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LETTER TO THE EDITOR

Expression of *CEBPA* is reduced in *RUNX1*-mutated acute myeloid leukemia

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CEBPA (CCAAT/enhancer-binding protein alpha) is a member of the C/EBP family of bZIP transcription factors encoding two different translational protein isoforms. The CEBPA transcription factor is involved in cell cycle arrest, repression of self-renewal and myeloid differentiation during normal hematopoiesis. In acute myeloid leukemia (AML), mutations in *CEBPA* result in a cellular differentiation block.[1] They occur in around 8% of normal karyotype AML (CN-AML). Approximately half of the patients harbor two *CEPBA* mutations associated to a favorable prognosis. *CEBPA* function can also be affected by promoter methylation[2] or alterations in other oncogenes, for example through the *t*(8;21)(q22;q22)/*RUNX1–RUNX1T1*, which suppresses *CEBPA* mRNA expression. The *t*(8;21)(q22;q22) translocation replaces the C terminus, including the transactivation domain (TAD) of *RUNX1*, with *RUNX1T1*.[3] *RUNX1–RUNX1T1* in *t*(8;21) blocks *CEBPA*-dependent activation of its own (*CEBPA*) promoter and thereby inhibits autoregulation.[4] Other leukemic fusion proteins involving core-binding factor (CBF) family members, for example *t*(3;21)(q26;q22)/*RUNX1–EVI1* or inv(16)(p13q22)/*CBFB–MYH11*, did not suppress *CEBPA* mRNA, indicating a *RUNX1–RUNX1T1*-specific effect on *CEBPA* transcriptional control.[1]

Intragenic *RUNX1* mutations confer an adverse prognosis in AML. Previously, we identified *RUNX1* mutations in 32.7% of CN-AML or with non-complex chromosomal imbalances.[5] *RUNX1* mutations are absent in CBF–AML and acute promyelocytic leukemia.[6] They are inversely correlated with *CEBPA* mutations.[5,6]

To clarify whether intragenic *RUNX1* mutations such as *RUNX1–RUNXT1* fusions also result in *CEBPA* mRNA downregulation, we investigated 359 AML patients consisting of two independent cohorts: cohort 1 with 209 AML cases (109 males/100 females; median age 65.4 years; 19.7–88.1 years) from different cytogenetic subgroups (normal karyotype ($n=93$), *t*(8;21)(q22;q22)/*RUNX1–RUNX1T1* ($n=16$), *t*(15;17)(q22;q12)/*PML-RARA* ($n=15$), sole +8 ($n=12$), sole +13 ($n=10$), complex karyotypes ($n=10$) and other rare noncomplex genetic abnormalities ($n=53$)). Cohort 2 comprised 150 normal karyotype patients selected according to *RUNX1* mutation status (92 males/58 females; median age, 69.7 years; 18.3–88.1 years; 81/150 (54.0%) *RUNX1* mutated) with survival data in 124 cases. Bone marrow and/or peripheral blood samples were sent to the MLL Munich Leukemia Laboratory in 2005–2011. All patients gave their written informed consent to genetic analysis and scientific studies.

Chromosome-banding analysis was performed in all cases, when needed, combined with fluorescent *in situ* hybridization. *RUNX1* mutations were analyzed by Sanger sequencing or an amplicon-based high-throughput deep-sequencing assay (454 Life Sciences, Branford, CT, USA). *CEBPA* (mRNA) expression was quantified in cohort 1 by gene expression microarray profiling (Affymetrix HG-U133 Plus 2.0 microarrays; Santa Clara, CA, USA). The gene expression raw data were processed according to the manufacturer's recommendations. Detection calls, that is present, marginal, or absent expression, were determined by default parameters. For measurement of *CEBPA* expression in cohort 2, a quantitative real-time reverse transcriptase PCR (RT-PCR) assay was established (Taqman, Life Technologies, Carlsbad, CA, USA; CEBPA TaqMan Gene Expression Assay: HS00269972_S1). mRNA expression of *CEBPA* was normalized against expression of *ABL1*; ratios were given as %*CEBPA*/*ABL1*.

