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LncRNA HOTTIP promotes proliferation and cell cycle progression of acute myeloid leukemia cells

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Abstract. – OBJECTIVE: The aim of this study was to elucidate the biological function of long non-coding RNA (lncRNA) HOTTIP (HOXA transcript at the distal tip) in the development of acute myeloid leukemia (AML), and to investigate the potential mechanism.

PATIENTS AND METHODS: Relative expression levels of HOTTIP, microRNA-608 and DDA1 in AML patients were determined by quantitative Real-time polymerase chain reaction (qRT-PCR). Meanwhile, the expressions of these genes in AML cell lines were detected as well. The regulatory effects of HOTTIP, microRNA-608 and DDA1 on the proliferative ability and cell cycle progression of AML cells were examined by cell counting kit-8 (CCK-8) and flow cytometry, respectively. Dual-luciferase reporter gene assay was performed to confirm the binding condition of microRNA-608 to HOTTIP and DDA1. Finally, the specific role of HOTTIP/microRNA-608/DDA1 axis in the development of AML was verified through a series of rescue experiments.

RESULTS: HOTTIP was highly expressed in AML-M5 patients than normal controls. No significant difference in HOTTIP expression was found between patients with other subtypes of AML (M0, M1, M2, M3, M4 and M6) and normal controls. HOTTIP expression was significantly up-regulated in AML cell lines U-937 and THP-1. Up-regulation of HOTTIP remarkably promoted the proliferative potential and cell cycle progression of AML cells. Dual-luciferase reporter gene indicated that HOTTIP could bind to microRNA-608, which was lowly expressed in AML-M5 patients. Overexpression of microRNA-608 significantly inhibited the proliferative ability and cell cycle progression of U-937 and THP-1 cells. More importantly, microRNA-608 could partially reverse the regulatory effect of HOTTIP on AML cells. Meanwhile, DDA1 was verified as the target of microRNA-608. Subsequent experiments elucidated that DDA1 significantly accelerated the proliferation and cell cycle of AML cells. Furthermore, DDA1 could reverse the inhibitory effect of microRNA-608 on proliferative ability and cell cycle progression of AML cells.

CONCLUSIONS: HOTTIP accelerated the proliferative ability and cell cycle of AML cells via up-regulating DDA1 expression by sponging microRNA-608.

Key Words:
Acute myeloid leukemia (AML), HOTTIP, MicroRNA-608, DDA1.

Introduction

Acute myeloid leukemia (AML) is the most common hematological tumor with poor prognosis. Accumulation of leukemia cells resulted from differentiation block and maturation disorder of hematopoietic stem cells eventually leading to hematopoietic failure[1]. In recent years, environmental changes have gradually increased the incidence of AML. Meanwhile, the prognosis of AML is far from unsatisfactory. It is estimated that the 5-year survival of AML patients is only 20-40%[2]. Both cytogenetic abnormalities and gene mutations play an important role in the pathogenesis of AML. Therefore, it is of clinical significance to elucidate the molecular mechanism of AML, thus developing therapeutic strategies to improve its prognosis. Long non-coding RNA (lncRNA) is a kind of RNAs with more than 200 bp in length. LncRNA is involved in the regulation of various signaling pathways and biological functions. Meanwhile, lncRNAs can serve as oncogenes or tumor-suppressor genes to mediate tumor proliferation, invasion and metastasis. Currently, lncRNAs have been concerned as diagnostic and therapeutic targets of tumors[3,4]. Previous studies[5] have demonstrated that some certain lncRNAs exert potential values as biological hallmarks for AML. Their expression levels are closely related to the prognosis of AML. For example, lncRNA HOTAIR plays an oncogenic role in AML by adsorbing miR-193a[6]. In AML cell line HL-60, up-regulation

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of CCAT1 promotes the growth and differentiation of cells, which may become a new therapeutic target for AML[7]. HOXA transcript at the distal tip (HOTTIP) has been identified in various tumors, such as lung cancer, pancreatic cancer, liver cancer, etc.[8-10]. However, the exact role of HOTTIP in AML has not been elucidated yet.

