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*Original Article*

# FoxM1 is involved in the development of acute myeloid leukemia by targeting Bcl-2 expression

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**Abstract:** Background and aim: Forkhead box M1 (FoxM1) is frequently upregulated in human malignancies, including acute myeloid leukemia (AML). However, the biological function of FoxM1 in AML remains unclear. Methods: FoxM1 expression was determined by quantitative real-time PCR (qRT-PCR) and immunofluorescence analysis in AML-*de novo* patients, AML-complete remission (CR) patients, and AML-refractory relapse (RR) patients, alongside healthy individuals. The proliferation rate of HL60 and U937 cells transfected with FoxM1 or negative control (NC) siRNA was determined by MTT and colony formation assays, and the cellular apoptotic rate was determined by flow cytometry. The correlation between FoxM1 and B-cell leukemia/lymphoma-2 (Bcl-2) was analyzed using Pearson correlation. FoxM1 and Bcl-2 expression at mRNA and protein levels were determined by qRT-PCR and western blotting, respectively. The activity of the Bcl-2 promoter was examined using a luciferase reporter assay with FoxM1 targeting. Results: FoxM1 expression was increased in AML-*de novo* patients compared with that in healthy individuals and was downregulated in AML-CR patients compared with that in AML-*de novo* patients (all $P<0.05$). Silencing of FoxM1 decreased the proliferation of HL60 and U937 cells ($P<0.01$) and increased the apoptotic rate ($P<0.01$). Additionally, Bcl-2 mRNA level was significantly increased ($P<0.01$) in AML-*de novo* patients and showed a significant positive correlation with FoxM1 expression ($r=0.7458$, $P<0.001$). Luciferase activity of the Bcl-2 promotor was suppressed following FoxM1 binding ($P<0.01$). Conclusion: Silencing of FoxM1 inhibits proliferation and promotes apoptosis of AML cells. FoxM1 is involved in the development of AML by regulating Bcl-2 expression.

**Keywords:** Acute myeloid leukemia, forkhead box M1 (FoxM1), apoptosis, B-cell leukemia/lymphoma-2 (Bcl-2)

## Introduction

Acute myeloid leukemia (AML) is characterized by clonal expansion of undifferentiated myeloid precursors, resulting in impaired hematopoiesis and bone marrow failure [1]. Despite improvements in treatment protocols, a large number of AML cases is resistant to chemotherapy and has poor prognosis [2, 3]. The long-term overall survival (OS) rate for AML patients under the age of 60 years is 30-40%, whereas the OS rate for 60 years or older is under 10% [4]. Therefore, understanding the underlying mechanisms of AML is important to develop new therapies for AML.

FoxM1 (Forkhead box M1) is a typical proliferation-associated transcription factor that is also intimately involved in oncogenesis [5-7]. FoxM1 is a member of the FOX (Forkhead box) transcription factor family, which is characterized by a conserved DBD (DNA-binding domain) also called the forkhead domain or forkhead box [8]. Emerging evidence suggests that FoxM1 signaling is frequently upregulated in human malignancies [9]. However, few studies have reported the biological function of FoxM1 in AML [10]. The B-cell leukemia/lymphoma-2 (Bcl-2) family of proteins includes key regulators of cell death that can either suppress or promote apoptosis [11, 12]. Overexpression of Bcl-2 is frequently observed in several types of cancer, including breast, lung, and ovarian cancers, as well as acute myeloid leukemia, and is often associated with unfavorable outcome [13-15]. Whether FoxM1 regulates the biological effects of Bcl-2 in AML is worthy of study.

This study aims to explore the role of FoxM1 in the pathogenesis of AML and investigate its

underlying mechanism, to find a novel therapeutic target of AML.

## Materials and methods

### *Enrollment*

A total of 87 patients with AML (aged 19-73 years) were enrolled from July 2014 to December 2015 at The Jingzhou Central Hospital. These patients included 30 AML-*de novo* patients, 30 AML-complete remission (AML-CR) patients after treatment and 27 AML-refractory relapse (AML-RR) patients, alongside 30 healthy individuals. All patients signed the informed consent form, and the study protocol was approved by the Ethics Committee of the hospital. Each patient was re-evaluated and met the 2014 WHO diagnostic criteria for AML. Mononuclear cells were isolated from each patient following the standard Ficoll procedure and viably frozen. Patients with extramedullary disease and those who received allogeneic stem cell transplantation were excluded. Patients with acute promyelocytic leukemia (M3) were also excluded because of different treatment regimens.