First, we investigated 209 AML cases from different cytogenetic subgroups using gene expression microarray profiling (Table 1a). The *RUNX1* mutation status was analyzed in 178 cases (*RUNX1–RUNX1T1* or *PML-RARA*-mutated cases had been excluded), in 41/178 (23%) of patients *RUNX1* was mutated. The median *CEBPA* expression intensity value in all patients was 670 (range 48–5244). *RUNX1*-mutated cases showed a lower *CEBPA* expression than *RUNX1* wild-type cases ($n=41$ vs 137, mean ± s.d. 429 ± 395 vs 998 ± 717; $P<0.001$). Cases harboring a *t*(8;21)/*RUNX1–RUNX1T1* presented a lower *CEBPA* expression than patients without ($n=16$ vs 193, mean ± s.d. 292 ± 216 vs 950 ± 808; $P<0.001$), whereas *t*(15;17)/*PML-RARA*-mutated cases showed enhanced *CEBPA* expression ($n=15$ vs 194, mean ± s.d. 1940 ± 1290 vs 819 ± 690; $P=0.005$) (Figure 1a). As reported previously, cases with a sole +13 showed lower expression than cases without ($n=10$ vs 199, mean ± s.d. 326 ± 406 vs 929 ± 803; $P=0.020$); however, all +13 cases were *RUNX1*-mutated.

For validation, an independent cohort of 150 normal karyotype AML was investigated for *RUNX1* mutations (Table 1b), *CEBPA* expression was quantified using real-time RT-PCR. *RUNX1*

Table 1a. *CEBPA* expression levels were determined by microarray gene expression analysis in cohort 1 including 209 AML patients from different cytogenetic subgroups

Cytogenetic subgroup	*Status*	*No.*	*Mean CEBPA expression (± s.d.)*	P-*value*
Normal karyotype	1	93	995 ± 671	0.122
	0	116	823 ± 883	
t(8;21)/*RUNX1–RUNX1T1*	1	16	292 ± 216	<0.001
	0	193	950 ± 808	
t(15;17)/*PML-RARA*	1	15	1940 ± 1290	0.005
	0	194	819 ± 690	
+8 as sole alteration	1	12	877 ± 755	0.918
	0	197	901 ± 803	
+13 as sole alteration	1	10	326 ± 406	0.020
	0	199	929 ± 803	
Complex karyotype	1	10	885 ± 786	0.951
	0	199	901 ± 801	
Other genetic abnormalities	1	53	738 ± 711	0.087
	0	156	955 ± 821	
Total cohort	209	209	900 ± 799	—

Abbreviations: AML, acute myeloid leukemia; *CEBPA,* CCAAT/enhancer-binding protein alpha; No., number of patients. The *P*-values result from comparison of the *CEBPA* expression levels depending on whether the patients had the respective cytogenetic alteration or not. (status: 1 means evidence of the respective cytogenetic alteration, 0 means no evidence of the respective alteration).