MicroRNAs (miRNAs) are endogenous, non-coding RNAs with approximately 22 nucleotides in length. They regulate target gene expression by completely or incompletely binding to the 3' untranslated region (3'UTR) of target transcripts. Once bound to target mRNAs, miRNAs can inhibit or degrade them to further regulate the expression of downstream genes. Since first discovery in 1993, miRNAs have been confirmed to participate in the regulation of a variety of biological and cellular processes as endogenous regulators. In recent years, abnormally expressed miRNAs are observed in a large number of malignant tumors[11]. Several abnormally expressed miRNAs have been found to participate in the progression of AML[12]. For example, miR-34a exhibits potential immunotherapeutic effects on AML by targeting PD-L1[13]. MiR-128a knockdown induces Lin28a expression and reverses the arrest of myeloid differentiation in AML[14]. MicroRNA-608 is a recently discovered miRNAs involved in the progression of different diseases. In colon cancer, microRNA-608 inhibits tumor progression by targeting NAA10[15]. In gastric cancer, microRNA-608 is adsorbed and degraded by NORAD, which up-regulates the expression of target genes. Eventually, microRNA-608 may impair its inhibitory effect on tumor development[16]. Although microRNA-608 has been reported in multiple tumors, its function in AML has rarely been reported.

In this study, we elucidated that the plasma level of HOTTIP was significantly increased in AML-M5 patients, whereas microRNA-608 expression was down-regulated. The aim of this study was to elucidate the potential functions of HOTTIP and microRNA-608 in regulating the progression of AML.

Patients and Methods

Sample Collection

Bone marrow blood samples were collected from 80 AML patients (5 cases of M0 subtype, 6 of M1 subtype, 11 of M2 subtype, 12 of M3 subtype, 5 of M4 subtype, 31 of M5 subtype, 10 of M6 subtype) and 24 healthy controls. Collected blood samples were centrifuged at 2500 rpm for 3 min, and the supernatant was preserved in a 1.5 mL Eppendorf (EP) tube. The remaining precipitate was incubated in isodose phosphate-buffered saline (PBS) and preserved in an EP tube containing 4 mL of Ficoll-Hypaque. Then, the precipitate was centrifuged at 2500 rpm for 20 min, and the intermediate monocyte layer was aspirated for 2 mL preserving in a new EP tube. Subsequently, monocytes were centrifuged at 1500 rpm for 5 min, and diluted in 1 mL of PBS, followed by centrifugation again at 1500 rpm for 5 min. This process was repeated twice. Finally, the precipitate was diluted in 1 mL of TRIzol (Invitrogen, Carlsbad, CA, USA) and preserved at -80°C. Patients volunteered to participate in this study, and signed written informed consent was obtained from each subject before the study. This study was approved by the Ethics Committee of The Second People's Hospital of Liaocheng.

Cell Culture

AML cell lines (U-937, THP-1) and normal monocyte cell line (SC) were provided by Cell Bank, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in a 37°C, 5% CO_2 incubator following the culture methods of suspension cells.

Cell Transfection

Cells were first seeded one day prior to transfection. Subsequently, cells were transfected with relative plasmids or miRNA mimics at a final dose of 50-100 nM according to the instructions of Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA).

RNA Extraction

Tissues or cells were lysed in 1 mL of TRIzol. After maintenance for 5 min, 200 μL of chloroform was added, followed by incubation at room temperature for 5 min. The supernatant was transferred into a new RNase-free centrifuge tube after centrifugation at 4°C, 12000 rpm for 15 min. Isopropanol with the same volume of supernatant was added to harvest RNA precipitate by centrifugation. Extracted RNA was air dried, quantified (A260/A280=1.8-2.0) and dissolved in 10-20 μL of diethyl pyrocarbonate (DEPC) water (Beyotime, Shanghai, China).

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

RNA was subjected to a reverse transcription system using PrimeScript RT reagent Kit. Next, com-

