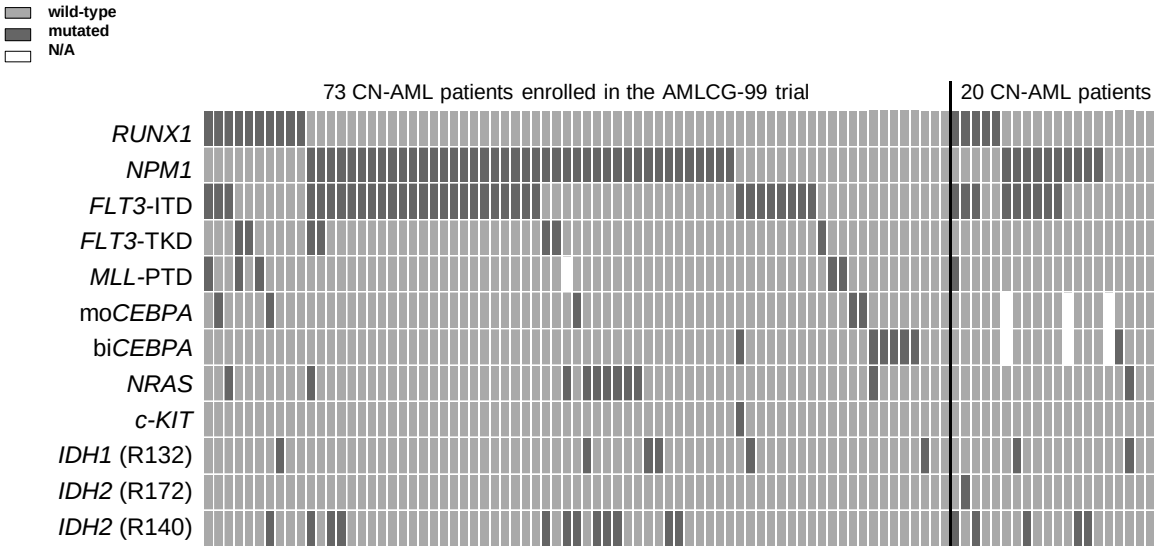

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Figure 2 A. Infl uence of *RUNX1* mutations on overall survival.

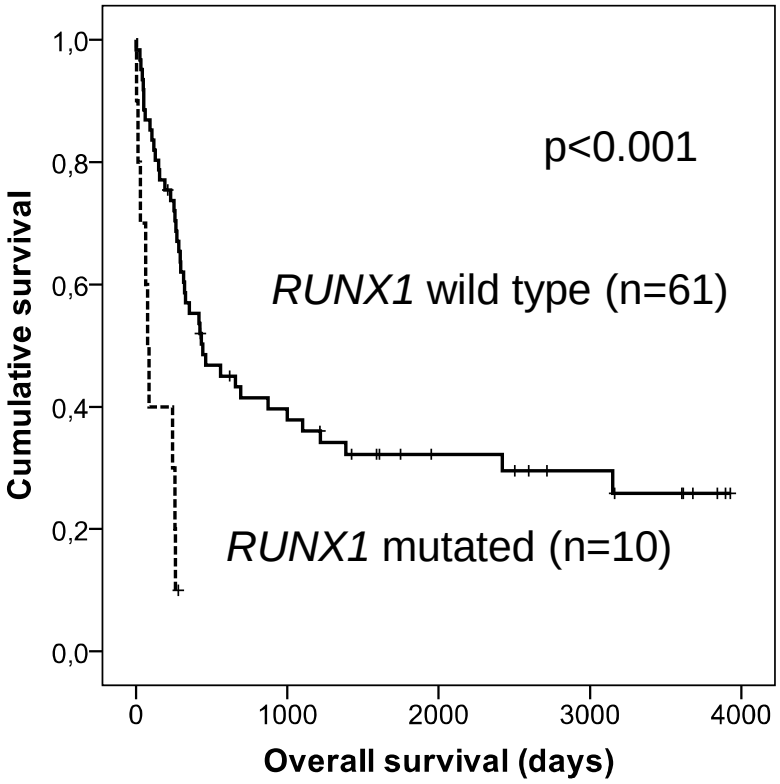

Figure 2 B. In fluence of *RUNX1* mutations on overall survival in ELN Intermediate I patients.