### *Immunofluorescence*

The protein expression levels of FoxM1 and Bcl-2 in bone marrow aspirate smears from individuals with AML and negative controls were detected by immunofluorescence assay. Briefly, bone marrow aspirate smears were fixed in 1:1 acetone methanol at 4°C, and allowed to air dry for 20 minutes. Smears were then permeabilized in 0.1% Triton X-100 and blocked in 1% bovine serum albumin (BSA) for 30 minutes. After incubation, FoxM1 (1:150 dilution) and Bcl-2 (1:100 dilution) antibodies were added at 4°C for 12 hours. The smears were then washed 3 times with phosphate-buffered saline. The coverslips were incubated with a fluorescein isothyocyanate-conjugated secondary goat anti-rabbit immunoglobulin G (Fab2) antibody (1:40, Supertechs, Bethesda, MD) for 30 minutes at room temperature in the dark. After washing, slides were mounted using mounting media (ab104139, Abcam) that contained DAPI to outline the nuclear contours. The coverslips were analyzed and photographed using a conventional fluorescence microscope (Axioskop; Zeiss, Jena, Germany).

### *Cell culture and transfection*

The human AML cell lines HL60 and U937 were purchased from the Beinachuanglian Biotechnology Research Institute (Beijing, China). All cells were maintained at 37°C in a humidified environment containing 5% $CO_2$ in RPMI-1640 (Gibco Company, USA) with 10% fetal bovine serum (FBS, Gibco). The siRNA targeting FoxM1 was synthesized by GenePharma Co. Ltd. (Shanghai, China). Transfection of siRNAs was performed using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Cells were grown in plates at a density of $1 \times 10^5$ cells/volume, and then transfected with FoxM1 siRNA or negative control (NC) siRNA using Lipofectamine™ 2000 (500 μL Opti-MEM + 5 μL Lipofectamine™ 2000 + 5 μL FoxM1 or NC siRNA). The FoxM1 siRNA sequence was: 5'-GACAACUGUCAAGUGUACCACUCUU-3' and the NC siRNA sequence was: 5'-CCUACAUCCCGAUCGAUGAUGUUGA-3'. The siRNA-transfected cells were incubated for 48 hours and then harvested for experiments.

### *Cell proliferation and colony formation assays*

Cell proliferation was monitored using the Cell Proliferation Reagent Kit I (MTT) (Sigma). FoxM1 siRNA or NC siRNA transfected HL60 and U937 cells were grown in 96-well plates. Cell proliferation was documented every 24 hours following the manufacturer's protocol, and a cell growth curve was drawn. Additionally, cell proliferation was monitored via the colony formation assay. FoxM1 siRNA or NC siRNA transfected HL60 and U937 cells (100/well) were grown in 24-well plates and maintained in 10% FBS containing media, with the media being replaced every 4 days. After 10 days, cells were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet (Beyotime Biotechnology, Jiangsu, China). Visible colonies were manually counted. All experiments were performed in triplicate.

### *Quantitative real-time PCR*

Total RNA was isolated from bone marrow mononuclear cells and cultured cells with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. QPCR assays were performed to detect the expression levels of FoxM1 and Bcl-2 using the PrimeScript RT reagent Kit and SYBR Premix Ex

Taq (GeneCopoeia, USA) according to the manufacturer's instructions. All PCR reactions were assessed in triplicate. Amplification of the target genes was normalized using the amplification levels of GAPDH as the endogenous control. Relative mRNA expression levels were determined according to the $2^{-\Delta\Delta Ct}$ method. The primer pairs are listed (5'-3') as follows: GAPDH-F: AATCCCATCACCATCTTCCA; GAPDH-R: CCTGCTTCACCACCTTCTTG; FoxM1-F: TGCAGCTAGGATGTGAATCTTC; FoxM1-R: GGAGCCCAGTCCATCAGAACT; Bcl-F: GGTGAACTGGGGGAGGATTG; Bcl-R: GTGCCGGTTCAGGTACTCAG.

First-strand cDNA was synthesized followed by subjection to qRT-PCR on a Roche Lightcycler 480 Real Time PCR System (95°C for 1 minute and 40 cycles at 95°C for 15 seconds, 60°C for 60 seconds).

