European Review for Medical and Pharmacological Sciences 2023; 27: 9145-9151

The effect of small molecule inhibitor NSC348884 on nucleophosmin 1-mutated acute myeloid leukemia cells

J.-F. ZHU, L.-L. HAN, Y. MA, Y.-X. WANG, F. ZHANG

Department of Hematology, The First Affiliated Hospital of Bengbu Medical College, Bengbu, Anhui Province, China

Abstract. – OBJECTIVE: Nucleophosmin 1 *(NPM1)* is a common shuttling protein. Mutation in the *NPM1* gene is the most frequent gene alteration in acute myeloid leukemia (AML). This study aims to explore the inhibitory effects of small molecule NSC348884 on wild-type and *NPM1*-mutated AML cells.

MATERIALS AND METHODS: Immunofluorescence was used to determine the intracellular localization of *NPM1* protein in wild-type (OCI-AML2) and *NPM1*-mutated (OCI-AML3) AML cell lines. The oligomerization state of *NPM1* was assessed by Western blot analysis, and the inhibitory effect of NSC348884 on the proliferation of AML cells was evaluated by Cell-counting kit-8 (CCK-8). Flow cytometry was used to detect the proapoptotic effect of NSC348884 on AML cells.

RESULTS: Western blot results showed a significant reduction in the levels of the oligomeric *NPM1* protein after the treatment with NSC348884. NSC348884 had an inhibitory effect on the proliferation of both wild-type and *NPM1*-mutant AML cells. The inhibitory effect on OCI-AML3 cells was stronger, compared to OCI-AML2 cells. Flow cytometry showed that NSC348884 could significantly induce AML cell apoptosis and had a stronger proapoptotic effect on OCI-AML3 cells.

CONCLUSIONS: NSC348884 had inhibitory and proapoptotic effects on both wild-type and *NPM1*-mutated AML cells. The effect of NSC348884 on AML cells, carrying *NPM1* mutation was significantly stronger.

Key Words:
NSC348884, NPM1, AML, Proliferation, Apoptosis.

Introduction

Nucleophosmin 1 (*NPM1*) is a widely expressed nuclear phosphoprotein that is mainly located in the nucleolus and shuttles between the nucleoplasm and the cytoplasm[1]. Its main functions include ribosome generation, mRNA processing, chromatin remodeling, apoptosis regulation and DNA repair[1,2]. Recent studies[3,4] found that the expression of *NPM1* is increased in solid tumors such as liver, ovarian, and colon cancers, and overexpression of *NPM1* is closely related to tumor proliferation, invasion, and patient prognosis. *NPM1* is also the most common mutated gene in acute myeloid leukemia (AML). Approximately 35% of AML patients have frameshift mutations in the 12th exon of the *NPM1* gene[4].

The mutation changes the nucleolar localization signal (NoLS) of *NPM1* into the nuclear export signal (NES), resulting in the cytoplasmic localization of the *NPM1*-mutated protein NPMc+[2-4]. In 2016, WHO[5] updated the identification of unique biomarkers associated with some myeloid neoplasms and acute leukemias and classified myeloid tumors as a subtype with reproducible genetic abnormalities. While *NPM1* mutation is an important cause of AML, there is still a lack of drugs specifically targeting *NPM1*[4,5]. Small molecule inhibitor NSC348884 can act on *NPM1*, affecting its function by impacting the oligomerization state of the protein[3,5]. Multiple studies[5-7] have shown that NSC348884 has anti-tumor effects. The aim of this study is to compare the inhibitory effects of NSC348884 on wild-type and *NPM1*-mutated AML cells, providing some reference for developing more effective clinical treatment of leukemia.

