Clin. Lab. 2020;66:XXX-XXX

ORIGINAL ARTICLE

The Expression and Clinical Significance of MicroRNA-409-3p in Acute Myeloid Leukemia

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SUMMARY

Background: **MicroRNA-409-3p, is down-regulated in a variety of malignant diseases. However, the expression level and clinical value of microRNA-409-3p in acute myeloid leukemia has not yet been systematically studied.**
Methods: **We collected 88 bone marrow samples derived from 73 patients with acute myeloid leukemia and 15 healthy controls. Then we evaluated the expression of microRNA-409-3p by quantitative real-time PCR.**
Results: **The results revealed that compared with the healthy controls, microRNA-409-3p expression in a newly diagnosed group was significantly lower ($p < 0.001$). In addition, the microRNA-409-3p expression in the complete remission group was strikingly higher compared to that of the newly diagnosed group ($p < 0.001$). There was a correlation between microRNA-409-3p expression and white blood cells ($p = 0.021$). Most importantly, the microRNA-409-3p low expression group indicated a shorter event-free survival compared with microRNA-409-3p high expression group by using Kaplan-Meier analysis ($p < 0.0438$).**
Conclusions: **The microRNA-409-3p expression level could be a novel potential biomarker for acute myeloid leukemia diagnosis and prognosis, providing a new therapeutic strategy for acute myeloid leukemia treatment.**
(Clin. Lab. 2020;66:xx-xx. DOI: 10.7754/Clin.Lab.2019.191107)

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KEY WORDS

acute myeloid leukemia, microRNA-409-3p, qPCR, biomarker, prognosis

LIST OF ABBREVIATIONS

AML - acute myeloid leukemia
AUC - Area under curve
ceRNAs - competing endogenous RNAs
circRNAs - circular RNAs
CR - complete remission
EFS - event-free survival
HB - haemoglobin
lncRNAs - long non-coding RNAs
MiRNAs - microRNAs
MicroRNA-409-3p - miR-409-3p
PLT - platelet
ROC - receiver operating characteristic curve
WBC - white blood cell count

INTRODUCTION

Manuscript accepted December 1, 2019

Acute myeloid leukemia (AML) is a highly heterogeneous disease, characterized by myeloid precursor cells failing to undergo final differentiation [1]. It is the most universal type of leukemia, with an incidence of 3 - 4 per 100,000 people per year. With the advancement of medical technology, the complete remission rate of patients with AML is rising, but recurrence is still the major threat to long-term survival. There is an urgent need to identify novel biomarkers, which contribute to the early diagnosis of AML patients and to find a new treatment strategy, further improving the survival rate of AML patients [2].

MicroRNAs (miRNAs) are a class of endogenous single-stranded non-coding small RNA including approximately 22 nucleotides, which negatively regulate the expression of target genes at the transcriptional or post-transcriptional level by binding to the 3´-UTR of target mRNAs [3]. Increasingly, miRNAs have been widely studied after two miRNAs, lin-4 and let-7, were found for the first time, and they play a vital role in *Caenorhabditis elegans* [3,4]. The first study about the deregulation of miRNA in cancer is that both miR-15 and miR-16 gene, located at 13q14, are usually missing or down-regulated in chronic lymphoid leukemia (CLL) [5]. As far as the current research on miRNAs is concerned, the miRNAs, as tumor oncogenes or suppressors, are involved in various biological processes, including proliferation, differentiation, apoptosis [6], and hematopoiesis [7].

Dysregulated miRNA expressions could influence normal hematopoiesis, resulting in the occurrence and development hematological malignancies. Recently, emerging studies reported that numerous miRNAs play significant roles in hematological malignancies including AML. For example, in AML cells, miR-143 overexpression impeded cell growth, partially promoted differentiation, and induced apoptosis by directly targeting ERK5 [8]. In another study, Wang et al. [9] found that the expression of miR-340 was downregulated in bone marrow samples from patients with AML, which was correlated with poor outcome of patients. Recently, Fu et al. [10] found that in the chemotherapy group, AML patients with miR-338 overexpression have a poorer prognosis compared with those with low miR-338 expression. Moreover, miR-15b, as a tumor suppressor, promoted the differentiation of acute promyelocytic leukemia (APL) cells and attenuated cell proliferation via targeting cyclinE1 (CCNE1) [11].

