

# LncRNA H19 regulates ID2 expression through competitive binding to hsa-miR-19a/b in acute myelocytic leukemia

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**Abstract.** Acute myelocytic leukemia (AML) is the most common type of acute leukemia. Long non-coding RNAs (lncRNAs) serve an important role in regulating gene expression through chromatin modification, transcription and post-transcriptional processing. LncRNA H19 was considered as an independent prognostic marker for patients with tumors. The expression of lncRNA H19 was identified to be significantly upregulated in bone marrow samples from patients with AML-M2. Furthermore, it was demonstrated that the knockdown of lncRNA H19 resulted in increased expression of hsa-microRNA (miR)-19a/b and decreased expression of inhibitor of DNA binding 2 (ID2) in AML cells. The knockdown of lncRNA H19 inhibited the proliferation of AML cells *in vitro*, which could be partially reversed by ID2 overexpression. Furthermore, the results of the bioinformatic analysis revealed potential hsa-miR-19a/b-3p binding sites in lncRNA

H19 and ID2. Altogether, the results of the present study suggest that lncRNA H19 regulates the expression of ID2 through competitive binding to hsa-miR-19a and hsa-miR-19b, which may serve a role in AML cell proliferation.

## Introduction

Acute myelocytic leukemia (AML), a heterogeneous clonal disorder of hematopoietic cells, is the most common acute leukemia in adults (1). AML was characterized as an abnormal proliferation of myeloid progenitor cells and an arrest in cellular differentiation. According to the French-American-British classification system, AML was classified into eight major AML subtypes (FAB, M0-M7). Despite great progress achieved in this field, the etiology and pathogenesis of AML is still not fully understood.

In the past, non-coding RNA (ncRNA) had been considered as transcriptional noise. Recently, it was suggested that ncRNAs participated in a wide range of processes including protein translation, mRNA splicing, chromatin organization, and the regulation of gene expression. According to their size, ncRNAs are generally classified into small ncRNAs (sncRNAs; <200 nucleotides, including microRNAs, Piwi-interacting RNAs and small nucleolar RNAs) and long ncRNAs (lncRNAs, >200 nucleotides) (2,3). The pivotal regulatory role of ncRNAs in the development of solid tumors and hematological malignancy has attracted increasing attention. LncRNAs are often expressed in a temporally and spatially specific manner (4,5), which also play a critical role in cancer development (6,7).

LncRNA H19 is one of the first discovered lncRNAs. LncRNA H19 is highly expressed during embryonic and fetus stages, while it is downregulated in most of tissues shortly after birth (8). It was found that the silence of lncRNA H19 inhibited tumor cell proliferation, invasion and proliferation, while the overexpression of lncRNA H19 conferred growth

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advantage to tumor cell (9,10). Moreover, it was suggested that lncRNA H19 expression was negatively related to survival rate of tumor patients (11,12). Level of lncRNA H19 in plasma was stable in gastric cancer, which could be used as a biomarker of cancer diagnosis, especially for early cancer screening and dynamic monitoring of tumor after surgery (13,14). Zhang *et al* have proved that the level of lncRNA H19 overexpression was positively related to Tumor-Node-Metastasis (TNM) stage and tumor size in non-small cell lung cancer, which could be also used as an independent prognostic factor of overall survival of patients (15). However, there is little research about the expression of lncRNA H19 in AML patients, and the role of lncRNA H19 in AML pathogenesis is still unknown.

In this study, it was showed that the expression of lncRNA H19 was significantly upregulated in bone marrow samples from AML-M2 patients. Knockdown of lncRNA H19 inhibited AML cell proliferation, which gave rise to the upregulated expression of hsa-miR-19a/b and the downregulated expression of inhibitor of DNA binding 2 (ID2), one of the target genes of hsa-miR-19a/b.

## Materials and methods

**Cell culture and transfection.** HL-60 cells, KG-1a cells and HEK-293T cells were cultured in RPMI-1640 medium (Gibco, Grand Island, NY, USA) supplied with 10% fetal bovine serum, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin, at 37°C in a 5% CO<sub>2</sub> atmosphere. Has-miR-19a/b mimic, Has-miR-19a/b inhibitor and their respective scramble controls were obtained from Guangzhou RiboBio Co., Ltd. (Guangzhou, China). lncRNA H19 siRNA was purchased from Invitrogen Life Technologies (Waltham, MA, USA). The sequences of mimics and siRNAs were listed in Table I.

