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Research article

Naringenin induces the cell apoptosis of acute myeloid leukemia cells by regulating the lncRNA XIST/miR-34a/HDAC1 signaling

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ABSTRACT

Acute myeloid leukemia (AML) is a life-threatening aggressive malignancy of the bone marrow and has posed a great challenge to the clinic, due to a lack of fully understanding of the molecular mechanism. Histone deacetylase 1 (HDAC1) has been reported to be a therapeutic target for treating AML. Naringenin (Nar) may act as an anti-leukemic agent and suppress the expression of HDACs. However, the potential underlying mechanism of Nar in suppressing the activity of HDAC1 remains unclear. Here, we found that Nar induced the apoptosis, decreased the expression of lncRNA XIST and HDAC1, and increased the expression of microRNA-34a in HL60 cells. Sh-XIST transfection could induce cell apoptosis. On the contrary, the forced expression of XIST might reverse the biological actions of Nar. XIST could sponge miR-34a, which targeted to degrade HDAC1. The forced expression of HDAC1 could effectively reverse the effects of Nar. Thus, Nar can induce cell apoptosis by mediating the expression of lncRNA XIST/miR-34a/HDAC1 signaling in HL60 cells.

1. Introduction

Acute myeloid leukemia (AML), a life-threatening aggressive malignancy of the bone marrow, is characterized by aberrant cell proliferation and dysregulated differentiation of immature myeloid cells [1]. Several risk factors, such as aging, radiation, mental diseases, and hematological malignancies, contribute to the pathogenesis and development of AML [2]. Approximately 6,660 patients (including 3,740 males and 2,920 females) were diagnosed with AML in the USA in 2022 [3]. AML is associated with poor prognosis, as well as high recurrence in young patients due to drug resistance [4]. Mechanistically, genetic mutations, epigenetic abnormalities, and molecular heterogeneity play critical roles in AML development [2]. Thus, an in-depth understanding of the pathophysiological mechanisms of AML is essential for the better therapeutic management of AML.

Long noncoding RNAs (lncRNAs), a set of RNA molecules measuring >200 nucleotides in length, have been reported to have various biological functions in the pathogenesis and progression of cancer [5]. The aberrant expression profile of transcriptome-wide lncRNAs has been reported in AML, and lncRNA LOC285758 has been found to be closely associated with poor prognosis in patients with AML [6]. The expression of lncRNA nuclear paraspeckle assembly transcript 1 (NEAT1) has been shown to be decreased and

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correlated with the expression of the phosphate and tension homology deleted on chromosome ten gene but not p53 and Bcl-2 genes in patients with AML [7]. LncRNA ubiquitin-specific protease 30 antisense 1 (USP30-AS1) is highly upregulated and related to poor prognosis in AML. Another study indicates that USP30-AS1 may enhance the survival of AML cells by *cis*-regulating the expression of USP30 and ankyrin repeat domain 13A [8]. In the study of lncRNA-microRNA (miR)-NA-mRNA network in cytogenetically normal AML, three lncRNAs, including X inactive specific transcript (XIST), taurine upregulated 1, and GABPB1 antisense RNA 1, are found to have higher node degrees and higher numbers of lncRNA-miRNA pairs and total pairs [9]. XIST expression in AML KG-1 cells is increased, and the increase is associated with drug resistance through the sponging of miR-29a expression and an increase of MYC proto-oncogene, basic helix-loop-helix (bHLH) transcription factor proto-oncogene, and bHLH transcription factor MYC expression [10]. However, the molecular mechanism of XIST in inhibiting AML cell apoptosis requires further investigation.

HDACs act as epigenetic regulators and play an important role in tumor development. Targeting HDACs has become a potential therapeutic strategy for the clinic management of tumors, including AML [11]. The expression of HDAC1 can be regulated by lncRNAs and miRNAs. It has been reported that lncRNA ANRIL can upregulate HDAC1-mediated ASPP2 expression by sponging miR-34a in AML cells [12]. In addition, lncRNA MEG3 may promote chronic myeloid leukemia development by downregulating miR-147 expression and upregulating HDAC1 expression [13]. Inhibition of HDAC1 expression may suppress cell proliferation and induce apoptosis in AML [14]. However, the regulating mechanisms of HDAC1 in the development of AML still need further elucidation.

Naringenin (4′,5,7-trihydroxy flavanone, Nar), often found in various citrus fruits and tomatoes, has multiple pharmacological activities, including anti-oxidant properties, anti-inflammatory properties, anti-cancer activities, anti-microorganism properties, and neuroprotective properties [15]. The anti-cancer activities of Nar have been comprehensively discussed [16–18]. Recently, it has been reported that Nar may act as an anti-leukemic agent to inhibit the proliferation and cell cycles in THP-1 cells by mediating Sirt2/p53/p21/cyclin E1 signaling [19]. Nar has been reported to suppress tumor progression by mediating the expression of HDACs [20]. However, how Nar suppresses the activity of HDACs is still unknown. Whether Nar exhibits protective activity against AML development by downregulating XIST and HDAC1 expression should be explored. Thus, we mainly explored the potential molecular mechanism of Nar in mediating AML cell apoptosis by mediating the XIST/miR-34a/HDAC1 axis in this study.

2. Materials and methods

2.1. General

The experimental protocol (GMU201539) was approved by the Ethics Committee of the First Affiliated Hospital of Gannan Medical University, according to the Declaration of Helsinki. This study was performed in accordance with the internationally accepted principles in the European Community guidelines (EEC Directive of 1986; 86/609/EEC). Nar was obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Cell culture

HL60 and KG-1 human AML cell lines (Procell Life Science & Technology Co., Ltd., Wuhan, China) and THP-1 human normal monocyte line (Procell Life Science & Technology Co., Ltd.) were cultured in the RPMI 1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 U/ml streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.) and 100 U/ml penicillin (Invitrogen). Cells were cultured at 37 °C in an incubator with 5% CO_2. Cells were sub-cultured once every three days and prepared for further experiments when they were in log-phase growth.

2.3. Cell viability evaluation

A Cell Counting Kit-8 (CCK-8; Beyotime Institute of Biotechnology, Shanghai, China) was used to measure cell viability [21]. Cells (1×10^4/well) were cultured in 96-well plates at 37 °C for 48 h. Then, cells were treated with various concentrations of Nar (5, 10, 20, 40, and 80 μM) for 24 h. After that, 10% CCK-8 was added into the medium and then co-incubated at 37 °C for 2 h. A wavelength of 450 nm was selected for the detection of absorbance using a microplate reader (Thermo Fisher Scientific, Inc.).

