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Regular paper

MALAT-1 regulates the AML progression by promoting the m6A modification of ZEB1

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Metastasis-associated lung adenocarcinoma transcript 1 (MALAT-1) is abnormally upregulated in various human cancers. However, the role of MALAT-1 in acute myeloid leukemia (AML) remains unclear. This study investigated the expression and function of MALAT-1 in AML. MTT assay was used to determine cell viability, qRT-PCR was applied to determine the RNA levels. Western blot was performed to detect the protein expression. Flow cytometry was conducted to measure cell apoptosis. RNA pull-down assay was carried out to detect the interaction between MALAT-1 and METTL14. RNA FISH assay was performed to determine the localization of MALAT-1 and METTL14 in AML cells. Our results have revealed the key role of MEEL14 and m6A modification in AML. Besides, MALAT-1 was significantly up-regulated in AML patients. MALAT-1 knockdown inhibited the proliferation, migration and invasion of AML cells, and induced cell apoptosis; additionally, MALAT-1 binding to METTL14 promoted the m6A modification of ZEB1. Besides, ZEB1 overexpression partially reversed the effect of MALAT-1 knockdown on the cellular functions of AML cells. Taken together, MALAT-1 promoted the aggressiveness of AML through regulating m6A modification of ZEB1.

Keywords: AML, MALAT1, MEEL14, m6A, ZEB1

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Abbreviations: AML, acute myeloid leukemia; MALAT-1, metastasis-associated lung adenocarcinoma transcript 1; lncRNAs, long non-coding RNAs; m6A, N6-methyladenosine; ZEB1, the zinc finger E-box binding homeobox 1; EMT, epithelial-mesenchymal transition; PI, propidium iodide

INTRODUCTION

Acute myeloid leukemia (AML) is a malignant disease of myeloid hematopoietic stem/progenitor cells (Cai & Levine, 2019). Before maturation, leukemic cells abnormally proliferate and accumulate in bone marrow and blood, causing loss of hematopoietic function (Acheampong *et al.*, 2018; Mueller *et al.*, 2018). AML is highly popular among adults, accounting for more than 30 percents of all leukemia cases worldwide (Zimta *et al.*, 2019). Current studies have shown that chromosome abnormalities, uncontrolled gene expression, and non-coding RNA such as long non-coding RNAs (lncRNAs) could promote acute myeloid leukemia (Mer *et al.*, 2018; Wang *et al.*, 2019). Clinically, the AML recuperation rate is less than 45%, while 50% relapse and the late survival rate is only at about 10% (Pan *et al.*, 2017). Because of the poor prognosis of AML, it is of great importance to identify new markers and therapeutic targets for AML.

LncRNA is a category of transcripts larger than 200bp and lacks protein-coding function (Yang *et al.*, 2019). Recent studies have illustrated the crucial roles of the lncRNAs in cell proliferation, survival, apoptosis, and differentiation (Ferre *et al.*, 2016; Ju *et al.*, 2019). However, most lncRNAs underlying mechanisms are yet to be studied. The lncRNA MALAT-1 is involved in the development of various tumors (Zhang *et al.*, 2017; Liu *et al.*, 2019; He *et al.*, 2019). Previous studies have confirmed that the overexpression of MALAT-1 can promote the invasion and migration of lung cancer, cervical cancer, and ovarian cancer (Xia *et al.*, 2018; Guo *et al.*, 2018; Yu *et al.*, 2019). It is also an essential marker for the early prognosis of lung adenocarcinoma, gastrointestinal cancer, and B-cell lymphomas (Li *et al.*, 2016; Xu *et al.*, 2018). However, the underlying mechanism of MALAT-1 in AML is not clear.

N6-methyladenosine (m6A) is the most accustomed mRNA modification; this modification affects all steps of RNA metabolism (Yu *et al.*, 2018). Emerged evidence showed that m6A is involved in cancer progression and proliferation; it is also engaged in cancer stem cells' maintenance and differentiation (Sun *et al.*, 2019). However, the mechanism of m6A in AML remains to be studied.

