European Review for Medical and Pharmacological Sciences 2018; 22: 7364-7370

Long non-coding RNA LINC00899 as a novel serum biomarker for diagnosis and prognosis prediction of acute myeloid leukemia

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Abstract. – OBJECTIVE: Recently, several long non-coding RNAs (lncRNAs) have been implicated in acute myeloid leukemia (AML). However, the clinical significance of lncRNAs in AML patients still remains unclear. We aimed to evaluate the expression level of lncRNA LINC00899 (LINC00899) and its potential for diagnosis and prognosis in AML.

PATIENTS AND METHODS: Expression levels of LINC00899 in bone marrow and serum obtained from AML patients and healthy controls were assessed by quantitative real-time PCR. Receiver operating characteristic (ROC) curves were used to evaluate the sensitivity and specificity of serum LINC00899. The association between serum LINC00899 expression and clinicopathological factors as well as the overall survival were analyzed.

RESULTS: We found that the levels of serum LINC00899 were frequently upregulated in the bone marrow and serum of AML patients. Higher expression of serum LINC00899 was positively associated with FAB classification ($p = 0.002$) and cytogenetics ($p = 0.005$). Moreover, ROC curve analyses showed that serum LINC00899 could discriminate AML patients from healthy controls with the area under the curve (AUC) of 0.807 (95% CI, 0.7262- 0.8752). In addition, the serum LINC00899 expression level was significantly reduced when the patients achieved complete remission. Kaplan-Meier analysis showed that patients with high serum LINC00899 expression had a shorter overall survival compared with the low serum LINC00899 expression group ($p = 0.0013$). Finally, Cox proportional hazards analysis showed that high serum LINC00899 expression was an independent prognostic marker of poor outcome.

CONCLUSIONS: We firstly found that serum LINC00899 might be a potential and useful non-invasive biomarker for the early clinical detection and prognosis of AML.

Key Words:
LncRNA LINC00899, Prognosis, Diagnosis, Acute myeloid leukemia.

Introduction

Acute myeloid leukemia (AML) is an aggressive malignant disorder of hematopoietic cells characterized by uncontrolled proliferation of hematopoietic progenitor cells[1,2]. According to the French-American-British classification system, AML involving the granulocytic lineage accounts for 85% (M1 to M5 subtypes) of all patients[3]. During the development of leukemia, somatic gene mutations and nonrandom chromosomal translocations occur in leukemic stem cells[4]. Despite the advancements in the treatment of leukemia, the prognosis of AML remains poor, with a 5-year survival rate less than 30%[5,6]. Therefore, the identification of novel and sensitive biomarkers which can recognize these AML patients who are at the risk of poor prognosis is warranted to optimize treatment strategies. Long noncoding RNA (LncRNA) is a class of non-protein coding transcripts of >200 nucleotides and <100 kb[7]. LncRNAs are usually transcribed by RNA polymerase II and formed

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by fragmentation and modification of the precursor RNA[8]. Over the past several years, significant progress has been made in elucidating the function of lncRNAs in various biological processes, such as development, differentiation and determination of cell fate, as well as disease pathogenesis[9,10]. In addition, aberrant expressed lncRNAs are frequently observed in many diseases, particularly in cancers[11,12]. Up to date, more and more lncRNAs have been identified to be important biological regulator in various tumors[13,14]. However, in AML, there are only preliminary studies of some known lncRNAs, such as HOTAIR[15], MALAT-1[16] and NEAT1[17]. Other lncRNAs, especially unknown lncRNAs that are involved in the regulation of AML, remain largely unclear. LINC00899, a newly identified lncRNA, is located in 22q13.31. RNA-seq showed that LINC00899 was ubiquitously expressed in human tissues, such as ovary, endometrium and bone marrow, indicating that LINC00899 may play an important biological role in human[18]. Feng et al[19] firstly reported that LINC00899 was abnormally expressed in AML patients by microarray and bioinformatics. To our best knowledge, this is the only study about the expression of LINC00899 in disease. Based on previous work, we further detected the expression levels of LINC00899 in bone marrow and serum of AML patients from our hospital and explored its clinical significance in AML patients.

