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ORIGINAL ARTICLE

BAALC expression: a suitable marker for prognostic risk stratification and detection of residual disease in cytogenetically normal acute myeloid leukemia

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High *brain and acute leukemia, cytoplasmic* (*BAALC*) expression defines an important risk factor in cytogenetically normal acute myeloid leukemia (CN-AML). The prognostic value of *BAALC* expression in relation to other molecular prognosticators was analyzed in 326 CN-AML patients (<65 years). At diagnosis, high *BAALC* expression was associated with prognostically adverse mutations: *FLT3* internal tandem duplication (*FLT3*-ITD) with an *FLT3*-ITD/*FLT3* wild-type (wt) ratio of $\geqslant 0.5$ ($P = 0.001$), partial tandem duplications within the *MLL* gene (*MLL*-PTD) ($P = 0.002$), *RUNX1* mutations (mut) ($P < 0.001$) and *WT1*mut ($P = 0.001$), while it was negatively associated with *NPM1*mut ($P < 0.001$). However, high *BAALC* expression was also associated with prognostically favorable biallelic *CEBPA* ($P = 0.001$). Survival analysis revealed an independent adverse prognostic impact of high *BAALC* expression on overall survival (OS) and event-free survival (EFS), and also on OS when eliminating the effect of allogeneic stem cell transplantation (SCT) (OS^{TXcens}). Furthermore, we analyzed *BAALC* expression in 416 diagnostic and follow-up samples of 66 patients. During follow-up, *BAALC* expression correlated with mutational load or expression levels, respectively, of other minimal residual disease markers: *FLT3*-ITD ($r = 0.650$, $P < 0.001$), *MLL*-PTD ($r = 0.728$, $P < 0.001$), *NPM1*mut ($r = 0.599$, $P < 0.001$) and *RUNX1*mut ($r = 0.889$, $P < 0.001$). Moreover, a reduction in *BAALC* expression after the second cycle of induction chemotherapy was associated with improved EFS. Thus, our data underline the utility of *BAALC* expression as a marker for prognostic risk stratification and detection of residual disease in CN-AML.

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INTRODUCTION

Acute myeloid leukemia (AML) is a heterogeneous disease with respect to clinical picture and therapeutic outcome, partly reflected by differences in cytogenetics and molecular genetics. In approximately 55% of patients with AML, cytogenetic aberrations can be used for risk stratification, but there is still a large subgroup of patients who lack informative chromosome markers.[1] Differential prognosis of cytogenetically normal AML (CN-AML) has been allocated by the discovery of specific molecular abnormalities.[2] The most useful markers implicated in prognostication are *NPM1* mutations (mut), *FLT3* internal tandem duplication (*FLT3*-ITD),[3–7] biallelic *CEBPA* mutations (bi*CEBPA*),[8] partial tandem duplications within the *MLL* gene (*MLL*-PTD), *RUNX1*mut and *ASXL1*mut.[9–12]

Besides mutations, deregulated expression of genes involved in cell proliferation, survival and differentiation, for example, *brain and acute leukemia, cytoplasmic* (*BAALC*),[13,14] *ERG*,[15] *MN1*,[15] *WT1*[16] and *EVI1*,[17] have been identified as prognostic markers. An elevated expression of the *BAALC* gene was originally discovered in a gene expression profiling study of AML with trisomy 8, but was later also found in other AML and in acute lymphoblastic leukemia.[18,19] High *BAALC* expression was shown to correlate with *FLT3*-ITD, *NPM1* wild-type (wt) and high expression levels of *ERG* and *MN1* and has been linked to poor prognosis, especially in CN-AML.[13–15,20] The function of *BAALC* in the hematopoietic system as well as its contribution in leukemogenesis is not fully understood. Recently, it was proposed that *BAALC* blocks myeloid differentiation and thus requires a second mutation, which gives a proliferative advantage to induce leukemia.[21]

Risk stratification based on pretreatment genetic signatures has become a critical step in the therapeutic decision-making process. For instance, a large study of 3638 patients has revealed that only low- or intermediate-risk but not good-risk group patients would benefit from allogeneic cell transplantation in first complete remission.[22,23] Moreover, the detection of mutations during the course of therapy is an important prognostic tool, since the outgrowth of minimal residual disease (MRD) cells is responsible for relapse.[24,25] Nowadays, highly sensitive and fast methods (for example, quantitative real-time PCR) facilitate molecular monitoring and early detection of relapse, thereby allowing direct treatment intervention. In spite of the great progress in understanding the biology of AML, only 40–45% of patients younger than 60 years achieve long-term survival.[26,27]

Therefore, it is of great interest to further analyze the recently described biomarkers to refine risk-adapted models. The objective of this study was to evaluate the prognostic impact of *BAALC* expression on clinical outcome in the context of other relevant molecular prognosticators and to examine its utility as a marker for detection of residual disease in CN-AML.

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PATIENTS AND METHODS

Patients

All bone marrow (BM) ($n = 524$) or peripheral blood (PB) ($n = 152$) samples included in the study were referred to our laboratory for diagnostic or follow-up assessment of AML between September 2005 and September 2012. AML was diagnosed according to the FAB (French-American-British) and WHO (World Health Organization) classifications.[28,29] To the best of our knowledge, all patients had *de novo* AML without any preceding malignancy or myelodysplastic syndrome. The characteristics of the 326 patients analyzed at diagnosis are summarized in Table 1. Of these, 290 patients received intensive treatment according to German standard AML protocols. Before therapy, all patients gave their informed consent for scientific evaluations, after having been advised about the purpose and investigational nature of the study. The study was approved by the Internal Review Board of the MLL and adhered to the tenets of the Declaration of Helsinki.

Cytomorphology, cytogenetics and immunophenotyping

Cytomorphologic assessment was based on May-Grünwald-Giemsa stains, myeloperoxidase reaction, and non-specific esterase using alpha-naphthyl-acetate as described previously and was performed according to the criteria defined in the FAB and the WHO classifications in 325 patients.[28–30] Cytogenetic studies were performed in all cases after short-term culture. Karyotypes, analyzed after G-banding, were described according to the International System for Human Cytogenetic nomenclature.[31] The minimum number of analyzed metaphases was 20 per case except for 20 patients, where only 9–19 metaphases could be analyzed. Cytogenetic results were available for all patients in the study. Immunophenotyping was performed in 155 cases as described previously.[32,33]

Examination time points

BAALC mRNA expression was analyzed in 326 *de novo* AML patients (<65 years) with CN-AML by the use of quantitative real-time PCR (qPCR). Median follow-up was 2.7 years. In 66 cases follow-up samples were available, with 57 cases showing high and 9 cases showing low *BAALC* expression at diagnosis. In total, 350 follow-up samples from different time points during therapy were analyzed. A total of 2–20 samples (median, 5; mean, 6.3) were analyzed per patient.

Molecular analysis and *BAALC* determination

Mononuclear cells from PB or BM were separated by *Ficoll* density gradient. Either mRNA or RNA was extracted with the MagnaPureLC mRNA Kit I (Roche Applied Science, Mannheim, Germany) or with the MagNA Pure 96 Cellular RNA Large Volume Kit (Roche Applied Science). The cDNA synthesis from mRNA or RNA from an equivalent of $2.5–5 \times 10^6$ cells was performed using 300 U Superscript II (Life Technologies, Darmstadt, Germany) and random hexamer primers (Roche Applied Science). qPCR was performed by the use of the Applied Biosystems 7500 Fast Real Time PCR System (Life Technologies). Each sample was analyzed at least in duplicates. *BAALC* expression was determined using previously described primers and probes.[13] For detection of *ABL1*, the following primers and probe were used: ABL_ex6-7R, GCAGCAAGATCTCTGTGGATGAAGT, ABL2_exon5F, ATGACCTACGGGAACCTCCT and ABL-Probe_ex6, CTGCCGG TTGCACTCCCTCAGGTA. Amplification was performed after initial incubation at 95 °C for 1 min in a 2-step cycle procedure (95 °C, 15 s and 60 °C, 30 s) for 40 cycles.

To calculate *BAALC* and *ABL1* copy numbers, standard curves for both assays were generated in every run by 10-fold dilution series of five different plasmid concentrations. The expression of *BAALC* was normalized against the expression of the control gene *ABL1* to adjust for variations in mRNA quality and varying efficiencies of cDNA synthesis.