plementary deoxyribose nucleic acid (cDNA) was obtained. QRT-PCR was carried out in accordance with the instructions of SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA). Total qRT-PCR system was 10 μL, and specific reaction procedure was as follows: pre-denaturation at 95°C for 10 min, denaturation at 95°C for 15 s, annealing at 60°C for 1 min and extension at 72°C for 1 min, for a total of 40 cycles. Primer sequences used in this study were as follows: MicroRNA-608, F: 5′-ACACTCCAGCTGGGAGGGGTGGTGTTGGGACAG-3′, R: 5′-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGACGGAGCT-3′; U6, F: 5′-CTCGCTTCGGCAGCAGCACATATA-3′, R: 5′-AAATATGGAACGCTTCACGA-3′; HOTTIP, F: 5′-CACACTCACATTCGCACACT-3′, R: 5′-TCCAGAACTAAGCCAGCCATA-3′; GAPDH, F: 5′-GAAGAGAGAGACCCTCACGCTG-3′, R: 5′-ACTGTGAGGAGGGGAGATTCAGT-3′; STON2, F: 5′-ACCATGTGATTGCCACCCAC-3′, R: 5′-AGCTCTCGGACTGGTCTGG-3′; DDA1, F: 5′-GCCCTCAGTCTACCTGCCTA-3′, R: 5′-TCCTGGTCTCTCTTCTTGGC-3′.

Dual-Luciferase Reporter Gene Assay

The transcript 3'UTR sequences of HOTTIP/DDA1 were cloned into pGL3 vector containing luciferase reporter gene, namely HOTTIP/DDA1 WT group. HOTTIP/DDA1 MUT group was constructed by mutating the core binding sequences using site-directed mutagenesis kit. Cells were co-transfected with microRNA-608 mimics and HOTTIP/DDA1 WT or HOTTIP/DDA1 MUT, respectively. 24 hours after transfection, cells were lysed for luciferase activity determination.

Cell Cycle

24 h after transfection, cells were digested, re-suspended in 70% alcohol and fixed. After washing with PBS for three times, cells were incubated with 1 mL of DNA Staining Solution and 50 μg/mL Propidium Iodide (PI) at 37°C for 30 min (Cell Signaling Technology, Danvers, MA, USA). Finally, cells were subjected to flow cytometry analysis.

Cell Proliferation

Cells were first seeded into 96-well plates at a density of $3\text{-}5\times10^3$ cells per well. 10 replicates were set for each group. At the appointed time points, 10 μL of cell counting kit-8 (CCK-8) (Dojindo, Kumamoto, Japan) was added each well, followed by incubation for 2 h in dark. Absorbance at 450 nm was detected by a micro-plate reader, and growth curve was plotted.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 19.0 (IBM, Armonk, NY, USA) was used for all statistical analysis. Data were represented as mean ± SD (Standard Deviation). *t*-test was used to compare the differences between two groups. $p<0.05$ was considered statistically significant.

Results

HOTTIP was Highly Expressed in AML-M5 Patients and AML Cell Lines

Firstly, we determined HOTTIP expression in peripheral blood of AML patients (n=80) and healthy controls (n=24) by qRT-PCR. Results indicated that HOTTIP was highly expressed in AML-M5 patients than controls. No significant difference in HOTTIP expression was observed between other subtypes of AML (M0, M1, M2, M3, M4 and M6) and controls (Figure 1A). Moreover, HOTTIP expression in peripheral blood of AML-M5 patients was markedly higher than that of non-M5 patients (Figure 1B). Identically, HOTTIP expression in AML cell lines U-937 and THP-1 was significantly higher than normal monocyte cell line SC (Figure 1C). Based on the expression level of HOTTIP, AML-M5 patients were divided into high-level and low-level groups. Survival analysis indicated that the 5-year survival of AML-M5 patients in high-level group was significantly lower than those in low-level group ($p=0.0031$, HR=1.992, Figure 1D).

HOTTIP Promoted Proliferative Ability and Cell Cycle of AML Cells

To investigate the potential function of HOTTIP in AML, we constructed pcDNA-HOTTIP and pcDNA-NC. Transfection of pcDNA-HOTTIP remarkably up-regulated HOTTIP expression in U-937 cells and THP-1 cells (Figure 2A, 2B). CCK-8 assay indicated that the proliferation of AML cells overexpressing HOTTIP was significantly elevated (Figure 2C, 2E). Besides, transfection of pcDNA-HOTTIP remarkably accelerated cell cycle progression of U-937 cells and THP-1 cells (Figure 2D, 2F). The above data confirmed that HOTTIP might promote AML progression through accelerating proliferation and cell cycle progression.

