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Long noncoding RNA LINC00265 predicts the prognosis of acute myeloid leukemia patients and functions as a promoter by activating PI3K-AKT pathway

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Abstract. – OBJECTIVE: Mounting evidence suggests that long noncoding RNAs (lncRNAs) function in multiple cancers. This study aimed to determine the expression, clinical significance, and possible biological function of a novel lncRNA LINC00265 in acute myeloid leukemia (AML).

PATIENTS AND METHODS: The expression levels of LINC00265 were systematically evaluated in TCGA datasets. RT-PCR was performed to examine the expression level of LINC00265 in bone marrow and serum obtained from AML patients and healthy controls. The clinical data were interpreted by x^2 test, Kaplan-Meier analyses, univariate analysis, and multivariate analysis. The functional role of LINC00265 was verified using cell experiments. Western blotting was used to examine the modulatory effect of LINC00265 on AKT/PI3K pathway in AML.

RESULTS: LINC00265 was significantly highly expressed in the bone marrow and serum of AML patients. High serum LINC00265 was significantly associated with FAB classification and cytogenetics. ROC analyses showed that serum LINC00265 levels were reliable in distinguishing patients with AML from normal controls. Clinical assay indicated that AML patients with higher serum LINC00265 expression suffered poorer overall survival. Functionally, overexpression of LINC00265 suppressed the capability of proliferation, migration and invasion in AML cell lines. By using Western blot, we further illustrated that LINC00265 activated PI3K/AKT signaling in AML cell lines.

CONCLUSIONS: Our findings not only demonstrated that LINC00265 contributes to AML proliferation, migration and invasion via modulation of PI3K/AKT signaling, but also suggested the potential value of LINC00265 as a clinical prognostic and a diagnostic marker for AML.

Key Words:
Long noncoding RNA, LINC00265, Acute myeloid leukemia, Biomarker, Metastasis.

Introduction

Acute myeloid leukemia (AML) is a group of heterogeneous malignant disorders characterized by clonal proliferation of myeloid progenitor cells and differentiation arrest[1,2]. It is a heterogeneous disease featuring cytogenetic aberrations, recurrent somatic mutations and alterations in gene expression[3]. Recent evidence confirms that AML results from mutations in multiple genes involved in cell proliferation, survival and apoptosis[4]. Approximately 23000 patients will be diagnosed with AML in China during 2018, but less than 35% of them will survive beyond 5 years despite improved supportive therapies[5]. This highlights the need for more thorough knowledge of AML.

Long non-coding RNAs (LncRNAs) are a class of non-protein coding transcripts of >200 nucleotides and < 100 kb, which do not have open reading frames and thus do not translate to form proteins[6]. Widespread evidence shows that lncRNAs serve important roles in regulating various biological processes, including cell proliferation, cell invasion, cell differentiation and chromosome inactivation[7,8]. It has been reported that the dysregulation of lncRNAs occurs in numerous diseases, including cancers, and affects tumor development and progression[9,10]. Up to date, more and more lncRNAs, such as, lncRNA SUMO1P3, lncRNA CASC9 and lncRNA SNHG1, were identified to act as oncogenes or tumor suppressors in tumor progression by regulating cells proliferation, metastasis and apoptosis[11-13]. However, the expression pattern and biological function of lncRNAs in AML remain largely unclear. LINC00265 is a new identified lncRNA in human cancers. Up-regulation of LINC00265 has been reported in lung adenocarcinoma and it may

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act as a prognostic biomarker for this disease[14]. However, its expression pattern and function in other tumors remains largely unknown. In this study, by analyzing The Cancer Genome Atlas (TCGA), we found that LINC00265 expression was significantly up-regulated in AML compared to healthy controls. Then, we performed a series of experiments to explore the expression, biological function and clinical significance of LINC00265 in AML progression. This study advances our understanding of the role of LINC00265 as regulator of AML pathogenesis.

Patients and Methods

Patients

A total of 135 patients with AML from the Affiliated Huaian NO.1 People's Hospital of Nanjing Medical University were enrolled in this study. The bone marrow specimens and peripheral blood of the AML patients were collected from March 2010 to April 2013. Written informed consent was signed by all participants. This study was approved by the Ethics Committee of the Affiliated Huaian No. 1 People's Hospital of Nanjing Medical University (Nanjing, China). None of the patients had received radiotherapy and chemotherapy before surgery. The collected tissue samples were stored at -80°C for later use. The clinicopathological information of the patients was listed in Table I. The control group consisted of 35 healthy volunteers with no clinical symptoms of cancer.