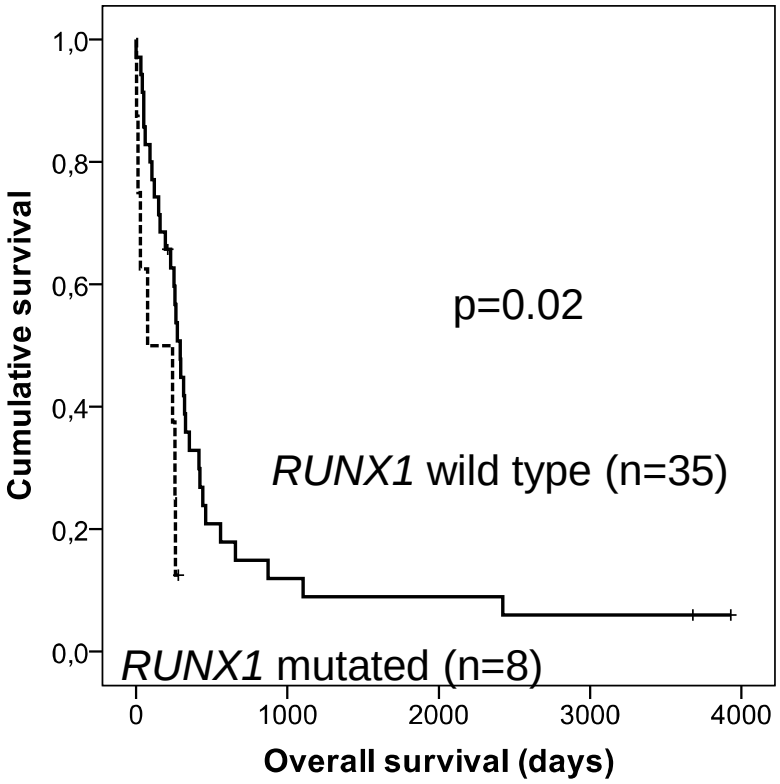

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Figure 2 C. Influence of *RUNX 1* mutations on overall survival in elderly AML patients (≥ 60 years).