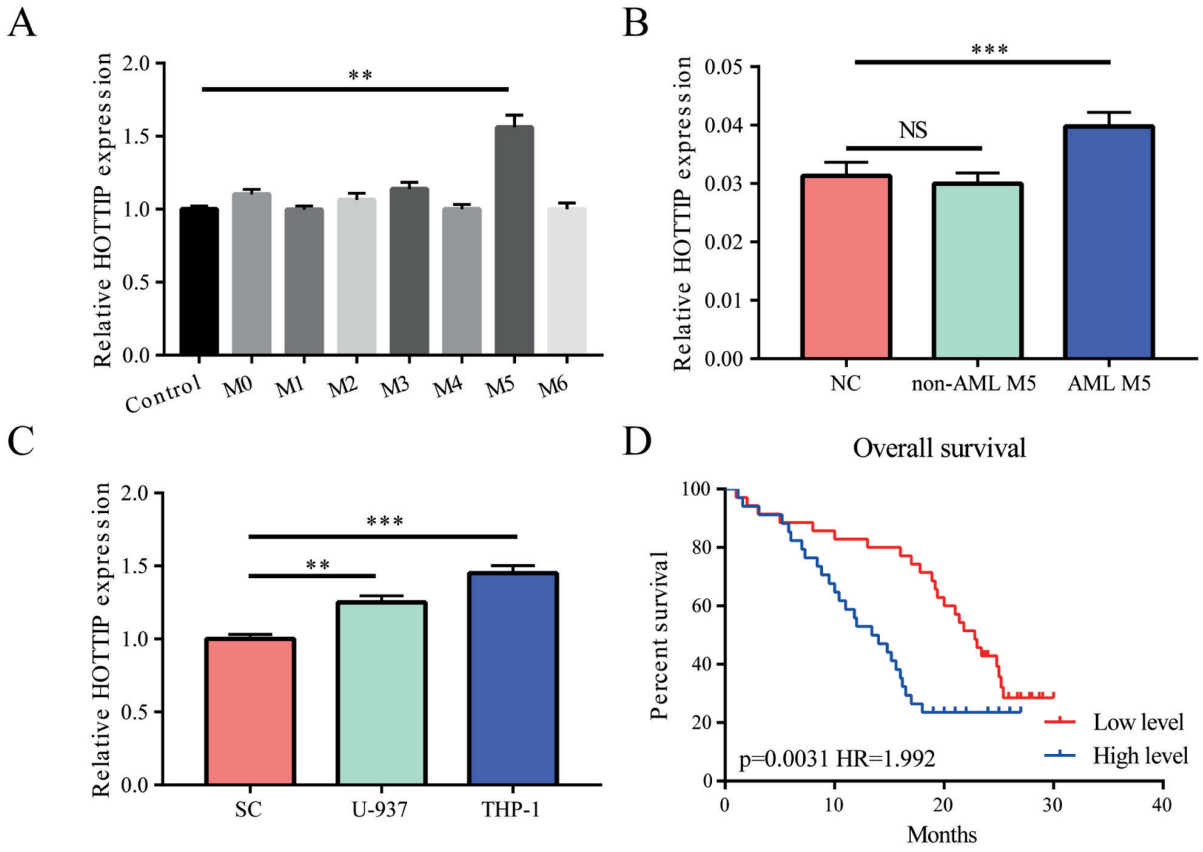

Figure 1. HOTTIP was highly expressed in AML-M5 patients and AML cell lines. ***A,*** HOTTIP expression in peripheral blood of AML-M5 patients was significantly higher than that of healthy controls. No significant difference was found in HOTTIP expression between other subtypes of AML (M0, M1, M2, M3, M4 and M6) and healthy controls. ***B,*** HOTTIP expression in peripheral blood of AML-M5 patients was markedly higher than that of non-M5 patients. ***C,*** HOTTIP was highly expressed in AML cell lines U-937 and THP-1 relative to normal monocyte line SC. ***D,*** Survival analysis indicated the 5-year survival of AML-M5 patients in high-level group was significantly lower than those in low-level group (p=0.0031, HR=1.992).

MicroRNA-608 was the Target Gene of HOTTIP

Current evidence proved that lncRNAs can serve as ceRNAs by absorbing target miRNAs. In this study, online websites predicted that microRNA-608 was the target gene of HOTTIP (Figure 3A). Dual-luciferase reporter gene assay further verified the binding condition of microRNA-608 to HOTTIP (Figure 3B, C). Compared with non-M5 patients, microRNA-608 was lowly expressed in AML-M5 patients (Figure 3D). Identically, microRNA-608 expression in AML cells was significantly lower than that of SC cells (Figure 3E). Meanwhile, microRNA-608 overexpression significantly inhibited the viability of AML cells. More importantly, microRNA-608 overexpression reversed the promotion of HOTTIP on proliferative rate (Figure 3F, 3G). Flow cytometry also revealed the inhibitory effect of microRNA-608 on cell cycle progression of AML cells. Furthermore, transfection of microRNA-608 mimics partially reversed the effect of HOTTIP on cell cycle progression of AML cells (Figure 3H, 3I). Therefore, we believed that HOTTIP exerted its function in AML by degrading microRNA-608.