Cell Culture

The human leukemia cell lines HL-60 were purchased from the Chinese Academy of Sciences Cell Bank (Xuhui, Shanghai, China). The AML cell lines NB4 and human normal stromal cells HS-5 were purchased from the American Type Culture Collection (ATCC, Manassas, MA, USA). All the cell lines were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Meilun Biotechnology, Dalian, Liaoning, China) supplemented with 10% fetal bovine serum (FBS; Excell Bio, Taicang, Jiangsu, China), 2 mM L-glutamine (Sangon, Pudong, Shanghai, China) and Penicillin-Streptomycin Solution for cell culture (Beyotime, Shanghai, China) in a humidified incubator at 37°C with 5% CO_2.

Table I. Association of serum LINC00265 level with clinical characteristics of 135 newly diagnosed AML patients.

Variable	n	LINC00265 expression		p
		High	Low	
Gender				0.540
Male	65	30	35	
Female	70	36	34	
Age				0.329
< 60	61	27	34	
≥ 60	74	39	35	
WBC				0.715
< 10	45	21	24	
≥ 10	90	45	45	
Blast in BM				0.528
< 50%	61	28	33	
≥ 50%	74	38	36	
FAB classification				**0.033**
M1-M6	97	53	44	
M7	38	13	25	
Extramedullary disease				0.598
Absent	87	44	43	
Present	48	22	26	
Cytogenetics				**0.002**
Favorable	38	8	30	
Intermediate	58	30	28	
Unfavorable	39	28	11	
Complete remission				0.359
Yes	56	30	26	
No	79	36	43	

Cell Transfection

Small interfering RNAs (siRNA) specific against LINC00265 (siRNA-1 and siRNA-2) and negative control siRNAs (NC) were synthesized by GenePharma Co., Ltd. (Pudong, Shanghai, China). The cell transfection was conducted by Lipofectamine 2000 reagent (Invitrogen Co., Carlsbad, CA, USA) according to the manufacturer's protocols. In brief, after HL-60 or NB4 cells (4×10^5 cells per well) were maintained in 6-well plates (NEST, Wuxi, Jiangsu, China) for 24 h, 16 μl siRNAs (20 μM; siRNA-1, siRNA-1 or NC) were mixed with 16 μl of Lipofectamine 2000 reagent in the Opti-MEM (500 μl; Invitrogen, Carlsbad, CA, USA) for 10-15 min at room temperature. Thereafter, the mixtures were added into the cultured cells and the medium were changed after culturing for 5 h.

Reverse Transcription-Quantitative Polymerase Chain Reaction (qRT-PCR)

Total RNA was isolated from cultured cells or AML samples using TRIzol reagent (Leagene Biotechnology, Fengtai, Beijing, China) according to the manufacturer's instructions. Subsequently, appropriate 2 μg total RNA was reverse transcribed to cDNA using ABScript II cDNA First Strand Synthesis Kit (ABclonal, Wuhan, China) and qRT-PCR assay was conducted using ExCell (MB) MB000-3043 2 × HotStart SYBR Green qPCR Master Mix (Excell Bio, Taicang, Jiangsu, China). The qRT-PCR assay was performed on a Stratagene MX3005P Real-time PCR instrument (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal references for mRNA and the $2^{-\Delta\Delta Ct}$ method was used to analyze the mRNA relative expression. The primer sequences were as follows: GADPH: 5'-GTCAACGGATTTGGTCTGTATT-3' (forward), 5'-AGTCTTCTGGGTGGCAGTGAT-3' (reverse); LINC00265: 5'-GGAAGAGAGACTGACTGGGC-3' (forward), 5'- GTTTCGCTGTCACCCCTCTG -3' (reverse).

Western Blot Assay

Radioimmunoprecipitation assay (RIPA) lysis buffer (Yeasen Pudong, Shanghai, China) supplemented with protease inhibitor cocktail (Cwbio, Changping, Beijing, China) were utilized to extracted the total proteins of the HL-60 and NB4 cells. The protein concentration was determined by a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Subsequently, the protein extracts were separated using 8-12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto the polyvinylidene difluoride (PVDF) membranes (Thermo Fischer Scientific, Waltham, MA, USA). Membranes were then incubated with 5% BSA in TBST at room temperature for 1 h. After washing with PBS containing 0.05% Tween for three times, the membranes were incubated with primary antibodies overnight. The primary antibodies against p-PI3K, PI3K, p-AKT and AKT were purchased from Cell Signaling Technology Co., Ltd. (Danvers, MA, USA). The primary antibody against GAPDH was obtained from Protein Tech Co., Ltd. (Wuhan, Hubei, China). Afterwards, the protein bands were revealed by corresponding secondary antibodies, ECL chromogenic substrate (Beyotime, Pudong, Shanghai, China) using Odyssey Infrared Imaging System (LI-COR, Lincoln, NE, USA). In addition, ImageJ software (NIH, Bethesda, MD, USA) was utilized to assess the optical density of the protein bands.

