Am J Transl Res 2021;13(4):2308-2319
www.ajtr.org /ISSN:1943-8141/AJTR0119709

Original Article

Overexpression of KDM4D promotes acute myeloid leukemia cell development by activating MCL-1

Wei Wu[1], Xiaonian Cao[2], Luxia Mo[3]

[1]Department of Hepatopancreatobiliary Surgery, The Central Hospital of Wuhan, Tongji Medical College, Huazhong University of Science and Technology, Gusao Tree Road No. 16 of Jianghan District, Wuhan 430000, Hubei Province, China; [2]Department of Thoracic Surgery, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430040, China; [3]Wuhan Children's Hospital (Wuhan Maternal and Child Healthcare Hospital), Tongji Medical College, Huazhong University of Science & Technology, Wuhan, China

Received August 6, 2020; Accepted January 20, 2021; Epub April 15, 2021; Published April 30, 2021

Abstract: Acute myeloid leukemia (AML) is regarded as a fatal cancer in the world. The overall survival in adult patients with AML is still poor. As lysine demethylases, the KDM4 family is found highly expressed in many kinds of tumors. In this study, we demonstrate that KDM4D is overexpressed in AML and knockdown of KDM4D not only inhibits the proliferation of AML cells, but also induces cell cycle arrest and apoptosis. Furthermore, our research shows that KDM4D can regulate the expression of MCL-1 by demethylating H3K9me3 at the promoter region in AML cells. Besides, we find that high expression of KDM4D is correlated with poor overall survival in AML patients. Taken together, our study demonstrated that KDM4D can promote MCL-1 expression in AML and may serve as a novel target for the treatment of AML.

Keywords: KDM4D, MCL-1, proliferation, cell cycle, AML, apoptosis

Introduction

Acute myeloid leukemia (AML) is a heterogeneous malignancy characterized by aberrant proliferation and blocked apoptosis in the bone marrow [1, 2]. Although recent treatment advances in anticancer and targeted drug therapies, the prognosis of AML still remains poor due to progress of resistance and high rates of recurrence after treating with the available chemotherapy [3, 4]. Thus, it is urgent to identify effective therapies to improve the overall survival.

The basal subunits of chromatin are histones, regulatory proteins, genomic DNA and non-coding RNAs [5]. Nucleosome is a primary component of chromatin where 146 bp of DNA is wrapped around a histone, an octamer consisting of core histone proteins: H2A, H2B, H3 and H4 [6, 7]. These histones can be reversibly modified as many forms, such as phosphorylation, acetylation, methylation, and ubiquitination, et al [5, 6, 8]. The methylation of each of H3 has particular functions, as H3K4me3 and H3K36me3 are required for activating transcription, while the methylation of H3K9 and H3K27 are mainly linked to transcription repression [9, 10]. Abnormal histone methylation has been related to many human diseases including malignant tumor [11-13].

Histone lysine methylation is a reversible post-translational modification which is regulated via the interplay between the lysine methyltransferases and lysine demethylases (KDMs) [14]. It has been reported that a large family of JmjC-domain containing lysine demethylases (KDM) can demethylate lysine [15]. The KDM4 family (KDM4A-D, also known as JMJD2A-D) is one of KDMs subfamily in humans, where KDM4A-C are ~120 kDa proteins and possess double PHD and Tudor domains, but KDM4D encodes only half the size of others and lacks PHD and Tudor domains [7, 16]. KDM4A-C catalyze demethylation of histone substrates H3K9Me3/Me2, H3K36Me3/Me2, and H1.4K26Me3, while KDM4D demethylate H3K9Me3/Me2, H3K36Me3/Me2 and both the demethylated and trimethylated forms of H1.4K26 [11, 17-19]. The KDM4 proteins play an important role in many different processes, such as regu-

lation of gene transcription, epigenetic silencing, heterochromatin formation, genomic imprinting and DNA repair [20]. Accordingly, irregulation of KDM4 proteins can raise the risk of oncogenesis [21, 22]. For instance, it has been uncovered that KDM4B and KDM4C are overexpressed in breast cancer [23, 24]; knockdown KDM4B can suppress the growth of human gastric cancer cells [25]; Furthermore, there is evidence that KDM4A was associated with poor prognosis in non-small cell lung cancer patients [26].

But to date, we know little about the physiological role of KDM4D in the occurrence and progression of tumor. There has been reported that KDM4D promotes hepatic fibrogenesis by modulating Toll-Like Receptor 4 signaling pathway [27]. Also, scholars have proved that KDM4D forms a complex with the p53 tumor suppressor in vivo and interacts with the DNA binding domain of p53 in vitro [20]. Moreover, through the study of clinic data, high expression of KDM4D was linked to earlier recurrence in pancreatic carcinomas [7]. However, the function of KDM4D in AML remains elusive.

Myeloid Cell Leukemia-1 (MCL-1), one of the antiapoptotic Bcl-2 family members, is required for the survival of a variety of cell lines and has a very short half-life [28]. There is evidence that MCL-1 plays an important role in AML. For instance, ABT-199, the inhibition of XPO1, can enhance cell death in AML via MCL-1 [29]. It is observed that overexpression of MCL-1 is identified as playing an important role in several hematologic malignancies including in AML cell survival and treatment resistance [30]. In addition, recent studies have proved that MCL-1 is one amplified chromosomal region in human cancers and is the reason for resistance to various chemotherapeutic drugs [31-36]. We have known that MCL-1 protein levels are regulated by phosphorylation, ubiquitination and protease cleavage [39], But in our study, we demonstrated that MCL-1 expression could be controlled by KDM4D via demethylation of H3K9me3 at the promoter region in AML cells.

Materials and methods

Cell lines and culture

Human AML cell lines HL-60, MOLM-13 and NB4 were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). All cells were cultured in RPMI-1640 media supplemented with 10% FBS. The above cell lines were cultured at 37°C in a 5% CO_2 atmosphere.

Patients

All the blood samples from AML patients (n= 13) and the normal volunteers (n=5) were obtained from The Central Hospital of Wuhan. AML patients were clinically diagnosed based on the French-American-British (FAB) classification criteria. CD34+ cells were isolated by positive selection for the cell surface marker CD34 using MojoSort™ Nanobeads (BioLegend) in accordance with the manufacturer's instructions. All patients and volunteers have signed a written form of consents. Sample acquisition and experimental protocols were approved by the Ethics Committee of The Central Hospital of Wuhan.

RNA isolation and quantitative RT-PCR

Total RNAs were extracted from cells using TRIzol reagent (Takara). Reverse transcriptase reactions were immediately performed using the 5× Primescript RT Master Mix (Takara). The mRNA levels of specific genes were analyzed by qRT-PCR with 2× SYBR Green Mix (Takara). The primers are showed as follows: GGGCAGGGGTGTTTACTCAAT (KDM4D forward), TGTTTGCCAAATGGCGATACT (KDM4D reverse). The relative level of mRNA was calculated by the ΔΔCT method.

Western blotting

Cells were washed with ice cold PBS, harvested and lysed with radioimmuoprecipitation assay buffer (RIPA buffer) (50 mmol/L Tris-HCL, pH 7.4, 1% NP40, 0.25% sodium deoxycholate, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L dithiothreitol and 1 mmol/L phenylmethylsulfony fuoride). Equal quality of proteins was separated by SDS-PAGE, and then transferred onto PVDF membranes (Millipore, Bedford, MA). The membranes were blocked in 5% non-fat powdered milk in Tris-buffered saline with 0.1% Tween 20 (Sigma) for 1 hour. Blots were incubated with primary antibodies at 4°C overnight. On the next day, the membranes were washed with TBST buffer 3 times, and then subsequently probed with a horseradish perox-

idase-conjugated secondary antibody. Protein bands were detected by SuperSignal West Dura Chemiluminescent Substrate (Thermo). For immunoblotting, antibodies against KDM-4D (1:1000; Santa Cruz Biotechnology), MCL-1 (1;1000, CST), GAPDH (1:1000; Santa Cruz Biotechnology), H3K9me3 (1;1000, CST), H3 (1;1000, CST) were used.

