miR-3151 interplays with its host gene *BAALC* and independently impacts on outcome of patients with cytogenetically normal acute myeloid leukemia

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Referat / Abstract

High expression levels of the gene *BAALC* (brain and acute leukemia, cytoplasmic) are associated with poor prognosis in acute myeloid leukemia (AML) patients, but the underlying mechanisms are not yet understood. We evaluated the prognostic significance of expression levels of *miR-3151*, a newly discovered microRNA embedded in intron 1 of the *BAALC* gene, in a cohort of 179 older (≥60 years) cytogenetically normal AML (CN-AML) patients, in the context of established molecular markers and especially with regard to the possible interplay with its host gene *BAALC*. In multivariable analyses, high *miR-3151* was associated with shorter disease-free and overall survival (OS), while higher *BAALC* expression strongly predicted failure of complete remission attainment and OS. Patients exhibiting both high *miR-3151* and *BAALC* expression had worse outcome than patients expressing low levels of either one of the genes or both. Next, gene - and microRNA-expression profiles associated with *miR-3151* expression were derived using microarrays, and a pathway analysis of the *miR-3151* associated gene signature was performed using Ingenuity software. High *miR-3151* expressers showed downregulation of genes involved in transcriptional regulation, post-translational modifications and cell-cycle control. Two genes of the ubiquitination pathway, *FBXL20* and *USP40,* were experimentally validated as direct *miR-3151* targets. In summary, we identified high expression levels of the intronic *miR-3151* as a novel, independent prognosticator for poor outcome in CN-AML. Interestingly, *miR-3151* impacted differently on outcome than its host gene *BAALC*; and the combination of both markers identified a patient subset with the poorest outcome, suggesting that the microRNA and its host gene contribute to clinical and prognostic features of CN-AML independently and through distinct mechanisms. This is the first example of the interplay of an intronic miR and its host gene in leukemia. Its discovery may have important biologic implications for future targeted treatment strategies.

Einführung / Introduction

Acute Myeloid Leukemia (AML) is a complex disease, which is characterized by the uncontrolled proliferation of a leukemic clone in the bone marrow. In recent years, new insights into the biology have significantly improved our understanding of AML. But, despite this progress, the outcome of the majority of AML patients still remains poor and only a fraction of the patients achieves long term survival.[1-3] The outcome is especially poor in patients aged 60 years or older, of whom only ~15% achieve a long term survival.[1-3]

Very soon it became clear, that the cytogenetical and molecular heterogeneity may be the reason for the different outcomes of the patient, since each chromosomal and/or molecular abnormality in the leukemic clone may lead to a more or less aggressive disease course. Today, numerous clinical, cytogenetic and molecular variables have been found to be associated with AML outcome.[4-16] Their discoveries raised hopes to better predict the individual chances of treatment response and potentially lead to more personalized, risk-adapted therapeutic strategies for AML patients. Among the prognostic markers, mutations in the (nucleolar phosphoprotein B23, numatrin) (*NPM1*)[15] and CCAAT/enhancer binding protein C/EBP, alpha (*CEBPA*)[8] genes are associated with favorable outcome and have been included as provisional entities in the World Health Organization classification of AML.[17] These two mutations as well as the presence of internal tandem duplications of the fms-related tyrosine kinase 3 gene (*FLT3*-ITD)[5] that has been associated with unfavorable outcome, have been incorporated into a standardized reporting system for genetic abnormalities suggested by an international expert panel on behalf of the European LeukemiaNet (ELN),[18] and should be determined in every AML patient.

In addition to the presence of gene mutations, the differential expression of specific genes has also been proven to be of prognostic significance in AML. Examples for those gene expression markers are, among others, the v-ets erythroblastosis virus E26 oncogene homolog (avian) (*ERG*),[19] meningioma (disrupted in balanced translocation) 1 (*MN1*),[16] the dominant negative helix-loop-helix protein *ID1,*[20] and the surface marker *CD200.*[7] It is important to know, that the underlying (leukemogenic) mechanisms for

some of these markers are known, while for others their connection to leukemia pathogenesis has not been discovered yet.

Another gene, whose high expression levels have been shown to associate with poor prognosis in AML patients is the *BAALC* (brain and acute leukemia cytoplasmic) gene, which was identified by cDNA-based representational difference analysis in leukemia patients.[21] The prognostic impact of *BAALC* expression has been most extensively studied in AML patients with a normal karyotype (CN-AML),[19, 22-24] with high expression levels being associated with a lower complete remission (CR) rate, as well as shorter disease-free (DFS) and overall survival (OS) of the patients.[19,22] Although recent data suggest that *BAALC* contributes to leukemogenesis by interfering with normal patterns of myeloid differentiation,[23] the function of this gene and the mechanism(s) through which its high expression levels impact negatively on clinical outcome remain(s) unknown. Taking together the known published data on *BAALC*'s impact on outcome of AML patients, deciphering *BAALC*'s function may be of high interest to the scientific community. But, since multiple attempts of various study groups failed so far, new avenues and research approaches may have to be taken to gain more insights into the leukemogenic properties of the *BAALC* locus.

MicroRNAs (miRs) are noncoding RNAs that downregulate gene expression by inhibiting translation or promoting mRNA degradation.[26] Their targets can be various and may vary by tissue- and cell type; potentially providing simultaneous regulation of multiple genes to regulate a specific pathway. They are not only involved in such biological processes as cellular differentiation, proliferation and survival but also play an essential role in the development of solid tumors and AML.[26-30] miR genes can be found throughout the genome, but about one third of mammalian miRs are located within introns of host genes,[31,32] and most of them are believed to be co-expressed and processed from the same precursor mRNA in which they reside,[33-35] while approximately 26% of intronic miRs have their own promoters.[36,37] The functional relationship of miR genes and their hosts is vastly unknown. Most intronic miRs are thought to strengthen the function of its host gene, which may happen in a direct manner by targeting genes within the same cellular pathway,[38,39] while other miRs have

been shown to silence genes which are acting antagonistic to their hosts.[40] Additionally, it could be shown that some miRs may even be made accountable for effects which have been thought to be caused by their host gene and that they can be achieved independent of the expression of their host gene.[41]

Recently, deep sequencing of melanoma[42] and acute lymphoblastic leukemia samples[43] led to the discovery of a novel miR, *miR-3151*, which was found to be embedded in intron 1 of *BAALC*.

We therefore hypothesized, that *miR-3151* might also be aberrantly expressed in patients with high expression levels of its host gene *BAALC* and might also – dependently or independently - impact on outcome of AML patients. Furthermore, since the function of *BAALC* is still unknown, we hypothesized that *miR-3151* may either be the crucial partner needed for an unknown leukemogenic function of *BAALC*, or even be the element predominantly responsible for the adverse outcome of patients with high *BAALC* expression levels. Thus, *miR-3151* may be a novel oncomiR.

In our here presented study, we measured the expression levels of *miR-3151* and its host gene *BAALC* in a set of 179 CN-AML patients aged 60 years and older by RT Real-Time PCR. To compare the prognostic impact of *miR-3151* expression levels to the impact of other well-established molecular markers, we additionally analyzed all patients for the presence or absence of *FLT3*-ITD,[5] FLT3 tyrosine kinase domain mutations (*FLT3*-TKD),[6] partial tandem duplication of the myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila) gene (*MLL*-PTD),[4] mutations in the *NPM1*,[15] *CEBPA*,[8] tet methylcytosine dioxygenase 2 (*TET2*),[12] additional sex combs like 1 (Drosophila) (*ASXL1*),[13] DNA (cytosine-5-)-methyltransferase 3 alpha (*DNMT3A*),[10] runt-related transcription factor 1 (*RUNX1*),[11] Wilms tumor 1 (*WT1*),[14] and isocitrate dehydrogenase 1 (NADP+), soluble (*IDH1*) and isocitrate dehydrogenase 2 (NADP+), mitochondrial (*IDH2*) [9] genes, and expression levels of *ERG*[19] and *MN1*[16].

Additionally, gene- and microRNA expression profiling was performed with the aim to analyze the derived gene expression signatures for enrichments of specific pathways and/or biologic functions. Finally, we aimed to validate two of the most important genes of the signature as direct targets of *miR-3151*, thereby giving first insights into the downstream biology of *miR-3151*.

Publikation /Publication

Plenary paper

miR-3151 interplays with its host gene *BAALC* and independently affects outcome of patients with cytogenetically normal acute myeloid leukemia

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High *BAALC* expression levels are associated with poor outcome in cytogenetically normal acute myeloid leukemia (CN-AML) patients. Recently, *miR-3151* was discovered in intron 1 of *BAALC*. To evaluate the prognostic significance of *miR-3151* expression levels and to gain insight into the biologic and prognostic interplay between *miR-3151* and its host, *miR-3151* and *BAALC* expression were measured in pretreatment blood of 179 CN-AML patients. Gene-expression profiling and miRNA-expression profiling were performed using microarrays. High *miR-3151* expression was associated with shorter disease-free and overall survival, whereas high *BAALC* expression predicted failure of complete remission and shorter overall survival. Patients exhibiting high expression of both *miR-3151* and *BAALC* had worse outcome than patients expressing low levels of either gene or both genes. In gene-expression profiling, high *miR-3151* expressers showed down-regulation of genes involved in transcriptional regulation, posttranslational modification, and cancer pathways. Two genes, *FBXL20* and *USP40*, were validated as direct *miR-3151* targets. The results of the present study show that high expression of *miR-3151* is an independent prognosticator for poor outcome in CN-AML and affects different outcome end points than its host gene, *BAALC*. The combination of both markers identified a patient subset with the poorest outcome. This interplay between an intronic miR and its host may have important biologic implications. (*Blood*. 2012;120(2):249-258)