Cell Counting Kit-8 (CCK-8) Assay

The proliferative rates of HL-60 and NB4 cells under various conditions were evaluated by a CCK-8 assay kit (MedChemExpress, Pudong, Shanghai, China) in accordance with the manufacturer's protocols. In short, HL-60 or NB4 cells (1×10^3 cells per well) were plated in 96-well plates (NEST, Wuxi, Jiangsu, China). Then, 10 μl CCK-8 solution were added into each well. After maintaining at 37°C for an additional 1-2 h, microplate reader (Infinite M200; Tecan, Männedorf, Switzerland) was employed to detect the absorbance of each well at the wavelength of 450 nm.

Cell Apoptosis and Cell Cycle Analysis

An Annexin-V-FITC apoptosis detection kit (BD, Franklin Lakes, NJ, USA) was employed to analyze the cell apoptosis of HL-60 and NB4 cells. Briefly, cells were collected, washed with ice-cold PBS, and resuspended in appropriate binding buffer. Afterwards, the cells were stained using Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) for 15-20 min in the dark. Then, the results were measured by a FACSCalibur system (BD Biosciences, Franklin lakes, NJ, USA) and analyzed with Flow Jo software (Version 7.6.1; Tree Star Inc., Ashland, Oregon, USA). For cell cycle analysis, the cells were harvested, fixed with 70% ethanol at 4°C, and then stained with PI/RNase A mixture solu-

tion according to the protocols of the Cell Cycle Analysis Kit (KeyGEN BioTech., Nanjing, Jiangsu, China). Next, the cells were kept in the dark at 4°C for 30 min. Then, the cell cycle stages were analyzed using a FACSCalibur system (BD Biosciences, Franklin Lakes, NJ, USA).

Transwell Assays

Transwell assays were applied to determine the migratory and invasive abilities of HL-60 and NB4 cells. The cells (100 µl; 5×10^4 cells), which were suspended in serum-free medium, were plated on uncoated (for migration assays) or Matrigel (1:8, BD Biosciences, Franklin Lakes, NJ, USA) coated (for invasion assays) upper chambers of transwell inserts (8 µM pore sized, BD Biosciences, Franklin Lakes, NJ, USA), respectively. Subsequently, the lower compartments were filled with RPMI-1640 complete medium. After 24 h of incubation, the upper surface of the membrane was removed with a cotton swab, and the cells that traversed the membrane were fixed with 70% ethanol at room temperature, stained with 0.3% crystal violet solution (Sigma-Aldrich, St. Louis, MO, USA). After washing with PBS twice, the invading cells were photographed using an inverted microscope (IX71, Olympus, Tokyo, Japan).

Statistical Analysis

All data were generated from tripartite independent experiments and presented as the mean ± standard error of the mean (SEM). SPSS 20.0 software (IBM Corp., IBM SPSS Statistics for Windows, Armonk, NY, USA) and GraphPad Prism Software (version 7; GraphPad Software, Inc., La Jolla, CA, USA) were utilized for all statistical analyses. The differences between groups were evaluated by using Student's t-test and one-way ANOVA. Tukey's post-hoc test was used to validate the ANOVA for comparing measurement data between groups. Survival curve was generated using the Kaplan-Meier method, and assessed by a log-rank test. The Cox proportional hazards model was used in the multivariate analysis. Statistically significance was considered as $p < 0.05$.

Results

LINC00265 Was Highly Expressed in AML Patient and Cell Lines

In order to reveal the role of LINC00265 in progression of AML, we screened abnormally expressed lncRNAs using TCGA data. As shown in Figurer 1A, we found that LINC00265 was significantly highly expressed in AML patients. Then, results of RT-PCR showed that the expression level of bone marrow LINC00265 was significantly higher in AML patients compared with healthy controls ($p < 0.01$, Figure 1B). Similarly, compared with the healthy individuals, serum LINC00265 expression in AML patients was significantly up-regulated ($p < 0.01$, Figure 1C). In addition, we also detect the expression of LINC00265 in several AML cell lines. As shown in Figure 1D, we found that higher expression level of LINC00265 was detected in four AML cell lines when compared with HS-5. Furthermore, we performed ROC curve to explore the diagnostic value of serum LINC00265 in AML. As shown in Figure 1E, the AUC value was 0.8131 ($p < 0.001$; 95% CI, 0.7256 to 0.9007). At the optimal diagnostic cut-off point of serum LINC00265 level was 4.63, the sensitivity was 72.43% and the specificity was 91.45%. Taken together, our findings suggested that LINC00265 was dysregulated in AML may be a useful marker for disease status.