Patients and Methods

Patients

AML patient samples were obtained from the Department of Hematology at the Affiliated Hospital of Shandong University of Traditional Chinese Medicine between June 2010 and August 2013. The diagnoses were made according to standard morphological criteria based on immunohistochemistry and cytogenetic studies, according to the AIEOP-2002 AML pediatric protocol[20]. According to the French-American-British (FAB) classification, 10 patients had AML M0, 41 had M1/M2, 43 had M3, 12 had M4/M5 and 47 had M6/M7. Clinical data, including baseline clinical characteristics, angiographic, laboratory features, and clinical outcomes, were recorded for all AML patients. In addition, 54 people without AML or other malignancies were recruited to act as healthy controls. Written informed consent was obtained from all patients prior to participation in the study. The medical Ethics Committee of The Second Affiliated Hospital of The Affiliated Hospital of Shandong University of Traditional Chinese Medicine approved the study.

RNA Extraction and Quantitative Real-Time PCR

Venous blood samples and bone marrow tissues were collected from 153 AML patients and 54 normal healthy controls. Serum samples were then stored at -80°C until further processing. Total RNA was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA was reverse transcribed into cDNA using Superscript III transcriptase, according to the manufacturer's protocol (Thermo Fisher Scientific, Waltham, MA, USA). qRT-PCR assays were performed to detect LINC00899 expression using the Prime Script RT reagent Kit and SYBR Premix ExTaq (TaKaRa, Otsu, Shiga, Japan) according to the manufacturer's instructions. The results were normalized to the expression of glyceraldehydes-3-phosphate dehydrogenase (GAPDH). The primers were as follows: LINC00899 sense: 5'- CCCAACAGGAAGGTCTGGT -3'; LINC00899 antisense: 5'- TCAGTGCTGGGTCATTCTTG -3'; GAPDH sense: 5'- AGAAGGCTGG GGCTCATTTG-3'; GAPDH antisense: 5'-AGGGGCCATCCACAGTCTTC-3'. Fold-change in expression was calculated by the relative quantification ($2^{-\Delta\Delta Ct}$) method.

Statistical Analysis

Statistical analyses were performed using SPSS 18.0 (SPSS Inc., Chicago, IL, USA) or Prism 5.0 (GraphPad Software, La Jolla, CA, USA) software. Mann-Whitney's U-test, Pearson's x^2 analysis and Fisher's exact test were used to compare the difference of continuous variables and categorical variables between the groups, respectively. Receiver operating characteristic (ROC) curve and area under the ROC curve (AUC) analyses were performed to assess the diagnostic value of LINC00899 in patients with AML and healthy controls. Survival analysis was performed by Kaplan-Meier method with the log-rank test. The Cox proportional hazards regression model was employed for univariate and multivariate analyses to estimate the prognostic factors for survival prediction. A p-value (two-side) < 0.05 was considered statistically significant.

Results

Upregulation of LINC00899 in Patients With AML

We firstly compared the expression levels of LINC00899 in bone marrow from 153 AML and 54 healthy controls. As shown in Figure 1A, the results of RT-PCR showed that the expression level of bone marrow LINC00899 was significantly higher in AML patients compared with healthy controls ($p < 0.01$). Moreover, we also detected the levels of serum LINC00899 in AML patients and healthy controls. As shown in Figure 1B, we found that serum LINC00899 levels were significantly upregulated in AML patients compared with that in the controls ($p = 0.001$). Thus, it was concluded that upregulation of LINC00899 might play important roles in AML progression.

The Diagnostic Significance of LINC00899 in AML

A receiver operating characteristic (ROC) curve was built to estimate the diagnostic value of serum LINC00899. As shown in Figure 1C, we found that serum LINC00899 was a potential biomarker for screening AML patients from healthy controls with the AUC of 0.807, and the serum LINC00899 level at 4.67 was the clear cutoff value to distinguish AML patients from healthy controls. At the optimal expression cutoff value of 4.67, the sensitivity was 92.7% and specificity was 83.4%.

Association Between LINC00899 Expression and Clinicopathologic Features of AML

We next analyzed the correlation between the expression of serum LINC00899 and clinicopathological characteristics of AML. The 153 human AML was classified into high serum LINC00899 group (n=75) and low serum LINC00899 group (n=78) according to the median serum LINC00899 expression level. As shown in Table I, serum LINC00899 levels were dramatically correlated with some clinicopathological parameters, including FAB classification ($p = 0.002$) and cytogenetics ($p = 0.005$). However, there was no significant correlation of LINC00899 expression with other clinical features such as gender, age, WBC Blast in BM, extramedullary disease and complete remission (all $p > 0.05$, Table I).

Table I. Relationship between serum LINC00899 expression and clinicopathologic features in AML.

