The prognostic Impact of *microRNA-181a* expression levels in patients with cytogenetically normal acute myeloid leukemia

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41 Seiten, 53 Literaturangaben, 2 Tabellen, 1 Abbildung

Vorbemerkung / Preliminary Remarks

The following synopsis serves the purpose to summarize the peer-reviewed publication that I contribute to the scope of my thesis. The structure and extent of the synopsis follows the regulations of the Medizinischen Fakultät, Universität Leipzig. For detailed introduction, methods, results and discussion please see the publication (Schwind et al. J Clin Oncol. 2010 Dec 20; 28(36): 5257-64) and the supplemental material, which are inserted in their complete form in section 'Publication', starting on page 13.

Referat / Abstract

Despite advances in the understanding of cancer biology, most patients with acute myeloid leukemia (AML) still die of their disease. Improving risk-stratification and identifying new targets are important steps towards personalized medicine and outcome improvement. MicroRNAs, short non-coding RNAs that hybridize to their target messenger RNAs (mRNAs) and repress the expression of the encoded proteins, are known to be involved in physiological processes like cellular differentiation, proliferation and cell survival but also play an essential role in cancer, including AML.

In this thesis we demonstrated that higher expression of a single microRNA - *miR-181a* - was associated with clinical outcome in cytogenetically normal AML (CN-AML) patients. In multivariable models, higher expression of *miR-181a* was associated with achievement of complete remission (CR), with longer disease-free (DFS) and overall survival (OS) even in consideration of other validated prognostic clinical and molecular variables. Measurement of pretreatment levels of this microRNA may improve risk-stratification for AML patients. A genome-wide gene-expression signature gave biological insights into *miR-181a* associated AML, and provides a basis for further functional studies. Furthermore, as higher *miR-181a* expression associated with improved treatment response, increasing *miR-181a* levels by delivering synthetic *miR-181a* or by agents increasing endogenous levels of this microRNA in AML blasts may represent a novel and personalized therapeutic approach in AML.

Einführung / Introduction

Acute myeloid leukemia (AML) belongs to the most common malignant hematological disorders with a median age at diagnosis in the late 60's.[1,2] The disease is characterized by uncontrolled proliferation of hematopoietic progenitor cells and a differentiation block in bone marrow and blood. AML presents itself cytogenetically and molecularly heterogeneous.[3-7] Despite recent progress in deciphering the biology of the disease and advances in the development of new investigational therapies, the outcome of most AML patients remains vastly improvable.[8,9]

Determination of the best therapy for individual patients remains an every-day challenge for clinicians. Today, specific clinical, cytogenetic and molecular prognosticators for outcome may be used as tools to provide the physician with objective information which may aid in the task of making informed treatment decisions.[7-10] Such information allows decisions on intensification or de-escalation of therapy, enrollment in studies testing new agents or experimental treatment approaches, as well as on the appropriateness of an allograft procedure in first complete remission (CR) with its consequent treatment-related morbidity, mortality and financial costs.

Among the strongest prognostic factors in AML are chromosomal aberrations (i.e. numerical or structural abnormalities) within the malignant clones.[3] An abnormal karyotype is observed in approximately 60% of AML patients and certain abnormalities are highly associated with outcome, following today's standard of care.[3,4] Since these

cytogenetic changes are associated with outcome of AML patients they are used to assign patients to certain risk categories (Table 1).[3,4]

Table 1: Adapted from Fröhling et al.;[4] Cytogenetic Classification Systems used to define prognostic groups in the respective cooperative groups (MRC, Medical Research Council; SWOG/ECOG, Southwest Oncology Group/Eastern Cooperative Group; CALGB, Cancer and Leukemia Group B [did not include t(15;17)]).

Risk	MRC	SWOG/ECOG	CALGB
Favorable	t(8;21) inv(16)/t(16;16) t(15;17)	t(8;21) without del(9q) or complex karyotype inv(16)/t(16;16)/ del(16q) t(15;17)	t(8;21) inv(16)/t(16;16) del(9q)
Intermediate	Normal karyotype del(7q) +8 del(9q) abn(11q23) +21 +22 All other Aberrations	Normal karyotype -Y +6 +8 del(12p)	Normal karyotype -Y del(5q) del(7q) t(9;11) +11 del(11q) abn(12p) +13 del(20q) +21
Unfavorable	abn(3q) -5/del(5q) -7 ≥ 5 aberrations	abn(3q) -5/del(5q) t(6;9) -7/ del(7q) t(9;22) abn(9q) abn(11q) abn(17p) abn(20q) abn(21q) ≥ 3 aberrations	inv(3)/t(3;3) t(6;9) t(6;11) t(11;19) ≥3 aberrations

Even though for some of these abnormalities the assigned risk is lacking agreement among the cooperative groups [e.g. the risk associated with del(5q) or del(9q)], some general statements can be derived (Table 1).

About 25% of AML cases that arise in younger adults will have cytogenetic abnormalities associated with favorable outcome.[3] These are i.e. the translocation t(15;17)(q22;q21) in acute promyelocytic leukemia (APL) and the core-binding factor (CBF) leukemias inv(16)(p13q22) / t(16;16)(p13;q22) and t(8;21)(q22;q22).[3,4,10] Such favorable karyotype patients have a good prognosis with CR rates exceeding 90%, a

5-year survival of at least 65% and relapse rates too low and salvage rates too high to benefit from routine use of an allograft in first CR.[11-17]

On the other hand, in about 10–20% of patients with AML so called adverse cytogenetics are found. They typically include monosomies of chromosome 5 and/or 7 (−5/−7), abnormalities of 3q [abn(3q)] or complex karyotypes. The definition of the latter ones is based on the presence of at least three (or five) unrelated cytogenetic abnormalities according to different cooperative groups (Table 1).[3,4] In general, these patients can expect CR rates of approximately 60% and a 5-year survival as low as 10%.[3,4] Due to the very poor prognosis with current standard therapies, an allograft procedure in first CR or an experimental treatment approach may be justified.[3,4]

These risk classifications show that cytogenetic information is important for informed decisions on appropriate treatment strategies. However, for some AML patients, cytogenetic analysis may not be sufficient for risk-adapted stratification. For example, approximately 40% of the patients with AML harbor a normal karyotype with no numerical or structural abnormalities [cytogenetically normal (CN)-AML].[3,4] But despite the homogeneous chromosomal appearance, and their general association with an intermediate risk, this group of patients presents with a very heterogeneous clinical outcome.[3,4]

Within the past years, in addition to karyotype information, the presence or absence of certain recurrent gene mutations have been proven very helpful in further refining the

risk-classifications of AML patients. A growing body of evidence suggests that two gene mutations in AML are representing primary genetic lesions in the disease: mutations in the *NPM1* (nucleophosmin 1) gene and in the *CEBPA* [CCAAT/enhancer binding protein (C/EBP), alpha] gene have been recognized as two new provisional entities by the World Health Organization (WHO).[10] Mutations in a third gene, coding for the fms-related tyrosine kinase 3 (FLT3) are found in many AML subtypes and are believed to confer to a proliferation and/or survival advantage of the myeloid blasts. AML with *FLT3* mutations are not considered a distinct entity, but the WHO recommends determining the presence of such mutations due to their prognostic significance.[10] Today these three mutations are recommended for assessment in newly diagnosed AML cases for standard risk stratification.

Following these recommendations, recently an expert panel on behalf of the European LeukemiaNet (ELN) suggested a standardized reporting system for genetic abnormalities.[11] This reporting system includes data from cytogenetic analysis and from the mutation analysis of the three aforementioned genes (i.e. *NPM1*, *CEBPA* and *FLT3*).[11] The guidelines are classifying AML in four genetic groups according to this information (Table 2).[11]

Table 2: Adapted from Döhner et al.;[11] standardized reporting for correlation of cytogenetic and molecular genetic data in AML.

ELN Genetic Group	Subsets
Favorable	**t(8;21)(q22;q22); *RUNX1-RUNX1T1***
	inv(16)(p13q22) or t(16;16)(p13q;22); *CBFB-MYH11*
	Mutated *NPM1* without *FLT3*-ITD (normal karyotype)
	Mutated *CEBPA* (normal karyotype)
Intermediate-I	**Mutated *NPM1* and *FLT3*-ITD (normal karyotype)**
	Wild-type *NPM1* and *FLT3*-ITD (normal karyotype)
	Wild-type *NPM1* without *FLT3*-ITD (normal karyotype)
Intermediate-II	**t(9;11)(p22;q23); *MLLT3-MLL***
	Cytogenetic abnormalities not classified as favorable or adverse
Adverse	**inv(3)(q21q26.2) or t(3;3)(q21;q26.2); *RPN1-EVI1***
	t(6;9)(p23;q34); *DEK-NUP214*
	t(v;11)(v;q23); *MLL* rearranged
	-5 or del(5q); -7; abnl(17p); complex karyotype

CN-AML patients are classified into the Favorable or the Intermediate-I ELN Genetic Group based on the mutational status of the *CEBPA*, *NPM1* and *FLT3* genes.[11] The ELN Favorable Genetic Group comprises CN-AML patients with *CEBPA* mutation and/or *NPM1* mutation that do not have internal tandem duplications of the *FLT3* gene (*FLT3*-ITD), whereas the Intermediate-I Genetic Group encompasses CN-AML patients with wild-type *CEBPA* and either *NPM1* mutation with *FLT3*-ITD or wild-type *NPM1* and/or *FLT3*-ITD.[11]