*Western blotting*

Total protein was extracted from HL60 and U937 cells with 1% RIPA Lysis Buffer (Beyotime, Jiangsu, China). Equal amounts of proteins were separated by SDS-PAGE and transferred onto PVDF membranes, followed by blocking with Tris buffer containing 0.1% Tween-20 and 5% nonfat milk for 30 minutes. Then, the membranes were probed with FoxM1 (1:1000, Abcam) or Bcl-2 (1:1000, Abcam) antibodies overnight at 4°C, followed by incubation with secondary antibodies (Pierce, 1:5000) for 1 hour at 24°C. Immunoreactive bands were detected using the ECL detection system and ImageJ software 6.0 was used to analyze the expression of target proteins in comparison to β-actin (1:5000, Abcam).

*Cell apoptosis*

Cells transfected with FoxM1 or NC siRNA were used to determine cell apoptosis. Apoptotic cells were counted using a commercially available Annexin V-APC/PI Apoptosis Detection Kit (Abnova, Shanghai, China), performed according to the manufacturer's recommended protocol. Cells were collected, washed, and dispersed in 500 μL binding buffer and then stained with 5 μL APC-conjugated Annexin V and 5 μL propidium iodide (PI) for 10 minutes. The tagged apoptotic cells were sorted using the FACSCalibur flow cytometer for 1 hour and analyzed using CellQuest software (Accuri C6; BD Biosciences).

*Plasmid construction and luciferase reporter assay*

To assess whether FoxM1 regulates the activity of Bcl-2, the sequence containing the Bcl-2 promoter was inserted into the pRL-TK plasmid (Promega, USA). For the luciferase reporter assay, HL60 and U937 cells were cultured in 6-well plates and co-transfected, using Lipofectamine 2000, with 50 nmol/L of FoxM1 or NC siRNA along with either the pRL-TK plasmid with Bcl-2 promoter or the empty pRL-TK plasmid. Luciferase activity was assessed in each sample 48 hours after transfection with a luciferase assay kit (Promega, USA). Each experiment was repeated in triplicate.

*Statistical analysis*

All statistical analyses were performed using SPSS version 21.0 software (SPSS Inc., Chicago, IL). GraphPad Prism 5.0 (GraphPad Software Inc., CA) was used for plotting graphs. Data are mean ± standard deviation (SD) from three independent experiments. Group comparisons were carried out by unpaired two-tailed Student t-tests. Pearson correlation analysis was used to evaluate the relationship between FoxM1 and Bcl-2 expression. Values of $P<0.05$ were considered statistically significant.

## Results

*FoxM1 expression is specifically upregulated in AML-de novo and AML-RR patients*

The expression level of FoxM1 in AML-*de novo*, AML-CR, and AML-RR patients, alongside 30 healthy individuals was examined by qRT-PCR. As shown in **Figure 1A**, FoxM1 expression was upregulated in AML-*de novo* patients compared with that in healthy individuals ($P<0.05$). However, when compared to that in AML-*de novo* patients, FoxM1 expression was downregulated ($P<0.05$). Moreover, FoxM1 expression was significantly higher in AML-RR patients compared with that in AML-CR patients ($P<0.05$). As shown in **Figure 1B**, cellular immunofluorescence analysis revealed that the protein level of FoxM1 was consistent with the mRNA expression level in AML patients and healthy individuals. These findings supported the idea that FoxM1 is involved in tumorigenesis, development, and response to chemother-