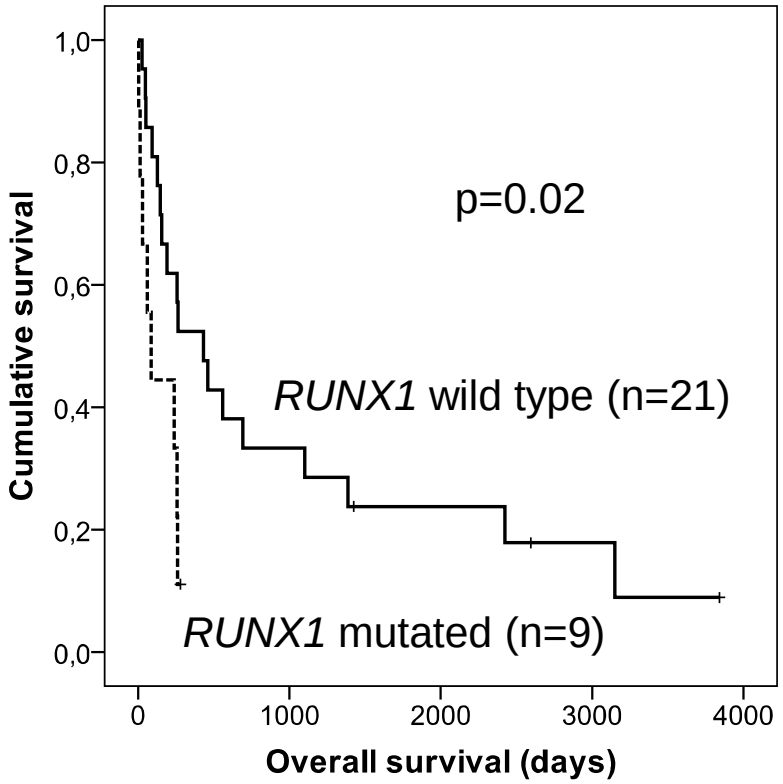

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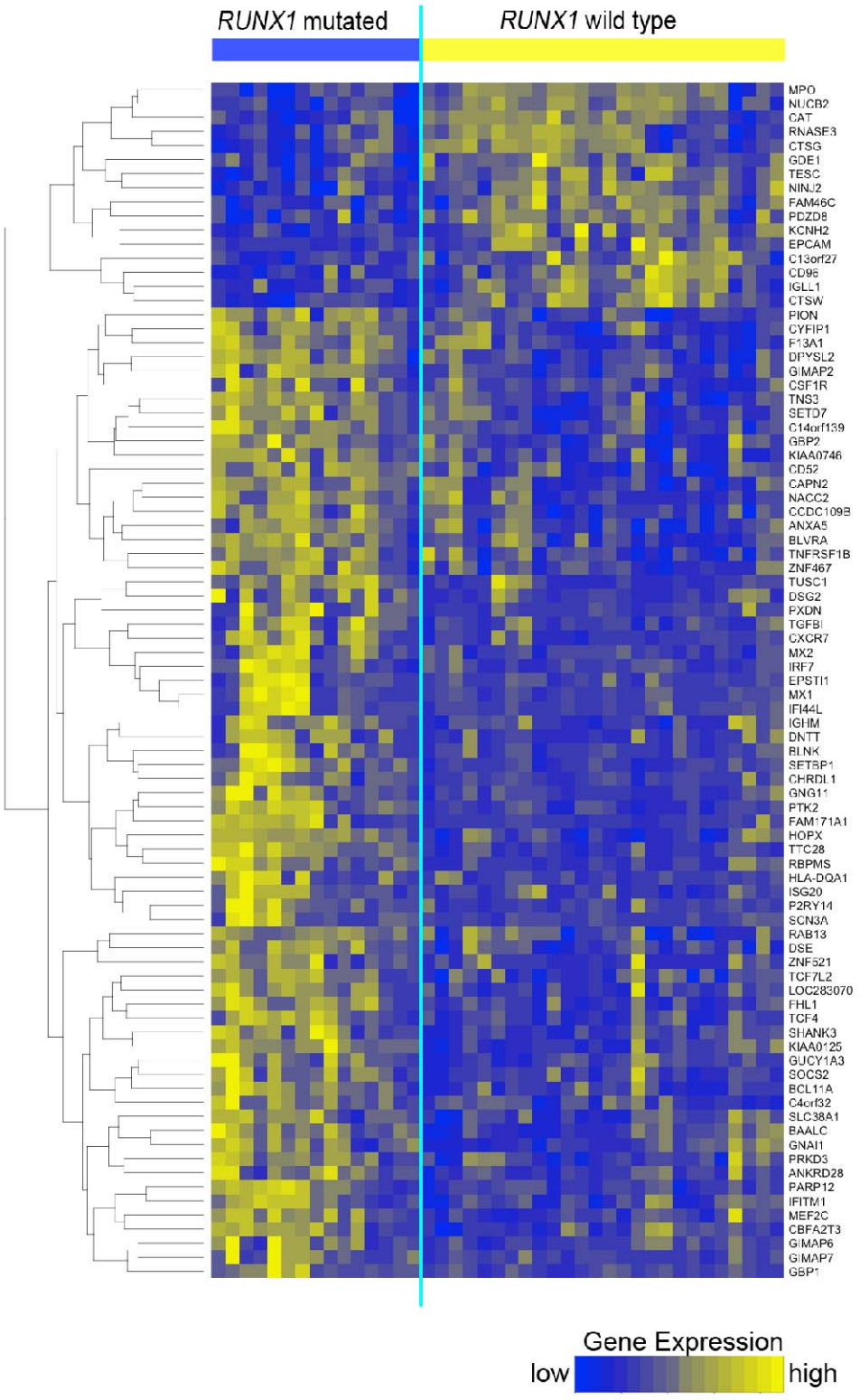
Figure 3. Heatmap of genes differentially expressed between *RUNX1*-mutated and *RUNX1*-wild type patients.



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Supplementary Table S1

Primers targeting the coding exons of RUNX1

cDNA sequence: uc010gmu.1 (NM_001754.4)
Genomic sequence: hg18_chr21:35080967-35344437_rev

NAME	LEFT_PRIMER	NAME	RIGHT_PRIMER	PRODUCT_SIZE
RUNX1-exon_2F	GTCTTGGTTTTCGCTCCG	RUNX1-exon_2R	CATTTCATTACAGGCAAAGCTG	197
RUNX1-exon_3F	AACCACGTGCATAAGGAACAG	RUNX1-exon_3R	GCAGAAACAGCCTTAATTATTTGG	363
RUNX1-exon_4F	TGGTAGGAGCTGTTTGCAGG	RUNX1-exon_4R	CATCCCAAGCTAGGAAGACC	459
RUNX1-exon_5F	TCACTACACAAATGCCCTAAAAG	RUNX1-exon_5R	TTGAAATGTGGGTTTGTTGC	292
RUNX1-exon_6F	AGATATGTTCAGGCCACCAAC	RUNX1-exon_6R	TCTGAGACATGGTCCCTGAG	243
RUNX1-exon_7F	AAGAAAAGCCCCAGTTTTAGG	RUNX1-exon_7R	AGTTGGTCTGGGAAGGTGTG	387
RUNX1-exon_8F	GAACAAGGGCCACTCATTTC	RUNX1-exon_8R	TGGACCTTCCACCCCAG	301
RUNX1-exon_9F	CTCCGCAACCTCCTACTCAC	RUNX1-exon_9Ra	CCCACCATGGAGAACTGGTA	342
RUNX1_exon_9Fa	CACGCGCTACCACACCTAC	RUNX1-exon_9R	CCTGACCTACAGCGAGATCC	484

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Supplementary Table S2

List of probe sets differentially expressed in RUNX1-mut (n=15) vs. RUNX1-wt (n=26) CN-AML patients. The analysis was restricted to NPM1-wt patients. 85 probe sets out of 17109 were significant (*q* value<.05). The *p* value of a Global test for difference between the two groups was .03.

