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Long non-coding RNA LINC00641 promotes cell growth and migration through modulating miR-378a/ZBTB20 axis in acute myeloid leukemia

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Abstract. – OBJECTIVE: Many researchers have revealed that long noncoding RNAs (lncRNAs) acted as modulators in tumor biology. LncRNA LINC00641 (LINC00641), a newly discovered tumor-related lncRNA, has been reported to act as a modulator in several tumors. Hence, our study aimed to examine the expression and function of LINC00641 in acute myeloid leukemia (AML).

PATIENTS AND METHODS: The expression pattern of LINC00641 in AML specimens and cell lines was explored using a gene expression profiling interactive analysis (GEPIA) tool and RT-PCR assays. The cell counting kit-8 (CCK-8) assays, transwell migration, and invasion assays were used for the functional study of cell viability, cell migration, and invasion. The influence of LINC00641 on cell cycle and apoptosis was determined using Flow cytometry detection. The regulating associations between LINC00641, miR-378a, and ZBTB20 were investigated in AML cells using the Luciferase reporter assays and RT-PCR assays

RESULTS: We found that LINC00641 was highly expressed in AML specimens and cell lines. Functionally, the silence of LINC00641 inhibited the proliferation, migration, invasion, and cell cycle arrest in AML cells while inducing their apoptosis. The results using bioinformatics assays predicted the complementary binding sites within LINC00641 and miR-378a, which was demonstrated by the use of the Luciferase reporter assays. In addition, we also demonstrated that ZBTB20 was a direct target of miR-378a. Moreover, the inhibition of miR-378a could rescue the ZBTB20 protein level decrease induced by LINC00641 knockdown.

CONCLUSIONS: We firstly identified LINC00641 as a novel AML-related lncRNA whose knockdown inhibited cell proliferation, migration, invasion, and promoted apoptosis by modulating miR-378a/ZBTB20 axis in AML.

Key Words:

LncRNA LINC00641, MiR-378a, ZBTB20, Acute myeloid leukemia, Proliferation, Migration.

Introduction

Acute myeloid leukemia (AML) is a clonal disorder, which originates from an uncommon population of leukemia stem cells with the anomalous proliferation of myeloblasts in the medulla ossium[1,2]. This disease can occur in both children and adults and its incidence in recent years is rising, partly due to a growing prevalence of therapy-associated AML and house decoration[3,4]. With the advances in chemotherapy, the immune modulation therapies, and bone marrow transplants, the 5-year survival for patients diagnosed at an early stage has been improved[5,6]. However, the relapse remains one of the fundamental challenges for treatment failure, and an estimated 5-year survival rate is found in those patients[7]. Therefore, for the optimization of the current treatment system, a better understanding of the pathogenesis of AML is necessary for the basis research. Some reports have suggested long noncoding RNAs (lncRNAs) as novel modulators in AML progression.

LncRNAs, residing in the nucleus or cytoplasm, represent a subset of noncoding RNAs longer than 200 nucleotides without the biological functions in coding proteins[8]. The positive associations between lncRNAs and various biological progresses have been reported in growing studies, which show that they participate in the modulation of cells growth, differentiation, and apparent regulation[9,10]. Given their important functions in cellular progression, the potential modulation of lncRNAs in various diseases has attracted attention[11,12]. In tumor researches[13,14], it has been confirmed that lncRNAs serve as anti-tumor regulators or oncogenes in various tumors based on the types of organizations. Of note, the interactions with proteins and miRNAs are pivotal molecular mechanisms for lncRNAs to display their effects[15-17]. In addition, many clinical assays[18,19]

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indicate the significant potential of lncRNAs as novel tumor-related biomarkers for the screening of early tumor and the prediction of clinical outcome of tumor patients. However, the studies on the expression and functions of lncRNAs in AML were limited.

LncRNA LINC00641, a functional lncRNA firstly discovered by Liang et al[20], was a disease-associated lncRNA. This lncRNA has been reported to be a potential prognostic biomarker for glioblastoma patients. Recently, Wang et al[21] indicated that LINC00641 could modulate autophagy, and thus are involved in the pathogenesis of intervertebral disc degeneration. In bladder cancer, LINC00641 was shown to be overexpressed and acted as a tumor suppressor by regulating miR-197-3p[22]. However, little is known about the correlations between LINC00641 expressions and cellular progression in AML.

Patients and Methods

Clinical Samples and Cells

Twelve pediatric AML specimens and normal blood samples from 19 healthy controls were collected from Brain Hospital of Liaocheng People's Hospital from June 2011 to January 2014. The samples were immediately frozen using liquid nitrogen and preserved at -80°C. Written informed consents were obtained from the patients, and the procedures have been approved by the Ethics Committee of the Brain Hospital of Liaocheng People's Hospital. HS-5 cells (as control) and AML cancer cell lines (MV-4-11, NB4, THP1, HL-60, and U937) were bought from Mingjie Biological corporation (Dalian, Liaoning, China). The cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 media (10% fetal bovine serum) at 37°C and 5% CO_2.

SiRNA, miRNA, Plasmids, and Transfection

Small interfering RNAs (siRNA) targeting LINC00641 (si-lnc#1, si-lnc#2), Zinc finger, and BTB domain-containing 20 (ZBTB20) (siRNA#1, siRNA#2), and control siRNAs, miR-378a mimics and inhibitors, control miRNA mimics and inhibitors, were all bought from Sangong Biological corporation (Songjiang, Shanghai, China). LINC00641 or ZBTB20 sequence was separately cloned into pcDNA3.1 empty vector (pcDNA3.1-LINC00641, pcDNA3.1-ZBTB20) to continuously express LINC00641 or ZBTB20. The cell transfection was conducted by the use of Lipofectamine 3000 reagent kits (Furui, Jinan, Shandong, China) in accordance with the protocols in the kits.