Plasmid constructs

The construction of the pcDNA3.1-KDM4D vector was performed as follows: Total RNA was extracted from 293T using TRIzol reagent (Takara) and was immediately used for cDNA synthesis using the 5× Primescript RT Master Mix (Takara). The specific primers of the KDM4D's OFR designed by Gene Runner software, were amplified using the Taq DNA Polymerase Mix. The PCR products were subcloned into the pcDNA3.1 vector. The primers are as follows: Forward: ATGGAAACTATGAAGTCTAAGGCC; Reverse: TTAGGGCACAGGGGCCCAGCT. The plasmid transfection was performed with Lipofectamine 2000 (Thermo) following the manufacturer's instruction.

Stable AML cell establishment

For stable knockdown, pLKO-puro vectors encompassing shRNAs targeting KDM4D or a nontarget control were stably transfected into AML cell lines, HL-60, MOLM-13, NB4. To obtain viral particles, human kidney 293T cells were co-transfected with shKDM4D, phelper and Evn using $Cacl_2$ and Herbs. About 48 h-72 h after transfection, cell supernatants containing infectious lentiviral particles were harvested, then filtered through 0.45 μm membrane. The filtered supernatant was used to infect AML cell lines, HL-60, MOLM-13, NB4. After 24 hours, 1 μg/ml of puromycin was added to start select stable cell lines.

BrdU assay

The effect of KDM4D on cell proliferation was assessed by the 5-Bromo-2-deoxyUridine (BrdU) assay. 10,000-20,000 cells were cultured in 24-well plates overnight at 37°C in a 5% CO_2 atmosphere. Each well was added with culture medium containing DMSO and cultured for 48 hours. The cells were incubated with BrdU (Abcam, USA, 10 μg/ml) for 1 h and fixed in 4% paraformaldehyde for 20 min, then washed with PBS for 3 times. After treated with 0.5% Triton X-100 at 37°C for 2 h, the cells were washed three times with PBS buffer. Cells were incubated with a primary antibody against BrdU (1:1000) at 4°C overnight, then washed with PBS for 3 times. The nuclei were stained with 0.5 ug/ml DAPI. The percentage of BrdU staining was calculated from at least 10 microscopic fields.

Cell counting-kit 8

The viability of HL-60, MOLM-13 and NB4 cells was assessed by the use of a CCK-8 kit (Dojindo Laboratories, Kumamoto, Japan). The cells were inoculated into 96-well plates with 1640 medium at 37°C without CO_2 for 24 h. Then, the cells were incubated with 10 uL of CCK-8 reagent per well at 37°C without CO_2 for 4 h. The optical density value of each wells was measured at 450 nm using a microplate reader.

Flow cytometry assay

To determine the cell cycle arrest and apoptosis, flow cytometry assays were performed. The cells with indicated treatment for 24 h or 48 h were harvested. For apoptosis examination, the cells were stained with Annexin V-FITC/PI Apoptosis Detection Kit (BD, San Jose, CA, US) according to the manufacturer's instruction. For cell cycle profiles, the cells were fixed in 75% ethanol at 4°C overnight. After being washed with PBS, the cells were resuspended in PBS with PI and RNase A (Sigma Aldrich, USA) at 37°C in dark for 1 hour. Cell apoptosis and cell cycle distribution was performed with an FACS analysis system.

Luciferase activity assay and ChIP assay

Cells were seeded in triplicate in 24-well plates and allowed to settle for 24 h. Indicated plasmids plus pRL-TK Renilla plasmid were transfected into the cells using Lipofectamine 3000 (Thermo Fisher Scientific). Twenty-four hours after transfection, Luciferase activity assay was performed using the Dual-Luciferase Assay System according to the manufacturer's instructions. ChIP assays were performed using the ChIP assay kit (Billerica, MA, USA) according to the manufacturer's instructions. In all, we cultured 10^6 cells in the 6 well cell culture cluster, then lysed the cells, and chroma-

tins were produced by sonication. Anti-KDM4D or anti-IgG was used to precipitate the DNA-protein complexes overnight at 4°C. Magnetic beads were used to recover the DNA-protein complexes, which were followed by washing and eluting. Cross-links were reversed at 65°C overnight. The complexes were examined by PCR and qRT-PCR. Primers used for ChIP assay were as follows: MCL-1 Forward: 5'-GATCCCCATGGCAGCAGTAAAGCAAG-3', Reverse: 5'-GTTGCGCACGGCACCTACCAGCTG-3'. As a negative control, precipitated DNA was magnified via a primer in an off-target region that was the upstream of the MCL-1 transcriptional start sites.

Statistical analysis

SPSS19.0 software was used to analyze all data, and the results were presented as mean ± SD. All experiments were repeated at least three times. The student's t test or ANOVA multiple comparisons obtain the statistical significance of differences between groups in GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA), with $P<0.05$ considered as significant.

Result

KDM4D is highly expressed in AML and associated with poor survival

To demonstrate the prognostic value of KDM4D gene expression in AML, we carried out a cohort survival analysis on data from two independent public databases with transcriptome analysis results. Through the SurvExpress program, patients in the Metzeler Buske CM-AML GSE12417-GPL97 dataset generated by Metzeler and Buske were divided into "Low Risk" and "High Risk" groups according to the prognostic index. It predicted that the "High Risk" group of patients with AML exhibited a significantly higher KDM4D expression level than the "Low Risk" group (**Figure 1A**). In the available microarray datasets of GEPIA, we discovered that higher KDM4D expression predicted significantly lower OS in AML patients (**Figure 1B**). We also identified the expression profiles of KDM4D in serum samples from 13 AML patients and 5 healthy volunteers by qRT-PCR (Supplementary Figure 1). Results showed that KDM4D expression level was significantly increased in AML patients compared with healthy donors. To identify the expression of KDM4D in AML patients and normal PBMCs, we examined mRNA level of KDM4D in PBMCs from 2 healthy donors and 3 human AML cell lines (HL-60, MOLM-13, NB4). Corresponding with the result from the public microarray dataset, in the HL-60, MOLM-13 and NB4 cell lines, the expression of KDM4D is significantly higher than that in normal PBMCs (**Figure 1C**). We also measured the protein levels of KDM4D in these cells and found that HL-60, MOLM-13 and NB4 cell lines had higher protein level of KDM4D than that of PBMCs (**Figure 1D**). Furthermore, we have compared the expression of KDM4D in normal counterpart of AML cells (normal CD34+ cells) with AML cell lines (Supplementary Figure 2). Taken together, our results indicate that KDM4D is upregulated in AML cell lines.

KDM4D promotes the proliferation of AML cells

We specifically up-regulated KDM4D expression in AML cells by infecting them with KDM4D over-expression plasmids, and the expression of KDM4D was validated by western blot (**Figure 2A**). The CCK8 assay demonstrated that overexpression of KDM4D obviously enhanced the proliferation of all three cell lines (**Figure 2B**). Similarly, A BrdU staining assay showed that KDM4D increased the percentage of BrdU positive cells compared with the control (**Figure 2C** and **2D**). The MTT data indicated that over-expression of KDM4D promoted the proliferation of the AML cells (Supplementary Figure 3A-C). These results suggest that KDM4D remarkably promote human AML cells proliferation.