MicroRNA-409-3p (miR-409-3p) is located at chromosome 14q32.31 which was first found in embryonic stem cells [12]. MiR-409-3p, has been found to play vital roles in a variety of cancers, such as: bladder cancer [13], breast cancer [14], prostate cancer [15], lung cancer [16], colorectal cancer [17], gastric cancer [18], etc. The functions of miR-409-3p are involved in cellular proliferation, differentiation, invasion, and metastasis. In 2014, it was found that the expression of miR-409-3p was reduced in intermediate risk-AML (IR-AML) [19]. However, in AML, the diagnostic and prognostic significance of miR-409-3p is not clearly understand yet.

In this paper, we aimed to detect the expression of miR-409-3p in the bone marrow of patients with AML and analyze the clinical significance of miR-409-3p in AML to provide a novel biomarker of AML diagnosis and prognosis.

MATERIALS AND METHODS

Patients

We obtained 73 bone marrow (BM) samples of AML patients including 33 newly diagnosed, 29 complete remission (CR), and 11 relapse, as well as 15 bone marrow samples of healthy controls from The Affiliated Hospital of Qingdao University from April 2018 to June 2019. According to French-American-British (FAB) classification [20], a group of 33 newly diagnosed AML patients where 5 had AML M1, 7 had M2, 7 had M3, 6 had M4, and 8 had M5. All patients were diagnosed by bone marrow routine, immunophenotyping, fusion gene, and chromosome analysis. The diagnosis was in accordance with the 2016 WHO classification [21]. The patients' treatment plans are as follows: AML-M1, M2, M4, and M5 all use IA (Idabisin, cytarabine), DA (daunorubicin, cytarabine), and HA (Homoharringtonine, cytarabine) treatment programs; AML-M3 adopts all-trans retinoic acid combined with daunorubicin treatment. All samples were collected with appropriate informed consent from the patients and approved by the Ethics Committee of the Affiliated Hospital of Qingdao University. A summary of patients' characteristics is presented in Table 1.

RNA isolation and reverse transcription

By using standard Ficoll-Hypaque density centrifugation, the mononuclear cells were isolated from BM samples and then put into EP tubes. According to the manufacturer's protocol, total RNA was exacted from isolated bone marrow mononuclear cells (BMNCs) using Trizol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). For the determination of miR-409-3p expression, reverse transcription was performed using M-MLV reverse transcriptase (Invitrogen). MiR-409-3p-RT primer: GTCGTATCCAGTGCAGGGTCCGAGGTATTCG CACTGGATACGACAGGGGT
(Beijing Genomics institution).

Real-time quantitative polymerase chain reaction

qRT-PCR was performed using the SYBR-Green method (ABI) with U6 small nuclear RNA as an internal control. qRT-PCR was performed using an ABI 7500 real-time PCR system (Applied Biosystems: Thermo Fisher Scientific, Inc.) with incubation at 95°C for 30 seconds (predenaturation) followed by 40 cycles of 95°C for 30 seconds and 60°C for 30 seconds. The primers were as follows: U6, forward:

5'-CGCTTCGGCAGCACATATACTA-3' and reverse: 5'-GGAACGCTTCACGAATTTGC-3'; miR-409-3p, forward: 5'-CCGAATGTTGCTCGGTGAAC-3' and reverse: 5'-CCAGTGCAGGGTCCGAGGT-3' (Beijing Genomics institution). U6 small nuclear RNA was used to normalize the relative gene expression. We used the $2^{-\Delta\Delta Ct}$ method to compute the relative gene expression.