Real Time-PCR

AML cells transfected with siRNAs, miRNAs or corresponding vectors were collected 48 h later after treatment. Then, the total RNAs were extracted using TRIzol kits (Detang, Qingdao, Shandong, China) for Real Time-PCR analyses. Reverse transcription was then conducted by applying the Tiangen cDNA synthesis kits (Saiheng, Chengdu, Sichuan, China). The cDNAs were then subjected to qPCR detection with the use of SYBR Green qPCR kits (Junhong, Hefei, Anhui, China) in accordance with the kits' protocols. For miR-378a detection, Qiagen miRNA extraction kits (CeKunBio, Changsha, Hunan, China) were employed. The levels of miRNAs were determined by applying Qiagen miRNA detection kits (CeKunBio, Changsha, Hunan, China) according to the kits' protocols. The expressing levels of lncRNA, gene and miRNA were defined based on the threshold cycle (Ct), and calculated using the $2^{-\Delta\Delta CT}$ method. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and U6 were used as references for lncRNA, gene, or miRNA. The primers were shown in Table I.

Western Blotting

The protein extraction kits (FengLeBio, Haimen, Jiangsu, China) were applied for extracting the total proteins from AML cells after LINC00641 siRNAs treatment. The proteins were separated by 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to polyvinylidene difluoride membranes after the concentrations were determined. Subsequently, 5% bovine serum albumin solution was utilized for blocking the membranes, followed by washing twice using TBST buffer. Then, anti-caspase 3/9 primary antibodies were respectively incubated with the membranes at 4°C overnight. On the second day, after washing with TBST buffer, the membranes were probed with the corresponding secondary antibodies. Finally, the proteins were detected by ECL kits (SunTeng, Qingdao, Shandong, China). The anti-caspase 3/9 primary antibodies were bought from Heng Yuan Biological corporation (Hangzhou, Zhejiang, China).

Cell Proliferation Detection

The cell proliferation was determined by applying CCK-8 kits (AndiWe, Xiamen, Fujian,

Table I. The used sequences of primer for RT-PCR.

Primer name	Sequences
LINC00641: Forward	TCAAGTCGTGTTGGCTATCT
LINC00641: Reverse	GCACTGAGCCTGTATGACC
miR-378a: Forward	GCGCACTGGACTTGGAGTC
miR-378a: Reverse	GCAGGGTCCGAGGTATTC
ZBTB20: Forward	GACAGGATCTACTCGGCACTC
ZBTB20: Reverse	ACTGCGCCGCTGTAAAAAGA
GAPDH: Forward	ACAACTTTGGTATCGTGGAAGG
GAPDH: Reverse	GCCATCACGCCACAGTTTC
U6: Forward	CTCGCTTCGGCAGCACA
U6: Reverse	AACGCTTCACGAATTTGCGT

China). In short, HL-60 and THP-1 cells were placed into ninety-six well plates (1000 cells per well). At designated timepoint, CCK-8 reagents were added into the cells (15 μl/well). After incubating at 37°C in 5% CO_2 for 2.5 h, the absorbance was measured at 450 nm on a microplate reader.

Flow Cytometry Detection

The cell cycle and apoptosis were determined by flow cytometric analyses. For the cell cycle determination, the cells were treated with LINC00641 siRNAs. Forty-eight hours later, the AML cells were collected in a centrifuge tube and washed with phosphate-buffered saline (PBS) twice. Then, the cells were resuspended in 450 μl PBS, and propidium iodide (PI) and RNaseA (30 mg/ml) were added into the cells. After incubating for 30 min in the dark, cell cycles were measured using flow cytometry machine. Similarly, for cell apoptosis detection, the collected cells were labeled with Annexin V-FITC and PI for 25 min in the light-proof condition. After washing using PBS, the cell apoptosis was determined using flow cytometry machine. The PI, RNase A, Annexin V-FITC/PI apoptotic detection kits were bought from Beyotime corporation (Qingpu, Shanghai, China).

Transwell Migration and Invasion Assays

The AML cell migratory ability and invasive capability were respectively assessed by the transwell migration and invasion assays. The LINC00641 siRNAs treated AML cells in 250 μl media without serum were placed into the upper chambers of the transwell inserts (1×10^5 cells/well). For the transwell invasion assays, the inserts needed to be pre-coated with Matrigel. The lower place was added with media with 15% FBS. The cells were allowed to grow for 24 h. Then, the media was pipette out and the cells were treated with 70% ethanol and 0.1% crystal violet. After washing twice using PBS, the images of the migratory or invasive cells were photographed by a microscope.

Subcellular Fractionation Location

Nuclear or cytoplasm fraction of AML cells were obtained using Thermo Fisher Scientific' PARIS kits (Kaigene, Fuzhou, Fujian, China) according to the kits' protocols. The RNAs were then isolated from the nuclear or cytoplasm fractions, and U6, GAPDH, and LINC00641 were determined by qPCR analyses as described above.

RNA-Pull Down

LINC00641 was *in vitro* transcribed and labeled with biotin (biotin-LINC00641) by Yuheng Biological corporation (Changsha, Hunan, China). We employed Thermo Fisher Scientific RNA-pull down kits (Pudong, Shanghai, China) to conduct the RNA-pull down experiments. In brief, biotin-LINC00641 or biotin-control were mixed with AML cell lysates. Then, the complexes were incubation with streptavidin agarose beads for 1.5 h at room temperature. Lastly, the eluted miR-378a was measured by qPCR analyzed as described above.