The zinc finger E-box binding homeobox 1 (ZEB1) leads to epithelial-mesenchymal transition (EMT) and confers properties of 'stemness', such as self-renewal in cancer. ZEB1 is involved in myogenesis (Postigo & Dean, 1999), neuronal development and differentiation (Wang *et al.*, 2019;), gastrulation (Funahashi *et al.*, 1993), and T fine cell development (Higashi *et al.*, 1997). Copious evidence suggests that ZEB1 regulates stem cell self-renewal in cancer (Brabletz & Brabletz, 2010). Although ZEB1 is a renowned regulator of the expression of different stem cell/cancer-related transcription factors such as BMI1, KLF4, and SOX2, the deletion of ZEB1 promotes cell differentiation during the development of embryonic CNS (Singh *et al.*, 2016) and skeletal muscle (Li *et al.*, 2017). The comprehensive role of ZEB1 in normal stem cell progression remains unclear.

In the present study, we explored the underlying mechanism of MALAT-1 in AML. We found that the expression of MALAT-1 was significantly up-regulated in AML patients, and MALAT-1 promoted the m6A modification of ZEB1 through binding with METTL14, thereby promoting the proliferation and inhibiting the apoptosis of AML cells.

MATERIALS AND METHODS

Patient samples

40 AML patients and 40 healthy controls were recruited from Ningbo First Hospital. Among the AML patients, male: 26, female:14; age: 35.21±6.14; physical activity state score, 0-1: 27, 2-4: 13; Type, M1: 3; M2: 5; M3: 11; M4: 13; M5: 6; M6: 2. The peripheral blood was collected and stored in liquid nitrogen immediately until needed. The patients who received chemotherapy or radiotherapy were excluded from this study. This study was approved by the Research Ethics Committee of Ningbo First Hospital20190126, January 26th, 2019, and each patient has signed an informed consent form.

Cell culture and transfection

The human bone marrow stromal cell line HS-5 and human AML cell lines HL60 and THP-1 were obtained from the Cell Bank of the Chinese Academy of Science (Shanghai, China). All cells were maintained in RPMI-1640 medium (Hyclone, Logan, UT, USA) and were supplemented with 10% fetal bovine serum (FBS, Hao Yang Bio) in a humidified atmosphere of 5% CO_2 at 37°C. The MALAT-1 overexpression plasmids, si-MALAT1, si-METTL14, and ZEB1 overexpression plasmids were synthesized by GenePharma (Shanghai, China). Lipofectamine 2000 (Invitrogen, USA) was used for cell transfection following the manufacturers' instructions.

Quantitative real-time RCR (qRT-PCR)

The RNA was extracted from HS-5, HL60, and THP-1 cells using TRIzol reagent (nitrogen source). The cDNA was synthesized using a reverse-transcription kit (TRAGEN). The real-time quantitative PCR was conducted on 20ng of cDNA by SYBR Green mixture (Shanghai Yisheng) and GAPDH was used as an internal control. The primer sequences are as follows:
MALAT1:
Forward 5'-AGGCGTTGTGCGTAGAGGA-3',
reverse 5'-GGATTTTTACCAACCACTCGC-3';
ZEB1:
Forward 5'-GATGATGAATGCGAGTCAGATGC-3',
reverse 5'-ACAGCAGTGTCTTGTTGTTGT-3'.

MTT assay

MTT assay was used to determine the HL60 and THP-1 cells' viability. 1×10^4 cells were seeded into 96-well plates. Next, we added 16 μL/well MTT solution (5 mg/mL, Sigma) and incubated for 4 h at 37°C. The cell viability was determined by a microplate reader (Bio-Tek, Winooski, USA) at 570 nm.

Cell apoptosis assay

Annexin V-FITC Apoptosis Detection kit (YEASEN, Shanghai) was used to evaluate cell apoptosis. In brief, after treating HL60 and THP-1 cells for 72h, the cells were washed using PBS; Then, the cells were mixed with the binding buffer, Annexin V-FITC and propidium iodide (PI) and were incubated in dark for 15 min. The apoptotic rates were determined using flow cytometry (BD).

Transwell

The transwell assay was performed to detect the cell invasion, while transwell without Matrigel was performed to analyze the cell migration. About 0.6 mL Complete medium was added into lower chambers. Then, the cells were added into the upper chambers. After incubating for 48 h, the non-migrated and non-invaded cells were removed with a swab. The 4% paraformaldehyde was used to immobilize the cells on the bottom surface and 0.1% crystal violet was used to stain the cells. A light microscope was used to observe the cells.

