

Expression and Clinical Significance of miRNA-495 in the Peripheral Blood of Acute Myeloid Leukemia Patients

Lingjuan Huang^{1*}, Jinqian Dai²

¹Department of General Practice, the First Affiliated Hospital of Xi'an Medical University, Xi'an 710077, Shaanxi Province, China

²Department of Hematology, Xi'an Central Hospital, Xi'an 710003, Shaanxi Province, China

*Corresponding author: Lingjuan Huang, huanglingjuan2006@163.com

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Abstract: *Objective:* To analyze the expression and clinical significance of miRNA-495 in the peripheral blood of patients with acute myeloid leukemia. *Methods:* Fifty-six patients with acute myeloid leukemia and 56 healthy controls were selected. Fasting venous blood was drawn and centrifuged, and the plasma was collected. The target miRNA was directly amplified and reverse transcribed into cDNA. The expression of plasma miR-495 was detected by qRT-PCR. *Results:* The expression level of miRNA-495 in newly diagnosed acute myeloid leukemia (AML [ND]) and relapsed/refractory acute myeloid leukemia (AML [RR]) was significantly lower than that in complete remission (AML [CR]) and normal control group (Control) ($p < 0.0001$). There was no significant difference between AML (ND) group and AML (RR) group ($p > 0.05$). The area under the ROC curve (AUC) of miRNA-495 was 0.9503, the 95% confidence interval was 0.9113–0.9892 ($p < 0.0001$), the standard error was 0.020, the sensitivity and specificity were 91.1% and 92.9%, respectively, and the Jordan index was 0.857. There was no significant difference between the expression level of miRNA-495 and gender, age, leukocyte count, hemoglobin, and platelet count ($p > 0.05$). However, it was found related to the proportion of primitive bone marrow cells in patients ($p = 0.017$). *Conclusion:* The decreased expression of plasma miR-495 in AML patients can be used as a new indicator for the diagnosis and prognosis of AML.

Keywords: miR-495; Acute myeloid leukemia; Expression; Clinical significance

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1. Introduction

Acute myeloid leukemia (AML) is the most common hematological malignancy in adults. In recent years, various immunotherapies and new molecular targeted therapies have greatly improved the prognosis of patients, but drug resistance and recurrence are still the dilemma of current treatment; moreover, its pathogenesis and treatment targets are still being explored [1,2]. Studies have found that microRNAs (miRNAs/miRs) are abnormally expressed in various human tumors [3,4]. It can be used as a new marker for early diagnosis and prognosis evaluation of various tumors, including acute leukemia [5]. As a member of the miRNA family, miR-495 has been reported to play the role of tumor suppressor gene in most malignant tumors. On the other hand, it also plays the role of oncogene in some tumors, promoting the occurrence and progression of tumors [6]. At present, there are several reports about the expression of miR-495 in acute myeloid leukemia at home and abroad. This study aimed to explore the characteristics of miR-

495 expression in the plasma of patients with different types of acute leukemia and disease states.

2. Methods

2.1. Study population

The peripheral blood samples of 56 patients with newly diagnosed AML (AML [ND]) who were hospitalized in Xi'an Central Hospital under the care of the Department of Hematology from January 2020 to December 2021 and confirmed by bone marrow puncture, immunotyping, chromosome analysis, and fusion gene examination were collected. At the same time, the peripheral blood samples of 56 healthy controls who were matched by gender and age and selected as controls were collected. The AML patients were classified according to the French-American-British (FAB) classification of AML: M0 1 case, M1 3 cases, M2 12 cases, M4 18 cases, M5 20 cases, and M6 2 cases. At the same time, peripheral blood samples were collected from 11 patients with relapse/refractory AML (AML [RR]) (5 of whom were from the AML [ND] group) and 16 patients with complete remission of AML (AML [CR]) (all from the AML [ND] group). Among the 56 newly diagnosed AML patients, 37 had abnormal chromosome karyotype. The design of this study met the ethical requirements and was approved by the hospital clinical research ethics committee. All subjects voluntarily participated in the study and signed the informed consent form after being informed of the research content by professionals before enrollment.