Transfection of HL-60 cells and KG-1a cells were electroporated with Neon<sup>®</sup> Transfection System (Invitrogen Life Technologies), and transferred to RPMI-1640 medium supplied with 10% fetal bovine serum. Cell viability of the electroporated cells was more than 70%. HEK-293T Cells were transfected with Lipofectamine<sup>®</sup> 2000 (Invitrogen Life Technologies) according to the the manufacturer's protocol.

**Cell proliferation assay.** Cells were seeded in a 96-well plate and cultured in normal medium. At 0, 24, 48 and 72 h after transfection, the cells were incubated in 0.1 mg/ml MTT at 37°C for 4 h and lysed in dimethylsulphoxide (DMSO) at room temperature for 10 min. The absorbance in each well was measured by a microplate reader (Bio-Rad, Hercules, CA, USA). Data are the mean  $\pm$  standard deviation of three independent experiments.

**Soft agar colony formation assay.** A 2-ml layer of 0.7% agar in RPMI-1640 medium with 10% FBS was poured into 6-well plates. Cells were resuspended in 0.3% agar in RPMI-1640 medium with 10% FBS, and 1 ml cell suspension was poured on top of the base layer. The suspension was allowed to solidify and incubated at 37°C in 5% CO<sub>2</sub> for 14 days. The experiment was performed in triplicate and repeated three times.

**Patients and tissue collection.** Bone marrow samples were obtained from 107 patients with AML (58 female/49 male,

mean age: 40.14 $\pm$ 17.94 years) and 53 matched healthy subjects (26 female/27 male, mean age: 40.89 $\pm$ 11.75 years). The 107 patients included 46 AML patients with maturation (AML-M2), 20 AML patients with AML-M3 (acute promyelocytic leukemia, APL) and 41 AML patients with other types of AML. The diagnosis of leukemia was made by morphologic and cytochemical studies of bone marrow smears. All patients were not taking any anti-leukemic therapy at the moment of blood sampling and were newly diagnosed with AML. The study was approved by the Ethics Review Committee for Human Studies of the Peking University Health Science Center (Beijing, China). All participants provided written informed consent before any blood sampling.

**Plasmid construction.** In order to generate a reporter construct, the 3' untranslated region (3'UTR) of the ID2 gene was cloned into the psiCHECK-2 luciferase vector (Promega Corp., Madison, WI, USA). In order to generate ID2 expression construct, the coding sequence (CDS) of the ID2 gene were cloned into pcDNA4 (Addgene Inc., Cambridge, MA, USA). The sequences of primers were listed in Table I.

**RNA extraction and quantitative polymerase chain reaction.** RNA was extracted from bone marrow samples and AML cells using TRIzol (Invitrogen Life Technologies) following the manufacturer's protocol. 1  $\mu$ g RNA was reverse transcribed into complementary DNA (cDNA) using the GoScript<sup>™</sup> Reverse Transcription System (Promega Corp.) following the manufacturer's instructions. The ABI StepOne Plus (Applied Biosystems, Foster City, CA, USA) was used to perform the amplification reaction.

Primers for amplification of lncRNA H19, ID2, and GAPDH were synthesized by Invitrogen Life Technologies. qPCR was performed using 96 well plates (Bio-Rad) in 20  $\mu$ l mixture containing 10  $\mu$ l SYBR-Green qPCR Master Mix (Bio-Rad), 10 pmol forward primers and reverse primers, and the appropriate amount of cDNA. The samples were denatured at 95°C for 10 min, and then amplified during 40 cycles at 95°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec on an iQ5 Real-Time thermal cycler (Bio-Rad). Each sample was assayed in triplicate, and C<sub>quantification</sub> cycle (Cq) values were measured. A mean Cq value for each triplicate measurement was calculated. Relative gene expression was then calculated using 2<sup>- $\Delta\Delta$ Cq</sup> method. The sequences of primers were also listed in Table I.

**Western blot analysis.** Proteins were extracted from cells with RIPA lysis buffer (KenGen Biotech, Nanjing, China) and were quantified using a BCA Protein Assay kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA). A total of 30 mg protein lysates were loaded to SDS-PAGE gel, and transferred to PVDF membranes (Millipore Corp., Billerica, MA, USA). The membrane was blocked in 5% nonfat milk and incubated with diluted antibodies against ID2 (1:500; Invitrogen Life Technologies), followed by incubation with a horseradish peroxidase-conjugated secondary antibody (1:5,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA).  $\beta$ -actin was used as a control (1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA).