RNA FISH

The RNA FISH probe for MALAT1 and METTL14 was purchased from Biosearch Technologies (Novato, CA, USA) and used to visualize RNA in the interchromatin granule clusters. Hybridizations were performed according to the manufacturer's instructions. Briefly, slides were hybridized overnight with 50 pmol probe in 20 μl hybridization buffer, washed in RNAse-free PBS, dehydrated in ethanol, post-fixed in 1% paraformaldehyde (Life Technologies, Darmstadt, Germany) in PBS, and treated for 2 min with 0.1% Triton-X100 (Serva, Heidelberg, Germany).

RNA immunoprecipitation

RNA experiments were performed using a Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, USA) according to the manufacturer's instructions.

Western blotting

HL60 and THP-1 cells were placed in an ice-cold lysis buffer for 30 min (Beyotime). The supernatants were collected by centrifugation at 14000 r/min for 20 min at 4°C. Protein concentration was calculated with a BCA Kit (Pierce, USA). Then the protein was separated with 10% SDS-PAGE and transferred onto PVDF membranes. Afterwards, the membranes were blocked in 5% non-fat milk; next, the membranes were incubated with primary antibodies: anti-ZEB1 (1:1000), anti-METTL14 (1:1000), and anti-METTL3 (1:1000) (Boster Biological Technology, China) overnight at 4°C. On the next day, the membranes were incubated with the HRP-labeled secondary antibodies (Abcam, Shanghai) and treated with BeyoECL Plus (Beyotime, China). The bands were visualized using CC-D camera (Bio-Rad, USA).

RNA pull-down

RNA pull-down assay was performed to confirm whether METTL14 or METTL3 can be pulled down by MALAT-1 in HL60 and THP-1 cells. The biotin-labeled METTL14 and METTL3 were transcribed with the Biotin RNA Labeling Mix (Roche) and T7 RNA polymerase (Roche). After 2d of transfection, cells were lysed and then mixed with biotin-labeled METTL14 and METTL3 and streptavidin agarose magnetic beads at 4°C for 1 h. Western blot was used to detect the protein levels of the retrieved Ago2.

Statistical analysis

Data were analyzed using SPSS17.0 software (SPSS, Inc.) and expressed as mean ± S.D. One-way ANOVA followed by Duncan's post hoc test was used to evaluate the differences among groups. The differences between the two groups were analyzed by Student's *t*-test. $P<0.05$ was defined as significant.

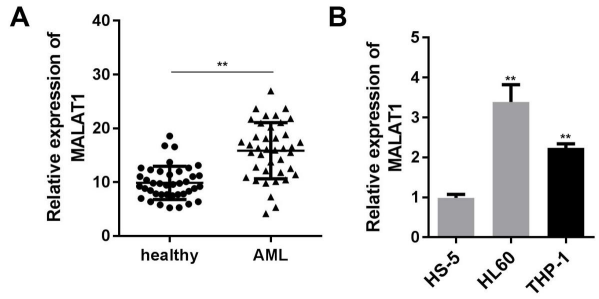

Figure 1. MALAT1 was upregulated in AML patients and AML cell lines.
(**A**) The expression of MALAT1 in serum samples from AML patients and healthy volunteers. (**B**) The expression of MALAT1 in AML in the human bone marrow stromal cell line HS-5 and human AML cell lines HL60 and THP-1. ***P*<0.01 *vs* HS-5.

RESULTS

lnc-MALAT-1 was up-regulated in AML patients and cells

Firstly, we collected serum samples from 40 healthy participants and 40 AML patients, and then examined the expression of MALAT-1 in serum by RT-qPCR method. The expression level of MALAT-1 in the serum of patients with AML was significantly higher than normal controls (*P*<0.05; Fig. 1A). It was consistent with in vitro assay, the expression of MALAT-1 in HL60 and THP-1 cells were significantly increased compared with HS-5 cells (*P*<0.05; Fig. 1B).

MALAT-1 knockdown inhibited proliferation of AML cells

To explore the role of MALAT-1 in AML, HL60, and THP-1 cells were transfected with si-MALAT-1 or MALAT-1 overexpression plasmids, and then we examined the effects of MALAT-1 on cell proliferation. Results, in Fig. 2A, revealed that compared with the si-NC group the expression of MALAT-1 was significantly reduced after transfection with si-MALAT1. Compared with the vector group, the expression of MALAT-1 was remarkably promoted after transfection with MALAT1 overexpression plasmids (*P*<0.01). As shown in Fig. 2B–C, knockdown of MALAT-1 significantly suppressed the cell viability of HL60 and THP-1, whereas overexpression MALAT-1 remarkably promoted the AML cell viability (*P*<0.05).