Luciferase Reporter Assay

The fragments including Wild-type miR-378a binding position of LINC00641 (LINC00641 wt), mutant-type miR-378a binding position of LINC00641 (LINC00641 mut), 3'UTR of ZBTB20 containing wild-type miR-378a binding site (wt ZBTB20), 3'UTR of ZBTB20 containing mutant-type miR-378a binding site (mut ZBTB20), were respectively cloned into pGL3 Luciferase reporter vectors. The AML cells (1×10^4 cells/well) were placed in ninety-six well

plates and co-transfected with Luciferase reporters and miR-378a mimics using Lipofectamine 3000. Forty-eight hours post-transfection, the Luciferase activity of the cells was determined by the use of Promega luciferase activity detection kits (XuheBio, Hefei, Anhui, China) on a microplate reader.

Statistical Analysis

SPSS 20.0 software (IBM Corp., Armonk, NY, USA) was employed to analyze the data. The analyses of the different groups were performed using One-way ANOVA or two-tailed Student's *t*-test. Tukey's post hoc tests were applied to validate the ANOVA for comparing measurement data between groups. A $p<0.05$ was regarded to be statistically significant.

Results

LINC00641 Was Highly in AML Specimens and Cells

To characterize the roles of LINC00641 in AML, we analyzed the RNA-Seq data (from TCGA) using a novel tool (GEPIA). As shown in Figure 1A, we found that the levels of LINC00641 were significantly increased in the AML specimens. To demonstrate the analysis results, we carried out qRT-PCR experiments to examine the LINC00641 levels in AML patients. The results showed that the LINC00641 levels were distinctly increased in AML specimens compared with normal blood samples (Figure 1B). Besides, the levels of LINC00641 in five AML cell lines were also examined. As presented in Figure 1C, LINC00641 expressions were distinctly upregulated in five AML cells compared with HS-5 cells. Our results firstly suggested LINC00641 as an AML-associated lncRNA.

Silencing LINC00641 Inhibited the Growth and Metastasis of AML Cells

To uncover the influences of LINC00641 on cellular growth and apoptosis of AML cells, we conducted loss of function studies using LINC00641 siRNAs. Real Time-PCR analyses demonstrated that LINC00641 siRNAs (si-lnc#1 and si-lnc#2) successfully silenced the levels of LINC00641 in AML cells (Figure 2A). Subsequently, the data from CCK-8 assays revealed that the proliferation abilities of AML cells were remarkably impeded by depressing LINC00641 expression (Figure 2B). Moreover, the cell cycles of AML cells treated with LINC00641 siRNAs were examined by flow cytometry analyses. The data confirmed that G0/G1 cell cycle arrest was induced when LINC00641 in AML cells was knocked down (Figures 2C and D). Besides, flow cytometry analyses were also utilized for the determination of cell apoptosis. The results suggested that inducing apoptosis happened in AML cells when their LINC00641 expression was repressed (Figure 2E). Accordingly, the levels of cellular apoptosis relevant molecules such as caspase-3 and caspase-9 were determined by Western blotting. The results elucidated that the protein levels of caspase-3 and caspase-9 were markedly elevated in AML cells when their LINC00641 expression was silenced (Figure 2F). Furthermore,

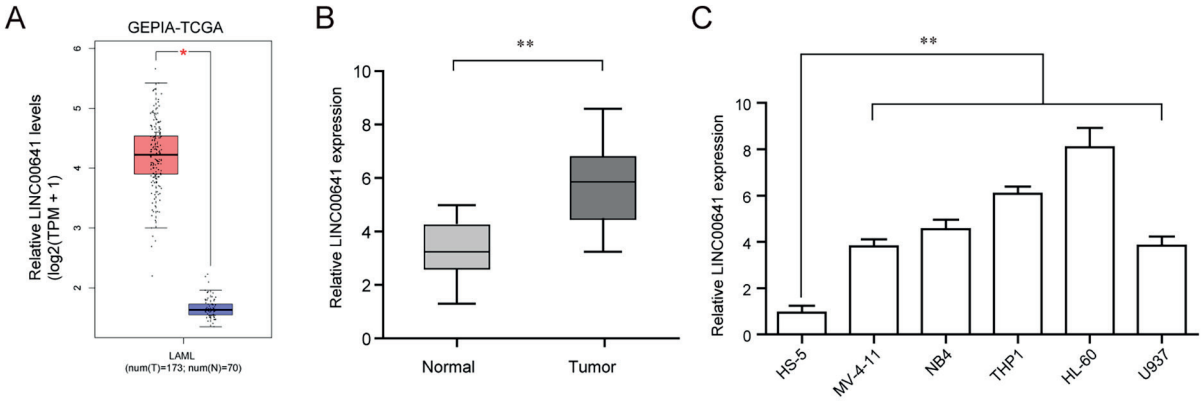

Figure 1. LINC00641 was highly expressed in AML samples and cells. ***A,*** A GEPIA tool was used to determine whether LINC00641 was dysregulated in AML. ***B,*** RT-PCR was performed for the examination of LINC00641 levels in AML patients and healthy controls. ***C,*** Comparing differences in the expression levels of LINC00641 between AML cell lines and normal HS-5 cells. *$p<0.05$, **$p<0.01$.

