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Original Article

MicroRNA-181a-3p as a Diagnostic and Prognostic Biomarker for Acute Myeloid Leukemia

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Abstract. ***Background:*** **Micro (mi) RNAs play an important role in the pathogenesis and development of acute myeloid leukemia (AML), and their abnormal expression may be sufficient to predict the prognosis and outcomes in AML patients. We evaluated the clinical diagnostic value of miRNA-181a-3p in predicting prognosis and outcomes in patients with AML.**
Methods: **A total of 119 newly diagnosed adult patients with AML and 60 healthy controls were recruited. Blood specimens were obtained from all AML patients at diagnosis, and 10 blood specimens were obtained on day 28 after induction chemotherapy. The controls also provided blood samples. Relative gene expression was quantified by PCR and determined using the comparative Ct method. Publicly available clinical data and gene expressions for 188 patients with AML were downloaded from TCGA data portal.**
Results: **Compared with healthy controls, the expression of miRNA-181a-3p was significantly increased in patients with AML. MiR-181a-3p expression could be used to discriminate AML patients from controls, with up-regulated expression correlating with favorable prognosis. Moreover, miRNA-181a-3p expression was significantly decreased in patients who achieved a complete response after induction chemotherapy. The multivariate Cox analysis highlighted the prognostic value of miR-181a-3p for patients with AML. Finally, we found that miR-181a-3p expression was negatively correlated with the expression of the NF-κB essential modulator (NEMO/IKBKG).**
Conclusions: **MiR-181a-3p may be clinically useful as a disease marker for AML, and enhanced the prediction of patient outcomes to chemotherapy.**

Keywords: MicroRNAs; MiR-181a-3p; Biomarkers; Leukemia, Myeloid, Acute; Treatment outcome.

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Introduction. Acute myeloid leukemia (AML) is one of the most common adult leukemias. It is a molecularly heterogeneous disease that is generally associated with poor outcomes. AML patients are classified into distinct risk categories for risk-adjusted chemotherapy, on the basis of cytogenetic and molecular abnormalities.[1] Patients with complex karyotype abnormalities or unfavorable molecular characteristics often have an unfavorable prognosis. However, not all AML patients carry cytogenetic alterations, so new genomic approaches to improve risk stratification are needed.

Micro (mi)RNAs are small, noncoding RNAs that bind their target mRNAs and inhibit the expression of encoded proteins.[2] MiRNAs have critical biological functions, including in hematopoietic cell proliferation, differentiation, and apoptosis, and may also play an essential role in the pathogenesis and development of AML.[3] Several studies have identified that distinctive miRNA profiles are associated with cytogenetic subtypes, mutations, and clinical outcomes of AML.[3-5] For example, decreased miR-196b expression is associated with the absence of FLT3-ITD and *NPM1* mutations, and high miR-196b expression acts as a predictive factor of poor prognosis.[4-5] Therefore, miRNA expression levels may be suitable to predict prognosis and outcomes in AML patients.

The miR-181 family is thought to be involved in a number of biological processes, including transcription, translation, and signaling transduction.[6] In humans, the miR-181 family has four mature homologs (hsa-miR-181a, hsa-miR-181b, hsa-miR-181c and hsa-miR-181d).[7] MiR-181a-3p belongs to miR-181a mature homologs, acts as a negative post-transcriptional regulator of Nuclear Factor kappa-B (NF-κB) signaling pathway by directly targeting NF-κB essential modulator (NEMO/IKBKG) in Human Umbilical Vein Endothelial Cells (HUVECs).[8] Our previous study showed that abnormal expression of miR-181a-3p was associated with human monocytic leukaemia cell line THP-1 cell.[9] However, few studies have focused on the clinical role of miR-181a-3p in AML patients. Therefore, the present study examined the expression of miR-181a-3p in AML patients prior to treatment to evaluate its clinical diagnostic value and predictive role in the prognosis and outcomes of AML patients.

Materials and Methods.