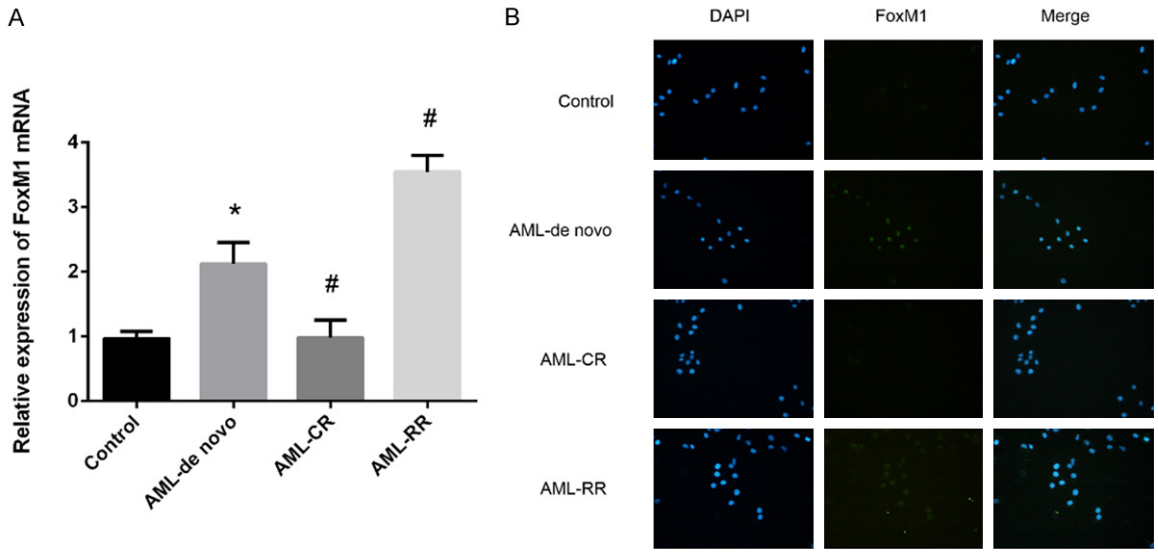


**Figure 1.** FoxM1 was upregulated in AML-*de novo* and AML-RR patients. A: The expression of FoxM1 mRNA in AML patients and healthy individuals (indicated as Control) detected by qRT-PCR. B: Cellular immunofluorescence analysis of FoxM1 protein in AML patients and healthy individuals (Control). Note: *P<0.05 *vs* Control, #P<0.05 *vs* AML-*de novo*.

