Department of Hematology[1], Qinghai Province People's Hospital; Qinghai University[2], Xining City, China

Decreased miR-144 expression as a non-invasive biomarker for acute myeloid leukemia patients

Qiangqiang Zhao[1], Jianping Li[1], Shaobin Chen[1], Kuo Shen[1], Guo Ai[1], Xin Dai[1], Bangyou Xie[1], Yannan Shi[1], Shuxia Jiang[2], Jianming Feng[1], Wenqian Li[1,*]

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**Corresponding author: Dr. Wenqian Li, Department of Hematology, Qinghai Province People's Hospital, No. 2, Republican Road East District, Xining city, Qinghai Province, P.R. China, 810007*
m18254831270_2@163.com

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MicroRNAs are found to be stable in blood and they demonstrated tissue specific expression patterns. Thus, they may be used as potential non-invasive biomarkers of specific cancers. In the current study, we mainly focused on miR-144, which has never been studied in acute myeloid leukemia (AML). The expression of miR-144 was explored in the bone marrow and peripheral blood of AML patients and healthy control. The correlation between peripheral blood miR-144 level and key clinical parameters, including overall survival and prognostic value, was further explored. We showed that miR-144 was markedly reduced in both the peripheral blood and bone marrow of AML patients compared with healthy controls. Further study revealed that there is a significant correlation between peripheral blood miR-144 level and FAB classification (p=0.0023) and cytogenetics (p=0.001). More importantly, a lower expression of peripheral blood miR-144 level was found to be positively correlated with poorer overall survival rate. In summary, peripheral blood miR-144 may be utilized as a potential novel non-invasive biomarker for AML screening.

1. Introduction

Acute myeloid leukemia (AML) is a malignant disorder, which is characterized by the maturation arrest and rapid proliferation of immature precursors (Chen et al. 2010). Due to large genetic abnormalities, the clinicopathological features and prognosis of AML patients are very variable. It is reported that genetic mutations, including FLT3, NPM1, CEBPA, KIT, MLL, WT1, NRAS, and KRAS genes, can be used as the prognosis marker for AML patients (Verhaak and Valk 2010; Khasawneh and Abdel-Wahab 2014). In the past decades, the patients are largely treated through chemotherapy-based regimen and allogeneic stem cell transplantation (Foran 2010). However, due to the relapse, the overall 5-year survival rate is still low, especially considering the elderly (Estey 2013). It is therefore urgent and necessary to identify novel biomarkers for the prediction of AML patients, thereby decreasing disease risk and improving the treatment method.

MicroRNAs (miRNAs) are small, noncoding RNA molecules, which negatively modulate target gene expression through incomplete base pairing mechanism (Elkayam et al. 2012). In the past years, miRNAs are widely reported to be involved in tumorigenesis. And numerous studies have found the aberrant expression of miRNAs in patients with AML. For instance, miR-29a was identified to be decreased in AML patients compared with healthy control (Zhu et al. 2013). Reduced miR-29a expression was correlated with the poor prognosis of pediatric AML patients (Zhu et al. 2013), and miR-378 was demonstrated to be increased in AML patients and was negatively associated with the prognosis (Qian et al. 2013). Furthermore, decreased miR-124-1 levels were shown to correlate with positive AML prognosis (Chen et al. 2014).

Recently, miRNAs were found to be stable in blood and demonstrated tissue specific expression patterns (Zhi et al. 2013). Thus, they may be used as potential non-invasive biomarkers of specific cancers. In the current study, we mainly focused on miR-144, which is widely explored in different tumors. For instance, miR-144 was found to repress renal cancer cell proliferation mainly by binding the 3'untranslated region (3'UTR) of mTOR (Xiang, Cui et al. 2016). And in laryngeal squamous cell carcinoma, miR-144 was shown to inhibit cell growth and metastasis by suppressing IRS1 (Wu et al. 2016). However, the specific role of miR-144 has never been explored in AML. Therefore, the present study aims to evaluate the expression of miR-144 in the peripheral whole blood of the AML patients and in healthy control, thereby investigating the potential of blood miR-144 as a non-invasive AML diagnostic biomarker.

2. Investigations and results

2.1. Decreased miR-144 in the peripheral blood and bone marrow of AML patients

Firstly, we explored the expression of miR-144 in the peripheral blood. The expression of miR-144 was significantly reduced in the peripheral blood of AML patients compared with that of healthy individuals (P<0.001) (Fig. 1A). The relative expression of miR-144 was 0.21±0.12 for AML patients, and 1±0.48 for healthy controls. Furthermore, the expression of miR-144 was found to be reduced in the bone marrow of AML patients (0.36±0.012) compared to that of healthy individuals (1±0.38) (P<0.01) (Fig. 1B).

