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Upregulation of serum exosomal miR-21 was associated with poor prognosis of acute myeloid leukemia patients

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Abstract

Accumulating reports have showed that altered expression of blood-circulating (miRNAs) can serve as diagnostic biomarkers of cancer, but descriptions of serum exosomal miRNAs in AML are still lacking. The present study was designed to measure serum exosomal miR-21 levels in AML patients and explore its correlation with clinical variables and prognosis. In this study, blood samples were collected from 135 AML patients and 60 controls. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was used to detect serum exosomal miR-21 expression levels. The data showed that serum exosomal miR-21 levels were significantly higher in AML patients than those in healthy controls. In addition, serum exosomal miR-21 levels were significantly decreased at one month and three months after their treatment. Further receiver operating characteristic (ROC) curve analysis showed that serum exosomal miR-21 level could identify AML patients from normal controls with relative high sensitivity and specificity. Moreover, serum exosomal miR-21 upregulation was closely associated with worse clinical features and shorter survival. In multivariate Cox regression analysis, serum exosomal miR-21 expression showed a significance correlation with survival. In conclusion, serum exosomal miR-21 could be used to predict the diagnosis and prognosis of AML patients.

Keywords: biomarker; miR-21; exosome; acute myeloid leukemia.

Practical Application: Serum exosomal miR-21 could be used to predict the diagnosis and prognosis of AML patients.

1 Introduction

Acute myeloid leukemia (AML) is the most common hematological malignancy in adults and characterized by the rapid proliferation of leukemic blasts (Wen et al., 2018; Döhner et al., 2015). According to cytogenetic information, AML patients can be divided into three risk-based categories: favorable, intermediate, and poor (Gregory et al., 2009). Although great advance has been made for AML therapy over the past decades, the prognosis of AML patients is still very difficult to accurately evaluate (Gregory et al., 2009). The survival of AML patients varies greatly from a few weeks to complete remission and cure (Estey, 2013). Since early detection of this malignancy can reduce the mortality rates of AML patients, identification of novel and effective biomarkers are urgently required to improve the prognosis of AML patients.

MicroRNAs (miRNAs) are small single-stranded non-coding molecules with 18-25 nucleotides in length. MiRNAs are involved in various biological processes, such as cell proliferation, differentiation, development and apoptosis (Han & Sun, 2017; Cech & Steitz, 2014). Previous studies had demonstrated that miRNAs can serve as either oncogenes or tumor suppressors to suppress translation or induce mRNA degradation (Bai et al., 2016; Chen, 2005). Exosomes are 30-100 nm membrane vesicles and carry proteins, lipids, mRNA, and miRNA (Colombo et al., 2014). Serum exosomal miRNAs could be stably detected and used as diagnostic biomarkers for early detection of various types of cancer.

MiR-21 has been shown its oncogenic functions in many cancer types including AML. MiR-21 expression in AML was significantly increased compared to normal bone marrow cells, and miR-21 upregulation or KLF5 inhibition markedly promoted the carcinogenesis *in vitro* and stimulated tumor growth *in vivo* (Li et al., 2019). Besides AML, upregulation of miR-21 was found to correlate with some cancers, such as hepatocellular carcinoma (Wang et al., 2015a; Meng et al., 2007; Wang et al., 2014), gastric cancer (Komatsu et al., 2013; Zhang et al., 2012), esophageal squamous cell cancer (Chen et al., 2019), epithelial ovarian cancer (Lou et al., 2010), osteosarcoma (Ren et al., 2016), renal cell carcinoma (Liu et al., 2017), pancreatic ductal adenocarcinoma (Abue et al., 2015), bladder cancer (Tao et al., 2011), intrahepatic cholangiocarcinoma (Wang et al., 2015b), oral squamous cell carcinoma (Zheng et al., 2018) and colorectal cancer (Toiyama et al., 2013). However, studies of serum exosomal miR-21 expression in AML have not been found. Here, we analyzed serum exosomal miR-21 expression in AML patients and then evaluated its clinical significance in AML.