Upregulation of LINC00265 Was Correlated With Poor Prognosis

In order to investigate the association between LINC00265 expression and clinicopathological features in AML, the median expression level of serum LINC00265 was used as a cutoff point to divide all AML patients into two groups (High expression group and Low expression group). As shown in Table I, we found that high serum LINC00265 was significantly associated with FAB classification ($p = 0.033$) and cytogenetics ($p = 0.002$). However, there was no significant correlation of serum LINC00265 expression with other clinical features such as gender, age, tumor size, WBC, blast in BM, extramedullary disease and complete remission ($p > 0.05$). Then, we further explored the prognostic value of LINC00265 using TCGA, finding that LINC00265 expression was significantly correlated with AML patients' overall survival (Figure 2A). Moreover, in our 135 AML patients, we also found that patients with high LINC00265 expression had shorter overall survival compared with the low LINC00265 group ($p = 0.0082$). Finally, in multivariate analysis, we confirmed that the expression of serum LINC00265 was an independent prognostic factor for overall patient survival (RR: 3.237, CI: 1.158-4.564, $p = 0.008$, Table II).

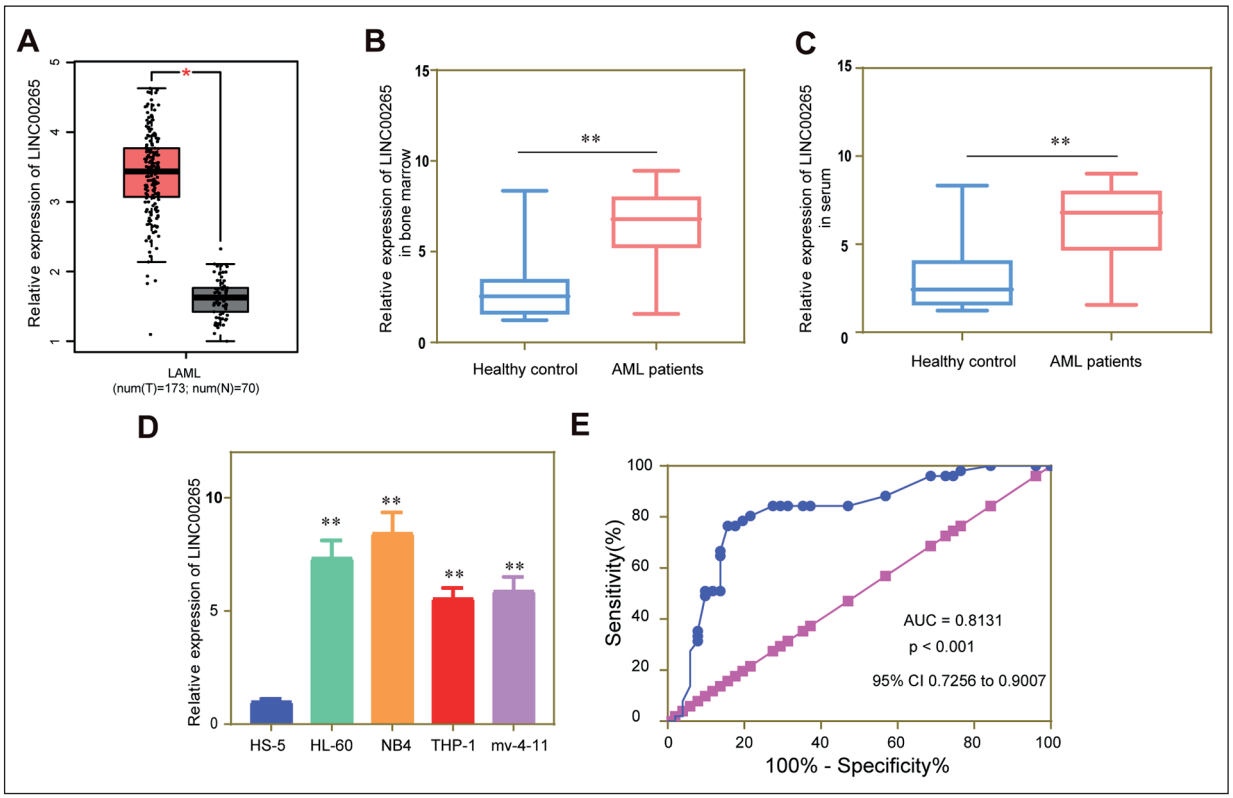

Figure 1. The expression of LINC00265 in AML patient samples and cell lines. ***(A)*** Data mining of LINC00265 expression levels in AML patients nad healthy controls from TCGA. ***(B)*** The expression levels of bone marrow LINC00265 in AML patients and healthy controls by RT-PCR. ***(C)*** The expression levels of serum LINC00265 in AML patients and healthy controls by RT-PCR. ***(D)*** The expression levels of LINC00265 in HL-60, NB-4, THP-1 and HS-5 cells were detected by qRT-PCR. ***(E)*** Diagnostic value of serum LINC00265 level in patients with AML. *$p < 0.05$, **$p < 0.01$.