Silencing of KDM4D declines the proliferation of AML cells

To validate the important role of KDM4D on the proliferation in AML cells, we specifically down-regulated KDM4D expression by infecting them with a lentivirus expressing specific shRNA targeting KDM4D. According to Western blot results, KDM4D was successfully knocked down in all three cell lines (**Figure 3A**). The CCK8 assay demonstrated that knockdown of KDM4D obviously reduced the proliferation of all three cell lines (**Figure 3B**). Consisting with the CCK8 assay, the BrdU staining assay showed that the percentage of BrdU positive cells was decreased in KDM4D knock-down AML cells (**Figure 3C** and **3D**). The MTT data indicated that low expression of KDM4D inhibited the proliferation of the AML cells (Supple-

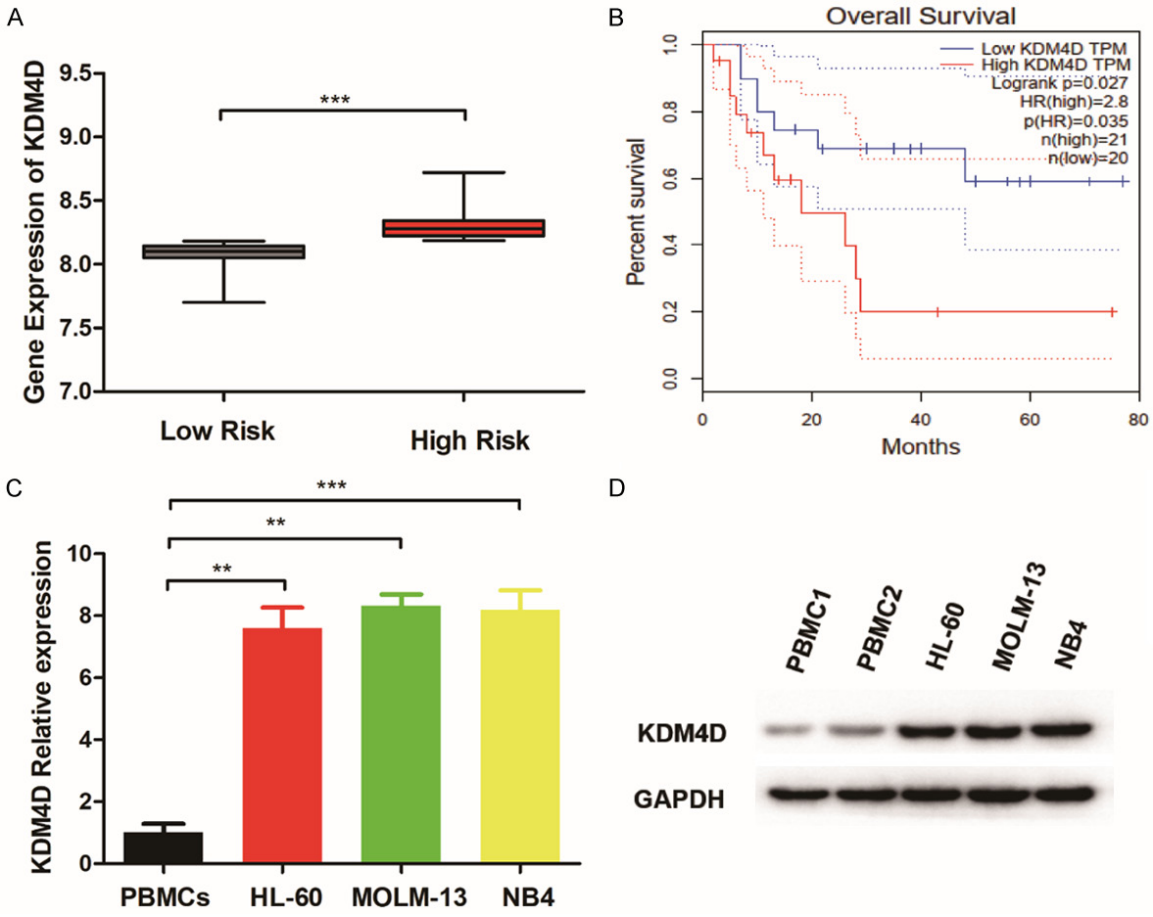


Figure 1. The expression of KDM4D was elevated in AML cells. A. Box plot showing the KDM4D expression level by risk groups in the AML GSE12417-GPL97 dataset generated by Metzeler and Buske (from SurvExpress program), **P<0.01. B. The Kaplan-Meier curve comparing the KDM4D high expression population (red) and KDM4D low expression population (blue) of AML patients was created from datasets of GEPIA, *P<0.05. C. Expression level of KDM4D in PBMCs from 2 healthy donors and 3 human AML cell lines (HL-60, MOLM-13, NB4), **P<0.01, ***P<0.001. D. Western blotting analysis of protein level of KDM4D in PBMCs, HL-60, MOLM-13, NB4.

mentary Figure 3D-F). These data clearly indicated that KDM4D plays an important role in AML cell proliferation.

Silencing of KDM4D induces cell cycle arrest and apoptosis of AML cells

The apoptotic arrest and subsequently uncontrolled cell cycle are common phenotypes of cancer cells. Therefore, we next investigated whether KDM4D could regulate cell cycle and apoptosis in AML cells. As shown in **Figure 4A** and **4C**, the downregulation of KDM4D in HL-60 and MOLM-13 cells significantly decreased the proportion of cells in S phase. In addition, we examine the effect of KDM4D on cell apoptosis. Annexin V-APC/7-amino-actinomycin D double staining was performed, followed by flow cytometry analysis. We found that knockdown of KDM4D in HL-60 and MOLM-13 cells significantly increased the percentage of apoptotic cells (**Figure 4B** and **4D**). These data together with the aforementioned results suggested that KDM4D might act as a potential oncogene in AML.

KDM4D transcriptionally activates MCL-1 expression via demethylation of H3K9me3 at the promoter region in AML cells

The critical role of MCL-1 in tumor progression motivated us to identify the genes that are regulated by KDM4D. In the available microarray datasets of GEPIA, we found that MCL-1 has a positive correlation with KDM4D (**Figure 5A**). To investigate the mechanism of KDM4D regulation of MCL-1 expression, Chromatin immunoprecipitation (ChIP) analysis was carried out to

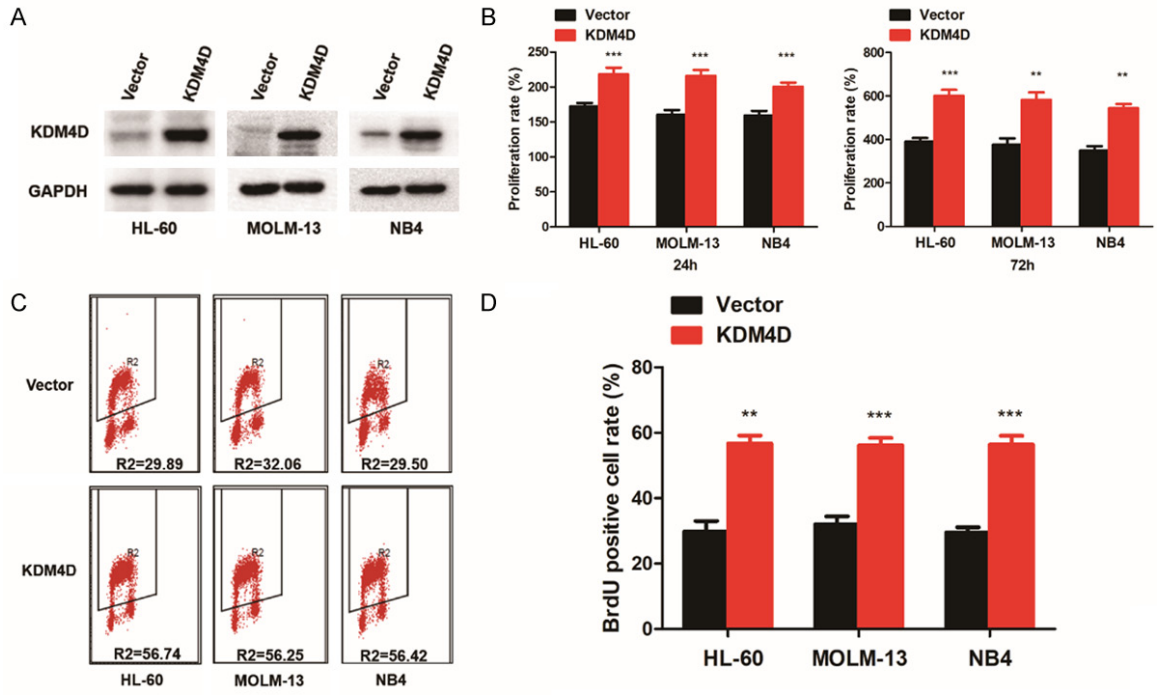


Figure 2. KDM4D promotes the proliferation of AML cells. A. Western blotting analysis confirmed upregulation of KDM4D in HL-60, MOLM-13, NB4 cells. B. Proliferation of all three AML cells (HL-60, MOLM-13, NB4) overexpressing KDM4D detected by CCK-8 method. C and D. Bromodeoxyuridine assay in AML cells determining proliferation capacity on KDM4D overexpression.