2 Materials and methods

2.1 Patients and samples

The current study was conducted on 135 blood samples obtained from *de novo* AML patients who were confirmed by biopsy and bone marrow aspiration in our hospital. There were

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71 men and 64 women in the patient group, with aging between 28.3 and 63.6 years old. All AML patients were diagnosed according to the French-America-British (FAB) and World Health Organization criteria. The research was approved by Ethics Committee of The First Affiliated Hospital of Zhengzhou University, and signed informed consent was obtained from all of the patients before bone marrow puncture. Sixty healthy volunteers with no clinical symptoms of cancer or other diseases were enrolled and used as the control group. Overall survival (OS) was defined as the time from diagnosis to death or the last follow-up. Relapse free survival (RFS) was defined as the time from diagnosis to removal from the study due to relapse or death or the last follow-up. The demographic characteristics of all AML patients were presented in Table 1.

Peripheral blood samples were withdrawn from all participants at room temperature for 30 min, and centrifuged at 2,000 g for 10 min, then centrifuged at 12,000 g for 10 min. The supernatant was stored at -80 °C until use.

Table 1. Clinical variables of 135 newly diagnosed AML patients and serum exosomal miR-21 expression.

Variables	Patients	Serum exosomal miR-21		P-value
		Low	High	
Age (years)				0.4201
< 60	98	50	48	
≥ 60	37	16	21	
Gender				0.4300
Men	71	37	34	
Women	64	29	35	
Platelet (×10^9/L)				0.2432
< 50	77	41	36	
≥ 50	58	25	33	
Hemoglobin (g/L)				0.1177
< 80	83	45	38	
≥ 80	52	21	31	
WBC (×10^9/L)				0.0029
< 10	41	28	13	
≥ 10	94	38	56	
BM blasts				0.0204
< 50%	54	33	21	
≥ 50%	81	33	48	
Cytogenetics				0.0086
Favorable	18	11	7	
Intermediate	93	50	43	
Unfavorable	24	5	19	
FAB subtype				0.1012
M1/M2	57	34	23	
M4/M5	71	29	42	
Other	7	3	4	
Extramedullary disease				0.1300
No	94	50	44	
Yes	41	16	25	

2.2 Serum exosome isolation

Exosome isolation from serum samples were performed using the ExoQuick Exosome Precipitation Solution (SBI, Mountain View, CA, USA) according to the manufacturer's protocol. Briefly, serum samples were thawed on ice and centrifuged at 3000 g for 15 min to remove possible residual cell debris. Then, one-fourth volume of ExoQuick solution was mixed with the supernatants. After incubation at 4 °C for 30 min, the mixture was centrifuged at 2000 g for 30 min. The exosome pellet was dissolved in 50 μL PBS and stored at –80 °C for further experiments.

2.3 RNA isolation and quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

Exosomal RNA was isolated with a mirVana PARIS RNA isolation kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The RNA concentration was assessed using Nanodrop 2000 thermo scientific spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). In the RNA isolation step, 20 fmol of synthetic Caenorhabditis elegans *Cel-miR-39* (RiboBio, Guangzhou, China) was added to each sample as a spike-in control. For cDNA synthesis, TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) was used following the manufacturer's protocol. Then, qRT-PCR was performed on ABI PRISM 7900 Sequence Detection System (Applied Biosystems) and Taqman Universal PCR Master Mix (Applied Biosystems). The relative serum exosomal miR-21 expression was normalized to *Cel-miR-39* and calculated using the $2^{-\Delta\Delta Ct}$ method. Each experiment was performed in triplicate.

2.4 Statistical analysis

All the data analyses were performed using MedCalc 10.4.7 software (MedCalc, Mariakerke, Belgium) and GraphPad Prism 8.0.1 software (GraphPad Software, Inc., USA). The Mann-Whitney U test or Kruskal-Wallis test were performed to determine the significance in serum exosomal miR-21 levels between different groups. The Chi-squared test was used to assess the correlations between serum exosomal miR-21 expression and clinical factors. Receiver-operator characteristic (ROC) curves and the areas under the ROC curves (AUCs) were calculated to evaluate the AML detection potential of serum exosomal miR-21. Survival analysis was conducted for the AML cases using Kaplan-Meier methods plus log-rank test. Multivariate survival analyses (OS and RFS) were performed using Cox proportional hazards regression model. A statistical significance was defined when $P < 0.05$.