Materials and Methods

Wild-type AML cell line OCI-AML2 and *NPM1*-mutated AML cell line OCI-AML3 were purchased from Triton Company (Purchase, NY, USA). NSC348884 was purchased from MCE Company (NJ, USA). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), *NPM1*, B-cell lymphoma

Corresponding Author: Feng Zhang, MD; e-mail: 17705520517@163.com

protein 2 (*Bcl-2*) and *Bcl-2*-associated X (*Bax*) antibodies were purchased from Proteintech Group Inc. (Rosemont, IL, USA). RPMI-1640 culture medium, and penicillin/streptomycin solution were purchased from Invitrogen Company (Waltham, MA, USA). Fetal bovine serum (FBS) was purchased from Sichuan Sijiqing Company (Sichuan, China). The CCK-8 proliferation kit was purchased from Vazyme Company (Nanjing, China), and the Annexin V-FITC/PI cell apoptosis detection kit was purchased from ServiceBio Company (Wuhan, China). The study was approved by the Ethics Committee of the First Affiliated Hospital of Bengbu Medical College with No. BY2022026, date: 2022-March-05th.

Cell Culture

OCI-AM2 and OCI-AML3 cells were cultured in RPMI-1640 complete medium containing 15% FBS, 1% penicillin and streptomycin, and incubated at 37°C, 5% CO_2 with saturated humidity.

Immunofluorescence

OCI-AML2 and OCI-AML3 cells were centrifuged at 800 rpm for five minutes, and the pellets were washed with phosphate-buffered saline (PBS). Centrifugation and washing with PBS were repeated twice. One hundred microliters of cell suspension were put onto a glass slide, fixed with 4% paraformaldehyde for 30 minutes, permeabilized with 0.5% Triton x-100 for 15 minutes, and blocked with 5% BSA for one hour. Cells were incubated with the *NPM1* primary antibody overnight at 4°C, followed by one hour incubation with the fluorescent secondary antibody (in the dark). Nuclear DNA was labeled by 4',6-diamidino-2-phenylindole (DAPI) staining for 15 minutes and confocal fluorescence microscopy was carried out using FV-1000 (Olympus, Tokyo, Japan).

Cell Apoptosis Detection

OCI-AML2 and OCI-AML3 cells were cultured on a six-well plate and treated with different concentrations (0 μM, 1 μM, 2 μM, 4 μM) of NSC348884 for 24 hours. Cells were then transferred into a 5 ml centrifuge tube, pelleted by centrifugation and washed twice with ice-cold PBS. Five microliters of Annexin V-FITC and Propidium Iodide (PI) staining solutions were added to each well while protecting from light, and cells were incubated at room temperature for 10 minutes in the dark. After incubation, 400 microliters of binding buffer were added, and cells were resuspended into a single-cell suspension. Apoptosis was detected by flow cytometry using FACSCanto™ II (BD Biosciences, Jose, CA, USA).

CCK-8 Cell Proliferation

OCI-AML2 and OCI-AML3 cells were cultured on the 96-well plate, 12,000 cells per well. Cells were incubated with 2 μM of NSC348884 for 12, 24, 36, and 48 hours, or with different concentrations (0 μM, 1 μM, 3 μM, 6 μM, 10 μM) of NSC348884 for 12 hours to test the dose-dependent effect, and 10 microliters of CCK-8 reagent were then added to each well, for 2 hours. The absorbance of CKK-8 at 450 nm was measured using a microplate reader Varioskan LUX (Thermo Scientific™, Waltham, MA, USA). All experiments were performed in triplicates.

Western Blot

After 12 hours of treatment with 2 μM NSC348884, cells were collected by centrifugation, washed twice with PBS, lysed using RIPA buffer [50 mMTris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 0.1% SDS, Beyotime Institute of Biotechnology, Jiangsu, China], and the protein concentration was measured by bicinchoninic acid assay method. Protein samples (50 μg) were separated by polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene fluoride (PVDF) membrane, and blocked with 5% skim milk for one hour. Membranes were then incubated with the appropriate primary antibody overnight at 4°C, followed by the incubation with the secondary antibody labeled with horseradish peroxidase (Proteintech, Rosemont, IL, USA) at room temperature for two hours. Proteins were visualized using ECL chemiluminescence kit (Thermo Scientific, Waltham, MA, USA). *GAPDH* was used as a loading control.

Statistical Analysis

Categorical variables were compared *via* Fisher's exact test or Chi-squared tests. Continuous variables are expressed as means ± SD (standard deviation) and were compared *via* Student's *t*-tests or Mann-Whitney's U tests. $p<0.05$ was the significance threshold. SPSS v26.0 (IBM Corp., Armonk, NY, USA) was used for all statistical testing.