Today reporting systems like the ELN system may also aid in the task of risk stratification and may help guiding the physician to choose the most appropriate treatment option.[18] Furthermore, much effort has been undertaken to pharmacologically

target some of these molecular markers (e.g. alterations of the *FLT3* gene), thereby leading to new treatment avenues in AML.[19]

However, the limitations of today's prognostic assessment and treatment approaches are reflected by the fact that only approximately 30% of all newly diagnosed AML patients survive in the long term.[1,2,18] The outcome is even more dismal in older patients (≥60 years), who represent a large group of AML patients. Among them, only approximately 7 to 15% achieve long-term survival.[1,2,18] Thus, novel markers are needed to improve identification of those patients that would be better served with more intense or experimental treatment approaches. Furthermore the identification of new molecular alterations would increase the understanding of the disease biology and aid in finding novel, targetable pathways in AML. Ultimately this in turn could lead to new treatment approaches for those patients that fail current therapeutic strategies, as demonstrated by the development of therapies targeting aberrant FLT3 activation in AML.[19]

In addition to mutational markers, certain aberrantly expressed genes have been associated with outcome in AML patients (e.g. the expression of the *BAALC*, *ERG* or *MN1* gene).[20-31] The measurement of the expression levels of genes or of a panel of genes may harbor the advantage that potentially several different mutations or epigenetic events funnel into the net expression of a certain gene or gene panel. Thus the determination of expression levels of genes could substitute for investigating multiple genes for the presence of mutations and/or epigenetic changes. Once

standardized methods to quantify the expression levels of RNAs have been developed these expression markers might be able to provide a practical and financial advantage over the determination of multiple mutations in the future.

Recently the differential expression of so called microRNAs (miRs) have been shown to be deeply involved in the development and maintenances of a variety of human cancers, including AML.[32-42] MicroRNAs are short (on average 18-24 nucleotide long) non-coding RNA molecules that hybridize to their target messenger RNAs (mRNAs) and repress the expression of the encoded proteins.[32,33] The mature miRs are derived from precursor molecules that are transcribed from their respective encoding genomic DNA sequences similarly to other coding mRNA molecules. Following processing of the precursor molecules the mature miRs are incorporated into the RNA-induced silencing complex (RISC) in which the miR and the respective targeted mRNA interact, which ultimately leads to a decreased expression of the encoded protein.[32,33]

In AML expression-signatures of miRs have been associated with certain cytogenetic aberrations,[36-42] as well as recurrent mutations, such as the afore mentioned alterations of the *NPM1*,[38,41] *FLT3*[38] and *CEBPA*[43] genes. Furthermore, it has been demonstrated that deregulated miR expression may also associate with outcome of CN-AML patients.[36,42] Using miR-expression profiling in CN-AML patients with unfavorable molecular features (i.e. the presence of *FLT3*-ITD and/or *NPM1* wild-type) a prognostic miR-signature was discovered.[36] However, to our knowledge, the independent prognostic impact of expression levels of a single miR outside of miR-expression

profiles, which would be easier to measure for molecular risk assessment of individual patients has not been demonstrated in AML so far. Among the miRs in the described signature, the upregulation of the *miR-181* family associated with favorable outcome in these molecular high-risk CN-AML patients.[36] Interestingly, *miR-181a* has also been associated with certain morphological sub-types of AML cases.[44] Thus to us *miR-181a* appeared to be a very promising candidate to pursue further investigations regarding its individual use for risk assessment. The genes encoding *miR-181a* and its close relative *miR-181b* are located in two clusters in close proximity on chromosome 1q32.1 and chromosome 9q33.3 of the human genome. In order to gain an impression of the differential *miR-181a* expression in AML patients, we measured the expression of the two precursor molecules *miR-181a-1* (derived from chromosome 1q32.1) and *miR-181a-2* (derived from chromosome 9q33.3) in bone marrow samples of 75 AML patients with pretreatment material available that were enrolled on treatment protocols of the Ostdeutsche Studiengruppe für Hämatologie und Onkologie (OSHO) at the University of Leipzig. All of the enrolled patients received allogeneic hematopoietic cell transplantation after reduced-Intensity conditioning. The expression of the two precursors showed a good correlation (R^2=0.8377, Figure 1A), which allowed us to use the mean expression of the two precursors for our preliminary investigation. At the time of this preliminary analysis, outcome data for a subset (n=22) of these 75 patients following the allograft procedure was available. Using a median cut to define low and high expressers of *miR-181a,* we analyzed the event-free survival (EFS) following allogeneic transplantation. Even though no statistical significance was reached, likely due to the limited number of patients with outcome data available at the time of

analysis, we observed a very promising trend (*P*=.07; log-rank test) towards a longer EFS following the allogeneic transplantation for patients that expressed *miR-181a* at high levels (Figure 1B).

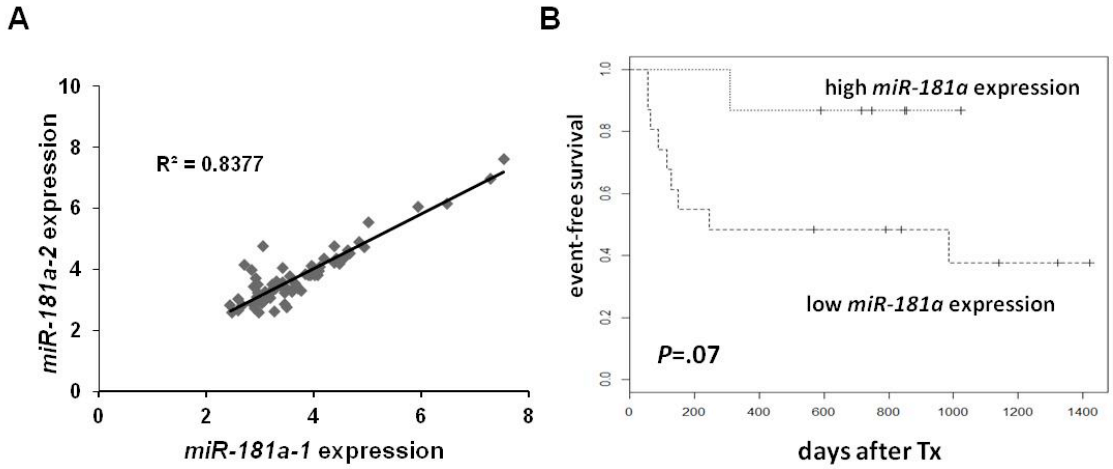

Figure 1: A) Scatter plot of relative *miR-181a-1* and *miR-181a-2* expression, normalized to *18S* in pretreatment bone marrow samples of 75 AML patients treated at the University of Leipzig. B) Event-free survival of a subset (n= 22) with outcome data available following the allogeneic transplantation according to *miR-181a* expression levels.

These data provided initial support for the usefulness of the expression level of this individual miR to predict outcome in AML.

In the here presented study we asked the question whether the expression levels of a single miR could provide prognostic information in CN-AML patients independent of a comprehensive panel of other established clinical and molecular predictors.

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ORIGINAL REPORT

Prognostic Significance of Expression of a Single MicroRNA, *miR-181a*, in Cytogenetically Normal Acute Myeloid Leukemia: A Cancer and Leukemia Group B Study

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Authors' disclosures of potential conflicts of interest and author contributions are found at the end of this article.

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ABSTRACT

Purpose
To evaluate the prognostic significance of expression levels of a single microRNA, *miR-181a*, in the context of established molecular markers in cytogenetically normal acute myeloid leukemia (CN-AML), and to gain insight into the leukemogenic role of *miR-181a*.

Patients and Methods
miR-181a expression was measured in pretreatment marrow using Ohio State University Comprehensive Cancer Center version 3.0 arrays in 187 younger (< 60 years) adults with CN-AML. Presence of other molecular prognosticators was assessed centrally. A gene-expression profile associated with *miR-181a* expression was derived using microarrays and evaluated by Gene-Ontology analysis.

Results
Higher *miR-181a* expression associated with a higher complete remission (CR) rate ($P = .04$), longer overall survival (OS; $P = .01$) and a trend for longer disease-free survival (DFS; $P = .09$). The impact of *miR-181a* was most striking in poor molecular risk patients with *FLT3*-internal tandem duplication (*FLT3*-ITD) and/or *NPM1* wild-type, where higher *miR-181a* expression associated with a higher CR rate ($P = .009$), and longer DFS ($P < .001$) and OS ($P < .001$). In multivariable analyses, higher *miR-181a* expression was significantly associated with better outcome, both in the whole patient cohort and in patients with *FLT3*-ITD and/or *NPM1* wild-type. These results were also validated in an independent set of older (≥ 60 years) patients with CN-AML. A *miR-181a*-associated gene-expression profile was characterized by enrichment of genes usually involved in innate immunity.

Conclusion
To our knowledge, we provide the first evidence that the expression of a single microRNA, *miR-181a*, is associated with clinical outcome of patients with CN-AML and may refine their molecular risk classification. Targeted treatments that increase endogenous levels of *miR-181a* might represent novel therapeutic strategies.