2.4. Measurement of ROS production

The generation of intracellular ROS was detected using an oxidant-sensitive probe DCFH-DA (ROS Assay Kit; Beyotime) by in situ fluorescence assays [22]. Simply, cells (5×10^4/mL) in log-phase growth were seeded in confocal dishes and cultured at 37 °C for 24 h. After cells were washed with PBS, DCFH-DA (10 μM) was added to co-incubate with cells for 30 min in the darkness. Then, the fluorochrome was removed, and cells were washed with PBS. A Leica DM3000 microscope with a camera (Leica Microsystems GmbH, Wetzlar, Germany) was used to detect the fluorescence intensity with a wavelength of 502 nm for excitation and a wavelength of 523 nm for emission.

2.5. TUNEL/DAPI staining assays

The kit for TUNEL/DAPI staining assays was obtained from Beyotime and used to measure AML cell apoptosis [23]. Simply, cells

were grown under standard conditions and treated with Nar. 48 h later, cells were harvested and fixed with 4% paraformaldehyde at 4 °C for 0.5 h. Next, phosphate-buffered saline (PBS) was used for washing 3 times. Subsequently, 1% Triton X-100 was added for co-incubation at room temperature for 5 min, followed by washing with PBS three times. 50 μl of equilibration buffer was then used to equilibrate at room temperature for 10 min, and 50 μl of terminal deoxynucleotidyl transferase working solution was then added for incubation in the darkness at 37 °C for 1 h. Stained cells were detected for apoptosis, according to the manufacturer's instructions. The fluorescent images were captured using a Leica DM3000 microscope with a camera (Leica).

2.6. Cell transfection

Three short hairpin RNAs (shRNAs; sh-XIST-1, sh-XIST-2, and sh-XIST-3) and a negative control scrambled shRNA (sh-NC) were prepared by Shanghai GeneChem Co., Ltd (Shanghai, China). The sh-XISTs (100 nM) and sh-NC (100 nM) were, respectively, inserted into pGPH1/Neo (40 nM; Shanghai GenePharma Co., Ltd., Shanghai, China). Then, 75 pmol of constructed pGPH1/Neo were transfected into AML cells using Lipofectamine 3000 (Invitrogen), according to the manufacturer's instructions. Briefly, the plasmid pGPH1/Neo/shRNA and Lipofectamine 3000 were diluted with serum-reduced medium (Opti-MEM) and incubated for 5 min at room temperature (RT). The two transfection mixtures were mixed, incubated at RT for 20 min, and then added to each well for incubation at 37 °C. Neomycin (400 μg/μL) was employed to select the stably transfected cells for 4 weeks [24].

MiR-34a mimics (sense, 5′-UGGCAGUGUCUUAGCUGCUUGU-3'; antisense, 5′-ACAACCAGCUACGACACUGCCA-3′) and miR-NC (sense, 5′-UUCUCCGACCGUGUCACGUTT-3'; antisense, 5′-ACGUGACACGUUCGCAGAATT-3′) were obtained from Guangzhou RiboBio Co., Ltd. (Guangzhou, China). AML cells (1×10^5/well) at an ~60% confluence were transfected using Lipofectamine 3000 (Invitrogen), according to the manufacturer's instructions. The final concentrations of miR-34a mimics or miR-NC in the system were 50 nM. The transfected cells were incubated at 37 °C and collected after 48 h for further experiments.

In addition, pcDNA3.1-XIST and pcDNA3.1-HDAC1 (Guangzhou RiboBio Co., Ltd.) were constructed by cloning the open reading frame of XIST and HDAC1, respectively, into the pcDNA3.1 vectors. pcDNA3.1-XIST and pcDNA3.1-HDAC1 were transfected into AML cells, respectively, using Lipofectamine 3000 (Invitrogen), according to the manufacturer's instructions. The transfected cells were incubated at 37 °C and collected after 48 h for further experiments.

2.7. Reverse transcription-quantitative PCR (RT-qPCR)

TRIzol (Invitrogen) was used for total RNA extraction. The mRNA concentrations were detected by an SMA 1000 UV Spectrophotometer (Merinton, Beijing, China). Reverse transcription to cDNA was performed using M-MLV [25]. A miRNA first-strand cDNA synthesis kit, purchased from Sangon Biotech, was used for miRNA expression detection. RT-qPCR was performed on the ABI PRISM7500 sequence detection system. The reaction conditions of PCR were set as follows: 10 min at 94 °C and then 45 cycles of 12 s at 90 °C, 35 s at 60 °C, and 10 s at 70 °C. The primers used (Biomics, Beijing, China) were as follows: XIST, 5′-AGCTCCTCGCACAGCTCTAA-3′ forward and 5′-CTCCAGATCGCTGGCAACC-3′ reverse; miR-34a, 5′-GGGTGGCAGTCTCTTAGC-3′ forward and 5′-GTGCACGTCCGAGGT-3′ reverse; HDAC1, 5′-TCA AGA TGG CGT GAG CAA GG-3′ forward and 5′-TGT GCG CTG GTC CCT ATC TA-3′ reverse; Bcl-2, 5′-AGGATTGTCGCCTTCTTTGAG-3′ forward and 5′-AGCCAGGAGCAATCAAACAGAG-3′ reverse; caspase-3, 5′-AGAACTGCACTGTGGCATTGAG-3′ forward and 5′-GCTTGTCGCCATACTGTTTCAG-3′ reverse; GAPDH, 5′-AGGTGAAGCTCGCAGTCAACG-3′ forward and 5′-AGGCGTGATTGATGGCAACA-3′ reverse; U6, 5′-CTCGCTTCGCCAGCACA-3′ forward and 5′-AACGCATCACGAATTTGCGT-3′ reverse. GAPDH and U6 were used as the internal control for mRNA and miRNA normalization, respectively, and measured in fold changes using the $2^{-\Delta\Delta Ct}$ method [26].