Table I. Sequences of primers and siRNA used in this study.

Names	Sequences
qPCR primers	
H19-F	5'-CGGACACAAAACCCTCTA-3'
H19-R	5'-CTAGCTTCACCTTCCAGA-3'
GAPDH-F	5'-GAAGGTGAAGGTCGGAGTC-3'
GAPDH-R	5'-GAAGATGGTGATGGGATTTTC-3'
ID2-F	5'-CATCCCACTATTGTTCAGC-3'
ID2-R	5'-AATTCAGAAGCCTGCAAG-3'
siRNA	
H19 siRNA	5'-CCCGUCCCUUCUGAAUUUATT-3'
Plasmid construction	
ID2-3'UTR reporter-F	CCGCTCGAGATAAGCGGTGTTTCATGAT
ID2-3'UTR reporter-R	ATAAGAATGCGGCCGCTTTGACTTCAGACATGTTT
ID2-pcDNA4-F	CCCAAGCTTATGAAAGCCTTCAGTCCCGTGA
ID2-pcDNA4-R	CGGGATCCTCAGCCACACAGTGCTTTGCT

F, forward; R, reverse; qPCR, quantitative polymerase chain reaction; siRNA, small interfering RNA; ID2, inhibitor of DNA binding 2; 3'UTR, 3' untranslated region.

**Dual luciferase reporter assay.** Cotransfection was conducted in HEK-293T cells with 0.8  $\mu$ g plasmid and 50  $\mu$ M miRNA mimic. After transfected for 24 h, the cells were harvested and treated according to instructions of Promega Dual-Luciferase<sup>®</sup> Reporter activity assay kit (Promega Corp., Fitchburg, WI, USA). The luciferase activity was then detected by a single photon detector. The relative luciferase activity was calculated as firefly luciferase activity value divided by Renilla reniformis luciferase activity. The experiment was repeated three times.

**Statistical analysis.** The expression differences between bone marrow samples of AML patients and health individuals were analyzed using paired samples t-test. Pearson's coefficient correlation was used for expression correlation assay. MTT assays were analyzed using ANOVA. The statistical analyses were performed using SPSS (Version 19.0; SPSS, Inc., Chicago, IL, USA). A two-sided  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**LncRNA H19 expression was upregulated in the bone marrow samples of AML-M2 patients.** Many studies have shown that lncRNA H19 expression was upregulated in various tumorigenesis (9-16), which suggest that lncRNA H19 could serve as an oncogene in cancer. The expression level of lncRNA H19 in the bone marrow of AML-M2 patients (M2) was also higher (Fig. 1). It suggested that the aberrant expression of lncRNA H19 may be involved in the progression of AML.

Dalton *et al* have shown that HL-60 cell line was derived from a patient with FAB-M2 and not FAB-M3 (17). Therefore, HL-60 and KG-1a cell lines were chosen for further investigation in this study. The HL-60 cell line was transfected with negative control or si-lncRNA H19. Three different siRNAs

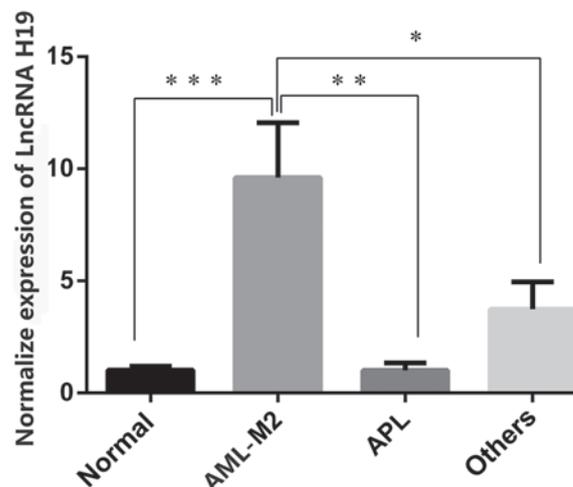


Figure 1. The expression of long non-coding RNA (lncRNA) H19 in bone marrows of acute myelocytic leukemia (AML) patients. Compared with healthy subjects (NORMAL), patients with acute promyelocytic leukemia (APL) and other types (OTHERS), relative expression of lncRNA H19 in AML-M2 (M2) was higher ( $P < 0.05$ ;  $**P < 0.01$ ;  $***P < 0.001$ ).

were designed against H19 (named as si-H19a, si-H19b, si-H19c). Two of them can efficiently knockdown H19 expression, and eventually si-H19a was chosen for its best efficiency in the following experiments (Fig. 2A). The MTT assays and the soft agar colony formation assays showed that proliferation of HL-60 cell transfected with si-lncRNA H19 was also markedly decreased (Fig. 2B and C). Similar results were obtained with KG-1a cell line (Fig. 3).