Introduction

Acute myeloid leukemia (AML) is a clinically, cytogenetically, and molecularly heterogeneous disease. Despite recent advances in our understanding of the mechanisms of leukemogenesis and the identification of markers that allow molecular-based stratification to risk-adapted therapies, the majority of patients with AML are not cured.[1] The clinical outcome is particularly poor in older (≥ 60 years of age) patients, who have long-term survival rates of only 7%-15%.[2]

To date, the prognostic impact of molecular genetic markers has been studied most extensively in patients with cytogenetically normal AML (CN-AML).[3] In this large cytogenetic subset, several molecular markers have been found to be associated with outcome.[3-9] Among them, mutations in the nucleophosmin (nucleolar phosphoprotein B23, numatrin; *NPM1*) and CCAAT/enhancer binding protein (C/EBP), apha (*CEBPA*) genes are associated with favorable outcome and have been included as provisional entities in the World Health Organization classification of AML.[10] In addition, these 2 mutations and the presence of internal tandem duplications of the fms-related tyrosine kinase 3 gene (*FLT3*-ITD) that has been associated with unfavorable outcome have been incorporated into a standardized reporting system for genetic abnormalities suggested by an international expert panel on behalf of the European LeukemiaNet (ELN).[11]

Another strong prognostic factor associated with treatment resistance and poor outcome is high expression of the brain and acute leukemia cytoplasmic (*BAALC*) gene, which was identified by cDNA-based representational difference analysis in leukemia patients.[12] The prognostic impact of *BAALC* expression has been most extensively studied in CN-AML patients.[13-16] Although recent data suggest that *BAALC* contributes to leukemogenesis by interfering with normal patterns of myeloid differentiation,[17] the function of this gene and the mechanism(s) through which its high expression levels affect clinical outcome negatively remain(s) unknown.

Recently, small RNA deep sequencing of melanoma[18] and pediatric acute lymphoblastic leukemia samples[19] identified a new miR, *miR-3151*, embedded in intron 1 of the *BAALC* gene. miRs are short, noncoding RNAs that hybridize to and regulate the expression of targeted mRNAs[20] and have been implicated in

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leukemogenesis.[21] Furthermore, expression levels of miRs may be used to refine risk assessment in AML in CN-AML patients.[22,23]

miR genes are located throughout the genome, but approximately one-third of mammalian miRs reside within the introns of host genes.[24,25] Some of these intronic miRs have been found to act in functional synergism with their host genes.[26] These findings led us to hypothesize that *miR-3151* could be overexpressed in patients with elevated *BAALC* levels and thus might contribute to the adverse prognostic impact of its host gene, either acting in concert with *BAALC* or perhaps even being the element predominantly responsible for the adverse outcome observed in *BAALC*-overexpressing AML patients.

In the present study, we measured *miR-3151* and *BAALC* expression in a cohort of molecularly well-characterized de novo CN-AML patients to assess the impact of *miR-3151* expression alone and in combination with the expression of its host *BAALC* on outcome and also explored some downstream effects of *miR-3151* expression.

Methods

Patients, treatment, and cytogenetic studies

One-hundred-seventy-nine patients 60 years of age or older with de novo CN-AML who were treated with intensive cytarabine/daunorubicin-based regimens on Cancer and Leukemia Group B (CALGB) frontline clinical protocols (for details see supplemental Methods, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article), were included in this study. All study protocols received institutional review board approval at the participating institutions. Cytogenetic analyses were performed on pretreatment BM samples by CALGB-approved institutional cytogenetic laboratories as part of CALGB 8461, a prospective cytogenetic companion study.[27] The diagnosis of normal cytogenetics was based on the analysis of ≥ 20 BM metaphase cells and confirmed by central karyotype review.[28] All patients gave informed consent for the research use of their specimens in accordance with the Declaration of Helsinki.

Molecular analyses

Pretreatment peripheral blood samples were analyzed for *miR-3151* and *BAALC* expression levels by real-time RT-PCR. The TaqMan assays were carried out for each sample in triplicate using TaqMan Primer-Probe sets for *BAALC* and *miR-3151* and the respective housekeeping genes *18S* and *RNU44* (Life Technologies Corporation/Applied Biosystems) according to protocol instructions (see supplemental Methods).

Additional molecular markers were analyzed centrally in pretreatment BM or peripheral blood samples as described previously and included: *FLT3*-ITD,[29] *FLT3* tyrosine kinase domain mutations (*FLT3*-TKDs),[30] partial tandem duplication of the myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, *Drosophila*) gene (*MLL*-PTD),[31] mutations in the *NPM1*,[32] *CEBPA*,[33] tet methylcytosine dioxygenase 2 (*TET2*),[6] additional sex combs like 1 (*Drosophila* [*ASXL1*]),[7] DNA (cytosine-5-)-methyltransferase 3 alpha (*DNMT3A*),[8] runt-related transcription factor 1 (*RUNX1*),[9] Wilms tumor 1 (*WT1*),[5] and isocitrate dehydrogenase 1 ($NADP^+$), soluble (*IDH1*) and isocitrate dehydrogenase 2 ($NADP^+$), mitochondrial (*IDH2*)[4] genes, and expression levels of the v-ets erythroblastosis virus E26 oncogene homolog (avian; *ERG*)[16] and meningioma (disrupted in balanced translocation) 1 (*MN1*)[34] genes.

GEP and MEP

Gene-expression profiling (GEP) of samples was performed using the Affymetrix U133 plus 2.0 array (Affymetrix) and miR-expression profiling (MEP) was performed using The Ohio State University custom miR array (OSU_CCC Version 4.0), as described previously (see supplemental Methods for details).[32,35] The miR microarray data have been deposited in ArrayExpress under accession number E-MTAB-1074 and the microarray expression data under accession number E-MTAB-1075.

Validation of *FBXL20* and *USP40* as direct *miR-3151* targets

For stable expression of *miR-3151*, the *miR-3151* stem-loop was cloned into a lentiviral expression vector, as described in supplemental Methods, using lentiviral miR-scramble as the respective control for all experiments. To analyze the effects of forced *miR-3151* expression on the predicted target genes, we assessed the expression of *FBXL20* and *USP40* on mRNA level, as described in the supplemental materials, and compared it with the effects of cells infected with scramble control using real-time RT-PCR. Western Blotting to test the effects of *miR-3151* on protein level was performed as described in detail in the supplemental materials. The 3′-untranslated regions of *FBXL20* and *USP40* were cloned into a luciferase reporter vector (wild-type vs mutated *miR-3151*–binding sequence, see supplemental Table 3 for details) and luciferase activity was assessed as described in supplemental Methods.

Definition of clinical end points and statistical analysis

The main objective of this study was to evaluate the prognostic impact on clinical outcome in older CN-AML patients of *miR-3151* expression alone and in combination with its host gene, *BAALC*. Median expression levels of *miR-3151* and *BAALC* were used to define low and high *miR-3151* and *BAALC* expressers, respectively, for all analyses (see supplemental Methods for details).

Definitions of clinical end points (ie, complete remission [CR], disease-free [DFS], and overall survival [OS]) and details of statistical analyses, including variable selection for statistical modeling, are provided in supplemental Methods. Associations between patients with low and high expression of *miR-3151* for baseline demographic, clinical, and molecular features were compared using the Fisher exact and Wilcoxon rank-sum tests for categorical and continuous variables, respectively. Estimated probabilities of DFS and OS were calculated using the Kaplan-Meier method, and the log-rank test was used to evaluate differences between survival distributions. Multivariable logistical regression models were constructed to analyze factors related to the probability of achieving CR using a limited backward selection procedure. Multivariable proportional hazards models were constructed for DFS and OS to evaluate the impact of *miR-3151* expression by adjusting for other variables using a limited backward selection procedure. For achievement of CR, estimated odds ratios (ORs) and for survival end points, hazard ratios (HRs) with their corresponding 95% confidence intervals were obtained for each significant prognostic factor.

For the GEP and MEP, summary measures of gene and miR expression, respectively, were computed, normalized, and filtered (supplemental Methods). The profiles were derived by comparing gene expression between low and high *miR-3151* expressers. Univariable significance levels of $P = .001$ for GEP ($P = .005$ for MEP) were used to determine the probe sets (probes) that comprised the signatures.

All clinical analyses were performed by the Alliance for Clinical Trials in Oncology Statistics and Data Center.

Results

Associations of *miR-3151* expression with clinical and molecular characteristics

Patients with high *miR-3151* expression had lower percentages of circulating blasts ($P = .02$), and were more likely to be *NPM1* wild-type ($P < .001$), belong to the ELN Intermediate-I Genetic Group,[11] and harbor mutations of *RUNX1* ($P < .001$) than low expressers ($P = .05$; Table 1). In addition, high *miR-3151* expression was associated with high expression levels of its host gene, *BAALC* ($P < .001$), and also of *MN1* ($P = .05$). Approximately

Table 1. Clinical and molecular characteristics according to *miR-3151* expression status in CN-AML patients 60 years of age or older