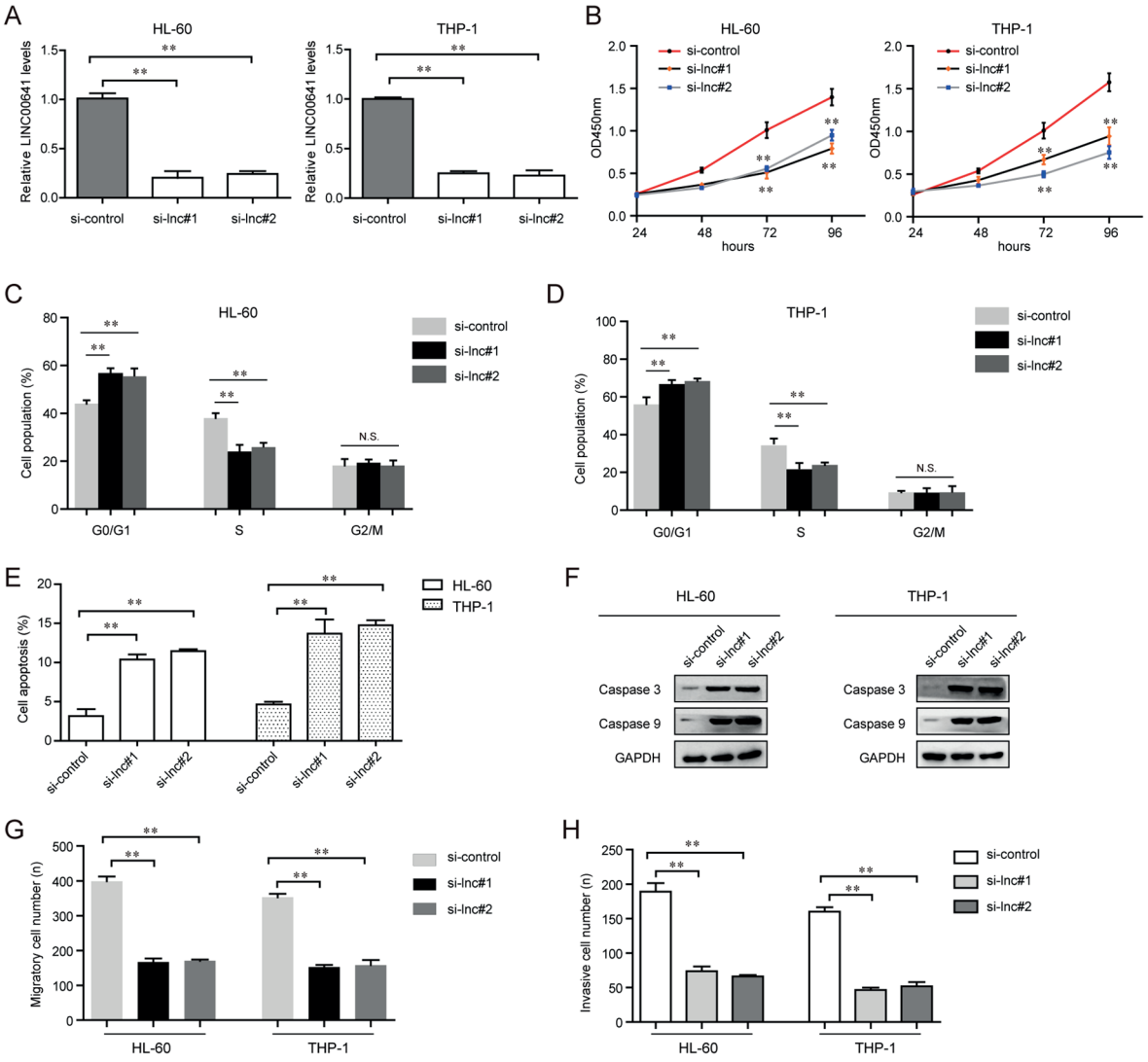

Figure 2. LINC00641 promoted malignant progression of AML cells. ***A,*** LINC00641 levels in HL-60 and THP-1 were measured by qPCR analyses. ***B,*** CCK-8 assays detected the cellular growth. ***C,*** and ***D,*** Cell cycle was determined by flow cytometry. ***E,*** Cell apoptosis was determined by flow cytometry. ***F,*** Western blot evaluated caspase 3/9 protein levels. ***G,*** Transwell migration assays. ***H,*** Transwell invasion assays. *$p<0.05$, **$p<0.01$.

AML cell mobility was also evaluated by transwell migration assays, and the data validated that repressing LINC00641 expression contributed to a remarkable reduction of migratory AML cell number (Figure 2G). Additionally, the transwell invasion assays were applied to detect the changes of the invasive capabilities in AML cells after their LINC00641 was silenced. The results certified that the invasive cell number was significantly reduced in AML cells by introduction of LINC00641 siRNAs (Figure 2H). In summary, these data demonstrated that LINC00641 knockdown inhibited AML cell malignant phenotypes, indicating that LINC00641 played essential roles in modulating the development and progression of AML.

LINC00641 Acted as a ceRNA Via Sponging MiR-378a in AML Cells

Next, we attempted to elucidate the mechanisms by which LINC00641 facilitated the AML tumorigeneses. Many studies revealed that lncRNAs might be involved in the progression of diverse cancer types *via* competitively binding to miRNAs, particularly when the lncRNAs were located in the cytoplasm. Therefore, we

next first sought to determine the localization of LINC00641 in HL-60 and THP-1 cells. The data from subcellular localization assays validated that LINC00641 was mainly exhibited in the cytoplasm of AML cells (Figure 3A). Then, the "Starbase" program showed that there was a binding site of miR-378a, a previously reported tumor suppressor in various cancer types, in the sequence of LINC00641 (Figure 3B)[23,24]. In fact, qPCR assays were performed to determine