RNA extraction, real-time PCR and miR-181a-3p Expression Analyses. Total RNA was extracted from blood cells using TRIzol (Invitrogen). Reverse transcription and quantitative real-time PCR (RT-qPCR) for gene expression were performed using the SYBR Green PCR Kit (GenePharma, shanghai, PR China). The primer sequences were as follows. MiR-181-a-3p forward (5'-3'): AGAATTACACCATCGACCGTTG; MiRNA-181-a-3p reverse (5'-3'): TATGCTTGTTCTCGTCTCTGTGTC. U6 forward (5'-3'): ATTGGAACGATACAGAGAAGATT; U6 reverse (5'-3'): GGAACGCTTCACGAATTTG. NF-κB forward (5'-3'): CTGAACCAGGGCATACCTGT; NF-κB reverse (5'-3'): GAGAAGTCCATGTCCGCAAT. NEMO/IKBKG forward (5'-3'): TACTGGGCGAAGAGTCTCC; NEMO/IKBKG reverse (5'-3'): AGAATCTGGTTGCTCTGCC.
Analysis of relative gene expression was using $2^{-\triangle\triangle CT}$ method.

TCGA data set. Publicly available clinical, gene expression data for 188 patients with AML were downloaded from TCGA data portal.

Data analysis. We used SPSS (version 24), GraphPad Prism (version 7) software and R language to analyze the data. The unpaired Student's t test (two-tailed) was performed to compare differences in miRNA expression between different groups. Chi-square tests were used to test the association between the expression of miRNAs and clinicopathological characteristics. Cox proportional hazards models were used to analyze the prognostic utility of miRNA expression for disease-free survival (DFS) and overall survival (OS) in AML patients.

Results.

Patient clinical characteristics and treatment. From September 2014 through December 2016, a total of 119 untreated, newly diagnosed adult AML patients [age range 15-83 years; male, 52.1%] in The First Affiliated Hospital of University of Science and Technology of China were recruited , together with 60 heath controls [age range 16-81 years; male, 57%] with no hematologic disease. The patients represented the major French-American-British (FAB) subtypes: 1M0, 9 M1, 30 M2, 20 M3, 16 M4, and 43 M5. Of the 119 AML patients, 20 had PML-RARa rearrangements, 24 had AML1-ETO rearrangements, 30 had other molecular genetic abnormalities, and 45 had normal karyotypes. 20 M3 patients received ARTA plus anthracycline-based induction chemotherapy with or without ATO, and 99 AML patients received traditional 7+3 induction chemotherapy. A total of 81 patients achieved a complete response (CR), 30 did not, and 8 patients died within 30 days of receiving

Table 1. Clinical characteristics of AML patients at diagnosis.

Characteristic	N(%)
Gender	
Male	62 (52.1)
Female	57 (47.9)
Age	
< 60 years	81 (68.1)
≥60 years	38 (31.9)
WBC	
< 100×109/L	113 (95.0)
≥100×109/L	6 (5.0)
FAB	
M0	1 (0.8)
M1	9 (7.6)
M2	30 (25.2)
M3	20 (16.8)
M4	16 (13.4)
M5	43(36.1)
Risk groups	
Low and intermediate risk	73 (61.3)
High risk	46 (38.7)
Molecular abnormalities	
PML/RARa	20 (16.8)
AML1/ETO	24 (20.2)
FLT3-ITD or -TKD	12 (10.1)
CEBPA	5 (4.2)
NPM1	5 (4.2)

Abbreviations: WBC = white blood cell; FAB = French-American-British; PML/RARa = PML-RARa rearrangement; AML1/ETO = AML1-ETO rearrangement; FLT3-ITD or -TKD = FLT3-ITD or TKD rearrangement.

induction chemotherapy. The patients' clinical characteristics were summarized in **Table 1**.

The clinical value of miRNA-181a-3p in the diagnosis of AML. To examine whether miRNA-181a-3p was abnormally expressed in patients with AML, we detected miRNA expression in 60 healthy controls and 119 adult patients with newly diagnosed AML. Compared with healthy controls, the expression of miRNA-181a-3p (P<0.001, **Figure 1 A**) was significantly increased in AML patients, and in the samples of M1, M2, M3, and M4 subtypes **(Figure 1 B)**. Furthermore, we compared the expression of microRNAs in 4 subtypes base on molecular genetic abnormalities. Compared with healthy controls, the expression of miRNA-181a-3p was significantly increased in all four subtypes **(Figure 1 C)**.