**Restored expression of ID2 partially reversed the effect of lncRNA H19 on AML cell proliferation.** The possible mechanism involved in the regulation of AML cell proliferation by lncRNA H19 was unclear. It was proved that the

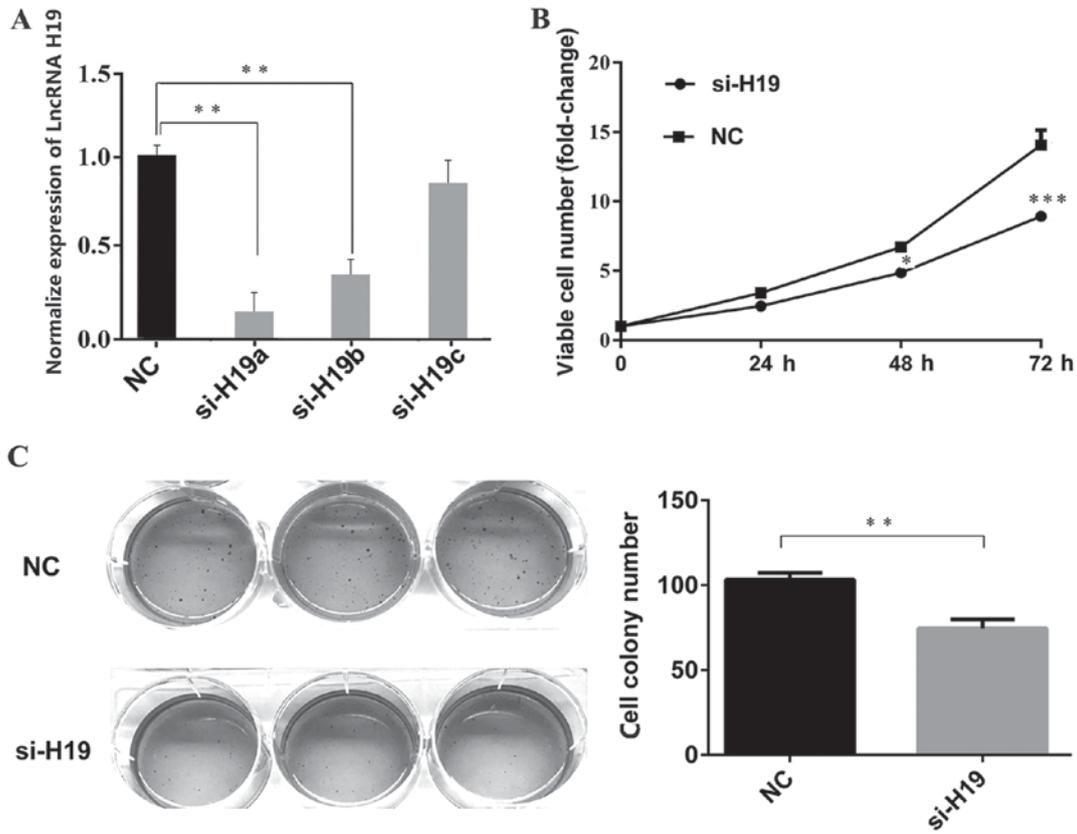


Figure 2. Knockdown of long non-coding RNA (lncRNA) H19 inhibited acute myelocytic leukemia cell proliferation. (A) si-lncRNA H19 could significantly reduce the expression of lncRNA H19. si-lncRNA H19 (si-H19) and negative control (NC) were transfected into HL-60 cell lines. lncRNA H19 expression was detected by qPCR. (B) Cell proliferation assay was performed by MTT assay. After transfected with si-H19 and NC for 72 h, the rates of cell proliferation in the treatment of si-H19 were significantly slower than NC. (C) Cell proliferation assay was performed by soft agar colony formation assay. After transfected with si-H19 and NC for 72 h, the number of colony formation in in the treatment of si-H19 was significantly slower than NC (\* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ).