Results

Localization of NPM1 Protein in AML Cells and the Effect of NSC348884 on NPM1 Oligomerization

Localization of the *NPM1* in OCI-AML2 (expressing unmutated *NPM1* protein) and OCI-AML3 (carrying an *NPM1* mutation) cells was first detected by immunofluorescence. As shown in Figure 1,

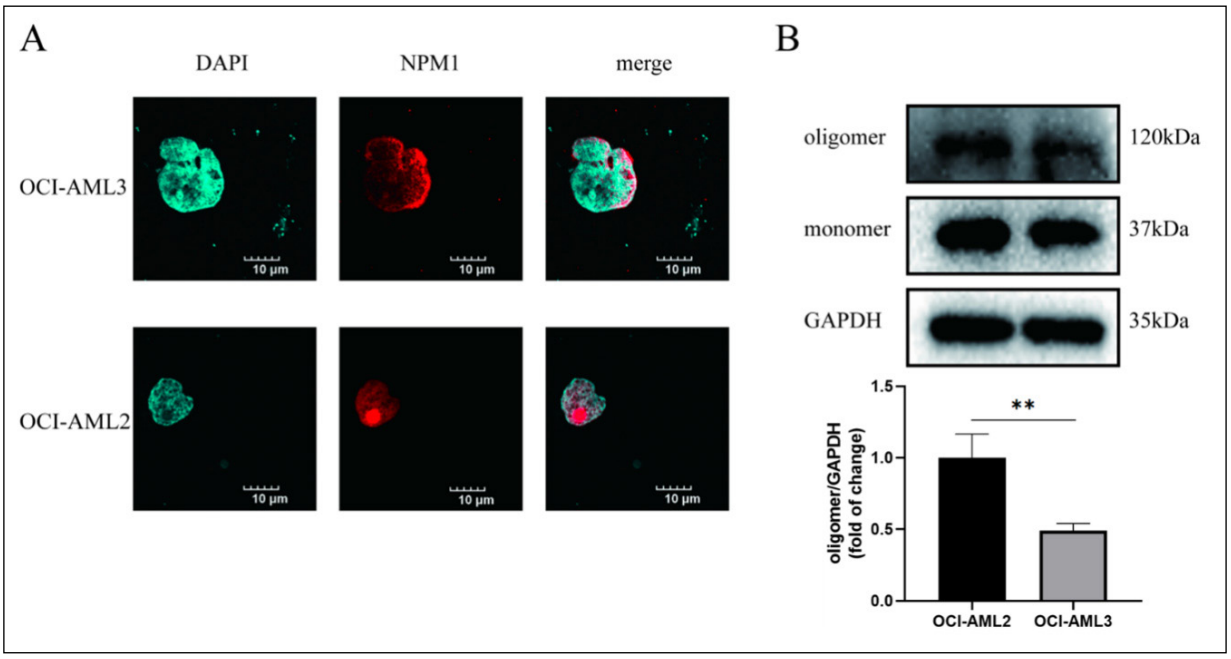

Figure 1. Localization of NPM1 protein in OCI-AML2 and OCI-AML3 cell lines and the effect of NSC348884 on NPM1 oligomerization. **A**, The localization of NPM1 protein (red) in AML cell lines was detected by laser confocal microscopy. Cellular DNA was stained with DAPI (green), **B**, Levels of NPM-1 oligomer in OCI-AML2 and OCI-AML3 cells, treated with 2 μM NSC348884 for 12 hours were detected by Western blotting (**p<0.01).

in OCI-AML2 cells, *NPM1* was mainly localized in the nucleus and nucleolus. In contrast, the localization of *NPM1* protein in OCI-AML3 cells changed from nuclear to cytoplasmic (Figure 1A).

To investigate whether the inhibitory effect of NSC348884 on AML cells is exerted by affecting *NPM1* oligomerization, cells were incubated with 2 μM of NSC348884 for 12 hours. Western blot analysis showed that after 12 hours of NSC348884 treatment, levels of oligomeric *NPM1* protein in OCI-AML3 cells were significantly reduced, as shown in Figure 1B.