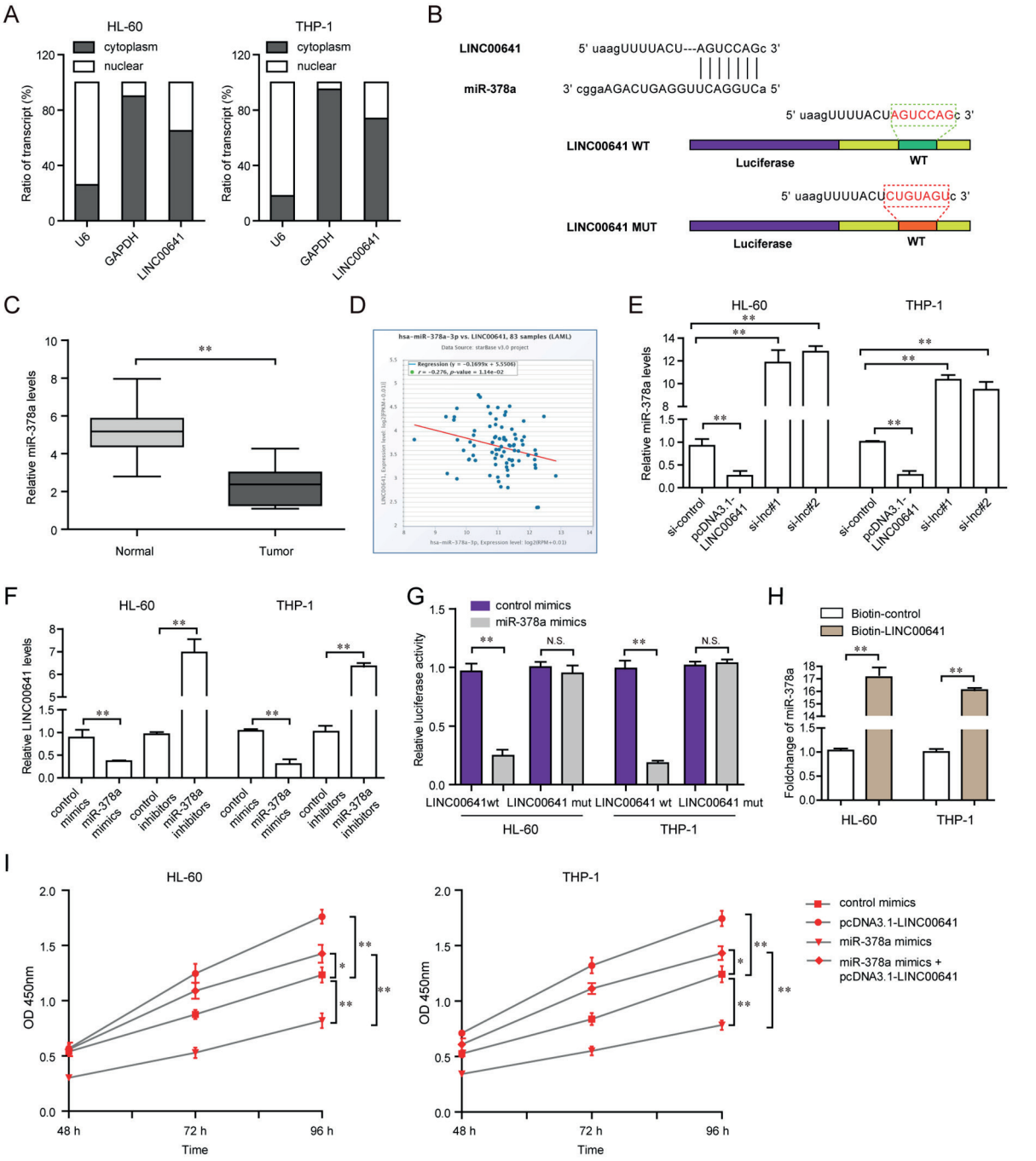

Figure 3. MiR-378a was a direct target of LINC00641 in AML cells. **A**, Subcellular fractionation location assay. ***B,*** "StarBase" predicted binding site between miR-378a and LINC00641. **C**, qPCR detected miR-378a levels in twelve pediatric AML specimens and matched normal samples. ***D,*** "StarBase" program analyzed the expressing correlation of miR-378a and LINC00641. ***E,*** qPCR detected miR-378a levels. ***F,*** qPCR detected LINC00641 levels. ***G,*** Luciferase activity detection. ***H,*** RNA-pull down. ***I,*** CCK-8 assays assessed the proliferation of AML cells after various treatments. *$p < 0.05$, **$p < 0.01$.

the miR-378a levels in AML tumor samples and paired normal specimens. The data suggested that miR-378a levels in AML tumor samples were markedly lower than that of paired normal specimens (Figure 3C). In addition, the data from "StarBase" program also indicated that the levels of LINC00641 and miR-378a were reversely correlated in AML tumor specimens (Figure 3D). Afterwards, we forced or silenced the expression of LINC00641 in AML cells, and qPCR assays revealed that miR-378a expression was decreased by ectopic expression of LINC00641, while its levels were elevated by LINC00641 knockdown (Figure 3E). Vice versa, the forced expression of miR-378a notably reduced LINC00641 levels, while repressing miR-378a levels remarkably increased LINC00641 expression (Figure 3F). Therefore, these data further demonstrated that LINC00641 expression was inversely associated with miR-378a levels, indicating that miR-378a might directly interact with LINC00641. For further directly confirming that, we next performed Luciferase activity detection analyses. The results certified that co-transfection with miR-378a mimics and LINC00641 wt but not LINC00641 mut significantly depressed the Luciferase activities in AML cells, which validated that miR-378a directly interacted with LINC00641 (Figure 3G). For further clarifying that, RNA-pull down analyses were conducted and the results proved that LINC00641 could predominantly precipitate miR-378a in HL-60 and THP-1 cells (Figure 3H). Hence, these above data demonstrated that miR-378a was a target of LINC00641. Next, we aimed to investigate whether LINC00641 was able to restore the inhibitory impact of miR-378a on malignant phenotypes. To achieve that, CCK-8 assays were carried out in AML cells after various treatments. As the results presented in Figure 3I, LINC00641 could accelerate AML cells growth, and enhancing miR-378a expression led to significant inhibition of AML cell proliferation, while re-introduction of LINC00641 was capable to reverse the impeding influences of miR-378a on cellular proliferation. In summary, these results displayed that LINC00641 modulated AML malignant phenotypes *via* sponging miR-378a.