2.8. Western blotting

AML cells were harvested to extract the total protein using RIPA lysis buffer (Beyotime Institute of Biotechnology). A BCA protein assay kit (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) was used to detect the protein concentration. Western blotting was performed as follows [27]: the extracted protein (30 μg) was subjected to 10% SDS-PAGE and then transferred onto the PVDF membranes. Tris-buffered saline containing 5% nonfat milk was used to block blots at 37 °C for 1 h. After being washed with Tris-buffered saline with 1% Tween 20 (TBST) three times, the membranes were then co-incubated with the primary antibodies against HDAC1 (dilution, 1:1000; Cell Signaling Technology, Cat.no.34589), Bcl-2 (dilution, 1:1000; Cell Signaling Technology, Cat.no.4223), cleaved caspase-3 (dilution, 1:1000; Cell Signaling Technology, Cat.no.9654), GAPDH (dilution, 1:1000; Cell Signaling Technology, Cat.no.5174), and the secondary antibody HRP-labeled Goat Anti-Rabbit IgG (dilution, 1:5000; Beyotime Institute of Biotechnology, Cat.no.A0208). The enhanced chemiluminescence detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.) and Quantity One software (Bio-Rad Laboratories, Inc., Hercules, CA, USA) were used to quantify the protein expression.

2.9. Dual luciferase reporter assays

StarBase v2.0 at the website http://starbase.sysu.edu.cn and TargetScan 7.2 at the website http://www.targetscan.org were the two prediction systems employed in this study to predict the possible targets of XIST and miR-34a, respectively [24]. The sequences of XIST (wild type) and the 3′-untranslated region (UTR) of HDAC1 were cloned into a pGL-3 luciferase basic vector, constructing a recombinant luciferase plasmid. Additionally, mutant (MUT)-XIST and MUT-HDAC1 were, respectively, designed to have mutant binding sites for miR-34a. These designed plasmids were transfected into AML cells with miR-34a mimics and miR-NC using

Lipofectamine 3000, respectively. Following transfection at 37 °C for 48 h, the activities of firefly and Renilla luciferase were measured using the Glomax 96 luminometer, according to the manufacturer's instructions (Promega Corporation). The activity of firefly luciferase reporter was calculated by normalizing to the activity of Renilla luciferase.

2.10. RNA immunoprecipitation (RIP) assays

The interaction between XIST and miR-34a was investigated by RIP assays using a Magna RIP kit (MilliporeSigma; Merck KGaA, Darmstadt, Germany), according to the manufacturer's instructions. Briefly [28], RIPA lysis buffer was used to lyse AML cells (~2×10^7 cells). The supernatant was collected for RIP assays using an EZ-Magna RIPTM RNA-Binding Protein Immunoprecipitation Kit (MilliporeSigma). 10% cell extract was used as the input, and 100 μL cell extract was incubated with 50 μL protein A/G conjugated magnetic beads precoated with antibodies against Argonaute2 (Ago2) or IgG at 4 °C overnight. TRIzol was used for RNA extraction,

