Long non-coding RNA LINC00460 serves as a potential biomarker and oncogene via regulation of the miR-320b/PBX3 axis in acute myeloid leukemia

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Abstract. Long non-coding RNA 00460 (LINC00460) has been reported to be involved in the tumorigenesis of various cancer types. However, the function of LINC00460 in acute myeloid leukemia (AML) remains elusive. Therefore, the present study aimed to investigate the role of LINC00460 in AML. The expression of LINC00460 in the serum of 80 diagnosed patients with AML and 67 healthy controls was measured via reverse transcription-quantitative polymerase chain reaction, and the results were compared with clinical features and patient outcomes. The expression of LINC00460 in 45 patients with cytogenetically normal-AML (CN-AML) was also assayed. Receiver operating characteristic (ROC) curves were generated to evaluate the sensitivity and specificity of serum LINC00460. In addition, the effects of LINC00460 on the viability, cell cycle distribution and apoptosis of AML cells were investigated. Bioinformatics tools were used to identify the possible mechanisms of how LINC00460 affects AML cells. It was found that the expression of LINC00460 was significantly upregulated in the serum of patients with AML and those with CN-AML. Higher expression of serum LINC00460 was positively associated with French-American-British classification and cytogenetics. Furthermore, ROC curve analyses demonstrated that serum LINC00460 could differentiate patients with AML from healthy individuals with an area under the curve of 0.8488 (95% CI, 0.7697-0.9279). The serum LINC00460 expression was also significantly decreased when the patients achieved complete remission. Kaplan-Meier analysis indicated that patients with high serum LINC00460 expression had a shorter overall survival time compared with the low serum LINC00460 expression group. Knockdown of LINC00460 inhibited viability, while inducing cell cycle arrest and apoptosis in AML cells. LINC00460 was also a decoy of microRNA (miR)-320b, which can further inhibit the expression of PBX homeobox 3 (PBX3). Collectively, the results suggested that LINC00460 may be applied as a potential diagnostic and prognostic biomarker for patients with AML. It was identified that LINC00460 may exert its effects, at least partly, via the miR-320b/PBX3 axis in AML.

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Introduction

Acute myeloid leukemia (AML) is a heterogeneous malignant disorder characterized by uncontrolled proliferation of hematopoietic progenitor cells and differentiation arrest (1). AML is the most common form of acute leukemia and the second most common type of leukemia diagnosed worldwide (2). The incidence of AML is directly proportional to an increase in age (3). Previous studies have reported that AML results from mutations in different genes involved in the processes of cell proliferation, survival and apoptosis (4). For instance, mutations in RAS, tyrosine kinase signaling pathways (*CBL, FLT3, JAK2, KIT, PTPN11*), chromatin modifiers (*ASXL1/2, BCOR/L1, TET2*) and an additional 50 genes have been found to be associated with occurrence of AML (5). According to cytogenetic information, patients with AML can be divided into three risk-based subgroups: Favorable, intermediate and poor (6). Although great progress has been made in chemotherapy-based regimens and hematopoietic stem cell transplantation, the prognosis of AML remains unsatisfactory, and the 5-year survival rate is <30% (7). Early diagnosis of AML can increase the overall survival rate of patients with AML (3). Therefore, it is necessary to identify novel and sensitive biomarkers for the diagnosis and prognosis of patients with AML.

Long non-coding RNAs (lncRNAs) are a group of non-coding RNAs that are between 200 bp and 100 kb in

size (8). Due to the absence of open reading frames, lncRNAs lack the ability to encode proteins (9). Mounting evidence has suggested that lncRNAs serve essential roles in the regulation of various cellular biological processes, such as proliferation, differentiation, metabolism and programmed cell death (10). Aberrant expression of non-coding RNAs has been observed in multiple diseases, including cancer (11). To date, numerous lncRNAs, such as nuclear paraspeckle assembly transcript 1 (NEAT1), HOX transcript antisense RNA (HOTAIR) and metastasis-associated lung adenocarcinoma transcript 1 (MALAT1), have been identified as important factors in the regulation of AML (12). lncRNA 00460 (LINC00460) is a newly discovered lncRNA located on human chromosome 13q33.2 with a transcript size of 935 bp (13). Previous studies have revealed that LINC00460 functions as an oncogene in various malignancies, such as lung cancer, colorectal cancer, gastric cancer, ovarian cancer and osteosarcoma (14-18). However, the status of LINC00460 expression and its prognostic significance in AML remain elusive. To address this question, the present study aimed to investigate the diagnostic and prognostic value of serum LINC00460 in patients with AML.

MicroRNAs (miRNAs/miRs) are another type of non-coding RNA, with a size of ~20 nucleotides, that can inhibit target mRNAs at the post-transcriptional level (19). Previous studies have reported that miRNAs serve essential roles in the initiation and progression of various cancer types, including AML (20). Among the various miRNAs, miR-320b has been implicated in the tumorigenesis of different cancer types, such as glioma, colorectal cancer, lung cancer and prostate cancer (21-24). However, knowledge regarding the role of miR-320b in AML is currently lacking.

PBX homeobox 3 (PBX3) is a homeodomain-containing transcription factor that belongs to the pre-B cell leukemia family (25). The role of PBX3 has been extensively studied in AML. For instance, enhanced expression of PBX3 with its cofactor Meis homeobox 1 (MEIS1) can transform normal hematopoietic stem cells into AML in mice (26). Furthermore, silencing PBX3 can increase the sensitivity of AML cells to chemotherapy agents (27). However, to the best of the authors' knowledge, there are no reports investigating the association between LINC00460, miR-320b and PBX3.

The present study examined the prognostic and biological roles of LINC00460 in AML. The *in vivo* expression of LINC00460 was measured by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The effects of LINCO00460 on the viability, cell cycle distribution and apoptosis were assayed *in vitro*. Furthermore, the mechanisms underlying the functions of LINCO00460 were also investigated. The findings suggested that LINC00460 might be a prognostic biomarker and therapeutic target for AML.

Materials and methods

Patient samples. A total of 80 diagnosed AML cases were recruited between December 2018 and January 2010 at the First Affiliated Hospital of Wenzhou Medical University, which were classified as favorable-risk cytogenetic (20 subjects), intermediate-risk cytogenetic (42 subjects) and poor-risk cytogenetic (18 subjects). The patients consisted of 38 females and 42 males, aged 37-87 years. The median value of expression of LINC00460 was 2.36 and was used as a cutoff value. All patients were divided into two groups: High LINC00460 expression group (>2.36; n=36) and low LINC00460 expression group (≤2.36; n=44). Of the 80 patients with AML, 45 cases were classified as cytogenetically normal-AML (CN-AML). The diagnosis of patients with AML was made according to the French-American-British (FAB) and World Health Organization criteria combined with immunophenotyping and cytogenetic analysis (28). Additionally, 67 healthy volunteers without any type of malignancy or other benign disease were enrolled as controls.

Firstly, the serum (0.5 ml) was collected from all patients before any treatment. After receiving different treatments [chemotherapy and (n=41)/or targeted therapy (n=1)], 42 patients achieved complete remission (CR), the serum (0.5 ml) of these patients was collected again.

This research was approved by the Ethics Committee of Wenzhou Medical University. Written informed consent was obtained from all patients prior to participation in the study.