ZBTB20 Was a Target Gene of MiR-378a in AML Cells

It is well known that miRNAs exerted their functions via directly targeting tumorigenesis-related genes[25]. Hence, we next attempted to discover which gene was able to be targeted by miR-378a in AML cells. We firstly employed three classical miRNA-targets predicting algorithms (miRDB, TargetScan, and StarBase) to predict the possible target genes of miR-378a, and then we intersected these potential targets with genes upregulated in AML tumor samples. We found that only five genes were common expressed (Figure 4A). Among these five genes, ZBTB20, a previously reported tumor promoter, attracted our attention[26]. Bioinformatics analyses using "StarBase" website revealed that ZBTB20 levels were negatively correlated with miR-378a levels in AML tumor specimens (Figure 4B). Additionally, "GEPIA" program analyses using TCGA data suggested that ZBTB20 levels were markedly upregulated in AML tumor samples (Figure 4C). Furthermore, we performed qPCR assays to detect ZBTB20 levels in twelve paired AML tumor samples and normal specimens, and we found that ZBTB20 was also upregulated in AML tumor samples (Figure 4D). Moreover, we next investigated the functions of ZBTB20 on AML cells through loss-of-function studies. For that purpose, siRNAs targeting ZBTB20 were synthesized and the knockdown efficiency was determined by qPCR analyses (Figure 4E). Then, CCK-8 assays were conducted and the data indicated that the depression of ZBTB20 resulted in dramatically inhibition of AML cell growth (Figure 4F). Thereafter, the transwell invasion assays were also performed and we discovered that ZBTB20 knockdown remarkably reduced the invasive AML cells (Figure 4G). These data suggested that ZBTB20 could modulate the malignancies of AML. Therefore, we next sought to clarify whether ZBTB20 was a target of miR-378a. The predicted binding position between miR-378a and ZBTB20 was shown in Figure 4H. We, then, constructed Luciferase reporters, respectively containing predicted Wild-type binding position (wt ZBTB20) or mutated-type binding position (mut ZBTB20). Subsequently, the Luciferase reporters were separately co-transfected with miR-378a mimics into AML cells. The data from the Luciferase activity detection assays revealed that the Luciferase activities were significantly decreased in AML cells co-transfected with wt ZBTB20 and miR-378a mimics, which validated that miR-378a directly interacted with ZBTB20 mRNA 3'UTR. Taken together, these data demonstrated that ZBTB20 was a target gene of miR-378a in AML cells.