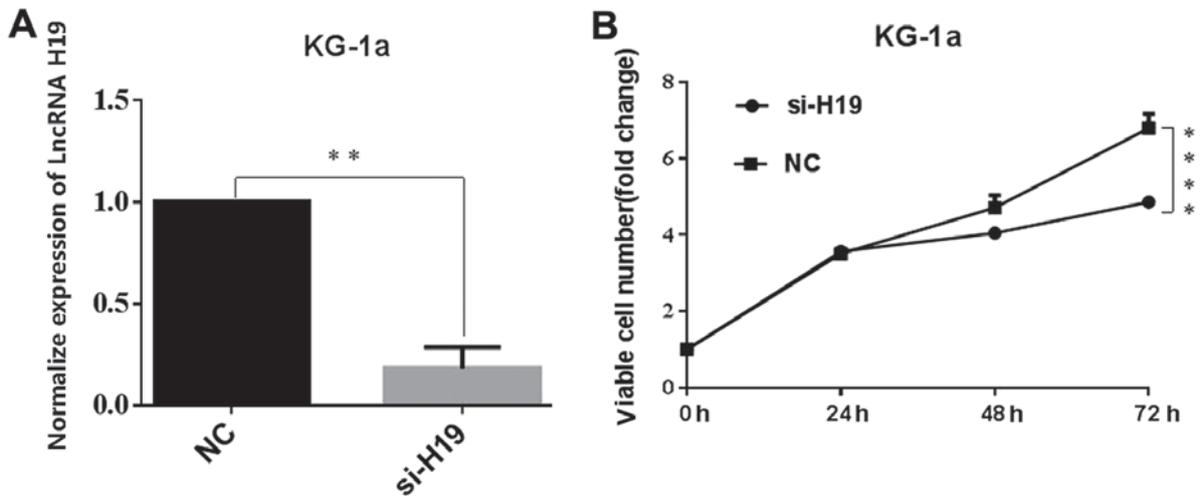


Figure 3. Knockdown of long non-coding RNA (lncRNA) H19 inhibited KG-1a cell proliferation. (A) Si-lncRNA H19 could significantly reduce the expression of lncRNA H19. Si-lncRNA H19 (si-H19) and negative control (NC) were transfected into KG-1a cell lines. lncRNA H19 expression was detected by qPCR. (B) Cell proliferation assay was performed by MTT assay. After transfected with si-H19 and NC for 72 h, the rates of KG-1a cell proliferation in the treatment of si-H19 were significantly slower than NC. (\* $P < 0.05$ ; \*\* $P < 0.01$ ).

expression of lncRNA H19 was related to the expression of ID2 in bladder cancer, which may promote bladder cell proliferation (18). Many studies have shown that expression of ID2 protein is increased in a large variety of human tumors, including bladder cancer, breast cancer, and squamous cell

carcinoma (18-20). lncRNA H19 could serve as a competing endogenous RNA (ceRNA) by sponging hsa-miR-19a-3p in gastric cancer (21). Bioinformatics analysis indicated that the binding sites of hsa-miR-19a-3p and hsa-miR-19b-3p existed in the lncRNA H19 and 3'-untranslated region of ID2