Analyses for mutations of *ASXL1*, *CEBPA*, *FLT3*-TKD, *IDH1*R132, *IDH2*R140, *IDH2*R172, *NPM1*, *NRAS*, *RUNX1*, *WT1*, *TET2* and *TP53*, as well as *MLL*-PTD and *FLT3*-ITD were described previously.[5,9,12,34–40] For *CEBPA*, the term 'biallelic' (bi*CEBPA*) was used for patients with one N-terminal and one bZIP gene mutation, since it has been published that these mutations are usually biallelic and no wt *CEBPA* is expressed in these cases.[8] Samples showing only one mutation were referred as to monoallelic *CEBPA* (mono*CEBPA*).

Statistical analysis

First, *BAALC* expression was analyzed as a continuous variable. Subsequently, the median expression level was calculated and used to dichotomize the total patient cohort into low and high expressers. Overall survival (OS) was the time from diagnosis to death or last follow-up. Event-free survival (EFS) was defined as the time from diagnosis to treatment failure, relapse, death, or last follow-up. To eliminate the effect of allogeneic stem cell transplantation (SCT), OS was also recalculated by censoring patients at the day of transplantation (OS^{TXcens}). Survival curves were calculated for OS, EFS and OS^{TXcens} according to Kaplan–Meier and compared using the two-sided log-rank test. Cox regression analysis was performed for OS, EFS and OS^{TXcens} with different parameters as covariates. Median follow-up was calculated taking the respective last observations in surviving cases into account and censoring non-surviving cases at the time of death. Results were considered as significant at $P < 0.05$. Parameters that were significant in univariate analyses were included in multivariate analyses. Dichotomous variables were compared between different groups using the Fisher's exact test and continuous variables by Student's *t*-test. Correlation coefficient was specified as Spearman's rank correlation. All reported *P*-values are two-sided. No adjustments for multiple comparisons were performed. SPSS software version 19.0.0 (IBM corporation, Armonk, NY, USA) was used for statistical analysis.

RESULTS

Association of *BAALC* expression with patient characteristics and molecular mutations at diagnosis

At diagnosis, *BAALC* expression of 326 patients ranged from 0.1 to 8019.9% *BAALC*/*ABL1* with a median of 33.1%. With regard to patient characteristics, no correlation between *BAALC* expression levels and sex, white blood cell (WBC) count, PB blasts, BM blasts or hemoglobin levels was found (Table 1). There was a trend of high *BAALC* expressers to be of younger age than the low expressers (49.6 vs 51.9 years, $P = 0.063$). In terms of molecular characteristics, patients with high *BAALC* expression were more likely to harbor *FLT3*-ITD (71/163, 43.6% vs 53/163, 32.5%, $P = 0.052$), *MLL*-PTD (21/163, 12.9% vs 5/163, 3.1%, $P = 0.002$) and to carry mutations in *RUNX1* (31/163, 19.0% vs 2/162, 1.2%, $P < 0.001$), *CEBPA* (23/163, 14.1% vs 7/163, 4.3%, $P = 0.003$) or *WT1* (22/163, 13.5% vs 5/162, 3.1%, $P = 0.001$), whereas *NPM1*mut was negatively correlated (71/163, 43.6% vs 138/163, 84.7%, $P < 0.001$). Separation of *FLT3*-ITD according to its mutation load revealed a strong correlation of high *BAALC* expression to *FLT3*-ITD with an *FLT3*-ITD/*FLT3*wt ratio of $\geqslant 0.5$, as compared with low *BAALC* expression (50/163, 30.7% vs 24/163, 14.7%, $P = 0.001$). For *CEBPA*, a significant correlation of high *BAALC* expression with bi*CEBPA* could be shown (16/163, 9.8% vs 2/163, 1.2%, $P = 0.001$).

To confirm these correlations, *BAALC* expression was also analyzed as a continuous variable, thereby comparing mean expression levels of *BAALC* within the molecular groups (Figure 1). In this analysis, patients with *RUNX1*mut and *WT1*mut revealed higher mean *BAALC* expression levels than patients without these mutations (1227.8 vs 106.2, $P = 0.001$ and 462.6 vs 198.1, $P = 0.051$, respectively). Mean expression levels of *BAALC* in patients with *NPM1*mut were significantly lower as compared with those in patients with *NPM1*wt (54.4 vs 514.3, $P < 0.001$). Analyzing *BAALC* expression with regard to *FLT3*-ITD, mean *BAALC* expression levels were almost identical in *FLT3*-ITD-positive patients compared with *FLT3*wt patients, also when analyzing *FLT3*-ITD according to its mutation load (*FLT3*-ITD/*FLT3*wt < 0.5 vs *FLT3*-ITD/*FLT3*wt $\geqslant$ 0.5: 218.9 vs 221.4, n.s.). However, subdivision of patients according to their *NPM1* and *FLT3*-ITD mutational status revealed significantly lower *BAALC* expression in the group of patients with *NPM1*mut and *FLT3*wt or *FLT3*-ITD/*FLT3*wt < 0.5 expression compared to patients with *FLT3*-ITD/*FLT3*wt $\geqslant$ 0.5, irrespective of their *NPM1* mutational status (39.4 vs 374.8, $P < 0.001$). In contrast, mean expression levels of *BAALC* in patients concomitantly harboring *MLL*-PTD were comparable to those of *MLL*-PTD-negative patients (217.9 vs 237.4, n.s., data not shown). When comparing *BAALC* expression of patients with bi*CEBPA* to that of patients with mono*CEBPA* or *CEBPA*wt, the mean expression levels did not differ between these groups (281.0 vs 215.9, n.s.).

Table 1. Pretreatment clinical characteristics and molecular features at diagnosis according to *BAALC* expression status in CN-AML

	Total cohort	*Low BAALC expression*	*High BAALC expression*	P
	n = *326*	n *(%)*	n *(%)*	
Sex, no. (%)				0.825
Female	167 (51.2)	85 (52.1)	82 (50.3)	
Male	159 (48.8)	78 (47.9)	81 (49.7)	
Age (n = 326), years (range)				0.063
Median	52.9 (18.3–64.8)	53.8 (18.5–64.8)	52.4 (18.3–64.5)	
Mean	50.8	51.9	49.6	
WBC count (n = 256), x10^9/l (range)				0.807
Median	24.4 (0.6–400.0)	32.1 (0.7–313.2)	18.0 (0.6–400.0)	
Mean	53.8	54.9	52.8	
Hb levels (n = 243), g/dl (range)				0.700
Median	9.2 (2.8–16.3)	9.2 (2.8–16.3)	9.1 (4.0–14.4)	
Mean	9.3	9.3	9.2	
Platelet count (n = 243), x10^9/l (range)				0.939
Median	64.0 (6.0–454.0)	64.0 (6.0–342.0)	65.0 (6.0–454.0)	
Mean	90.8	91.2	90.4	
PB blasts (n = 156), % (range)				0.285
Median	50.5 (0–100)	49.5 (0–100)	52.5 (0–97)	
Mean	48.9	45.8	51.5	
BM blasts (n = 269), % (range)				0.951
Median	68.5 (6–99)	73.6 (6–99)	66.0 (9–99)	
Mean	63.8	63.9	63.7	
NPM1, no. (%)				< 0.001
Mutated	209 (64.1)	138 (84.7)	71 (43.6)	
Wild type	117 (35.9)	25 (15.3)	92 (56.4)	
FLT3-ITD, *no. (%)*				0.052
Present	124 (38.0)	53 (32.5)	71 (43.6)	
Absent	202 (62.0)	110 (67.5)	92 (56.4)	
*NPM1*mut and *FLT3*wt or *FLT3*-ITD/*FLT3*wt < 0.5, no. (%)	151 (46.3)	114 (69.9)	37 (22.7)	< 0.001
FLT3-ITD/*FLT3*wt < 0.5, no. (%)	252 (77.3)	139 (85.3)	113 (69.3)	0.001
FLT3-ITD/*FLT3*wt ⩾ 0.5, no. (%)	74 (22.7)	24 (14.7)	50 (30.7)	
FLT3-TKD, no. (%)				0.058
Present	31 (9.5)	21 (12.9)	10 (6.1)	
Absent	295 (90.5)	142 (87.1)	153 (93.9)	
MLL-PTD, *no. (%)*				0.002
Present	26 (8.0)	5 (3.1)	21 (12.9)	
Absent	300 (92.0)	158 (96.9)	142 (87.1)	
RUNX1, no. (%), (n = 325)				< 0.001
Mutated	33 (10.2)	2 (1.2)	31 (19.0)	
Wild type	292 (89.8)	160 (98.8)	132 (81.0)	
ASXL1, no. (%)				0.257
Mutated	13 (4.0)	4 (2.5)	9 (5.5)	
Wild type	313 (96.0)	159 (97.5)	154 (94.5)	
CEBPA, no. (%)				0.003
Mutated	30 (9.2)	7 (4.3)	23 (14.1)	
Wild type	296 (90.8)	156 (95.7)	140 (85.9)	
Monoallelic and wild type, no. (%)	308 (94.5)	161 (98.8)	147 (90.2)	0.001
Biallelic (*n* = 332), no. (%)	18 (5.5)	2 (1.2)	16 (9.8)	
*IDH1*R132, *no. (%), (n = 324)*				0.397
Mutated	39 (12.0)	22 (13.7)	17 (10.4)	
Wild type	285 (88.0)	139 (86.3)	146 (89.6)	
*IDH2*R140, *no. (%), (n = 325)*				0.121
Mutated	48 (14.8)	29 (17.9)	19 (11.7)	
Wild type	277 (85.2)	133 (82.1)	144 (88.3)	
*IDH2*R172, *no. (%), (n = 325)*				0.030
Mutated	6 (1.8)	0 (0.0)	6 (3.7)	
Wild type	319 (98.2)	162 (100.0)	157 (96.3)	
NRAS, no. (%)				0.166
Mutated	50 (15.3)	20 (12.3)	30 (18.4)	
Wild type	276 (84.7)	143 (87.7)	133 (81.6)	
TET2, no. (%), (n = 80)				0.593
Mutated	18 (22.5)	10 (26.3)	8 (19.0)	
Wild type	62 (77.5)	28 (73.7)	34 (81.0)	
WT1, no. (%)				0.001
Mutated	27 (8.3)	5 (3.1)	22 (13.5)	
Wild type	298 (91.7)	157 (96.9)	141 (86.5)	