Characteristic	Low *miR-3151** (n = 90)	High *miR-3151** (n = 89)	*P*†
Age, y			.99
Median	68	68	
Range	60-79	60-81	
Sex, n (%)			.88
Male	48 (53)	49 (55)	
Female	42 (47)	40 (45)	
Race, n (%)			.10
White	79 (88)	83 (95)	
Nonwhite	11 (12)	4 (5)	
Hemoglobin, g/dL			.47
Median	9.3	9.5	
Range	6.0-11.7	6.5-15.0	
Platelet count, × 10^9/L			.36
Median	60	69	
Range	4-481	11-850	
WBC count, × 10^9/L			.10
Median	26.2	38.7	
Range	1.4-450.0	1.1-434.1	
Percentage of blood blasts			.02
Median	65	39	
Range	0-96	0-99	
Percentage of BM blasts			.52
Median	70	69	
Range	11-97	4-96	
Extramedullary involvement, n (%)	21 (24)	24 (28)	.73
***NPM1*, n (%)**			< .001
Mutated	71 (79)	43 (49)	
Wild-type	19 (21)	45 (51)	
***FLT3*-ITD, n (%)**			1.00
Present	33 (37)	33 (38)	
Absent	57 (63)	55 (63)	
***CEBPA*, n (%)**			.83
Mutated	12 (13)	13 (15)	
Single mutated	7	8	
Double mutated	5	5	
Wild-type	78 (87)	76 (85)	
ELN Genetic Group, n (%)‡			.05
Favorable	50 (56)	35 (40)	
Intermediate-I	40 (44)	52 (60)	
***RUNX1*, n (%)**			< .001
Mutated	4 (5)	19 (24)	
Wild-type	80 (95)	59 (76)	
***FLT3*-TKD, n (%)**			1.00
Present	7 (8)	7 (8)	
Absent	82 (92)	79 (92)	
***WT1*, n (%)**			.08
Mutated	3 (3)	9 (10)	
Wild-type	87 (97)	79 (90)	
***TET2*, n (%)**			.33
Mutated	24 (27)	30 (35)	
Wild-type	65 (73)	56 (65)	
***MLL*-PTD, n (%)**			.50
Present	3 (5)	6 (8)	
Absent	63 (95)	67 (92)	
***IDH1*, n (%)**			1.00
R132 mutated	12 (13)	10 (11)	
V71I mutated	0	1 (1)	
Wild-type	77 (87)	76 (87)	
***IDH2*, n (%)**			.71
IDH2	20 (22)	17 (20)	
R140 mutated	20	14	
R172 mutated	0	3	
Wild-type	69 (78)	70 (80)	

*The median expression value was used as the cutoff point.

†*P* values for categorical variables are from the Fisher exact test; *P* values for continuous variables are from the Wilcoxon rank-sum test.

‡The ELN Favorable Genetic Group comprises patients with mutated *CEBPA* and those with mutated *NPM1* without *FLT3*-ITD; the ELN Intermediate-I Genetic Group includes patients with *CEBPA* wild-type who are *FLT3*-ITD–positive and *NPM1*-mutated, *FLT3*-ITD–negative and *NPM1* wild-type, or *FLT3*-ITD–positive and *NPM1* wild-type.

Table 1. (continued)

Characteristic	Low *miR-3151** (n = 90)	High *miR-3151** (n = 89)	*P*†
***ASXL1*, n (%)**			.13
Mutated	9 (10)	16 (19)	
Wild-type	79 (90)	69 (81)	
***DNMT3A*, n (%)**			.74 (mut vs wt)
Mutated	27 (31)	29 (35)	
R882	15	19	.56 (R882 vs wt)
Non-R882	12	10	1.00 (non-R882 vs wt)
Wild-type	59 (69)	55 (65)	
ERG* expression group, n (%)			.40
High	42 (58)	35 (50)	
Low	31 (42)	35 (50)	
BAALC* expression group, n (%)			< .001
High	34 (39)	56 (66)	
Low	54 (61)	29 (34)	
MN1* expression group, n (%)			.05
High	18 (37)	31 (56)	
Low	31 (63)	24 (44)	

*The median expression value was used as the cutoff point.

†*P* values for categorical variables are from the Fisher exact test; *P* values for continuous variables are from the Wilcoxon rank-sum test.

‡The ELN Favorable Genetic Group comprises patients with mutated *CEBPA* and those with mutated *NPM1* without *FLT3*-ITD; the ELN Intermediate-I Genetic Group includes patients with *CEBPA* wild-type who are *FLT3*-ITD–positive and *NPM1*-mutated, *FLT3*-ITD–negative and *NPM1* wild-type, or *FLT3*-ITD–positive and *NPM1* wild-type.

two-thirds of the patients had a concordant expresser status for *miR-3151* and *BAALC* expression levels, whereas one-third of the patients exhibited up-regulation of only 1 of the 2 markers.

Prognostic value of *miR-3151* expression in CN-AML

Patients with high *miR-3151* expression had a lower CR rate ($P = .005$, 62% vs 81%) compared with low expressers. With a median follow-up time for living patients of 5.1 years (range, 4.1-11.6), high *miR-3151* expressers had a shorter DFS ($P = .003$, HR = 1.76; Figure 1A). At 3 years after CR achievement, only 7% of high *miR-3151*–expressing patients were disease free compared with 26% of low expressers. High *miR-3151* expressers also had a shorter OS ($P < .001$, HR = 1.86; Figure 1B). Three years after diagnosis, 10% of high *miR-3151* expressers were still alive compared with 32% of low expressers.

Because *miR-3151* expression levels were associated with the expression levels of its host gene, *BAALC*, we analyzed the impact on outcome end points of both genes using bivariable models. Analyses showed that high expression levels of either marker had significant adverse impact on CR (*miR-3151*, $P < .001$, OR = 0.47; *BAALC*, $P < .001$, OR = 0.3), DFS (*miR-3151*, $P = .01$, HR = 1.68; *BAALC*, $P = .003$, HR = 1.82), and OS (*miR-3151*, $P = .002$, HR = 1.68; *BAALC*, $P < .001$, HR = 2.01). Therefore, *miR-3151* and *BAALC* independently added information for determination of all outcome end points (supplemental Table 1).

Despite the strong association of *miR-3151* and *BAALC* expression levels, approximately one-third of patients were discordant in expresser status of the 2 markers (Table 1). Therefore, we next investigated whether their combination (*miR-3151/BAALC*, high/high, high/low, low/high, low/low) would reveal differences in impact on outcome end points. Patients who had high expression of both *miR-3151* and *BAALC* demonstrated the lowest CR rates (50%), whereas patients who highly expressed only 1 of the 2 markers had intermediate CR rates (low *miR-3151*/high *BAALC*, 71%; high *miR-3151*/low *BAALC*, 79%), and low expressers of both markers had the highest CR rate (87%, $P < .001$). Patients with high expression of both *miR-3151* and *BAALC* had significantly shorter DFS and OS than those expressing both markers at low levels ($P < .001$ for both DFS and OS; Figure 2A-B) or those who exhibited high expression of only one of the markers (DFS, $P = .03$, OS, $P = .01$). Of patients who expressed both the miR and its host gene at high levels, only 1 survived longer than 3 years after diagnosis.

In multivariable analyses (Table 2), after adjustment for *BAALC* expression status and WBC count, patients with high *miR-3151* expression had a trend toward a lower CR rate ($P = .13$, OR = 0.56). High *miR-3151* expressers had shorter DFS ($P < .001$,

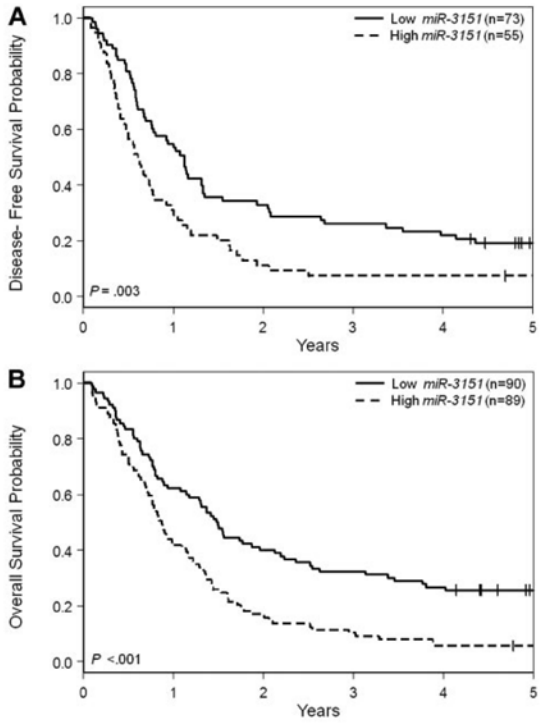

Figure 1. Outcome of CN-AML patients 60 years of age or older with respect to *miR-3151* expression. (A) Disease-free survival. (B) Overall survival.

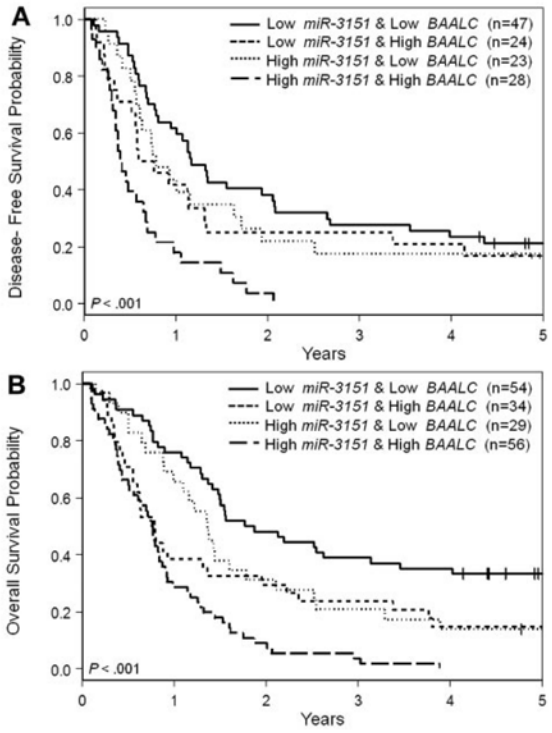

Figure 2. Outcome of CN-AML patients 60 years of age or older with respect to *miR-3151* and *BAALC* expression. (A) Disease-free survival. (B) Overall survival.