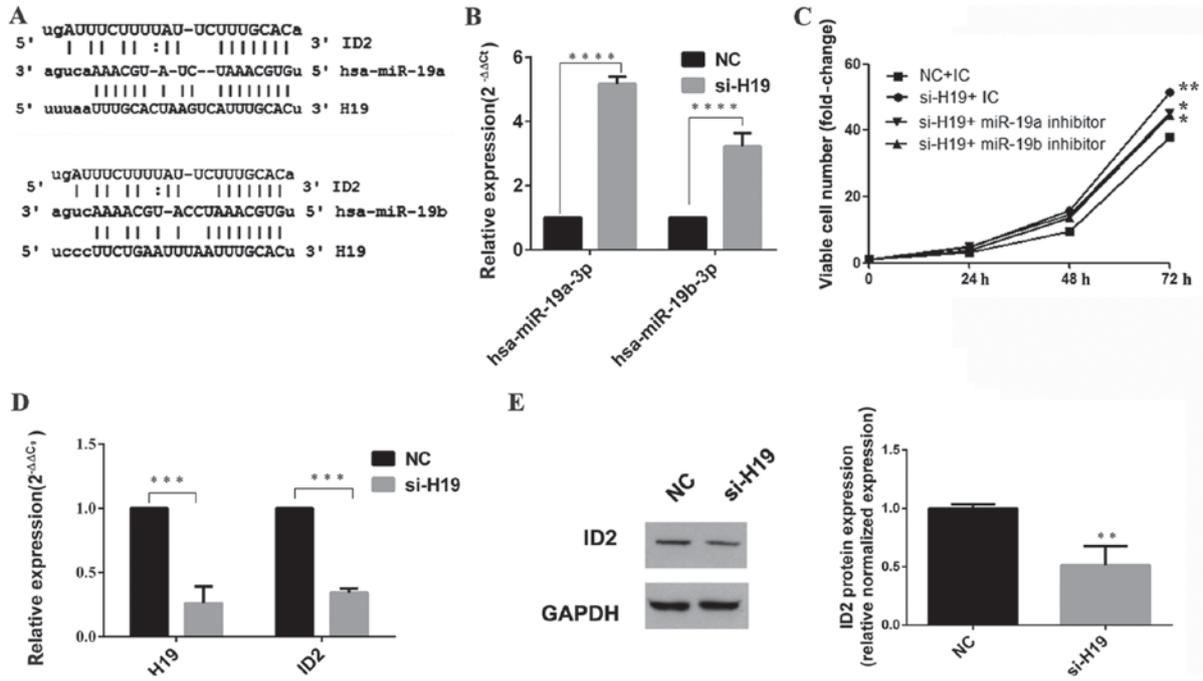


Figure 4. Knockdown of long non-coding RNA (lncRNA) H19 decreased inhibitor of DNA binding 2 (ID2) expression and increased hsa-miR-19a-3p and hsa-miR-19b-3p expression. (A) Seed match of hsa-miR-19a-3p, lncRNA H19 and ID2. (B) Hsa-miR-19a-3p expression in HL-60 cells transfected with negative control (NC) or si-lncRNA H19 (si-H19) was detected by qPCR. (C) Cell proliferation assay was performed by MTT assay. After transfected with si-H19, miR-19a/b inhibitor and related controls for 72 h, the rates of cell proliferation in the treatment of si-H19 were significantly restored by miR-19a/b inhibitor. (D) Expression level of ID2 mRNA in HL-60 cells transfected with NC or si-H19 was detected by qPCR. (E) LncRNA H19 knockdown reduced the ID2 protein expression. GAPDH was used as internal loading control (\*\*P<0.01; \*\*\*P<0.001).