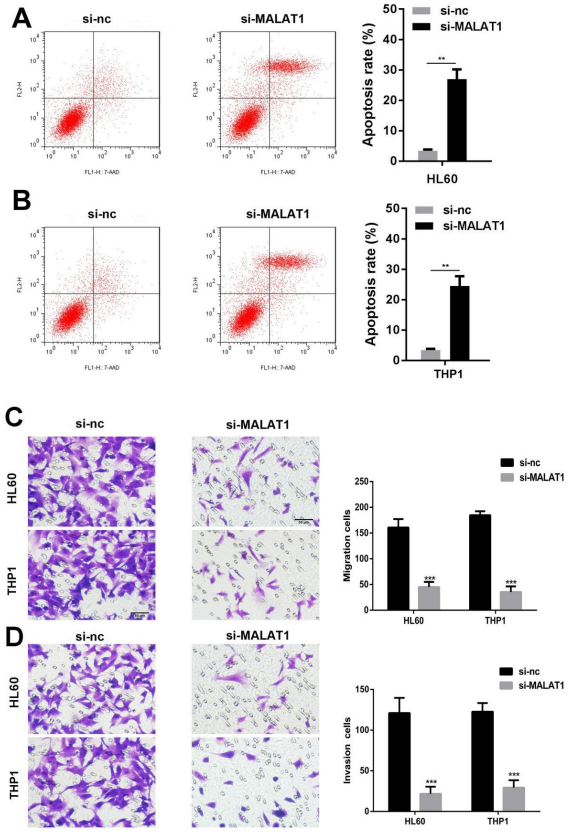

Figure 3. Effects of MALAT1 knockdown on apoptosis of AML cells.
After HL60 and THP-1 cells were transfected with si-MALAT1 or MALAT1 for 48 h, cell apoptosis (**A–B**), migration (**C**) and invasion (**D**) were evaluated by flow cytometry assay. ***P*<0.01. ****P*<0.001.

MALAT-1 knockdown promoted AML cells apoptosis

To further explore the function of MALAT-1 in AML, after HL60 and THP-1 cells transfection with si-MALAT-1 or MALAT-1 overexpression plasmids, we examined the effects of MALAT-1 on cell apoptosis, migration, and invasion. Flow cytometry results showed that compared with the si-NC group, the apoptosis of HL60 and THP-1 cells was significantly increased in si-MALAT1 group (*P*<0.05; Fig. 3A–B). Besides, the results of Transwell exhibited that compared with the si-NC group, the migration and invasion abilities of the HL60 and THP-1 cells were significantly decreased in

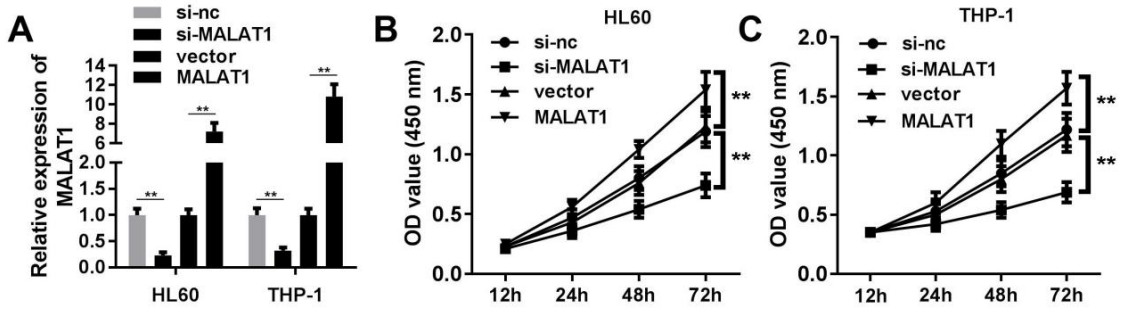

Figure 2. Effects of MALAT1 on proliferation of AML cells.
(**A**) After AML cells were transfected with si-MALAT1 or MALAT1 for 48 h, the transfection efficiency was evaluated by qRT-PCR. (**B and C**) MTT assay was conducted to determine cell proliferation in HL60, and THP-1 cells transfected with si-MALAT1 or MALAT1. ***P*<0.01.

