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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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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.

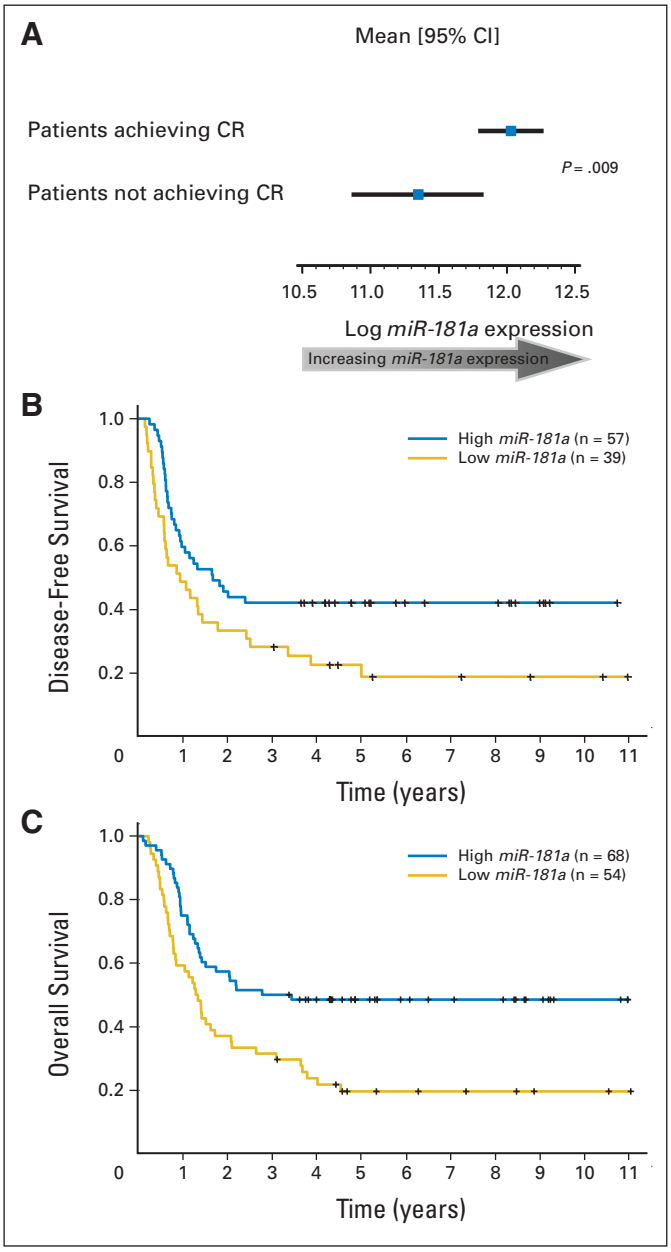

Fig 1. Favorable outcome of patients with *FLT3*-ITD and/or *NPM1*wt and higher *miR-181a* expression levels. (A) *miR-181a* expression in patients who achieved a complete response (CR) versus patients who did not achieve a CR; (B) disease-free and (C) overall survival according to *miR-181a* expression 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

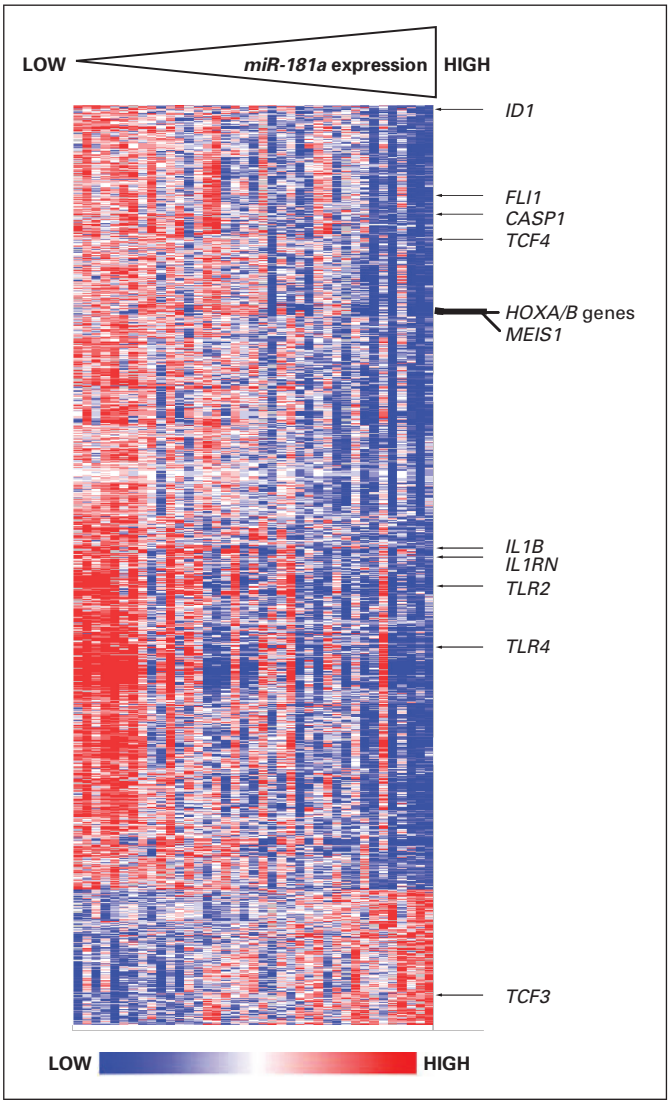

Fig 2. Heat map of the derived gene-expression signature correlated with *miR-181a* expression. Rows represent probe sets and columns represent patients. Probe sets are ordered by hierarchical cluster analysis. Patients are ordered from left to right by increasing *miR-181a* expression. Expression values 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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