Microarray probe set	Gene symbol	Gene name	Fold change: *RUNX1*-mutated vs. *RUNX1*-wild type	*q* value
GC04M057210_at	HOPX	HOP homeobox	4,10	0,0079
GC10P098054_at	DNTT	deoxynucleotidyltransferase, terminal	3,38	0,0231
GC08P026491_at	DPYSL2	dihydropyrimidinase-like 2	2,71	0,0122
GC01P026516_at	CD52	CD52 molecule	2,67	0,0114
GC14P105461_at	KIAA0125	KIAA0125	2,65	0,0079
GC01P078858_at	IFI44L	interferon-induced protein 44-like	2,63	0,0301
GC10M097941_at	BLNK	B-cell linker	2,58	0,0151
GC06M006089_at	F13A1	coagulation factor XIII, A1 polypeptide	2,51	0,0182
GC07M047281_at	TNS3	tensin 3	2,46	0,0079
GC12P092466_at	SOCS2	suppressor of cytokine signaling 2	2,40	0,0127
GC04M122868_at	ANXA5	annexin A5	2,39	0,0315
GC05P135392_at	TGFBI	transforming growth factor, beta-induced, 68kDa	2,29	0,0273
GC10M015294_at	FAM171A1	family with sequence similarity 171, member A1	2,23	0,0079
GC21P041720_at	MX1	myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)	2,14	0,0278
GC08P104222_at	BAALC	brain and acute leukemia, cytoplasmic	2,14	0,0122
GC11M000602_at	IRF7	interferon regulatory factor 7	2,13	0,0231
GC07P093388_at	GNG11	guanine nucleotide binding protein (G protein), gamma 11	2,09	0,0118
GC11P000303_at	IFITM1	interferon induced transmembrane protein 1 (9-27)	2,09	0,0262
GC12M044867_at	SLC38A1	solute carrier family 38, member 1	2,08	0,0251
GC07P079602_at	GNAI1	guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 1	2,07	0,0231
GC01M152220_at	RAB13	RAB13, member RAS oncogene family	2,01	0,0452
GC18M051045_at	TCF4	transcription factor 4	2,00	0,0120
GC04P110700_at	CCDC109B	coiled-coil domain containing 109B	1,99	0,0150
GC07P150015_at	GIMAP2	GTPase, IMAP family member 2	1,99	0,0164
GC03M152412_at	P2RY14	purinergic receptor P2Y, G-protein coupled, 14	1,96	0,0216
GC04P156807_at	GUCY1A3	guanylate cyclase 1, soluble, alpha 3	1,96	0,0153
GC03M015700_at	ANKRD28	ankyrin repeat domain 28	1,93	0,0251
GC01M089345_at	GBP2	guanylate binding protein 2, interferon-inducible	1,93	0,0158
GC07M149953_at	GIMAP6	GTPase, IMAP family member 6	1,92	0,0315
GC18M020895_at	ZNF521	zinc finger protein 521	1,91	0,0496
GC10U900364_at	LOC283070	hypothetical LOC283070	1,89	0,0153
GC15P020444_at	CYFIP1	cytoplasmic FMR1 interacting protein 1	1,87	0,0301
GC07M139370_at	PARP12	poly (ADP-ribose) polymerase family, member 12	1,84	0,0114
GC13M042358_at	EPSTI1	epithelial stromal interaction 1 (breast)	1,83	0,0496
GC01P012161_at	TNFRSF1B	tumor necrosis factor receptor superfamily, member 1B	1,81	0,0493
GC06P116708_at	DSE	dermatan sulfate epimerase	1,79	0,0153
GC09M025668_at	TUSC1	tumor suppressor candidate 1	1,79	0,0150
GC02P237143_at	CXCR7	chemokine (C-X-C motif) receptor 7	1,77	0,0122
GC15P086983_at	ISG20	interferon stimulated exonuclease gene 20kDa	1,77	0,0363
GC18P040535_at	SETBP1	SET binding protein 1	1,76	0,0079
GC22P049402_at	SHANK3	SH3 and multiple ankyrin repeat domains 3	1,76	0,0150