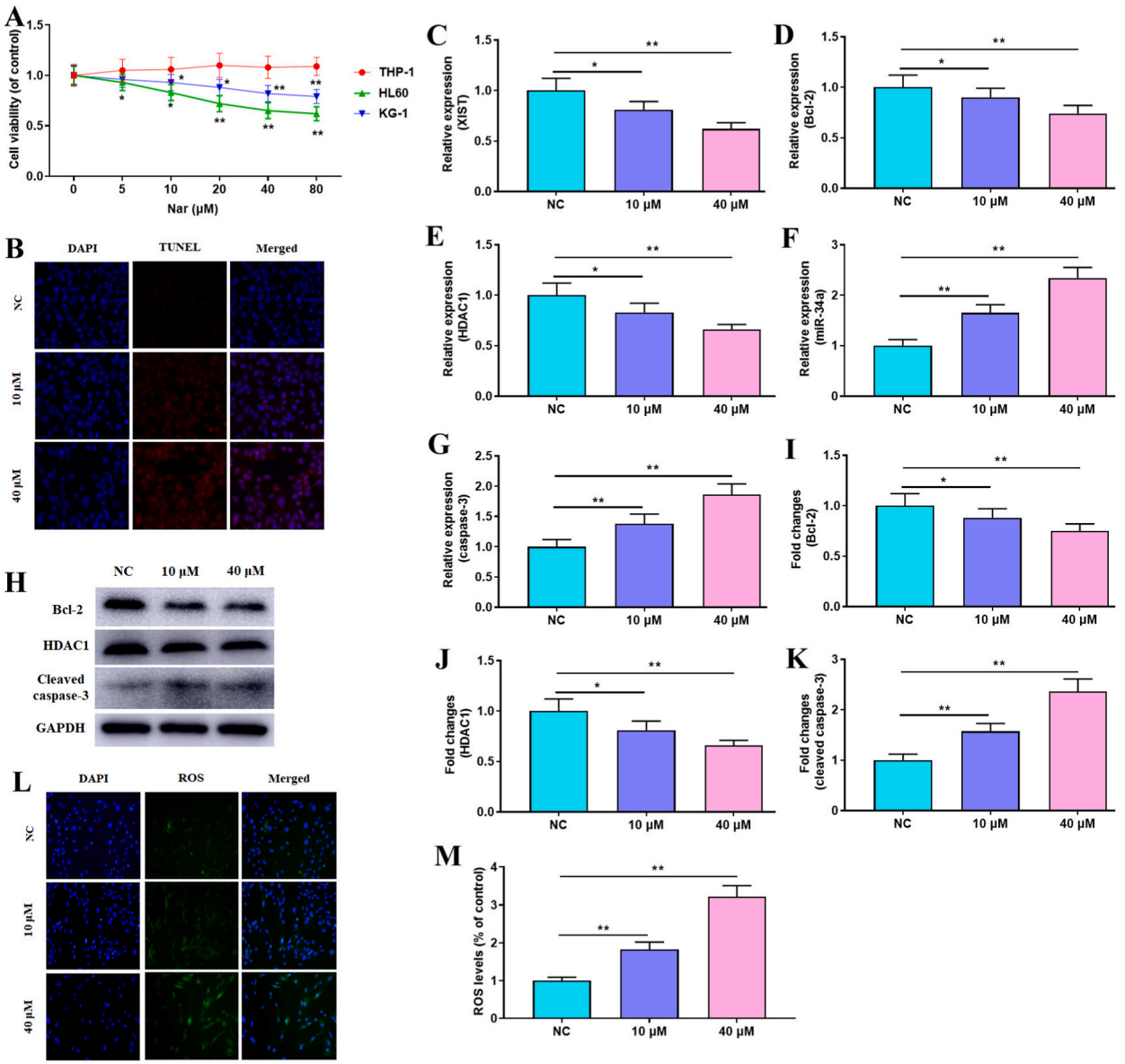

Fig. 1. Nar increased the apoptosis and inhibited the expression of XIST/miR-34a/HDAC1 in AML cells. (A) Cell viability was tested using CCK-8 assay on THP-1, HL60, and KG-1 cell lines. (B) A TUNEL/DAPI assay was performed for apoptosis detection, according to the manufacturer's instructions. RT-qPCR was used to determine the gene expression of (C) XIST, (D) Bcl-2, (E) HDAC1, (F) miR-34a, and (G) caspase-3. Western blotting was conducted to detect the protein expression of (H–I) Bcl-2, (H–J) HDAC1, and (H–K) cleaved caspase-3. All experiments were conducted separately three times. (L–M) The production of ROS was detected by ROS Assay Kit. *P < 0.05, **P < 0.01. NC, negative control; 10 μM, 10 μM Nar; 40 μM, 40 μM Nar.

and RT-qPCR was used for detection. The expression of XIST and miR-34a was analyzed in the anti-Ago2 and *anti*-IgG groups, respectively.

2.11. Statistical analysis

Data are presented as the mean ± standard error of the mean. GraphPad Prism v8 software (GraphPad Software, Inc., La Jolla, CA, USA) was used for statistical analysis using one-way ANOVA and Student's t-test. $P < 0.05$ was considered a statistically significant

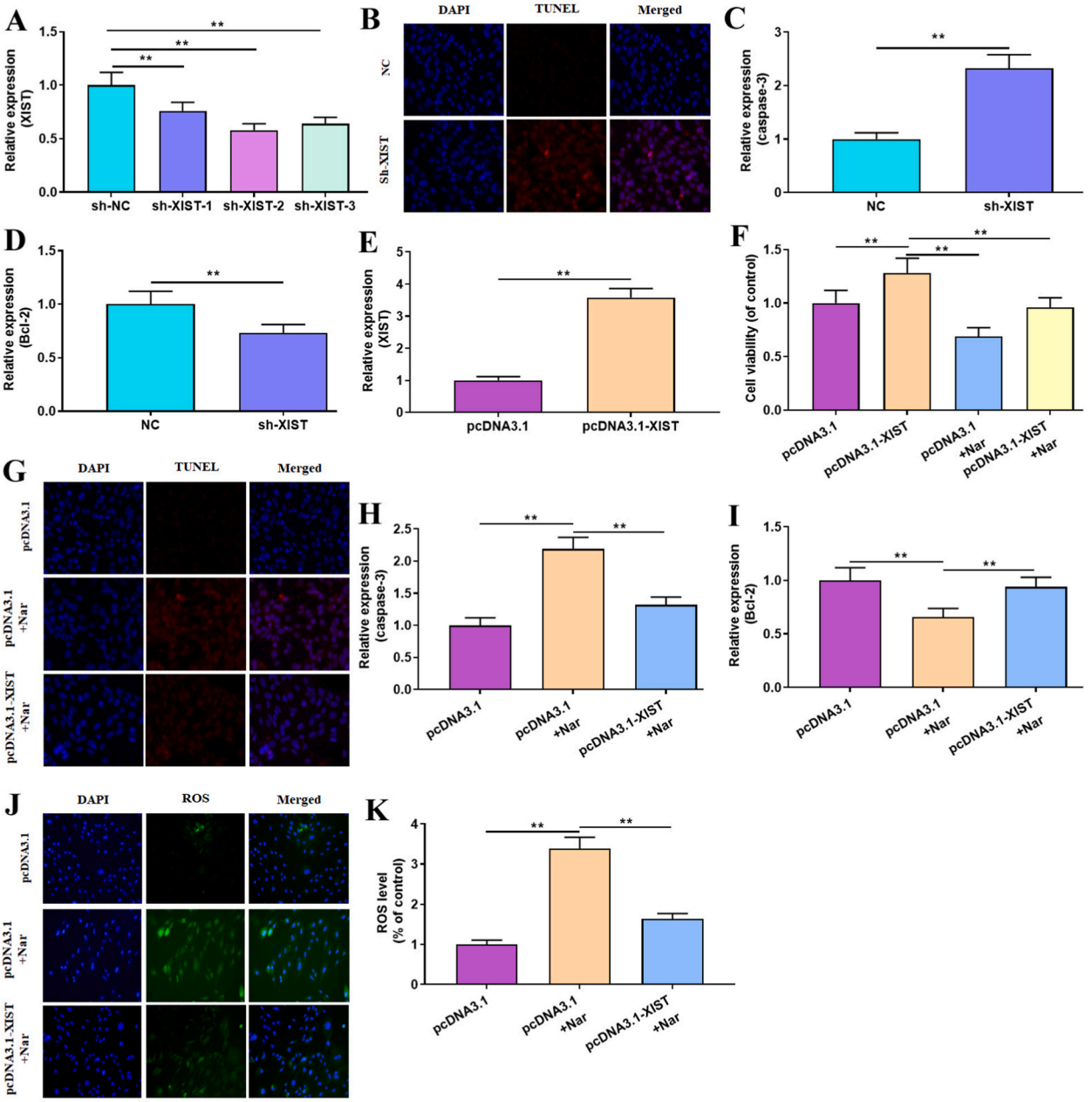

Fig. 2. Nar promoted HL60 cell apoptosis by inhibiting XIST expression. (A) XIST expression was detected in HL60 cells transfected with three sh-XISTs and sh-NC, respectively. (B) TUNEL/DAPI staining was used to evaluate the apoptosis induced by sh-XIST transfection. The expression of (C) caspase-3 and (D) Bcl-2 was determined by RT-qPCR. (E) XIST expression was detected in HL60 cells transfected with pcDNA3.1-XIST. (F) A CCK-8 assay was performed to determine the effects of Nar in XIST-overexpressed HL60 cells. (G) TUNEL/DAPI staining was conducted. The mRNA expression of (H) caspase-3 and (I) Bcl-2 was detected in XIST-overexpressed HL60 cells. (J–K) The production of ROS was detected by ROS Assay Kit. **$P < 0.01$. NC, negative control.

difference.