Abbreviations: *BAALC*, brain and acute leukemia, cytoplasmic; BM, bone marrow; CN-AML, cytogenetically normal acute myeloid leukemia; *FLT3*-ITD, *FLT3* internal tandem duplication; Hb, hemoglobin; PB, peripheral blood; WBC, white blood cell; wt, wild type.

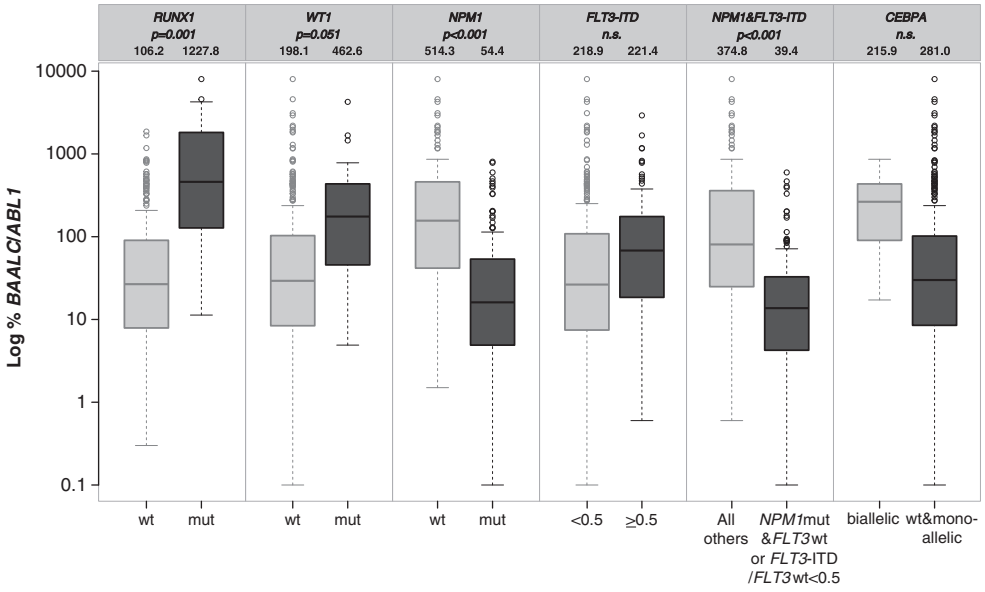

Figure 1. Box plot of *BAALC* expression levels across different genetic subgroups. Mean %*BAALC*/*ABL1* expression levels were compared with Student's *t*-test. Depicted are *BAALC* expression levels in *RUNX1*, *WT1*, *NPM1*, *FLT3*-ITD, *NPM1*&*FLT3*-ITD, and *CEBPA* mutated and wt cases separately. Also mean *%BAALC/ABL1* values are given in the heading of the respective genetic subgroups.

BAALC expression as a prognostic marker

Patients with high *BAALC* expression had significantly shorter EFS and OS (Figures 2a and b). The estimated 3-year EFS rates for high and low *BAALC* expressers were 31.2 and 47.4% ($P=0.006$) and the estimated 3-year OS rates for the two groups were 46.2 and 71.1% ($P=0.002$), respectively. Additionally, when analyzing OS and censoring patients at the day of transplantation, thereby eliminating the effect of allogeneic SCT (OS^{TXcens}) high *BAALC* expression was related to considerably shorter survival (OS^{TXcens} at 3 years: 48.6 vs 76.1%, $P=0.002$; Figure 2c).

Univariate- and multivariate analysis

As we observed significant correlations of *BAALC* expression with several molecular markers, we performed multivariate analysis to clarify whether *BAALC* expression is an independent prognostic factor in CN-AML (Table 2). In univariate analysis, a significant negative impact on OS was shown for higher age ($P<0.001$, hazard ratio (HR) per decade: 1.37), higher WBC count ($P<0.001$, HR per 10×10^9/l: 1.07), *ASXL1*mut ($P=0.047$, HR: 2.18), *FLT3*-ITD/*FLT3*wt$\geqslant$0.5 ($P<0.001$, HR: 2.54), *MLL*-PTD ($P<0.001$, HR: 2.77), *WT1*mut ($P=0.029$, HR: 1.96) and high *BAALC* expression ($P=0.002$, HR low vs high *BAALC* expression: 1.85). A significant negative impact on EFS was found for higher age ($P=0.001$, HR per decade: 1.24), higher WBC count ($P<0.001$, HR per 10×10^9/l: 1.05), *FLT3*-ITD/*FLT3*wt$\geqslant$0.5 ($P<0.001$, HR: 1.96), *WT1*mut ($P<0.001$, HR: 2.58) and high *BAALC* expression ($P=0.006$, HR low vs high *BAALC* expression: 1.53). When censoring the effect of allogeneic SCT the following parameters revealed a negative impact on OS^{TXcens}: higher age ($P<0.001$, HR per decade: 1.50), higher WBC count ($P<0.001$, HR per 10×10^9/l: 1.11), *ASXL1*mut ($P=0.009$, HR: 2.83), *FLT3*-ITD/*FLT3*wt$\geqslant$0.5 ($P<0.001$, HR: 3.29), *IDH2*R140mut ($P=0.029$, HR: 1.89), *MLL*-PTD ($P=0.001$, HR: 3.25), *WT1*mut ($P=0.024$, HR: 2.26) and high *BAALC* expression ($P=0.002$, HR low vs high *BAALC* expression: 2.10).

In multivariate analysis, high *BAALC* expression revealed an independent prognostic impact on OS ($P=0.013$, HR: 1.77), EFS ($P=0.011$, HR: 1.59) and also on OS^{TXcens} ($P=0.018$, HR: 2.00). In addition, age ($P<0.001$, HR per decade: 1.45), higher WBC count ($P<0.001$, HR per 10×10^9/l: 1.06), *MLL*-PTD ($P=0.017$, HR: 2.23) and *WT1*mut ($P=0.042$, HR: 2.11) had an independent impact on OS.