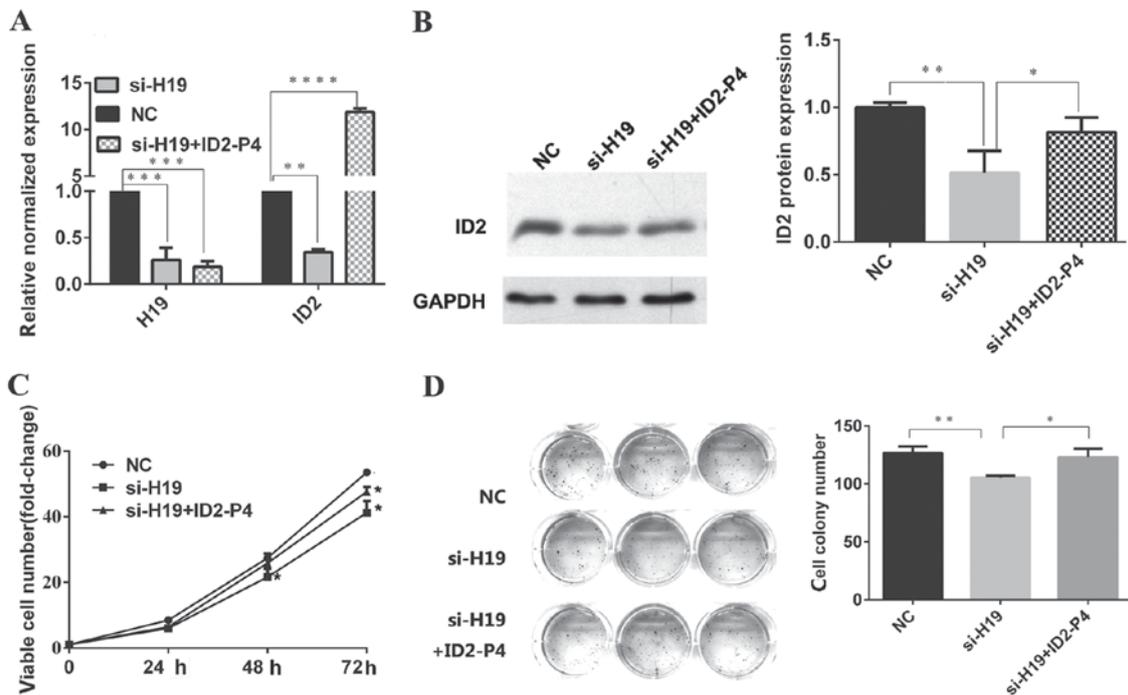


Figure 5. Restored inhibitor of DNA binding 2 (ID2) partially restored the effect of long non-coding RNA (lncRNA) H19 in AML cell proliferation. (A) LncRNA H19 and ID2 expression were detected by qPCR. (B) LncRNA H19 and ID2 expression were detected by western blotting. (C) MTT assay showed the effect of lncRNA H19 in cell proliferation partially reversed by restored ID2. (D) Colony formation assay showed the effect of lncRNA H19 in cell proliferation partially reversed by restored ID2 (\*P<0.05; \*\*P<0.01; \*\*\*P<0.001).

(Fig. 4A). Hsa-miR-19a-3p and hsa-miR-19b-3p expressions were upregulated in HL-60 cells transfected with si-lncRNA H19 (Fig. 4B). Both inhibitors of hsa-miR-19a-3p and

hsa-miR-19b-3p reversed the effect of lncRNA H19 on AML cell proliferation (Fig. 4C), while both mRNA and protein of ID2 was decreased (Fig. 4D and E).