2.2. Correlation between peripheral blood miR-144 expression with AML clinical parameters

After determining the average expression of miR-144 in AML patients and healthy control, the median expression of peripheral blood miR-144 (0.21-fold) was employed as the cut-off points to define high or low expression level. According to the Table, no correlation between miR-144 and gender, age, leukocyte, extramedullary disease and complete remission exists. Strikingly, there is a significant correlation between peripheral blood miR-144 level and FAB classification (p=0.0023) and cytogenetics (p=0.001) (Table).

2.3. Expression of miR-144 as a potential diagnostic tool

Furthermore, we analyzed the ROC curve and found that miR-144 was a potential biomarker for screening pediatric AML patients from healthy controls (AUC, 0.984; 95% CI, 0.956-1.000; P<0.001) (Fig. 2).

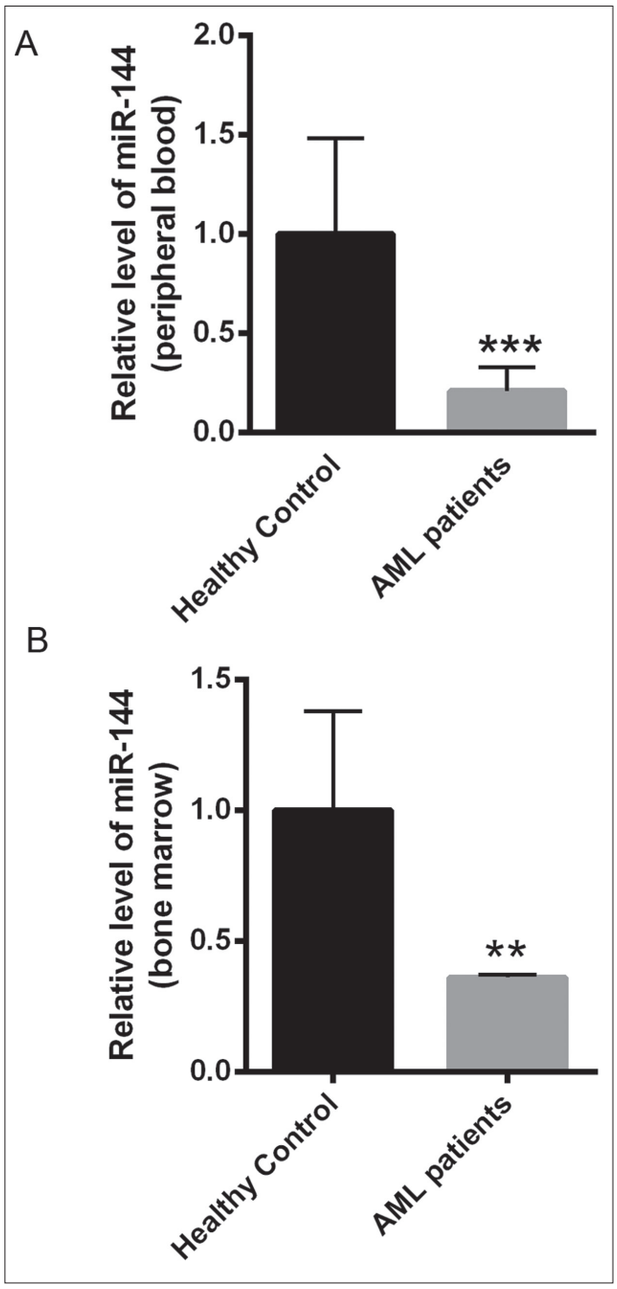

Fig. 1: Decreased miR-144 in the peripheral blood and bone marrow of AML patients. (A) The expression of miR-144 was significantly reduced in the peripheral blood of AML patients (n=120) compared with that of healthy control individuals (n=30). (B) The expression of miR-144 was also found to be reduced in the bone marrow of AML patients (n=120) compared than that of healthy control individuals (n=30). *** P<0.001; ** P<0.01 vs. healthy control.