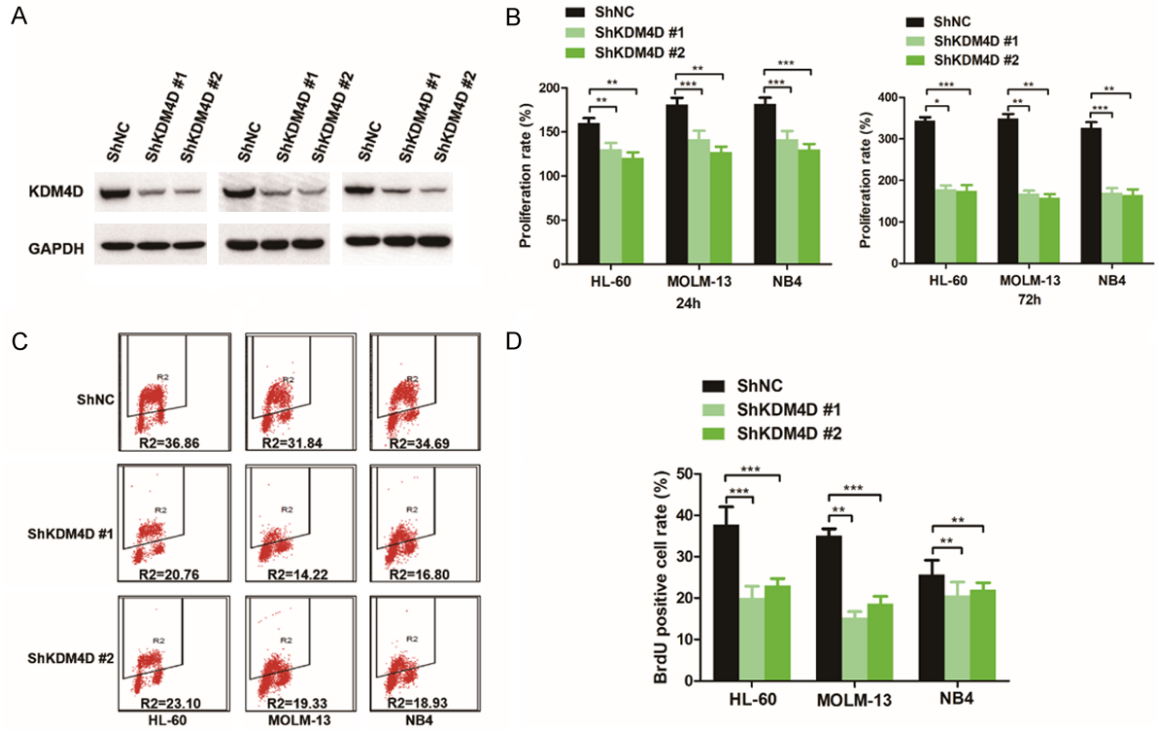


Figure 3. Silencing of KDM4D declines the proliferation of AML cells. A. Western blotting analysis confirmed knockdown of KDM4D in HL-60, MOLM-13, NB4 cells. B. Proliferation of all three AML cells (HL-60, MOLM-13, NB4) low expressing KDM4D detected by CCK-8 method. C and D. Bromodeoxyuridine assay in AML cells determining proliferation capacity on KDM4D knockdown. **P<0.01, ***P<0.001.