Cell culture and transfection. Human bone marrow stromal cells (HS-5) and human AML cells (THP-1, KG-1, K562 and HL-60) were purchased from the Cell Bank of Shanghai Institute of Biological Sciences, Chinese Academy of Sciences. All cells were cultured in RPMI-1640 medium (HyClone; Cytiva) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 μg/ml streptomycin (Thermo Fisher Scientific, Inc.) at 37˚C with 5% CO_2.

For transfection, small interfering RNA (siRNA) against LINC00460 (si-LINC00460), negative control siRNA (si-NC), pcDNA3.1 vector, LINC00460 overexpression vector (pcDNA-LINC00460), miR-320b mimic, miR-NC, miR-320b inhibitor, miR-NC inhibitor and PBX3 overexpression vector (pcDNA-PBX3) were synthesized by Suzhou GenePharma Co., Ltd. The target sequences were as follows: si-LINC00460, 5'-CACACUUCTCGGCUAAG-3'; si-NC, 5'-AACAGGCAU CCUACGACGCCA-3'; miR-320b mimics, 5'-AAAGCUGGG UUGAGAGGGCAA-3'; miR-NC, 5'-AAUUCUCCGAAC GUGUCACTT-3'; miR-320b inhibitor, 5'-UUGCCCUCU CAACCCAGCUUU-3'; and miR-NC inhibitor, 5'-AAACAC GGUUAUAUCACCAUCGCAUUA-3'. All transfections were conducted using Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Cells were seeded into 6-well plate at the density of $3x10^5$ cells/well in 2 ml complete medium. After 24 h of incubation at 37˚C, the medium was changed to 300 μl Opti-MEM (Gibco; Thermo Fisher Scientific, Inc.) containing 2 μg pcDNA-PBX3 or 2 μg pcDNA-LINC00460 with 50 mM siRNA. To modulate the expression of miR-320b, 20 mM miRNA mimics or 20 mM miRNA inhibitor in 200 μl Opti-MEM containing 5 μl Lipofectamine® 2000was added into each well. A total of 4 h after incubation, 1.5 ml complete medium was added into each well and the cells were continually incubated at 37˚C for 48 h for following experiments. Experiments were repeated ≥3 times.

Cell viability assay. Cell viability was measured using the MTT assay as described previously (29). Cells were seeded in 6-well plates at a density of $1x10^5$ cells/well.

After transfection for the indicated time, 20 μl 0.5 mg/ml MTT (Beyotime Institute of Biotechnology) was added to the medium and incubated for another 4 h at 37˚C. Before measurement, 150 μl DMSO was added, and a microplate reader (SpectraMax 190; Molecular Devices, LLC) was used to measure the optical density at 450 nm. Experiments were repeated ≥3 times.

Cell cycle measurement. Cell cycle status was detected via flow cytometry (FACScan; Becton, Dickinson and Company) and analyzed with FlowJo v10.4 software (FlowJo LLC). Following the different transfections as described above, the cells were harvested and cell pellets were fixed in cold ethanol (75%) overnight at -20˚C. The fixed cells were then resuspended in 1 mg/ml RNase A (Sigma-Aldrich; Merck KGaA) in PBS and incubated for 1 h at 37˚C. The cells were stained with 50 μg/ml PI (Sigma-Aldrich; Merck KGaA) for 0.5 h at room temperature in the dark. The cells were then analyzed, and the results are presented as the mean values from three independent measurements. Experiments were repeated ≥3 times.

Cellular apoptosis measurement. The apoptosis of cells was measured using the Cell Death Detection ELISA^{plus} kit (Roche Diagnostics GmbH) according to the manufacturer's instructions. The amount of histone-coupled DNA was quantified by measuring the absorbance at 405 nm by the BioTek synergy multimode microplate reader (BioTek Instruments, Inc.). The results were analyzed by the Gen5 v1.0 (BioTek Instruments, Inc.). Experiments were repeated ≥3 times.

Caspase-3 activity assay. The activity of caspase-3 was measured using the caspase-3 Activity Assay kit (Fluorometric; Abcam) according to the manufacturer's instructions. Experiments were repeated ≥3 times.

RNA purification and RT-qPCR. Whole blood samples (5 ml) were obtained from the healthy controls and patients with AML before receiving any therapy. All serum specimens were centrifuged at 500 x g for 5 min and then centrifuged at 3,000 x g for 5 min, both at 4˚C. Serum samples were then stored at -80˚C until further analysis.

Total RNA was isolated using TRIzol® reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The quality and concentration of RNA were measured using a NanoDrop ND-1000 (Thermo Fisher Scientific, Inc.). Total RNA was reverse transcribed into cDNA using the PrimerScript™ RT reagent kit (Takara Biotechnology Co., Ltd.). The reverse transcription was conducted at 65˚C for 10 min. qPCR was performed on an Applied Biosystems 7500 Detection System (Applied Biosystems; Thermo Fisher Scientific, Inc.) using SYBR Premix ExTaq (Takara Biotechnology Co., Ltd.) according to the manufacturer's instructions. The thermocycling conditions were: 95˚C for 10 min, followed by 40 cycles of 95˚C for 5 sec and 55˚C for 5 sec and 72˚C for 5 sec, followed by 73˚C for 30 sec as a final extension. The relative expression of LINC00460 was assessed using the comparative $2^{-\Delta\Delta Cq}$ method (30). The expression levels of LINC00460 and miR-320b were normalized to the expression levels of GAPDH and U6, respectively. The following primers were used: LINC00460 forward, 5'-GGATGAACCACCATTGCC-3' and reverse, 5'-CCCACGCTCAGTCTTTCT-3'; miR-320b forward, 5'-TCCGAAACGGGAGAGTTGG-3' and reverse, 5'-GTGCAGGGTCCGAGGT-3', GAPDH forward, 5'-AGAAGGCTGGGGCTCATTTG-3' and reverse, 5'-AGGGGCCATCCACAGTCTTC-3'; and U6 forward, 5'-GCTTCGGCAGCACATATACTAAAAT-3' and reverse, 5'-CGCTTCACGAATTTGCGTGTCAT-3'. Experiments were repeated ≥3 times.

Western blot analysis. Total cell lysates were collected using RIPA buffer (Beyotime Institute of Biotechnology). The concentration of proteins was measured using a BCA kit (Beyotime Institute of Biotechnology). Equal amounts of protein (20 μg) were subjected to 12% SDS-PAGE and then transferred to PVDF membranes (Beyotime Institute of Biotechnology). The membranes were blocked with 10% skimmed milk for 1 h at room temperature and then incubated with the primary antibodies at 4˚C overnight. The following primary antibodies were used: Bcl-2 (cat. no. 1507; 1:1,000), Bcl-xl (cat. no. 2762; 1:1,000), caspase-3 (cat. no. 14220; 1:1,000), PBX3 (cat. no. ab183849; 1:1,000; Abcam, USA), GAPDH (cat. no. 5174; 1:5,000). The membranes were washed with PBS and then incubated with secondary antibody at room temperature for 1 h. The following secondary antibodies were used: HRP linked anti-mouse antibody (cat. no. 7076; 1:5,000), HRP linked anti-rabbit antibody (cat. no. 7074; 1:5,000). All antibodies were purchased from Cell Signaling Technology, Inc., and diluted at the ratio recommended by the manufacturer. Finally, the protein bands were visualized using the ECL reagent (Beyotime Institute of Biotechnology). Images were analyzed using Chemidoc Touch Imaging System v1.2 (Bio-Rad Laboratories, Inc.). The experiments were repeated ≥3 times.