2.2. Detection methods

2.2.1. Plasma separation and total RNA extraction

From the AML patients included in the study, 2 ml of peripheral blood was drawn on an empty stomach, anticoagulated with ethylenediaminetetraacetic acid (EDTA), and centrifuged at $2000 \times g$ for 10 minutes at 4°C ; the supernatant was then drawn into a new sterile enzyme-free Eppendorf (EP) tube. Subsequently, it was centrifuged at $16000 \times g$ for 10 minutes at 4°C , and the supernatant was then transferred into another sterile enzyme-free EP tube. It was frozen at -80°C for standby. RNA was extracted using the Ultrapure RNA Kit (DNase I) (Beijing Kangwei Century Biological Co., Ltd.).

2.2.2. RNA reverse transcription reaction

HiFiScript cDNA Synthesis Kit (Beijing Kangwei Century Biological Co., Ltd.) was used in this step. The concentration of RNA samples was adjusted to $1000 \text{ ng}/\mu\text{L}$ with RNase-free Water. $1.0 \mu\text{L}$ RT Primer Mix, $1.0 \mu\text{L}$ PrimeScript RT Enzyme Mix I, $4.0 \mu\text{L}$ 5X PrimeScript Buffer 2 (for Real Time) were used, and the reverse transcription reaction system was prepared with $1.0 \mu\text{L}$ RNA RNase-free Water. After the preparation, it was mixed well, followed by centrifugation to collect the mixed liquid at the bottom of the pipe as much as possible. The reverse transcription conditions were 37°C for 60 minutes and 95°C for 5 minutes. The synthesized cDNA was then stored in a refrigerator at 20°C for standby.

2.2.3. Real time fluorescent quantitative polymerase chain reaction (PCR)

The primers used in the experiment were designed and synthesized by Qingke Company. The primer sequence is shown in **Table 1**. The PCR reaction system $25 \mu\text{L}$ (PCR Forward Primer $1.0 \mu\text{L}$, PCR Reverse Primer $1.0 \mu\text{L}$, $2 \times$ UltraSYBR Mixture $25 \mu\text{L}$, Template DNA $2.0 \mu\text{L}$, and ddH_2O $21 \mu\text{L}$) was centrifuged at $4500 \times g$ for 3 minutes at 4°C and placed in BIORAD CFX96 real-time PCR instrument for amplification. The reaction conditions were 94°C for 2 minutes, 94°C for 20 seconds, and 60°C for 30 seconds, with 40 cycles in total. Each sample was set with 3 repeat holes, and the experiment was carried out for 3 times. The relative expression of miR-495 was calculated by using the formula $2^{-\Delta\Delta\text{Ct}}$.

Table 1. Primer sequence

Gene	3')	3')
miR-495	ACACTCCAGCTGGGGAAGTTGCCCATGTT	CTCAACTGGTGTTCGTGGA
GAPDH	TGTTTCGTCATGGGTGTGAAC	ATGGCATGGACTGTGGTCAT

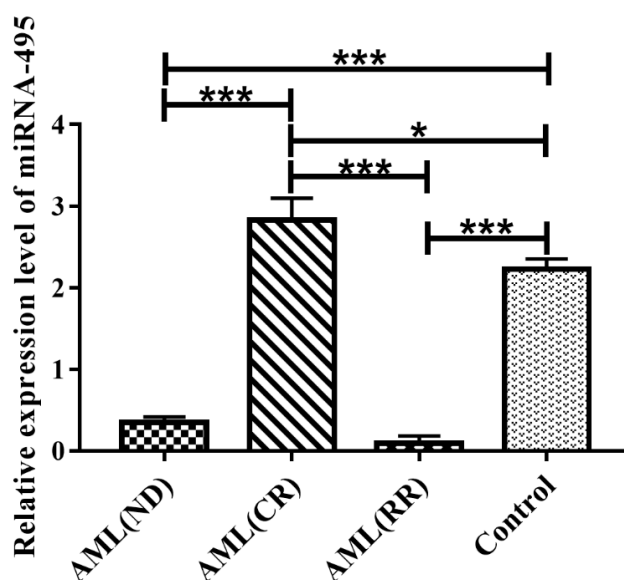
2.3. Statistical analysis

Statistical analysis was carried out by using SPSS 22.0. When the measurement data obeyed the normal distribution, mean \pm standard deviation was used to express the data, and the Bonferroni method was used for the comparison between groups; the median (range) was used in contrasting situation. Categorical variables were expressed in rates, and chi-square test was used. For rank data, rank sum test was used. The difference was considered statistically significant when $p < 0.05$.