In multivariate analysis for EFS, additional independent factors were age ($P<0.001$, HR per decade: 1.31), higher WBC count ($P=0.013$, HR per 10×10^9/l: 1.03), *FLT3*-ITD/*FLT3*wt$\geqslant$0.5 ($P=0.030$, HR: 1.61) and *WT1*mut ($P=0.001$, HR: 2.52). In multivariate analysis for OS^{TXcens}, additional independent factors were age ($P=0.001$, HR per decade: 1.57), higher WBC count ($P<0.001$, HR per 10×10^9/l: 1.10), *ASXL1*mut ($P=0.038$, HR: 2.91), *FLT3*-ITD/*FLT3*wt$\geqslant$0.5 ($P=0.014$, HR: 2.08), *IDH2*R140mut ($P=0.022$, HR: 2.15), *MLL*-PTD ($P=0.020$, HR: 2.56) and *WT1*mut ($P=0.013$, HR: 2.85).

Evaluation of *BAALC* expression for disease monitoring

Before evaluating the utility of *BAALC* overexpression as a novel biomarker for molecular monitoring, we performed experiments to exclude *BAALC* expression in general from being modulated by the treatment regimen. For this purpose, serial follow-up samples of nine patients showing an *NPM1*mut and low *BAALC* expression at diagnosis were analyzed. *BAALC* expression levels of the diagnostic samples were correlated with those of the first sample showing complete molecular remission (CMR) defined by *NPM1* mutational status. In these nine patients, no significant difference of *BAALC* expression levels could be observed during treatment (mean $\pm$ s.e.m. at diagnosis vs mean $\pm$ s.e.m. at first CMR: 6.2 ± 2.2 vs 13.8 ± 3.0, $P=0.082$; Figure 3a). In contrast, in 13 patients with *BAALC* overexpression at diagnosis a strong reduction in mean *BAALC* expression levels at first CMR could be shown (mean $\pm$ s.e.m. at diagnosis vs mean $\pm$ s.e.m. at first CMR: 121.5 ± 32.5 vs 9.7 ± 1.6, $P=0.005$; Figure 3b). Furthermore, in the patients with low *BAALC* expression at diagnosis the detected *BAALC* expression levels remained below the previously defined cutoff. These results indicate that *BAALC* expression is not generally modulated by the treatment.

Next, we investigated the stability of *BAALC* overexpression between diagnosis and relapse in paired samples of 14 patients. As demonstrated in Figure 3c, no significant difference in mean *BAALC* expression levels between both time points was found (mean $\pm$ s.e.m. at diagnosis vs mean $\pm$ s.e.m. at relapse: 384.4 ± 146.6 vs 286.3 ± 119.5, $P=0.319$). These results also indicate that the expression levels of *BAALC* at relapse were in the range of those at diagnosis. Moreover in 4 of these 14 patients a characterization

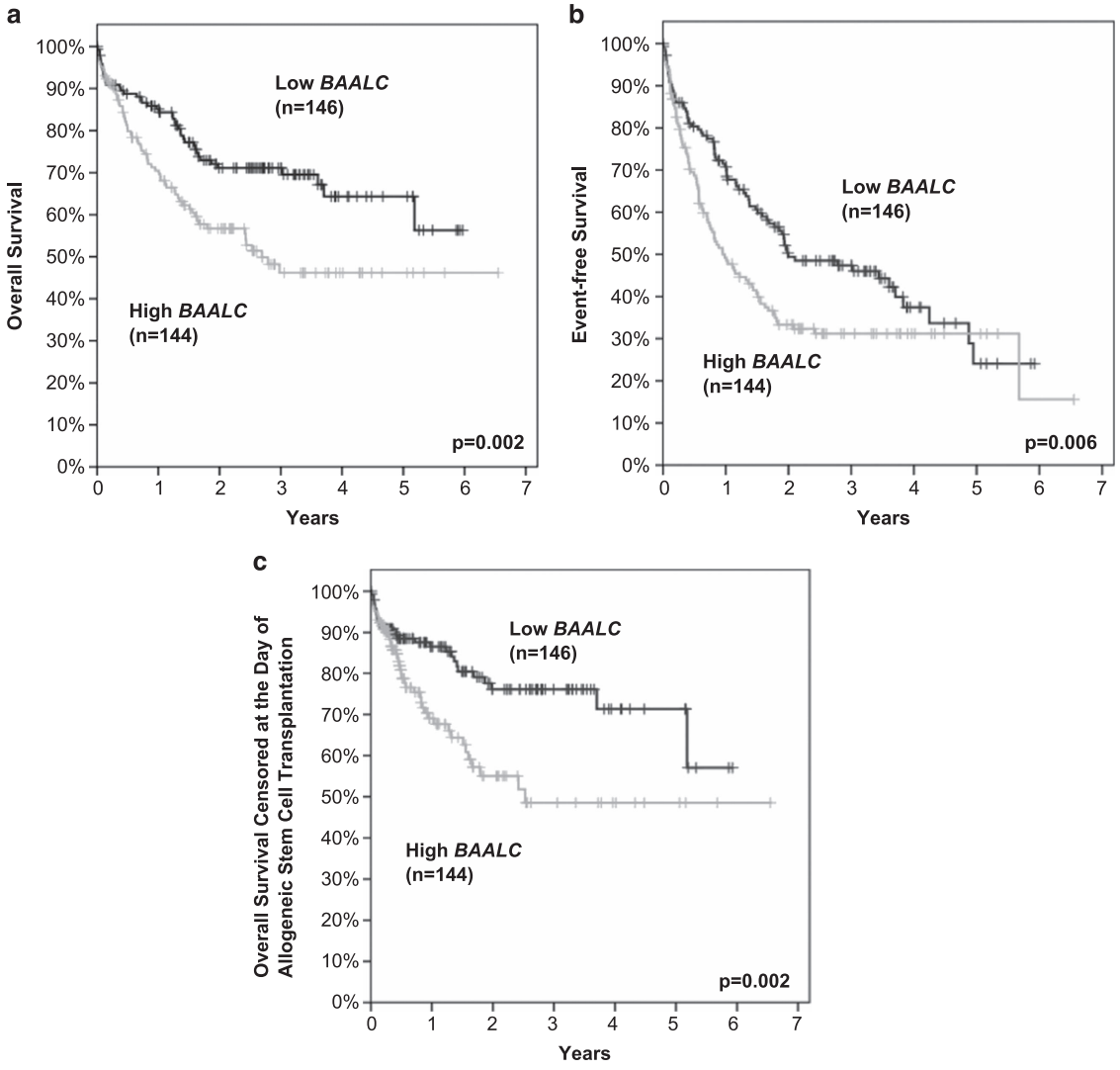

Figure 2. Outcome of 290 intensively treated CN-AML patients aged younger than 65 years with respect to *BAALC* expression. The median expression level was used to dichotomize the total patient cohort into low (black) and high (gray) *BAALC* expressers. (**a**) Overall survival; at 3 years: 46.2 vs 71.1%, $P = 0.002$, (**b**) event-free survival; at 3 years: 31.2 vs 47.4%, $P = 0.006$, (**c**) overall survival censored at the day of allogeneic SCT; at 3 years: 48.6 vs 76.1%, $P = 0.002$.

Table 2. Univariate and multivariate Cox regression analyses on OS, EFS and OS^{TXcens}

Parameter	*Overall survival*				*Event-free survival*				OS^{TXcens}			
	Univariate		*Multivariate*		*Univariate*		*Multivariate*		*Univariate*		*Multivariate*	
	HR	P	*HR*	P	*HR*	P	*HR*	P	*HR*	P	*HR*	P
Age	1.37[a]	<0.001	1.45[a]	<0.001	1.24[a]	0.001	1.31[a]	<0.001	1.50[a]	<0.001	1.57[a]	0.001
WBC count	1.07[b]	<0.001	1.06[b]	<0.001	1.05[b]	<0.001	1.03[b]	0.013	1.11[b]	<0.001	1.10[b]	<0.001
*ASXL1*mut	2.18	0.047	2.01	0.142	1.63	0.155	—	—	2.83	0.009	2.91	0.038
FLT3-ITD[c]	1.97	0.001	n.a.	n.a.	1.38	0.045	n.a.	n.a.	2.78	<0.001	n.a.	n.a.
*NPM1*mut and *FLT3*wt or *FLT3*-ITD/*FLT3*wt<0.5	0.47	<0.001	n.a.	n.a.	0.67	0.009	n.a.	n.a.	0.36	<0.001	n.a.	n.a.
FLT3-ITD/*FLT3*wt⩾0.5	2.54	<0.001	1.65	0.061	1.96	<0.001	1.61	0.030	3.29	<0.001	2.08	0.014
*IDH2*R140	1.36	0.247	—	—	1.19	0.422	—	—	1.89	0.029	2.15	0.022
MLL-PTD	2.77	<0.001	2.23	0.017	1.58	0.102	—	—	3.25	0.001	2.56	0.020
*WT1*mut	1.96	0.029	2.11	0.042	2.58	<0.001	2.52	0.001	2.26	0.024	2.85	0.013
High *BAALC* expression	1.85	0.002	1.77	0.013	1.53	0.006	1.59	0.011	2.10	0.002	2.00	0.018

Abbreviations: *BAALC*, brain and acute leukemia, cytoplasmic; EFS, event-free survival; *FLT3*-ITD, *FLT3* internal tandem duplication; HR, hazard ratio; *MLL*-PTD, partial tandem duplications within the *MLL* gene; n.a., not applicable; OS, overall survival; OS^{TXcens}, overall survival censored at the day of allogeneic SCT; SCT, stem cell transplantation; wt, wild type. [a]Per 10 years of increase. [b]Per 10×10^9/l. [c]Not analyzed because *FLT3*-ITD was analyzed according to its mutation load.