HR = 2.38), after adjustment for *FLT3*-TKD and *ERG* expression status, and shorter OS ($P = .009$, HR = 1.69) after adjustment for *DNMT3A* R882 mutations and *ERG* and *BAALC* expression status.

miR-3151 expression in the context of ELN Genetic Groups

The ELN Genetic Groups have been shown to be associated with outcome in older CN-AML patients.[36] Moreover, recent studies have demonstrated that the addition of selected new molecular markers can further improve prognostication within the ELN Genetic Groups.[6,7,34] Therefore, we investigated whether *miR-3151* expression levels could improve outcome prediction within those ELN Genetic Groups that include CN-AML. Within the ELN Favorable Genetic Group, there were no differences in outcome between high and low *miR-3151*–expressing patients. However, in the ELN Intermediate-I Genetic Group, high *miR-3151* expression levels identified patients with particularly poor prognosis for all 3 outcome end points. Only 52% of high *miR-3151* expressers in this ELN group achieved a CR, compared with 78% of low *miR-3151* expressers ($P < .001$). Likewise, patients with high *miR-3151* expression levels had significantly shorter DFS (< .001; Figure 3A) and OS ($P < .001$; Figure 3B) compared with low *miR-3151* expressers. For all 3 end points, the outcome of the low *miR-3151* expressers classified in the ELN Intermediate-I Group was similar to that of both high and low *miR-3151*–expressing patients in the ELN Favorable Genetic Group (Figure 3A-B and supplemental Table 2).

Biologic insights

To gain biologic insights into *miR-3151*–associated leukemia, we derived a gene-expression signature comparing high versus low *miR-3151* expressers. High *miR-3151* expresser status was associated with the differential expression of 597 probe sets, representing 374 annotated genes. Of these, 192 probe sets (116 annotated genes) were up-regulated and 405 probe sets (258 annotated genes) were down-regulated (Figure 4A and supplemental Tables 4 and 5).

High *miR-3151* expressers exhibited up-regulation of genes previously associated with worse outcome in CN-AML, including the transcriptional coregulator *MN1*,[34] the dominant negative helix-loop-helix protein *ID1*,[37] the *miR-3151* host *BAALC*, and the surface marker *CD200*.[38] In addition, we observed up-regulation of genes encoding several kinases, such as *DDR1*, which has been shown to be important for cell growth and differentiation by activation of *NOTCH1*,[39] and *PRKCE*,[40] which has been shown to be involved in apoptosis and several cellular signaling pathways.

Among the most down-regulated genes in high *miR-3151* expressers was the *HOX* cofactor *MEIS1*, which is a key regulator in developmental processes and the absence of which is known to cause disturbances in the colony-forming ability of hematopoietic stem cells,[41] and the tumor suppressor *PDCD4*, which has been shown to contribute to retinoic acid-induced granulocytic differentiation.[42] Furthermore, down-regulation of 23 genes encoding different zinc finger proteins (*ZNF*s), which are known to be involved in transcriptional regulation, was found in high *miR-3151* expressers. Of the 258 down-regulated genes, 73 were in silico

Table 2. Multivariable analysis for outcome according to the *miR-3151* expression status in older patients with CN-AML

End point	Variables in final models	OR/HR	95% CI	*P*
CR*	*miR-3151*, high vs low	0.56	0.27-1.19	.13
	BAALC, high vs low	0.23	0.10-0.52	< .001
	WBC, continuous, 50-unit increase	0.65	0.48-0.88	.01
DFS†	*miR-3151*, high vs low	2.38	1.47-3.85	< .001
	FLT3-TKD, positive vs no TKD	0.28	0.11-0.73	.009
	ERG, high vs low	2.41	1.50-3.86	< .001
OS‡	*miR-3151*, high vs low	1.69	1.14-2.50	.009
	ERG, high vs low	1.71	1.16-2.53	.007
	DNMT3A			
	R882 *v* wild-type	1.66	1.04-2.66	.04
	Non-R882 vs wild-type	1.11	0.61-2.01	.74
	BAALC, high vs low	1.93	1.32-2.82	< .001

CR indicates complete remission; OR, odds ratio; HR, hazard ratio; OS, overall survival; DFS, disease-free survival; OR > 1.0, a higher CR rate for the higher values of the continuous variables and the first category listed for the categorical variables; OR < 1.0, a lower CR rate for the higher values of the continuous variables and the first category listed for the categorical variables; HR > 1.0, a higher risk for an event for the first category listed for the categorical variables; HR < 1.0, a lower risk for an event for the first category listed for the categorical variables; and 95% CI, 95% confidence interval..

*Variables considered in the model based on univariable analyses were: *miR-3151* expression (high vs low; median cut), *BAALC* expression (high vs low; median cut), *ERG* expression (high vs low; median cut), *FLT3*-ITD (positive vs no ITD), *NPM1* (mutated vs wild-type), *WT1* (mutated vs wild-type), *MLL*-PTD (present vs absent), *ASXL1* (mutated vs wild-type), *RUNX1* (mutated vs wild-type), WBC (continuous, 50-unit increase), platelets (continuous, 50-unit increase), and age (continuous, 10-year increase).

†Variables considered in the model based on univariable analyses were: *miR-3151* expression (high vs low; median cut), *BAALC* expression (high vs low; median cut), *ERG* expression (high vs low; median cut), *FLT3*-ITD (positive vs no ITD), *FLT3*-TKD (positive vs no TKD), *NPM1* (mutated vs wild-type), *DNMT3A* (R882 vs Non-R882 vs wild-type), *RUNX1* (mutated vs wild-type), *WT1* (mutated vs wild-type), and WBC (continuous, 50-unit increase).

‡Variables considered in the model based on univariable analyses were: *miR-3151* expression (high vs low; median cut), *BAALC* expression (high vs low; median cut), *ERG* expression (high vs low; median cut), *FLT3*-ITD (positive vs no ITD), *NPM1* (mutated vs wild-type), *ASXL1* (mutated vs wild-type), *RUNX1* (mutated vs wild-type), *WT1* (mutated vs wild-type), *DNMT3A* (R882 vs non-R882 vs wild-type), platelets (continuous, 50-unit increase), and WBC (continuous, 50-unit increase).

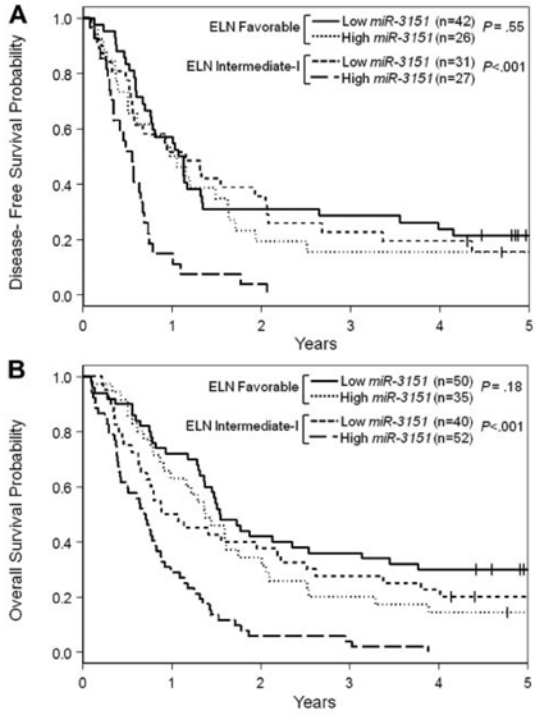

Figure 3. Outcome of CN-AML patients 60 years of age or older with respect to *miR-3151* expression and ELN Genetic Group. (A) Disease-free survival. (B) Overall survival.

predicted targets of *miR-3151* (www.microrna.org; supplemental Table 5).

Pathway analysis of the *miR-3151*–associated expression signature showed an enrichment of genes involved in transcriptional regulation, posttranslational modification, cell-cycle control, cellular development, and cancer pathways, suggesting an impact of *miR-3151* on basic regulatory functions (http://ingenuity.com; supplemental Table 6).

To further investigate the networking processes of known miRs in which *miR-3151* might be involved, we derived miR-expression signatures associated with *miR-3151* expresser status (Figure 4B). We found 15 differentially expressed probes, representing 14 miRs, 5 up-regulated and 9 down-regulated in high compared with low *miR-3151* expressers. Among the down-regulated miRs were *let-7a, let-7b*, and *let-7c*, which are known to suppress tumorigenesis by participating in many cell-proliferation pathways[43] and the down-regulation of which has been previously associated with AML leukemogenesis.[22] We also observed down-regulation of *miR-10a* and *miR-10b*, which are miRs embedded in *HOX* gene clusters, and *miR-99a* and *miR-100*, the reduced expression of which has been implicated in tumor progression in cervical and prostate cancers.[44,45]

To gain initial insights into the downstream effects of high *miR-3151* expression, we sought to validate 1 or 2 of the down-regulated genes in the *miR-3151*–associated gene expression signature as a direct target of *miR-3151*. For the identification of potential candidate genes, we searched among the in silico predicted targets for probe sets of annotated genes that showed at least a 25% down-regulation with $P < .0001$. Only 6 of the 73 genes fulfilled these criteria (see supplemental Table 5 gray highlights). Among these, we selected as potential candidates those with a described function that was associated with the pathways shown to be preferentially involved (supplemental Table 6). Strikingly, 2 of the 6 genes, the F-box and leucine-rich repeat protein 20 (*FBXL20*) and the ubiquitin-specific protease 40 (*USP40*), are involved in the ubiquitination pathway, suggesting an impact of *miR-3151* on this important posttranslational regulatory process. *FBXL20* is a member of the F-box gene family.[46] As a part of the SCF (Skp, Cullin, F-box containing) ubiquitin ligase complex, it is responsible for the ubiquitination of proteins, thereby labeling them for consecutive proteasomal degradation. *USP40* belongs to the family of cysteine proteases that function as deubiquitinating enzymes.[47]

To validate *FBXL20* and *USP40* as direct targets of *miR-3151*, we stably overexpressed *miR-3151* in KG1 cells using a lentiviral system. Forced expression of *miR-3151* resulted in significant down-regulation of the *FBXL20* and *USP40* transcripts (*FBXL20*, 85% decrease, Figure 5A; *USP40*, 66% decrease, Figure 5B; both $P < .001$) compared with scramble control. *miR-3151* also down-regulated FBXL20 on the protein level (Figure 5D). However, none of the commercially available USP40 Abs showed a band at the predicted protein size of 140 kDa. To demonstrate that *FBXL20* and *USP40* are direct targets of *miR-3151*, the respective 3′-untranslated regions of both genes with the sequence containing the predicted *miR-3151*–binding sites were cloned into luciferase reporter vectors. The luciferase assays demonstrated a 54% and 33% decrease in luciferase activity for the *FBXL20* and *USP40* constructs, respectively, after addition of *miR-3151* compared with scramble control (Figure 5E-F). The observed down-regulations were abrogated after mutation of the seed sequence of the predicted *miR-3151*–binding sites (Figure 5E-F). These results demonstrate that *FBXL20* and USP40 are direct targets of *miR-3151*.