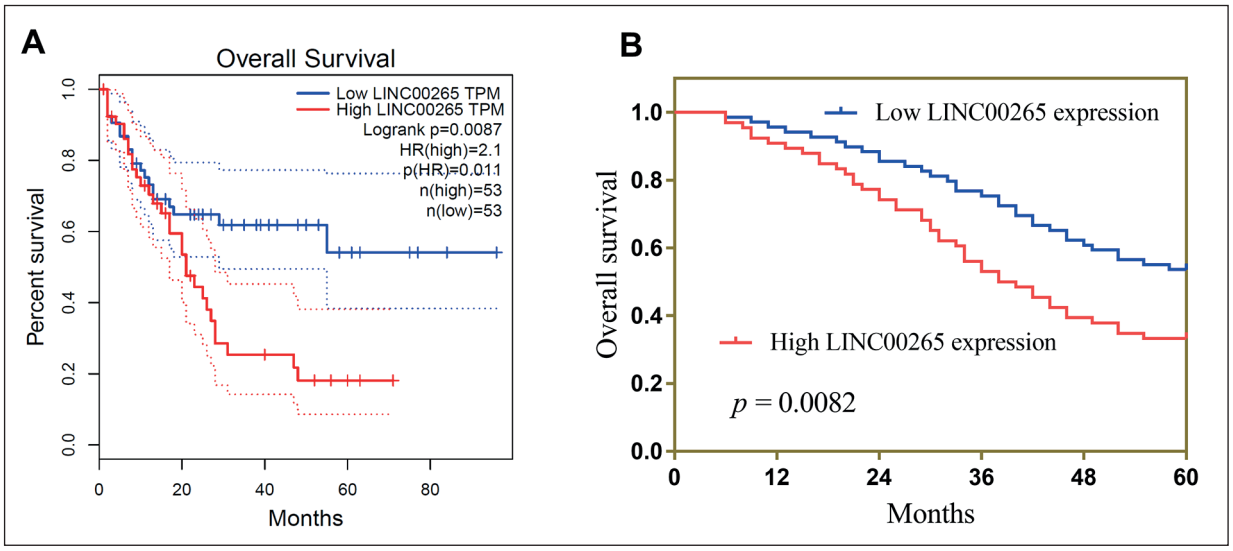

Figure 2. Up-regulated expression of LINC00265 was correlated with poor prognosis of AML. ***(A)*** Kaplan-Meier survival plots demonstrated that higher LINC00265 abundance correlated with a poorer OS, using microarray data from 106 AML patients. ***(B)*** Kaplan-Meier overall survival curves according to LINC00265 expression levels in 135 AML patients.

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

Variables	Univariate analysis			Multivariate analysis		
	RR	95% CI	*p*-value	RR	95% CI	*p*-value
Age	1.562	0.782-2.233	0.415	1.493	0.944-2.039	0.327
Gender	1.662	0.672-2.234	0.215	-	-	-
Age	1.346	0.833-2.548	0.359	-	-	-
WBC	1.675	0.734-2.541	0.187	-	-	-
Blast in BM	1.358	1.133-2.189	0.165	-	-	-
FAB classification	3.213	1.342-4.782	**0.014**	2.893	1.142-4.166	**0.027**
Extramedullary disease	1.421	0.895-2.155	0.194	-	-	-
Cytogenetics	3.895	1.437-6.233	**0.001**	3.136	1.137-5.348	**0.006**
Complete remission	1.554	0.785-2.169	0.219	-	-	-
Serum LINC00265 expression	3.667	1.327-5.438	**0.003**	3.237	1.158-4.564	**0.008**

The Effects of LINC00265 on the Proliferation, Apoptosis, Cell Cycle, Migration and Invasion of HL-60 and NB4 Cells

Given that LINC00265 was highly expressed in AML tissues and cell lines, as well as it was associated with poor prognosis, we asked whether LINC00265 played essential roles in regulating the development and progression of AML cells. Hence, loss-of-function studies using siRNAs specific against LINC00265 (siRNA-1 and siRNA-2 were performed to investigate the effects of LINC00265 on the biological functions of HL-60 and NB4 cells. The results of qRT-PCR assays showed that both siRNA-1 and siRNA-2 could reduce the expression of LINC00265 effectively in HL-60 and NB4 cells (Figure 3A). Next, the influence of LINC00265 depletion on the proliferation of HL-60 and NB4 cells was assessed using CCK-8 assays. The data suggested that knockdown of LINC00265 resulted in a remarkably decreased proliferative rates of the HL-60 and NB4 cells compared to the corresponding negative control (NC) group (Figure 3B and C). Furthermore, flow cytometry analysis was carried out to evaluate the alteration of cell cycle and apoptosis of HL-60 and NB4 cells after LINC00265 was knockdown. The results indicated that depression of LINC00265 dramatically accelerated the apoptosis of HL-60 and NB4 cells (Figure 3D). The cell cycle analysis revealed that supersession of LINC00265 in HL-60 and NB4 cells promoted G0/G1 cell cycle arrest (Figure 3E). Additionally, the effects of LINC00265 on migration and invasion were also investigated using transwell assays. The data of the transwell migration assays confirmed that knockdown of LINC00265 led to a significant reduction of migratory capabilities of HL-60 and NB4 cells (Figure 3F). Besides, the transwell invasion assays demonstrated that transfection of LINC00265 siRNAs notably impaired the invasive abilities of HL-60 and NB4 cells (Figure 3G). Taken together, our data provided evidence that LINC00265 played essential roles in modulating the biological functions of AML cells.