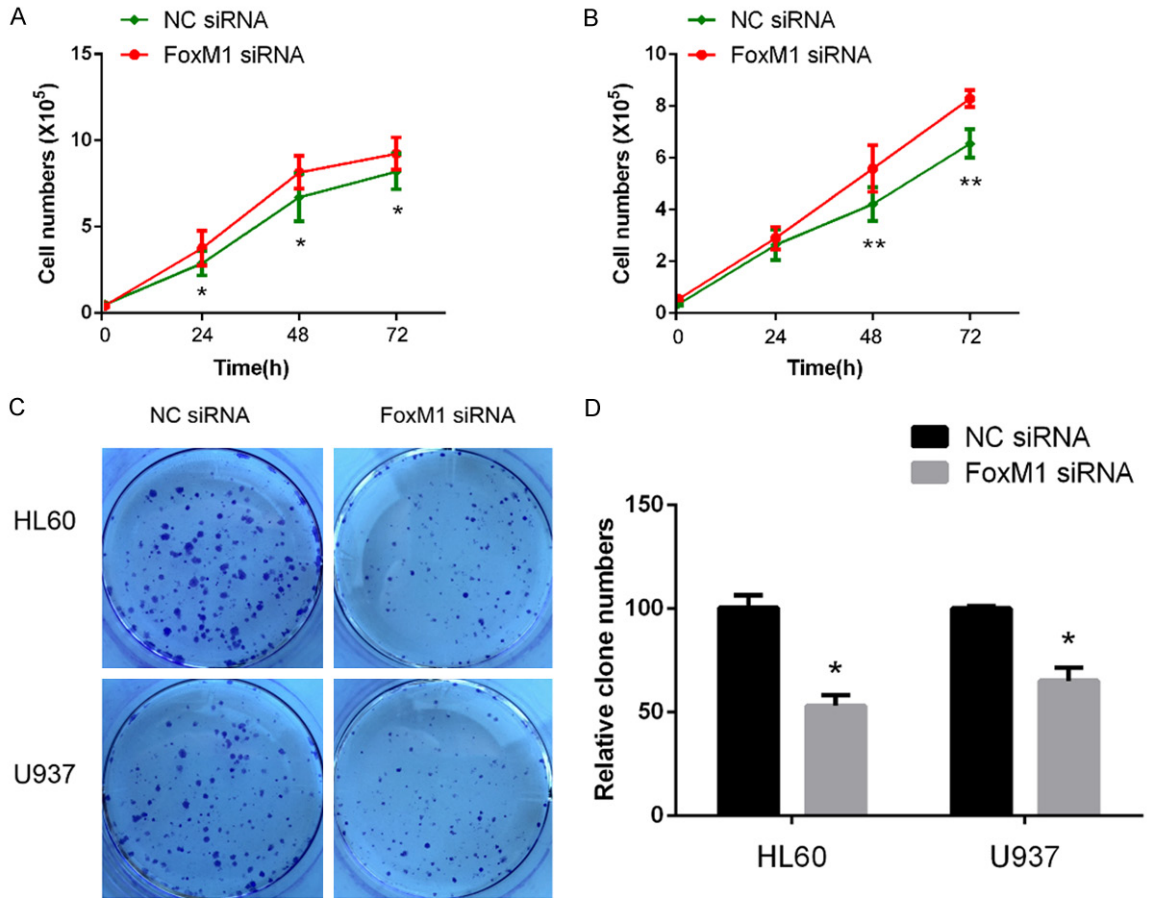


**Figure 2.** Silencing FoxM1 expression inhibits proliferation in AML cells. (A, B) Cell growth curves were drawn according to the MTT assay on HL60 (A) and U937 (B) cells transfected with FoxM1 or NC siRNA. (C, D) Effects of FoxM1 silencing on colony formation of HL60 cells and U937 cells transfected with FoxM1 or NC siRNA. Data represent mean ± SD. *P<0.05, **P<0.01.

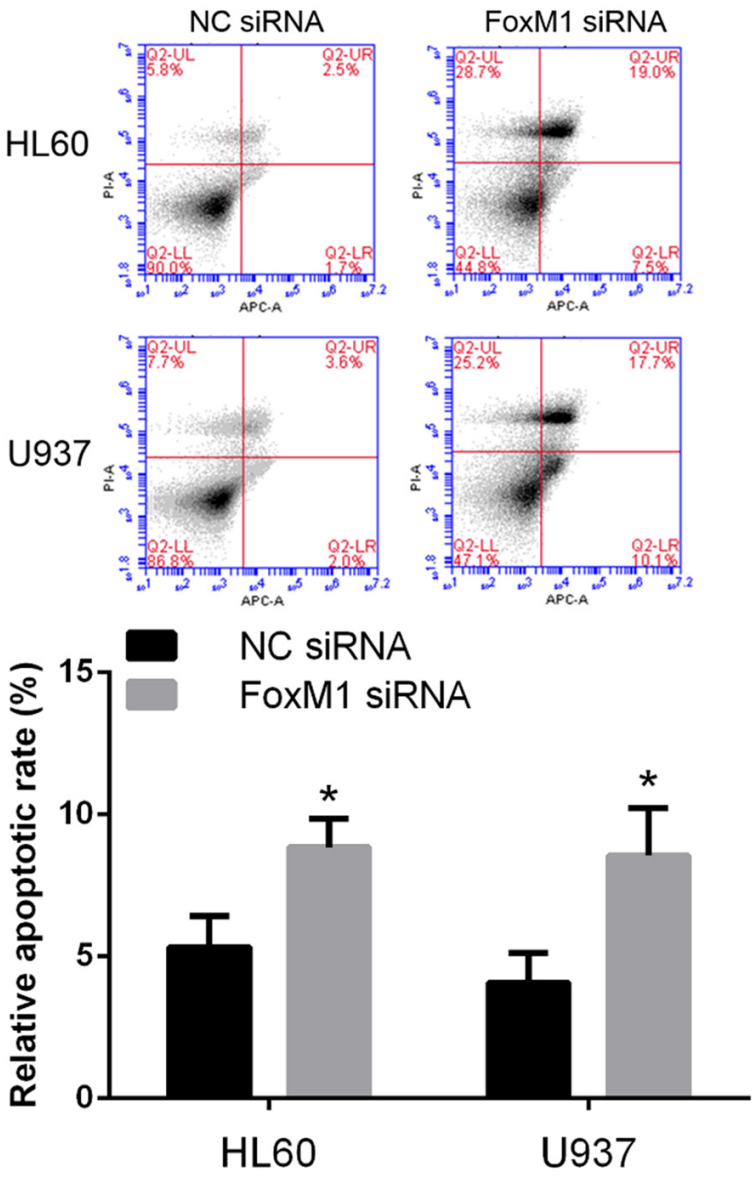


**Figure 3.** FoxM1 silencing promotes cell apoptosis in AML cells. Flow cytometry was performed with Annexin V-allophycocyanin (APC) and Propidium Iodide (PI) double staining to determine the apoptotic rate of HL60 and U937 cells transfected with FoxM1/NC siRNA. *P<0.05 vs NC siRNA.

apy, and may be a potential novel treatment target for AML. Consequently, we aimed to investigate the exact mechanism for FoxM1 in AML.