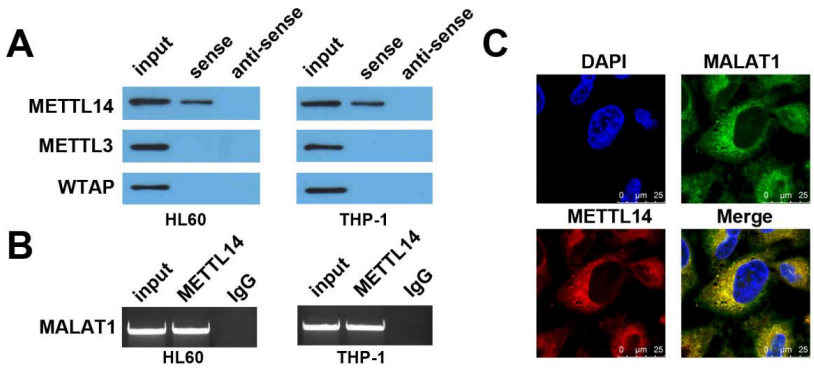

Figure 4. MALAT1 bound with METTL14 in AML cells.
(**A**) RNA pull-down assay. (**B**) RNA immunoprecipitation assay. (**C**) MALAT1 and METTL14 location detecting by RNA FISH in AML cells.

the si-MALAT1 group (P<0.05; Fig. 3C–D). These data revealed that MALAT1 knockdown promoted AML cell apoptosis and inhibited AML cell migration and invasion.

METTL14 bound with MALAT-1

m6A Modification affects almost all steps of RNA metabolism, including lncRNA. Therefore, RNA pull-down assay, RIP experiment, and RNA FISH were used to verify whether m6A binds with MALAT-1. The results of the RNA pull-down assay verified the interaction between MALAT-1 and METTL14 (Fig. 4A). Furthermore, METTL14 directly bound with MALAT-1 (Fig. 4B). Similarly, RNA FISH results also confirmed the mutual binding of MALAT-1 and METLL14 (Fig. 4C). Taken together, these data confirmed that METLL14 bound with MALAT-1.

MALAT-1 regulated m6A modification of ZEB1

Previous research has shown that mRNA translation is regulated by lncRNA and m6A modification. Therefore, we explored the effects of MALAT-1 and m6A modification on ZEB1 expression. In Fig. 5A, ZEB1 produced significant m6A methylation modification in both AML cells, compared with the control group. MALAT-1 knockdown remarkably reduced the enrichments of ZEB1 in HL60 and THP-1 cells (P<0.05; Fig. 5B). As shown in Fig. 5C, overexpression of MALAT-1 significantly promoted the enrichments of ZEB1 compared with the control group. However, these changes would be partly reversed in the MALAT-1+si-METTL14 group (P<0.05; Fig. 5C). After transfection with MALAT-1 overexpression plasmids or si-METTL14, we detected the expression of ZEB1 in AML cells. Results showed that the expression levels of ZEB1 were promoted by MALAT-1 overexpression plasmids, compared with the control group. The expression levels of ZEB1 were significantly reduced by MALAT-1+si-METTL14 compared with the MALAT-1+si-NC group (P<0.05; Fig. 5C). As shown in Fig. 5D, after transfection with MALAT-1 overexpression plasmids, the stability of ZEB1 expression was promoted, compared with the vector+si-NC group, which was partly reversed by si-METTL14 (Fig. 5D). Overexpressed MALAT-1 promoted the protein expression of ZEB1 compared with the vector+si-NC group, while the protein expression of ZEB1 was decreased in the MALAT-1+si-METTL14 group compared with the MALAT-1+si-NC group (P<0.05; Fig. 5E).