GC02M165652_at	SCN3A	sodium channel, voltage-gated, type III, alpha subunit	1,76	0,0311
GC14M105389_at	IGHM	immunoglobulin heavy constant mu	1,75	0,0394
GC01M089290_at	GBP1	guanylate binding protein 1, interferon-inducible, 67kDa	1,75	0,0466
GC14M094943_at	C14orf139	chromosome 14 open reading frame 139	1,74	0,0127
GC06P032649_at	HLA-DQA1	major histocompatibility complex, class II, DQ alpha 1	1,74	0,0420
GC05M088051_at	MEF2C	myocyte enhancer factor 2C	1,71	0,0153
GC0XP135057_at	FHL1	four and a half LIM domains 1	1,71	0,0114
GC08P030361_at	RBPMS	RNA binding protein with multiple splicing	1,70	0,0122
GC21P041655_at	MX2	myxovirus (influenza virus) resistance 2 (mouse)	1,69	0,0390
GC05M149413_at	CSF1R	colony stimulating factor 1 receptor	1,69	0,0479
GC22M026704_at	TTC28	tetratricopeptide repeat domain 28	1,69	0,0079
GC16M087468_at	CBFA2T3	core-binding factor, runt domain, alpha subunit 2; translocated to, 3	1,66	0,0284
GC04M025425_at	KIAA0746	KIAA0746 protein	1,64	0,0301
GC07P043764_at	BLVRA	biliverdin reductase A	1,64	0,0301
GC07M149094_at	ZNF467	zinc finger protein 467	1,64	0,0280
GC07P149842_at	GIMAP7	GTPase, IMAP family member 7	1,62	0,0311
GC04P113286_at	C4orf32	chromosome 4 open reading frame 32	1,61	0,0479
GC02M037389_at	PRKD3	protein kinase D3	1,59	0,0448
GC10P114700_at	TCF7L2	transcription factor 7-like 2 (T-cell specific, HMG-box)	1,59	0,0273
GC08M141737_at	PTK2	PTK2 protein tyrosine kinase 2	1,58	0,0079
GC09M138039_at	NACC2	NACC family member 2, BEN and BTB (POZ) domain containing	1,58	0,0388
GC04M140646_at	SETD7	SET domain containing (lysine methyltransferase) 7	1,57	0,0270
GC07M076779_at	PION	pigeon homolog (Drosophila)	1,57	0,0264
GC02M060589_at	BCL11A	B-cell CLL/lymphoma 11A (zinc finger protein)	1,56	0,0210
GC01P221966_at	CAPN2	calpain 2, (m/II) large subunit	1,55	0,0301
GC18P027332_at	DSG2	desmoglein 2	1,54	0,0420
GC0XM109724_at	CHRDL1	chordin-like 1	1,53	0,0401
GC02M001606_at	PXDN	peroxidasin homolog (Drosophila)	1,53	0,0374
GC07M150272_at	KCNH2	potassium voltage-gated channel, subfamily H (eag-related), member 2	0,65	0,0420
GC12M000543_at	NINJ2	ninjurin 2	0,65	0,0231
GC16M019422_at	GDE1	glycerophosphodiester phosphodiesterase 1	0,64	0,0479
GC13M102219_at	C13orf27	chromosome 13 open reading frame 27	0,63	0,0463
GC10M119033_at	PDZD8	PDZ domain containing 8	0,60	0,0322
GC02P047425_at	EPCAM	epithelial cell adhesion molecule	0,57	0,0394
GC01P117860_at	FAM46C	family with sequence similarity 46, member C	0,56	0,0278
GC11P034417_at	CAT	catalase	0,52	0,0153
GC12M115961_at	TESC	tescalcin	0,52	0,0122
GC03P112743_at	CD96	CD96 molecule	0,52	0,0311
GC11P017255_at	NUCB2	nucleobindin 2	0,45	0,0122
GC11P065405_at	CTSW	cathepsin W	0,41	0,0114
GC14P020429_at	RNASE3	ribonuclease, RNase A family, 3 (eosinophil cationic protein)	0,37	0,0432
GC22M022239_at	IGLL1	immunoglobulin lambda-like polypeptide 1	0,36	0,0311
GC14M024112_at	CTSG	cathepsin G	0,30	0,0231
GC17M053702_at	MPO	myeloperoxidase	0,29	0,0127