3 Results

3.1 Serum exosomal miR-21 is highly expressed in AML

The serum exosomal miR-21 levels in AML patients and healthy individuals were measured using qRT-PCR analysis. As illustrated in Figure 1, serum exosomal miR-21 levels were significantly higher in AML subjects than controls. In addition, serum exosomal miR-21 expression was significantly reduced in AML patients with lower WBC and <50% BM blasts. AML patients in unfavorable cytogenetic risk group showed higher serum

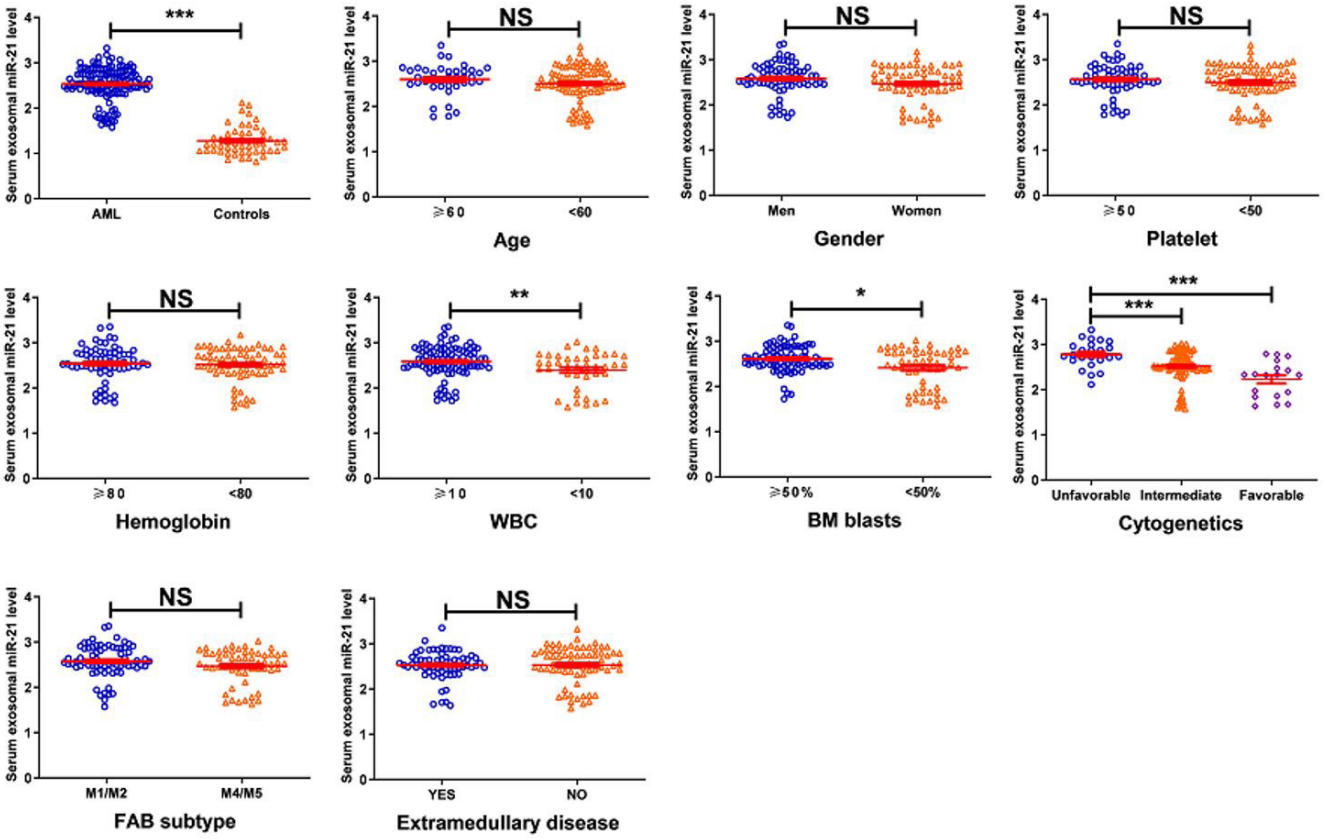

Figure 1. Serum exosomal miR-21 expression levels were significantly higher in AML patients. Serum exosomal miR-21 expression levels were significantly higher in AML patients with ≥ 10 WBC and with ≥ 50% BM blasts. Serum exosomal miR-21 expression levels were significantly higher in AML patients with unfavorable cytogenetic abnormalities.

exosomal miR-21 levels than those in intermediate and favorable cytogenetic risk group.