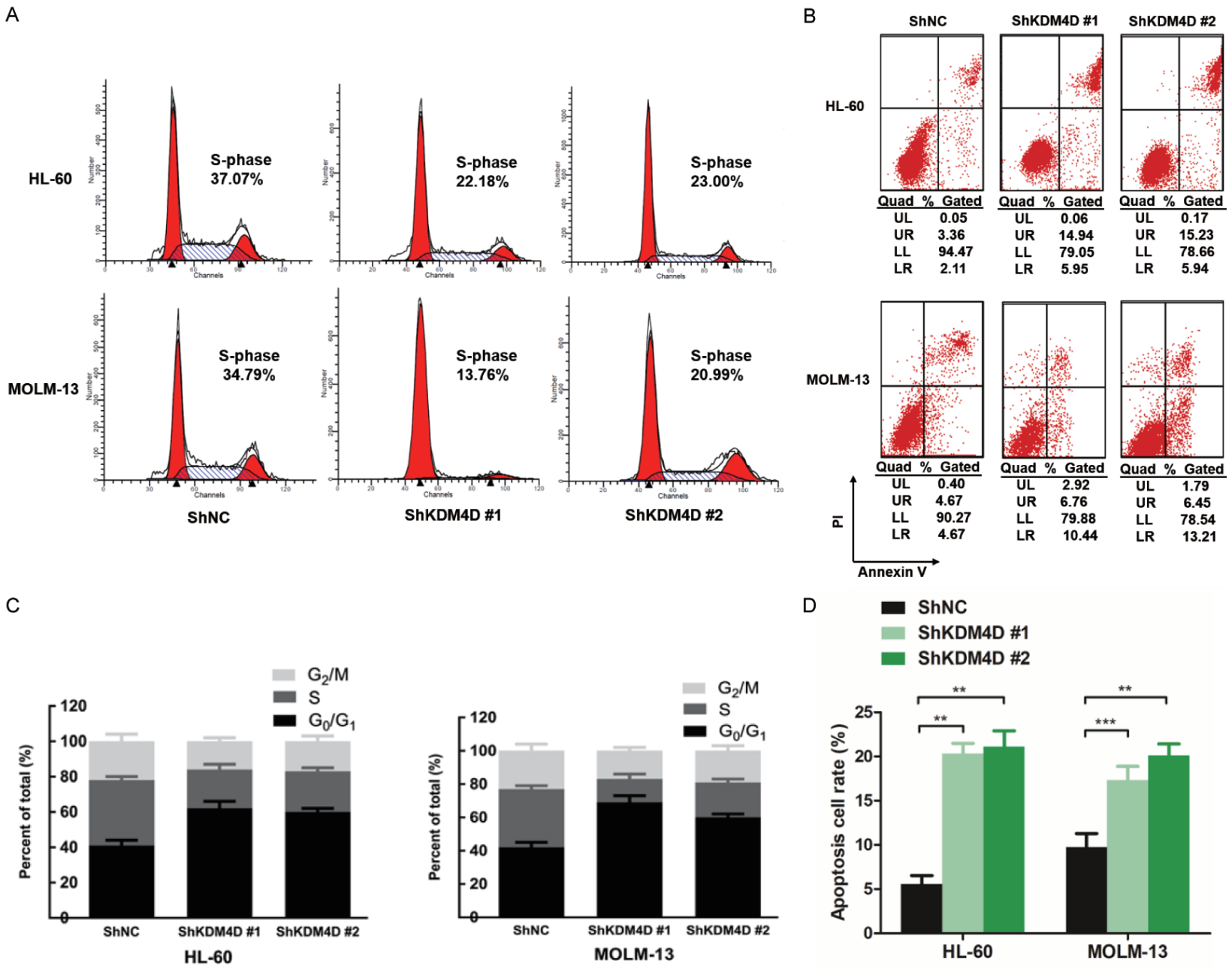

A
HL-60
S-phase 37.07%
S-phase 22.18%
S-phase 23.00%
MOLM-13
S-phase 34.79%
S-phase 13.76%
S-phase 20.99%
ShNC
ShKDM4D #1
ShKDM4D #2
B
ShNC
ShKDM4D #1
ShKDM4D #2
HL-60
Quad % Gated
UL 0.05
UR 3.36
LL 94.47
LR 2.11
Quad % Gated
UL 0.06
UR 14.94
LL 79.05
LR 5.95
Quad % Gated
UL 0.17
UR 15.23
LL 78.66
LR 5.94
MOLM-13
Quad % Gated
UL 0.40
UR 4.67
LL 90.27
LR 4.67
Quad % Gated
UL 2.92
UR 6.76
LL 79.88
LR 10.44
Quad % Gated
UL 1.79
UR 6.45
LL 78.54
LR 13.21
PI
Annexin V
C
G2/M
S
G0/G1
Percent of total (%)
ShNC
ShKDM4D #1
ShKDM4D #2
HL-60
MOLM-13
D
ShNC
ShKDM4D #1
ShKDM4D #2
Apoptosis cell rate (%)
HL-60
MOLM-13

Figure 4. Silencing of KDM4D induces cell cycle arrest and apoptosis of AML cells. (A and C) KDM4D knockdown induces the cell cycle arrest in HL-60 and MOLM-13 cells. HL-60 and MOLM-13 cells were infected with lentivirus carrying control shRNA or shRNA targeting KDM4D for 48 hours, then cell cycle analysis was performed. The representative (A) and quantitative results (C) are shown. (B and D) KDM4D knockdown induces apoptosis in HL-60 and MOLM-13 cells. The representative (B) and quantitative results (D) are shown. The percentage of cells in the UR region represents the apoptotic cells. **P<0.01, ***P<0.001.

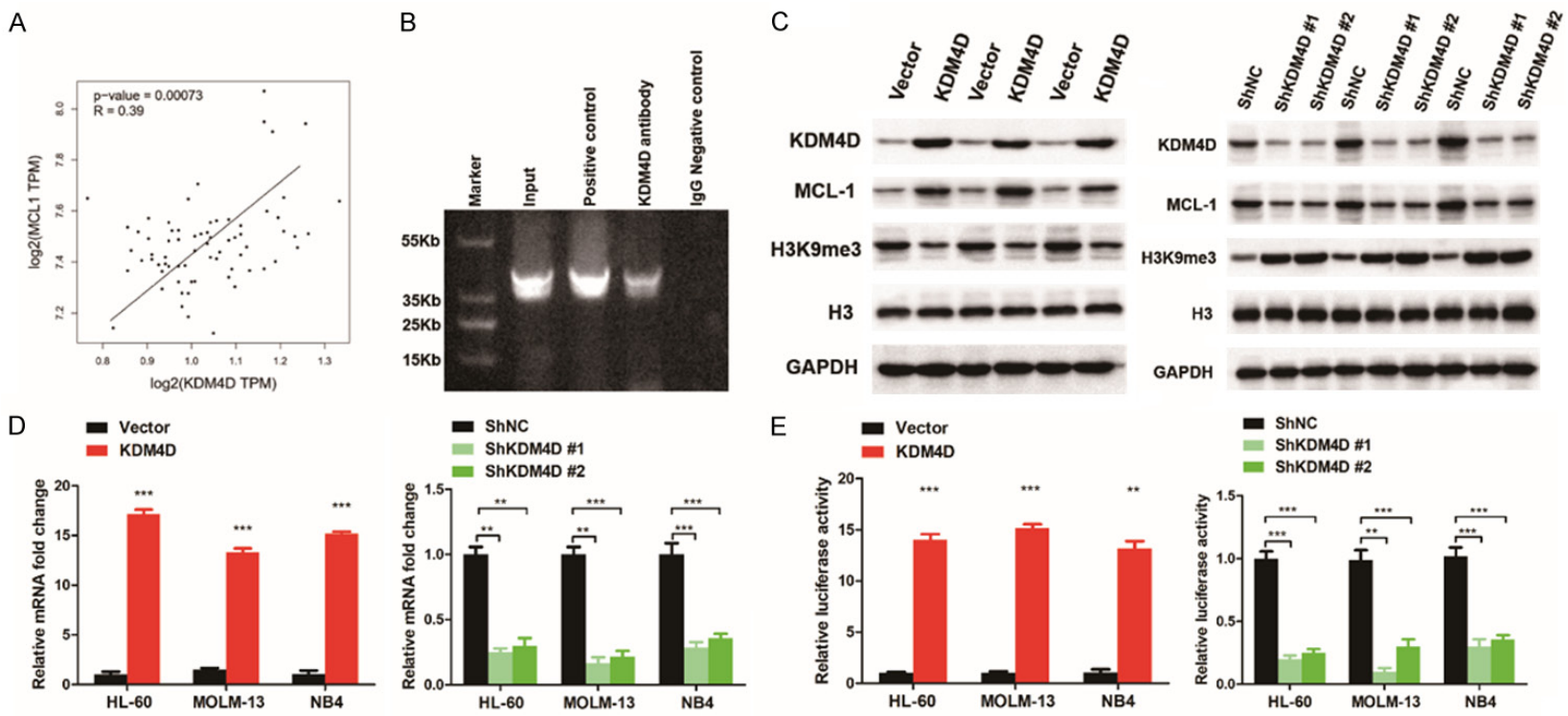


Figure 5. KDM4D transcriptionally activates MCL-1 expression via demethylation of H3K9me3 at the promoter region in AML cells. A. The expression patterns of KDM4D and MCL-1 in the Leukemia cell line of GEPIA datasets. B. ChIP analysis in HL-60 cells 24 hours after infected with control shRNA or shRNA targeting KDM4D, using anti-KDM4D and anti-rabbit-IgG antibodies. C. ChIP and quantitative real-time PCR analysis in HL-60 cells after infected with vector, KDM4D overexpression plasmids (left), control shRNA or shRNA targeting KDM4D, using anti-KDM4D and anti-rabbit-IgG antibodies (right). D. Luciferase assay in HL-60 cells 72 hours after infected with vector, KDM4D overexpression plasmids (left), control shRNA or shRNA targeting KDM4D, **P<0.01, ***P<0.001. E. HL-60, MOLM-13 and NB4 cells were infected with vector, KDM4D overexpression plasmids (left), control shRNA or shRNA targeting KDM4D. The protein levels of MCL-1, H3K9me3, H3, GAPDH were detected by western blot. Positive control: MCL-1 antibody. **P<0.01, ***P<0.001.