Dual-luciferase assays. The wild-type (WT) and mutant (MUT) fragments of LINC00460 and the 3'-untranslated region (UTR) of PBX3 containing the miR-320b targeting sequences were synthesized and inserted into the pGL3 promoter vector (Promega Corporation). Cells were seeded into 6-well plate at the density of $3x10^5$ cells/well in 2 ml complete medium. After 24 h of incubation at 37˚C, the medium was changed to 500 μl Opti-MEM (Gibco; Thermo Fisher Scientific, Inc.) containing 1 μg plasmid, 20 mM miRNA mimics and 5 μl Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Cells were then cultured at 37˚C. Finally, 48 h after transfection, cells were collected and the Dual-Luciferase Reporter Assay system (Promega Corporation) was used to measure luminescence. *Renilla* luciferase activity was used to normalize the firefly luciferase activity. Experiments were repeated ≥3 times.

Bioinformatic analysis. StarBase Ver 3.0 (http://starbase.sysu.edu.cn) and TargetScan Ver 3.1 (http://www.targetscan.org/mamm_31/) were used to predict the potential binding miRNAs of LINC00460 and targets of miR-320b.

Statistical analysis. Statistical analyses were performed using SPSS 18.0 (SPSS, Inc.) or GraphPad 8.0 (GraphPad Software,

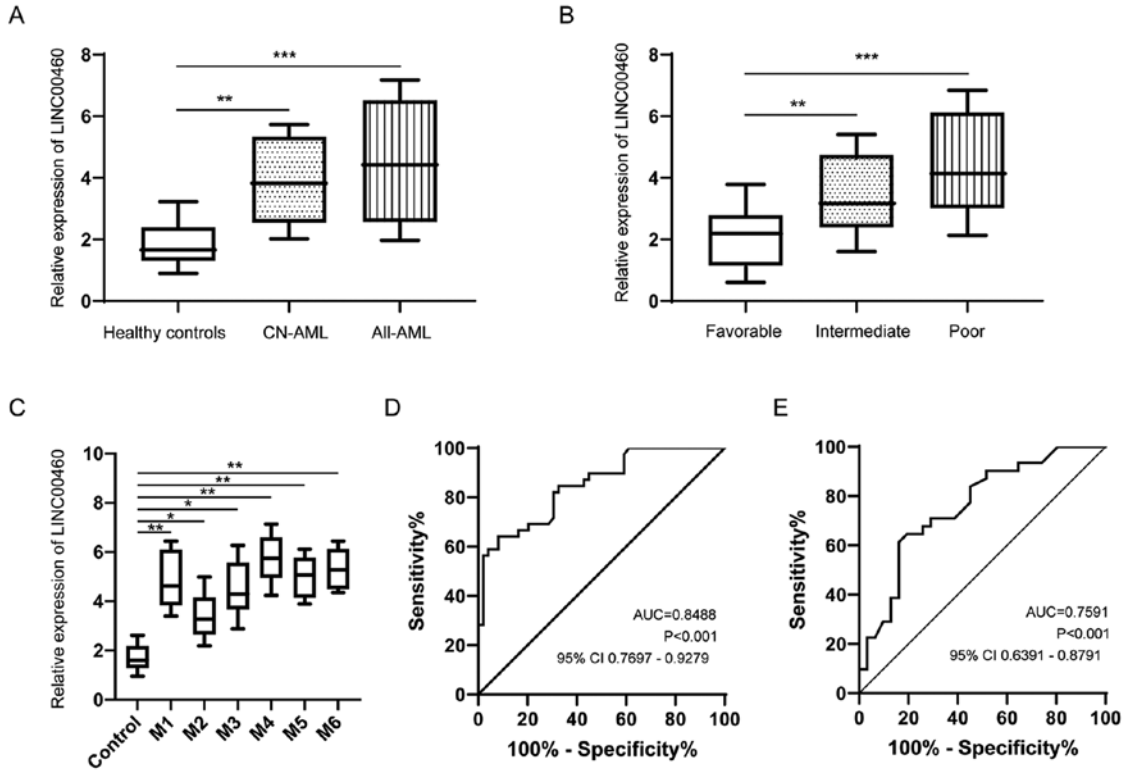

Figure 1. Expression of LINC00460 is significantly upregulated in patients with AML. (A) Expression of LINC00460 was compared between healthy controls, patients with CN-AML and patients with total AML. (B) Expression of LINC00460 was compared between different cytogenetic groups. (C) Expression of LINC00460 was compared between different French-American-British subtype groups (M1: Acute myeloblastic leukemia with minimal maturation; M2: Acute myeloblastic leukemia with maturation; M3: Acute promyelocytic leukemia; M4: Acute myelomonocytic leukemia; M5: Acute monocytic leukemia; M6: Acute erythroid leukemia; M7: Acute megakaryoblastic leukemia). (D) ROC analysis for the diagnosis of patients with AML using serum LINC00460 in patients with AML and healthy controls. (E) ROC analysis for the diagnosis of patients with CN-AML using serum LINC00460 in patients with AML and healthy controls. *P<0.05; **P<0.01; ***P<0.001. AML, acute myeloid leukemia; LINC00460, long non-coding RNA 00460; AUC, area under the curve; CN-AML, cytogenetically normal-AML; ROC, Receiver operating characteristic.

Inc.) software. Data were presented as mean ± standard deviation. The difference in the relative serum LINC00460 expression between groups was determined using the Mann-Whitney U test or Kruskal-Wallis test followed by Dunn's post hoc test. The median value of LINC00460 expression was used as the cut-off value to designate the patients with AML into a high LINC00460 group and a low LINC00460 group. A receiver operating characteristic (ROC) curve and the area under the curve (AUC) were used to assess the diagnostic value of serum LINC00460 expression in patients with AML and healthy controls. The Pearson χ^2 test was used to assay intergroup differences. The Cox proportional hazards regression model was applied for univariate and multivariate analyses to estimate the prognostic factors for survival prediction. Survival curves were constructed via Kaplan-Meier survival analysis with the log-rank test. Paired Student's t-test and One-way ANOVA followed by the post hoc Tukey test were applied for comparing the difference between two groups or multiple groups, respectively. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of LINC00460 is upregulated in patients with AML. First, the expression of LINC00460 in serum from 80 patients with AML and 67 healthy controls was measured via RT-qPCR. LINC00460 expression was significantly upregulated in patients with AML or CN-AML compared with those in healthy controls (Fig. 1A). Next, the patients with AML were divided into favorable, intermediate and poor groups according to their cytogenetic features (31). It was identified that LINC00460 expression in patients with AML with intermediate or poor cytogenetic risk subtypes was greatly increased compared with that in patients with favorable cytogenetic risk subtypes (Fig. 1B). Moreover, the expression of LINC00460 was compared among different FAB subtypes, but there was no significant difference among the various subtypes (Fig. 1C). ROC curve analysis demonstrated that serum LINC00460 could differentiate patients with AML from healthy controls with an AUC value of 0.8488 (Fig. 1D). In addition, serum LINC00460 could serve as a reliable biomarker for differentiating patients with CN-AML from healthy controls with an AUC value of 0.7591 (Fig. 1E).