To assess the clinical diagnostic value of miR-181a-3p in discriminating AML patients from healthy controls, we performed receiver operating characteristic (ROC) curve analyses. The Area Under Curve (AUC) of miR-181a-3p was (0.654, 95% CI, 0.575 to 0.732, P<0.001, **Figure 1D**). Compared with health controls, miR-181a-3p showed significant difference in the samples of FAB M1, M2, M3 subtypes **(Figure 1E)** and PML/RARa, AML1/ETO subtypes **(Figure 1F)** compared with controls, suggesting that it had value in discriminating patients from controls. The AUC of miR-181a-3p in the samples of different subtypes was showed in **Table 2**.

The expression of miRNA-181a-3p was decreased when patients achieved CR after induction chemotherapy. To examine whether miRNA-181a-3p was decreased in patients who achieved CR after induction chemotherapy, we detected its expression from blood specimens in 10 patients. On day 28 after induction chemotherapy, miRNA-181a-3p expression was significantly decreased in 80% of patients (P<0.001, **Figure 2)**. Moreover, on day 28 after induction chemotherapy, miR-181a-3p was expressed at higher levels compared with healthy controls (P<0.05, **Figure 2A).**

Association of miR181a-3p expression with AML patient outcome. A total of 119 adult patients with newly diagnosed AML were recruited, 8 patients died within 30 days after chemotherapy, finally 111 patients were included for statistical analysis. A total of 81 patients achieved complete remission (CR), whereas 30 patients failed to. To graphically display the association of miR181a-3p expression with CR achievement, we compared expression levels in patients who achieved CR (n=81) with those who failed to achieve CR (n=30) (**Fig 3A**). At the time of diagnosis, miRNA-181a-3p expression level was correlated with the response to induction chemotherapy (P<0.05).

Table 2. The AUC of miR-181a-3p in the samples of different subtypes. P-values calculated by Unpaired student's t test. AUC = The Area Under Curve. 95% CI = 95% confidence interval.

	miR-181a-3p		
	AUC	95%CI	*P*
M1	0.736	0.537-0.935	P<0.05
M2	0.805	0.701-0.908	P<0.0001
M3	0.759	0.612-0.907	P<0.001
M4	0.588	0.408-0.767	P>0.05
M5	0.521	0.405-0.637	P>0.05
AML1/ETO	0.732	0.606-0.859	P<0.001
PML/RARa	0.759	0.612-0.907	P<0.001