DDA1 was the Target Gene of microRNA-608

Online prediction and analysis indicated that DDA1 was the target gene of microRNA-608 (Figure 4A). The binding condition between DDA1 and microRNA-608 was further confirmed by dual-luciferase reporter gene assay (Figure 4B, 4C). Subsequently, we determined DDA1 expression in AML patients and cell lines. No significant difference in DDA1 expression was found between healthy controls and AML patients with

other subtypes except for M5. However, DDA1 expression in peripheral blood of AML-M5 patients was significantly higher than than of non-M5 patients (Figure 4D). Meanwhile, DDA1 was highly expressed in AML cell lines when compared with SC cell line (Figure 4E). CCK-8 assay and flow cytometry indicated that DDA1 overexpression remarkably enhanced the proliferative ability and cell cycle progression. Interestingly, DDA1 could partially reverse the inhibitory effect of microRNA-608 on cellular behaviors of AML cells (Figure 4F-4I). To sum up, HOTTIP promoted the proliferative ability and cell cycle of AML cells by absorbing microRNA-608 to up-regulate DDA1.

Discussion

In 2010, ceRNA hypothesis proposed that some mRNAs and their pseudo-genes contained the same miRNA response elements (MREs) in their respective 3'UTRs. Meanwhile, they can be regulated by competitively binding to miRNAs[17]. The ceRNA hypothesis complements traditional gene regulation patterns, proposing a novel regulatory network as ceRNA-miRNA-mRNA axis[18]. In this theory, ceRNA regulates RNAs with the same MRE. Multiple types of genes can be served as ceRNAs, and lncRNAs as ceRNAs have been well explored.

In this study, we first examined HOTTIP expression in peripheral blood of AML patients by qRT-PCR. The results showed that HOTTIP was highly expressed in peripheral blood of AML-M5 patients when compared with other subtypes of AML. Identically, HOTTIP was also highly expressed in AML cell lines of U-837 and THP-1. Survival analysis indicated that the 5-year survival rate of AML patients with high level of HOTTIP was significantly lower than