Association between the expression of LINC00460 and clinicopathologic features of AML. The correlation between the expression of serum LINC00460 and the clinicopathological characteristics of AML was analyzed. The 80 patients with AML were grouped into a high serum LINC00460 group (n=36) and a low serum LINC00460 group (n=44) according to the median serum LINC00460 expression. As presented in Tables I and II, serum LINC00460 levels were significantly associated with clinicopathological features, such as FAB

Table I. Relationship between serum LINC00460 expression and clinicopathologic features in acute myeloid leukemia.

Clinicopathological features	No.	Serum Linc0062 expression		P-value
		High	Low	
Sex				
Male	42	18	24	0.685
Female	38	18	20	
Age, years				
<60	47	21	26	0.945
≥60	33	15	18	
BM blasts, %				
<50	41	15	26	0.079
≥50	39	21	18	
PLT counts, $x10^9$/l				
<50	34	18	16	0.220
≥50	46	18	28	
WBC counts, $x10^9$/l				
<10	45	16	29	0.054
≥10	35	20	15	
Extramedullary disease				
No	52	22	30	0.509
Yes	28	14	14	
Complete remission				
Yes	51	24	27	0.624
No	29	12	17	
Cytogenetics				
Favorable	20	5	15	0.018
Intermediate	42	20	22	
Unfavorable	18	11	7	

BM, bone marrow; PLT, platelet; WBC, white blood cells.

classification and cytogenetics. However, there was no significant correlation of LINC00460 expression with other clinical features, including sex, age, white blood cell count, BM blasts, extramedullary disease and CR (Table I).

Association between serum LINC00460 expression and treatment response. After receiving different treatments (chemotherapy and/or targeted therapy), 42 patients achieved CR. To further investigate the effect of serum LINC00460 on the progression of AML, the expression of serum LINC00460 in patients with AML was compared before and after achieving CR. It was demonstrated that serum LINC00460 expression was markedly decreased in patients with AML after treatment, suggesting that serum LINC00460 expression was closely associated with treatment response (Fig. 2).

Prognostic evaluation of LINC00460 expression in patients with AML. Clinical follow-up was conduct to determine the prognostic value of serum LINC00460 expression in patients with AML. The log-rank test and Kaplan-Meier analysis were performed. The results demonstrated that the patients with AML in the low LINC00460 expression group had a significantly longer 5-year overall survival time (Fig. 3A) and recurrence-free survival (Fig. 3B) compared with those in the high expression group. Similar results were obtained in non-M3 subtype cases (non-acute promyelocytic leukemia; Fig. 3C and D). In addition, among the 45 patients with CN-AML, higher expression of LINC00460 was associated with shorter 5-year overall survival (Fig. 3E) and recurrence-free survival (Fig. 3F) compared with the lower expression group.

Univariate analysis identified that FAB classification, cytogenetics and serum LINC00460 expression were significantly correlated with poor overall survival of patients with AML ($P<0.05$; Table II). Further multivariate analysis demonstrated that serum LINC00460 expression, FAB classification and cytogenetics were independent prognostic indicators of the overall survival of patients with AML (Table II). These data suggested that LINC00460 was a potential prognostic biomarker for patients with AML.

Knockdown of LINC00460 inhibits viability, as well as induces cell cycle arrest and apoptosis in AML cells. Next,

Table II. Univariate and multivariate analyses of prognostic factors in acute myeloid leukemia.

	Univariate analysis			Multivariate analysis		
Variables	Hazard ratio	95% CI	P-value	Hazard ratio	95% CI	P-value
Sex Male vs. female	1.332	0.691-2.412	0.372	-	-	-
Age, years <60 vs. ≥60	1.448	0.732-2.578	0.228	-	-	-
WBC counts x10^9/l <10 vs. ≥10	1.541	0.811-2.635	0.175	-	-	-
Blast in BM, % <50 vs. ≥50	1.493	0.824-2.674	0.213	-	-	-
Extramedullary disease No vs. Yes	1.712	0.631-2.215	0.302	-	-	-
Complete remission Yes vs. No	1.515	0.703-2.113	0.117	-	-	-
FAB classification M1-M5 vs. M6-M7	3.126	1.643-5.189	0.004	2.916	1.352-4.715	0.009
Cytogenetics Unfavorable vs. favorable/intermediate	3.372	1.769-5.376	0.003	2.747	1.116-4.032	0.017
Serum LINC00899 expression High vs. low	3.762	1.818-6.177	0.001	3.015	1.462-5.018	0.004

LINC00460, long non-coding RNA 00460; FAB, French-American-British; BM, bone marrow; WBC, white blood cells.

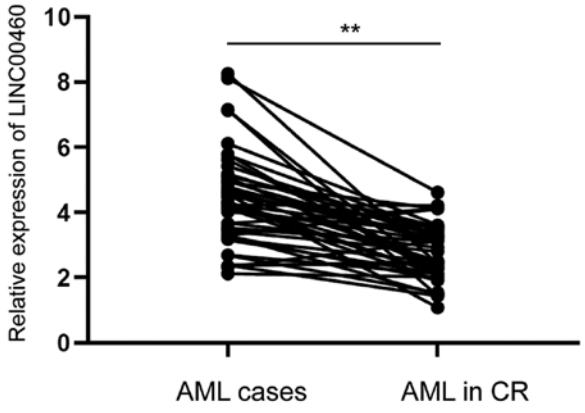

Figure 2. Serum LINC00460 expression levels in patients with AML before and after CR. Serum LINCO00460 expression was measured by reverse transcription-quantitative PCR. **P<0.01, AML vs AML following complete remission. CR, complete remission; AML, cytogenetically normal-AML; LINC00460, long non-coding RNA 00460.

the function of LINC00460 were investigated *in vitro*. The expression of LINC00460 was measured in a normal human bone marrow stromal cell line (HS-5) and AML cells (THP-1, KG-1, ME-1 and HL-60) via RT-qPCR. It was found that the expression of LINC00460 was significantly upregulated in AML cells (Fig. 4A).

To further address the biological functions of LINC00460 in AML cells, siRNA-mediated knockdown of LINC00460 was performed in AML cells. The si-LINC00460-transfected cells demonstrated a significant decrease in LINC00460 expression compared with cells transfected with si-NC (Fig. 4B). The MTT assay indicated that the viability of AML cells was significantly inhibited after silencing LINC00460 compared with the control group (Fig. 4C). Cell cycle distribution analysis identified that knockdown of LINC00460 caused cell cycle arrest at the G_2 phase in AML cells (Fig. 4D). Moreover, a significant increase in apoptotic AML cells was observed after silencing LINC00460 (Fig. 4E). Western blotting and caspase-3 activity results also suggested that knockdown LINC00460 induced apoptosis in AML cells (Fig. 4F and G). Taken together, these data indicated that knockdown LINC00460 inhibited viability, as well as induced cell cycle arrest and apoptosis in AML cells.