NSC348884 Inhibits the Proliferation of NPM1-Mutated AML Cells

We next investigated whether NSC348884 inhibits AML proliferation by affecting *NPM1* oligomerization. To determine the dose-dependent effect, OCI-AML2 and OCI-AML3 cell lines were incubated with varying concentrations (0 μM, 1 μM, 3 μM, 6 μM, 10 μM) of NSC348884 for 12 hours (Figure 2A). Alternatively, to test the time-dependent effect, cells were incubated with 2 μM NSC348884 for 12, 24, 36, and 48 h (Figure 2B). Cell proliferation was measured by CCK-8. NSC348884 had a significant inhibitory effect on both OCI-AML2 and OCI-AML3 cells in a concentration- (Figure 2A) and time-dependent (Figure 2B) manner. At the concentration of 2 μM, NSC348884 had a significantly stronger inhibitory effect on OCI-AML3 cells compared to OCI-AML2 cells (Figure 2) (p<0.05).

NSC348884 Induces Apoptosis in AML Cells

To investigate the proapoptotic effect of NSC348884 on AML cells, cells were treated with 2 μM NSC348884 for 12 hours, and the expression of apoptosis-related proteins *Bax* and *Bcl-2* in OCI-AML2 and OCI-AML3 cells was detected by immunoblotting. The results showed that the expression level of *Bax* in OCI-AML3 cells was significantly higher (Figure 3A), and the expression level of *Bcl-2* was significantly lower than that of OCI-AML2 cells (Figure 3B) (p<0.05). After NSC348884 treatment, the expression of anti-apoptotic protein *Bcl-2* in OCI-AML3 cells was significantly reduced (Figure 3B) (p<0.05). Flow cytometry analysis showed that NSC348884 had a proapoptotic effect on both OCI-AML2 and OCI-AML3 cells. As the concentration increased, cell survival decreased, and the proapoptotic effect was enhanced. The proapoptotic effect of NSC348884 on the OCI-AML3 cell line was significantly higher compared to OCI-AML3 cells (Figure 3D and E) (p<0.05).

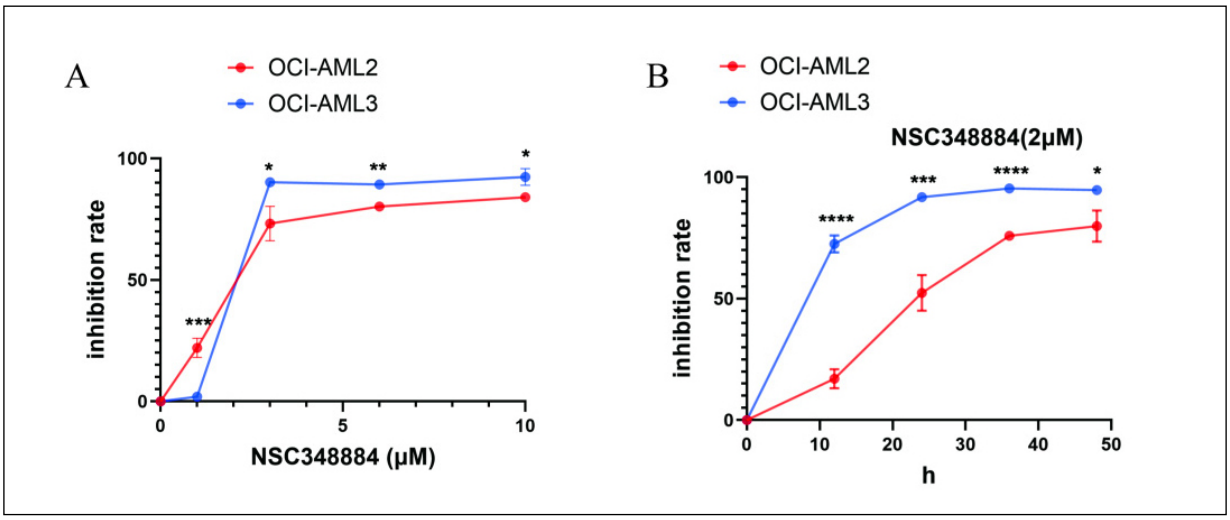

Figure 2. The proliferative inhibitory effect of NSC348884 on OCI-AML2 and OCI-AML3 cell lines was detected by CCK-8. **A**, Different concentrations of NSC348884 inhibit the proliferation of AML cells; (**B**) The inhibitory effect of 2 μM NSC348884 on AML cell proliferation at different time points (all data are shown as mean ± SD. Of three replicates: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