LINC00641 Regulated ZBTB20 Expression Via MiR-378a in AML Cells

We next sought to investigate whether LINC00641 could regulate ZBTB20 expression

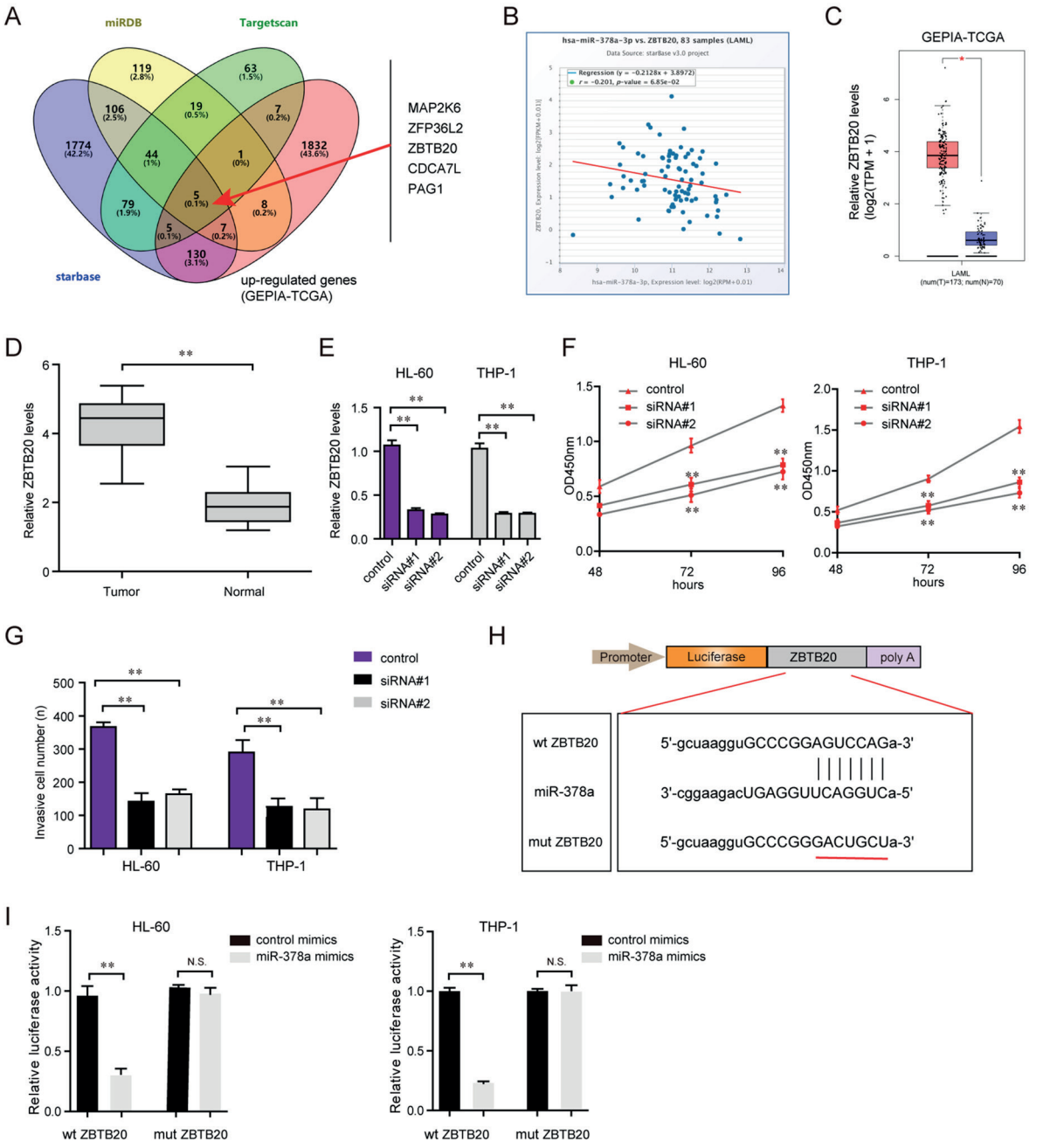

Figure 4. MiR-378a directly interacted with ZBTB20 in AML cells. ***A,*** Venn diagram of "miRDB", "TargetScan" and "StarBase" prediction, and upregulated genes in AML tumor samples analyzed by "GEPIA". ***B,*** "StarBase" program analyzed the expressing correlation of miR-378a and ZBTB20. ***C,*** "GEPIA" analyzed the ZBTB20 levels in AML specimens. ***D,*** qPCR detected ZBTB20 levels in twelve pediatric AML specimens and matched normal samples. ***E,*** qPCR examined ZBTB20 levels in AML cells after transfection with ZBTB20 siRNAs (siRNA#1, siRNA#2). ***F,*** CCK-8 assays. ***G,*** Transwell invasion assays. ***H,*** "StarBase" predicted miR-378a binding site in 3'UTR of ZBTB20 mRNA. ***I,*** Luciferase activity detection. *p<0.05, **p<0.01.

via miR-378a. Firstly, using "GEPIA" algorithm, we found that LINC00641 and ZBTB20 expression in AML samples was positively correlated, indicating there might be a modulatory relationship between LINC00641 and ZBTB20 (Figure 5A). To validate that, a series of experiments were conducted. Real Time-PCR analyses demonstrated that both LINC00641 and ZBTB20 levels

were repressed in HL-60 cells after ectopic expression of miR-378a (Figure 5B). Accordingly, LINC00641 and ZBTB20 levels were elevated by transfection of miR-378a mimics. In addition, ZBTB20 levels were remarkably elevated in AML cells by forced expression of LINC00641, while its expression was impeded in the cells by LINC00641 knockdown (Figure 5C). Vice versa, LINC00641 expression was also depressed or promoted by ZBTB20 overexpression or knockdown (Figure 5D). Of note, qPCR assays clarified that the ectopic expression of ZBTB20 could significantly reverse the levels of LINC00641, which was repressed by miR-378a (Figure 5E). Vice versa, enhancing LINC00641 expression was also able to restore the inhibiting influence of miR-378a on ZBTB20 (Figure 5F). Collectively, these data showed that LINC00641 regulated ZBTB20 expression via miR-378a in AML cells.

Discussion

AML is a heterogeneous malignancy and has become a problem for human health. The tremendous successes have been achieved in stem cell researches and enormous, extensive genomic assays, which have greatly promoted our understanding of the potential molecular mechanisms involved in the pathogenesis of AML[27,28]. However, the effects for treatment plans are limited. Thus, it is urgent to discover novel targets to override the proliferation and metastasis of AML cells for the improvements of clinical prognosis of AML patients.

With the advancement of RNA-Seq technologies, growing dysregulated lncRNAs that are intimately associated with AML leukemogenesis have been recognized[29,30]. In addition, several lncRNAs have been functionally characterized in AML[31]. In this study, we discovered a novel AML-related lncRNA LINC00641. Using RT-PCR and the data from TCGA datasets, the overexpression of LINC00641 was observed in both AML specimens and cells. Functional experiments revealed that the knockdown of LINC00641 suppressed proliferation, induced G1-phase arrest, and accelerated apoptosis in HL60 and THP-1 cells. In addition, the metastatic abilities of HL60 and THP-1 cells were suppressed by the downregulation of LINC00641. Our finding indicated that LINC00641 served as a promoter during AML carcinogenic process. Previously, Li et al[22] reported that LINC00641 was lowly expressed in bladder cancer. In their *in vitro* assays,