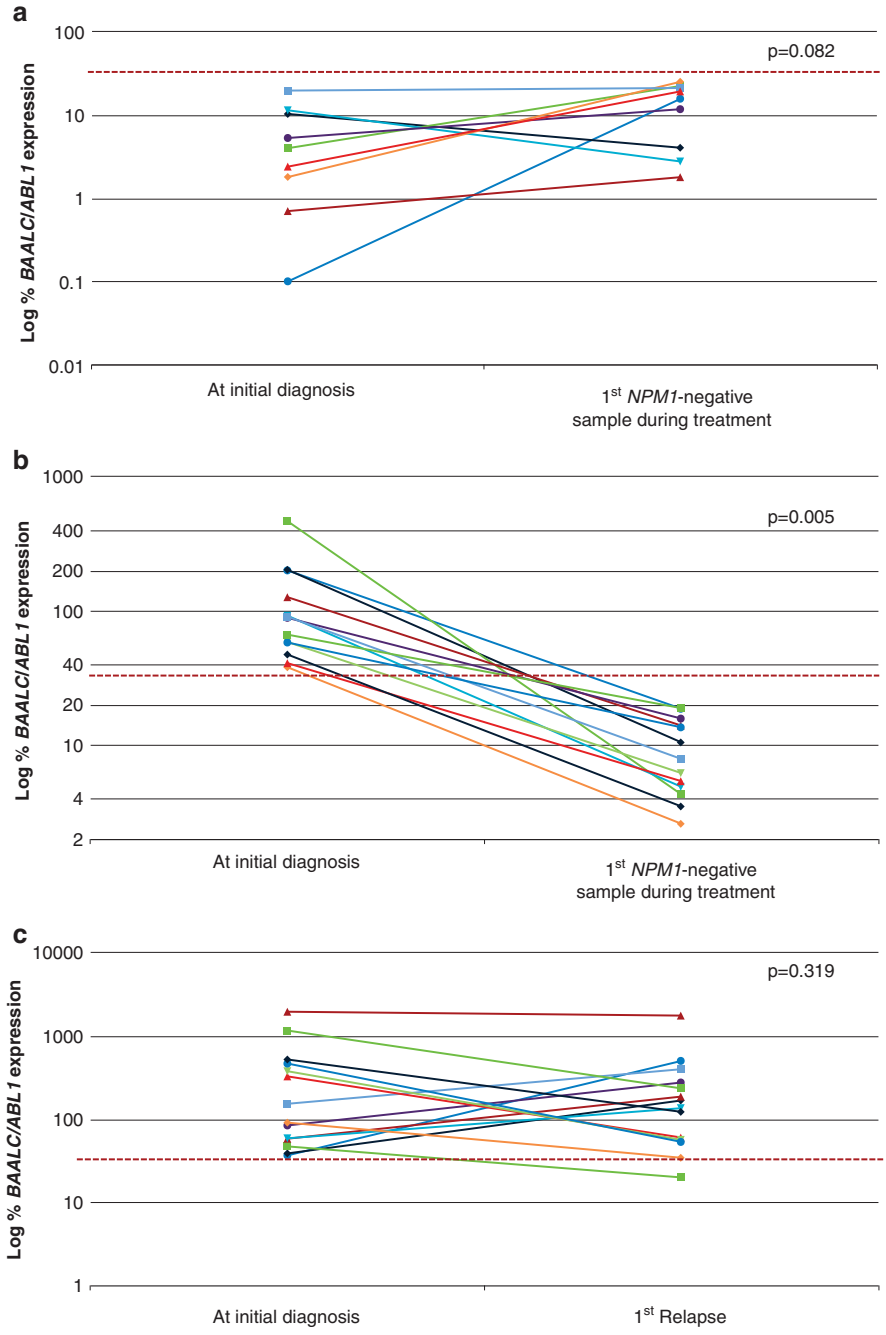

Figure 3. Analysis of 36 diagnostic and 36 follow-up samples. (**a**) Nine *NPM1*mut patients with low *BAALC* expression at diagnosis, (**b**) 13 patients with *NPM1*mut and high *BAALC* expression levels at diagnosis. Figures (**a**) and (**b**) represent *BAALC* expression levels at diagnosis and at first CMR defined by undetectable *NPM1*mut. (**c**) Fourteen patients with high *BAALC* expression at diagnosis and samples of first relapse available. *P*-values were derived by paired Student's *t*-test. The dashed line represents the median *BAALC* expression level (33.1% *BAALC/ABL1*) of the diagnostic cohort.

according to cytomorphologic criteria during follow-up was available. In these four cases a molecular relapse was detected, based on elevated *BAALC* expression levels (32.4–65.5%*BAALC/ABL1)* within 37–149 days before morphological relapse.

To further evaluate the utility of *BAALC* expression to monitor therapy response, *BAALC* expression was analyzed in 358 diagnostic and follow-up samples of 57 patients showing high *BAALC* expression at diagnosis. *BAALC* expression levels were correlated with either the mutational status or the expression levels of other follow-up markers: *FLT3*-ITD, *MLL*-PTD, *NPM1*mut and *RUNX1*mut. *BAALC* expression at diagnosis ranged from 11.3 to 8019.9 (median: 459.8) in patients with *RUNX1*mut and from 1.5 to 1177.7 (median: 140.4) in patients with *MLL*-PTD. Initial *BAALC* expression in *NPM1*-mutated patients ranged from 0.1 to 806.2 (median: 16.1) and in *FLT3*-ITD-positive patients from 0.4 to 4256.3 (median: 44.3). Spearman's rank correlation coefficient revealed a strong correlation of mutational status of %*RUNX1* and %*MLL*-PTD/*ABL1* with %*BAALC/ABL1* levels ($r = 0.889$, $P < 0.001$ and $r = 0.728$, $P < 0.001$, Figures 4a and b). But, less consistency in correlation of *NPM1* mutation load and *FLT3*-ITD expression with %*BAALC/ABL1* levels ($r = 0.448$, $P < 0.001$ and $r = 0.445$, $P < 0.001$) was found. These conflicting results might be due to the lower overall *BAALC*

expression levels in the *NPM1*mut group at diagnosis, which only allows detection of *BAALC* expression during follow-up within one log range (Figure 1). To confirm this hypothesis, in a second analysis only patients with high %*BAALC/ABL1* levels (⩾100; ⩾200; ⩾300) at diagnosis were correlated with mutational status of *NPM1*. This comparison revealed a good correlation of the %*BAALC/ABL1* levels with the *NPM1* mutation load (levels ⩾100: $r=0.599$, $P<0.001$, Figure 4c and levels ⩾200: $n=38$, $r=0.601$, $P<0.001$; levels ⩾300: $n=24$, $r=0.698$, $P<0.001$). *FLT3*-ITD mutational load was detected by Genescan analysis, which is a semi-quantitative approach. Furthermore, copy-number neutral loss of heterozygosity at 13q, frequently occurring in AML, results in the prevalence of the *FLT3*-ITD mutant allele over the *FLT3*wt allele. Both influences the linearity and therefore the precision of the *FLT3*-ITD expression analysis and might account for the constrained correlation of %*BAALC/ABL1* with *FLT3*-ITD expression. Consequently, exclusion of loss of heterozygosity cases showed good correlation of %*BAALC/ABL1* with *FLT3*-ITD expression ($r=0.650$, $P<0.001$, Figure 4d).