3. Results

3.1. Nar induced AML cell apoptosis

To investigate the potential protective activity of Nar against the development of AML, CCK-8 assays were performed. The results showed that the viability of THP-1 cells was not obviously affected after treatment with various concentrations of Nar for 24 h. However, Nar at doses of 10, 20, 40, and 80 μM exerted strong cytotoxicity against the viability of HL60 and KG-1 AML cell lines. In addition, Nar exhibited higher cytotoxicity against the viability of HL60 cells (Fig. 1A). Thus, HL60 cells were used in the subsequent experiments. In this study, 10 and 40 μM of Nar were selected as the low and high doses, respectively. In TUNEL/DAPI staining assays (Fig. 1B), Nar significantly induced apoptosis in HL60 cells in a dose-dependent manner. Furthermore, Nar at the dose of 40 μM downregulated the expression of XIST (0.62-fold; $^{**}P < 0.01$; Fig. 1C), Bcl-2 (0.74-fold; $^{**}P < 0.01$; Fig. 1D) and HDAC1 (0.66-fold, $^{**}P < 0.01$; Fig. 1E), and upregulated that of miR-34a (2.34-fold, $^{**}P < 0.01$; Fig. 1F) and caspase-3 (1.86-fold, $^{**}P < 0.01$; Fig. 1G). At the protein level, 40 μM Nar decreased the expression of Bcl-2 (0.75-fold, $^{**}P < 0.01$; Fig. 1H–I) and HDAC1 (0.66-fold, $^{**}P < 0.01$; Fig. 1H–J), and increased that of cleaved caspase-3 (2.36-fold, $^{**}P < 0.01$; Fig. 1H–K). In addition, Nar at the concentration of 40 μM also enhanced the levels of ROS dose-dependently (3.22-fold, $^{**}P < 0.01$; Fig. 1L-M). These findings indicated that Nar could effectively induce AML cell apoptosis.

3.2. Nar promoted HL60 cell apoptosis by inhibiting XIST expression

To study the roles of XIST in HL60 cell apoptosis, sh-XIST transfection-induced XIST knockdown was conducted (Fig. 2A). sh-XIST-2 had stronger activity in decreasing XIST expression than sh-XIST-1 and sh-XIST-1 and was selected for the following experiments. XIST expression knockdown promoted the apoptosis of HL60 cells, as indicated by increased TUNEL staining (Fig. 2B), increased caspase-3 expression (2.32-fold, $^{**}P < 0.01$; Fig. 2C), and decreased Bcl-2 expression (0.73-fold, $^{**}P < 0.01$; Fig. 2D). To explore the possible actions of XIST in the induction of cell apoptosis by Nar, XIST overexpression was induced in Nar-treated HL60 cells (Fig. 2E). Of note, XIST overexpression abolished the promoting effects of Nar (40 μM) on HL60 apoptosis, as shown by increased cell viability (Fig. 2F), reduced TUNEL staining (Fig. 2G) and caspase-3 expression (2.19-fold → 1.32-fold, $^{**}P < 0.01$; Fig. 2H), and increased Bcl-2 expression (0.66-fold → 0.94-fold, $^{**}P < 0.01$; Fig. 2I). In addition, XIST overexpression might compromise the Nar-increased ROS production (3.39-fold → 1.64-fold, $^{**}P < 0.01$; Fig. 2J–K). These findings suggested that Nar promoted HL60 cell apoptosis by inhibiting XIST expression.

3.3. XIST could interact with miR-34a

To further investigate the possible roles of XIST in HL60 cell apoptosis, the potential miRNAs were predicted by StarBase v2.0 software. miR-34a was included in the potential targets of XIST (Fig. 3A). To verify this prediction, dual luciferase reporter assays were conducted (Fig. 3B). The activity of luciferase in the reporter containing wild-type XIST was decreased >60% ($^{**}P < 0.01$). However, no obvious differences were observed in the relative activities of luciferase between the NC and mutant groups. Further study via RIP assays (Fig. 3C) showed that XIST could physically interact with miR-34a. Thus, miR-34a may be a potential target of XIST.

To explore the effects of miR-34a on HL60 cell apoptosis, miR-34a mimics were transfected into HL60 cells. The transfection efficiency was determined using RT-qPCR (Fig. 4A). miR-34a overexpression in HL60 cells significantly downregulated the expression of XIST (0.42-fold, $^{**}P < 0.01$; Fig. 4B). In addition, miR-34a overexpression increased cell apoptosis, as shown by increased TUNEL staining (Fig. 4C), increased expression of caspase-3 (1.98-fold, $^{**}P < 0.01$; Fig. 4D), and decreased expression of Bcl-2 (0.79-fold, $^{**}P < 0.01$; Fig. 4E) in HL60 cells. Collectively, these results suggested that XIST inhibited HL60 cell apoptosis by sponging miR-34a.

HDAC1 is a direct target of miR-34a. To further explore the biological roles of miR-34a in HL60 cells, the potential target of miR-34a was predicted by the online software TargetScan v7.2 (Fig. 5A). HDAC1 could be a possible target gene of miR-34a, which was verified by a dual luciferase reporter assay (Fig. 5B). The relative luciferase activity in the reporter involving the wild type binding site of HDAC1 decreased by >60% ($^{**}P < 0.01$). However, the relative luciferase activities in both the NC reporter and the reporter

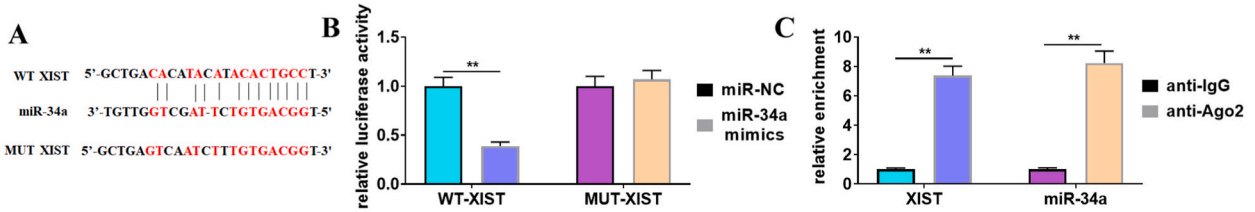

Fig. 3. XIST interacted with miR-34a. (A) The possible interaction between XIST and miR-34a was predicted by StarBase v2.0 software. (B) The relative luciferase activity was determined in HL60 cells co-transfected with wild type/mutant XIST and miR-34a mimics/miR-NC. (C) RIP assays were performed to verify the interaction between XIST and miR-34a. $^{**}P < 0.01$.