Next, blood samples were collected from all AML patients one month and three months after threatment. Serum exosomal miR-21 expression levels were measured using qRT-PCR. Serum exosomal miR-21 levels were markedly decreased at one month after treatment ($P < 0.0001$, Figure 2). Likewise, serum exosomal miR-21 levels continued to be decreased at three months after threatment ($P = 0.0045$, Figure 2).

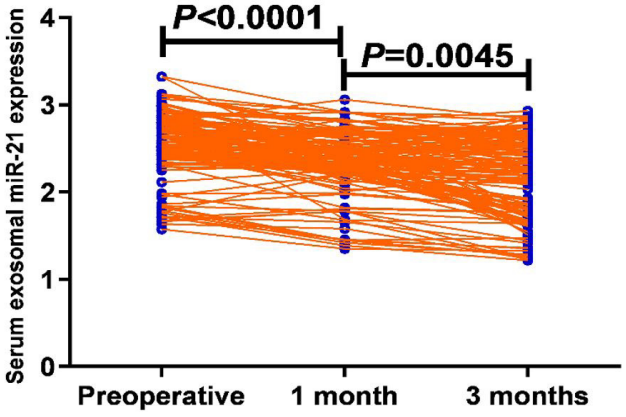

Figure 2. Serum exosomal miR-21 expression levels continued to be decreased at one month and three months after treatment.

Diagnostic value of serum exosomal miR-21 expression in AML

Then, ROC curve was constructed to analyze the diagnostic accuracy of serum exosomal miR-21 in AML. As shown in Figure 3, ROC analysis revealed an AUC value of 0.844 with 85.2% sensitivity and 84.0% specificity. The results demonstrated that serum exosomal miR-21 was a potential biomarker for screening AML patients from healthy volunteers.

3.2 Serum exosomal miR-21 upregulation associated with advanced clinical variables of AML

The serum exosomal miR-21 levels were detected to evaluate the relationship with clinicopathologic features. All AML patients were divided into high expression group (n = 69) and low expression group (n = 66) according to the median serum exosomal miR-21 expression levels. As shown in Table 1, high serum exosomal miR-21 expression was significantly correlated with white blood cells (WBC) ($P = 0.0029$), bone marrow (BM)

blasts ($P = 0.0204$) and cytogenetics ($P = 0.0086$). However, no significant correlation was observed between serum exosomal miR-21 expression and age ($P = 0.4201$), gender ($P = 0.4300$), platelet ($P = 0.0953$), hemoglobin ($P = 0.1417$), FAB subtype ($P = 0.1012$) and extramedullary disease ($P = 0.0997$).

3.3 Serum exosomal miR-21 upregulation associated with poor prognosis in AML patients

The association of serum exosomal miR-21 expression levels with OS and RFS of AML patients was further analyzed. The data suggested that AML patients with high serum exosomal miR-21 expression group had worse OS and RFS than those in low serum exosomal miR-21 expression group ($P = 0.0225$, Figure 4A; $P = 0.0008$, Figure 4B).

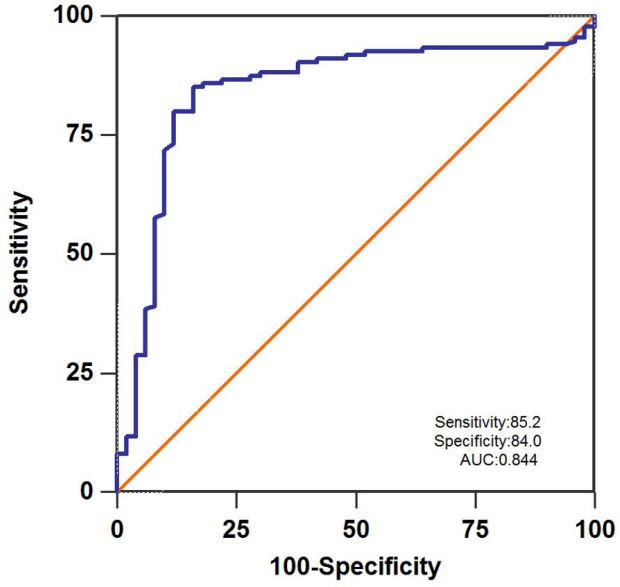

Figure 3. ROC analysis showed an AUC of 0.844 in identifying AML patients from controls.