Depression of LINC00265 Regulated the Activity of PI3K/Akt Signaling in AML Cells

To identify key downstream signaling pathways that were modulated by LINC00265 involved in the development and progression of AML, we focused on PI3K/Akt signaling which had diverse functions in tumor progression. Therefore, Western blot assays were performed to determine the alteration of proteins involved in PI3K/Akt signaling. As the data shown in Figure 4A, transfection of LINC00265 siRNAs resulted in a remarkable decline of phosphorylated PI3K (p-PI3K) as well as phosphorylated AKT (p-AKT) in HL-60 cells, whereas there was no significant impact on the protein levels of PI3K and AKT. Similarly, silencing the expression of LINC00265 also reduced the expression of p-PI3K and p-AKT in NB4 cells, while no significantly suppressed expression of PI3K and AKT was observed in LINC00265 siRNAs transfected NB4 cells (Figure 4B). To sum up, our results uncovered that LINC00265 knockdown could attenuate the activation of the PI3K/Akt signaling pathway in AML cells, and these results further indicated that LINC00265 served as critical roles in regulating the development and progression of AML by affecting PI3K/Akt signaling pathway.

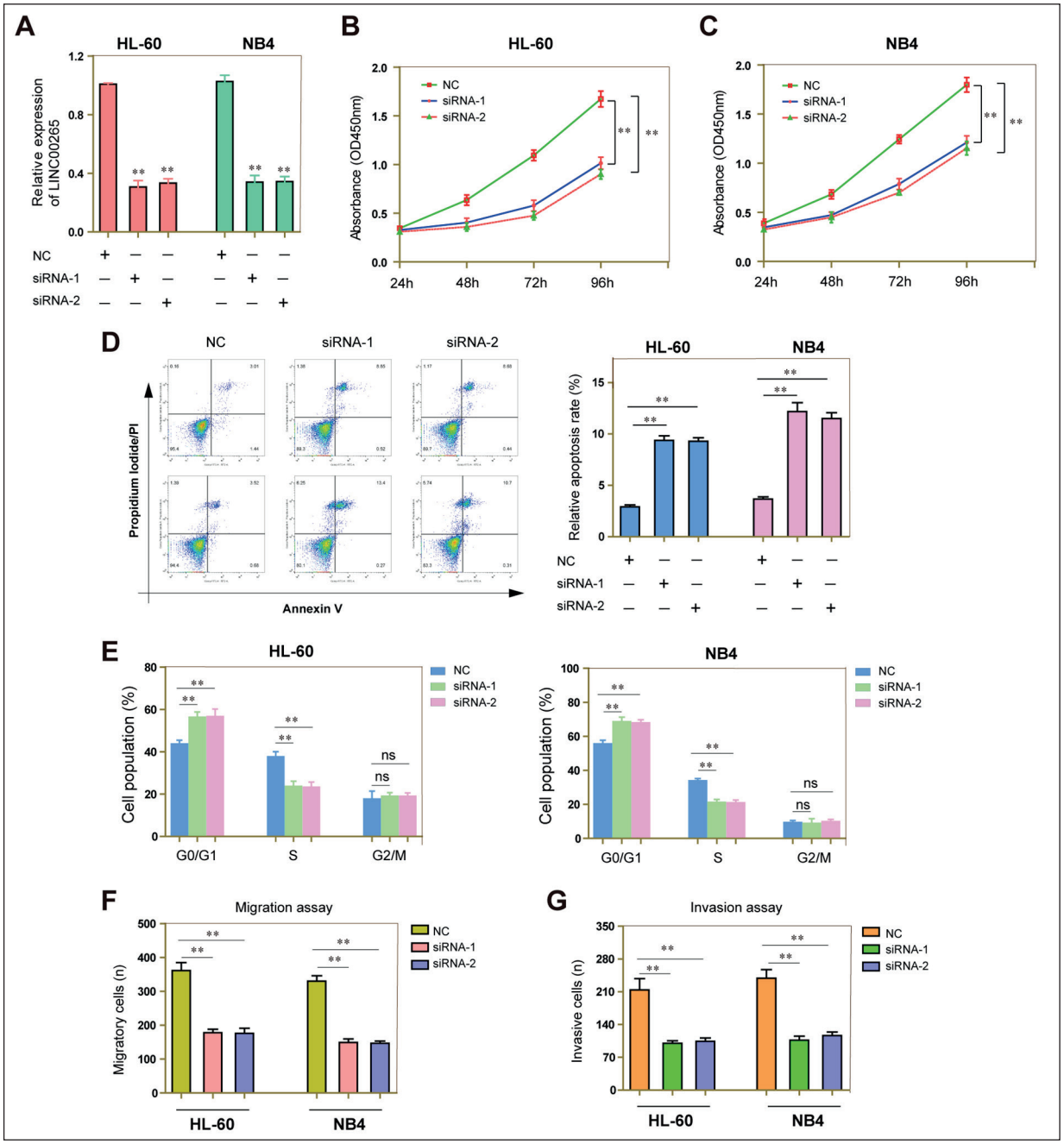

Figure 3. Knockdown of LINC00265 affected the proliferation, cell cycle, apoptosis, migration and invasion of HL-60 and NB4 cells. ***(A)*** Expression of LINC00265 in HL-60 and NB4 cells was determined by qRT-PCR assays. ***(B*** and ***C)*** Cell viabilities of HL-60 and NB4 cells transfected with siRNAs were determined by the cell CCK-8 assays at different time points (24, 48, 72 and 96 h). ***(D)*** The apoptotic rates of HL-60 and NB4 cells transfected with LINC00265 siRNAs (siRNA-1 or siRNA-2) or negative control siRNAs (NC) were determined by flow cytometry analysis. ***(E)*** Cell cycle analysis of HL-60 and NB4 cells after transfection with siRNAs. ***(F)*** The migratory abilities of HL-60 and NB4 cells were assessed by transwell migration assays. ***(G)*** The transwell invasion assays determined the invasive cell number of HL-60 and NB4 cells. *$p < 0.05$, **$p < 0.01$.

Discussion

AML is the most common form of hematological malignant tumor that threatens human health[15]. Up to date, there are two systems that are widely used in the classification of AML, the French-American-British (FAB) and the World Health Organization (WHO) systems[16,17]. These two systems are frequently used to predict the prognosis of AML patients. However, the sen-