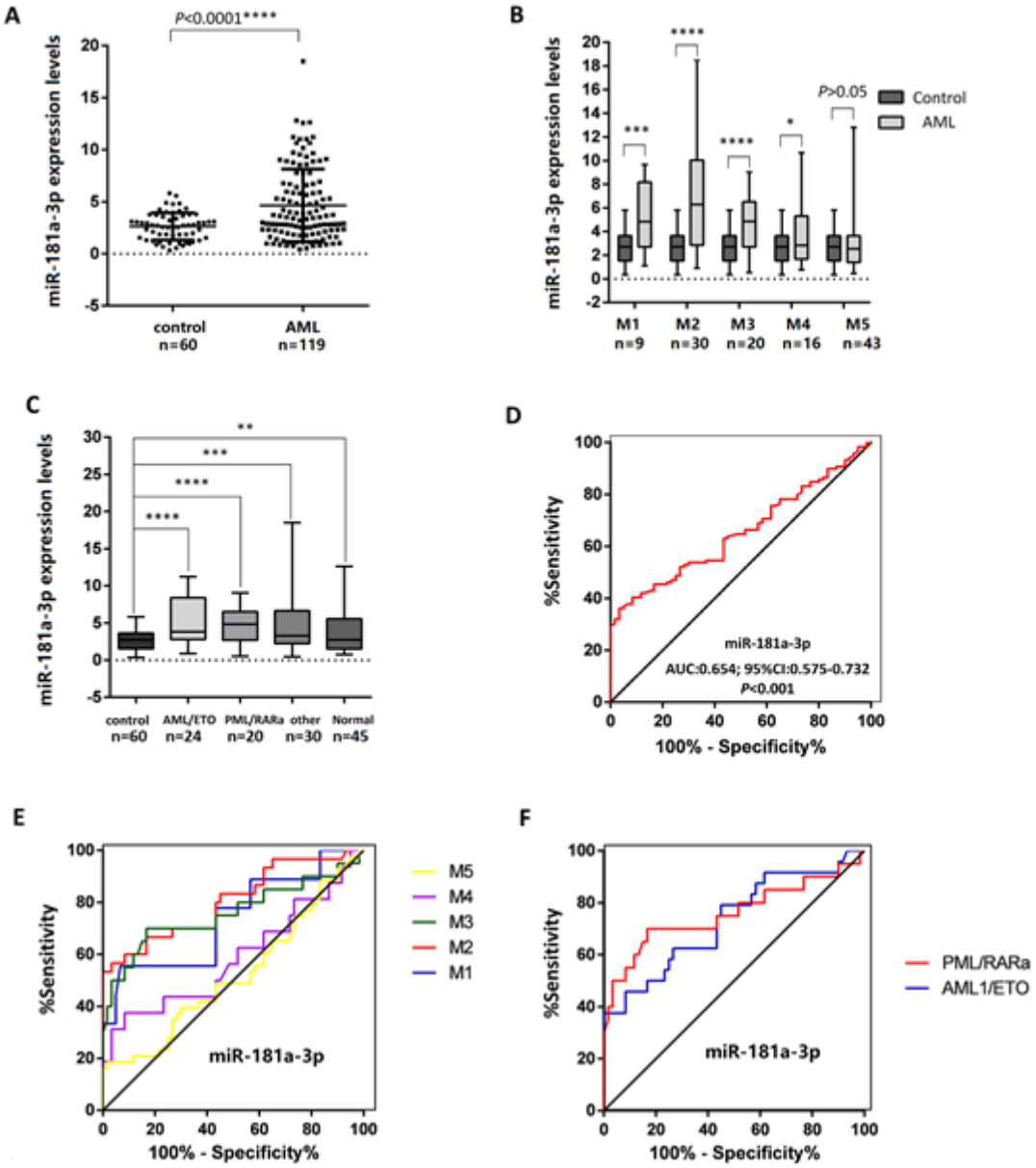

Figure 1. Evaluation of miR-181a-3p expression levels in patients with AML. (**A**) Expression levels of miR-181a-3p in peripheral blood mononuclear cells derived from AML patients and healthy controls. (**B**) Expression levels of miR-181a-3p in AML patients of major FAB subtypes (from M1 to M5 subtypes), respectively. (**C**) Expression levels of miR-181a-3p in subtypes grouped according to molecular genetic abnormalities, respectively. (**D**) AUC of miR-181a-3p comparison between AML and health controls. (**E**) AUC of miR-181a-3p comparison between FAB subtypes of AML and health controls. (**F**) AUC of miR-181a-3p comparison between PML/RARa and AML1/ETO subtypes of AML and health controls. P-values calculated by Unpaired student's t test. * = $P<0.05$, ** = $P<0.01$, *** = $P<0.001$, **** = $P<0.0001$.