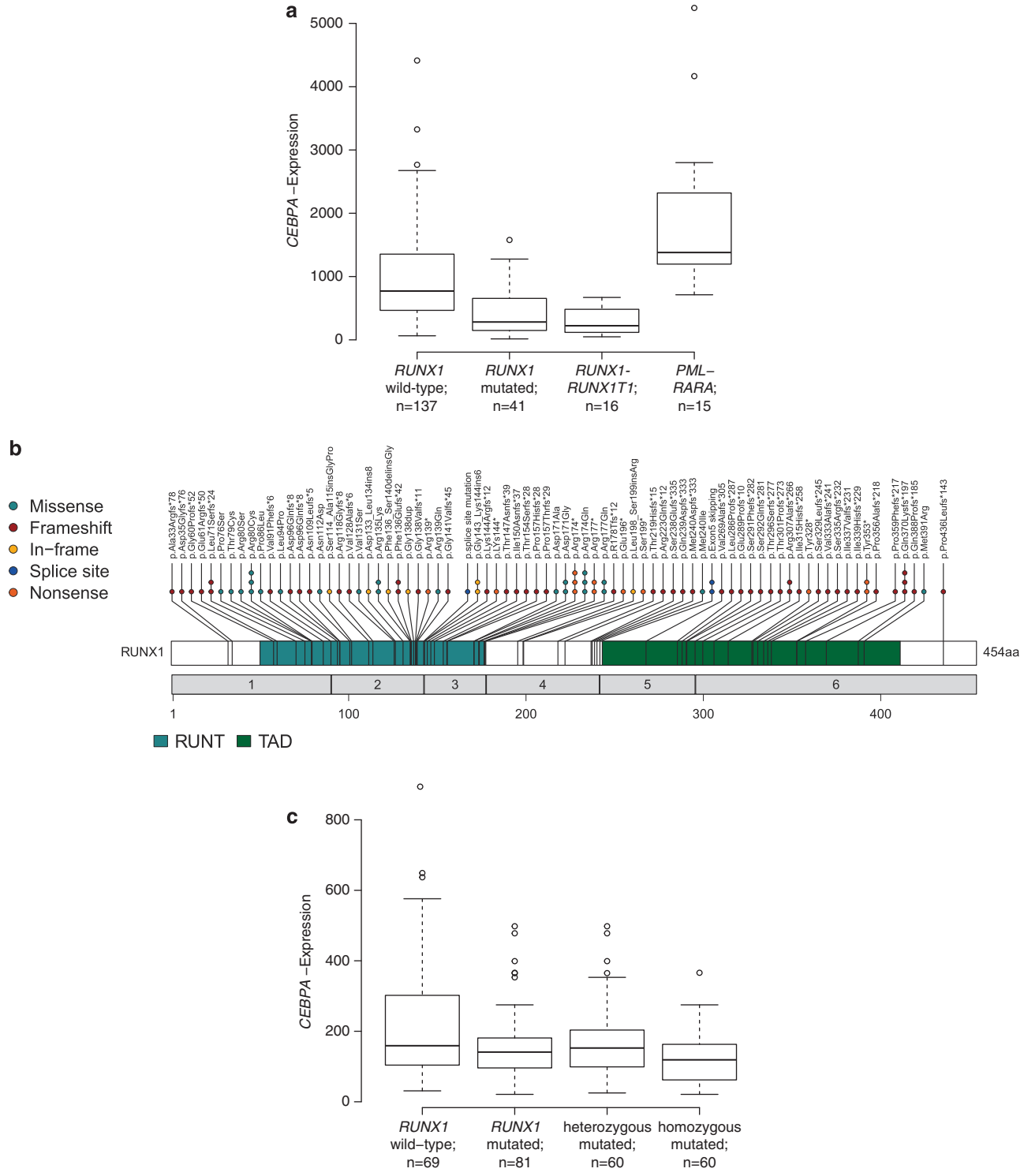

Figure 1. (**a**) *CEBPA* expression as determined by microarray gene expression analysis in different genetic subgroups of cohort 1. (**b**) Distribution of the *RUNX1* mutations in cohort 2 with regards to the different domains. (**c**) *CEBPA* expression as determined by real-time PCR in cohort 2.

mutations were detected in 81/150 (54.0%) (Figure 1b). Although this cohort was selected according to karyotype and *RUNX1* mutation status, we compared the overall survival (OS) and event-free survival (EFS) from *RUNX1*-mutated and *RUNX1* wild-type cases. Median OS was 19.9 and 12.2 months, OS at 3 years was 35.3 and 17.4%, $P = 0.049$; median EFS was 18.8 and 6.9 months, OS at 3 years was 34.2 and 4.7%, $P = 0.007$. Median *CEBPA* expression intensity was 148 (range: 21–960). Correspondingly to the data obtained from the first cohort, *CEBPA* expression was lower in *RUNX1*-mutated cases as compared with *RUNX1* wild-type patients (mean ± s.d. 155 ± 98 vs 222 ± 183; $P = 0.007$) (Figure 1c). When separating the cohort in cases with $\leqslant 148$ and > 148 *CEBPA* expression (using the median *CEBPA* expression as threshold in the cohort), we observed no association with OS and EFS (median