Discussion

In the present study, we report an intronic miR as an independent prognostic factor for outcome in older CN-AML patients. Furthermore, our results suggest that *miR-3151* may act in concert with its host gene, the established molecular prognostic marker *BAALC*.

In considering previous findings of a similar nature, the interplay of *miR-33* and its *SREBP* host gene is an illustrative example. It has been shown that the intronic *miR-33* targets the ATP-binding cassette transporter A1 (*ABCA1*), an important regulator of high-density lipoprotein synthesis and reverse cholesterol transport, thereby acting in synergism with *SREBP* to control cholesterol homeostasis.[26] Recently, cooperation between intronic miRs and their host genes has been also demonstrated in prostate cancer[48] and hepatocellular carcinoma.[49] However, to our knowledge, similar mechanisms have not been reported so far in AML. Even though it was not the aim of our study to determine a functional mechanism of *miR-3151* and *BAALC* interaction, our data suggest that both markers contribute independently to poor outcome in CN-AML patients.

We also found that, like *BAALC*, higher expression levels of *miR-3151* were associated with poor prognosis. However, the hosted miR and the hosting gene have an independent clinical significance and had different effects on specific outcome end points. Whereas *miR-3151* expression did not remain an independent predictor in the multivariable model for achievement of CR, *BAALC* expression remained a strong prognostic factor for CR. This is consistent with the findings of our group and others that CR

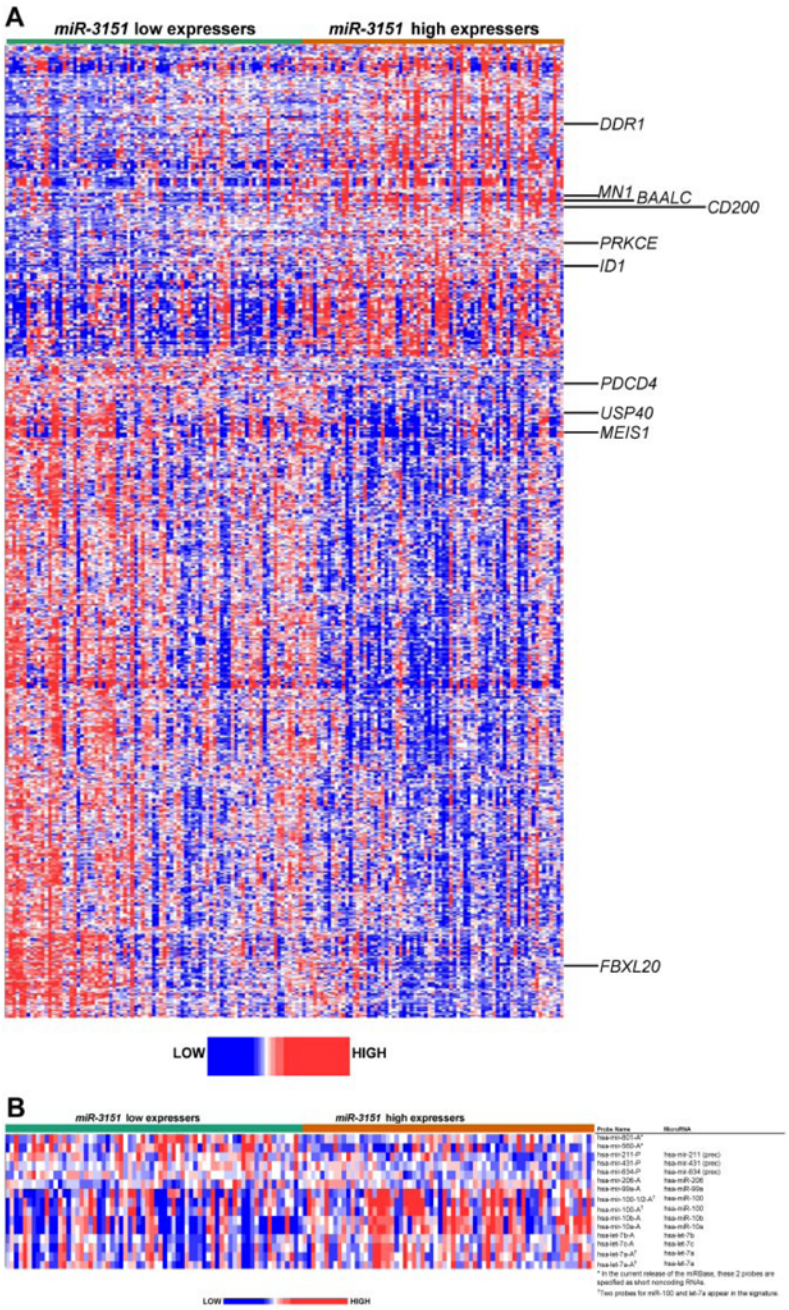

Figure 4. Heat map of the derived gene- and miR-expression signature associated with *miR-3151* expression in CN-AML patients 60 years of age or older. Expression values of the probe sets (probes) are represented by color, with blue indicating expression less than and red indicating expression greater than the median value for the given probe set (probe). (A) GEP: up-regulated and down-regulated genes that are mentioned in the text are indicated along the side. (B) MEP: up-regulated and down-regulated miRs are indicated along the side.

rate is an outcome end point that is strongly affected by aberrant *BAALC* expression levels.[13,15,16] Conversely, high *miR-3151* expression and not *BAALC* expression was a strong predictor of DFS. Finally, both *miR-3151* and *BAALC* expression levels remained important prognostic factors for OS.

We also found that patients overexpressing both *miR-3151* and its host gene *BAALC* had particularly poor outcome for all outcome end points. In contrast, patients exhibiting up-regulation of only 1 of the 2 markers had a significantly better outcome; their DFS was comparable to that of low *miR-3151*/low *BAALC*-expressing patients and their OS was intermediate in comparison with OS of both groups of patients who had concordant *miR-3151* and *BAALC* expresser status. These findings suggest that *miR-3151* and *BAALC* act independently to affect outcome, thereby possibly creating a synergism to support leukemogenesis.

In the present study, by deriving a GEP signature comparing high and low *miR-3151*–expressing patients, we were able to gain initial insights into the biology and possible downstream effects of *miR-3151* in CN-AML patients. We showed that high *miR-3151*-expressing patients also had up-regulation of genes that are known prognosticators of worse outcome in AML patients, such as *MN1*, *ID1*, and *CD200* and the *miR-3151* host gene *BAALC*. Among the down-regulated genes associated with high *miR-3151* expression, we found several that were implicated in hematopoietic differentiation, including *MEIS1*, the absence of which has been shown to

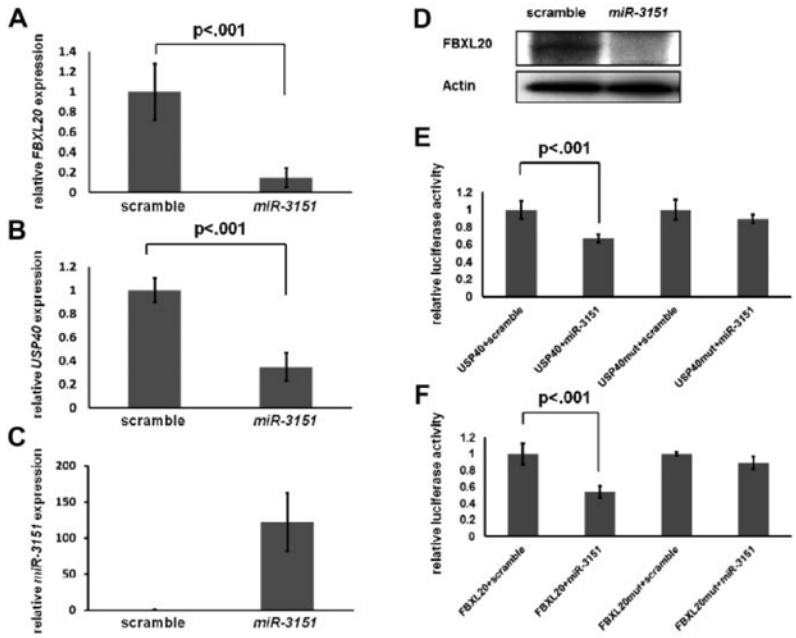

Figure 5. Validation of *FBXL20* and *USP40* as direct targets of *miR-3151*. Expression levels of *FBXL20* and *USP40* as determined by RT-PCR in KG1 cells infected in triplicate with *miR-3151* compared relative to scramble control are shown. Forced *miR-3151* expression resulted in an 85% decrease of *FBXL20* (± SD; A) and a 66% decrease of *USP40* expression levels (± SD; B, both $P < .001$; expression levels are displayed relative to scramble control). (C) Validation of increased *miR-3151* expression levels after lentiviral infection. (D) Western blot of FBXL20 expression comparing *miR-3151* infected KG1 cells versus scramble control. *miR-3151* expression results in elimination of the FBXL20 protein. The effect of *miR-3151* on luciferase activity is shown for the *FBXL20* (E) and *USP40* (F) 3′-UTRs (cloned 3′ of the luciferase gene). The addition of *miR-3151* resulted in a decrease of luciferase activity compared with scramble control of 54% for *FBXL20* and of 33% for *USP40* (± SD; both $P < .001$). This effect was abrogated after mutation of the respective binding sequences of the predicted *miR-3151*–binding sites.

interfere with the normal development of hematopoietic precursors.[42] In addition, we observed a down-regulation of multiple genes encoding ZNF proteins, suggesting a role for *miR-3151* in pretranscriptional regulation.