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INTRODUCTION

Several recent studies have revealed that microRNAs, short noncoding RNAs that hybridize to their target mRNAs and repress the expression of the encoded proteins,[1] are not only involved in such biologic processes as cellular differentiation, proliferation, and survival, but also play an essential role in the development of solid tumors and acute myeloid leukemia (AML).[2-6] In AML, genome-wide microRNA-expression profiling has revealed distinctive microRNA-expression signatures capable of differentiating among specific cytogenetic subtypes, such as core-binding factor (CBF) -AML with t(8;21), CBF-AML with inv(16) or t(16;16), and acute promyelocytic leukemia with t(15;17), and setting them apart from other AML subtypes.[7-9] Moreover, microRNA expression signatures have been associated with mutations of *NPM1*,[7,10] *FLT3*,[7,10,11] and *CEBPA*,[7,12] which are genetic alterations known to affect clinical outcome of patients belonging to the largest subset of AML—cytogenetically normal AML (CN-AML).[13,14]

Furthermore, we have recently demonstrated that deregulated microRNA expression may also be associated with outcome in CN-AML.[5,11] Using

microRNA-expression profiling in patients with CN-AML with unfavorable molecular features—*FLT3*-ITD and/or *NPM1* wild-type (*NPM1*wt)—we discovered a prognostic microRNA signature consisting of 12 microRNA probes, five of which corresponded to members of the *miR-181* family.[5] Although these data provided initial support for the usefulness of microRNAs for assessment of molecular risk in AML, microRNAs have been linked to prognosis in AML mainly in the context of genome-wide profiling. This approach, however, is based on population analysis, and therefore, is relatively difficult to implement for prospectively assessing the molecular risk of individual patients. Thus new strategies are needed to increase the clinical applicability of microRNA expression–based prognostication in AML.

To our knowledge, the independent prognostic impact of expression levels of individual microRNAs, which are relatively easy to measure for molecular risk assessment of individual patients at diagnosis, has not been demonstrated in CN-AML outside of microRNA expression profiles. Thus, we sought evidence here that the expression levels of a single microRNA, *miR-181a,* could provide prognostic information in patients with CN-AML independently from a comprehensive panel of other established clinical and molecular predictors, and therefore, be readily applicable as a risk-stratification tool. We show that expression of *miR-181a* is strongly associated with outcome, which suggests that *miR-181a* expression could be used for individual patients' molecular risk assessment and perhaps as a potential therapeutic target.

PATIENTS AND METHODS

Patients, Treatment, and Cytogenetic Analysis

A total of 187 adult patients younger than 60 years (range, 18 to 59 years) with untreated, primary CN-AML and material available for analysis were included. Patients were treated similarly with intensive induction chemotherapy and consolidation with autologous peripheral blood stem-cell transplantation on Cancer and Leukemia Group B (CALGB) protocols 9621 (n = 89) and 19808 (n = 98).[15,16] Of those who achieved a complete remission (CR), 82% received an autologous transplant. Cytogenetic analyses of pretreatment bone marrow (BM) samples were performed by CALGB-approved institutional cytogenetic laboratories as part of CALGB 8461, a prospective cytogenetic companion study, and centrally reviewed.[17,18] All patients gave informed consent for the research use of their specimens, in accordance with the Declaration of Helsinki. No patient received allogeneic stem-cell transplantation in first CR.

A cohort of 122 CN-AML patients age 60 years or older, treated on first-line CALGB protocols (Appendix, online only), constituted an independent validation set for outcome analyses.

Molecular Analyses

The presence or absence of additional molecular markers such as *FLT3*-ITD, *FLT3* tyrosine kinase domain mutations (*FLT3*-TKD), mutations in the *NPM1*, *CEBPA*, *WT1*, *IDH1,* and *IDH2* genes, *MLL* partial tandem duplication (*MLL*-PTD), and *BAALC* and *ERG* expression levels were assessed centrally, as previously reported.[12,19-29]

miR-181a *Expression Analyses*

For microRNA expression, total RNA was extracted from pretreatment BM or blood mononuclear cells, and biotinylated first-strand complementary DNA was synthesized and hybridized to microRNA microarray chips.[5] Images of the microRNA microarray chips were acquired, and calculation, normalization, and filtering of signal intensity for each microarray spot and batch-effect adjustment were performed.[5] *miR-181a* expression was measured using Ohio State University Comprehensive Cancer Center version 3.0 arrays. Log intensities for *miR-181a* probes were averaged and used as a continuous variable for analyses. To validate measurements of *miR-181a* expression made using the microRNA microarrays, quantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR) was performed in a subgroup of younger patients (Appendix).

Gene Expression Profiling

To gain further insight into the biologic processes associated with *miR-181a* in CN-AML, we performed gene-expression profiling using the AffymetrixU133 plus 2.0 array (Affymetrix, Santa Clara, CA), and Gene Ontology analysis as reported previously,[30] and described in the Appendix.

Definition of Clinical End Points and Statistical Analysis

The main objective of our study was to evaluate the impact of *miR-181a* expression on outcome (for definition of clinical end points, see Appendix).

The associations of *miR-181a* expression, considered as a continuous variable, with baseline clinical, demographic, and molecular features were analyzed using one-way analysis of variance. Univariable logistic regression models were constructed to evaluate *miR-181a* expression for achievement of CR, and univariable Cox proportional hazards models were used to evaluate the associations of *miR-181a* expression with disease-free survival (DFS) and overall survival (OS). Multivariable logistic regression models were constructed to analyze factors related to the probability of achieving CR, and multivariable Cox proportional hazards models were constructed to analyze factors important for DFS and OS (multivariable analyses are detailed in the Appendix).

RESULTS

Associations of miR-181a Expression With Clinical and Molecular Characteristics in Patients With CN-AML

At diagnosis, higher expression of *miR-181a*, analyzed here as a continuous variable, was significantly associated with higher hemoglobin ($P = .05$) and percentage of circulating blasts ($P < .001$), French-American-British M1 and M2 subtypes ($P < .001$) and the absence of extramedullary disease, especially skin and gum involvement ($P = .04$; Table 1). Higher *miR-181a* expression was also significantly associated with higher frequency of wild-type *NPM1* ($P = .003$), *CEBPA* mutations ($P < .001$), *IDH1* mutations ($P = .007$), and lower *ERG* ($P = .02$) and higher *BAALC* ($P = .05$) expresser status (Table 1).

Prognostic Value of miR-181a Expression in CN-AML

Patients with higher *miR-181a* expression had a higher CR rate (odds ratio [OR], 1.38; $P = .04$). With a median follow-up time for patients alive at the last follow-up visit of 6.5 years (range, 3.1 to 11.0 years), higher *miR-181a* expressers had a trend for longer DFS ($P = .09$) and had longer OS (hazard ratio [HR], 0.82; $P = .01$; Table 2). The prognostic impact of *miR-181a* expression levels measured using microRNA microarrays was technically validated by outcome analyses in a subgroup of 30 patients for whom *miR-181a* expression was also determined using real-time RT-PCR (Appendix).

In multivariable analyses (Table 3), higher *miR-181a* expression levels were associated with an increased rate of CR (OR, 2.36; $P = .02$), after adjusting for *ERG* ($P = .008$) and *BAALC* expression status ($P = .01$) and age ($P = .01$). Higher *miR-181a* expression was also associated with longer DFS (HR, 0.8; $P = .02$), after adjusting for *CEBPA* ($P = .005$), *NPM1* ($P < .001$), *WT1* ($P = .003$), *FLT3*-ITD ($P < .001$) and *FLT3*-TKD ($P = .02$) mutational status, and with longer OS (HR, 0.81; $P = .01$), after adjusting for *CEBPA* ($P < .001$),

Table 1. Relationship of Clinical and Molecular Characteristics With *miR-181a* Expression in the Whole Group of 187 Younger Patients With Cytogenetically Normal Acute Myeloid Leukemia at Diagnosis

Characteristic	No.	%	*P**
Median age, years	45		.08 ↓
Range	18-59		
Sex			.39
Female	98	52	
Male	89	48	
Race			.91
White	163	88	
Nonwhite	23	12	
Median hemoglobin, g/L	9.3		.05 ↑
Range	4.6-13.6		
Median platelet count, $\times 10^9$/L	58		.29
Range	7-466		
Median WBC, $\times 10^9$/L	27.9		.13 ↓
Range	0.9-295.0		
Median blood blasts, %	62		< .001 ↑
Range	0-97		
Median bone marrow blasts, %	67		.58
Range	21-95		
FAB			< .001
M1/M2	**92**	**59**	
M4/M5	56	36	
Extramedullary involvement†			.04
No	**129**	**70**	
Yes	56	30	
FLT3-ITD			.94
Negative	117	63	
Positive	70	37	
FLT3-TKD			.06
Negative	**167**	**90**	
Positive	18	10	
NPM1			.003
Wild type	**67**	**36**	
Mutated	120	64	
CEBPA			< .001
Wild type	152	83	
Mutated	**32**	**17**	
WT1			.16
Wild type	**161**	**88**	
Mutated	22	12	
MLL-PTD			.59
Negative	175	94	
Positive	12	6	
IDH1			.007
Wild type	124	87	
Mutated	**19**	**13**	
IDH2			.88
Wild type	126	88	
Mutated	17	12	
ERG expression			.02
Low	**83**	**62**	
High	50	38	
BAALC expression			.05
Low	70	50	
High	**70**	**50**	

Abbreviations: FAB, French-American-British classification; *FLT3*-ITD, internal tandem duplication of the *FLT3* gene; *FLT3*-TKD, tyrosine kinase domain mutation of the *FLT3* gene; *MLL*-PTD, partial tandem duplication of the *MLL* gene.

**P* values are from the one-way analysis of variance overall F-test, evaluating the presence of any linear relationship between *miR-181a* expression and the variable tested. For tests with a *P* value < .20, ↑ indicates that higher values of the continuous variable associate with higher *miR-181a* expression and ↓ indicates that lower values of the continuous variable associate with higher *miR-181a* expression; for the categorical variables, those associated with higher *miR-181a* expression are indicated using bold type.