Multivariate analysis revealed that BM blasts (RR 1.73; 95% CI, 0.84-2.89; $P = 0.025$), WBC (RR 2.87; 95% CI, 1.51-4.48; $P = 0.008$), cytogenetics (RR 2.15; 95% CI, 1.13-3.35; $P = 0.017$), and serum exosomal miR-21 (RR 2.63; 95% CI, 1.48-4.22; $P = 0.011$) were independent prognostic factors affecting patient's OS. Likewise, BM blasts (RR 1.94; 95% CI, 1.02-3.11; $P = 0.021$), WBC (RR 3.32; 95% CI, 1.52-5.46; $P = 0.003$), cytogenetics (RR 2.36; 95% CI, 1.24-3.68; $P = 0.014$), and serum exosomal miR-21 (RR 3.06; 95% CI, 1.75-4.69; $P = 0.006$) were identified as independent predictors of RFS (Table 2).

4 Discussion

To the best of our knowledge, this was the first study to investigate prognostic significance of serum exosomal miR-21 in AML. In the present study, serum exosomal miR-21 was significantly increased in AML patients relative to healthy volunteers. Importantly, serum exosomal miR-21 levels were significantly decreased after treatment. In addition, high serum exosomal miR-21 expression was closely associated with higher WBC counts, higher BM blasts and unfavorable cytogenetic. ROC analysis showed serum exosomal miR-21 could be used to effectively differentiate AML patients from healthy controls. Moreover, Kaplan-Meier analysis demonstrated that patients with higher serum exosomal miR-21 expression had shown significantly shorter OS and RFS. Furthermore, multivariate Cox regression analysis revealed that high serum exosomal miR-21 expression was independently associated with poor survival. Our results indicated that serum exosomal miR-21 might be used as a reliable biomarker in AML.

The role of miR-21 in some other cancers also have been reported by previous studies. In hepatocellular carcinoma (HCC), miR-21 was frequently elevated in both HCC tissues and cell lines, and miR-21 overexpression significantly increased cancer cell proliferation, migration, and invasion through degrading NAV-3[12] or PTEN[13]. Moreover, HCC patients with higher miR-21 expression was closely associated with worse OS and DFS[14]. In gastric cancer (GC), miR-21 upregulation was highly associated with aggressive clinical variables and poorer survival