*Silencing FoxM1 expression inhibits cell proliferation and promotes cell apoptosis in AML*

To determine whether FoxM1 could regulate the proliferative ability of AML cell lines, we depleted FoxM1 or corresponding NC, using siRNAs. HL60 and U927 AML cell lines were cultured and then transfected with FoxM1 siRNA or NC siRNA. Cell growth curves suggested that the proliferative ability of HL60 and U937 cells transfected with FoxM1 siRNA was significantly stronger than cells transfected with NC siRNA (**Figure 2A**, **2B**, P<0.05). In addition, we performed a colony formation assay 72 hours after cell transfection. Images captured after 14 days indicated that cells transfected with FoxM1 siRNA showed fewer colonies than those with NC siRNA (**Figure 2C**). Data combined suggested that silencing FoxM1 expression in AML cell lines could inhibit cell proliferative ability. We also investigated the function of FoxM1 on cell apoptosis in AML using flow cytometry. As illustrated in **Figure 3**, remarkably, the apoptotic rates were increased in cells transfected with FoxM1 siRNA compared with those transfected with NC siRNA (P<0.05).

*FoxM1 directly targets Bcl-2 in AML*

Owing to our findings that FoxM1 has an important role in AML cell proliferation and apoptosis, we performed further studies to explore the underlying mechanism. Therefore, we investigated the expression of Bcl-2 in bone marrow specimens from AML-*de novo* patients and healthy individuals. Notably, expression of Bcl-2 mRNA, together with FoxM1 mRNA, was significantly higher in AML-*de novo* patients than in healthy individuals (**Figure 4A**). Results obtained from the Pearson correlation analysis indicated that FoxM1 mRNA expression was positively correlated with Bcl-2 levels (r=0.7458, $R^2$=0.6533, P<0.001) (**Figure 4B**). Furthermore, immunofluorescence indicated that the protein expression of Bcl-2 was higher in bone marrow

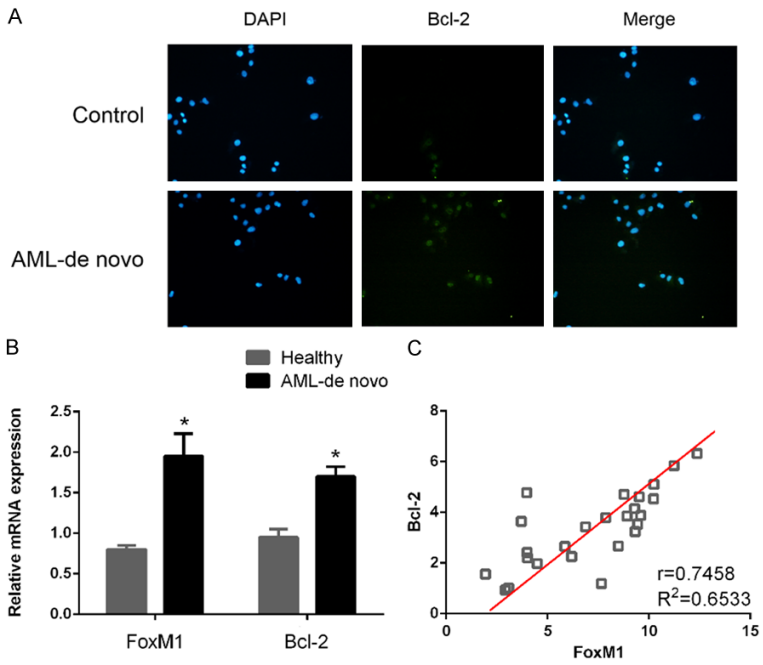


**Figure 4.** Positive correlation between FoxM1 and Bcl-2 expression in AML. A: The expression level of Bcl-2 protein was detected in healthy individuals (indicated as Control) and AML-*de novo* patients by cellular immunofluorescence analysis. B: The expression level of FoxM1 mRNA and Bcl-2 mRNA was determined by qRT-PCR. **P*<0.01 vs Control. C: Pearson correlation was used to analyze the relationship between FoxM1 and Bcl-2 mRNA.