Table: Correlation of peripheral blood miR-144 level with clinical characteristics of 120 AML patients

Variable	No. of patients	MiR-144 expression (n)		P value
		Low	High	
Gender				
Male	62	28	34	ns
Female	58	27	21	
Age				
<60	75	36	39	ns
≥60	45	22	23	
Leukocyte (/μL)				
>10,000	65	35	30	ns
≤10,000	55	28	27	
FAB classification				
M1-M6	102	57	55	0.0023
M7	18	10	8	
Extramedullary disease				
Absent	67	36	31	ns
Present	53	22	31	
Cytogenetics				
Favorable	34	13	23	0.001
Intermediate	67	37	30	
Unfavorable	19	10	9	
Complete remission				
Y	84	45	39	ns
N	36	21	15	

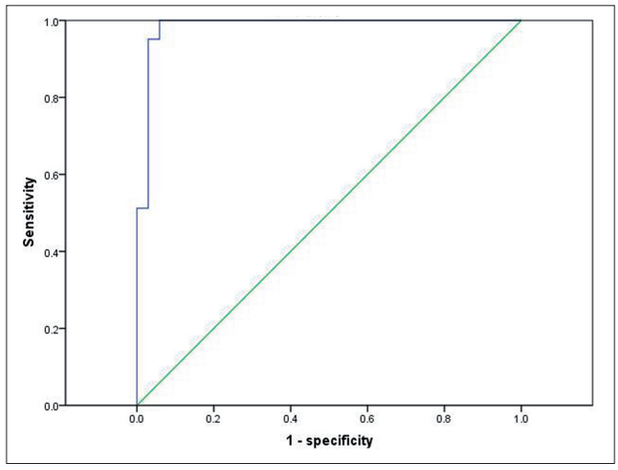

Fig. 2: ROC curve analysis revealed that miR-144 was a potential biomarker for screening pediatric AML patients from healthy controls.

2.4. Survival analysis

Next, the correlation between peripheral blood miR-144 level and the overall survival of AML patients was analyzed using Kaplan-Meier method. As shown in Fig. 3, low miR-144 expression demonstrated poor overall survival rate (p=0.018)).

2.5. mTOR was a target gene of miR-144

mTOR was reported as a target gene of miR-144. Therefore, we explored whether mTOR could be suppressed in HL60 cells. As shown in Fig. 4A, overexpression of miR-144 markedly repressed the phosphorylation level of mTOR, S6, PI3K and AKT. Further study demonstrated that transfection of miR-144 into HL60 cells significantly induced AML cell apoptosis (Figure 4B), indicating the tumor suppressor role of miR-144 in AML cells.

3. Discussion

As a malignant disease, AML leads to significant threat of the bone marrow and blood (Van Bockstaele et al. 2009). Among the pediatric AML patients, the 5-year survival rate is approximately 50% and higher mortality is identified among the elderly AML patients (Thol and Ganser 2010; Godley et al. 2011). Any disruption of miRNAs may cause dysregulation in oncogenesis and metastasis (Bouyssou et al. 2014). In leukemogenesis, dysregulation of miRNAs is widely reported. For instance, upregulation of miR-155 correlates with the initial progression and poor outcome in Chinese pediatric AML patients (Xu et al. 2015). Thus, investigation of miRNAs may provide

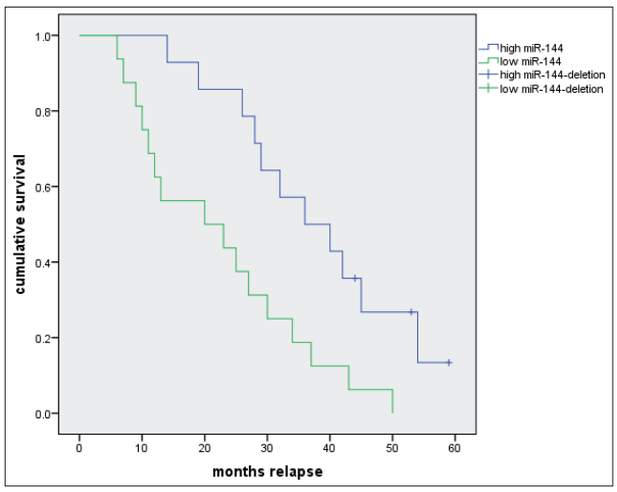

Fig. 3: The correlation between peripheral blood miR-144 level and the overall survival of AML patients was analyzed using Kaplan-Meier method.

a deep insight into the understanding of AML progression and help us to explore an effective diagnostic tool as potential biomarkers.