†Primarily extramedullary skin and gum involvement.

Table 2. Relationship Between *miR-181a* Expression and Outcome of Younger Patients With Cytogenetically Normal Acute Myeloid Leukemia

End Point	OR/HR	95% CI	*P*
Analyses in all CN-AML patients			
Complete remission	1.38	1.01 to 1.88	.04
Disease-free survival	—	—	.09
Overall survival	0.82	0.71 to 0.96	.01
Analyses in *FLT3*-ITD and/or *NPM1*wt patients			
Complete remission	1.64	1.12 to 2.42	.009
Disease-free survival	0.66	0.53 to 0.84	< .001
Overall survival	0.71	0.60 to 0.84	< .001

NOTE: An OR greater than 1.0 means a higher complete remission rate for higher values of *miR-181a* expression. An HR lower than 1.0 means longer survival for higher values of *miR-181a* expression. The sample size for the entire set was n = 187 for complete remission and overall survival and n = 154 for disease-free survival. The sample size for *FLT3*-ITD and/or *NPM1*wt patients was n = 122 for complete remission and overall survival and n = 96 for disease-free survival.

Abbreviations: HR, hazard ratio; OR, odds ratio.

NPM1 ($P < .001$), *WT1* ($P < .001$), and *FLT3*-ITD ($P = .003$) mutational status, and WBC ($P = .005$).

Association of miR-181a *Expression Levels With Outcome in Distinct CN-AML Molecular Groups*

The presence or absence of *FLT3*-ITD and *NPM1* mutations has been reported to stratify patients with CN-AML into prognostically distinct categories. Patients with *NPM1* mutations, but no *FLT3*-ITD had a more favorable outcome, whereas those with *FLT3*-ITD and/or *NPM1*wt had worse prognosis.[23] Thus, to better understand the prognostic significance of higher *miR-181a* expression levels in CN-AML, we analyzed their impact on the aforementioned prognostic subsets. While there was no prognostic impact of *miR-181a* expression on patients with *NPM1* mutations and no *FLT3*-ITD (n = 65; CR rate, $P = .58$; DFS, $P = .76$; and OS, $P = .66$), we observed that higher *miR-181a* expression levels were associated with a significantly higher CR rate (OR, 1.64; $P = .009$), and longer DFS (HR, 0.66; $P < .001$) and OS (HR, 0.71; $P < .001$) in patients with *FLT3*-ITD and/or *NPM1*wt (n = 122; Table 2).

In multivariable analysis restricted to patients with *FLT3*-ITD and/or *NPM1*wt (Table 3), higher *miR-181a* expression levels were associated with higher odds of achieving a CR (OR, 1.61; $P = .02$), after adjusting for age ($P = .009$), with longer DFS (HR = 0.74; $P = .02$), after adjusting for *CEBPA* ($P < .001$), *NPM1* ($P = .007$), and *FLT3*-ITD ($P = .02$) mutational status, and hemoglobin levels ($P = .04$), and with longer OS (HR, 0.74; $P = .002$), after adjusting for *CEBPA* ($P < .001$), *NPM1* ($P = .007$), and *WT1* ($P = .01$) mutational status, WBC ($P < .001$), and extramedullary involvement ($P = .01$).

In the aforementioned analyses, we used *miR-181a* expression values as a continuous variable. To graphically display the relationship between *miR-181a* expression and achievement of CR, we compared *miR-181a* expression in patients achieving CR with that of patients experiencing failure with induction therapy within the subgroup of patients with *FLT3*-ITD and/or *NPM1*wt (Fig 1A). Furthermore, to graphically display the relationship between *miR-181a* expression and DFS and OS, we dichotomized *miR-181a* expression values at the median, and present survival curves for the high and low *miR-181a* expressers within the subgroup of patients with *FLT3*-ITD and/or *NPM1*wt (Fig 1B and 1C).

Table 3. Multivariable Analyses Evaluating *miR-181a* Expression for Clinical Outcome in Younger Patients With CN-AML

Variables in Final Models	OR/HR	95% CI	*P*
Multivariable analyses in all patients with CN-AML			
CR[a]			
miR-181a expression	2.36	1.17 to 4.78	.02
ERG expression; low *v* high	5.86	1.60 to 21.52	.008
BAALC expression; low *v* high	6.69	1.56 to 28.74	.01
Age	0.36	0.17 to 0.78	.01
DFS[b]			
miR-181a expression	0.80	0.66 to 0.97	.02
CEBPA; mutated *v* wild type	0.38	0.19 to 0.75	.005
NPM1; mutated *v* wild type	0.42	0.24 to 0.75	< .001[c]
WT1; mutated *v* wild type	2.54	1.39 to 4.65	.003
FLT3-ITD; positive *v* negative	2.68	1.65 to 4.36	< .001[c]
FLT3-TKD; positive *v* negative	2.19	1.14 to 4.19	.02
OS[d]			
miR-181a expression	0.81	0.69 to 0.95	.01
CEBPA; mutated *v* wild type	0.32	0.16 to 0.62	< .001
NPM1; mutated *v* wild type	0.47	0.28 to 0.79	< .001[c]
WT1; mutated *v* wild type	2.65	1.54 to 4.57	< .001
FLT3-ITD; positive *v* negative	2.39	1.46 to 3.93	.003[c]
WBC	1.37	1.13 to 1.67	.005[c]
Multivariable analyses in patients with *FLT3*-ITD and/or *NPM1*wt			
CR[e]			
miR-181a expression	1.61	1.07 to 2.42	.02
Age	0.53	0.33 to 0.85	.009
DFS[f]			
miR-181a expression	0.74	0.57 to 0.96	.02
CEBPA; mutated *v* wild type	0.27	0.13 to 0.58	< .001
NPM1; mutated *v* wild type	0.33	0.14 to 0.79	.007[g]
FLT3-ITD; positive *v* negative	3.05	1.30 to 7.14	.02[g]
Hemoglobin	0.75	0.57 to 0.99	.04
OS[h]			
miR-181a expression	0.74	0.61 to 0.90	.002
CEBPA; mutated *v* wild type	0.29	0.14 to 0.59	< .001
NPM1; mutated *v* wild type	0.41	0.22 to 0.78	.007[g]
WT1; mutated *v* wild type	2.23	1.18 to 4.23	.01
WBC	1.40	1.15 to 1.71	< .001
Extramedullary involvement; absent *v* present	2.45	1.27 to 4.71	.01[g]

NOTE. Further details of the multivariable analyses are found in the Appendix (online only). ORs greater than 1.0 mean higher and those less than 1.0 mean lower CR rate for the higher values of the continuous variables and the first category listed for the categorical variables. HRs greater than 1.0 indicate higher and those less than 1.0 indicate lower risk for relapse or death (DFS) or death (OS) for the higher values of the continuous variables and the first category listed for the categorical variables.

Abbreviations: CN-AML, cytogenetically normal acute myeloid leukemia; CR, complete remission; DFS, disease-free survival; *FLT3*-ITD, internal tandem duplication of the *FLT3* gene; *FLT3*-TKD, tyrosine kinase domain of the *FLT3* gene; HR, hazard ratio; OS, overall survival; OR, odds ratio.

[a]Variables considered in the model based on univariable analyses were *miR-181a* expression, *ERG* expression (low *v* high), *FLT3*-ITD (positive *v* negative), *BAALC* expression (low *v* high), age (in 10-year increments), hemoglobin (in 2-unit increments), and WBC (in 50-unit increments).

[b]Variables considered in the model based on univariable analyses were *miR-181a* expression, *CEBPA* (mutated *v* wild type), *ERG* expression (low *v* high), *WT1* (mutated *v* wild type), *BAALC* expression (low *v* high), *FLT3*-ITD (positive *v* negative), *FLT3*-TKD (positive *v* negative), *MLL*-PTD (mutated *v* wild type), *NPM1* (mutated *v* wild type), WBC (in 50-unit increments), extramedullary involvement, and race.

[c]Does not meet the proportional hazards assumption. For DFS, the HR for *FLT3*-ITD and *NPM1* are reported at 9 months; for OS, the HR for *NPM1, FLT3*-ITD, and WBC are reported at 9 months.

[d]Variables considered in the model based on univariable analyses were *miR-181a* expression, *CEBPA* (mutated *v* wild type), *ERG* expression (low *v* high), *FLT3*-ITD (positive *v* negative), *WT1* (mutated *v* wild type), *BAALC* expression (low *v* high), *NPM1* (mutated *v* wild type), WBC (in 50-unit increments), age (in 10-year increments), hemoglobin (in 2-unit increments), platelet count, percentage of blood blasts, and extramedullary involvement.

[e]Variables considered in the model based on univariable analyses were *miR-181a* expression, age (in 10-year increments), hemoglobin (in 2-unit increments), and WBC (in 50-unit increments).

[f]Variables considered in the model based on univariable analyses were *miR-181a* expression, *CEBPA* (mutated *v* wild type), *ERG* expression (low *v* high), *WT1* (mutated *v* wild type), *FLT3*-ITD (positive *v* negative), *FLT3*-TKD (positive *v* negative), *NPM1* (mutated *v* wild type), hemoglobin (in 2-unit increments), WBC (in 50-unit increments), and race.