LINC00460 acts as a sponge of miR-320b. Considering that lncRNAs can act as sponges to bind miRNAs, two bioinformatic tools (TargetScan and StarBase 2.0) were applied for predicting putative miRNAs. The putative binding sites between LINC00460 and miR-320b are presented in Fig. 5A. It was found that the expression of miR-320b was significantly lower in AML cells compared with in HS-5 cells (Fig. 5B). The transfection efficiency of the miR-320b mimic is presented in Fig. 5C. The interaction between miR-320b and LINC00460 was then examined via measuring luciferase activity. The results demonstrated that overexpression of miR-320b

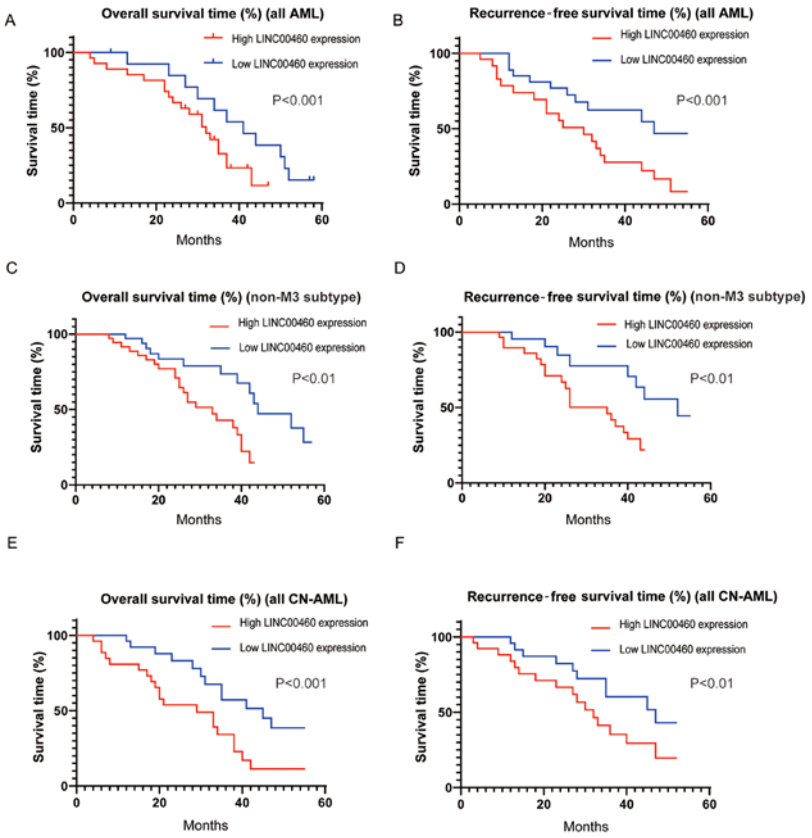

Figure 3. Kaplan-Meier analysis of the overall survival time and recurrence-free survival time of patients with AML with different expression levels of serum LINC00460. All patients with AML with higher serum LINC00460 expression had a (A) poorer overall survival and (B) poorer recurrence-free survival. Patients with non-M3 subtype AML with higher serum LINC00460 expression had a (C) poorer overall survival and (D) poorer recurrence-free survival. All patients with CN-AML with higher serum LINC00460 expression had a (E) poorer overall survival and (F) poorer recurrence-free survival. CN-AML, cytogenetically normal-AML; AML, acute myeloid leukemia; LINC00460, long non-coding RNA 00460.

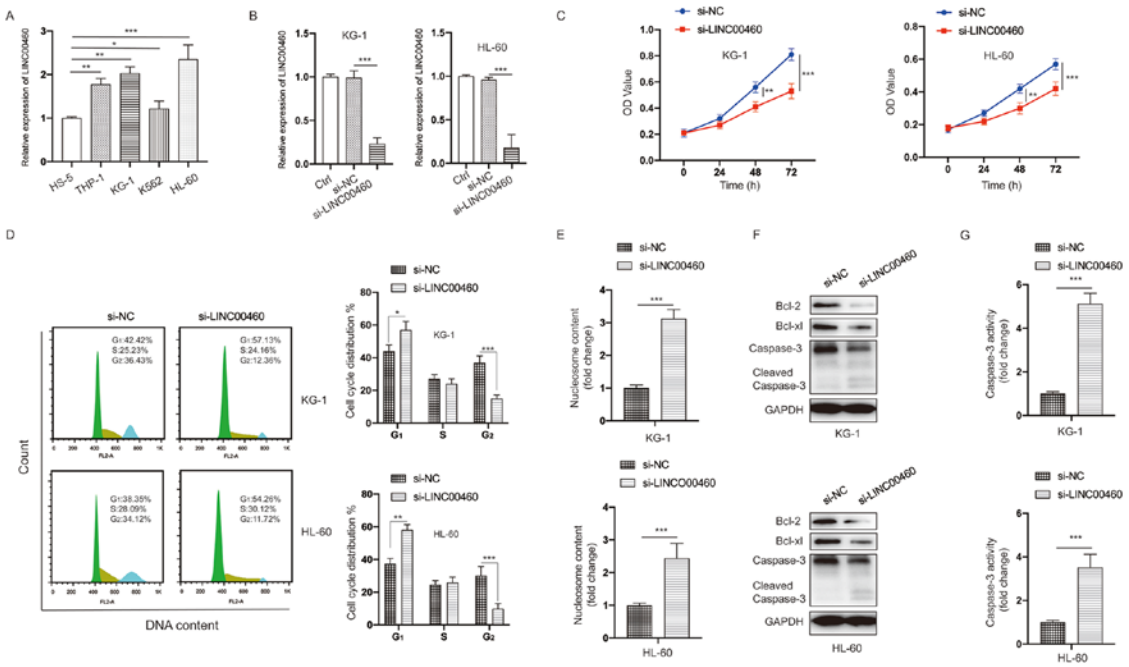

Figure 4. LINC00460 functions as an oncogene in AML cells. (A) Expression of LINC00460 was measured in bone marrow stromal cells (HS-5) and AML cells (THP-1, KG-1, K562 and HL-60). (B) AML cells were transfected as indicated, and the expression of LINC00460 was measured. (C) AML cells were treated as indicated, and cell viability was measured at different time points. (D) AML cells were treated as indicated, and cell cycle distribution was assayed. (E) AML cells were treated as indicated, and cell apoptosis was assayed. (F) AML cells were treated as indicated, and total cell lysates were subjected to western blotting with the specified antibodies. (G) AML cells were treated as indicated, and Caspase-3 activity was assayed. All experiments were conducted ≥3 times. Data are presented as the mean ± SD. *P<0.05; **P<0.01; ***P<0.001. NC, negative control; siRNA, small interfering RNA; AML, acute myeloid leukemia; LINC00460, long non-coding RNA 00460; OD, optical density.