Clinicopathological features	No. of cases	Serum LINC00899 expression		*p*-value
		High	Low	
Gender				NS
Male	91	41	50	
Female	62	35	27	
Age				NS
< 60	74	34	40	
≥ 60	79	41	38	
WBC				NS
< 10	46	21	25	
≥ 10	107	54	53	
Blast in BM				NS
< 50%	66	31	35	
≥ 50%	87	44	43	
Extramedullary disease				NS
No	111	51	60	
Yes	42	24	18	
Complete Remission				NS
Yes	94	41	53	
No	59	34	25	
FAB classification				0.002
M1-M5	106	43	63	
M6-M7	47	32	15	
Cytogenetics				0.005
Favorable	42	14	28	
Intermediate	73	35	38	
Unfavorable	38	26	12	

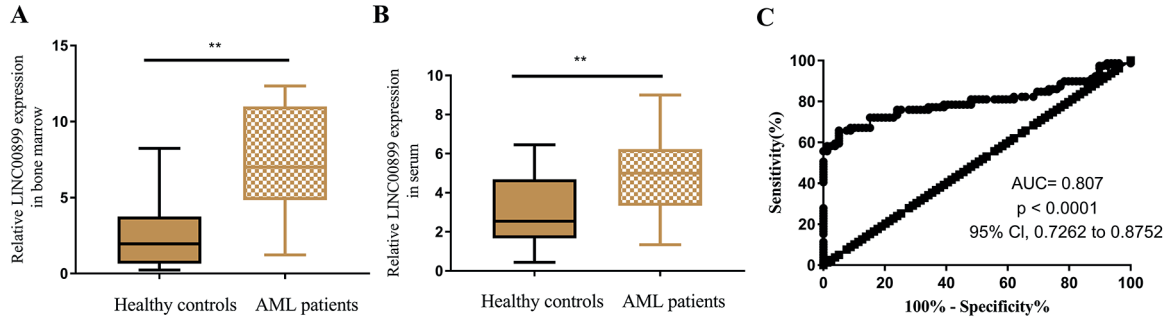

Figure 1. The expression of LINC00899 in AML patients and its diagnostic value. ***(A, B)*** up-regulation of LINC00899 in bone marrow and serum of AML patients. ***(C)*** ROC analysis for the diagnosis of AML patients using serum LINC00899 in AML patients and healthy controls. $^{**}p < 0.01$.

The Correlation Between Serum LINC00899 Expression Level and Treatment Response

In order to further explore the effect of serum LINC00899 in progression of AML, we compared the expression level of serum LINC00899 in AML patients before or after achieving a complete remission. As shown in Figure 2, we observed that serum LINC00899 expression level was significantly up-regulated when the AML patients achieved a complete remission.

Serum LINC00899 Upregulation Associates With Poor Prognosis in Patients With AML

To determine the prognostic value of serum LINC00899 expression in human AML, clinical follow-up was available for all patients. We performed log-lank test and Kaplan-Meier analysis to evaluate the association between serum LINC00899 expression level and clinical information in 153 AML patients mentioned above. As shown in Figure 3, we found that AML patients with high serum LINC00899 expression level had shorter overall survival than those with low serum LINC00899 expression level ($p = 0.0013$). Then, univariate analysis showed that FAB classification, cytogenetics and serum LINC00899 expression were significantly correlated with poor overall survival of AML patients ($p < 0.05$, Table II). Further multivariate analysis revealed that serum LINC00899 expression level, FAB classification and cytogenetics were independent prognostic indicators for the overall survival of AML patients (Table II). Those results indicated LINC00899 as a potential prognostic biomarker for AML patients.

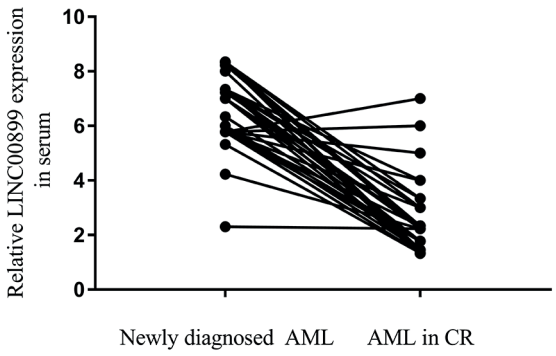

Figure 2. The association between serum LINC00899 expression level and treatment response in AML patients.