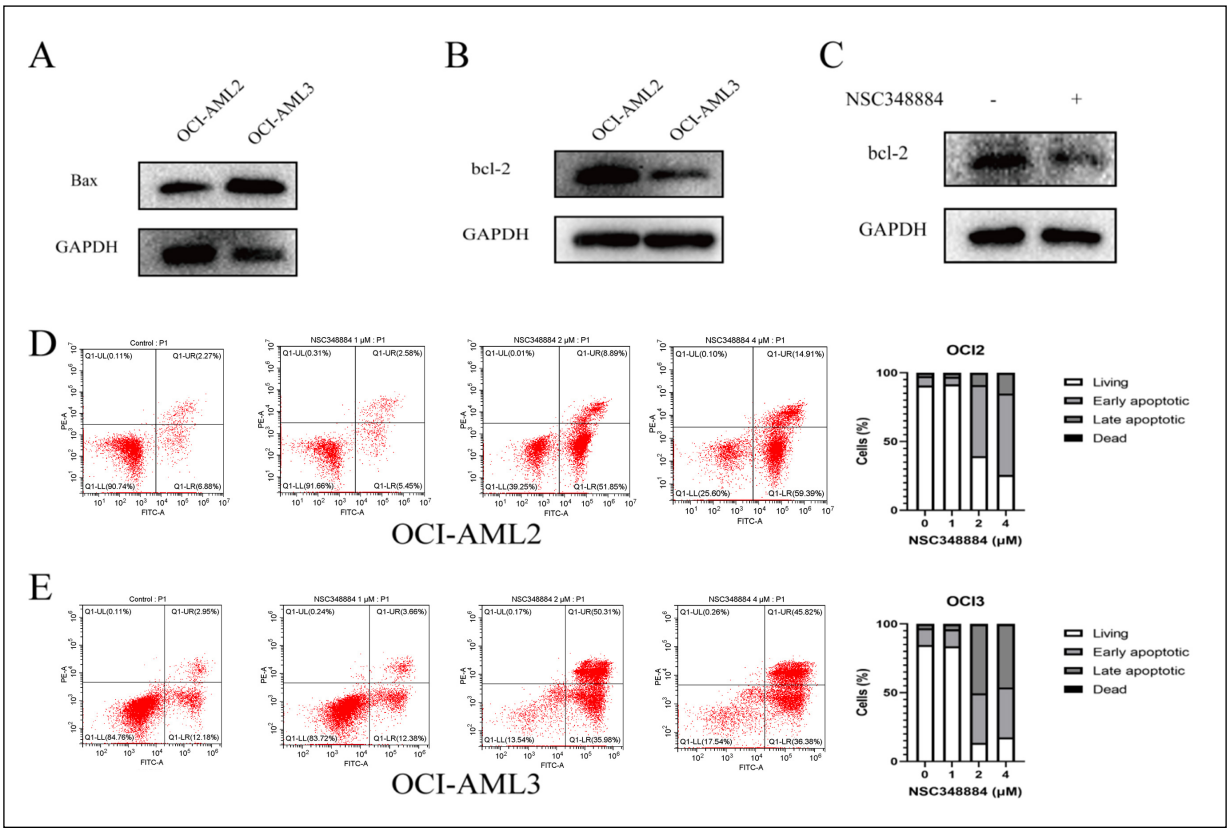

Figure 3. The proapoptotic effect of NSC348884 on AML cell lines. **A**, Western blot analysis of the expression of *Bax* protein in AML cells; (**B**) Western blot analysis of the expression of *Bcl-2* protein in AML cells; (**C**) Western blot analysis of the expression of *Bcl-2* protein in cells, treated with 2 μM of NSC348884; (**D**) Flow cytometry was used to detect the apoptotic effect of NSC348884 on OCI-AML2 cells; (**E**) Flow cytometry was used to detect the apoptotic effect of NSC348884 on OCI-AML3 cells (statistically significant differences was set as p<0.05, *p<0.05, **p<0.01, ***p<0.001).