Statistical analysis

Statistical analyses were performed using SPSS 21 (IBM, Armonk, NY, USA). Data was plotted using GraphPad Prism 7 software. Data are presented as median and 25th and 75th interquartile. Mann-Whitney's *U* test was used to evaluate the difference between the miR-409-3p expression in the newly diagnosed group and the healthy control group. Comparison of differences between multiple groups was done using the Kruskal-Wallis *H* test. Differences in expression of miR-409-3p before and after treatment were analyzed by Wilcoxon Matched-Pairs Signed-Ranks Test. Spearman's test was used to detect the correlation between miR-409-3p expression and clinicopathological parameters. Receiver operating characteristic (ROC) curve analysis was applied for assessing the value of miR-409-3p expression in discriminating patients with AML from control cohorts. The Kaplan-Meier method was used to plot the survival curves, and differences were compared using the log-rank test. In all cases, $p \leq 0.05$ was regarded as significant (*), ≤ 0.01 (**) and ≤ 0.001 (***) was considered as highly significant.

RESULTS

The expression of miR-409-3p was down-regulated in AML patients

We used qRT-PCR technology to detect miR-409-3p expressions in BM derived from 73 patients with AML and 15 healthy volunteers. We found that the expression level of miR-409-3p in BM of newly diagnosed patients was significantly downregulated compared with that of healthy individuals ($p < 0.001$, Figure 1A). There was no significant difference in the expression of miR-409-3p among M1 - M5 groups, and no significant differences between patients with abnormal karyotype in the newly diagnosed group and that with normal karyotype. The expression of miR-409-3p was different at the different stages of disease (newly diagnosed, CR, relapse). The expression of miR-409-3p in newly diagnosed patients and relapsed patients were markedly lower than that of the healthy controls (all $p < 0.001$, Figure 1B). In the CR phase, miR-409-3p expression was higher than newly diagnosed stage ($p < 0.001$, Figure 1B), and returned to newly diagnosed level when in the relapse phase. Comparing the miR-409-3p expression before and after treatment, we found that for the same patient, the miR-409-3p expression in BM specimens was upregulated after treatment ($p < 0.05$, Figure 1C). These results indicate that miR-409-3p may play a vital role in the progression of AML and may be valuable in the evaluation of therapeutic effects.

The relationship between clinical characteristics and miR-409-3p expression in primary AML patients

We used Spearman's analysis to analyze the correlation between miR-409-3p expression and clinical parameters of newly diagnosed AML patients. We used the median expression level of miR-409-3p (median = 0.0235) as the cutoff point to define the high expression level or low expression level, due to the non-normal distributed data. A significant difference was found between miR-409-3p and WBC ($p = 0.021$, Table 2). However, there was no correlation between miR-409-3p expression and clinical parameters including bone marrow blasts% ($p = 0.399$), PLT ($p = 0.902$), and HB ($p = 0.916$) (Table 2).

The clinical significance of miR-409-3p in BM from AML patients

Using ROC curve analysis, we determined that the diagnostic performance of miR-409-3p in filtering AML patients from healthy individuals had an AUC value of 0.931 (95% CI = 0.8618 ~ 1.000, $p < 0.0001$, Figure 2A). At the cutoff value of 0.812, the specificity and sensitivity were 87.9% and 93.3%, respectively. So, miR-409-3p may be a potential biomarker for discriminating between AML and controls.

A total of 33 newly diagnosed patients with AML received chemotherapy with available follow-up data. Based on the median value of miR-409-3p expression (median = 0.0235) in the newly diagnosed group, we divided the primary AML group into two groups: high miR-409-3p expression group (n = 16) and low miR-409-3p expression group (n = 17). Furthermore, by Kaplan-Meier analyses, we found patients with high miR-409-3p expression have longer event-free survival (EFS) compared with the low miR-409-3p expressing patients ($p < 0.0438$, Figure 2B). High miR-409-3p expression is a prognostic factor for favorable outcome in AML patients.