Discussion

AML is a malignant clonal disease, which is sustained by a subpopulation of long-term proliferative progenitor cells[21]. The poor prognosis of AML patients encouraged us to search novel treatment methods and sensitive biomarkers[22]. LncRNAs are key players in genetic programs that control differentiation and embryogenesis and a new group of potential biomarkers that may improve the diagnosis, prognosis and treatment monitoring of acute leukemias[23,24]. With the advancement of sequencing technology, it is easier to screen dysregulated lncRNAs. Currently, large part of the efforts focuses on identification of serum-based biomarkers for the diagnosis and prognosis of AML. Scholars[25-27] have illustrated that serum lncRNAs expression could be a novel potential biomarker for the diagnosis and prognosis of various cancers. However, only a few lncRNAs were reported to be abnormal-

Table II. Univariate and multivariate analyses of prognostic factors in AML.

Variables	Univariate analysis			Multivariate analysis		
	RR	95 % CI	*p*-value	RR	95 % CI	*p*-value
Gender Male *vs.* Female	1.224	0.672-2.231	0.346	-	-	-
Age < 60 *vs.* ≥ 60	1.562	0.782-2.667	0.216	-	-	-
WBC < 10 *vs.* ≥ 10	1.732	0.823-2.442	0.135	-	-	-
Blast in BM < 50% *vs.* ≥ 50%	1.452	0.932-2.667	0.273	-	-	-
Extramedullary disease No vs. Yes	1.569	0.678-1.994	0.136	-	-	-
Complete Remission Yes vs. No	1.774	0.895-2.672	0.114	-	-	-
FAB classification M1-M5 *vs.* M6-M7	3.564	1.428-5.372	0.003	2.983	1.273-4.664	0.008
Cytogenetics Unfavorable *vs.* Favorable/ Intermediate	3.236	1.267-4.557	0.006	2.894	1.139-4.023	0.013
Serum LINC00899 expression High *vs.* Low	3.784	1.475-6.283	0.001	3.198	1.288-5.275	0.003

ly expressed and act as regulators in AML progression. For instance, Chen et al[28] reported that lncRNA CCAT1 repressed monocytic differentiation and promoted cell growth of HL-60 by sequestering tumor suppressive miR-155, indicating lncRNA CCAT1 may be used as a treatment target. Sun et al[29] found that lncRNA UCA1 was highly expressed in AML cell lines, inhibited cell viability, migration, invasion, and prompted apoptosis of AML cells *in vitro* by via sponging miR-126. Mer et al[30] observed that AML patients in the ClinSeq cohort could be stratified into four distinct subtypes based on their lncRNA expression abundances and lncRNAs have enormous potential to be novel prognostic biomarkers. Feng et al[19] found that LINC00899 expression was significantly up-regulated in AML patients by microarray and bioinformatics. They also performed clinical assay and found that LINC00899 expression was significantly associated with overall survival of AML patients. However, there were no reports about the clinicopathologic and diagnostic significance of LINC00899 expression in AML. In addition, further deep clinical research was needed to confirm previous results. In this study, consistently with previous results, we also found that the expression level of LINC00899 was significantly higher in the bone marrow and serum of patients with AML than that of normal controls, and elevated LINC00899 expression was associated with FAB classification and cytogenetics, revealing that LINC00899 overexpression may promote an aggressive phenotype of AML. In addition, ROC curves indicated that serum LINC00899 level could be used to distinguish AML patients from healthy controls. Furthermore, our results demonstrated that patients with high serum LINC00899 expression had significantly poorer overall survival than those with low serum LINC00899 expression, providing evidence that increased expression of serum LINC00899 might facilitate an increased ma-

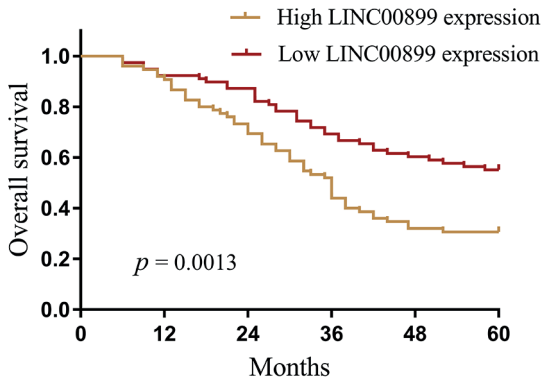

Figure 3. Kaplan-Meier analysis for the overall survival of AML patients with different expression of serum LINC00899. Patients with high serum LINC00899 expression had a shorter overall survival than those with low expression ($p = 0.0013$).

lignant and worse prognostic phenotype. More importantly, according to multivariate analyses, serum LINC00899 overexpression was an independent unfavorable prognostic biomarker for AML patients.

Conclusions

We demonstrated that LINC00899 was significantly downregulated in ESCC tissue and serum. It could serve as promising biomarker for both diagnosis and prognosis of AML. In the coming future, more large-scale and high-qualified investigations are needed to confirm our findings.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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