Next, single patients were analyzed serially during follow-up. The molecular courses of three patients with high and two patients with low *BAALC* expression which were also monitored for *FLT3*-ITD, *MLL*-PTD, *NPM1*mut or *RUNX1*mut are depicted in Figure 5. As expected, in low expressers the %*BAALC/ABL1* is stable during follow-up (Figure 5a). In contrast, in high expressers the %*BAALC/ABL1* during follow-up correlated well with the kinetics of *FLT3*-ITD, *MLL*-PTD and/or *RUNX1*mut (Figure 5b).

Prognostic significance of *BAALC* expression during follow-up

To investigate the predictive value of *BAALC* expression levels during follow-up, *BAALC* expression was analyzed in samples following the second cycle of induction chemotherapy of 27 patients with high *BAALC* expression levels at diagnosis. To separate low from high *BAALC* expression in this follow-up analysis, the initial cutoff (median expression of diagnostic cohort = 33.1%*BAALC/ABL1*) was used. During follow-up, 37% (10/27) of the patients had high *BAALC* expression levels, while 63% (17/27) of the patients exhibited low *BAALC* expression levels. Kaplan–Meier analysis revealed that low *BAALC* expression after the second cycle of induction chemotherapy was associated with higher EFS rates compared with high *BAALC* expression (median: not reached vs 218 days, $P=0.046$, Figure 6).

DISCUSSION

In this study, we investigated the prognostic value of *BAALC* expression levels on clinical outcome in the light of other currently

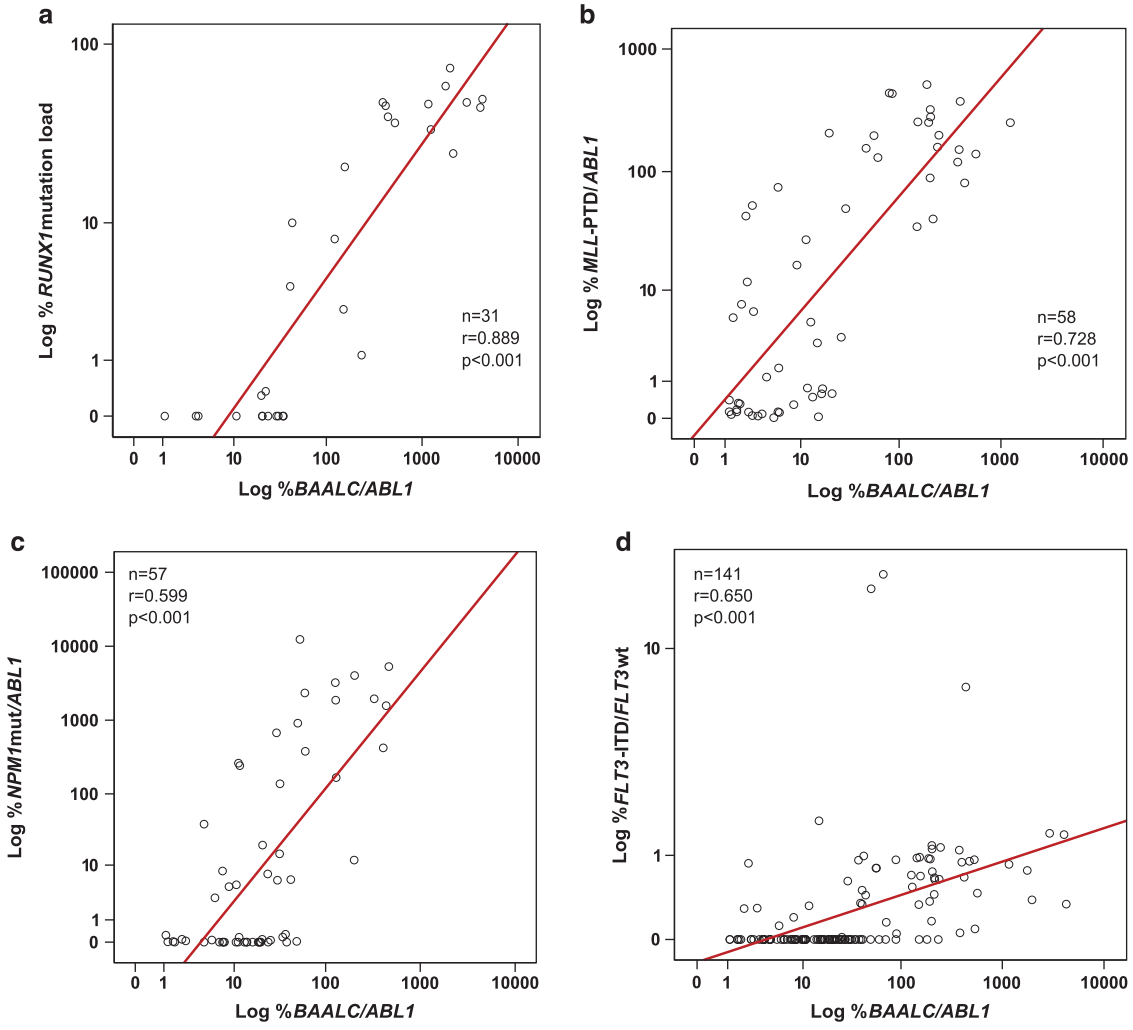

Figure 4. Correlation of *BAALC* expression levels with mutation load or expression levels of different mutations. (**a**) *RUNX*1 mutation load, (**b**) %*MLL*-PTD/*ABL1* expression, (**c**) %*NPM1/ABL1* mutation load (only patients with %*BAALC/ABL1* levels⩾100) and (**d**) *FLT3*-ITD (excluding cases showing loss of heterozygosity (LOH) by loss of *FLT3*wt).