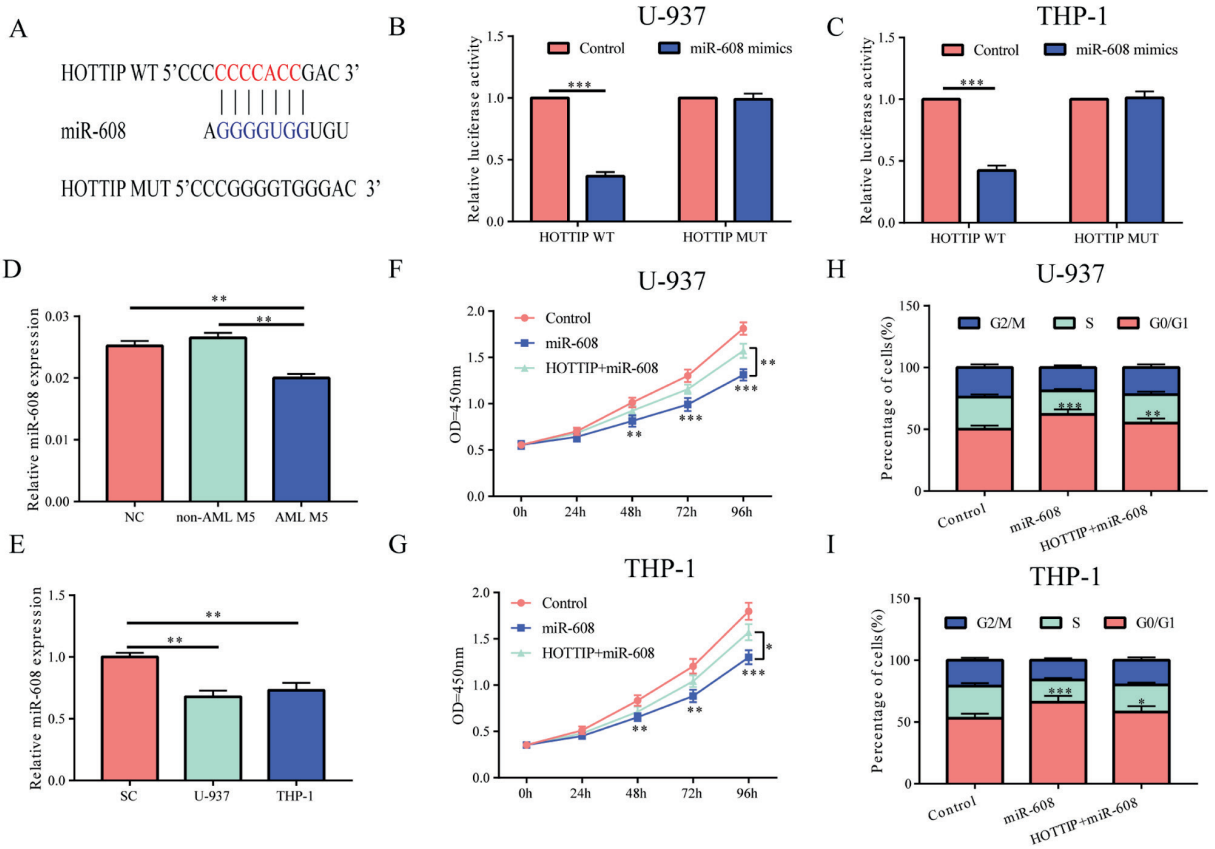

Figure 2. HOTTIP promoted proliferative ability and cell cycle of AML cells. ***A, B,*** Transfection of pcDNA-HOTTIP sufficiently up-regulated HOTTIP expression in U-937 cells (***A***) and THP-1 cells (***B***). ***C, E,*** CCK-8 assay indicated the viability of U-937 cells (***C***) and THP-1 cells (***E***) was remarkably elevated after transfection with pcDNA-HOTTIP. ***D, F,*** Flow cytometry indicated that transfection of pcDNA-HOTTIP significantly accelerated cell cycle progression of U-937 cells (***D***) and THP-1 cells (***F***).

those with lower level. The above results revealed that HOTTIP might be a potential tumor hallmark for AML-M5. We further investigated the regulatory effect of HOTTIP on AML cells. It was found that HOTTIP markedly accelerated the proliferative ability and cell cycle progression of AML cells.

We speculated that HOTTIP might function as a ceRNA in regulating AML progression. Firstly, bioinformatics predicted that microRNA-608 was the target gene of HOTTIP. QRT-PCR results revealed that microRNA-608 expression was lowly expressed in AML-M5 patients and AML cell lines. Furthermore, microRNA-608 could significantly inhibit the proliferative ability and cell cycle progression of AML cells. More importantly, the promoted effect of HOTTIP on AML cell behaviors was partially reversed by microRNA-608. We might conclude that HOTTIP exerted its biological function in AML by absorbing microRNA-608 as a ceRNA.

Online software predicted that DDA1 was the target gene of microRNA-608. DDA1 was highly expressed in AML-M5 patients and AML cell lines. DDA1 (DET1 and DDB1 associated 1) is a conservative gene exerting vital functions. Recent studies have shown that DDA1 is involved in ubiquitin-proteasome pathway, which also promotes the degradation of target proteins[19]. It is reported that DDA1 accelerates the progression of lung cancer by promoting cell cycle and proliferation[20]. To verify the role of DDA1 in AML, DDA1 was overexpressed in U-937 and THP-1 cells by liposome transfection. Subse-