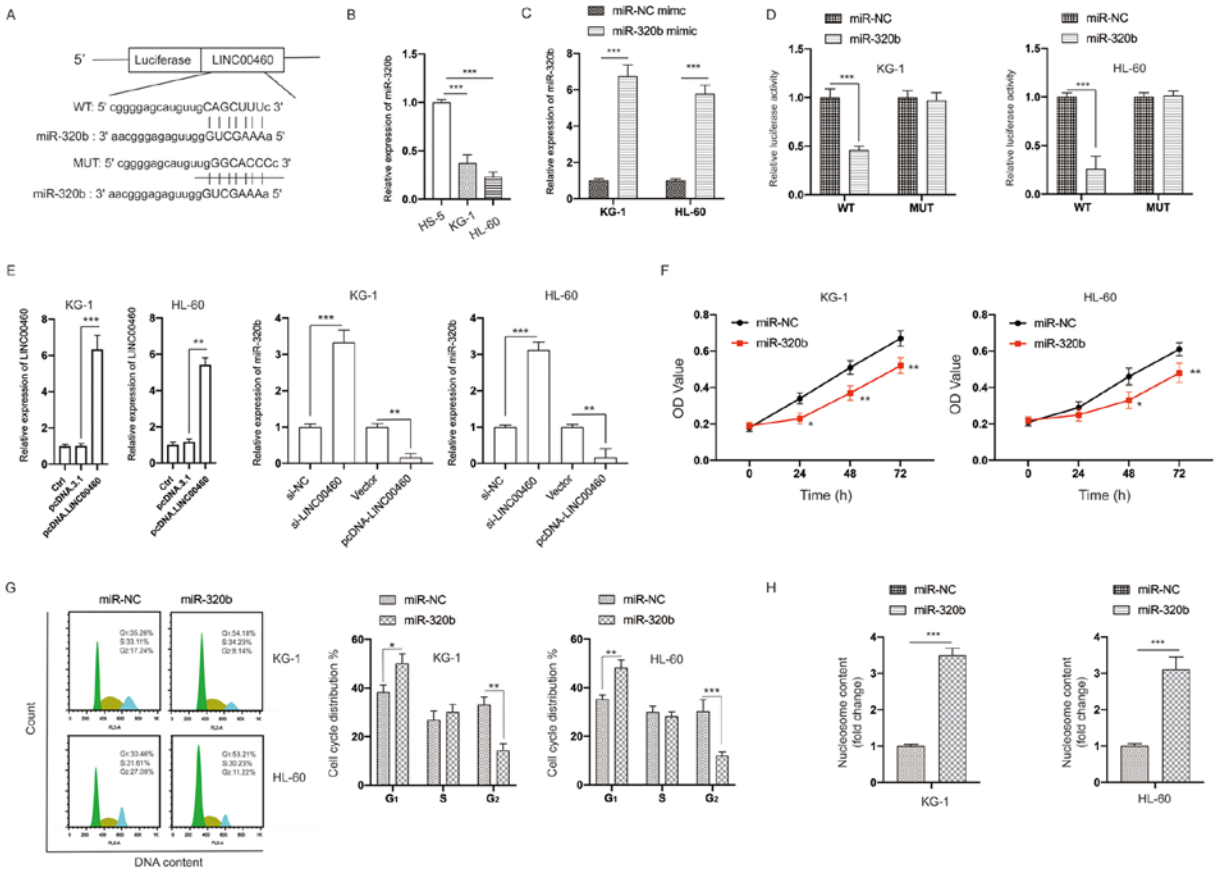

Figure 5. LINC00460 acts as a sponge of miR-320b. (A) Constructed luciferase reporter plasmids containing the predicted WT or MUT miR-320b binding sites on LINC00460. (B) Expression of miR-320b in HS-5, KG-1 and HL-60 cells was measured. (C) KG-1 and HL-60 cells were transfected with miR-NC mimic or miR-320b mimic, the expression of miR-320b was measured via reverse transcription-quantitative PCR. (D) A luciferase reporter assay was performed to measure the luciferase activity in AML cells after co-transfection of miRNA mimics and LINC00460 WT or MUT. (E) AML cells were transfected as indicated, and the expression of miR-320b was measured. (F) AML cells were transfected with miR-320b mimics or miR-NC mimics for the indicated times, and cell viability was measured. (G) AML cells were transfected as indicated, and the cell cycle distribution was assayed. (H) AML cells were transfected as indicated, and cell apoptosis was measured. All experiments were conducted ≥3 times. Data are presented as the mean ± SD. *P<0.05; **P<0.01; ***P<0.001. NC, negative control; miR, microRNA; AML, acute myeloid leukemia; LINC00460, long non-coding RNA 00460; OD, optical density; Ctrl, control; WT, wild-type; MUT, mutant.

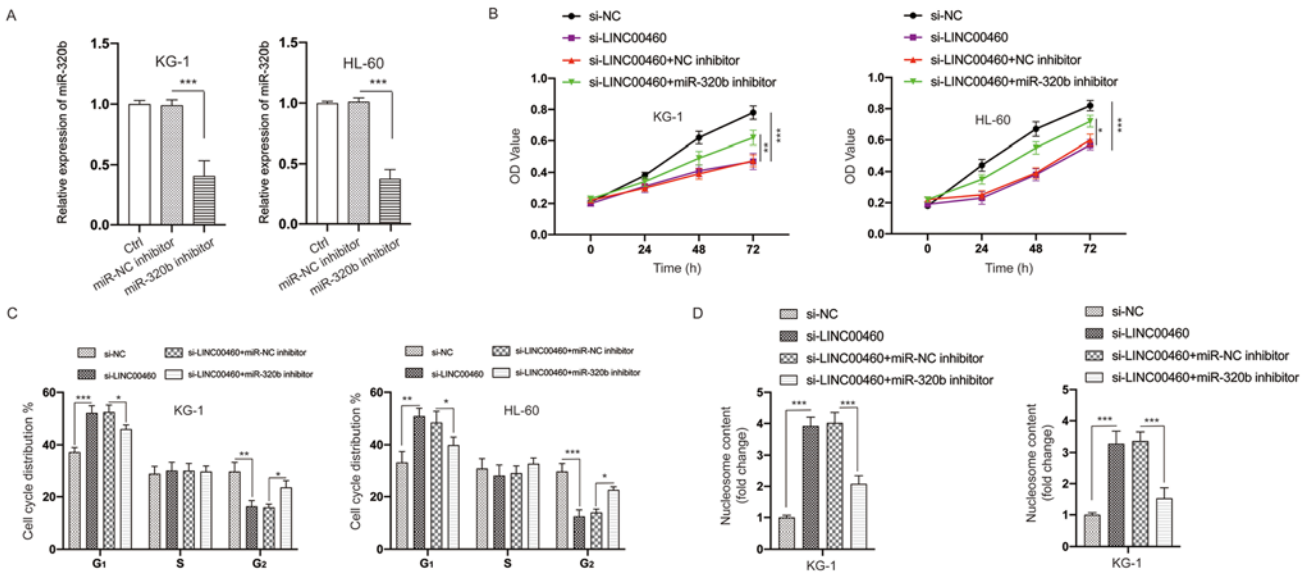

Figure 6. Knockdown of miR-320b suppresses the effects of silencing LINC00460 on AML cells. (A) AML cells were transfected as indicated, and the expression of miR-320b was measured. (B) AML cells were transfected as indicated, and the viability of cells was measured using the MTT assay. (C) AML cells were transfected as indicated, and the cell cycle distribution was detected. (D) AML cells were transfected as indicated, and cell apoptosis was evaluated. All experiments were conducted ≥3 times. Data are presented as the mean ± SD. *P<0.05; **P<0.01; ***P<0.001. NC, negative control; miR, microRNA; AML, acute myeloid leukemia; LINC00460, long non-coding RNA 00460; OD, optical density; Ctrl, control; siRNA, small interfering RNA.