Interestingly, pathway analysis of the *miR-3151*–associated gene-expression signature suggested an involvement of *miR-3151* in general regulatory processes on both the transcriptional and posttranslational levels. Identification of direct target genes of *miR-3151* belonging to these pathways may pinpoint a root cause for the downstream effects, including the pathophysiological consequences seen in high *miR-3151*–expressing CN-AML patients. Indeed, we were able to validate *FBXL20* and *USP40* as direct targets of *miR-3151*. Both genes are involved in the ubiquitination pathway, which is known to be important for cell-cycle control, cell growth, and a multitude of transcriptional and posttranscriptional regulatory processes.[46,47,50] Even though *FBXL20* and *USP40* are likely only the first of several important *miR-3151* targets, their involvement may initiate and direct future research into our understanding of the function of *miR-3151*.

The *miR-3151*–associated GEP signature shared some features with a recently described GEP signature associated with *BAALC* expression in older CN-AML patients, in which we also reported an up-regulation of *MN1* and *CD200* and down-regulation of *MEIS1*.[16] However, changes in the expression of other genes were unique to the GEP signature associated with *miR-3151* (eg, up-regulation of *ID1* and down-regulation of *ZNFs*). Moreover, key components of the *BAALC* signature (eg, up-regulation of *CD34* and *PROM1* and down-regulation of several *HOX* gene clusters) were not found in our *miR-3151*–associated signature, suggesting important differences in the biologic activities of *miR-3151* and *BAALC*.

Regarding the derived MEP signature, we found 14 miRs to be differentially expressed between high and low *miR-3151* expressers. Among these miRs, a key component was down-regulation of members of the *let-7* family, which are known tumor suppressors and the down-regulation of which is found in various types of cancer,[43] linking the *let-7* family members to the *HMGA2* and *RAS* pathways.

Comparing the derived MEP signature with the signature described to be associated with *BAALC* expression,[16] we observed interesting similarities but also differences. High *BAALC*-expressing patients exhibited down-regulation of *miR-99a*, *miR-100*, and *let-7b*, whereas down-regulation of *let-7a* and *let-7c* seemed to be uniquely associated with high *miR-3151* expression levels. Down-regulation of *miR-9* was only observed in the signature associated with high *BAALC* expression levels. No up-regulated miR was shared by both MEP signatures.

The fact that we were able to identify *miR-3151* as the second miR after *miR-181a*[21,23] that independently affects outcome of CN-AML patients provides further support to the importance of aberrantly expressed miRs as prognostic factors in CN-AML.

It is noteworthy that *miR-3151* is not simply a new molecular marker, but to our knowledge is the first example of how known factors (here *BAALC*) in AML leukemogenesis may be supported by miRs located in the locus itself. In the case of the *BAALC* gene, this finding is of special interest because the gene's function and therefore the reasons for its strong prognostic impact in CN-AML patients are unknown.

In conclusion, we have shown in the present study that high expression of *miR-3151* is an independent prognostic factor associated with poor outcome in older CN-AML patients. Th

partly discordant expresser status of *miR-3151* and its host gene *BAALC*, the independent impact of the 2 genes on outcome, and the fact that patients overexpressing both *miR-3151* and its host gene have significantly worse outcomes than those exhibiting up-regulation of only 1 of the genes suggest that the 2 genes contribute to the aggressiveness of the disease through different mechanisms. We conclude that determining the expression levels of *miR-3151* at diagnosis will help to improve the risk stratification of older CN-AML patients. The development of therapies targeting *miR-3151* up-regulation with synthetic inhibitors may provide novel, more effective strategies for personalized treatment of these patients.

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Authorship

Contribution: A.-K.E., G.M., K. Mrózek, S.M.T., A.d.l.C., and C.D.B. designed the study, analyzed the data, and wrote the manuscript; A.-K.E., S.S., H.B., S.P.W., K.H.M., J.H.M., Y.-Z.W., and R.P. performed the laboratory-based research; K. Maharry, M.D.R., D.N., and S.L. performed the statistical analyses; G.M., M.R.B., B.L.P., T.H.C., J.O.M., J.E.K., M.W., M.A.C., R.A.L., and C.D.B. were involved directly or indirectly in the care of patients and/or sample procurement; and all authors read and agreed upon the final version of the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

For a complete list of participating Alliance institutions, principal investigators, and cytogeneticists, please see the supplemental Appendix.

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Zusammenfassung / Conclusion

Since the here presented study evolved out of the aim to better understand the biology and function of *BAALC*, three main conclusions may be drawn.

1) We could, for the first time, demonstrate an independent prognostic impact of *BAALC*'s intronic microRNA *miR-3151*. In addition, we could validate the important prognostic impact of *BAALC* expression in older CN-AML patients, especially on the achievement of CR and OS.
2) Both genes impacted on different outcome endpoints and showed also differences in their associated gene-expression signatures. Thus, neither does *miR-3151* explain -in full - the impact of *BAALC* expression levels (*BAALC* is not only a meaningless cover gene), nor does *BAALC* account for the impact of *miR-3151* expression levels.
3) *BAALC* and *miR-3151* may have to be interpreted as an oncogenic locus, which interplays towards leukemogenesis.

Within the scope of this thesis we present a study that reported *miR-3151* as an independent prognostic factor for outcome in older patients with CN-AML. The impact of *miR-3151* expression on survival probability was independent from other, well-established molecular markers (eg, *NPM1* mutations and *FLT3*-ITD) and notably also independent from the expression status of its host gene *BAALC*. Importantly, higher expression of *miR-3151* impacted on different outcome endpoints than *BAALC*: The effect of *BAALC* was mainly on achievement of CR while that of *miR-3151* was mainly on outcome- once CR had been achieved (DFS); naturally then both *BAALC* and *miR-3151* affected OS in these older patients. Patients with high expression levels of both genes were identified as the patient subset with the poorest outcome. In general, patients who had high expression of both *miR-3151* and *BAALC* did worst, patients with low expression of both genes did best, and patients with discordant expresser status had intermediate outcomes.

The publication of a standardized reporting system for genetic abnormalities suggested by an international expert panel on behalf of the European LeukemiaNet (ELN) enabled

clinicians with a tool to stratify AML patients based on both cytogenetic abnormalities and the presence or absence of the mutational status of the established molecular markers *NPM1*, *CEBPA* and *FLT3*-ITD. Therefore, it was important to test the impact of *miR-3151* expression levels within those ELN Genetic Groups. Interestingly, *miR-3151* expression was able to identify a subset with an especially poor outcome within the ELN Intermediate-I Genetic Group. Notably, the DFS and OS rates of patients with low *miR-3151* expression levels were comparable to the survival rates of patients belonging to the ELN Favorable Genetic Group. In contrast, *miR-3151* expression levels were without influence on the outcome of patients belonging to the ELN Favorable Genetic Group. Thus, determination of *miR-3151* expression levels may be a useful tool to refine the risk stratification proposed in the guidelines of the ELN. But, even knowing about the important prognostic information which may be gained by the determination of *miR-3151* and *BAALC* expression levels in the pre-treatment peripheral blood of AML patients, it has to be kept in mind that the clinical use of those expression markers is so far still limited by the difficulties to standardize the methods for gene- and microRNA expression determination for individual patients and by the lack of absolute cut-offs to undoubtedly define high and low expressing patients. Once those technical difficulties are overcome, the usage of gene expression markers may be similar or even better than the one gained by the mutational analysis, since the expression markers may reflect the combined downstream effects of a plethora of molecular features, such as different gene mutations or epigenetic events. Besides the clinical observations, our aim was to gain first insights into the downstream biology of *miR-3151*-associated leukemia. Therefore, we performed a global gene-expression profiling in our patient cohort and were able to derive a distinct gene-expression signature associated with high *miR-3151* expression levels. Using ingenuity software, we next performed a detailed gene ontology analysis to identify genes and subsequent pathways which are enriched in high *miR-3151* expressing patients. This gene ontology analysis revealed pathways regulating gene expression, cell-cycle control and post-transcriptional regulation to be primarily affected by high *miR-3151* expression levels. As a next step, we aimed to validate two of the most downregulated genes of the *miR-3151*-associated signature as direct *miR-3151* targets. For identification of these genes, we used a significance-based

algorithm. Interestingly, 2 out of the 6 genes which fulfilled our selection criteria belonged to the ubiquitination pathway[44]- which goes in line with our previous observation that *miR-3151* impacts on post-transcriptional regulation pathways. Both genes, *FBXL20*[45] and *USP40*[46] could be validated as direct *miR-3151* target genes. FBXL20 belongs to the F-BOX proteins and is - as a part of the SCF (Skp, Cullin, F-box containing) -complex - responsible for the ubiquitination and consecutive degradation of its target genes.[45] The SCF-complex is a multiprotein complex catalyzing the ubiquitination of proteins destined for degradation, in which the F-box proteins (eg, FBXL20) contribute to the specificity of the complex, since they are the part which is binding to the target proteins. This protein- "capture" then allows the ubiquitin ligase (eg, E2) to attach the ubiquitin label (Figure 1).