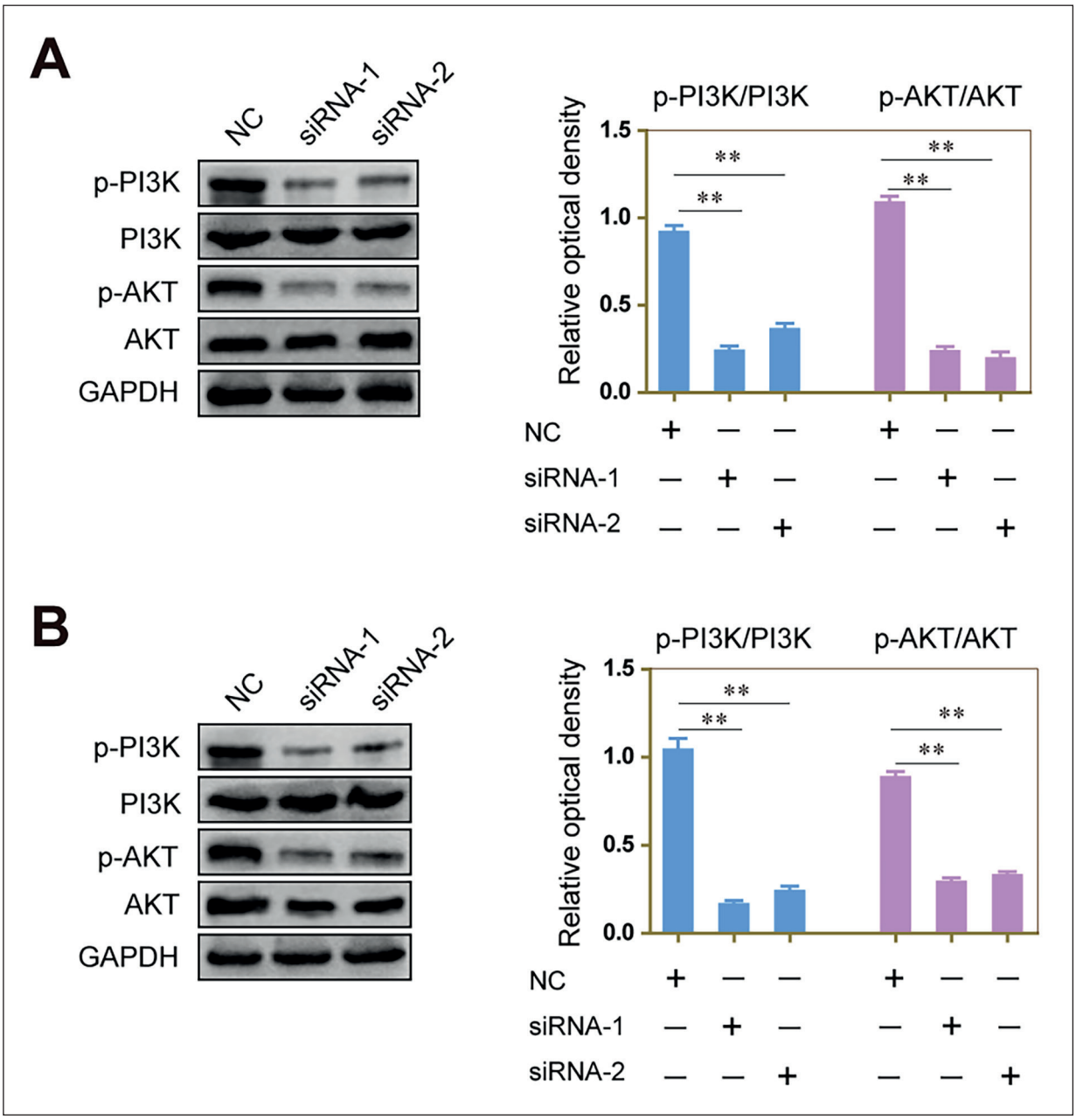

Figure 4. Effects of LINC01426 on PI3K/AKT signaling in AML. ***(A)*** The protein expression levels and optical density analysis of p-PI3K, PI3K, p-AKT and AKT in HL-60 cells detected by Western blot assays. ***(B)*** Western blot assays were performed to determine the protein expression levels p-PI3K, PI3K, p-AKT and AKT in NB4 cells, and the optical density of each protein bands were also analyzed. *$p < 0.05$, **$p < 0.01$.

sibility is low due to personality and individual differences. Recently, lncRNAs become a hot candidate for diagnosis and prognosis of tumor patients because of its important role in tumor progression and the development of chip technology, which makes it easy to screen dysregulated lncRNAs[18-20]. In this study, by online bioinformatics analysis, we focused on a novel oncogenic lncRNA, LINC00265, whose up-regulation was showed by RT-PCR assay. In addition, our results also showed that LINC00265 could act as potential diagnostic biomarkers for AML. Then, according to clinical assay, LINC00265 was observed to be associated with FAB classification and cytogenetics. Further survival assay revealed that patients with

high LINC00265 expression lived shorter than those with low LINC00265 expression. More importantly, our multivariant analysis revealed the LINC00265 was an independent prognosis factor for AML. Taken together, our findings highlighted LINC00265 as an important potential biomarker for diagnosis and prognosis of AML patients. As a newly identified lncRNA, the role of LINC00265 was rarely reported. Recent study by Li et al[14] reported that LINC00265 expression was up-regulated in lung adenocarcinoma and associated with advanced clinical progression, indicating that LINC00265 may be a tumor promoter in tumors. Recently, growing lncRNAs were reported to act as important regulators in AML cells