specimens from AML-*de novo* patients than in healthy individuals (**Figure 4C**). We performed qPCR to provide an *in vitro* experiment to validate that the effective function of FoxM1 siRNA on AML cells was through Bcl-2. As shown in **Figure 5A**, the relative expression of FoxM1 mRNA was significantly decreased when AML cells were transfected with FoxM1 siRNA. Notably, in agreement with the decreased signature of FoxM1 mRNA, we found that Bcl-2 mRNA was also downregulated in FoxM1-siRNA group (**Figure 5B**). Moreover, the relative expression of FoxM1 and Bcl-2 proteins were lower with FoxM1 siRNA (**Figure 5C**, P<0.01), than with NC siRNA. Our data suggested that Bcl-2 might be the downstream target of FoxM1. To further verify our hypothesis, a luciferase reporter assay containing the Bcl-2 promoter sequence was performed on HL60 and U937 cells transfected with either FoxM1 or NC siRNA. As shown in **Figure 5D**, the luciferase activity of the Bcl-2 promoter was suppressed by FoxM1 siRNA, but not by NC siRNA (P<0.01). Taken together, these findings suggested that Bcl-2 might be a target of FoxM1 in AML.

## Discussion

Cytotoxic treatment is the main intervention strategy for AML. Although these approaches produce cell death within a tumor, they can also cause severe side effects in patients [4, 16]. Therefore, therapies targeting key molecules in the development and progression of AML has recently been a new development in the management procedures for AML [4, 17]. Until now, molecular targeted therapy of AML has made great progress. For instance, adding rituximab to the acute lymphocytic leukemia (ALL) chemotherapy protocol improved the outcome for CD20-positive younger adults [18]. Additionally, several small molecule FLT3-tyrosine kinase inhibitors (TKIs) have been developed and examined in AML patients as single agents or in combination with chemotherapy [19]. However, AML is a heterogeneous hematological malignancy and recent discoveries of new potent therapeutic molecules cannot reach the clinic. There is still a great challenge for identifying and directing the treatment towards cancer-specific pathways aiming to improve patient outcome.

Our study determined the expression level of FoxM1 in AML-*de novo*, AML-CR, and AML-RR patients, alongside healthy individuals, and found higher levels of FoxM1 expression in AML-*de novo* and AML-RR patients, whereas, FoxM1 expression was significantly downregulated in AML-CR patients. The above finding suggested that FoxM1 might be a key regulator in leukemogenesis and might be closely associated with the effect of drug treatment.

FoxM1 mediates aberrant proliferation of cancer cells mainly through the transcription regulation of cell cycle-related proteins, including Aurora B, Cyclin B1, and cyclin dependent kinase (CDK) 2 [20]. Nakamura *et al.* suggested that FoxM1 is significantly expressed in AML