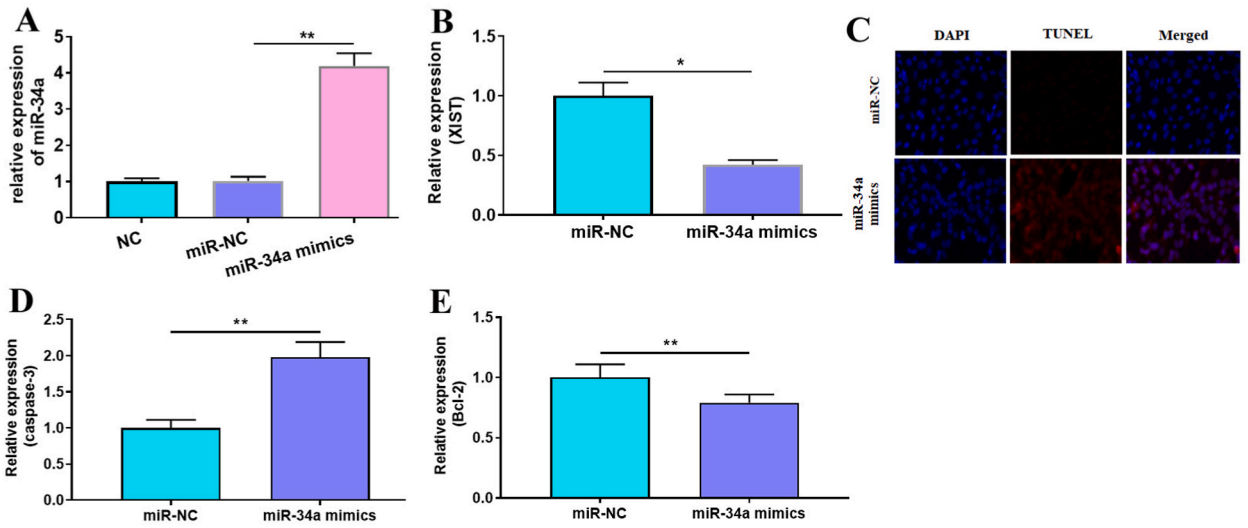

Fig. 4. miR-34a overexpression abrogated the effects of XIST on HL60 apoptosis. The expression of (A) miR-34a and (B) XIST was detected by RT-qPCR in HL60 transfected with miR-34a mimics. (C) TUNEL/DAPI staining was used to evaluate apoptosis. The expression of (D) caspase-3 and (E) Bcl-2 was determined by RT-qPCR. *P < 0.05, **P < 0.01.

involving the mutant site of HDAC1 did not exhibit any obvious differences. The expression of HDAC1 at the mRNA (0.41-fold, **P < 0.01; Fig. 5C) and protein levels (0.35-fold, **P < 0.01; Fig. 5D–E) was also detected, and it was shown that miR-34a significantly decreased the mRNA and protein expression of HDAC1. These findings suggested that HDAC1 might be the direct target of miR-34a.

3.4. HDAC1 overexpression abolished the inductive effects of Nar on HL60 cell apoptosis

To further investigate the underlying mechanisms of Nar in promoting HL60 cell apoptosis, HL60 cells were co-transfected with sh-XIST and pcDNA3.1-HDAC1. RT-qPCR was used to determine the successful transfection (Fig. 6A–B). HDAC1 overexpression may reverse the promoting effect of sh-XIST on cell apoptosis, as indicated by the attenuated TUNEL staining (Fig. 6C) and caspase-3 expression (0.58- and 0.66-fold for mRNA and protein, respectively, **P < 0.01; Fig. 6D, F and G) and enhanced Bcl-2 expression (1.88- and 2.26-fold for mRNA and protein, respectively, **P < 0.01; Fig. 6E, F and H). In addition, HDAC1 overexpression also decreased the levels of intracellular ROS (Fig. 6I–J). However, Nar (40 μM) might effectively block the co-transfection effects of both sh-XIST and pcDNA3.1-HDAC1, ameliorating the HDAC1-overexpression-inhibited HL60 cell apoptosis. In combination, these findings suggested that Nar might promote apoptosis by mediating the expression of the XIST/miR-34a/HDAC1 axis in HL60 cells.

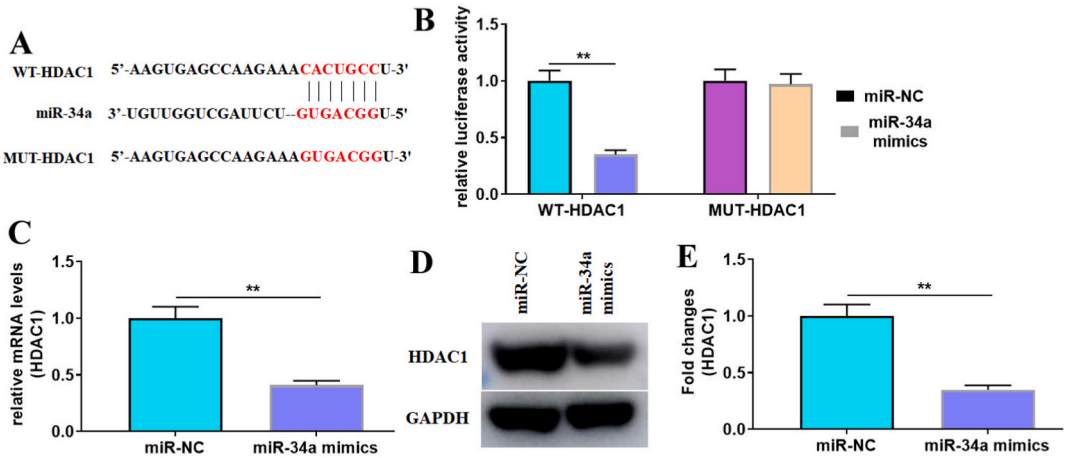

Fig. 5. HDAC1 was a direct target of miR-34a. (A) HDAC1 was predicted to be a potential target of miR-34a by TargetScan v7.2 software. (B) The relative luciferase activity was determined in HL60 cells co-transfected with wild type/mutant HDAC1 and miR-34a/miR-NC. The (C) mRNA and (D–E) protein expression was determined by RT-qPCR and western blotting, respectively, in miR-34a mimic-transfected HL60 cells. **P < 0.01.