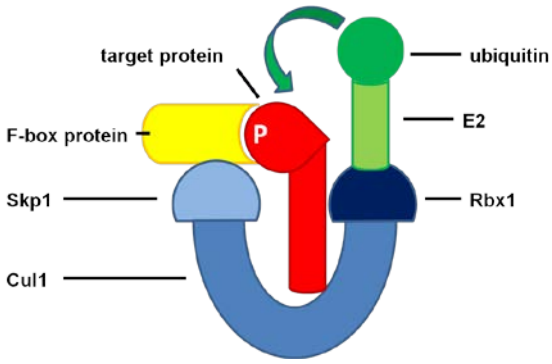

Figure 1. The SCF-(Skp, Cullin, F-box containing) complex. The F-box proteins (eg, FBXL20) define the specificity of the complex by providing the target gene sequence to bind the proteins destined for ubiquitination and proteasomal degradation.

Figure adapted from: Morgan, David "Protein Degradation in Cell-Cycle Control", The Cell Cycle; Principles of Control 2007

So far, nothing is known about specific FBXL20 targets. However, the identification of such genes may be an interesting topic of future research to further understand the downstream biology of high *miR-3151* expression levels and to potentially find new links in the complex networking of AML signaling pathways. Since we already have a successfully working antibody for FBXL20, which has been used for Western blotting in the publication, a next step may be the immunoprecipitation and consecutive mass-spectrometric analysis to identify the proteins bound to FBXL20.

The comparison of the *miR-3151*-associated gene- and microRNA-expression signatures with the gene- and microRNA-expression signatures previously reported to be associated with high expression levels of the host gene *BAALC* led to additional

important observations. Despite the strong association of the expression levels of both genes, interesting differences in the differentially expressed genes in both signatures could be observed. While high *BAALC* expression was associated with high expression of the stemness-markers *PROM1* and *CD34*, no such association could be observed in high *miR-3151* expressing patients. In contrast, downregulation of the *let-7* family, which was the key-feature of the *miR-3151*-associated microRNA-expression signature, has not been observed in high *BAALC* expressing patients.

Interestingly, in one of the initial studies of *BAALC*, Baldus et al. suggested that high *BAALC* expression may lose its prognostic importance in patients undergoing allogeneic hematopoietic stem cell transplantation (HCT).[22] Thus, as a pilot study, we measured pre-treatment *BAALC* and *miR-3151* expression levels in a total of 75 older AML patients with mixed karyotypes, which underwent allogeneic HCT at the University of Leipzig. In these patients we also assessed the *NPM1* mutation status. Similarly to the observations in the patients from the Cancer and Leukemia Group B (CALGB) study (presented study) we observed an association of high *BAALC* and high *miR-3151* expresser status – using a median cut for both genes to define high and low expressers - with *NPM1* wild-type (P=0.0024 and P=0.013, respectively). Furthermore, again validating our findings of the CALGB patient set, we observed an association between high *BAALC* and high *miR-3151* expresser status in the Leipzig patient set (P=.04). However, about 40% (n=30) of the patients had a discordant expresser status of the two genes. For a subset of the investigated Leipzig patients (n=22) follow-up data for a preliminary outcome analysis was available. Utilizing the Kaplan-Meier Method, we analyzed the OS according to the *BAALC* and *miR-3151* expresser status. In this analysis, both *miR-3151* and *BAALC* failed as a single marker to significantly separate the patients according to their overall survival (Figure 2A and B).

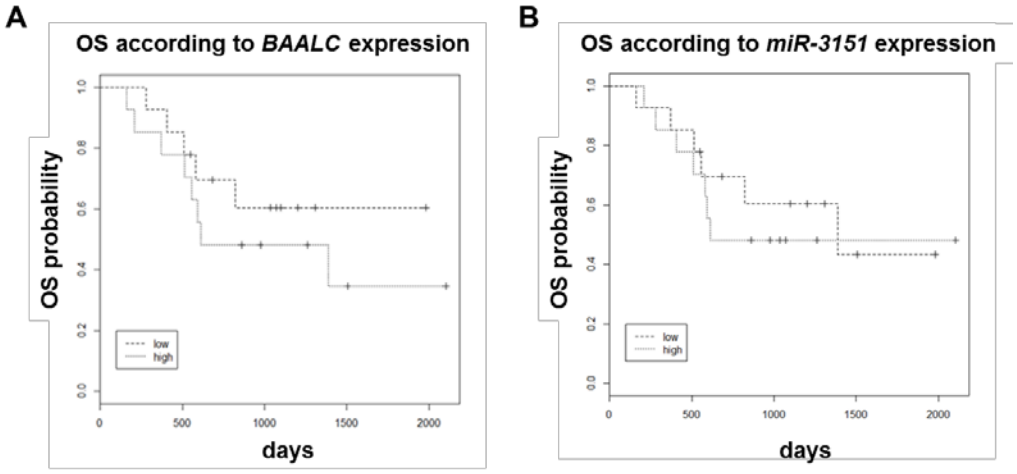

Figure 2: (A) OS according to *BAALC* expression, using the median cut to define high and low expressers treated at the University of Leipzig (n=22). (B) OS according to *miR-3151* expression, using the median cut to define high and low expressers in AML patients treated at the University of Leipzig (n=22).

However, when following the proposal to analyze the combined effect of the *BAALC/miR-3151* locus, we observed a significantly shorter OS (P=.05; log-rank test) in patients with high expression of *BAALC*, *miR-3151* or both when compared to patients expressing both genes on low levels (Figure 3).

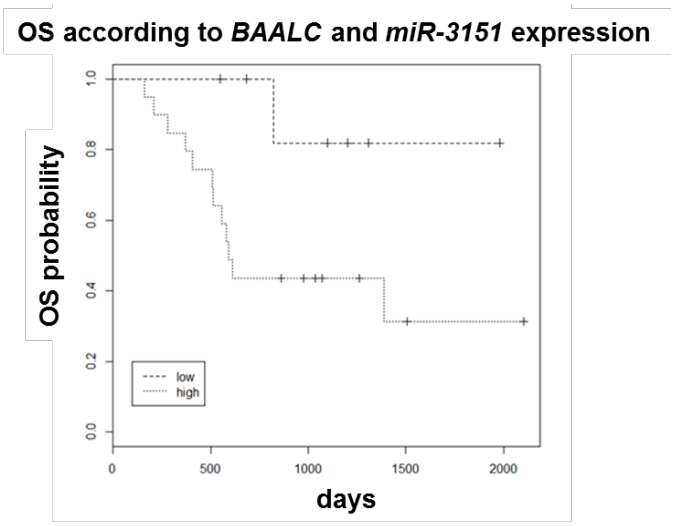

Figure 3: OS according to *BAALC* and *miR-3151*expression, using the median cut to define high and low expressers for *BAALC* and *miR-3151* expression, treated at the University of Leipzig (n=22). Patients with either high *BAALC* or high *miR-3151* expression or both demonstrated worse OS compared to those patients with low *BAALC* and low *miR-3151* expression (P=.05).

Even though the analyzed groups are very small, these data may give further support to the importance of the combined effect of the *BAALC/miR-3151* locus.

In conclusion, the here presented manuscript is not only the first report of the prognostic importance of a novel microRNA *miR-3151*, but it is the first example of an interaction between an intronic miR and its host gene in AML. Since so far the impact of intronic miRs on the effects of their host genes has been vastly overlooked, this discovery may initiate new research in the pathways of other human malignancies in which genes with intronic miRs are involved. In the case of *BAALC* and *miR-3151*, we may conclude that high expression of both oncogenes is important to promote leukemogenesis, since both are impacting on different downstream pathways and ultimately different outcome endpoints.

Nothing is known about the downstream targets about *BAALC* yet, but since we could validate two members of the ubiquitination pathway as direct *miR-3151* target genes, it may be tempting to speculate that *BAALC* may either also be involved in the ubiquitination pathway or that the repressed ubiquitination (and consecutive degradation) of the FBXL20 targets may directly or indirectly support the function of *BAALC*.

The further characterization of the interplay of *miR-3151* and its host gene *BAALC* may unravel novel pathways, which may lead to a better understanding of the pathogenesis of AML.

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Referenz der Publikation / Reference of the publication

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Komplette Publikationsliste / Complete List of Publications

Peer-Reviewed Publications

- Blum W, Schwind S, Tarighat SS, Geyer S, **Eisfeld AK**, Whitman S, Walker A, Klisovic R, Santhanam R, Wang H, Curfman JP, Jacob S, Caligiuri M, Chan K, Garr C, Kefauver C, Grever M, Perrotti D, Byrd J, Bloomfield CD, Garzon R, Marcucci G. *Clinical and Pharmacodynamic Activity of the Combination Bortezomib and Decitabine: a Phase I Trial in Patients with Acute Myeloid Leukemia (AML)*. **Blood** 2012; Jun 21;119(25):6025-31.

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Conference Proceedings and Abstracts

- **Eisfeld AK**, Marcucci G, Liyanarachchi S, Döhner K, Schwind S, Maharry K, Leffel B, Döhner H, Radmacher MD, Bloomfield CD, Tanner SM, de la Chapelle A.. *Heritable Polymorphism Predisposes to High Expression of BAALC in Cytogenetically Normal Acute Myeloid Leukemia (CN-AML).* (AACR Annual Meeting 2012, Abstract 1307).

- Schwind S, Blum W, Liu S, Tarighat SS, Geyer S, Klisovic R, **Eisfeld AK**, Walker A, Whitman SP, Santhanam R, Wu YZ, Jacob S, Caligiuri MA, Grever MR, Perrotti D, Byrd JC, Bloomfield CD, Garzon R, Marcucci G. *The Combination of Bortezomib (BOR) and Decitabine (DEC): a Phase I Trial in Patients (Pts) with Acute Myeloid Leukemia (AML) targeting FLT3 expression.* (AACR Annual meeting 2012, Abstract CT-06).