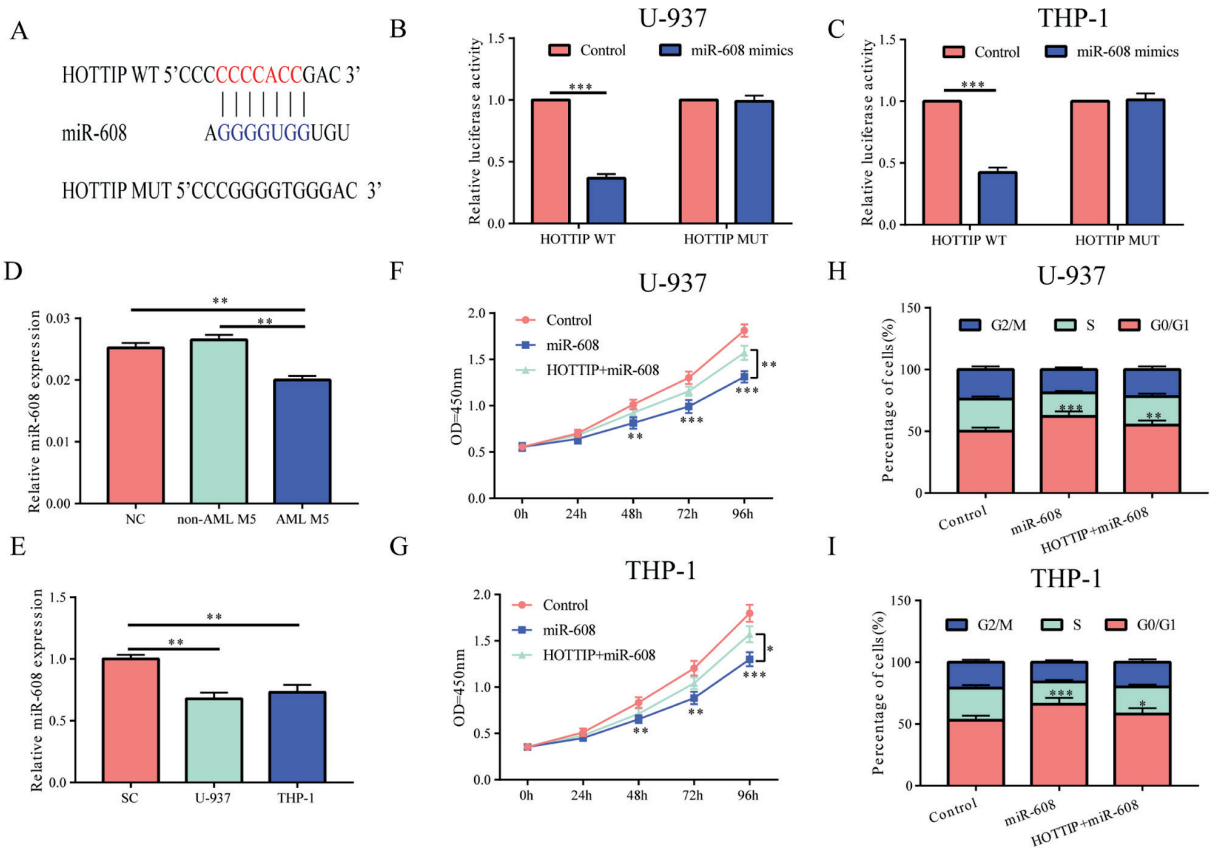

Figure 3. MiR-608 was the target gene of HOTTIP. ***A,*** Binding sites between miR-608 and HOTTIP. ***B, C,*** Dual-luciferase reporter gene assay further verified the binding condition of miR-608 to HOTTIP in U-937 cells (***B***) and THP-1 cells (***C***). ***D,*** MiR-608 was lowly expressed in AML-M5 patients than that of non-M5 patients. ***E,*** MiR-608 expression in AML cells was obviously lower than SC cells. ***F, G,*** MiR-608 overexpression in U-937 cells (***F***) and THP-1 cells (***G***) significantly inhibited cell viability, which reversed the promoted effect of HOTTIP on proliferative ability. ***H, I,*** Flow cytometry data revealed the inhibitory effect of miR-608 on cell cycle progression of U-937 cells (***H***) and THP-1 cells (***I***). Transfection of miR-608 mimics partially reversed the effect of HOTTIP on cell cycle progression of AML cells.