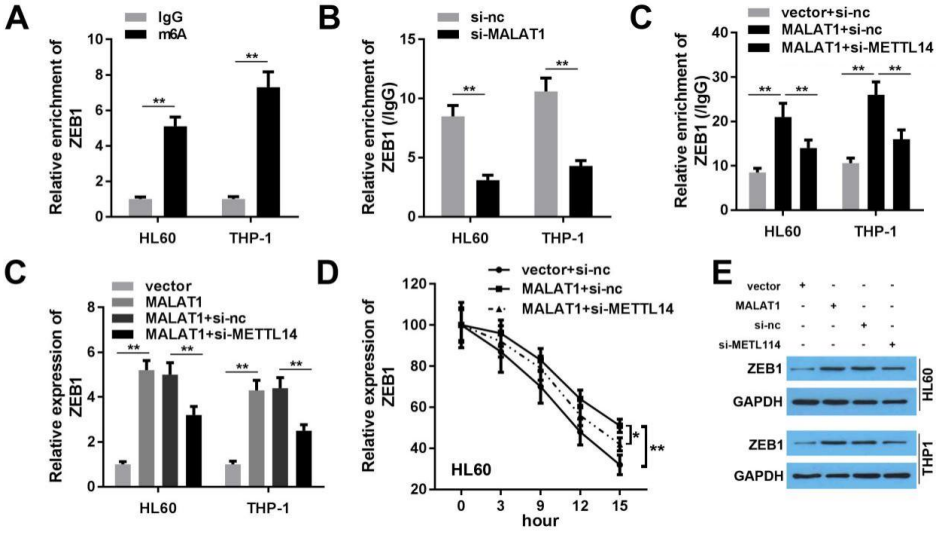

Figure 5. Effects of MALAT1 and METTL14 on ZEB1.
(**A**) ZEB1 produces m6A modification in AML cells. (**B**) After AML cells were transfected with si-MALAT1, the enrichment of ZEB1 was evaluated by qRT-PCR. (**C**) After AML cells were transfected with MALAT1 or si-METTL14, the enrichment and expression of ZEB1 was evaluated by qRT-PCR. (**D**) The stability of ZEB1. (**E**) The protein expression of ZEB1. **P<0.01.

Overexpression ZEB1 alleviated the effects of MALAT-1 knockdown on cellular function AML cells

To further explore the role of MALAT-1 and ZEB1, a rescue experiment was conducted in AML cells. As presented in Fig 6A, MALAT-1 knockdown significantly reduced the expression of ZEB1 compared with the vector+si-NC group, the expression levels of ZEB1 were promoted by ZEB1 overexpression plasmids compared with si-MALAT-1+si-NC group (P<0.05; Fig. 6A). Knockdown MALAT-1 significantly reduced cell viability of HL60 and THP-1 cells, whereas overexpression of ZEB1 remarkably reversed the effect of MALAT-1 knockdown (P<0.05; Fig. 6B). Knockdown of MALAT-1 significantly induced the apoptosis of HL60 and THP-1 cells, whereas overexpression of ZEB1 reversed the effect of MALAT-1 knockdown (P<0.05; Fig. 6C). What is more, knockdown of MALAT-1 significantly decreased the migration and invasion of the HL60 and THP-1 cells, whereas overexpressed ZEB1 remarkably reversed the effect of MALAT-1 knockdown on the migration and invasion of AML cells (P<0.05; Fig. 6D–E).

DISCUSSION

In this study, MALAT-1 was significantly upregulated in patients compared with the healthy control group. Besides, MALAT-1 knockdown inhibited HL60 and THP-1 cells' proliferation, migration, and invasion abilities, and promoted the apoptosis of HL60 and THP-1 cells. Moreover, MALAT-1 promoted m6A methylation modification of ZEB1 by binding with METTL14, thereby enhancing the proliferation, migration, and invasion ability of AML cells.