[g]Does not meet the proportional hazards assumption. For DFS, the HR for *FLT3*-ITD is reported at 1 year, *NPM1* is reported at 9 months; for OS, the HR for *NPM1* is reported at 1.5 years, extramedullary involvement is reported at 1 year.

[h]Variables considered in the model based on univariable analyses were *miR-181a* expression, *CEBPA* (mutated *v* wild type), *ERG* expression (low *v* high), *WT1* (mutated *v* wild type), *FLT3*-ITD (positive *v* negative), *NPM1* (mutated *v* wild type), hemoglobin (in 2-unit increments), WBC (in 50-unit increments), and extramedullary involvement.

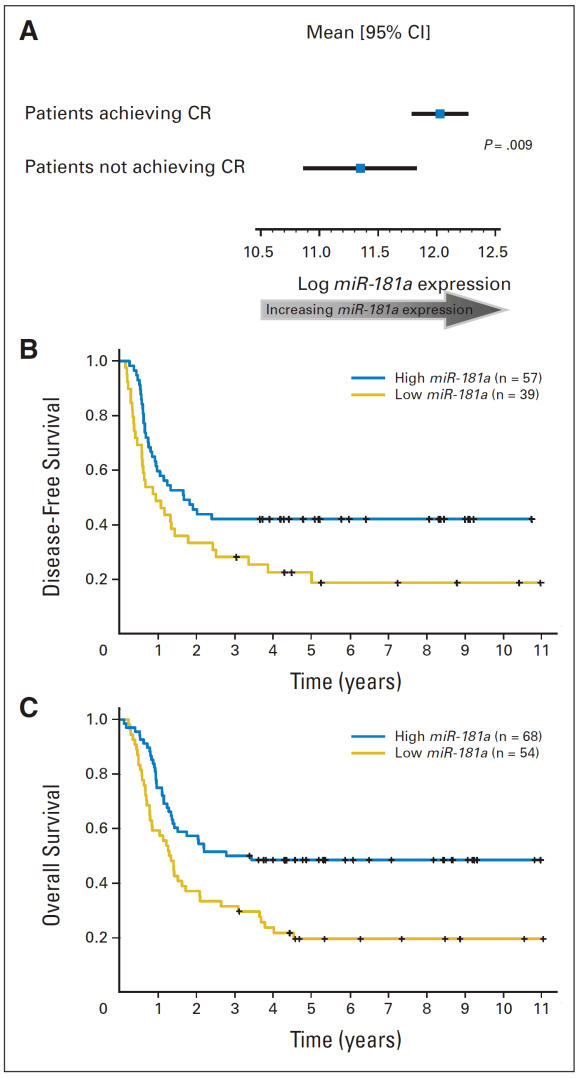

Fig 1. Favorable outcome of patients with *FLT3*-ITD and/or *NPM1*wt and higher *miR-181a* expression levels. (A) *miR-181a* expression in patients who achieved a complete response (CR) versus patients who did not achieve a CR; (B) disease-free and (C) overall survival according to *miR-181a* expression levels in patients with CN-AML dichotomized into high (above the median *miR-181a* expression value) or low (at or below the median *miR-181a* expression value) expression groups.

Importantly, an independent set of older patients with CN-AML with *FLT3*-ITD and/or *NPM1*wt (n = 122) was analyzed by microRNA microarray assays to validate the prognostic impact of *miR-181a* found in younger patients (Appendix). In this validation set, higher expression of *miR-181a*, used as a continuous variable, did not impact on the CR rate ($P = .52$), but was associated with longer DFS ($P = .04$) and with a trend for longer OS ($P = .08$). In multivariable models for this validation set, *miR-181a* was independently associated with longer DFS ($P = .04$) and OS ($P = .05$), even after adjusting for other clinical and molecular variables (Appendix Table A1, online only).

Biologic Insights

In order to gain insights into the functional contribution of *miR-181a* expression levels to the poor molecular risk CN-AML subset, we first derived a gene-expression signature associated with *miR-181a* expression in patients with *FLT3*-ITD and/or *NPM1*wt. We observed that the expression of 1,174 probe sets significantly correlated ($P < .001$) with that of *miR-181a*; 1,002 probe sets correlated negatively and 172 probe sets positively (Fig 2). Among other genes, we observed a negative correlation of *miR-181a* expression with the expression of the *HOXA* and *HOXB* clusters, as well as the *HOX* cofactor *MEIS1*. These genes are important for developmental processes and have also been linked to leukemogenesis and the self-renewal of leukemic stem cells.[31,32] We also observed a negative correlation of *miR-181a* expression with the expression of the

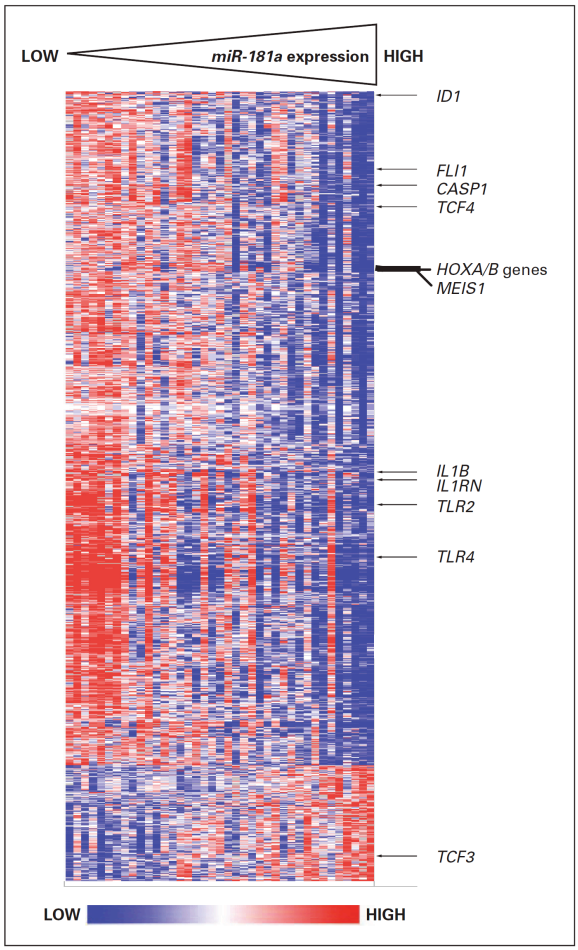

Fig 2. Heat map of the derived gene-expression signature correlated with *miR-181a* expression. Rows represent probe sets and columns represent patients. Probe sets are ordered by hierarchical cluster analysis. Patients are ordered from left to right by increasing *miR-181a* expression. Expression values of the probe sets are represented by color, with blue indicating expression less than and red indicating expression greater than the median value for the given probe set. Arrows indicate genes that are discussed in the text.

transcription coregulator *ID1*, which is able to prevent hematopoietic differentiation and has recently been associated with adverse outcome in AML[33]; the *FLI1* gene, a known suppressor of erythroid differentiation[34]; and the transcription factor *TCF4*, which contributes to neoplastic transformation as a downstream target of the WNT-pathway.[35] In contrast, we observed a positive correlation of *miR-181a* expression with the expression of *TCF3*, a gene encoding a transcription factor that has been shown to regulate the homeostasis of the hematopoietic stem cell pool and promote differentiation of hematopoietic progenitors.[36,37]

To further understand the potential functional role of *miR-181a* expression in CN-AML, we performed a Gene Ontology analysis. Biologic processes that relate to cytokine and native immunity-mediated processes, including those involving toll-like receptors (eg, *TLR4* and *TLR2*) and the interleukin pathways (eg, *IL1B, IL1RN,* and *CASP1*), were over-represented in the *miR-181a*-associated gene-expression signature (Table 4).

Table 4. GO Terms of Biological Processes Significantly Overrepresented in the *miR-181a*-Expression Profile

GO ID	GO Terms	Percentage of Members of the GO Term Present in the *miR-181a* Profile	*P*
50715	Positive regulation of cytokine secretion	83.33	< .001
50706	Regulation of interleukin-1 beta secretion	80	< .001
50716	Positive regulation of interleukin-1 secretion	80	< .001
50704	Regulation of interleukin-1 secretion	80	< .001
50718	Positive regulation of interleukin-1 beta secretion	80	< .001
50707	Regulation of cytokine secretion	77.78	< .001
45123	Cellular extravasation	66.67	< .001
50701	Interleukin-1 secretion	66.67	.001
50702	Interleukin-1 beta secretion	66.67	.001
7159	Leukocyte adhesion	66.67	.002
50663	Cytokine secretion	66.67	< .001
9595	Detection of biotic stimulus	62.5	< .001
50709	Negative regulation of protein secretion	60	.003
30593	Neutrophil chemotaxis	60	< .001
45408	Regulation of interleukin-6 biosynthetic process	57.14	.002
45576	Mast cell activation	57.14	.004
30149	Sphingolipid catabolic process	55.56	< .001
42226	Interleukin-6 biosynthetic process	50	.003
32635	Interleukin-6 production	50	.003
50714	Positive regulation of protein secretion	50	< .001
46466	Membrane lipid catabolic process	50	< .001

NOTE. Shown are significantly overrepresented GO terms with ≥ 50% of their assigned members represented in the gene expression signature associated with higher *miR-181a* expression. Gray shading identifies terms associated with genes encoding proteins in the interleukin-1β and toll-like receptor pathways (eg, *IL1B, IL1BRN, CASP1, TLR2, TLR4,* etc).
Abbreviation: GO, Gene Ontology.