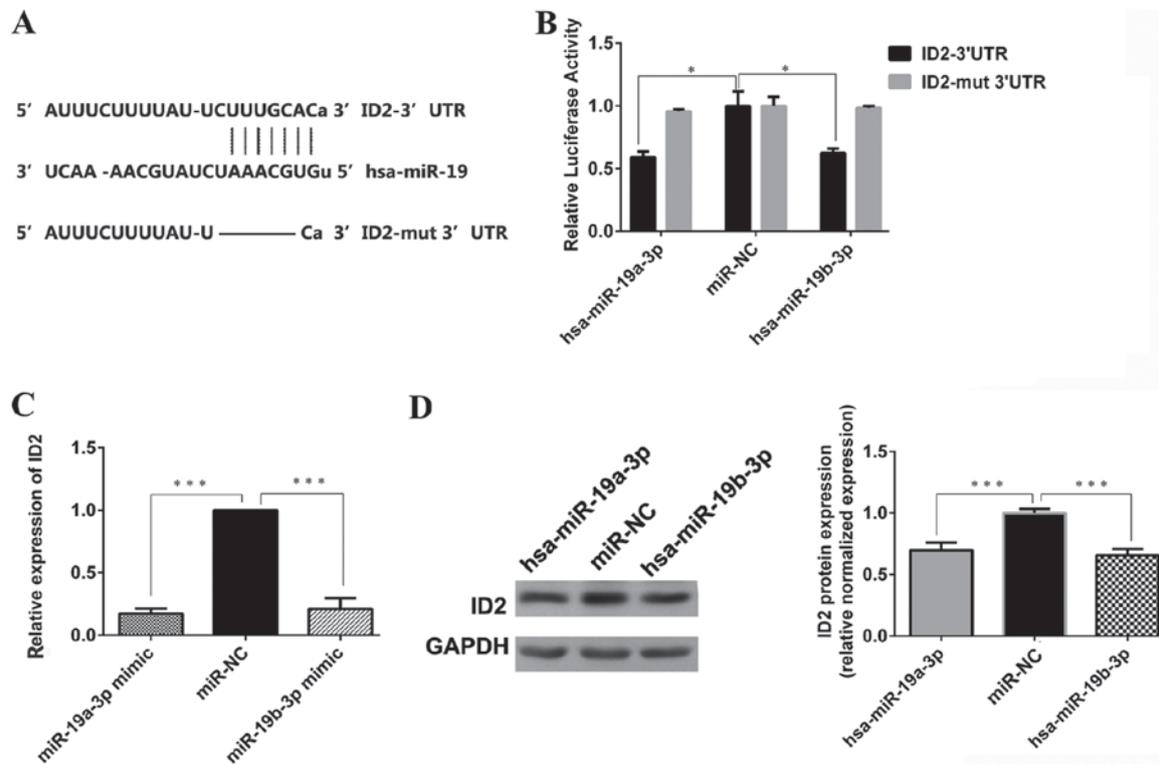


Figure 6. Inhibitor of DNA binding 2 (ID2) was a target of hsa-miR-19a-3p and hsa-miR-19b-3p. (A) Seed match of ID2 3' untranslated region (3'UTR) and hsa-miR-19a/b-3p. The 7 mer seed sequence in the 3'UTR of ID2 gene corresponding to hsa-miR-19a/b-3p binding site was marked, and the mutated-type fragments were generated by deletion of binding site. (B) Dual luciferase reporter assay showed that hsa-miR-19a-3p and hsa-miR-19b-3p mimics reduced relative luciferase activity of ID2-3'UTR rather than ID2-mut 3'UTR plasmid. (C) ID2 mRNA and protein expression were detected by qPCR. (D) hsa-miR-19a-3p and hsa-miR-19b-3p mimics significantly decrease the ID2 protein expression (\* $P < 0.05$ ; \*\*\* $P < 0.001$ ).

To confirm that lncRNA H19 may promote AML cell proliferation by regulating ID2 protein expression, knockdown of lncRNA H19 and overexpression of ID2 were performed in HL-60 cells simultaneously. As a result, restored expression of ID2 reversed the effect of lncRNA H19 on AML cell proliferation (Fig. 5). It was suggested that lncRNA H19 may increase AML cell proliferation by regulating ID2.

**Hsa-miR-19a-3p and hsa-miR-19b-3p inhibited ID2 expression.** One potential binding site for hsa-miR-19a-3p and hsa-miR-19b-3p was located in the ID2-3'UTR (Fig. 6A). Cotransfection with ID2-3'UTR and hsa-miR-19a/b-3p mimic caused a significant decrease in the luciferase activity compared with negative control. In contrast, the activity of the reporter construct containing the mutant site was not changed (Fig. 6B). Furthermore, ID2 mRNA and protein expression were reduced in transfected HL-60 cells (Fig. 6C and D). These results suggested that hsa-miR-19a-3p and hsa-miR-19b-3p exerted an inhibitory effect on ID2 expression via binding 3'UTR of ID2.

## Discussion

ID2 is an important regulator in cell proliferation and differentiation, which plays critical roles in development and cancer. It suggests that ID2 serves as an oncogene in cancer. There was high ID2 expression in immature granulocyte, and the level of ID2 protein expression in acute leukemia blast cells was significantly higher (22). Luo *et al* have also mentioned

that the expression of lncRNA H19 was positively correlated with that of ID2 in bladder cancer tissues. Furthermore, ID2 partially reversed the effect of lncRNA H19 on cell proliferation (18). In this paper, we also found that the expression of ID2 was downregulated in AML cells after lncRNA H19 was knockdown. Taken together, it suggests that lncRNA H19 might play a role in AML cell proliferation through regulating ID2 expression, but the specific mechanism is still needed to be elucidated.

MicroRNAs (miRNA) are small noncoding RNA fragments approximately 22 nucleotides long, which are involved in post-transcriptional regulation of gene expression. MiRNAs can bind transcripts through sequence complementarity to either promote mRNA degradation or prevent mRNA translation. Salmena *et al* have proposed a ceRNA hypothesis (23). Protein-coding and ncRNAs, which share one or more miRNA response elements (MREs), may compete for binding to miRNAs and regulate expression of each other. lncRNA H19 was considered as a ceRNA of miR-138, miR-200a, let-7, miR-106a, miR-141 and miR-22 (14,24,25). In this research, according to bioinformatic analysis, there were hsa-miR-19a/b-3p binding sites in lncRNA H19 and ID2, which indicated that lncRNA H19 regulate ID2 expression by binding hsa-miR-19a/b-3p.

In conclusion, we found that the expression of lncRNA H19 in bone marrow samples of AML-M2 patients is higher. Knockdown of lncRNA H19 resulted in upregulated expression of hsa-miR-19a/b-3p. lncRNA H19 inhibited ID2 mRNA and protein expression, which promoted AML cell proliferation.

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