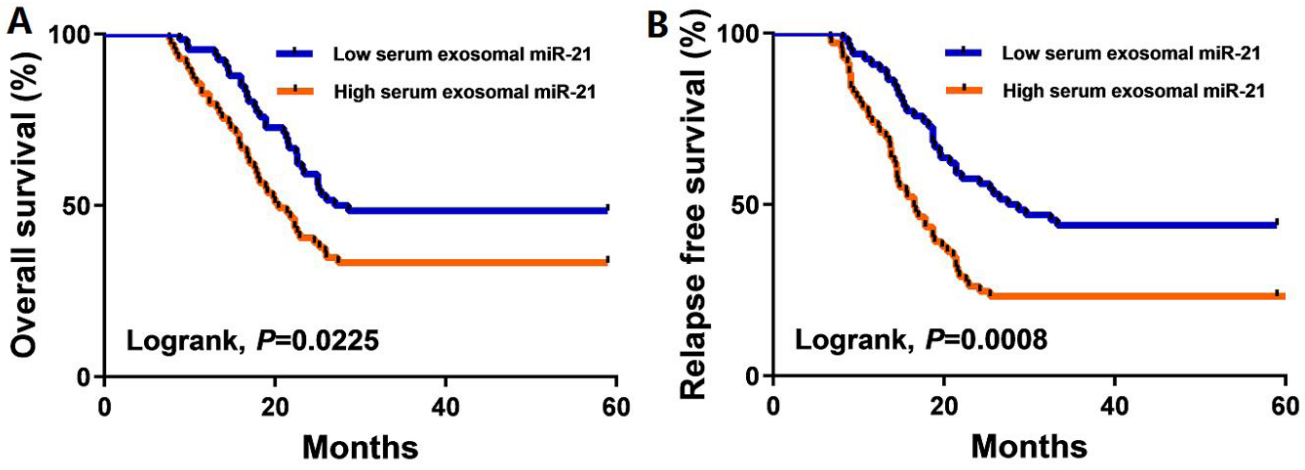

Figure 4. Analyses of OS and RFS according to serum exosomal miR-21 expression in AML patients.

Table 2. Multivariate Cox regression analysis for OS and RFS in AML patients.

Characteristics	Overall survival		Relapse free survival	
	Risk Ratio (95% CI)	P	Risk Ratio (95% CI)	P
BM blasts	1.73 (0.84 - 2.89)	0.025	1.94 (1.02 - 3.11)	0.021
WBC	2.87 (1.51 - 4.48)	0.008	3.32 (1.52 - 5.46)	0.003
Cytogenetics	2.15 (1.13 - 3.35)	0.017	2.36 (1.24 - 3.68)	0.014
Serum exosomal miR-21	2.63 (1.48 - 4.22)	0.011	3.06 (1.75 - 4.69)	0.006

rate. MiR-21 overexpression or PTEN inhibition remarkably enhanced GC cell viability and growth[15,16]. MiR-21 expression was significantly upregulated in esophageal squamous cell cancer tissues, reduced miR-21 expression markedly attenuated the carcinogenesis by targeting RASA1[17]. MiR-21 expression was dramatically increased both in tissues and cell lines of epithelial ovarian cancer, and miR-21 downregulation resulted in decreased cancer cell proliferation, migration and invasion[18]. Ren et al.demonstrated miR-21 expression was significantly higher in osteosarcoma tissues compared with adjacent normal tissues, high miR-21 expression was correlated with advanced clinical stage and metastasis[19]. In addition, miR-21 upregulation was associated with shorter survival of patients with renal cell carcinoma (RCC), and promoted RCC cell proliferation[20]. High plasma miR-21 expression was observed in pancreatic ductal adenocarcinoma patients, and closely correlated with advanced stage and lymph node metastasis[21]. MiR-21 was significantly elevated in bladder cancer tissues, restoration of miR-21 markedly stimulated the carcinogenesis and chemoresistance[22]. Furthermore, *in vitro* and *in vivo* analysis showed miR-21 downregulation significantly inhibited intrahepatic cholangiocarcinoma cell proliferation and tumor growth by inversely regulating PTPN14 and PTEN[23]. Zheng et al.showed miR-21 inhibition significantly suppressed cell proliferation, invasion and promoted apoptosis of oral squamous cell carcinoma, and PTEN was its downstream target gene[24]. In colorectal cancer (CRC), serum miR-21 expression was significantly higher in CRC patients and dramatically decreased after their surgery, and CRC patients with higher miR-21 expression had shorter survival[25]. Therefore, the data were in line with our results, and miR-21 upregulation involved in the progression of many cancer types.

In conclusion, serum exosomal miR-21 levels were markedly decreased in AML patients compared to healthy controls. High serum exosomal miR-21 expression was strongly correlated with aggressive clinical parameters and poor prognosis. Thus, data from this study validated that serum exosomal miR-21 could serve as a tumor biomarker for AML diagnosis and prognosis.

Ethical approval

The research was approved by Ethics Committee of The First Affiliated Hospital of Zhengzhou University (Approval No. 20170642X), and signed informed consent was obtained from all of the patients before bone marrow puncture.

Conflict of interest

All authors declare that they have no conflict of interest regarding this paper.

Author contributions

Xingang Li and Xia Zhang designed the study. Xingang Li, Xia Zhang, Hongxia Ma, Yang Liu, Shijia Cheng, Huili Wang and Jing Sun conducted the experiments, analyzed the data and wrote the manuscript. All authors have read and approved the manuscript.

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