DISCUSSION

We report here that expression levels of *miR-181a* constitute a strong prognostic factor in younger patients with CN-AML enrolled on similar CALGB first-line treatment protocols. We show that higher levels of *miR-181a* expression directly correlate with higher odds of achieving a CR and lower risk of experiencing relapse and/or death in patients with CN-AML. This study is the first to demonstrate that a single noncoding RNA associates with clinical outcome in CN-AML, even in the context of other well-established molecular markers including *CEBPA* and *NPM1* mutations, that were recently recognized by the WHO classification as defining markers for novel provisional AML entities,[38] and *FLT3*-ITD. Furthermore, we technically validated these results by using quantitative RT-PCR.

The prognostic impact was most striking in patients with *FLT3*-ITD and/or *NPM1*wt, which are associated with adverse outcome. These patients constitute approximately 65% of all CN-AML and one third of all AML patients younger than 60 years.[13] Notably, in this group, when other molecular prognostic markers were considered in multivariable models, higher expression of *miR-181a* was the only molecular marker that independently associated with higher odds of achieving CR, thereby suggesting a potential impact of this microRNA on mechanisms of resistance to chemotherapy-induced apoptosis. Higher expression of *miR-181a* was also associated with longer DFS after adjusting for the impact of *NPM1, CEBPA,* and *FLT3*-ITD mutational status and hemoglobin levels, and OS after adjusting for the impact of *NPM1, CEBPA,* and *WT1* mutational status, extramedullary involvement, and WBC. These results were validated by demonstrating the positive prognostic impact of higher *miR-181a* expression in an independent validation set of older patients with CN-AML.

Recently, a modified prognostic classification of CN-AML has been recommended by an international expert panel on behalf of the European LeukemiaNet, in which the intermediate I prognostic category also includes patients with *FLT3*-ITD and/or *NPM1*wt, but only those who lack *CEBPA* mutations; patients with *FLT3*-ITD and/or *NPM1*wt and *CEBPA* mutations are classified in the favorable category.[39] When we analyzed the prognostic significance of *miR-181a* expression in this European LeukemiaNet intermediate I prognostic category (n = 92), higher *miR-181a* expression levels were still associated with a significantly higher CR rate (OR, 1.56; $P = .04$), and longer DFS (HR, 0.72; $P = .03$) and OS (HR, 0.77; $P = .01$). Altogether, these data support a pivotal role of *miR-181a* expression levels for the response to treatment of patients with CN-AML, and suggest that since *miR-181a* expression provides additional prognostic information it can be used to further refine this newly devised molecular-risk classification of CN-AML.[39] Moreover, the identification of low levels of *miR-181a* as an adverse prognostic factor provides opportunity for potential therapeutic intervention with agents capable of increasing

low endogenous levels of *miR-181a* and/or with synthetic *miR-181a* compounds.

But how do changes of *miR-181a* expression levels in myeloid blasts affect the aggressiveness of the disease in patients with CN-AML? The biologic role of microRNAs may vary according to their expression in distinct cell populations of normal or neoplastic tissues. *miR-181a* has been described as a tumor suppressor in gliomas,[40] but also has been found elevated in hepatocellular carcinoma cells with features of hepatic cancer stem cells.[41] Currently, relatively little is known about the function of *miR-181a* in normal or malignant hematopoiesis. Previous studies reported that *miR-181* regulated B-cell development and influenced T-cell sensitivity to antigens by modulating T-cell receptor signaling strength.[42,43] Furthermore, *miR-181a* may also play a regulatory role in earlier steps of hematopoiesis.[44] Recently, it was shown that higher levels of *miR-181* are expressed during early erythroid differentiation.[45] In line with these findings, in this study, we observed a positive correlation between *miR-181a* expression and hemoglobin levels, and a negative correlation between *miR-181a* expression and expression of *FLI1*, a known suppressor of erythroid differentiation.[35] Furthermore, we found a negative correlation of *miR-181a* expression with the expression of *ID1*, an inhibitor of hematopoietic differentiation, and *TCF4*, a transcription factor promoting neoplastic transformation.[35] We also observed a negative correlation of *miR-181a* expression with the expression of the *HOXA* and *HOXB* clusters, as previously reported.[45] In contrast, we observed a positive correlation between *miR-181a* expression and *TCF3*, a transcription factor that seemingly promotes development of hematopoietic progenitors and contributes to regulating hematopoietic cell differentiation.[37]

In an effort to further understand how changes in *miR-181a* expression affect the aggressiveness of the disease, response to treatment, and outcome of patients with CN-AML, we used a Gene Ontology analysis. We show an over-representation of cytokine and native immunity-mediated processes in the *miR-181a*-associated gene-expression signature. The expression of the *TLR4*, *TLR2*, *IL1B*, *IL1RN*, and *CASP1* genes was negatively correlated with *miR-181a* expression, and we find some of these genes, namely *TLR4* and *IL1B* and *CASP1* to be predicted to be direct targets of *miR-181a*. Of these genes, *TLR4* and *IL1B* have previously been implicated in human cancer.[47-50] *TLR4* has been shown to promote tumor growth and interfere with response to chemotherapy in ovarian cancer,[46] and to contribute to the development of cytopenias in myelodysplastic syndromes.[47] In addition, TLR4 signaling has also been linked to blocking myeloid differentiation of hematopoietic stem and progenitor cells in severe sepsis.[48] IL-1β has been previously shown to be produced in an autocrine fashion and to stimulate the proliferation of AML blasts.[49,50] It is, therefore, tempting to speculate that high expression of *miR-181a* associates with a less aggressive disease by downregulating genes like *TLR4* and *IL1B*, that modulate the innate immune response to microbial pathogens in the normal host, but also when upregulated may support survival and proliferation of malignant blasts in AML patients.[47-50] However, the mechanisms through which the changes in levels of *miR-181a* expression contribute to different degrees of disease aggressiveness in patients with CN-AML and why *miR-181a* expression differs among individual patients remain to be elucidated.

In summary, we report here for the first time that the expression of a single microRNA, *miR-181a*, associates with clinical outcome in CN-AML. Moreover, it does so independently from other validated clinical and genetic variables, thus adding information useful for a better risk-stratification of patients with CN-AML. High *miR-181a* expression levels identify those patients with CN-AML who despite having molecular features associated with adverse outcome, such as *NPM1*wt and/or *FLT3*-ITD, might not need intensive treatment, such as allogeneic stem-cell transplantation. Moreover, for those patients with low *miR-181a* expression levels, it is hoped that the development of reliable methods of delivery of this microRNA directly to the leukemia cells and/or identification of agents capable of increasing endogenous levels of *miR-181a* may provide new therapeutic options. Further prospective studies should be done to confirm our findings. Establishment of standardized methods of microRNA quantification will allow prospective classification of patients according to their *miR-181a* levels. Finally, the combination of *miR-181a*-associated gene-expression profiling and Gene Ontology analyses provide insights into the leukemogenic role of genes that are either direct or indirect targets of *miR-181a*, and therefore should also be investigated as potential therapeutic targets in patients with CN-AML with low *miR-181a* expression.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

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Manuscript writing: All authors
Final approval of manuscript: All authors

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SUPPLEMENTAL MATERIAL

Definition of Clinical Endpoints

Complete remission (CR) was defined as bone marrow (BM) cellularity >20% with maturation of all cell lines, <5% leukemic blasts, undetectable Auer rods, and recovery of leukocyte (≥1,500/μL) and platelet (>100,000/μL) counts with no leukemic blasts in the blood, all of which had to persist for at least 1 month. Relapse was defined as ≥5% BM blasts, reappearance of circulating blasts or development of extramedullary leukemia.[1] Overall survival (OS) was measured from the date the patient was enrolled onto the study until the date of death, and patients alive at last follow-up were censored. Disease-free survival (DFS) was measured from the date of CR until the date of relapse or death; patients alive and relapse-free at last follow-up were censored. Pretreatment central nervous system, spleen, liver, skin, nodes, gum, or mediastinal mass involvement constituted extramedullary disease.

White blood count (WBC), BM, and peripheral blood (PB) blasts

Forty-four percent of the 423 CN-AML cases enrolled on the two protocols [Cancer and Leukemia Group B (CALGB) 9621 and 19808] were part of our study. WBC, and BM and PB blasts were significantly higher in the cases we studied (P<.001 for all three variables) versus the cases we did not. This is a result of selection criteria used to identify patients with adequate blast counts for reliable molecular studies. To explore this potential bias, we thoroughly evaluated WBC and blast percentage (BM and PB) in all our outcome multivariable models to ensure that the outcome association of our main

variable, *miR-181a* expression, did not change. *MiR-181a* stayed significant in all of the models that contained these variables (CR and DFS for WBC; CR, DFS, OS for BM and PB blasts) and neither WBC nor percent blasts (BM or PB) became significant for outcome.

miR-181a Expression Analyses

MiR-181a expression was measured using the OSUCCC v3.0 microRNA microarray chip. For quantification, the two *miR-181a* probes on the chip were averaged. These two probes correlated very strongly (r=.98, *P*<.001), and no outliers were observed.

BM samples were used to study *miR-181a* levels in the majority of our cases (72%). When we compared *miR-181a* levels across BM and blood samples, we did not see any differences across the tissue types.

Since *miR-181b* expression was also part of the reported prognostic microRNA signature, in CN-AML with unfavorable molecular features — *FLT3*-ITD and/or *NPM1* wild-type (*NPM1*wt) — we also analyzed the prognostic impact of *miR-181b* expression.[2] We found that the expression levels of *miR-181b* did not provide any additional information, with respect to outcome. Therefore we focused on *miR-181a* expression levels.