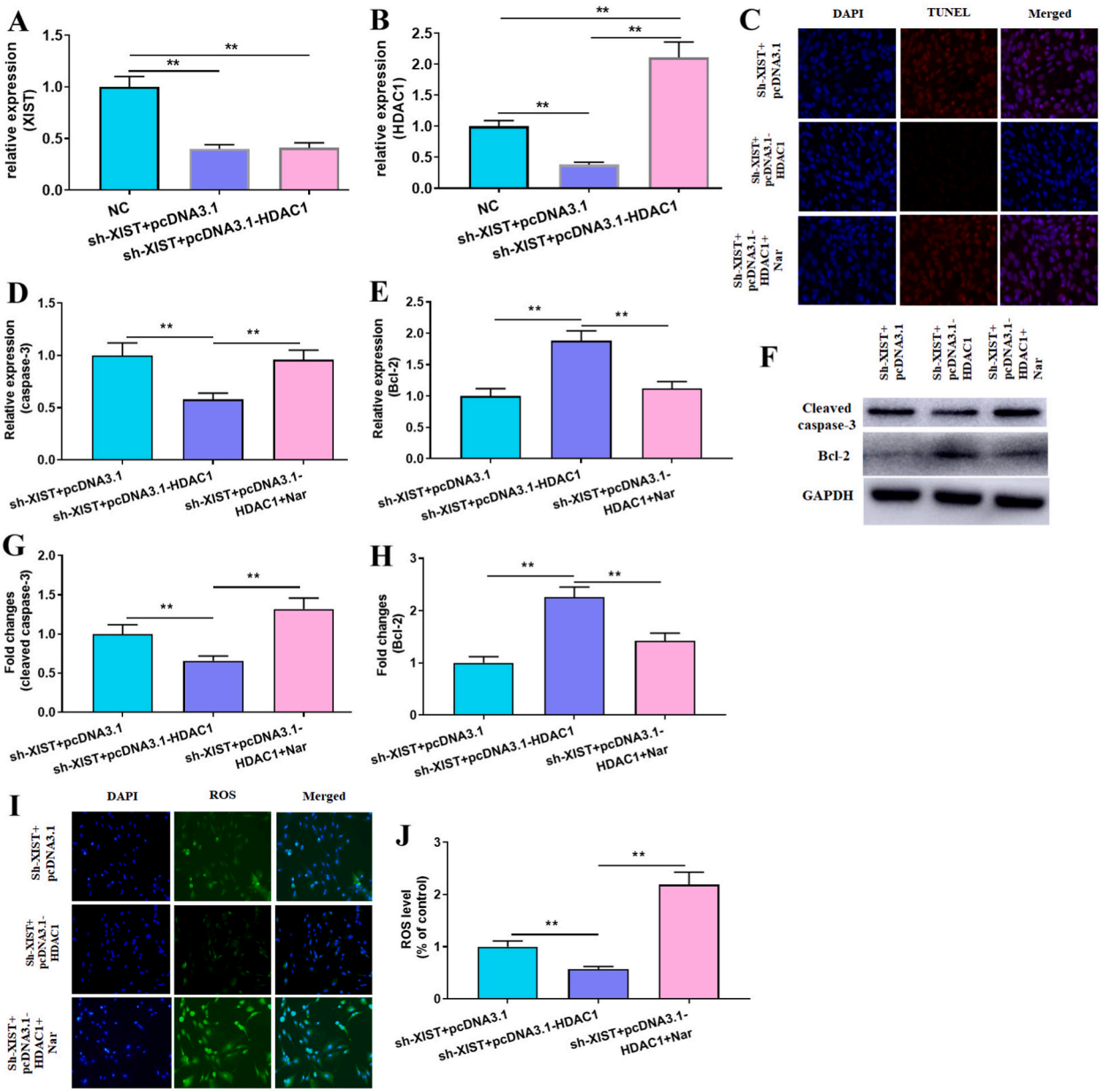

Fig. 6. HDAC1 overexpression abrogated the promoting effects of Nar on HL60 apoptosis. The gene expression of (A) XIST and (B) HDAC1 was detected in HL60 cells co-transfected with sh-XIST and pcDNA3.1-HDAC1. (C) TUNEL/DAPI staining was determined to evaluate cell apoptosis. The mRNA expression of (D) caspase-3 and (E) Bcl-2 was determined by RT-qPCR. The protein expression of (F, G) cleaved caspase-3 and (F, H) Bcl-2 was determined by western blotting. (I–J) The production of ROS was detected by ROS Assay Kit. **P < 0.01.

4. Discussion

Cell apoptosis induction can be employed in cancer treatment [29]. AML is a common type of cancer characterized by the highly proliferative activity of immature myeloid progenitors. Currently, the principal therapeutic strategy for AML in the clinic is chemotherapy and hematopoietic stem cell transplantation. However, these are associated with various adverse effects due to the misuse of cytarabine, which leads to unsatisfactory results [30]. Therefore, novel therapeutic targets and new drugs are urgently needed. Natural active compounds and traditional Chinese medicines have been extensively studied for various diseases treatment, particularly cancer. For example, emetine is a natural compound obtained from *Psychotria ipecacuanha* and has been reported to inhibit cell proliferation and induce apoptosis by mediating MAPKs, Wnt/β-catenin, PI3K/AKT, and Hippo/YAP signaling pathways in gastric cancer [31]. Resveratrol may inhibit epithelial sodium channels (ENaCs) activity and promote apoptosis by activating AMPK in human colon cancer cells [32].

Natural products may also provide a chemotherapeutic strategy for AML treatment. Thymoquinone exhibits protective activity against AML by mediating JAK/STAT signaling pathway, as shown by decreased proliferation and increased apoptosis in HL60 cells [33]. Britannin is an active ingredient from *Inula aucheriana* DC and exerts cytotoxic effects on K562 and U937 cell lines by promoting p21/p27-mediated apoptosis [34]. Honokiol, a biphenolic phytochemical from the mark of magnolia, has been shown to decrease the viability of AML cells and stimulate a non-canonical ferroptosis signaling pathway in THP-1 and U937 cell lines by enhancing lipid peroxidation and upregulating the expression of heme oxygenase 1 [35]. Acetylshikonin is a naturally occurring naphthoquinone compound isolated from *Lithospermum erythrorhyzon*. Acetylshikonin has been reported to induce HL60 cell death by triggering autophagy through the liver kinase B1/adenosine 5′-monophosphate (AMP)-activated protein kinase (AMPK) and PI3K/AKT-mediated mTOR signaling pathways [36]. In addition, combinational effects of Nar and curcumin on cell cycle arrest and apoptosis in THP-1 cells have been reported [37]. Consistently, our study also suggested the inductive effects of Nar on apoptosis in HL60 cells.