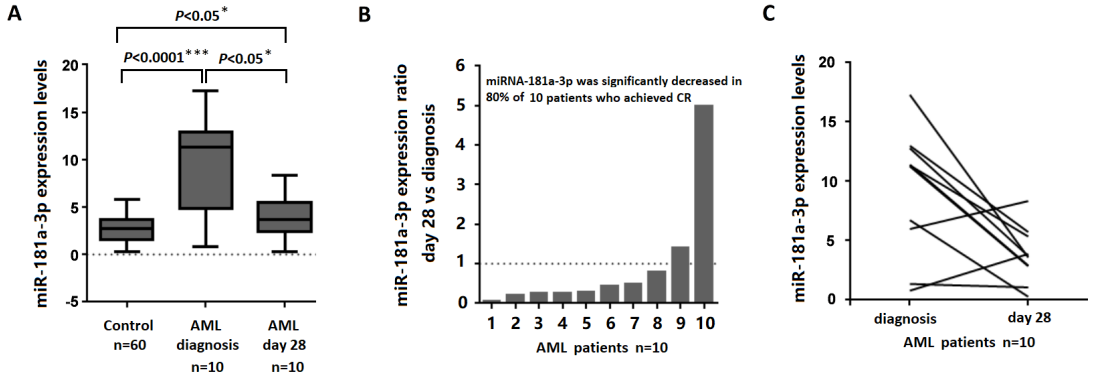

Figure 2. Evaluation of miR-181a-3p expression levels in patients with AML at diagnosis and on day 28 after induction chemotherapy. (**A**) Box plots of miR-181a-3p expression in AML patients at diagnosis and on day 28 after induction chemotherapy, and in healthy controls. P-values calculated by Unpaired student's t test. * = $P<0.05$, *** = $P<0.001$. (**B**) Bar graph of the miR-181a-3p expression level ratio on day 28 after induction chemotherapy compared with disease diagnosis. (**C**) Line graphs of miR-181a-3p expression levels in AML patients at diagnosis and on day 28 after induction chemotherapy.

According to miR-181a-3p expression, patients with AML were dichotomized into high (above median expression levels) and low (below or at median expression levels) groups. Kaplan–Meier survival curves showed that patients with higher miR-181a-3p expression levels at diagnosis presented with a better OS (*P*=0.014, **Fig 3D**) , but not a better DFS (*P*=0.062, **Fig 3C**), than those with lower expression levels. In the TCGA analysis, AML patients with higher miR-181a-3p also presented with a better OS (*P*=0.008, **Fig 3E**).

The Mantel–Cox test and Gehan–Breslow–Wilcoxon test were performed to determine the relationship between miR-181a-3p expression and OS or DFS (**Fig 3B**). The Gehan–Breslow–Wilcoxon test highlighted the prognostic value of increased miR-181a-3p expression at diagnosis both for disease relapse (HR: 1.597; 95% CI: 0.9804-2.559; *P*=0.03) and death (HR: 2.062; 95% CI: 1.160-3.456; *P*=0.02). Thus, a higher miR-181a-3p expression at diagnosis was significantly associated with patient outcome.

Finally, multivariate Cox analysis was performed to determine the relationship between independent prognostic value and OS or DFS. The multivariate Cox analysis was adjusted for patients' age, gender, WBC count and disease risk stratification. Multivariate analysis (Table 3) highlighted the of miR-181a-3p levels on AML diagnosis not for disease relapse (*P*>0.05) but for death (HR: 1.923; 95% CI: 1.035-3.574; *P*<0.05).