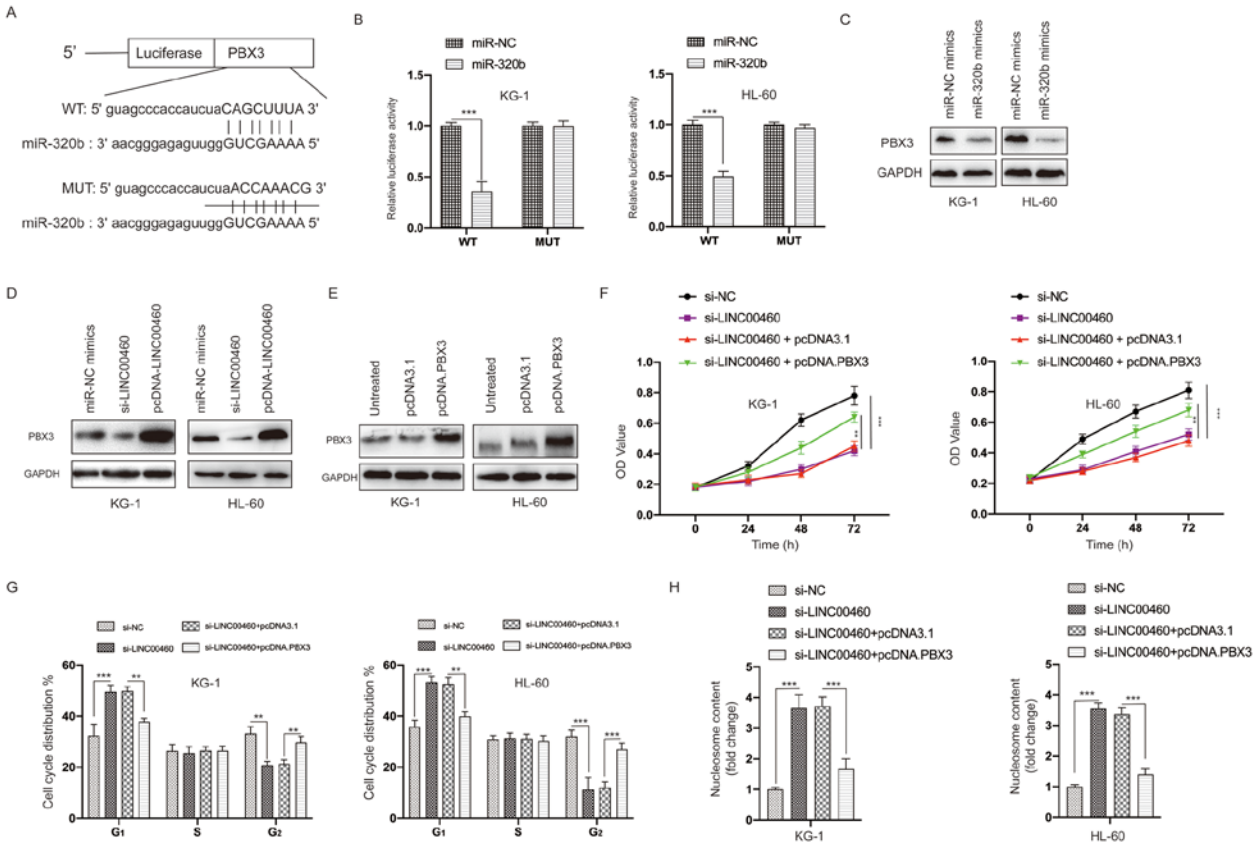

Figure 7. PBX3 is a direct target of miR-320b. (A) Constructed luciferase reporter plasmids containing the predicted WT or MUT miR-320b binding sites on PBX3. (B) A luciferase reporter assay was performed to measure the luciferase activity in AML cells after co-transfection of miRNA mimics and PBX3 WT or MUT. (C) AML cells were transfected with miR-NC mimics or miR-320b mimics and the protein levels of PBX3 measured by western blotting. (D) AML cells were transfected with miR-NC mimics, si-LINC00460 or pcDNA-LINC00460 and the protein levels of PBX3 measured by western blotting. (E) AML cells were transfected with pcDNA3.1 or pcDNA.PBX3 and the protein expression levels of PBX3 measured via western blotting. (F) AML cells were transfected as indicated, and cell viability was measured using the MTT assay. (G) AML cells were transfected as indicated, and the cell cycle distribution was detected. (H) AML cells were transfected as indicated, and cell apoptosis was examined. All experiments were conducted ≥3 times. Data are presented as the mean ± SD. **P<0.01; ***P<0.001. NC, negative control; miR, microRNA; AML, acute myeloid leukemia; LINC00460, long non-coding RNA 00460; OD, optical density; siRNA, small interfering RNA; PBX3, PBX homeobox 3; WT, wild-type; MUT, mutant.

significantly inhibited the luciferase activity of AML cells transfected with LINC00460-WT, while its efficacy was absent with respect to the LINC00460-MUT group (Fig. 5D). The expression of LINC00460 was significantly upregulated following transfection with pcDNA.LINC00460 (Fig. 5E). The expression of miR-320b was significantly decreased by LINC00460 overexpression, but was increased by LINC00460 knockdown in AML cells (Fig. 5E). Further analysis identified that the viability of AML cells was inhibited by transfection with the miR-320b mimic (Fig. 5F). Similar to the knockdown of LINC00460, overexpression of miR-320b caused cell cycle arrest at the G_2 phase and increased apoptosis of AML cells (Fig. 5G and H). Thus, these data indicated that LINC00460 acted as a sponge of miR-320b.

Inhibition of miR-320b partially blocks the effects of LINC00460 knockdown on AML cells. To further analyze the correlation between miR-320b and LINC00460, transfection of the miR-320b inhibitor was conducted, which successfully decreased the expression of miR-320b (Fig. 6A). It was found that knockdown of miR-320b partially reversed the effects of silencing LINC00460 on the viability of AML cells (Fig. 6B). Moreover, the cell cycle arrest at the G_2 phase (Figs. S1 and 6C) and apoptosis (Fig. 6D) induced by silencing LINC00460 could be partially rescued by knockdown of miR-320b. Therefore, these data further demonstrated the relationship between LINC00460 and miR-320b.

PBX3 is a direct target of miR-320b. Next, the possible direct targets of miR-320b were investigated. Using bioinformatics analysis, the 3'-UTR of the PBX3 gene was predicted to bind with miR-320b (Fig. 7A). The interaction between miR-320b and PBX3 was further validated via the dual-luciferase reporter assay, which indicated significantly decreased luciferase activity caused by miR-320b mimics in AML cells expressing the WT PBX3 3'-UTR sequence, while there was little change in luciferase activity in cells expressing the MUT PBX3 3'-UTR sequence (Fig. 7B). The transfection of miR-320b mimics decreased the protein expression level of PBX3 in AML cells (Fig. 7C). Moreover, knockdown and overexpression of LINC00460 led to decreased and increase of PBX3 expression in AML cells, respectively (Fig. 7D). This finding suggested that LINC00460 may exert its function via regulation of the miR-320b/PBX3 axis.