proliferation, migration and apoptosis[21,22]. In this study, we firstly explored the functional role of LINC00265 in human AML. Using siRNA, we successfully down-regulated LINC00265 in AML cell lines. Then, using several functional experiments, we showed that knockdown of LINC00265 significantly suppressed AML cells proliferation, migration and invasion, and promoted apoptosis, indicating that LINC00265 acted as a positively regulator in progression of AML. Our findings indicated that epigenetic regulator LINC00265 may be a potential therapeutic target for anti-cancer treatments for AML. The phosphatidylinositol-3-kinase (PI3K)/Akt/ signaling pathway plays an important role in cell proliferation and differentiation and therefore is frequently deregulated in various tumors[23,24]. There is growing evidence that PI3K-AKT pathway is frequently activated in AML and also responsible for modulating multiple processes in AML[25,26]. In order to explore the potential mechanism by which LINC00265 affected the biological progression of AML, we forced on the association between LINC00265 and the activation of PI3K-AKT pathway. We observed that knockdown of LINC00265 decreased the level of p-PI3K and p-Akt, but did not significantly decrease PI3K and Akt. The suppressed phosphorylations of PI3K and Akt indicated that LINC00265 displayed its oncogenic role by modulating PI3K-AKT pathway.

Conclusions

Our findings identified a novel lncRNA LINC00265, which was upregulated in AML and associated with poor prognosis of AML patients. Knockdown of LINC00265 suppressed AML cell proliferation, migration and invasion, promoted AML cells apoptosis by suppressing PI3K-AKT pathway. LINC00265 may be used as a prognostic and diagnostic factor and a therapeutic target for AML patients.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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