Discussion

This study compared the inhibitory effects of NSC348884 in AML cell lines with and without the mutation in *NPM1* and found that NSC348884 can inhibit the proliferation activity of AML cells in a time- and concentration-dependent manner. NSC348884 had a proapoptotic effect on both OCI-AML2 and OCI-AML3 cells, but this effect was stronger in cells carrying the *NPM1* mutation.

Mutations in the *NPM1* gene that result in the expression of the NPMc+ protein are unique to hematological tumors and occur in approximately one-third of acute myeloid leukemia cases[7]. Recent studies[7,8] suggest that the interaction between the mutated *NPM1* protein (NPMc+) and tumor suppressor protein alternative reading frame leads to abnormal localization of NPMc+ in the cytoplasm and its subsequent degradation. In agreement with this observation, our results demonstrated that NPMc+ localized in the cytoplasm of the AML cells, as opposed to no clearly localized wild-type NPM-1 protein. The interaction between NPMc+ and the deubiquitinating enzyme herpesvirus-associated ubiquitin-specific protease (HAUSP) of the tumor suppressor Phosphatase and TENsin homolog (PTEN) increases the degradation of PTEN[8]. NPMc+ also interacts with the E3 ubiquitin ligase Fbwγ of c-Myc and brings it to the cytoplasm to increase its degradation, thereby upregulating c-Myc protein expression[9]. In addition, the expression of the *HOX* gene, controlling the proliferation and differentiation of hematopoietic stem/progenitor cells, is upregulated in the *NPM1*-mutated AML cells[10]. This leads to impaired differentiation and maturation of hematopoietic cells, which may be an important cause of leukemia[10,11]. *NPM1* mutation is often accompanied by multiple additional mutations in various genes, such as *FLT3*, *DNMT3A*, *IDH1*, *IDH2*, *RUNX1*, etc[12]. While patients with *NPM1*-mutated AML have a good prognosis, the presence of additional gene mutations is associated with a poor prognosis[11,12].

Numerous studies[13-15] show that *NPM1* mutation provides an important basis for predicting the prognosis and hierarchical treatment of AML. Although *NPM1*-mutated AML responds well to standard chemotherapy, patients still face a risk of relapse and drug resistance after discontinuation of the treatment. So far, there is still a lack of drugs that specifically target *NPM1* and its exact role in the pathogenesis of AML has not been fully elucidated. Previous research[13,16] established that impaired oligomerization of NPMc+ affects the occurrence and development of AML. Since oligomerization is essential for *NPM1* function, NPMc+ oligomers are important targets for the treatment of *NPM1* mutant AML[17,18]. Studies[13-15] have found that NSC348884 can inhibit the formation of oligomers in *NPM1* by disrupting the N-terminal hydrophobic pocket required for oligomerization, and can inhibit the proliferation of various tumors and induce their apoptosis. However, the mechanism of action of NSC348884 on AML cells is still poorly understood. Our results showed that NSC348884 significantly reduced levels of NPMc+ oligomers in AML cells, carrying the *NPM-1* mutation, compared to AML cell lines expressing wild-type *NPM-1*. Studies[11,16] also show that NSC348884 can downregulate antioxidant Peroxiredoxin 6 (PRDX6) and increase the levels of reactive oxygen species in the body *via NPM1*. *NPM1* is the most significantly up-regulated gene in the atypical teratoma/rhabdoid tumor (AT/RT) tissues[16]. NSC348884 treatment was able to reduce the viability of the AT/RT cell line by blocking its cell cycle in the G1 phase[17]. NSC348884 can induce apoptosis in colorectal cancer cells by inhibiting *NPM1*, upregulating p53 protein, and inhibiting the AKT pathway, thereby enhancing the sensitivity of colorectal cancer cells with high expression levels of *NPM1* to chemotherapy[18]. Our results demonstrated that NSC348884 treatment markedly inhibited cell proliferation and induced apoptosis of AML cells. Mutation in *NPM-1* was associated with a significantly lower proliferation rate and higher extent of cell death compared to cells with the wild-type *NPM-1*.