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Supplementary Table S3:

Gene Set Enrichment Analysis, identifying sets of functionally related gene that are enriched in CN-AML patients with (n=15) or without (n=26) *RUNX1* mutations.
This analysis used the curated 'cannonical pathways' (c2:cp) collection of the Molecular Signatures Database, version 3.0, available online at http://www.broadinstitute.org/gsea/msigdb
Only gene sets comprising between 15 and 200 individual genes (654 of the 880 total gene sets) were included in the analysis. Gene sets were considered significant at a false discovery rate (FDR) of q<.10
128 gene sets were significant: 71 were enriched in *RUNX1*-mut patients and 51 in *RUNX1*-wt patients.

A) Gene sets enriched in *RUNX1*-mut patients compared to *RUNX1*-wt

Gene Set Name	No. of Genes in Gene Set	Normalized Enrichment Score	Nominal *p* Value	FDR *q* Value
BIOCARTA_EDG1_PATHWAY	27	1,97	<.0001	0,057
BIOCARTA_BCR_PATHWAY	34	1,96	0,001	0,034
REACTOME_TOLL_LIKE_RECEPTOR_4_CASCADE	28	1,95	0,001	0,025
REACTOME_TOLL_RECEPTOR_CASCADES	83	1,94	<.0001	0,022
BIOCARTA_CALCINEURIN_PATHWAY	18	1,94	<.0001	0,018
REACTOME_PROTEOLYTIC_CLEAVAGE_OF_SNARE_COMPLEX_PROTEINS	15	1,94	<.0001	0,015
KEGG_INTESTINAL_IMMUNE_NETWORK_FOR_IGA_PRODUCTION	38	1,89	<.0001	0,026
REACTOME_MYD88_CASCADE	19	1,89	0,001	0,025
REACTOME_SIGNALING_BY_BMP	22	1,89	0,003	0,023
REACTOME_INNATE_IMMUNITY_SIGNALING	103	1,89	<.0001	0,021
REACTOME_BOTULINUM_NEUROTOXICITY	17	1,88	0,002	0,019
REACTOME_TOLL_LIKE_RECEPTOR_9_CASCADE	23	1,88	<.0001	0,018
BIOCARTA_MEF2D_PATHWAY	18	1,87	0,002	0,020
KEGG_GAP_JUNCTION	84	1,86	0,0004	0,022
ST_GA12_PATHWAY	22	1,85	<.0001	0,021
REACTOME_PLATELET_ACTIVATION_TRIGGERS	56	1,85	0,0005	0,020
BIOCARTA_CXCR4_PATHWAY	24	1,84	0,002	0,023
SA_B_CELL_RECEPTOR_COMPLEXES	24	1,81	0,002	0,030
BIOCARTA_PAR1_PATHWAY	37	1,81	0,002	0,031
KEGG_TIGHT_JUNCTION	127	1,81	<.0001	0,029
BIOCARTA_VEGF_PATHWAY	28	1,80	0,003	0,029
BIOCARTA_TH1TH2_PATHWAY	18	1,79	0,002	0,031
BIOCARTA_PGC1A_PATHWAY	22	1,79	0,003	0,030
KEGG_ALLOGRAFT_REJECTION	25	1,79	0,003	0,030
BIOCARTA_BIOPEPTIDES_PATHWAY	42	1,78	0,001	0,032
BIOCARTA_CCR3_PATHWAY	23	1,78	0,004	0,032
KEGG_NOTCH_SIGNALING_PATHWAY	46	1,78	0,002	0,032
KEGG_GRAFT_VERSUS_HOST_DISEASE	23	1,77	0,002	0,032
BIOCARTA_SPPA_PATHWAY	21	1,77	0,002	0,033