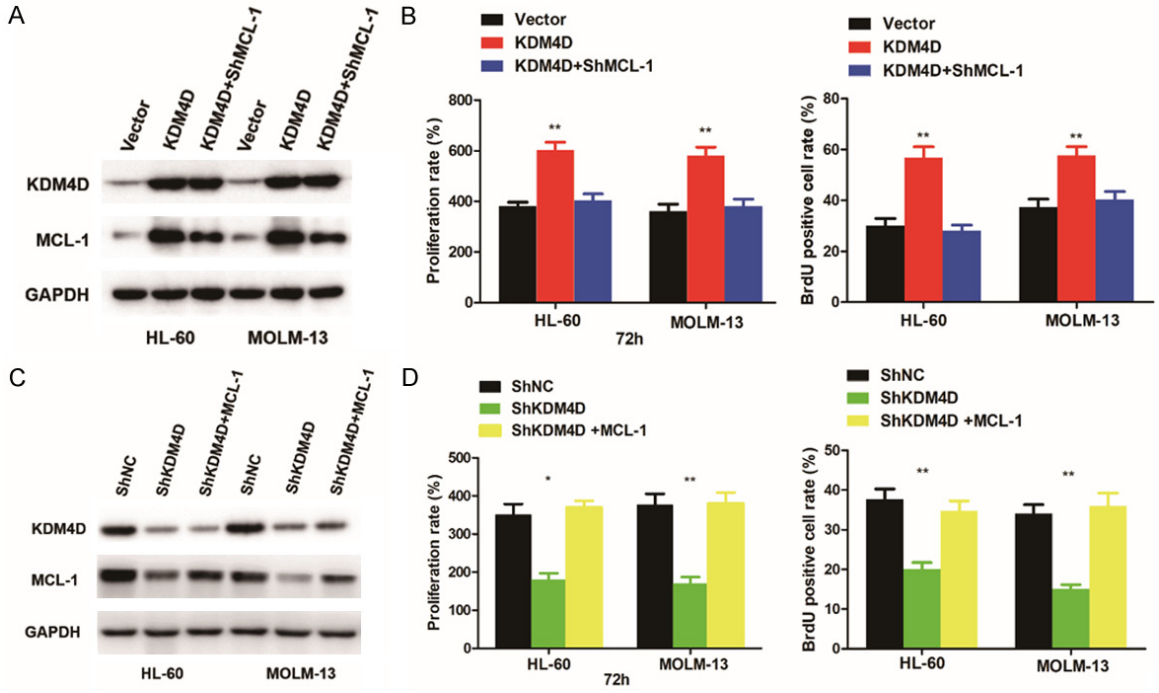


Figure 6. KDM4D regulates the survival of AML cells through MCL-1. A. HL-60 and MOLM-13 cells were infected with vector, KDM4D overexpression plasmids, KDM4D overexpression plasmids with ShMCL-1 lentivirus. The protein levels of KDM4D, MCL-1, GAPDH were detected by western blot. B. Proliferation capacity of HL-60, MOLM-13 cells with the corresponding treatment were detected by CCK-8 method (left) and Bromodeoxyuridine assay (right). C. HL-60 and MOLM-13 cells were infected with lentivirus carrying control shRNA, shKDM4D and shKDM4D with KDM4D overexpression plasmids. The protein levels of KDM4D, MCL-1, GAPDH were detected by western blot. D. Proliferation capacity of HL-60, MOLM-13 cells with the corresponding treatment were detected by CCK-8 method (left) and Bromodeoxyuridine assay (right). *P<0.05, **P<0.01.

show that KDM4D could bind to the promoter region of MCL-1 (**Figure 5B**). Western blot in KDM4D overexpression AML cells demonstrate that MCL-1 was upregulated and H3K9me3 was downregulated compared to control cells. While knocking down KDM4D, the results were adverse (**Figure 5C**). When KDM4D was upregulated in all three AML cells, the amount of immunoprecipitated DNA from the MCL-1 promoter was increased in ChIP assay, and the result was opposite when KDM4D knockdown (**Figure 5D**). We next constructed reporter plasmids containing the MCL-1 promoter, When KDM4D was overexpressed, MCL-1 promoter activity was significantly increased. In the same way, MCL-1 promoter activity was significantly decreased when KDM4D was knockdown (**Figure 5E**).

KDM4D regulates the survival of AML cells through MCL-1

To further investigate KDM4D's role on MCL-1 expression in AML progression, we performed a 'rescue' experiment by co-transfecting HL-60 and MOLM-13 cells with KDM4D and shMCL-1 vectors or shKDM4D and MCL-1 vectors. Western blot showed that the expression of MCL-1 has a positive correlation with the expression of KDM4D (**Figure 6A** and **6C**). Next, we found that inhibition of MCL-1 could reverse the effects of KDM4D overexpression on cell proliferation and apoptosis in CCK-8 and BrdU assay (**Figure 6B**). Likewise, overexpression of MCL-1 could partly reverse the effect of KDM4D knocked-down on cell proliferation and apoptosis (**Figure 6D**). Collectively, these results demonstrated that KDM4D regulates the survival of AML cells through MCL-1.

Discussion

Effective therapeutic strategies targeting AML are still scarce [40]. In this report, we have demonstrated that KDM4D plays an important role in AML progression. MCL-1 signaling is a well-known contributor of tumor anti-apoptosis, which is the most unique member of the

Bcl-2 family [41]. However, regulators involved in MCL-1 signaling during tumor progression remain less understood. It has been shown that KDM4 activities are required for MLL-AF9 translocated AML via using KDM4A, KDM4B, and KDM4C conditional triple-knockout mice [42]. Here we found KDM4D modulates proliferation and apoptosis via MCL-1 signaling. While KDM4D deficiency inhibits the proliferation of AML cells, induced expression of KDM4D promotes the proliferation and decreases apoptosis of AML cells. Our data provide KDM4D as a novel therapeutic target.

The function of KDM4D in tumor development is seldomly investigated. It has been claimed that KDM4D may modulate the transcriptional activity of p53, one of the most important tumor suppressors and found mutated in half of all human tumors [20]. However, to our best of knowledge it has not been reported as an independent factor that can influence the tumor progression in AML. In this study, we found that high KDM4D expression was significantly correlated with better AML cells survival and showed primary mechanism how KDM4D regulates the proliferation an apoptosis of AML. However, we did not find its prognostic value in AML and haven't demonstrated the effect of KDM4D in vivo. Though, we speculate that KDM4D may be a confounding factor to affect the prognostic value in AML.

In vitro assays demonstrated the oncogenic role of KDM4D in AML. Ectopic expression of KDM4D contributed to tumor growth in AML cells (HL-60, MOLM-13, NB4) by promoting cell proliferation and inhibiting apoptosis. On the other hand, lentivirus-mediated knockdown of KDM4D significantly inhibited cell proliferation. Moreover, we observed that ectopic expression of KDM4D led to demethylation of H3K9me3 and upregulation of MCL-1. All these changes in biomarkers suggest a strengthen of tumor growth. On the other hand, the knockdown of KDM4D impairs its ability in methylation of H3K9me3 and downregulating MCL-1. This is the first report on the role of KDM4D in tumor progression in AML.

It has been reported that nuclear KDM4D expression has a positive correlation with lung carcinoma, especially with squamous cell carcinoma [43]. Using different databases, we did see the similar changes in AML patients. Our results have suggested that KDM4D can function as an oncogene in AML, which is supported by the studies reported by others that KDM4D was required for proliferation and survival in colon cancer cell.

We further explore the downstream signaling pathway responsible for KDM4D oncogenic function in AML and found that the KDM4D-mediated tumor growth could be attributed to the activation of MCL-1 signaling via demethylation of H3K9me3, which is critical for the proliferation of AML. KDM4D has constantly been regarded as an important factor in cancer cells, and the overexpression is associated with increased cellular proliferation and anchorage-independent growth [44]. However, its mechanism in regulating signal pathway in human cancers is unknown. Our CHIP result indicated that KDM4D could interact with MCL-1 at the promoter region and enhance the demethylation of H3K9me3.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (81702264).

Disclosure of conflict of interest

None.