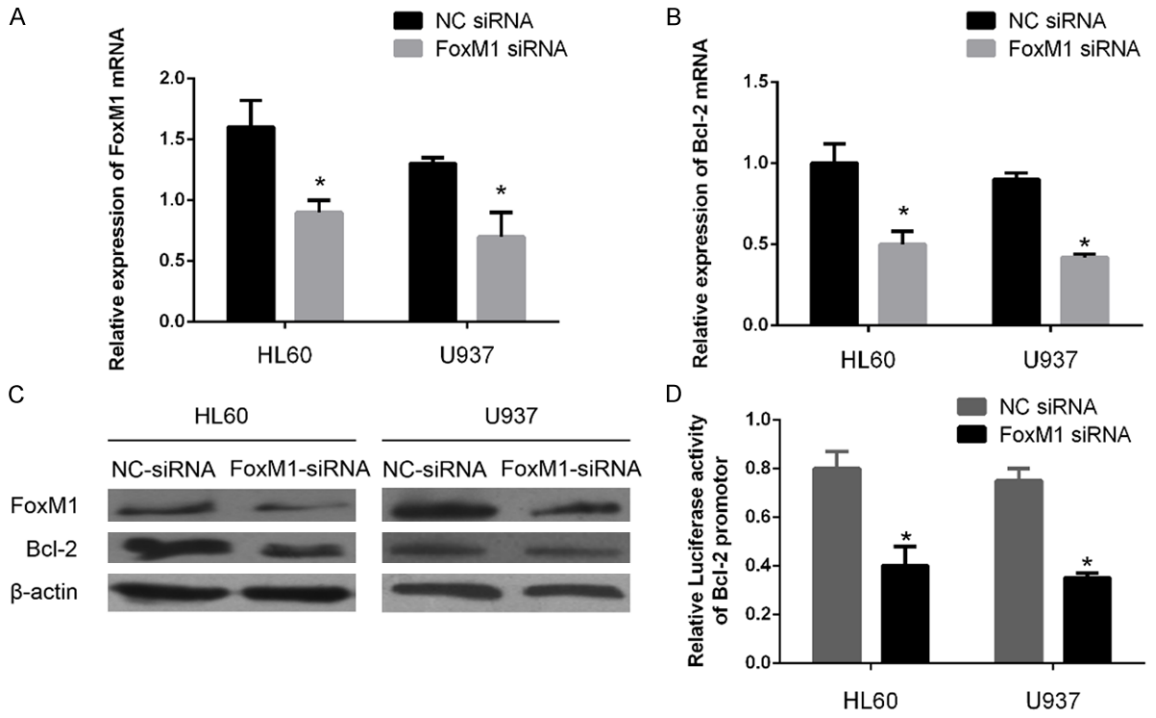


**Figure 5.** FoxM1 directly targeted Bcl-2 in AML cells. A and B: Relative expression of FoxM1 and Bcl-2 mRNA in HL60 cells and U937 cells transfected with FoxM1 or NC siRNA was detected by qRT-PCR. C: Western blot analysis of FoxM1 and Bcl-2 protein expression in the above cells. D: Luciferase reporter assay showed the luciferase activity of the Bcl-2 promotor with or without FoxM1 silencing. *$P$<0.01 vs NC siRNA.

cells and aberrant expression of FOXM1 induces AML cell proliferation through modulation of cell cycle progression [10]. Moreover, recent studies have indicated that FoxM1 could inhibit the activity of the c-Jun NH2-terminal kinase (JNK)/mitochondrial pathway, leading to its anti-apoptosis effect [21]. Zhang *et al.* found downregulation of FoxM1 by siRNA increased the susceptibility of AML cells to doxorubicin-induced apoptosis [22]. Additionally, inhibition of FoxM1 expression via RNA-interference or the proteasome inhibitor bortezomib has been shown to promote cell apoptosis induced by DNA damaging agents or oxidative stress [23]. Accordingly, we transfected FoxM1 specific siRNA into AML cell lines (HL60 and U937 cells) to suppress FoxM1 expression, and revealed that FoxM1 has a suppressive function on cell proliferation but increases cell apoptosis.

Bcl-2 suppresses apoptosis by binding to Bax or Bak, and inhibits autophagy by binding to the protein Beclin 1 [24]. Overexpression of the anti-apoptotic Bcl-2 proteins correlates with overall lower OS rates for AML patients [25]. Previous studies revealed that Bcl-2 was upregulated in AML cells and correlated with cell apoptosis and resistance to chemotherapy [13]. Zhang *et al.* co-suppressed MEK and mTOR signaling in conjunction with interference of anti-apoptotic Bcl-2 family members and found predominant induction of apoptosis in AML cells [26]. Owing to the important roles for FoxM1 and Bcl-2 on AML cells, we determined Bcl-2 expression in AML-*de novo* patients and found that it expressed at higher levels than in healthy individuals. Notably, the expression of Bcl-2 was significantly decreased when we suppressed the activity of FoxM1 by siRNA in AML cells. Furthermore, we showed that FoxM1 could affect the activity of Bcl-2 promoter, by a dual luciferase activity assay. This data supported the notion that FoxM1 in concert with Bcl-2 is involved in the development of AML, and that Bcl-2 may be a downstream target of FoxM1.

Our study has limitations in that we did not obverse the response of AML cells in proliferation and apoptosis with Bcl-2 depletion followed by incubation with FoxM1 siRNA. In addition, we performed *in vitro* experiments, but did

not verify our findings *in vivo*. Thus, it still needs more work to validate the biological function of FoxM1 in AML patients.

## Conclusion

Overall, this study preliminarily demonstrated that FoxM1 is aberrantly expressed in AML cells and involved in leukemogenesis. FoxM1 silencing could suppress the expression of Bcl-2, inhibit proliferation, and promote apoptosis of AML cells. Our data provides evidence that FoxM1 targeting agents may be an effective strategy for the treatment of AML.

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## Disclosure of conflict of interest

None.

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