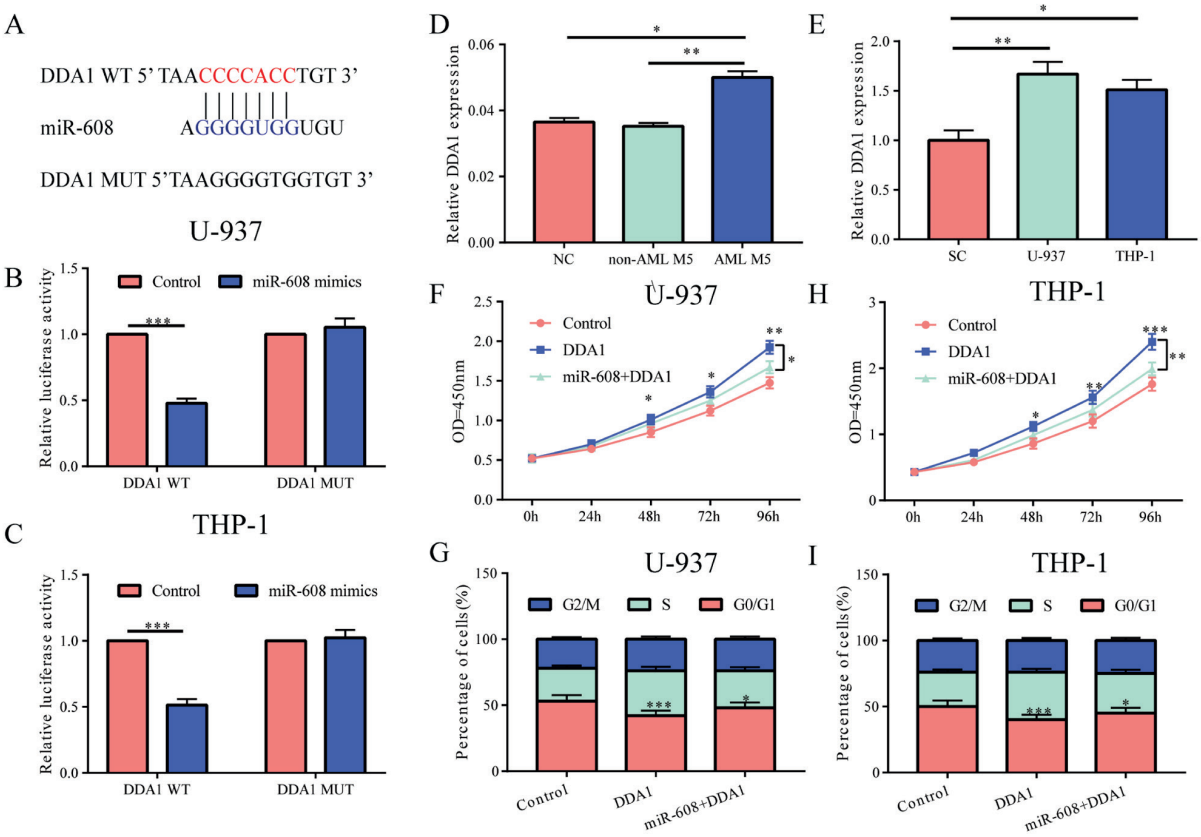

Figure 4. DDA1 was the target gene of miR-608. ***A,*** Binding sites between DDA1 and miR-608. ***B, C,*** Dual-luciferase reporter gene assay further verified the binding condition of DDA1 to miR-608 in U-937 cells (***B***) and THP-1 cells (***C***). ***D,*** DDA1 was highly expressed in AML-M5 patients than that of non-M5 patients. ***E,*** DDA1 expression in AML cells was significantly higher than SC cells. ***F, G,*** DDA1 overexpression in U-937 cells (***F***) and THP-1 cells (***G***) promoted cell viability, which reversed the inhibitory effect of miR-608 on proliferative ability. ***H, I,*** Flow cytometry data revealed the promoted effect of DDA1 on cell cycle progression of U-937 cells (***H***) and THP-1 cells (***I***). Transfection of pcDNA-DDA1 partially reversed the effect of miR-608 on cell cycle progression of AML cells.

quently, its effect on AML cell functions was verified by CCK-8 and flow cytometry. The results showed that DDA1 markedly promoted the proliferative ability and cell cycle of AML cells. More importantly, DDA1 reversed the inhibitory effect of microRNA-608 on AML cell behaviors. Therefore, it was suggested that microRNA-608 inhibited proliferative ability and cell cycle of AML cells by degrading DDA1.

Conclusions

We revealed that LncRNA HOTTIP is highly expressed in AML-M5. Meanwhile, it promotes the proliferative ability and cell cycle of AML cells by absorbing microRNA-608 to up-regulate DDA1. Our study may provide a novel therapeutic direction for clinical treatment of AML.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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