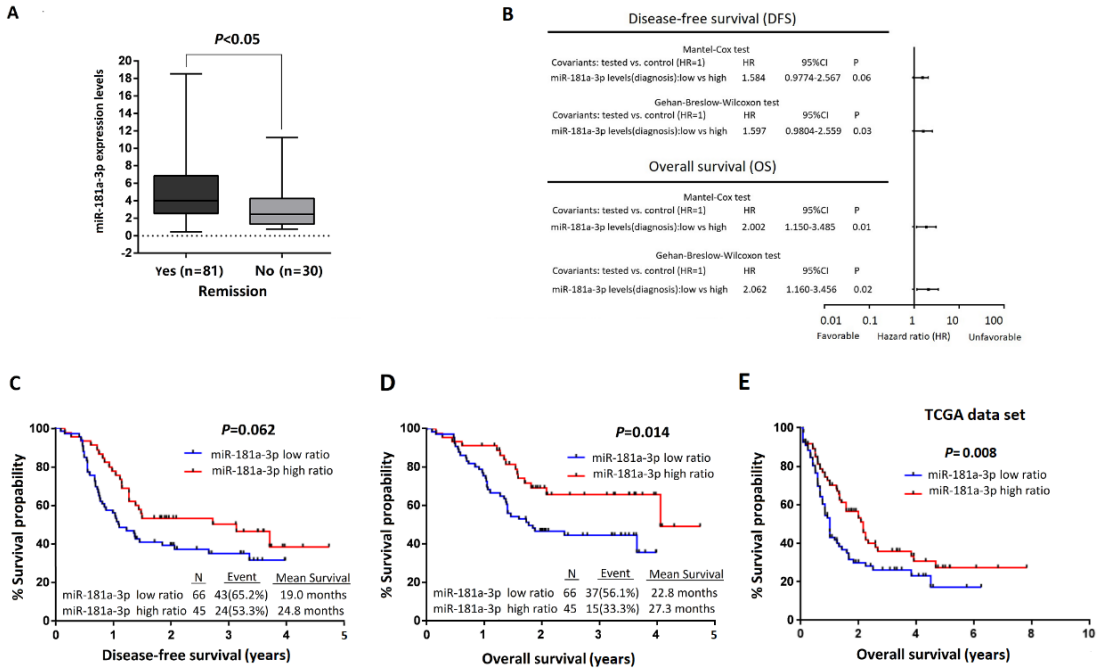

Figure 3. Association of miR-181a-3p expression in AML patients at diagnosis with clinical outcomes. (**A**) miR-181a-3p expression in patients who achieved CR versus those who failed. (**B**) Mantel–Cox test and Gehan–Breslow–Wilcoxon test for patient OS. HR: hazard ratio; 95%CI: 95% confidence interval. (**C**) DFS and (**D**) OS in patients with high and low miR-181a-3p expression. (**E**) OS in TCAG patients with high and low miR-181a-3p expression.

Table 3. Multivariate Cox regression analysis of independent risk factors influencing OS and DFS. P-values calculated by Unpaired student's t test. WBC = white blood cell. 95% CI = 95% confidence interval.

Multivariate Cox regression analysis			
Overall Survival (OS)			
Covariants : tested vs control (HR= 1)	HR	95%CI	*P*
miR-181a-3p levels (diagnosis): low vs high	1.923	1.035-3.574	*P* < 0.05
Age: ≥ 60 years vs <60 years	1.848	1.070-3.192	*P* < 0.05
High risk group vs low/intermediate risk	2.694	1.549-4.687	*P* < 0.0001
Gender: male vs female			*P* =0.204
WBC count: >100000 cells/µl vs <100000 cells/µl			*P* = 0.588
Disease-Free Survival (DFS)			
Covariants: tested vs control (HR=1)	HR	95%CI	*P*
miR-181a-3p levels (diagnosis): low vs high			*P* = 0.113
Age: ≥ 60 years vs <60 years			*P* = 0.098
High risk group vs low/intermediate risk	1.947	1.197-3.169	*P* < 0.001
Gender: male vs female			*P* = 0.970
WBC count: >100000 cells/µl vs <100000 cells/µ			*P* = 0.709

Assessment of the relationship between miR-181a-3p ectopic expression and IKBKG and NF-κB family. Since miR-181a-3p blocks the NF-κB signaling pathway by targeting NEMO/IKBKG in Human Umbilical Vein Endothelial Cells (HUVECs),[8] whether IKBKG and NF-κB expressions are affected by miR-181a-3p in AML cells needed to be determined. NF-κB family contains NF-κB1, NF-κB2, RelA, RelB and Rel. Firstly, we investigated the relation of miR-181a-3p ectopic expression with NEMO/IKBKG and NF-κB family in a set of primary TCGA AML patients. NEMO/IKBKG expression was positively correlated with expression of NF-κB family (NF-κB1, NF-κB2, RelA, RelB) (Person correlation= 0.377, P<0.01; Person correlation= 0.598, P<0.01; Person correlation= 0.557, P<0.01; Person correlation= 0.524, P<0.01). MiR-181a-3p expression was negatively correlated with expression of NF-κB family (NF-κB1, NF-κB2, RelA, RelB) (Person correlation= -0.209, P<0.05; Person correlation= -0.555, P<0.01, Person correlation= -0.19, P<0.05, Person correlation= -0.309, P<0.01) and NEMO/IKBKG (Person correlation= -0.313, P<0.01). Then we investigated the relation of miR-181a-3p expression with NEMO/IKBKG and NF-κB in 59 AML patients, and found that miR-181a-3p expression was negatively correlated with the expression of NF-κB (Person correlation= -0.2795, P=0.0321) and NEMO/IKBKG (Person correlation= -0.2613, P=0.0456). The linear correlation analysis in AML samples were showed in **Table 4**.