REACTOME_ERK_MAPK_TARGETS	21	1,77	0,003	0,032
BIOCARTA_MYOSIN_PATHWAY	31	1,75	0,002	0,037
KEGG_DORSO_VENTRAL_AXIS_FORMATION	23	1,75	0,003	0,038
BIOCARTA_HDAC_PATHWAY	28	1,74	0,003	0,039
KEGG_GLYCOSAMINOGLYCAN_BIOSYNTHESIS_HEPARAN_SULFATE	25	1,73	0,004	0,041
KEGG_LEISHMANIA_INFECTION	59	1,72	0,002	0,046
KEGG_B_CELL_RECEPTOR_SIGNALING_PATHWAY	74	1,72	0,0004	0,045
REACTOME_THROMBIN_SIGNALLING_THROUGH_PROTEINASE_ACTIVATED_RECEPTORS	25	1,71	0,006	0,047
REACTOME_PLC_BETA_MEDIATED_EVENTS	38	1,71	0,006	0,048
REACTOME_ACTIVATED_TLR4_SIGNALLING	24	1,71	0,010	0,047
BIOCARTA_AT1R_PATHWAY	32	1,71	0,005	0,047
KEGG_CHEMOKINE_SIGNALING_PATHWAY	173	1,70	<.0001	0,048
REACTOME_ACTIVATION_OF_KAINATE_RECEPTORS_UPON_GLUTAMATE_BINDING	30	1,69	0,006	0,053
KEGG_WNT_SIGNALING_PATHWAY	144	1,66	0,001	0,067
BIOCARTA_NFKB_PATHWAY	22	1,66	0,010	0,068
REACTOME_NUCLEAR_EVENTS_KINASE_AND_TRANSCRIPTION_FACTOR_ACTIVATION	24	1,65	0,006	0,074
BIOCARTA_FCER1_PATHWAY	38	1,65	0,010	0,072
BIOCARTA_AGR_PATHWAY	35	1,65	0,009	0,071
KEGG_AXON_GUIDANCE	128	1,65	0,001	0,072
REACTOME_G_ALPHA_12_13_SIGNALLING_EVENTS	53	1,65	0,005	0,071
BIOCARTA_TOLL_PATHWAY	35	1,65	0,006	0,070
BIOCARTA_ERK5_PATHWAY	17	1,64	0,017	0,070
BIOCARTA_NTHI_PATHWAY	24	1,64	0,018	0,072
KEGG_MELANOGENESIS	97	1,64	0,002	0,071
KEGG_PHOSPHATIDYLINOSITOL_SIGNALING_SYSTEM	74	1,64	0,002	0,070
REACTOME_CRMPS_IN_SEMA3A_SIGNALING	16	1,62	0,017	0,078
REACTOME_SEMAPHORIN_INTERACTIONS	65	1,62	0,007	0,081
REACTOME_SIGNAL_AMPLIFICATION	29	1,61	0,017	0,083
ST_WNT_BETA_CATENIN_PATHWAY	30	1,61	0,016	0,083
BIOCARTA_CDMAC_PATHWAY	16	1,61	0,022	0,084
REACTOME_TCR_SIGNALING	49	1,60	0,009	0,088
REACTOME_REGULATION_OF_LIPID_METABOLISM_BY_PEROXISOME_PROLIFERATOR_ACTIVATED_	55	1,60	0,008	0,091
REACTOME_OPIOID_SIGNALLING	81	1,59	0,005	0,090
REACTOME_G_PROTEIN_BETA_GAMMA_SIGNALLING	25	1,59	0,021	0,091
REACTOME_AXON_GUIDANCE	158	1,59	0,001	0,092
BIOCARTA_RARRXR_PATHWAY	15	1,58	0,031	0,100
BIOCARTA_CD40_PATHWAY	15	1,57	0,034	0,100
KEGG_GLYCOSAMINOGLYCAN_BIOSYNTHESIS_CHONDROITIN_SULFATE	20	1,57	0,031	0,099
KEGG_TYPE_I_DIABETES_MELLITUS	29	1,57	0,018	0,098
REACTOME_REGULATION_OF_INSULIN_SECRETION_BY_ACETYLCHOLINE	20	1,57	0,036	0,097
REACTOME_MAPK_TARGETS_NUCLEAR_EVENTS_MEDIATED_BY_MAP_KINASES	30	1,57	0,019	0,098
KEGG_LONG_TERM_DEPRESSION	66	1,57	0,011	0,099