Conclusions

In conclusion, our results showed that NSC348884 exerts inhibitory and proapoptotic effects on AML cells *in vitro*. However, the effect of NSC348884 on AML cells, carrying *NPM1* mutation is significantly stronger. Our results may provide a basis for identifying strategies to specifically target nucleophosmin and to develop new therapeutic approaches to treating AML.

Funding

This study is supported by the Key Project of Natural Science, Bengbu Medical College (2021byzd065).

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' Contributions
JZ conceived and designed the study. LH, YM and YW collected the data and performed the analysis. JZ was involved in the writing of the manuscript. FZ Edited the manuscript. All authors have read and approved the final manuscript.

Ethics Approval
The study was approved by the Ethics Committee of the First Affiliated Hospital of Bengbu Medical College with No. BY2022026, date: 2022-March-05th.

Informed Consent
Not applicable.

Conflict of Interest
The authors declare that they have no competing interests.

ORCID ID
Junfeng Zhu: 0000-0002-6739-6762
Lili Han: 0000-0002-6166-1502
Yue Ma: 0009-0009-7454-0871
Yuxian Wang: 0009-0002-2155-7804
Feng Zhang: 0009-0006-1057-6398

References

1) Döhner H, Wei AH, Roboz GJ, Montesinos P, Thol FR, Ravandi F, Dombret H, Porkka K, Sandhu I, Skikne B, See WL, Ugidos M, Risueño A, Chan ET, Thakurta A, Beach CL, Lopes de Menezes D. Prognostic impact of NPM1 and FLT3 mutations in patients with AML in first remission treated with oral azacitidine. Blood 2022; 140: 1674-1685.
2) Heath EM, Chan SM, Minden MD, Murphy T, Shlush LI, Schimmer AD. Biological and clinical consequences of NPM1 mutations in AML. Leukemia 2017; 31: 798-807.
3) Xu HS, Qin XL, Zong HL, He XG, Cao L. Cancer stem cell markers in glioblastoma - an update. Eur Rev Med Pharmacol Sci 2017; 21: 3207-3211.
4) Handschuh L, Kaźmierczak M, Milewski MC, Góralski M, Łuczak M, Wojtaszewska M, Uszczyńska-Ratajczak B, Lewandowski K, Komarnicki M, Figlerowicz M. Gene expression profiling of acute myeloid leukemia samples from adult patients with AML-M1 and -M2 through boutique microarrays, real-time PCR and droplet digital PCR. Int J Oncol 2018; 52: 656-678.
5) Arber DA, Orazi A, Hasserjian R, Thiele J, Borowitz MJ, Le Beau MM, Bloomfield CD, Cazzola M, Vardiman JW. The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. Blood 2016; 127: 2391-2405.
6) Damiano S, Sasso A, Accetta R, Monda M, De Luca B, Pavone LM, Belfiore A, Santillo M, Mondola P. Effect of Mutated Cu, Zn Superoxide Dismutase (SOD1G93A) on Modulation of Transductional Pathway Mediated by M1 Muscarinic Receptor in SK-N-BE and NSC-34 Cells. Front Physiol 2018; 9: 611.
7) Colombo E, Martinelli P, Zamponi R, Shing DC, Bonetti P, Luzi L, Volorio S, Bernard L, Pruneri G, Alcalay M, Pelicci PG. Delocalization and destabilization of the Arf tumor suppressor by the leukemia-associated NPM mutant. Cancer Res 2006; 66: 3044-3050.
8) Zhang XD, Sun GX, Guo JJ, Hu CC, Sun RC, Yu HC. Effects of PPARγ agonist pioglitazone on cardiac fibrosis in diabetic mice by regulating PTEN/AKT/FAK pathway. Eur Rev Med Pharmacol Sci 2021; 25: 812-819.
9) Sun XX, He X, Yin L, Komada M, Sears RC, Dai MS. The nucleolar ubiquitin-specific protease USP36 deubiquitinates and stabilizes c-Myc. Proc Natl Acad Sci U S A 2015; 112: 3734-3739.
10) Chevalier RL. Evolution, kidney development, and chronic kidney disease. Semin Cell Dev Biol 2019; 91: 119-131.
11) Neijts R, Deschamps J. At the base of colinear Hox gene expression: cis-features and trans-factors orchestrating the initial phase of Hox cluster activation. Dev Biol 2017; 428: 293-299.
12) Cancer Genome Atlas Research Network; Ley TJ, Miller C, Ding L, Raphael BJ, Mungall AJ, Robertson A, Hoadley K, Triche TJ Jr, Laird PW, Baty JD, Fulton LL, Fulton R, Heath SE, Kalicki-Veizer J, Kandoth C, Klco JM, Koboldt DC, Kanchi KL, Kulkarni S, Lamprecht TL, Larson DE, Lin L, Lu C, McLellan MD, McMichael JF, Payton J, Schmidt H, Spencer DH, Tomasson MH, Wallis JW, Wartman LD, Watson MA, Welch J, Wendl MC, Ally A, Balasundaram M, Birol I, Butterfield Y, Chiu R, Chu A, Chuah E, Chun HJ, Corbett R, Dhalla N, Guin R, He A, Hirst C, Hirst M, Holt RA, Jones S, Karsan A, Lee D, Li HI, Marra MA, Mayo M, Moore RA, Mungall K, Parker J, Pleasance E, Plettner P, Schein J, Stoll D, Swanson L, Tam A, Thiessen N, Varhol R, Wye N, Zhao Y, Gabriel S, Getz G, Sougnez C, Zou L, Leiserson MD, Vandin F, Wu HT, Applebaum F, Baylin SB, Akbani R, Broom BM, Chen K, Motter TC, Nguyen K, Weinstein JN, Zhang N, Ferguson ML, Adams C, Black A, Bowen J, Gastier-Foster J, Grossman T, Lichtenberg T, Wise L, Davidsen T, Demchok JA, Shaw KR, Sheth M, Sofia HJ, Yang L, Downing JR, Eley G. Genomic and epigenomic landscapes of adult de novo acute myeloid leukemia. N Engl J Med 2013; 368: 2059-2074.
13) Roboz GJ, Ravandi F, Wei AH, Dombret H, Thol F, Voso MT, Schuh AC, Porkka K, La Torre I, Skikne B, Zhong J, Beach CL, Risueño A, Menezes DL, Ossenkoppele G, Döhner H. Oral azacitidine prolongs survival of patients with AML in remission independently of measurable