In order to validate the microRNA microarray-based *miR-181a* quantification, we performed real-time RT-PCR for *miR-181a* in a subgroup of 30 patients, 15 of whom

had high and 15 low *miR-181a* expression levels according to the microarray data. *MiR-181a* expression was normalized to an internal control, *U48* small nucleolar RNA expression. Primers, probes and amplification conditions will be provided upon request. Outcome analysis of these 30 patients analyzed by RT-PCR reproduced the results obtained using the microRNA microarray. Using a median cut-off value for the real-time RT-PCR data to define high and low expressers, patients in the high *miR-181a* expression group had a significantly better DFS (P<.001) and OS (P<.001) than those in the low *miR-181a* expression group, thereby confirming the outcome results we observed using the microRNA microarray data for these 30 cases.

Multivariable Models

Multivariable logistic regression models were constructed to analyze factors related to the probability of achieving CR, and multivariable Cox proportional hazards models were constructed to analyze factors important for DFS and OS. Factors examined for inclusion in the CR models were *miR-181a*, *ERG* and *BAALC* expression, *FLT3*-ITD, *MLL* partial tandem duplication (*MLL*-PTD), *WT1* and *NPM1* mutation status, age, hemoglobin level, platelet count, WBC, percentage of blood and BM blasts, race, sex, induction regimen (ADE versus ADEP), and extramedullary involvement. For OS and DFS, factors examined for model inclusion were *miR-181a*, *ERG* and *BAALC* expression, *FLT3*-ITD, *FLT3* tyrosine kinase domain mutations (*FLT3*-TKD), *MLL*-PTD and *WT1*, *NPM1*, and *CEBPA* mutation status, age, hemoglobin level, platelet count, WBC, percentage of blood and BM blasts, race, sex, and extramedullary involvement. Of the above factors, those significant at α=.20 from the univariable models (see

footnotes in Table 3) were used in a limited backward selection procedure to build multivariable models, by retaining the main variable *miR-181a* throughout the model building. Variables remaining in the final models were significant at α=.05.

To further explore the impact of *miR-181a* expression level in the presence of other prognostic markers, we fitted additional multivariable models for outcome. All molecular markers were retained in these models irrespective of statistical significance. High *miR-181a* expression levels remained significantly associated with favorable outcome for all three end points. For the achievement of CR, the only molecular marker significant in the model was *miR-181a* expression (*P*=.01). For DFS, *miR-181a* expression (*P*=.02), *CEBPA* mutation status (*P*=.005), *FLT3*-ITD (*P*<.001), *FLT3*-TKD (*P*=.02), *NPM1* mutation status (*P*<.001), and *WT1* mutation status (*P*=.001) were all significant in the model. For OS, *miR-181a* expression (*P*=.03), *FLT3*-ITD (*P*<.002), and WT1 mutation status (*P*<.001) were the only significant predictors.

For all Cox models, the proportional hazards assumption was checked for each variable individually. If the proportional hazards assumption was not met for a particular variable for a given endpoint, an artificial time-dependent covariate was included in the model for that endpoint, which requires estimating the hazard ratios at specific time points for these variables as opposed to being able to provide one hazard ratio for the entire time period analyzed. For achievement of CR, estimated odds ratios (OR), and for survival endpoints, hazard ratios (HR) with their corresponding 95% confidence intervals (CI) were obtained for each significant prognostic factor.[3]

Missing Data

Missing data were handled using a complete case analysis approach.[3] This was required only for developing a CR model for the whole patient set.

Validation Analysis of Prognostic Significance of miR-181a Expression Levels

Older patients included in the validation set were treated on one of the following CALGB first-line treatment protocols: 8525,[5] 8923,[6] 9420,[7] 9720,[8] and 10201.[9] Per protocol, patients enrolled on this study did not receive stem cell transplantation in first CR.

In this set of older CN-AML patients with *FLT3*-ITD and/or *NPM1*wt (n=122), *miR-181a* expression levels were measured by microRNA microarray assays. *miR-181a* expression was used as a continuous variable in univariable and multivariable analyses. Due to the large number of predictors relative to the number of events in the analyzed data set, individual variables were evaluated along with *miR-181a* expression and based on these "bi-variable" results the final models were developed (see Supplemental Table S1).

Gene-Expression Profiling

As for the miRNA expression profiling, total RNA was extracted from pretreatment BM or blood mononuclear cells. Using AffymetrixU133 plus 2.0 GeneChips (Affymetrix, Santa Clara, CA), RNA samples from a group of cytogenetically normal acute myeloid leukemia patients treated on CALGB 9621 were analyzed (including data normalization

and computation of expression intensities), as previously reported.[10] Expression values were logged (base 2) before analysis. A filtering step was performed to remove probe sets that did not display significant variation in expression across arrays. In this procedure, a chi-square test was used to test whether the observed variance in expression of a probe set was significantly larger than the median observed variance in expression for all probe sets using α=.01 as the significance level. A total of 19,871 probe sets met the filtering criterion and were included in subsequent analyses. Thirty-nine patients with *FLT3*-ITD and/or *NPM1* wild-type and enrolled on CALGB 9621 were studied in the gene-expression profiling studies detailed above. These patient samples were used for the identification of the gene-expression profile associated with *miR-181a* expression. For this purpose, univariable correlation tests (using Pearson's correlation) were performed between the median *miR-181a* expression level and expression values of each Affymetrix probe set, using a univariable significance level of α=.001.

Analyses were performed using BRB-ArrayTools version 3.8.0 Beta 2 Release (R. Simon and A.P. Lam, National Cancer Institute, Bethesda, MD) and using the R version 2.9.0 (R Foundation for Statistical Computing, Vienna, Austria). Summary measures of gene expression were computed for each probe-set using the robust multichip average (RMA) method, which incorporates quantile normalization of arrays. For *in silico* target prediction of miRNAs, the online applications miRBase Targets Version 5 and Targetscan Release 5.0 were used.

Gene Ontology Analysis

We used GenMAPP version 2.1 and MAPPFinder version 2.0 to assess which biological processes (as designated by the Gene Ontology project at www.geneontology.org) were overrepresented among the genes that constituted the signature. An overrepresented biological process is one that has more associated genes (also referred to as members) in the gene-expression signature than is expected by chance. In our analysis, we considered only biological processes that were represented by ≥5 members among the genes that could be analyzed in our microarray-expression database. MAPPFinder uses a permutation procedure to determine overrepresented biological processes. An alpha level of .005 was used for identifying such biological processes. Furthermore, we only report the overrepresented biological processes for which at least half of their members (ie, genes) analyzed in our microarray-expression database were identified as part of the signature associated with *miR-181a* expression.

Table S1. Multivariable Analyses Evaluating *miR-181a* Expression for Clinical Outcome in Older Cytogenetically Normal Acute Myeloid Leukemia Patients with *FLT3*-ITD and/or Wild-Type *NPM1*

End Point	Variables in Final Models	HR	95% CI	*P*
DFS*	*miR-181a* expression	0.64	0.46, 0.90	.04†
	NPM1; mutated *v* wild-type	0.28	0.11, 0.71	.03†
	Platelets	1.45	1.14, 1.85	.002
	WBC	1.85	1.32, 2.61	<.001
OS*	*miR-181a* expression	0.78	0.63, 0.95	.05†
	FLT3-TKD; positive *v* negative	5.89	1.68, 20.58	.006
	NPM1; mutated *v* wild-type	0.49	0.24, 0.98	.03†
	Platelets	1.20	1.01, 1.35	.005
	WBC	1.15	1.02, 1.30	.02

NOTE: Hazard ratios greater than (less than) 1.0 indicate higher (lower) risk for relapse or death (DFS) or death (OS) for the higher values of the continuous variables and the first category listed for the categorical variables.

Abbreviations: CI, confidence interval; DFS, disease-free survival; *FLT3*-TKD, tyrosine kinase domain of the *FLT3* gene; HR, hazard ratio; OS, overall survival; WBC, white blood count.

* Variables considered in the models were *miR-181a* expression, *CEBPA* (mutated *v* wild-type), *FLT3*-ITD (positive *v* negative), *FLT3*-TKD (positive *v* negative), *NPM1* (mutated *v* wild-type), WBC (in 50 unit increments), platelet count (in 50 unit increments), and age (in 10 year increments).

† Does not meet the proportional hazards assumption. For DFS, the hazard ratios for *miR-181a* expression and *NPM1* mutation status are estimated at 18 months; for OS, the hazard ratios for *miR-181a* expression and *NPM1* mutation status are estimated at 24 months.

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Zusammenfassung / Conclusion

Within the scope of this thesis we present a study that reported on the prognostic impact of the expression levels of a single miR - *miR-181a* - in CN-AML patients. We showed that higher levels of *miR-181a* expression directly correlated with higher odds of achieving CR following standard cytarabine/daunorubicine based treatment protocols and lower risk of experiencing relapse and/or death. This is - to our knowledge - the first study that demonstrated that the expression of a single non-coding RNA associates with clinical outcome in AML, even in the context of other well established molecular markers. In the manuscript we also validated our results in an independent set of older (≥60 years) CN-AML patients. Higher *miR-181a* expression levels were also associated with a significantly higher CR rate, and longer disease-free (DFS) and overall survival (OS) within the ELN Intermediate-I Genetic Group, thus refining the ELN risk classification. Altogether, the presented data support a pivotal role of *miR-181a* expression levels for the response to treatment of CN-AML patients, and suggest that *miR-181a* expression might be used to further refine the risk in AML patients.[11] Additional studies to validate our prognostic findings are needed. Interestingly, very recently the favorable prognostic impact of higher *miR-181* could also be shown to in AML with cytogenetic abnormalities.[45] Moreover, validation studies are currently ongoing at the University of Leipzig, building upon our preliminary data (shown in the introduction) that suggest that higher pretreatment expression levels of *miR-181a* may also be associated with improved outcome following allogeneic hematopoietic cell transplantation after reduced-Intensity conditioning. If the results are confirmed and validated and once standardized RNA quantification methods are established, *miR-*

181a expression might be included in diagnostic panels, and used to improve risk-stratification of AML patients and help guide treatment decisions of physicians.