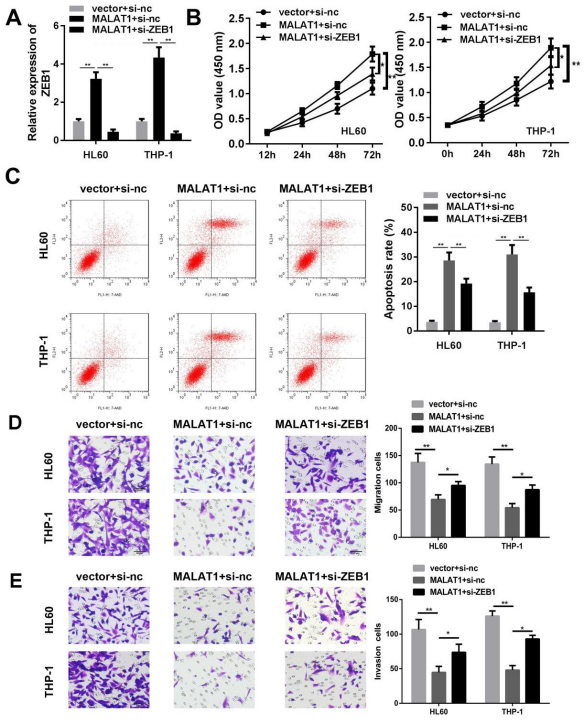

Figure 6. Knockdown ZEB1 reversed the effect of MALAT1 on AML cells.
(**A**) After AML cells were transfected with MALAT1 or si-ZEB1 for 48 h, the expression of ZEB1 was detected by qRT-PCR. (**B**) MTT assay was conducted to determine cell proliferation in HL60, and THP-1 cells transfected with MALAT1 or si-ZEB1. (**C**) After HL60 and THP-1 cells were transfected with MALAT1 or si-ZEB1 for 48 h, cell apoptosis was evaluated by flow cytometry assay. (**D–E**) After HL60 and THP-1 cells were transfected with MALAT1 or si-ZEB1 for 48 h, Transwell assay was performed to detect the migration and invasion. *P<0.05, **P<0.01.

MALAT-1 is a highly methylated LncRNA that is often abnormally upregulated in human cancer tissues (YiRen *et al.*, 2017; Shen *et al.*, 2019). Recently, abnormal expression of MALAT-1 can be a diagnostic marker for multiple myeloma at early stage (Hu *et al.*, 2018). Additionally, the abnormally high expression of lncRNA-MALAT-1 was associated with the poor prognosis of AML patients (Huang *et al.*, 2017). However, MALAT-1 knockout enhances cytarabine chemosensitivity of AML cells (Hu *et al.*, 2019). These findings suggest that MALAT-1 may function as an oncogene in AML. In this study, MALAT-1 was up-regulated in AML clinical samples and cells. Moreover, knockdown of MALAT-1 reduced AML cells' viability, migration, and invasion, and induced cells' apoptosis, which is a key "player" in the occurrence and development of AML. Nevertheless, the underlying molecular mechanisms remain unclear.

The m6A methylation modification mediates the 0.3–0.5% adenosine modification in human cell mRNA, and the main complexes responsible for the m6A modification of mRNAs are METTL3 and METTL14 (Ianniello *et al.*, 2019). The study found that METTL14 was overexpressed in AML, which was consistent with previous studies (Vu *et al.*, 2017; Barbieri *et al.*, 2017). Overexpression of METTL14 promotes the proliferation of AML cells, while knockout of METTL14 promotes AML cell apoptosis (Weng *et al.*, 2018). Barbieri *et al.* reveal that METLL14 is a crucial gene regulating the survival of AML cells (Barbieri *et al.*, 2017). METTL3/METTL14 methylation complex can promote the development of AML and maintain leukemia stem cells (Weng *et al.*, 2018). These results prove the oncogenic role of METTL14 in AML. In this study, MALAT-1 bound with methyltransferase METTL14, which suggests the possibility of m6A modification in AML cells.

ZEB1 is the primary regulator of EMT-related signal pathways related to cancer stem cells' growth, survival, and metastasis. For example, up-regulating ZEB1 improves the migration ability of HCC cells (Wang *et al.*, 2020). ZEB1 is highly expressed in AML patients and can be a marker for early diagnosis and prognosis of AML (Shousha *et al.*, 2019). However, ZEB1 knockout reduces the invasiveness of leukemia stem cells (Stavropoulou *et al.*, 2016). However, the reason that ZEB1 was overexpressed in AML cells is still unclear. In this study, we found that ZEB1 was rich in m6A level in AML cells. Additionally, MALAT-1 interacted with METTL14 to promote m6A modification of ZEB1, which induced the up-regulation of ZEB1 in AML cells. Besides, overexpression of ZEB1 promoted proliferation, migration, and invasion, and suppressed the apoptosis of AML cells.

In conclusion, MALAT-1 was overexpressed in AML. MALAT-1 bound with METTL14 to promote the m6A modification of ZEB1, which promoted the proliferation, migration, and invasion of AML cells. This might provide a new effective therapeutic strategy for the treatment of AML.

Declarations

Declaration of Competing Interest. No conflict of interest exits in the submission of this manuscript, and manuscript is approved by all authors for publication.

Acknowledgments. Not applicable.

Data Availability Statement. The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions. All authors participated in the design, interpretation of the studies, and analysis of the data, and review of the manuscript; LHF and PH conducted the experiments; JJ wrote the manuscript; WYF provided the idea.

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