Predicting target genes

To understand how miR-409-3p functioned, we utilized bioinformatic analysis to predict the target genes directly regulated by miR-409-3p. Three genes, cyclin G2 (CCNG2), cAMP responsive element binding protein 1 (CREB1), and a member RAS oncogene family (RAB-10) were initially filtered by overlapping the prediction results of miRNA recognition elements using miRbase, TargetScan, miRanda, and miRDB. In future studies, we will further strengthen our research by doing confirmatory assays (luciferase reporter assay, qRT-PCR, and western blot, etc.), as well as greater sample size in prospective studies.

Table 1. Clinical characteristics of patients with acute myeloid leukemia.

Variables	AML group
	n = 73, n (%)
Age	
Median	49
Range	14 ~ 73
Gender	
Male	34 (46.6)
Female	39 (53.4)
WBC, x 10^9/L	
> 10	25 (34.2)
≤ 10	48 (65.8)
Hemoglobin, g/L	
> 60	71 (97.3)
≤ 60	2 (2.7)
Platelets, x 10^{12}/L	
> 100	33 (45.2)
≤ 100	40 (54.8)
FAB subtypes	
M1	6 (8.2)
M2	18 (24.7)
M3	16 (21.9)
M4	9 (12.3)
M5	18 (24.7)
Karyotypes	
Normal	48 (65.7)
t(8;21)	2 (2.7)
t(15;17)	7 (9.6)
t(9;22)	
+8	3 (4.1)
Complex	4 (5.5)
Others	8 (11.0)
No data	1 (1.4)
BM blasts, %	
> 50	25 (34.2)
≤ 50	48 (65.8)
Clinical response	
De novo	33 (45.2)
Remission	29 (39.7)
Relapsed	11 (15.1)
Complete remission	
Yes	29 (39.7)
No	44 (60.2)

Abbreviations: WBC - white blood cell count, FAB - French-American-British, BM - bone marrow.
The classification was done according to the French-American-British system [20]. M1 - acute myeloblastic leukemia with minimal maturation, M2 - acute myeloblastic leukemia with maturation, M3 - acute promyelocytic leukemia (APL), M4 - acute myelomonocytic leukemia, M5 - acute monocytic leukemia.

Table 2. The association between miR-409-3p expression and clinical features of the newly diagnosed AML patients.

Clinical parameters	*r*	p-value
Blast (%)	-0.152	0.399
WBC (x 10^9/L)	-0.401	0.021 *
HB (g/L)	-0.019	0.916
PLT (x 10^9/L)	-0.022	0.902

Abbreviations: PLT - platelet, HB - haemoglobin, WBC - white blood cell count.
*** - Significant p < 0.05 (Spearman's correlation).**

DISCUSSION

In the current study, we revealed that the expression of miR-409-3p in the BM specimens derived from newly diagnosed AML patients was markedly down-regulated compared to that of healthy controls. Moreover, we found that at different stages of the disease, the expression of miR-409-3p has significant differences. The expression of miR-409-3p in AML patients at the initial diagnosis is significantly reduced, after complete remission is evidently increased, however, it is significantly reduced again after relapse. By ROC curve analysis, it was revealed that miR-409-3p could clearly screen AML patients from controls. We concluded that the patients in the low miR-409-3p group had a shorter EFS than those in the high miR-409-3p group according to the K-M survival curve. In addition, excepting WBC, there was no notable correlation between miR-409-3p expression and clinical characteristics including bone marrow Blast%, PLT, and HB. Collectively, these results showed that miR-409-3p may act as a potential biomarker for AML diagnosis and prognosis.

MiR-409-3p, plays a vital role in a variety of diseases whose low expression accelerates tumor generation. As early as 2016, Cao et al. [22] revealed that in breast cancer, miR-409-3p expression was downregulated, which was correlated with poorer prognosis of patients. Recently, the expression of miR-409-3p in osteosarcoma (OS) tissues and cell lines was diminished, which was associated with clinical stage and distant metastasis of patients. This miRNA plays a tumor suppressor role in OS by targeting zinc-finger E-box-binding homeobox-1 (ZEB1) [23]. In accordance with the previous studies, we showed that miR-409-3p expression was diminished in the BM of AML patients, suggesting its strong tumor suppressive function in AML.