LncRNAs and miRNAs are members of non-coding RNAs. The interaction between lncRNA and miRNA affects physiological and pathological activities [38]. LncRNAs play a critical role in the pathological development of various diseases, including malignant tumors [39]. It has been demonstrated that lncRNA LINC00630 can effectively inhibit the proliferation, migration, and invasion of cholangiocarcinoma cells by mediating the miR-199a/FGF7 axis [40]. LncRNA HOX transcript antisense RNA has been reported to play an important role in the progression of osteoarthritis, and it enhances mechanical stimulation-induced chondrocyte apoptosis by mediating the miR-221/BBC3 axis in C28/I2 cells [41]. LncRNA XIST has been shown to regulate tumor development at multiple levels. For example, XIST regulates tumor development and affects the prognosis by sponging miRNA and/or targeting proteins. In addition, XIST can interact with mRNA competitively by adsorbing miRNAs [42]. The important role of XIST in leukemia has been verified in cytogenetically normal AML by constructing a network of lncRNA-miRNA-mRNA [9]. Many important signaling pathways, such as PI3K/AKT signaling, forkhead box O signaling, p53 signaling, and Ras signaling, have been shown to be associated with XIST [9]. Consistently, the present study found that the expression of XIST was increased in HL60 cells. XIST knockdown could lead to increased cell apoptosis, and miR-34a overexpression may abrogate the effects of XIST on HL60 cells.

HDACs are a large enzyme family, which catalyzes the deacetylation at lysine residue in histone and non-histone proteins. HDAC1 is primarily located in the nucleus and has been shown to play an essential role in regulating gene expression [43]. HDAC1 may enhance the affinity of histone to the DNA backbone and then inhibit the transcriptional machinery/factors to access gene promoters by deacetylating lysine in histone proteins. Of note, HDAC1 plays important roles in the physiological and pathological activities of the human body [44]. The roles of HDAC1 in human lung adenocarcinoma cells have been shown to be an epigenetic regulator that promotes cell growth and docetaxel resistance development by suppressing the expression of miR-200b [45]. Another study showed that the upregulation of HDAC1 may result in the high activity of cell invasion and migration in MCF-7 and MDA-MB-231 breast cancer cells [46]. HDAC1 has also been reported to contribute to the pathological development of leukemia, and the neddylation and ubiquitination of HDAC1 have been shown to play a critical role in treating AML [47]. Consistently, HDAC1 knockout-induced cell apoptosis in imatinib-resistant chronic myeloid leukemia [48]. In the present study, the expression of HDAC1 was found to be enhanced in HL60 cells, and HDAC1 overexpression significantly decreased cell apoptosis. Of note, HDAC1 has been considered a therapeutic target for the treatment of AML [49].

Several inhibitors of HDAC1 have been explored. In addition, several synthetic or natural compounds that exert protective effects against AML by inhibiting HDAC1 activity have been studied [50,51]. Baicalein, a natural flavonoid, has been reported to decrease the viability of human leukemia cells by inhibiting the activity of HDAC1/8 [51]. Oroxylin A, a natural compound, may increase the expression of differentiation-associated CCAAT-enhancer-binding protein α and p21 by inhibiting the activity of HDAC1 in AML cells [52]. Overexpression of P-gp is associated with multidrug resistance and chemotherapy failure for leukemia. Seventeen thiosemicarbazone-containing compounds have been shown to suppress cell proliferation and induce apoptosis in drug-resistant K562/A02 cells by inhibiting the expression of HDAC1 and HDAC6 [53]. In the present study, it was found that Nar decreased the expression of HDAC1 in HL60 cells and that HDAC1 overexpression could abrogate the effects of Nar on HL60 cell apoptosis.

It should be noted that the highly divergent subtypes and mutation profiles in AML are often observed in the clinic. Most current studies are developed using cell lines; more studies should focus on the native AML blasts. Comparing experimental observations remains essential, at least for improving drug discovery and development. Conventional chemotherapy is being added to or replaced by targeted therapeutic strategies. However, therapy resistance in AML also becomes a challenging issue. The appearance of specific patterns of AML relapse indicates that different therapies are required to provide their distinct stem cell biology. Combinatorial therapeutic strategies may enhance the vulnerabilities of leukemia stem cells and then abolish relapse-triggering cells through different mechanisms [54]. The poor water solubility and bioavailability of Nar have posed challenges for its clinic applications. Methods to increase the bioavailability of Nar must be studied to increase the efficiency of Nar as a therapeutic. The drug delivery system for Nar in treating cancers has been explored [55]. Further study on the discovery of the therapeutic potentials of Nar with respect to human health promotion should be warranted. In addition, long-term and well-controlled clinical trials of Nar are still necessary.

5. Conclusion

Collectively, the findings of the present study showed that Nar could decrease the viability and apoptosis of HL60 cells by suppressing the expression of the lncRNA XIST/miR-34a/HDAC1 axis. However, our data were obtained from the experiments in vitro. It is essential to explore the pharmacological activity of Nar against AML in vivo. Our study sheds light on the molecular mechanisms of Nar and proposes its therapeutic potential. Investigating the critical signaling pathways underlying the suppressive effects of Nar against HL-60 cells provides a scientific idea that developing natural compounds as novel therapeutic alternatives for AML treatment with

fewer side effects might be valuable.

Author contribution statement

Chao Wen; Xiaoliang Lu; Yingyin Sun; Qi Li; Jing Liao: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Lin Li: Conceived and designed the experiments.

Data availability statement

Data included in article/supplementary material/referenced in article.

Additional information

No additional information is available for this paper.

Authors' Contribution

Lin Li provided the concept of this article. Chao Wen, Xiaoliang Lu, Yingyin Sun, Qi Li, and Jing Liao performed the experiments and wrote and & finalized the article. All authors approved the final paper.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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