- Huang X, Schwind S, **Eisfeld AK**, Jin Y, Yu B, Hickey CJ, Pang J, Santhanam R, Chan K, Perrotti D, Muthusamy N, Byrd JC, Blum W, Bloomfield CD, Liu S, Garzon R, Lee RJ, Lee LJ, Marcucci G: *Synthetic microRNA-181a nanoparticles (NP) target RAS and sensitize cells to daunorubicin (DNR) in acute myeloid leukemia (AML).* (AACR Annual meeting 2012 , Abstract 1111).

- **Eisfeld AK** , Marcucci G, Maharry K, Schwind S, Radmacher MD, Nicolet D, Becker H, Mrózek K, Whitman SP, Metzeler KH, Mendler JH, Wu YZ, Liyanarachchi S, Patel R, Baer MR, Powell BL, Carter TH, Moore JO, Kolitz JE, Wetzler M, Caligiuri MA, Larson RA, Tanner SM, de la Chapelle A, Bloomfield CD. MiR-3151*, a Novel MicroRNA Embedded in* BAALC*, Is Only Weakly Co-Expressed with Its Host Gene and Independently Impacts on the Clinical Outcome of Older Patients (Pts) with De Novo Cytogenetically Normal Acute*

Myeloid Leukemia (CN-AML). (ASH Annual Meeting 2011; **Blood** 118:635-635, Abstract 1462).

- **Eisfeld AK**, Marcucci G, Liyanarachchi S, Döhner K, Schwind S, Maharry K, Leffel B, Döhner H, Radmacher MD, Bloomfield CD, Tanner SM, de la Chapelle A. *The genomic basis for* BAALC *overexpression in AML* (OSUCCC-James 13th Annual Scientific Meeting 2011, Abstract P267)

- **Eisfeld AK**, Westerman M, Krahl R, Leiblein S, Liebert UG, Hehme M, Teupser D, Niederwieser D, Al-Ali HK. *Elevated serum hepcidin in patients with AML prior to and after allogeneic hematopoietic cell transplantation does not correlate with transfusional body iron, HFE genotype or Graf versus Host Disease and may protect from excessive parenchymal iron loading* (ASH Annual Meeting Abstracts 2009, Abstract P22974)

- **Eisfeld AK**, Krahl R, Leiblein S, Liebert UG, Edel E, Burkhardt R, Teupser D, Doehring C, Niederwieser D, Al-Ali HK . *Iron depletion pattern in patients with iron overload after allogeneic haematopoietic cell transplantation treated by phlebotomy* (EBMT Annual Meeting 2009, Abstract 1242)

- **Eisfeld AK**, Krahl R, Liebert UG, Edel E, Burkhardt R, Doehring C, Leiblein S, Teupser D, Doehring C, Niederwieser D, Al-Ali HK. *Donor HFE genotype influences the rate of iron depletion by phlebotomy for iron overload after allogeneic HCT.* Bone Marrow Transplantation (EBMT Annual Meeting Abstracts 2006, Vol. 37, Suppl.1 March 2006, Abstract O370)

- **Eisfeld AK**, Burkhardt R, Krahl R, Liebert UG, Edel E, Doehring C, Teupser D, Doehring C, Niederwieser D, Al-Ali HK. *HFE genotype is possibly always of donor origin and does not correlate with iron overload frequently present after HCT* (DGHO Annual Meeting 2006, Abstract P554)

- **Eisfeld AK**, Burkhardt R, Liebert UG, Krahl R, Edel E, Doehring C, , Doehring C, Teupser D, Thiery R, Niederwieser D, Al-Ali HK. *Excess body iron stores strongly correlate with acute graft-versus- host-disease of the liver and treatment related mortality after allogeneic hematopoientic cell transplantation* (Poster presentation) Bone Marrow Transplantation (EBMT Annual Meeting 2006, Vol. 37, Suppl.1 March 2006, Abstract P429)

- **Eisfeld AK**, Burkhardt R, Krahl R, Liebert UG, Edel E, Doehring C, Teupser D, Doehring C, Niederwieser D, Al-Ali HK. *Iron overload in patients after allogeneic does not correlate with HFE genotype which is always of donor origin.* Bone Marrow Transplantation (EBMT Annual Meeting 2005, Vol. 35, March 2005, Abstract O283)

- **Eisfeld AK,** Krahl R, Leiblein S, Liebert UG, Edel E, Burkhardt R, Teupser D, Doehring C, Niederwieser D, Al-Ali HK. *Iron overload is a frequent hidden complication after allogeneic HCT and treatable by phlebotomy .* Bone Marrow Transplantation (EBMT Annual Meeting 2005, Vol. 35, March 2005, Abstract O298)

Lebenslauf / Curriculum Vitae

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CURRENT POSITION

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CURRENT RESEARCH

Since 10/09	Hereditary changes in Acute Myeloid Leukemia
Since 11/10	MicroRNA *miR-3151*: Analysis of prognostic significance, upstream regulation and target gene validation in AML

RESIDENT WORK

Since 06/07	Department of Hematology and Oncology, University of Leipzig/Germany

GENERAL EDUCATION

08/00-05/07	Medical School University of Leipzig/ Germany (M.D.)
08/91-07/00	Hermann-Tast-Gymnasium, Husum/Nordsee

HONORS

08/2009 Fellowship of the Leukemia Clinical Research Foundation: "*Hereditary changes predisposing to Acute Myeloid Leukemia*", 3 year funding

05/2011 Pelotonia Postdoctoral Fellowship Award "*The genomic basis for* BAALC *overexpression in AML*", 2 year funding starting 09/2011

CONFERENCE PRESENTATIONS

03/2012 *"MiR-3151, a Novel MicroRNA Embedded in BAALC, Is Only Weakly Co-Expressed with Its Host Gene and Independently Impacts on the Clinical Outcome of Older Patients (Pts) with De Novo Cytogenetically Normal Acute Myeloid Leukemia (CN-AML)"* (Leukemia Correlative Science Symposium, ALLIANCE Meeting Chicago) .

02/2011 "*Heritable Polymorphism Predisposes to High Expression of BAALC in Cytogenetically Normal Acute Myeloid Leukemia (CN-AML)*" (Leukemia Symposium, Annual Meeting of the Comprehensive Cancer Center, February 28th 2011).

03/2009 *"Iron depletion pattern in patients with iron overload after allogeneic haematopoietic cell transplantation treated by phlebotomy"* (EBMT Annual Meeting 2009)

03/2006 "*Donor HFE genotype influences the rate of iron depletion by phlebotomy for iron overload after allogeneic HCT"* (EBMT Annual Meeting 2006)

03/2005 **"***Iron overload in patients after allogeneic does not correlate with HFE genotype which is always of donor origin"* (EBMT Annual Meeting 2005)

03/2005 **"***Iron overload is a frequent hidden complication after allogeneic HCT and treatable by phlebotomy"* (EBMT Annual Meeting 2005)

MEDICAL TEXTBOOKS/CONTRIBUTIONS

- *2. ÄP Examen Herbst 2009*, published by Georg Thieme Verlag KG, ISBN 978-3-13-153781-2

- *2. ÄP Examen Frühjahr 2010*, published by Georg Thieme Verlag KG, ISBN 978-3-13-154571-8

- *2. ÄP Examen Herbst 2010*, published by Georg Thieme Verlag KG, ISBN 978-3-13-156572-4

- *2. ÄP Examen Frühjahr 2011*, published by Georg Thieme Verlag KG, ISBN 978-3-13-158574-1

- *2. ÄP Examen Herbst 2011*, published by Georg Thieme Verlag KG, ISBN 978-3-13-162372-3

ACTIVE RESEARCH SUPPORT

2009-present Fellowship of the Leukemia Clinical Research Foundation: "*Hereditary changes predisposing to Acute Myeloid Leukemia*", 3 year funding started 10/2009

05/2011 Pelotonia Postdoctoral Fellowship Award "*The genomic basis for* BAALC *overexpression in AML*", 2 year funding starting 09/2011

COMPLETED RESEARCH SUPPORT

01/09 Novartis Young Investigator Foundation 02/01/09-06/01/09. Title: *Clinical relevance of HFE gene mutations of donor origin on iron metabolism of patients after allogeneic PBSCT.* PI: Haifa Katrin Al-Ali, MD, **Co-I: Ann-Kathrin Eisfeld, MD**

07/09 Novartis Young Investigators Foundation 07/01/09-12/01/09. Title: *Clinical relevance of serum hepcidin levels and detection of a possible predictive value for the development of GvHD, infections and relapse after allogeneic PBSCT.* PI: Haifa Katrin Al-Ali, MD, **Co-I: Ann-Kathrin Eisfeld, MD**

EXTRACURRICULAR ACTIVITIES

Since 08/09 Author for "Die Schwarze Reihe"/ Internal Medicine (Georg Thieme Verlag KG)

Since 10/05 Copyediting of „Die Schwarze Reihe" (Georg Thieme Verlag KG, literature for 1.+ 2. Medical State Examination)

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Erklärung über die eigenständige Abfassung der Arbeit

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig und ohne unzulässige Hilfe oder Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Ich versichere, dass Dritte von mir weder unmittelbar noch mittelbar geldwerte Leistungen für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen, und dass die vorgelegte Arbeit weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde zum Zweck einer Promotion oder eines anderen Prüfungsverfahrens vorgelegt wurde. Alles aus anderen Quellen und von anderen Personen übernommene Material, das in der Arbeit verwendet wurde oder auf das direkt Bezug genommen wird, wurde als solches kenntlich gemacht. Insbesondere wurden alle Personen genannt, die direkt an der Entstehung der vorliegenden Arbeit beteiligt waren.

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Supplement 1

Supplemental Material of Eisfeld et al. *miR-3151* interplays with its host gene *BAALC* and independently impacts on outcome of patients with cytogenetically normal acute myeloid leukemia. *Blood*. 2012 Jul 12;120(2):249-58),