Address correspondence to: Luxia Mo, Wuhan Children's Hospital (Wuhan Maternal and Child Healthcare Hospital), Tongji Medical College, Huazhong University of Science & Technology, 100 Xiang Gang Road, Jiang'an District, Wuhan 430000, Hubei, China. E-mail: molux87@126.com

References

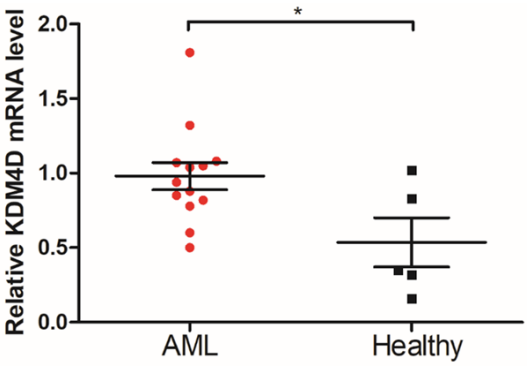
[1] Zhou P, Chen X, Li M, Sun X, Tan J, Wang X, Chu Y, Zhang Y, Cheng T, Zhou J, Wang G and Yuan W. Overexpression of PRDM5 promotes acute myeloid leukemia cell proliferation and migration by activating the JNK pathway. Cancer Med 2019; 8: 3905-3917.

[2] Binder S, Luciano M and Horejs-Hoeck J. The cytokine network in acute myeloid leukemia (AML): a focus on pro- and anti-inflammatory mediators. Cytokine Growth Factor Rev 2018; 43: 8-15.

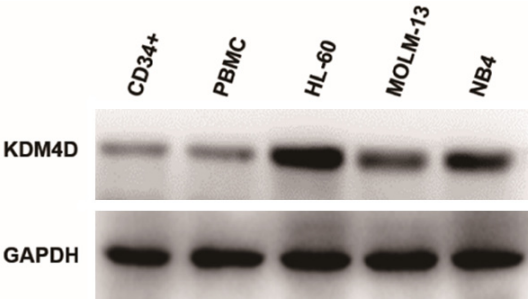
[3] Quintana J, Advis P, Becker A, Beresi V, Campbell M, Vinés EF, García H, Salgado C, Vargas L, Rojas J, Obando M, Neira L, Páez E and Zolezzi P. Acute myelogenous leukemia in Chile PINDA protocols 87 and 92 results. Leukemia 2005; 19: 2143-2146.

[4] Sun L, Li X, Sun Y, Huang W, Fang K, Han C, Chen Z, Luo X, Chen Y and Wang W. LncRNA ANRIL regulates AML development through modulating the glucose metabolism pathway of AdipoR1/AMPK/SIRT1. Mol Cancer 2018; 17: 127.
[5] Lomberk GA, Iovanna J and Urrutia R. The promise of epigenomic therapeutics in pancreatic cancer. Epigenomics 2016; 8: 831-842.
[6] Iwamori N, Zhao M, Meistrich ML and Matzuk MM. The testis-enriched histone demethylase, KDM4D, regulates methylation of histone H3 lysine 9 during spermatogenesis in the mouse but is dispensable for fertility. Biol Reprod 2011; 84: 1225-1234.
[7] Isohookana J, Haapasaari KM, Soini Y and Karihtala P. KDM4D predicts recurrence in exocrine pancreatic cells of resection margins from patients with pancreatic adenocarcinoma. Anticancer Res 2018; 38: 2295-2302.
[8] Kanwal R, Gupta K and Gupta S. Cancer epigenetics: an introduction. Methods Mol Biol 2015; 1238: 3-25.
[9] Martin C and Zhang Y. The diverse functions of histone lysine methylation. Nat Rev Mol Cell Biol 2005; 6: 838-849.
[10] Kouzarides T. Chromatin modifications and their function. Cell 2017; 128: 693-705.
[11] Greer EL and Shi Y. Histone methylation: a dynamic mark in health, disease and inheritance. Nat Rev Genet 2012; 13: 343-57.
[12] Black JC, Rechem CV and Whetstine JR. Hetstine histone lysine methylation dynamics: establishment, regulation, and biological impact. Mol Cell 2012; 48: 491-507.
[13] Zhang X, Wen H and Shi X. Lysine methylation: beyond histones. Acta Biochim Biophys Sin (Shanghai) 2011; 44: 14-27.
[14] Krishnan S and Trievel RC. Structural and functional analysis of JMJD2D reveals molecular basis for site-specific demethylation among JMJD2 demethylases. Structure 2013; 21: 98-108.
[15] Klose RJ, Kallin EM and Zhang Y. JmjC-domain-containing proteins and histone demethylation. Nat Rev Genet 2006; 7: 715-727.
[16] Jbara M, Raviv NG, Maity SK, Ayoub N and Brik A. Total chemical synthesis of methylated analogues of histone 3 revealed KDM4D as a potential regulator of H3K79me3. Bioorg Med Chem 2017; 25: 4966-4970.
[17] Young LC and Hendzel MJ. The oncogenic potential of Jumonji D2 (JMJD2/KDM4) histone demethylase overexpression. Biochem Cell Biol 2013; 91: 369-377.
[18] Williams ST, Walport LJ, Hopkinson RJ, Madden SK, Chowdhury R, Schofield CJ and Kawamura A. Studies on the catalytic domains of multiple JmjC oxygenases using peptide substrates. Epigenetics 2014; 9: 1596-603.
[19] Berry WL and Janknecht R. KDM4/JMJD2 histone demethylases: epigenetic regulators in cancer cells. Cancer Res 2013; 73: 2936-2942.
[20] Kim TD, Oh S, Shin S and Janknecht R. Regulation of tumor suppressor p53 and HCT116 cell physiology by histone demethylase JMJD2D/KDM4D. PLoS One 2012; 7: e34618.
[21] Chi P, Allis CD and Wang GG. Covalent histone modifications–miswritten, misinterpreted and mis-erased in human cancers. Nat Rev Cancer 2010; 10: 457-69.
[22] Cloos PA, Christensen J, Agger K and Helin K. Erasing the methyl mark: histone demethylases at the center of cellular differentiation and disease. Genes Dev 2008; 22: 1115-1140.
[23] Liu G, Fischer AB, Kreike B, Vijver MJ, Abrams J, Ethier SP and Yang ZQ. Genomic amplification and oncogenic properties of the GASC1 histone demethylase gene in breast cancer. Oncogene 2009; 28: 4491-500.
[24] Kawazu M, Saso K, Tong KI, McQuire T, Goto K, Son DO, Wakeham A, Miyagishi M, Mak TW and Okada H. Histone demethylase JMJD2B functions as a co-factor of estrogen receptor in breast cancer proliferation and mammary gland development. PLoS One 2011; 6: e17830.
[25] Li W, Zhao L, Zang W, Liu Z, Chen L, Liu T, Xu D and Jia J. Histone demethylase JMJD2B is required for tumor cell proliferation and survival and is overexpressed in gastric cancer. Biochem Biophys Res Commun 2011; 416: 372-378.
[26] Rechem CV, Black JC, Greninger P, Zhao Y, Donado C, Burrowes PD, Ladd B, Christiani DC, Benes CH and Whetstine JR. A coding single-nucleotide polymorphism in lysine demethylase KDM4A associates with increased sensitivity to mTOR inhibitors. Cancer Discov 2015; 5: 245-254.
[27] Dong F, Jiang S, Li J, Wang Y, Zhu L, Huang Y, Jiang X, Hu X, Zhou Q, Zhang Z and Bao Z. The histone demethylase KDM4D promotes hepatic fibrogenesis by modulating toll. EBioMedicine 2018; 39: 472-483.
[28] Pan Y, Zhang Y, Liu W, Huang Y, Shen X, Jing R, Pu J, Wang X, Ju S, Cong H and Chen H. LncRNA H19 overexpression induces bortezomib resistance in multiple myeloma by targeting MCL-1 via miR-29b-3p. Cell Death Dis 2019; 10: 106-14.
[29] Luedtke AD, Su Y, Liu S, Edwards H, Wang Y, Lin H, Taub JW and Ge B. Inhibition of XPO1 enhances cell death induced by ABT-199 in acute myeloid leukaemia via Mcl-1. J Cell Mol Med 2018; 22: 6099-6111.
[30] Tibes R and Bogenberger JM. Transcriptional silencing of MCL-1 through cyclin-dependent