residual disease status. Blood 2022; 139: 2145-2155.

14) Kumar D, Mehta A, Panigrahi MK, Nath S, Saikia KK. DNMT3A (R882) mutation features and prognostic effect in acute myeloid leukemia in Coexistent with NPM1 and FLT3 mutations. Hematol Oncol Stem Cell Ther 2018; 11: 82-89.

15) Qi W, Shakalya K, Stejskal A, Goldman A, Beeck S, Cooke L, Mahadevan D. NSC348884, a nucleophosmin inhibitor disrupts oligomer formation and induces apoptosis in human cancer cells. Oncogene 2008; 27: 4210-4220.

16) Šašinková M, Heřman P, Holoubek A, Strachotová D, Otevřelová P, Grebeňová D, Kuželová K, Brodská B. NSC348884 cytotoxicity is not mediated by inhibition of nucleophosmin oligomerization. Sci Rep 2021; 11: 1084.

17) Phi JH, Sun CH, Lee SH, Lee S, Park I, Choi SA, Park SH, Lee JY, Wang KC, Kim SK, Yun H, Park CK. NPM1 as a potential therapeutic target for atypical teratoid/rhabdoid tumors. BMC Cancer 2019; 19: 848.

18) Yu ACY, Chern YJ, Zhang P, Pasiliao CC, Rahman M, Chang G, Ren J, Tai IT. Inhibition of nucleophosmin 1 suppresses colorectal cancer tumor growth of patient-derived xenografts via activation of p53 and inhibition of AKT. Cancer Biol Ther 2021; 22: 112-123.