Discussion. In our study, we evaluated the clinical diagnostic value and role of miRNA-181a-3p in predicting prognosis and outcomes. We showed that miRNA-181a-3p expression was significantly increased in AML patients compared with controls, while ROC analyses verified the ability of miR-181a-3p to distinguish AML from control blood samples. The follow-up of 10 patients revealed a significant down-regulation of miR-181a-3p expression on day 28 of induction chemotherapy, while higher miR-181a-3p expression at diagnosis was correlated with favorable prognosis. Finally, we found that miR-181a-3p expression was negatively correlated with the expression of NEMO/IKBKG. Our findings should be confirmed using a larger sample size.

In recent years, novel high-throughput sequencing techniques have significantly advanced our understanding of the molecular pathogenesis of AML.[10-11] Several recurrent mutations in genes encoding epigenetic modifiers have been identified that affect not only disease phenotype but also a response to therapy[12-13]. MiRNAs play an important role in the pathogenesis and development of AML, and their abnormal expression is associated with specific cytogenetic subsets or mutations of AML, suggesting that they could be used as independent biomarkers for determining the outcomes of AML patients.[4,14-15]

MiR-181a belongs to the miR-181 family, its role in tumors is still controversial, and it may function as a tumor promoter or suppressor depending on tumor type.[16] In hematologic malignancies, miR-181a functions as a tumor suppressor in cellular division and differentiation. AML patients with higher miR-181 expression at diagnosis have a better prognosis than those with lower miR-181 expression, miR-181 may be a diagnostic biomarker and predictor of prognosis in AML patients.[17-19]

Precursor miR-181a can be processed into two mature strands: miR-181a-3p and miR-181a-5p. MiR-181a-3p is highly expressed in RPMI8226 cell-derived extracellular vesicles (R-EVs) and regulates cell proliferation.[20] Mir-181a-3p blocks the NF-κB signaling pathway by targeting NEMO/IKBKG in Human Umbilical Vein Endothelial Cells (HUVECs), and miR-181a-3p mimics treatment prevents myeloid cell recruitment and decreased the expression of TNF-α in apoE $^{-/-}$ mice.[8] NF-κB is an important transcription factor, which plays a crucial cancer-promoting role in Acute myeloid leukemia (AML).[21-22] It has been known that chromosomal translocations or gene mutations leading to the increase in NF-κB activity**.** NEMO/IKBKG acts as a crucial antiapoptotic transcription factor, which is crucial for the activation of NF-κB.[23] NF-κB family contains RelA, RelB, NF-κB1, NF-κB2 and Rel. We found that miR-181a-3p

Table 4. The linear correlation analysis in AML samples. P-values calculated by Pearson's Correlation. 95% CI = 95% confidence interval.

DATA from TCGA				
	vs NEMO/IKBKG		vs miR181a-3p	
	Person correlation	p-value	Person correlation	p-value
NF-κB1	0.377	2.723e-06	-0.209	1.12e-02
NF-κB2	0.598	1.653e-15	-0.555	3.66e-13
RelA	0.557	2.844e-13	-0.19	2.184e-02
RelB	0.524	1.15e-11	-0.309	1.478e-04
Rel	-0.013	8.799e-01	-0.121	1.45e-01
NEMO/IKBKG			-0.313	1.168e-04
59 patients with primary AML				
	vs NEMO/IKBKG		vs miR181a-3p	
	Person correlation	p-value	Person correlation	p-value
NF-κB	0.2601	0.0466	-0.2795	0.0321
NEMO/IKBKG			-0.2613	0.0456

expression was negatively correlated with the expression of NF-κB family (NF-κB1, NF-κB2, RelA, RelB) and NEMO/IKBKG in TCGA samples. In our study, miR-181a-3p expression was negatively correlated with the expression of NF-κB and NEMO/IKBKG in 59 AML patients. Maybe mir-181a-3p affects AML cell proliferation and apoptosis by targeting NEMO/IKBKG. We should make more efforts to test this hypothesis.

In summary, we reported a clinical role for miR-181a-3p in AML patients for the first time. MiRNA-181a-3p expression was shown to have value in discriminating AML patients from healthy controls, and to correlate with the response to induction chemotherapy. Patients with higher miR-181a-3p expression levels at diagnosis demonstrated an improved OS. Therefore, the miR-181a-3p expression may be an independent prognostic biomarker for AML patient outcomes.

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