In the current study, we collected peripheral blood and bone marrow tissues from AML patients and healthy controls. For the first time, we showed that miR-144 was markedly reduced in both the peripheral blood and bone marrow of AML patients compared with healthy control. Further study revealed that there is a significant correlation between peripheral blood miR-144 level and FAB classification (p=0.0023) and cytogenetics (p=0.001). More importantly, lower expression of peripheral blood miR-144 levels was found to be positively correlated with poorer overall survival rate, suggesting that peripheral blood miR-144 may be related to the progression of AML. Altogether, these data indicated that miR-144 may serve as a potential biomarker for AML diagnosis.

MiRNAs are highly conserved in mammals and they are reported to widely modulate gene expression, thereby regulating cell proliferation, survival, differentiation, and organ (Chen 2005). Upregulation or deregulation of miRNAs are rapidly being explored as potential diagnostic biomarkers in various tumors (Chen 2005; Gordon et al. 2005). MiR-144 is a small non-coding RNA that was identified to be dysregulated in tumors. In lung cancers, decreased miR-144

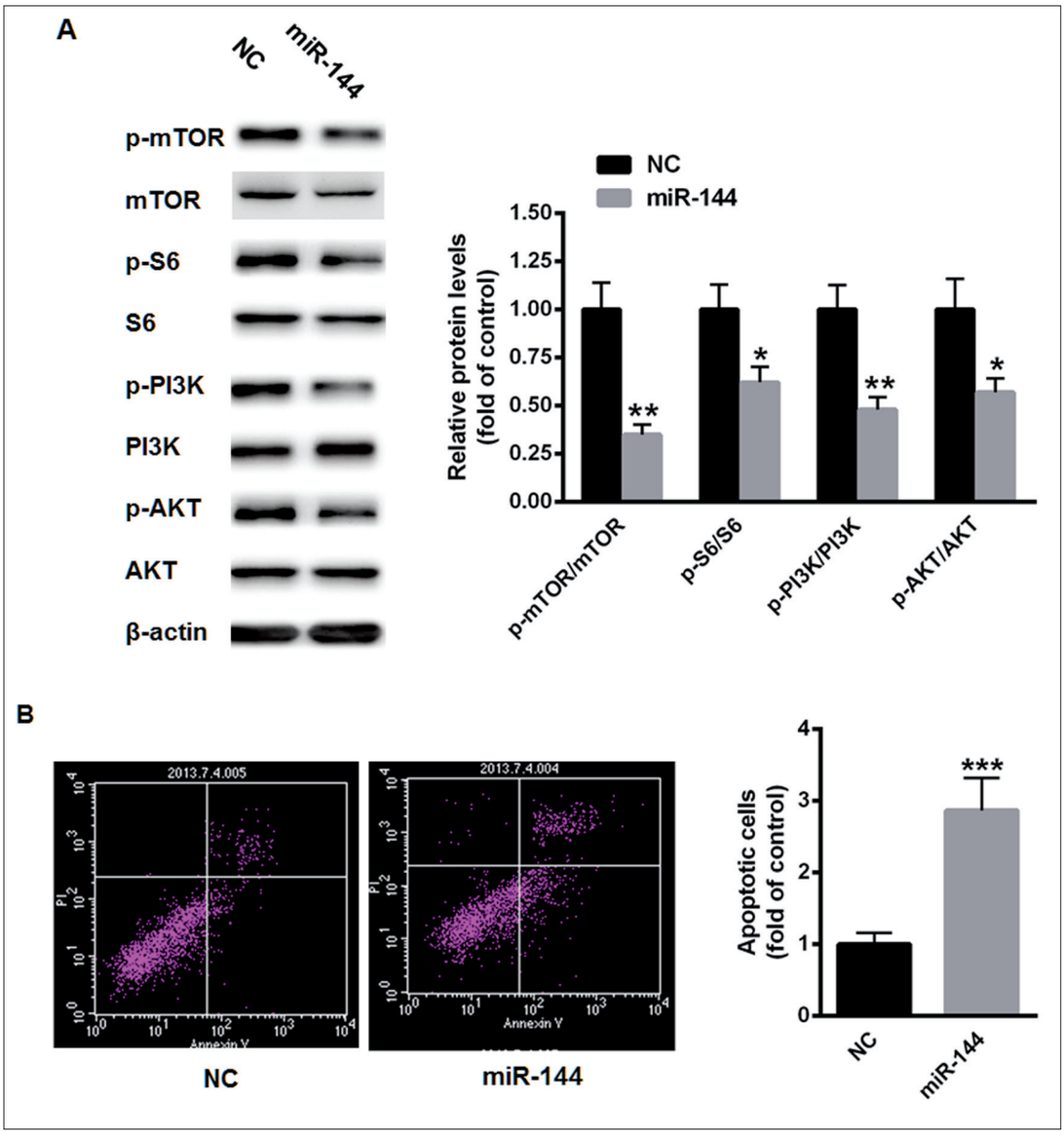

Fig. 4: mTOR was a target gene of miR-144. (A) Overexpression of miR-144 markedly repressed the phosphorylation level of mTOR, S6, PI3K and AKT. (B) Transfection of miR-144 into HL60 cells significantly induced AML cell apoptosis. *** P<0.001; ** P<0.01 vs. healthy control.

prompted cancer cell proliferation mainly by targeting GLUT1 (Liu et al. 2016). For esophageal squamous cell cancer, miR-144 was found to repress cancer cell proliferation and metastasis by binding the 3'UTR of cyclooxygenase-2 (Shao et al. 2016). These data suggested the tumor suppressive role of miR-144 in cancer progression. In line with the previous studies, we first showed that the expression of miR-144 was reduced in both the peripheral blood and bone marrow tissues of AML patients, suggesting its strong tumor suppressive function in AML.

The present study has a limited number of experimental samples, which may restrict the statistical significance of the study. Therefore, large prospective studies are required to confirm the role of miR-144 in AML patients. Despite this disadvantage, this study provides a novel potential non-invasive biomarker for AML detection.

3. Experimental

3.1. Patients and tissue samples

The present study was approved by Qinghai People's Hospital Ethics Committee. An informed consent was obtained from the patients in accordance with the guidelines of Qinghai People's Hospital, China. All peripheral whole blood samples of patients and specimens were handled and made anonymous between June 2014 and January 2016 according to the ethical and legal standards.