3. Results

3.1. Expression of plasma miR-495 in the peripheral blood of AML patients at different stages of onset and in healthy controls

The expression level of miR-495 in the peripheral plasma samples of 56 cases of AML (ND), 11 cases of AML (RR), 16 cases of AML (CR), and 56 healthy control (Control) was detected by fluorescence-based quantitative PCR. The results showed that compared with AML (CR) and Control groups, the relative expression level of miR-495 in AML (ND) and AML (RR) groups was significantly downregulated ($p < 0.0001$). The level of plasma miR-495 in AML (CR) group was slightly higher than that in the Control group (2.8303 ± 1.0642 versus 2.2260 ± 0.9407), and there was significant difference between the two groups ($p < 0.05$). However, there was no significant difference between the AML (ND) group and AML (RR) group ($p > 0.05$) (**Figure 1**).

**Figure 1.** Expression level of miR-495 in different disease states of AML

3.2. ROC curve analysis

ROC curve was used to evaluate the diagnostic value of miR-495 for AML. The results showed that miR-495 could be used as a potential molecular marker for the diagnosis of AML (AUC = 0.9503, 95% confidence interval 0.9113–0.9892, $p < 0.0001$) (**Figure 2**).

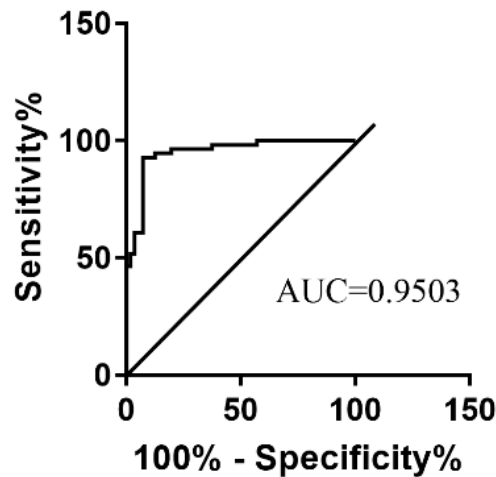


Figure 2. ROC diagnostic value of miR-495

3.3. Relationship between plasma miR-495 expression level and the clinical data of AML patients

According to the relative expression of miR-495 gene, taking a median of 0.0752, the 56 patients with newly diagnosed AML were divided into the low expression group (n = 28, expression range 0.0000–0.05578) and the high expression group (n = 28, expression range 0.0948–2.0346). By collecting and analyzing the clinical data of these 56 patients, it was found that the expression level of miR-495 had no significant difference with gender, age, leukocyte count, hemoglobin, and platelet count ($p > 0.05$). However, it was found related to the proportion of primitive bone marrow cells in patients ($p = 0.017$) (**Table 2**).

Table 2. Correlation between miR-495 expression level and the clinical data of newly diagnosed AML patients

Clinical features	Number of cases	miR-495 expression		χ	p
		High expression (n = 28)	Low expression (n = 28)		
<i>Gender</i>				0.031	0.861
Male	30	12 (21.4%)	18 (32.2%)		
Female	26	11 (19.6)	15 (26.8%)		
<i>Age</i>				0.638	0.425
< 60 years old	23	8 (14.3%)	15 (26.8%)		
≥ 60 years old	33	15 (26.8%)	18 (32.1%)		
<i>Proportion of primitive bone marrow cells (%)</i>				5.731	0.017
< 50	29	20 (35.7%)	9 (16.1%)		
≥ 50	27	10 (17.8%)	17 (30.4%)		
<i>White blood cell count ($\times 10^9/L$)</i>				0.160	0.689
< 100	31	12 (21.4%)	19 (33.9%)		
≥ 100	25	11 (19.7%)	14 (25.0%)		
<i>Hemoglobin (g/L)</i>				0.747	0.388
< 80	36	12 (21.4%)	24 (42.9%)		
≥ 80	20	9 (16.0%)	11 (19.7%)		