Additionally, in the here presented study, in order to gain insight into the biology of *miR-181a* associated leukemia, we derived a genome-wide gene expression profile associated with *miR-181a* expression levels. These results may aid in the task of elucidating the biology of *miR-181a* associated outcome differences in CN-AML patients, and provide a basis for further functional studies.

Since our prognostic study was published, additional evidence that underscore *miR-181a's* tumor suppressive effects and thus support our findings have emerged. In chronic lymphatic leukemia (CLL) lower *miR-181a* expression levels have been associated with a more aggressive subtype harboring chromosome 17p deletions and in gastric cancer higher *miR-181* expression was reported to be associated with increased chemosensitivity.[46,47] Furthermore, recently other groups have reported targets of *miR-181a* that may also have a role in AML. Shin *et al.* demonstrated that *miR-181a* may have tumor suppressor activity by targeting oncogenic K-Ras in oral squamous cancer,[48] and it has been described that high expression levels of *miR-181a* promote apoptosis in gliomas and astrocytomas by targeting members of the *BCL2* family.[49,50] Indeed, also *BCL2* itself has been suggested to be a direct target of *miR-181a* and Bai *et al.* showed that *miR-181a* directly targets anti-apoptotic *BCL2* in AML cell lines.[51,52] Overexpression of *miR-181a* in a cytarabine resistant HL-60 cell line decreased BCL-2 expression, induced apoptosis and sensitized the cells to cytarabine treatment.[52] The

findings that *miR-181a* is able to lower the endogenous apoptosis threshold of AML blasts supports our observation of an association of higher *miR-181a* expression with treatment response in AML and provided a functional basis for our clinical observations.

The identification of low levels of *miR-181a* as an adverse prognostic factor provides the opportunity for potential therapeutic intervention by artificially substituting *miR-181a*. Since free synthetic miRs are easily degraded in bio-fluids, have limited cellular uptake and are quickly cleared from blood the administration of unprotected synthetic miRs does not present a feasible approach. However, we recently presented a novel non-viral nanoparticle based targeted delivery system for miRs in AML, which may overcome these limitations.[53] In a proof-of-principal study, our preliminary results indicated that our nanoparticle-based delivered synthetic *miR-181a* is efficient in upregulation of mature *miR-181a* levels in AML cell lines and patient blasts. These increased *miR-181a* levels consequently downregulated *miR-181a* target genes (i.e. *NRAS* and *KRAS*) in *miR-181a*-nanoparticle treated AML cells.[53] Furthermore, the *miR-181a*-nanoparticle treatment had anti-leukemic activity and sensitized AML blasts to daunorubicine treatment *in vitro*,[53] which is in accordance with the findings in the here presented clinical study as well as the *in vitro* findings reported by others. These results show that *miR-181a* may not only have prognostic significance and improve risk stratification in AML, but our findings may also pave the way to a new miR-based personalized medicine for AML patients in the future.

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Ausgewählte Publikation / Selected Publication

The following peer-reviewed publication is submitted within the scope of this thesis.

Schwind S, Maharry K, Radmacher MD, Mrózek K, Holland KB, Margeson D, Whitman SP, Hickey C, Becker H, Metzeler KH, Paschka P, Baldus CD, Liu S, Garzon R, Powell BL, Kolitz JE, Carroll AJ, Caligiuri MA, Larson RA, Marcucci G, Bloomfield CD: Prognostic Significance of Expression of a Single microRNA, *miR-181a*, in Cytogenetically Normal Acute Myeloid Leukemia: A Cancer and Leukemia Group B Study, J Clin Oncol. 2010 Dec 20; 28(36): 5257-64.
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Komplette Publikationsliste / Complete List of Publications

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- Garzon R, Liu S, Fabbri M, Liu Z, Heaphy CEA, Callegari E, **Schwind S,** Pang J, Yu J, Natarajan MN, Havelange V, Volinia S, Blum W, Rush LJ, Perrotti D, Andreeff M, Bloomfield C.D, Byrd JC, Chan K, Wu LC, Croce CM, Marcucci G: *MicroRNA-29b* induces global DNA hypomethylation and tumor suppressor gene reexpression in acute myeloid leukemia by targeting directly DNMT3A and 3B and indirectly DNMT1. 2009; *Blood* 113:6411-6418.

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Schulbildung

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Ausbildung

1996 - 2003	Studium der Humanmedizin an der Freien Universität Berlin
1999	Famulatur in der Gefäßchirurgie Klinikum Reutlingen
2000	Famulatur in der Traumatologie, Universitätsklinikum Benjamin Franklin, Berlin
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2001	Famulatur in der Plastischen und Rekonstruktiven Chirurgie, Tygerberg Hospital, Kapstadt, Südafrika
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2002 - 2003	Erstes Tertial des Praktischen Jahres Innere Medizin, Kantonsspital Aarau, Schweiz
2003	Zweites Tertial des Praktischen Jahres Anästhesie und Intensive- und Notfallmedizin, Schmerztherapie, Krankenhaus Neukölln, Berlin
2003	Drittes Tertial des Praktischen Jahres Allgemein Chirurgie Universitätsklinikum Benjamin Franklin, Berlin

Berufliche Erfahrung

2004 – 2005	Medical Advisor/ Product Manager Internal Medicine Sanofi-Synthelabo, Berlin
seit November 2005	Assistenzarzt am Universitätsklinikum Leipzig Department Innere Medizin, Dermatologie und Neurologie Abteilung Hämatologie, internistische Onkologie, Hämostaseologische Ambulanz Mentor: Prof. Dr. Dr. Dietger Niederwieser
seit April 2008	Research Fellowship an der Ohio State University (OSU), Comprehensive Cancer Center; Columbus, OH; USA Mentoren: Prof. Dr. Clara D. Bloomfield und Prof. Dr. Guido Marcucci

Sonstige Tätigkeit

2000 - 2001	Interdisziplinärer Laborkurs in Zell- und Molekularbiologie, Charité-Universitätsmedizin, Berlin
2002	Kurs und praktisches Arbeiten mit Labor- / Versuchstieren; Zertifizierung durch GV-SOLAS (Society of Laboratory Animal Science), durchgeführt nach den FELASA-Richtlinien (Fed. of European Laboratory Animal Science Associations recommendations on the education and training of persons working with laboratory animals) Charité-Universitätsmedizin, Berlin
2002	Mitarbeit in der Non-Government Organisation “MSG” in Gambia, West-Africa (Malaria und Schüler-Hilfe Gambia)
2004	Seminar und Ausbildung für klinische Prüfungen mit Arzneimitteln in Übereinstimmung mit den ICH/GCP Richtlinien, Sanofi-Synthelabo, Berlin
2005	Praktikum in der Welt Gesundheitsorganisation (World Health Organisation; WHO) im “Roll-back malaria program” in Kairo, Ägypten

Auszeichnungen

2009	ASCO Cancer Foundation Merit Award
2010	AACR-GlaxoSmithKline Outstanding Clinical Scholar Award
2011	EHA-ASH Translational Research Training in Hematology Award
2012	AACR-Aflac, Incorporated Scholar-in-Training Award

Eingeladene Konferenz-Vorträge

2009	"*MicroRNA-181a* expression as a prognosticator in cytogenetically normal acute myeloid leukemia" Jahrestagung der American Society of Clinical Oncology (ASCO)
2009	"Higher *microRNA-181a* expression independently predicts better outcome in cytogenetically normal acute myeloid leukemia" Tagung der Cancer and Leukemia Group B (CALGB); Correlative Science Symposium
2009	"Lower *BAALC* and *ERG* expression levels are favorable independent prognosticators in older cytogenetically normal acute myeloid leukemia" Tagung der Cancer and Leukemia Group B (CALGB)
2009	"Aberrant gene expression of *BAALC* and *ERG* in older de novo cytogenetically normal acute myeloid leukemia: A Cancer and Leukemia Group B Study" Jahrestagung der American Society of Hematology (ASH)
2010	"High *MN1* expression as an independent prognosticator for poor outcome in older cytogenetically normal acute myeloid leukemia patients" Tagung der Cancer and Leukemia Group B (CALGB); Correlative Science Committee Meeting
2010	"Biologic, prognostic and therapeutic implications of *mircoRNA-181a* expression in acute myeloid leukemia"

	Interdisciplinary Research Symposium: Engineering Approaches to Translational Cancer Research; der Ohio State University
2012	“The combination of Bortezomib and Decitabine: a Phase I Trial in patients with acute myeloid leukemia targeting FLT3 expression” Tagung der Alliance for Clinical Trials in Oncology
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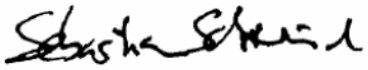
Erklärung über die eigenständige Abfassung der Arbeit

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.

24. July 2012 

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Datum Unterschrift

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