A total of 120 AML patients, including 62 males and 58 females, were collected from Qinghai People's Hospital. The diagnosis of AML was made according to a morphologic assessment of the Wright-Giemsa stained smears of the bone marrow aspirates along with special stains and immunophenotyping by flow cytometry. Laboratory investigation included conventional and molecular cytogenetic analyses. The median leukocyte count at diagnosis was 20,606/μL (range 420-352, 906/μL). The clinical characteristic of 120 AML patients is summarized in the Table. The healthy control group consisted of 30 healthy volunteers with no clinical symptoms of cancer or other diseases.

3.2. RNA extraction

The total RNA from the whole blood samples (5 ml) collected in tubes containing EDTA was extracted with RNAVzol LS (Vigorous Biotechnology, Beijing) in strict accordance with the manufacturer's instructions. The concentration and the purity of the RNA samples were determined by OD_{260}/OD_{280}.

3.3. Cell culture

Human AML cell lines HL-60 were derived from Institute of Hematology & Blood Diseases Hospital Chinese Academy of Medical Sciences & Peking Union Medical College (Tianjin, China). HL-60 was cultured in IMDM (Hyclone, Logan, UT, USA) supplemented with 20% fetal bovine serum (Gibco, Life, USA).

3.4. Reverse transcription (RT) and quantitative (q) polymerase chain reaction (PCR)

For synthesis of cDNA of the specific miR, Taq-Man MicroRNA Reverse Transcription Kit (Applied Biosystems) was used. To quantify the miR-144, a quantitative real-time PCR assay was performed using SYBR Green Supermix (Bio-Rad) in a BIO-RAD iCycleriQ real-time PCR detection system according to the instructions. The thermal cycling conditions were a hot start step at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The 2-delta delta Ct analysis method was used to determine the relative quantity of miR-144.

The specific primers used for reverse transcription were as follows (5'-3'): miR-144, CGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTTACA; U6, TCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAATATG.

The primers used for real-time PCR were as follows (5'-3'): miR-144 forward, GCTCTACAGTGCACGTGTC; U6 forward, GCGGATATCATCATATAC; Universal reverse primer, GTGCAGGGTCCGAGGT.

The raw data represent the relative quantity of target miRNA and RNU6 was used as the internal control. Each sample was examined in triplicate. Mean normalized gene expression ± standard deviation (SD) was calculated from independent experiments.

3.5. Statistical analysis

Data are presented as mean±SD. Differences were carried out with Student's t test. Differences were carried out with Student's t test. ROC curves were used to assess miR-144 as a biomarker, and the AUC was reported. $P < 0.05$ was considered as statistically significant difference.

Conflicts of interest: None declared.

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