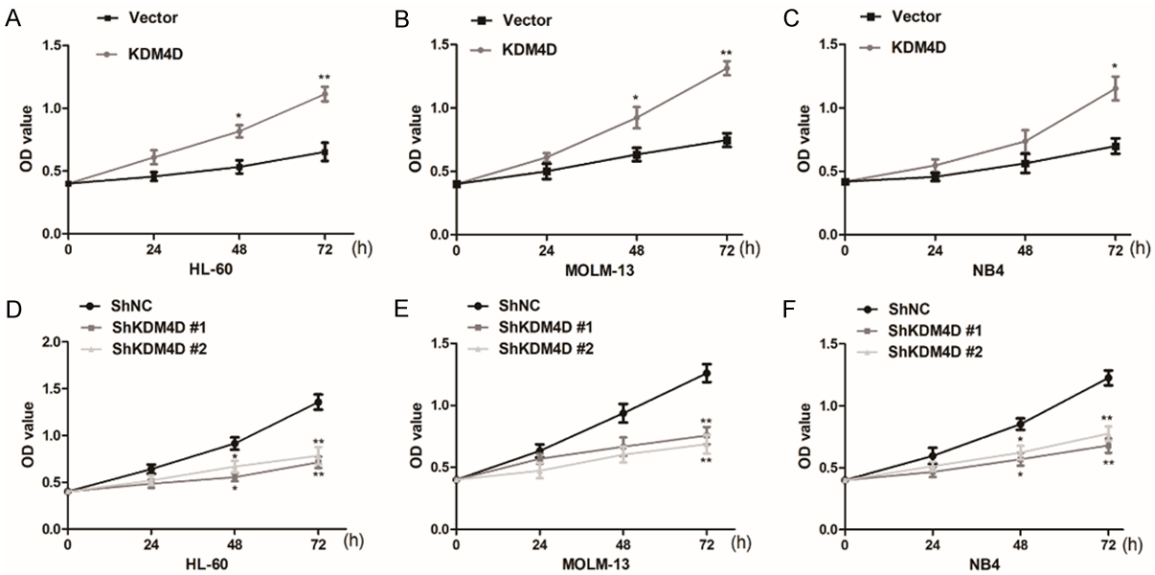
kinase inhibition in acute myeloid leukemia. Front Oncol 2019; 9: 1205.
[31] Wei G, Twomey D, Lamb J, Schlis K, Agarwal J, Stam RW, Opferman JT, Sallan SE, Boer ML, Pieters R, Golub TR and Armstrong SA. Gene expression-based chemical genomics identifies rapamycin as a modulator of MCL1 and glucocorticoid resistance. Cancer Cell 2006; 10: 331-342.
[32] Wei S, Dong K, Lin F, Wang X, Li B, Shen J, Zhang Q, Wang R and Zhang H. Inducing apoptosis and enhancing chemosensitivity to gemcitabine via RNA interference targeting Mcl-1 gene in pancreatic carcinoma cell. Cancer Chemother Pharmacol 2008; 62: 1055-1064.
[33] Hu S, Monette Z, Tzankov A, Green T, Wu L, Balasubramanyam A, Liu W, Visco C, Li Y, Miranda RN, Moreno SM, Dybkaer K, Chiu A, Orazi A, Zu Y, Bhagat G, Richards KL, Hsi ED, Choi WW, Zhao X, Krieken JH, Huang Q, Huh J, Ai W, Ponzoni M, Ferreri AJ, Zhou F, Slack GW, Gascoyne RD, Tu M, Variakojis D, Chen W, Go RS, Piris MA, Møller MB, Medeiros LJ and Young KH. MYC/BCL2 protein coexpression contributes to the inferior survival of activated B-cell subtype of diffuse large B-cell lymphoma and demonstrates high-risk gene expression signatures: a report from The International DLBCL Rituximab-CHOP Consortium Program. Blood 2013; 121: 4021-4031.
[34] Zack TI, Schumacher SE, Carter SL, Cherniack AD, Saksena G, Tabak B, Lawrence MS, Zhsng CZ, Wala J, Mermel CH, Sougnez C, Gabriel SB, Hernandez B, Shen H, Laird PW, Getz G, Meyerson M and Beroukhim R. Pan-cancer patterns of somatic copy number alteration. Nat Genet 2013; 45: 1134-1140.
[35] Wertz IE, Kusam S, Lam C, Okamoto T, Sandoval W, Anderson DJ, Helgason E, Ernst JA, Eby M, Liu J, Belmont LD, Kaminker JS, O'Rourke KM, Pujara K, Kohli PB, Johnson AR, Chiu ML, Lill JR, Jackson PK, Fairbrother WJ, Seshagiri S, Ludlam MJ, Leong KG, Dueber EC, Maecker H, Huang DC and Dixit VM. Sensitivity to antitubulin chemotherapeutics is regulated by MCL1 and FBW7. Nature 2011; 471: 110-114.
[36] Li L, Pongtornpipat P, Tiutan T, Kendrick SL, Park S, Persky DO, Rimsza LM, Puvvada SD and Schatz JH. Synergistic induction of apoptosis in high-risk DLBCL by BCL2 inhibition with ABT-199 combined with pharmacologic loss of MCL1. Leukemia 2015; 29: 1702-1712.
[37] Zhang H, Guttikonda S, Roberts L, Uziel T, Semizarov D, Elmore SW, Leverson JD and Lam LT. Mcl-1 is critical for survival in a subgroup of non-small-cell lung cancer cell lines. Oncogene 2011; 30: 1963-1968.
[38] Toumi SW, Robillard N, Gomez P, Moreau P, Gouill SL, Loiseau HA, Harousseau JL, Amiot M and Bataille R. Mcl-1 is overexpressed in multiple myeloma and associated with relapse and shorter survival. Leukemia 2005; 19: 1248-1252.
[39] Sieghart W, Losert D, Strommer S, Cejka D, Schmid K, Rockenschaub SR, Bodingbauer M, Crevenna R, Monia BP, Peck-Radosavljevic M and Wacheck V. Mcl-1 overexpression in hepatocellular carcinoma: a potential target for antisense therapy. J Hepatol 2006; 44: 151-157.
[40] Mojsa B, Lassot I and Desagher S. Mcl-1 ubiquitination: unique regulation of an essential survival protein. Cells 2014; 3: 418-437.
[41] Li C, Song Y and Li P. MCL1 regulates cell death, tumor growth and chemosensitivity to sabutoclax in ovarian adenocarcinoma. Cell Tissue Res 2020; 379: 625-633.
[42] Belmar J and Fesik SW. Small molecule Mcl-1 inhibitors for the treatment of cancer. Pharmacol Ther 2015; 145: 76-84.
[43] Uimonen K, Merikallio H, Pääkkö P, Harju T, Mannermaa A, Palvimo P, Kosma VM and Soini Y. GASC1 expression in lung carcinoma is associated with smoking and prognosis of squamous cell carcinoma. Histol Histopathol 2014; 29: 797-804.
[44] Lee DH, Kim GW, Jeon YH, Yoo J, Lee SW and Kwon SH. Advances in histone demethylase KDM4 as cancer therapeutic targets. FASEB J 2020; 34: 3461-3484.



Supplementary Figure 1. KDM4D was overexpression in AML patients. qRT-PCR analysis of KDM4D expression in serum samples from 13 AML patients and 5 healthy volunteers. *P<0.05.



Supplementary Figure 2. The expression of KDM4D was elevated in AML cells. Western blotting analysis of protein level of KDM4D in CD34+, PBMCs, HL-60, MOLM-13, NB4.



Supplementary Figure 3. The effect of KDM4D on cell proliferation verified by MTT assay. A-C. Proliferation of all three AML cells (HL-60, MOLM-13, NB4) overexpressing KDM4D detected by MTT method. D-F. Proliferation of all three AML cells (HL-60, MOLM-13, NB4) low expressing KDM4D detected by MTT method. *P<0.05, **P<0.01.