B) Gene sets enriched in *RUNX1*-wt patients compared to *RUNX1*-mut

Gene Set Name	No. of Genes in Gene Set	Normalized Enrichment Score	Nominal *p* Value	FDR *q* Value
REACTOME_ACTIVATION_OF_THE_PRE_REPLICATIVE_COMPLEX	29	-2,45	<.0001	<.0001
REACTOME_DNA_REPLICATION_PRE_INITIATION	68	-2,44	<.0001	<.0001
KEGG_DNA_REPLICATION	36	-2,43	<.0001	<.0001
REACTOME_DNA_STRAND_ELONGATION	30	-2,38	<.0001	<.0001
REACTOME_G1_S_TRANSITION	90	-2,33	<.0001	0,0002
REACTOME_SYNTHESIS_OF_DNA	81	-2,31	<.0001	0,0002
REACTOME_S_PHASE	93	-2,25	<.0001	0,001
REACTOME_MITOTIC_M_M_G1_PHASES	144	-2,19	<.0001	0,001
REACTOME_M_G1_TRANSITION	54	-2,17	<.0001	0,002
REACTOME_ACTIVATION_OF_ATR_IN_RESPONSE_TO_REPLICATION_STRESS	34	-2,16	<.0001	0,002
REACTOME_E2F_MEDIATED_REGULATION_OF_DNA_REPLICATION	30	-2,15	<.0001	0,002
REACTOME_EXTENSION_OF_TELOMERES	28	-2,11	<.0001	0,003
REACTOME_E2F_TRANSCRIPTIONAL_TARGETS_AT_G1_S	19	-2,11	0,001	0,003
REACTOME_G2_M_CHECKPOINTS	39	-2,09	<.0001	0,003
REACTOME_LAGGING_STRAND_SYNTHESIS	20	-2,07	0,001	0,004
REACTOME_REPAIR_SYNTHESIS_OF_PATCH_27_30_BASES_LONG_BY_DNA_POLYMERASE	15	-2,02	0,003	0,007
KEGG_CELL_CYCLE	115	-2,02	<.0001	0,007
REACTOME_ORC1_REMOVAL_FROM_CHROMATIN	56	-1,99	<.0001	0,008
BIOCARTA_MCM_PATHWAY	18	-1,99	<.0001	0,007
REACTOME_TRANSCRIPTION_COUPLED_NER	38	-1,99	0,001	0,007
REACTOME_CELL_CYCLE_CHECKPOINTS	97	-1,98	<.0001	0,008
KEGG_NUCLEOTIDE_EXCISION_REPAIR	40	-1,93	0,002	0,012
KEGG_BASE_EXCISION_REPAIR	31	-1,92	0,002	0,013
KEGG_MISMATCH_REPAIR	22	-1,87	0,003	0,021
REACTOME_ELECTRON_TRANSPORT_CHAIN	57	-1,87	<.0001	0,021
REACTOME_GLUCOSE_TRANSPORT	38	-1,86	0,002	0,022
KEGG_PORPHYRIN_AND_CHLOROPHYLL_METABOLISM	28	-1,86	0,003	0,021
REACTOME_CDT1_ASSOCIATION_WITH_THE_CDC6_ORC_ORIGIN_COMPLEX	45	-1,84	0,001	0,023
BIOCARTA_MITOCHONDRIA_PATHWAY	21	-1,77	0,010	0,042
REACTOME_GLOBAL_GENOMIC_NER	30	-1,77	0,003	0,042
REACTOME_MITOTIC_PROMETAPHASE	86	-1,77	0,001	0,041
REACTOME_NUCLEOTIDE_EXCISION_REPAIR	43	-1,75	0,001	0,048
KEGG_GLYCINE_SERINE_AND_THREONINE_METABOLISM	31	-1,74	0,003	0,047
REACTOME_METABOLISM_OF_CARBOHYDRATES	113	-1,74	<.0001	0,048
REACTOME_REGULATION_OF_APC_ACTIVATORS_BETWEEN_G1_S_AND_EARLY_ANAPHASE	61	-1,72	0,001	0,055
KEGG_TYROSINE_METABOLISM	38	-1,70	0,007	0,062
REACTOME_TRANSPORT_OF_THE_SLBP_INDEPENDENT_MATURE_MRNA	31	-1,69	0,007	0,066
REACTOME_REGULATION_OF_ORNITHINE_DECARBOXYLASE	40	-1,69	0,007	0,064
REACTOME_INFLUENZA_LIFE_CYCLE	85	-1,66	<.0001	0,076
REACTOME_TAT_MEDIATED_HIV1_ELONGATION_ARREST_AND_RECOVERY	27	-1,66	0,011	0,077
REACTOME_LATE_PHASE_OF_HIV_LIFE_CYCLE	82	-1,65	0,001	0,077
REACTOME_REGULATION_OF_GLUCOKINASE_BY_GLUCOKINASE_REGULATORY_PROTEIN	29	-1,65	0,011	0,078
REACTOME_SCF_SKP2_MEDIATED_DEGRADATION_OF_P27_P21	44	-1,65	0,006	0,077
REACTOME_HIV_LIFE_CYCLE	94	-1,63	0,003	0,086
REACTOME_GLYCOLYSIS	21	-1,63	0,023	0,084
REACTOME_P53_INDEPENDENT_DNA_DAMAGE_RESPONSE	36	-1,62	0,011	0,087
REACTOME_MTOR_SIGNALLING	25	-1,61	0,014	0,089
REACTOME_CDC20_PHOSPHO_APC_MEDIATED_DEGRADATION_OF_CYCLIN_A	53	-1,61	0,003	0,088
KEGG_RNA_POLYMERASE	26	-1,60	0,019	0,093
REACTOME_HIV1_TRANSCRIPTION_ELONGATION	35	-1,60	0,015	0,095
REACTOME_ABORTIVE_ELONGATION_OF_HIV1_TRANSCRIPT_IN_THE_ABSENCE_OF_TAT	21	-1,59	0,026	0,099
REACTOME_VPR_MEDIATED_NUCLEAR_IMPORT_OF_PICS	31	-1,58	0,015	0,098
KEGG_PHENYLALANINE_METABOLISM	18	-1,58	0,032	0,097
REACTOME_IRS_RELATED_EVENTS	75	-1,58	0,004	0,096
REACTOME_CYCLIN_E_ASSOCIATED_EVENTS_DURING_G1_S_TRANSITION_	49	-1,58	0,011	0,097
REACTOME_GLUCOSE_REGULATION_OF_INSULIN_SECRETION	135	-1,58	0,000	0,096
REACTOME_FRS2MEDIATED_CASCADE	25	-1,56	0,027	0,100