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Clinical features	Number of cases	miR-495 expression		x	p
		High expression (n = 28)	Low expression (n = 28)		
<i>Platelet</i> ($\times 10^9/L$)				0.054	0.817
< 50	32	15 (26.8%)	17 (30.4%)		
≥ 50	24	12 (21.4%)	12 (21.4%)		
<i>Prognostic stratification</i> ※ (n = 37)				2.190	0.334
Good prognosis	19	12 (32.4%)	7 (18.9%)		
Moderate prognosis	10	4 (10.8%)	6 (16.2%)		
Poor prognosis	8	3 (8.2%)	5 (13.5%)		

※: based on the 2017 European LeukemiaNet (ELN) consensus

4. Discussion

Evidence has shown that miRNAs can participate in the occurrence and development of tumors as oncogenes or tumor suppressor genes. Recent years have seen an increase in research on the role of miRNA in the pathogenesis of leukemia [7-9]. miR-15a/16-1, miR-188, miR-155, miR-29, miR-17-92, and let-7 are the most commonly differentially expressed miRNAs reported in leukemia [10]. In addition to the aforementioned molecules, it has been confirmed that the expression level of miR-495 in mixed lineage leukemia (MLL) is lower than that in non-MLL-rearranged AML [11]. However, studies on miR-495 in various types of leukemia and even various hematological malignancies have not been reported.

In this study, qRT-PCR was used to analyze the expression of miR-495 in AML. The results showed that compared with the healthy control group, the expression level of plasma miR-495 in the newly diagnosed group and recurrence group was significantly downregulated ($p < 0.0001$). However, it was significantly upregulated in the complete remission group, suggesting that plasma miR-495 has significance in the diagnosis and prognosis of AML. At present, bone marrow biopsy is still essential for the diagnosis of malignant hematological diseases, but this examination is a traumatic procedure. It is imperative to develop new diagnostic and evaluation methods for AML. More and more studies have confirmed that miRNA in serum or plasma can be used as a biomarker for the diagnosis of leukemia, prognosis evaluation, and treatment response monitoring. In this study, peripheral blood samples from AML patients were used to ensure minimal pain to the patients. Following the findings of previous experiments that miR-495 is differentially expressed in different stages of AML, ROC curve was used to evaluate the diagnostic value of miR-495. The results showed that miR-495 has certain diagnostic value (AUC = 0.9503, 95% confidence interval 0.9113–0.9892, $p < 0.0001$); hence, it could be used as an effective indicator to screen AML patients and normal healthy controls.

Previous studies have confirmed that miR-495 is underexpressed in most tumors, including gastric cancer [12], lung cancer [13], glioblastoma multiforme [14], and prostate cancer [15]. miR-495 acts as a tumor suppressor gene, inhibiting the proliferation, apoptosis, and migration of cancer cells, by regulating the expression of target genes. However, the expression of miR-495 is elevated in gallbladder cancer, which may promote the proliferation of cancer cells through miR-495/phlpp/akt/survivin [16]. At the same time, the study found that in breast cancer and liver cancer, miR-495 may play the role of both, tumor suppressor gene [17,18] and oncogene [19,20]. This study suggests that miR-495, as a tumor suppressor gene, may be involved in the occurrence of acute leukemia, which is consistent with the current research results of miR-495 in acute mixed leukemia [6].

In addition, this study also explored the relationship between the expression level of miR-495 and the clinical data of AML patients. It was found that the expression of miR-495 is not correlated with gender, age, leukocyte count, hemoglobin, and platelet count ($p > 0.05$). However, there was statistical difference

with the proportion of primitive bone marrow cells ($p = 0.017$). The results of this study may have some guiding significance for the evaluation and prognosis of AML.

In conclusion, miR-495 is differentially expressed in AML patients at different disease stages, and its expression level is correlated with the proportion of primitive bone marrow cells. This makes it possible as a molecular target for AML diagnosis, treatment monitoring, and prognosis evaluation. The limitation of this study lies in the small sample size, especially the comparison of plasma miR-495 levels between the AML (CR) group and healthy control group, which leads to different statistical results; this may be related to the small number of selected cases in the AML (CR) group. Moreover, the downstream target genes regulated by miR-495 were not detected. Hence, this part of work needs to be further demonstrated in future research.

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Disclosure statement

The authors declare no conflict of interest.

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