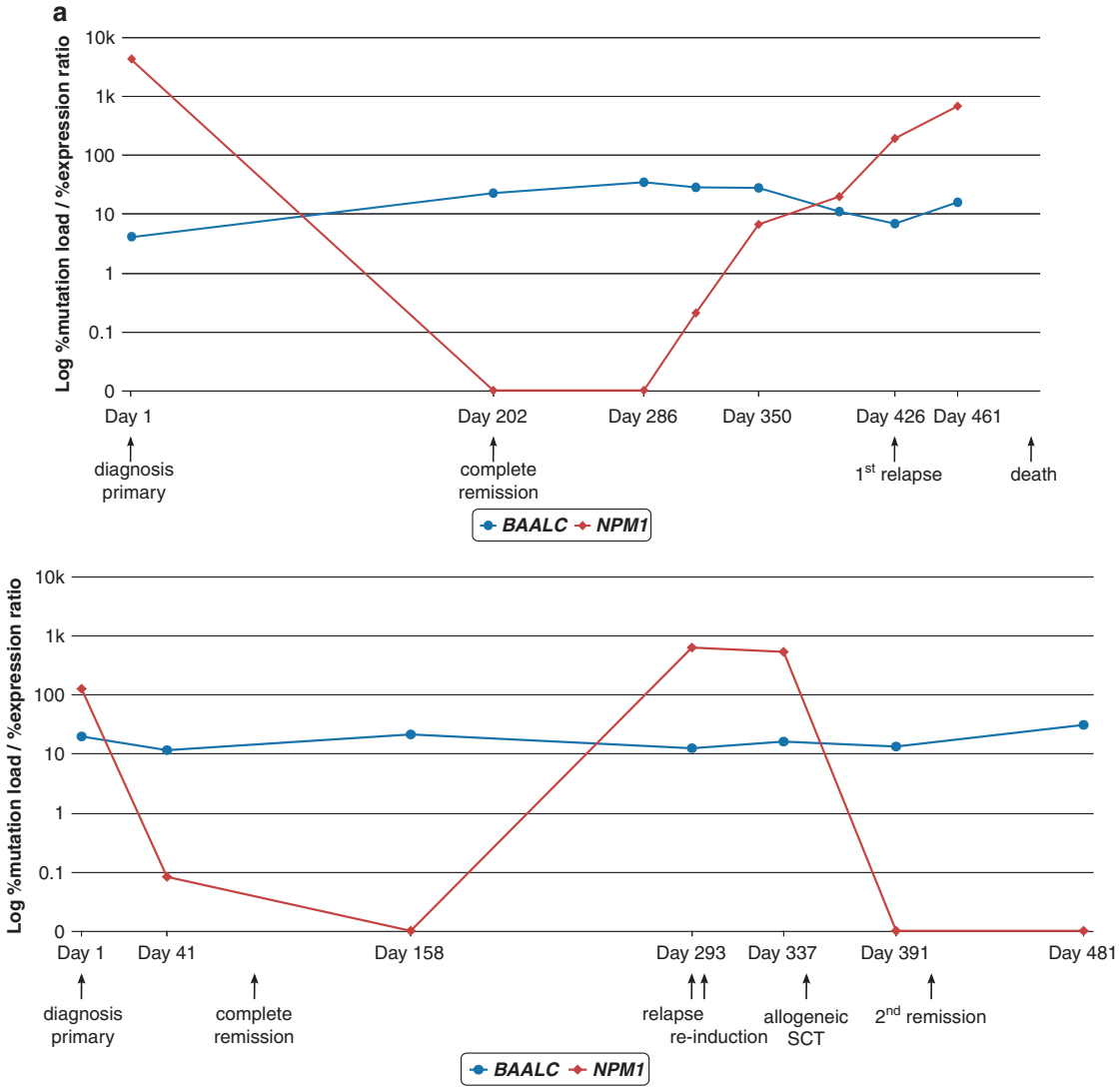

Figure 5. Clinical course of (**a**) two patients with *NPM1*mut (red) and low *BAALC* expression levels (blue) at diagnosis showing no difference in *BAALC* expression levels during the cause of the disease and (**b**) three patients with high *BAALC* expression levels (blue) at diagnosis concomitantly carrying *FLT3*-ITD (green), *MLL*-PTD and/or *RUNX1*mut (red) showing good concordance of these markers during follow-up.

relevant molecular prognosticators. Furthermore, we examined the utility of *BAALC* expression as a marker for detection of residual disease in CN-AML.

Previous studies have addressed the question whether to use BM or PB to analyze *BAALC* expression in AML. A strong correlation of *BAALC* expression levels in both specimens from AML patients has been reported.[41,42] This correlation was confirmed in seven of our patients from which pretreatment blood and marrow samples were available (data not shown) and therefore a combination of both specimens has been analyzed in this study.

Different associations of altered *BAALC* expression to specific molecular aberrations have been shown. For instance, high *BAALC* expression has been demonstrated to correlate with the mutation status of *FLT3*-ITD, *CEBPA*, *MLL*-PTD as well as to *NPM1*wt.[13–15,20] We here addressed the question whether *BAALC* expression also associates with recently described biomarkers such as *ASXL1*, *IDH1*R132, *IDH2*R140, *IDH2*R172, *NRAS*, *RUNX1*, *WT1* and *TET2*. In our cohort of 326 CN-AML patients, the correlation of high *BAALC* expression with *FLT3*-ITD, *CEBPA*mut, *MLL*-PTD and *NPM1*wt could be confirmed. Moreover, we were able to show a strong correlation of high *BAALC* expression with the recently described prognosticators *WT1*mut, *RUNX1*mut, bi*CEBPA* and also to *IDH2*R172mut, while no correlations to *ASXL1*mut, *IDH1*R132mut, *IDH2*R140mut or *TET2*mut were found.

An association of high *BAALC* expression with *WT1*mut has recently been described in a cohort of 196 young CN-AML patients by Paschka *et al.*[43] Here, we were able to corroborate this correlation of *BAALC* expression levels with the mutational status of *WT1*. This specific association might be age dependent since no correlation of high *BAALC* expression with *WT1*mut was found in a cohort of 158 CN-AML patients aged 60 years or older,[20] which could also be confirmed in our cohort, when analyzing a fairly small subset of 77 patients of at least 60 years of age (data not shown).

Furthermore, we show a strong correlation of *BAALC* expression with *RUNX1*mut, with 31 of 33 *RUNX1*-positive patients showing high *BAALC* expression. An association of *RUNX1*mut and high *BAALC* expression has also been shown in gene expression profiling experiments in a group of 93 CN-AML patients.[44] Moreover, recently Eisfeld *et al.*[45] reported two SNPs within exon 1 and 5′UTR of *BAALC* creating binding sites for RUNX1 as a predisposing genetic factor to overexpression of the *BAALC* gene. But, so far, it remains elusive, whether these SNPs also account for *BAALC* overexpression in *RUNX1*-mutated patients, since at least

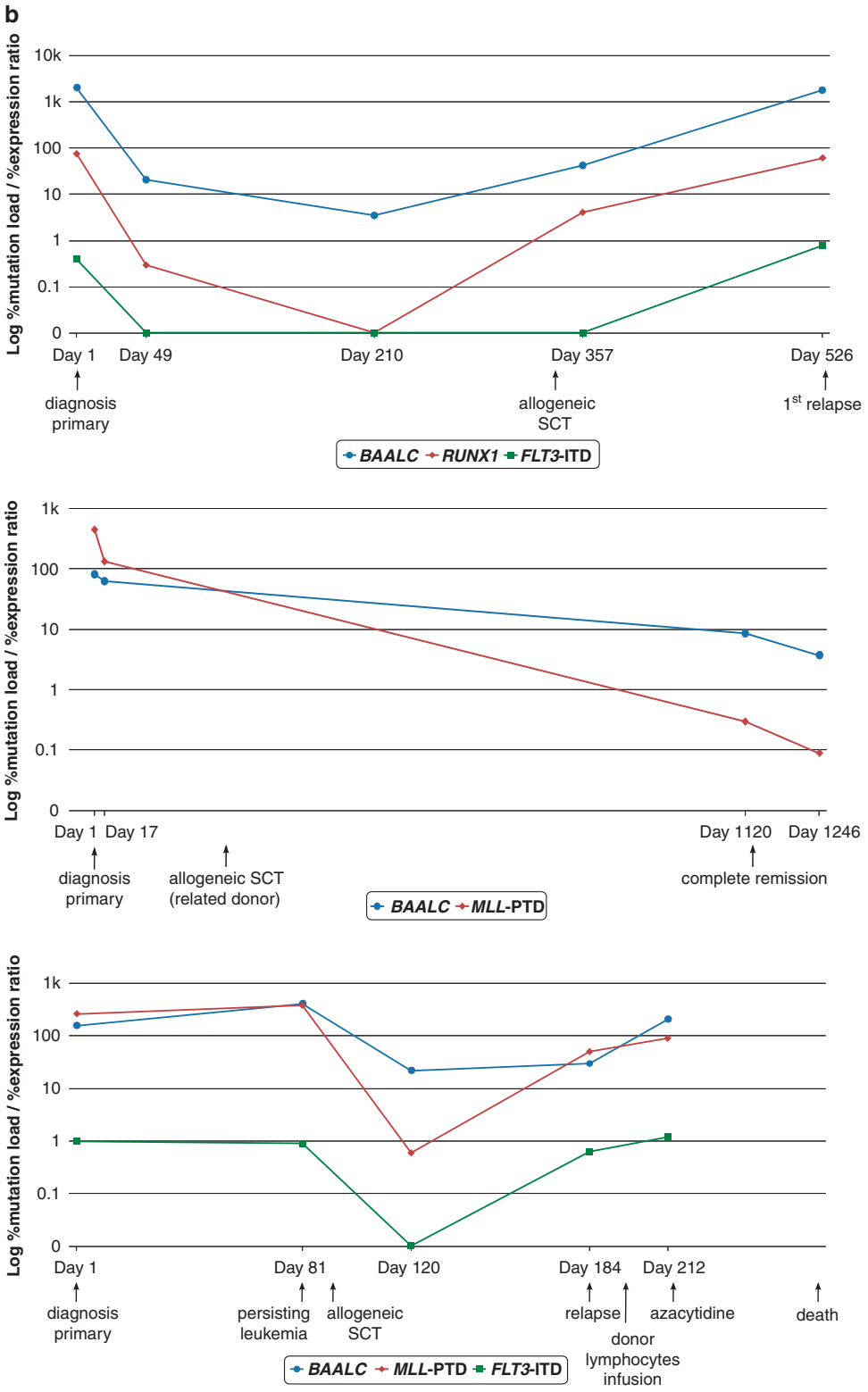

Figure 5. (Continued).

some *RUNX1*mut have been reported to lead to a loss of protein function by disruption of the DNA binding ability.[46] A correlation of high *BAALC* expression with distinct *RUNX1*mut was not found in our cohort (data not shown).