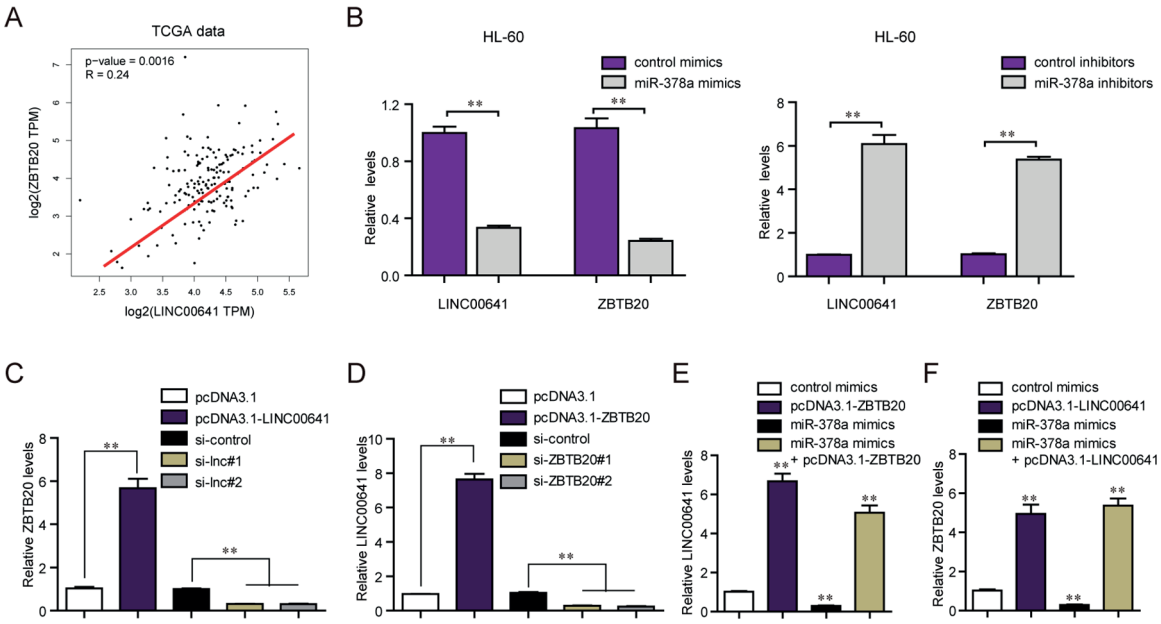

Figure 5. LINC00641 regulated miR-378a/ZBTB20 axis in AML cells. ***A,*** "GEPIA" analyzed the expressing correlation of LINC00641 and ZBTB20 in AML samples using TCGA data. ***B,*** qPCR examined LINC00641 and ZBTB20 levels in AML cells after miR-378a was ectopic expression or knockdown. ***C,*** qPCR examined ZBTB20 levels. ***D,*** qPCR analyses measured LINC00641 levels. ***E,*** and ***F,*** qPCR evaluated LINC00641 and ZBTB20 levels in AML cells. **p*<0.05, ***p*<0.01.

LINC00641 was demonstrated to markedly inhibit the proliferation and metastasis of tumor cells by the modulation of miR-197-3p/KLF10. Their results were not in line with our findings, which suggested that the function of LINC00641 varied in the different types of tumors.

Karreth and Pandolfi et al[32] discovered that some lncRNAs and circular RNAs (circRNAs) contain microRNA-binding elements and function as ceRNAs, suppressing functional miRNAs activities. For instance, lncRNA LINC00511 was indicted to accelerate the proliferation and invasion of glioma cells via targeting miR-124-3p[33]. LncRNA HOXA-AS2 was shown to be highly expressed in lung cancer, and its tumor-promotive roles in this tumor *via* sponging miR-520a-3p were also confirmed in *in vitro* assays[34]. In AML, lncRNA SBF2-AS1 was reported to modulate AML cell proliferation as a ceRNA of miR-188-5p[35]. In this study, we found that miR-378a may be a target of LINC00641 using the bioinformatics databases (StarBase v2.0). The levels of miR-378a were detected in AML samples and the results showed that its levels were lowly expressed in AML. In addition, we used online data to show that the expressions of LINC00641 were negatively associated with those of miR-378a in AML samples. Moreover, the results of the Luciferase assays validated the direct binding abilities of the response elements of miR-378a on the full-length LINC00641 transcript. More importantly, functional assays also confirmed the overexpression of miR-378a as a reverse regulator in modulating the suppressive roles in AML cells proliferation mediated by LINC00641 overexpression. Overall, these findings indicated that the knockdown of LINC00641 suppressed AML progression via miR-378a.

ZBTB20, located on 3q13.31, represents a novel member of the POK family of transcriptional suppressors[36]. The biological function of ZBTB20 mammals is complex and its preferential expression is observed in hippocampal progenitors[37]. In recent years, ZBTB20 was found to serve as an oncogene in the regulation of the pathogenesis and progression of bountiful kinds of diseases, including tumors[38,39]. However, its effects in AML remain unclear. Hence, our group performed functional investigations, finding that the downregulation of ZBTB20 inhibited the proliferation and invasion of AML cells. Interestingly, ZBTB20 was verified as a direct target of miR-378a in our bioinformatics assays. Furthermore, the data of the Luciferase reporter experiments demonstrated that miR-378a targeted ZBTB20 mRNA. These results suggested that miR-378a may display its function by targeting ZBTB20. However, whether LINC00641 was involved in the regulation of miR-378a/ZBTB20 remained unclear. In a series of experiments, we found that LINC00641 regulated the ZBTB20 expression *via* miR-378a in AML cells. Overall, our data demonstrated that LINC00641 may exert its functions through the miR-378a/ZBTB20 axis.

Some limitations of this study should be noted. First, the clinical significance of LINC00641 and ZBTB20 in AML patients has not been investigated. Further five-year follow-up for AML patients was needed. Second, this study just included the *in vitro* assays. Further *in vivo* experiments were necessary for the confirmation of the promotive function of LINC00641 in AML cells. Third, we did not unveil the downstream proteins, which LINC00641 modulated. The further in-depth exploration was essential for potential signaling pathway through which ZBTB20 exhibited its tumor-related effects.

Conclusions

LINC00641 was upregulated in AML and its overexpression could enhance AML cells growth and migration capacities, and reduced apoptosis of AML cells through miR-378a/ZBTB20 signaling. LINC00641 was likely to function as a capable therapeutic approach in respect of AML therapy.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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