To further verify the role of PBX3 in the function of LINC00460, the expression of PBX3 was successfully

overexpressed in AML cells via transfection of an expression vector (Fig. 7E). The MTT assays results demonstrated that overexpression of PBX3 partially blocked the inhibitory effects of LINC00460 knockdown on the viability of AML cells (Fig. 7F). Furthermore, overexpression of PBX3 reversed the effects of silencing LINC00460 on the cell cycle distribution (Figs. S2 and 7G) and apoptosis (Fig. 7H) of AML cells. Collectively, these data suggested that PBX3 was a direct target of miR-320b and that LINC00460 exerts its effects, at least, partially via regulation of the miR-320b/PBX3 axis.

Discussion

AML, characterized by a subpopulation of long-term proliferative progenitor cells, is a common type of hematological malignant tumor that threatens human life (32). Its poor prognosis means it is necessary to identify novel therapeutic agents and sensitive biomarkers of AML (33). In recent years, accumulating evidence has revealed that serum lncRNAs could be applied as potential biomarkers for the diagnosis and prognosis of different malignancies, including AML. For instance, both LINC00265 and LINC00899 are found to be upregulated in the serum of patients with AML and could be used as a potential biomarker for AML (34,35). In the current study, it was identified that serum LINC00460 expression in patients with AML was significantly upregulated compared with that in healthy controls. Moreover, serum LINC00460 expression was significantly increased in AML subjects with poor-risk cytogenetics. ROC analysis demonstrated that serum LINC00460 expression could effectively differentiate patients with AML from healthy controls. Moreover, downregulation of serum LINC00460 was detected in patients with AML with a CR. The present data also indicated that patients with high serum LINC00460 expression had a significantly poorer overall survival time and recurrence-free survival time compared with those with low serum LINC00460 expression. Additionally, serum LINC00460 could serve as an independent prognostic indicator for patients with AML. To the best of our knowledge, the present study was the first report on the clinical significance of serum LINC00460 in patients with AML.

The current findings are consistent with other studies showing that LINC00460 functioned as an oncogene in various types of cancer. For example, Feng *et al* (36) reported that LINC00460 was significantly upregulated in glioma tissues and cell lines compared with non-tumor healthy tissues. In epithelial ovarian cancer, LINC00460 expression is markedly increased in both cancer tissues and cell lines (17). Moreover, upregulated LINC00460 expression promotes tumorigenicity by binding miR-338-3p *in vitro* (17). LINC00460 was also observed to be upregulated in both head and neck squamous cell carcinoma (HNSCC) tissues and cell lines, as well as predicted a poor prognosis in patients with HNSCC (37). In breast cancer, upregulation of LINC00460 expression was observed in breast cancer tissues and was found to be associated with aggressive clinical characteristics (38). Furthermore, knockdown of LINC00460 results in the inhibition of viability, migration and invasion of breast cancer cells (38). In a recent study, LINC00460 was found to be notably upregulated in gastric cancer tissues compared with non-tumor tissues, and LINC00460 could be used as an independent prognostic marker in gastric cancer (39).

To date, accumulating evidence suggests that lncRNAs can be released into various body fluids, such as urine, saliva and serum, as a result of cancer cell excretion (40). The easy accessibility of serum and the stability of lncRNA in serum makes lncRNAs ideal biomarkers for the diagnosis and prognosis of cancer. There are several explanations regarding the stability of lncRNAs in serum: i) lncRNAs may be selectively enclosed into membrane-covered vesicles, which can provide protection against degradation; ii) lncRNAs may bind with proteins to avoid degradation; and iii) lncRNAs may fold into stable complex secondary and tertiary structures (41). Although the present study identified the oncogenic role of LINC00460 and its underlying mechanisms in AML, there remain some limitations. First, the clinical sample size was relatively small. Second, most participants were recruited from the same hospital. Therefore, further multicenter cohort studies should be performed in the future to further confirm the current findings. Moreover, it would be interesting to investigate LINC00460 in serum samples of patients with other malignant diseases.

The present study investigated the biological functions of LINC00460 *in vitro*. It was identified that knockdown of LINC00460 inhibited the viability, as well as induced cell cycle arrest and apoptosis of AML cells. These findings are in line with previous studies, which also reported that LINC00460 acted as an oncogene in lung, colorectal and ovarian cancer cells (22,42,43). Furthermore, Lian *et al* (15) revealed that inhibition of LINC00460 induced cell cycle arrest at the G^1 phase in colorectal cells, while the present study identified that silencing LINC00460 caused cell cycle arrest at the G^2 phase. This discrepancy may be caused by the use of different cell lines, and additional investigations are required to further elucidate the role of LINC00460 in the control of cell cycle progression.

It is well known that miRNAs participate widely in the progression of various human cancer types (44). In addition, lncRNAs can act as competing endogenous RNAs to bind and inhibit the functions of miRNAs (45). The present study demonstrated that LINC00460 acted as a sponge of miR-320b, which has been identified as a tumor suppressor in different human cancer types, such as colorectal cancer, glioma, lung cancer and prostate cancer (21-23). Previous studies have reported that the expression and functions of miR-320b in tumorigenesis could be regulated by lncRNAs. For instance, lncRNA X inactive specific transcript can regulate the progression of osteosarcoma by targeting and suppressing miR-320b expression (46). In addition, miR-320b is targeted and repressed by the lncRNA NR2F2-antisense 1 in modulating the tumorigenesis of lung cancer (23). Consistent with previous studies, the present results suggested that miR-320b could be a downstream target of LINC00460, which exerts its function at least partly via regulation of miR-320b.

PBX3 acts as a transcription factor and has been intensively studied in the pathogenesis of AML (26,27). More importantly, inactivation of PBX3 could suppress the stemness and survival of leukemia cells (47). PBX3 was also found to serve essential roles in the tumorigenesis of various solid cancer types, such as colorectal cancer, prostate cancer, gastric

cancer, cervical cancer and pancreatic cancer (48). Therefore, targeting PBX3 may be a potential strategy for the treatment of cancer, including AML. The present study identified novel mechanistic events involved in the regulation of PBX3, which may be applied in the field of treatment of various cancer types.

In conclusion, the present study provided evidence that serum LINC00460 expression was significantly upregulated in patients with AML, and was closely associated with poor clinical outcome and unfavorable clinical variables. To the best of our knowledge, the present study identified the role of LINC00460 in AML for the first time. Furthermore, it was suggested that PBX3, which may also be a potential target for AML, could be negatively regulated by miR-320b. The *in vitro* studies demonstrated that LINC00460 exerted its functions via the miR-320b/PBX3 axis in AML cells. Therefore, LINC00460 may be used as a potential indicator and target for the prognosis and treatment of AML.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

QZ performed the experiments and drafted the manuscript, ZJ performed the experiments, XZ conducted the statistical analysis, TJ repeated some of the experiments and LX designed the study and drafted the manuscript. All authors reviewed and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Wenzhou Medical University. Written informed consent was obtained from all patients prior to participation in the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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