CEBPA mutations have been shown to be associated with favorable outcome[47] and more recent studies suggest that this applies only to patients with bi*CEBPA*.[48] Here, we show that high *BAALC* expression correlates with bi*CEBPA*. However, when analyzing *BAALC* expression as a continuous variable, no significant difference in mean *BAALC* expression levels between bi*CEBPA* and mono*CEBPA* or wt patients could be observed. Due to the restricted number of bi*CEBPA*-mutated patients with high

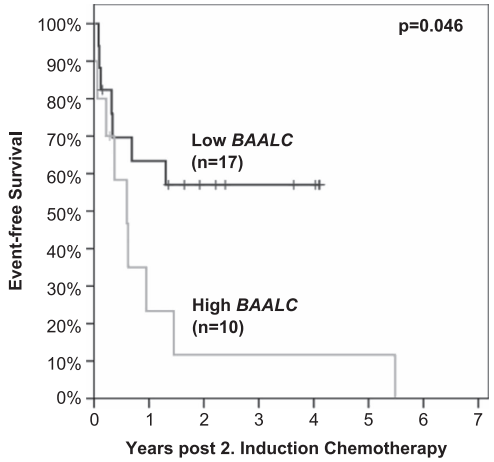

Figure 6. Kaplan–Meier plot of patients achieving low vs high *BAALC* expression levels after second cycle of induction chemotherapy. To separate low (black) from high (gray) *BAALC* expressers the median *BAALC* expression level (33.1%*BAALC*/*ABL1*) of the diagnostic cohort was used. This resulted in significant differences in EFS (median: not reached vs 218 days, $P = 0.046$).

BAALC expression in our cohort it has to be corroborated in a larger cohort, whether bi*CEBPA* patients with high *BAALC* expression levels define a separate prognostic group within the CN-AML.

Despite the specific correlation of altered *BAALC* expression with different well-defined molecular prognosticators, we were interested whether *BAALC* expression is only a surrogate marker or an independent factor for the prognostic allocation of intensively treated CN-AML patients. In our study, high *BAALC* expression presented with independent impact on EFS and OS. This is in part in accordance with previous studies where high *BAALC* expression was independently associated with lower CR rates,[14,20] shorter DFS[20] and shorter OS,[13,14,20] while some studies could not confirm this independent prognostic effect of *BAALC* expression on survival.[15,49] However, none of the aforementioned studies included the newly described prognosticators such as *RUNX1* or *WT1*, as we did, underlining the independent prognostic impact of high *BAALC* expression in CN-AML.

The pathogenetic impact of high *BAALC* expression and its association with different adverse prognosticators in the evolution of leukemia remains elusive, since neither the role of *BAALC* nor the role of most of the concomitant mutations in leukemogenesis have been fully clarified. A study of Heuser *et al.*[21] has shown that *BAALC* expression hinders cell differentiation, but does not promote cell proliferation. On the basis of the two-hit hypothesis,[50] a second event that induces proliferation would be indispensable for the onset of leukemia. At least for *FLT3*-ITD a proliferation promoting effect has been confirmed.[50] Therefore, the correlating mutations might synergize with *BAALC* expression in the development of AML.

Furthermore, *BAALC* overexpression seems to be specifically associated with certain subtypes of leukemia characterized by specific molecular features, since we found strong correlations with mutations in transcription factors and genes that induce proliferation, but no correlation with mutations in epigenetic regulators or genes associated with epigenetic pathways such as *TET2*, *ASXL1* and the *IDH* genes.[51–53] An exception is *IDH2*R172, which is correlated with high *BAALC* expression. Beside the fact that this result has to be interpreted with caution due to the small number of *IDH2*R172-mutated patients, it has already been described that this specific mutation differs in prognosis and appearance as compared with *IDH1*R132 and *IDH2*R140.[54]

Many studies have shown that assessment of MRD is of great importance for risk stratification and early detection of relapse in AML.[24,25] Most frequently, monitoring of PCR-based MRD was restricted to patients carrying specific genetic markers such as fusion genes[55–57] and gene mutations.[58,59] However, many patients lack such markers amenable to sensitive detection by PCR. For this reason, it is crucial to identify molecular targets that are appropriate to measure MRD in the majority of patients with AML. Up to now, only one study has addressed the molecular analysis of *BAALC* expression as a marker for molecular monitoring.[42] This study indicated the applicability of *BAALC* as an MRD target in a cohort of 34 AML and 11 acute lymphoblastic leukemia patients.

In our study, we were able to validate the applicability of *BAALC* expression as a marker for detection of residual disease in 57 patients. Parallel analysis of *BAALC* expression in a total of 358 diagnostic and follow-up samples revealed a significant correlation of *BAALC* expression levels with *FLT3*-ITD, *MLL*-PTD, *NPM1*mut and *RUNX1*mut, all of them being well-known MRD markers.[58–60] Moreover, in 14 patients with matched samples at diagnosis and relapse mean *BAALC* expression levels at first relapse were comparable to that of the diagnostic samples, indicating *BAALC* expression as a stable marker.

Depending on diagnostic *BAALC* expression levels, with our assay up to 2.4 log differences were assessable. Therefore, the sensitivity of our assay is comparable to that of *MLL*-PTD and *FLT3*-ITD detection assays. Using *BAALC* expression as a marker for molecular monitoring in patients with high initial %*BAALC*/*ABL1* levels of above 100 would make 28% (91/326) of our CN-AML patients accessible to molecular monitoring. A full molecular characterization according to the four MRD markers, *FLT3*-ITD, *MLL*-PTD, *NPM1*mut and *RUNX1*mut, was available for all of these 91 patients indicating a lack of mutation in 29% (26/91) of these cases with respect to the four established markers. Thus, despite good genetic characterization 29% of these patients lack a mutation-based MRD target and would benefit from molecular monitoring using a quantitative *BAALC* expression assay.

Recently, detecting *RUNX1*mut by next-generation deep sequencing has been proposed as a stable and sensitive method to monitor *RUNX1*mut during the cause of the disease.[60] However, this method is still quite cost intensive and not yet accessible to many diagnostics laboratories. In this respect, the analysis of *BAALC* expression by qPCR could be a suitable substitute to detected MRD in patients having *RUNX1*mut and high *BAALC* expression at diagnosis. In view of this fact, up to 43% (39/91) of patients with high initial %*BAALC*/*ABL1* levels could benefit from the quantitative assessment of *BAALC* expression during the course of the disease.

Furthermore, we were able to show that a reduction in *BAALC* expression levels below the initially defined cutoff after the second cycle of chemotherapy resulted in better EFS. This not only indicates the prognostic impact of *BAALC* detection for residual disease, but also further validates the cutoff set at diagnosis. However, in prospective studies larger numbers of patients should be analyzed to strengthen these data.

Taken together, our results reveal particular associations between *BAALC* expression and mutations in CN-AML. Despite these correlations, high *BAALC* expression was an independent predictor of shorter EFS and OS. Moreover, our data demonstrate the applicability of *BAALC* expression as a target for monitoring of residual disease. Therefore, future prospective studies should corroborate the prognostic impact of *BAALC*-based monitoring, since up to 43% of the CN-AML patients with high *BAALC* expression (%*BAALC*/*ABL* > 100) at diagnosis might benefit from *BAALC* detection during the course of their disease. Accordingly, our data may strongly affect the collective use of molecular markers in risk assessment and disease monitoring of CN-AML.

CONFLICT OF INTEREST

SS, WK, CH and TH are part owners of the MLL Munich Leukemia Laboratory. SW, TA, FD, SJ, NN, CE, AF, AK and MM are employed by the MLL Munich Leukemia Laboratory.

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AUTHOR CONTRIBUTIONS

SW investigated *BAALC* expression, analyzed the data and wrote the manuscript. SJ and FD contributed in writing the manuscript. CE, AF and MM did sequence analysis of *ASXL1*. AK performed next-generation sequencing. NN contributed to the data illustration. CH was responsible for chromosome banding analysis. WK was responsible for immunophenotyping. TH was responsible for cytomorphologic analysis. TA collected and analyzed clinical data. SS was the principle investigator of the study. All authors read and contributed to the final version of the manuscript.

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