Table 1b. Investigation of *CEBPA* expression as determined by quantitative real-time PCR in cohort 2 of 150 patients with normal karyotype AML

Genetic subgroup	*No.*	*Mean %CEBPA/ ABL1 (± s.d.)*	P-*value*
RUNX1 mutation	81	155 ± 98	0.007
RUNX1 wild-type	69	222 ± 183	
RUNX1mut			
Outside/within RUNT domain	46	161 ± 107	NS
Within TAD domain	35	148 ± 87	
RUNX1mut			
Missense	19	177 ± 114	NS
In-frame/frameshift/nonsense	62	148 ± 93	
RUNX1mut			
Single-mutated	60	165 ± 100	0.116
Double-mutated/homozygous	21	126 ± 89	

Abbreviations: AML, acute myeloid leukemia; *CEBPA,* CCAAT/enhancer-binding protein alpha; mut, mutation; No., number of patients; NS, nonsignificant; TAD, transactivation domain. *CEBPA* expression levels were compared depending on *RUNX1* mutation status and distinct *RUNX1* mutation characteristics.

OS was 16.8 and 18.8 months, OS at 3 years was 25.6 and 24.3%, $P = 0.507$; median EFS was 11.0 and 9.2 months, OS at 3 years was 19.1 and 15.0%; $P = 0.541$).

In addition, we investigated whether the localization of the *RUNX1* mutations had any impact on *CEBPA* expression. However, no significant difference was detected between *CEBPA* expression levels, when *RUNX1* mutations were located either outside or within the DNA-binding domain (RUNT) ($n = 46$ cases), or behind the RUNT and within the TAD ($n = 35$) (mean ± s.d. 161 ± 107 vs 148 ± 87; $P =$ NS), respectively. Also when comparing missense mutations ($n = 19$) against in-frame, frameshift and nonsense alterations ($n = 62$), no significant difference was detectable (mean ± s.d. 177 ± 114 vs 148 ± 93; $P =$ NS). In contrast, separating cases in single-mutated ($n = 60$) or double-mutated/homozygous ($n = 21$) *RUNX1* mutations, we observed a non-significant trend towards a lower *CEBPA* expression in cases with double-mutated/homozygous mutations (mean ± s.d. 126 ± 89 vs 165 ± 100; $P = 0.116$) (Figure 1c).

In murine experiments, *RUNX1* gene deletion was reducing *CEBPA* mRNA in lineage-negative marrow cells in granulocyte–monocyte progenitors or common myeloid progenitors.[7] Here, we demonstrated a negative effect of *RUNX1* mutations on *CEBPA* expression levels in AML patients, similar to the *RUNX1–RUNX1T1* fusion, which have previously been reported.[4] By gene expression profiling, downregulation of different hematological transcription regulators such as *CEBPA* or *ETV6* had been described by Silva *et al.*[8] in AML FAB M0, which is closely associated with *RUNX1* mutations. *RUNX1* mutation localization seems to have no impact on *CEBPA* expression. In summary, downregulation of *CEBPA* expression may contribute to leukemogenesis in *RUNX1*-mutated AML.

CONFLICT OF INTEREST

WK, SuS, TH and CH declare part ownership of the MLL Munich Leukemia Laboratory GmbH. VG, UB, AK, AR, SJ, FD and KB are employed by MLL Munich Leukemia Laboratory GmbH.

AUTHOR CONTRIBUTIONS

VG and CH performed study design. VG, UB, AK, AR and WK performed data analysis. UB and VG wrote the first manuscript draft. Molecular analyses were done by VG, AK, SJ, FD, KB and SuS. WK was responsible for immunophenotyping, SuS for molecular genetics, TH for cytomorphology and CH for cytogenetics. All authors contributed to writing of the manuscript and reviewed and approved the final version.

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