The miRNAs, at the center of ceRNA interplay, play vital roles in the progression of AML. CeRNA networks associate the performance of protein-coding mRNAs with that of noncoding RNAs such as miRNAs, long non-coding RNAs (lncRNAs), pseudogenic RNAs, and circular RNAs (circRNAs) [24]. Recently, several studies have shown that in AML, circRNAs [25] and lncRNAs [26,27] exert their effects by directly targeting

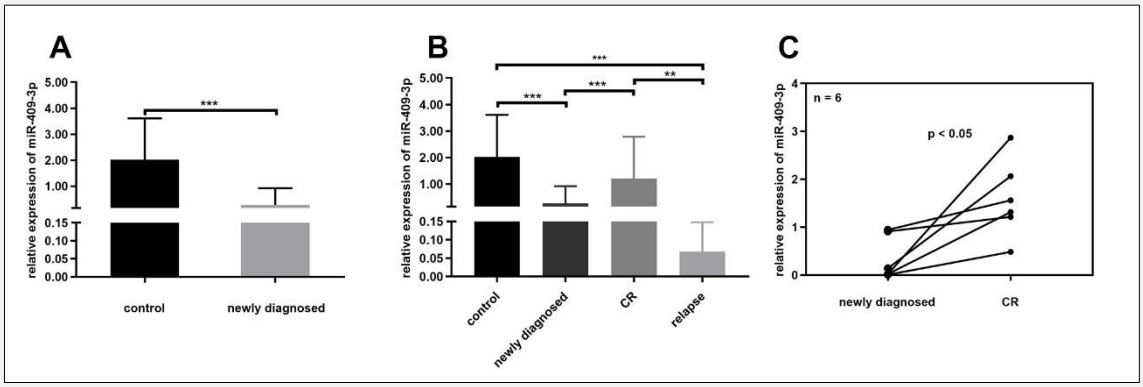

Figure 1. A. The expression of miR-409-3p was found to be significantly down-regulated in the BM of acute myeloid leukemia newly diagnosed patients compared to that of healthy controls. B. Comparison of the expression levels of miR-409-3p in the different stages (newly diagnosed group/remission group/relapse group). C. The dynamic change of miR-409-3p in 6 paired acute myeloid leukemia patients of different stages.

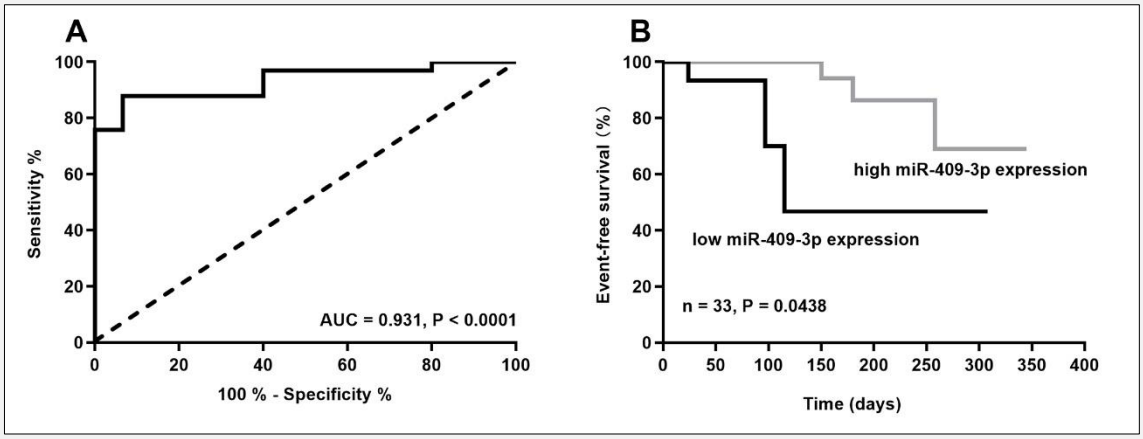

Figure 2. A. ROC curve analysis revealed that miR-409-3p was a potential biomarker for screening AML patients from healthy controls. B. Kaplan-Meier survival curve showing patients with low miR-409-3p expression had shorter EFS in newly diagnosed group (p = 0.0438).

miRNAs [28,29]. For instance, the circRNA-DLEU2 expression was greatly elevated which enforced AML tumor formation via inhibiting miR-496 and enhancing PRKACB expression *in vitro* and *vivo* [30]. Lei et al. [31] revealed that the circ_0009910 expression was strikingly increased in the BM specimen of AML patients, which enforced the proliferation of AML cells and repressed apoptosis by targeting miR-20a-5p and was closely correlated with the poorer prognosis of AML patients. The lncRNA SBF2-AS1 expression was highly elevated in AML cell lines. SBF2-AS1 acts as an oncogenic lncRNA enforcing the progression of AML by negatively regulating the miR-188-5p/ZFP91 axis and related to poor overall survival (OS) of patients [32]. Collectively, we will further explore the lncRNAs and circRNAs associated with miR-409-3p in AML,

and the potential mechanisms among them by utilizing the bioinformatic prediction tool.

Moreover, we will further investigate the miR-409-3p expression level in serum or plasma of acute leukemia patients, as well as the value of miR-409-3p in identifying AML patients and healthy controls. Studies indicated that the circulating miRNAs have the ability to reflect physiological and pathological states, can stably exist in patient samples, and are a novel class of biomarkers [33]. For example, in circulating exosomes derived from intermediate-risk acute myeloid leukemia patients, the expression of miR-125b was strikingly up-regulated, which predicted the higher risks of relapse and overall death [34]. In serum from pediatric AML patients, miR-192 underexpression was closely associated with poor outcome [35]. In AML and CN-AML patient sera, miR-34a expression was significantly diminished, which was closely correlated with unfavorable clinical features and poor outcome of patients [36]. Therefore, circulating miR-409-3p may also be correlated with AML diagnosis and prognosis.

Studies showed that some miRNA expression levels have significant differences in the FAB classification of AML (M0 ~ M7) as well as cytogenetics of AML. For example, in the BM and serum of AML patients, the expression level of miR-210 was evidently up-regulated. There was a marked difference found between serum miR-210 and FAB classification and cytogenetics [37]. However, due to the small sample size, we may not be able to detect some possible significant differences, so we will collect more samples and further study the mechanism of action of miR-409-3p in AML. According to the abnormal expressions of miRNAs in AML, correspondingly, over-expression or knockdown of miRNA expression levels will delay the development of AML. In the BM and serum from pediatric AML patients, miR-342 expression was markedly impaired. However, the expression of miR-342 enforced by transfecting the miR-342 mimic, attenuated the proliferation and G1/S transition of AML cell lines by targeting Naa-10p [38]. As for the miR-409-3p in AML, we will construct the miR-409-3p overexpression vector and transform into the animal model of AML to further verify the experimental results. It is still challenging to deliver a miRNA mimic, small interfering RNAs (siRNA), and antisense oligonucleotides into patients. Future technical improvements will provide more chances for AML treatment [39].

CONCLUSION

We found that the expression of miR-409-3p was notably down-regulated in bone marrow of acute leukemia patients, which was associated with poor prognosis in AML. Taken together, miR-409-3p might serve as a novel biomarker in acute leukemia progression and provide a potential therapeutic target for AML treatment.

Ethics Approval and Consent to Participate:
All samples were collected with appropriate informed consent from the patients and approved by the Ethics Committee of the Affiliated Hospital of Qingdao University.

Authors' Contributions:
MYL performed the experiments, analyzed the data, and drafted this manuscript. HZG provided direction and guidance throughout the preparation of this manuscript. HZG, XLC reviewed and made significant revisions to the manuscript. All authors approved this manuscript.

Acknowledgment:
None.

Source of Funding:
This work was supported by the Natural Science Foundation of Shandong Province (ZR2017MH042).

Declaration of Interest:
The authors declare that they have no competing interests.

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