


ARTICLE



Curcumin attenuates Adriamycin-resistance of acute myeloid leukemia by inhibiting the lncRNA HOTAIR/miR-20a-5p/WT1 axis

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Acute myeloid leukemia (AML) is a common subtype of leukemia, and a large proportion of patients with AML eventually develop drug resistance. Curcumin exerts cancer suppressive effects and increases sensitivity to chemotherapy in several diseases. This study aimed to investigate the mechanism by which curcumin affects the resistance of AML to Adriamycin by regulating HOX transcript antisense RNA (HOTAIR) expression. Cell viability, colony-formation, flow cytometry, and Transwell assays were used to assess cell proliferation, apoptosis, and migration. A dual-luciferase reporter assay was used to verify the interaction between microRNA (miR)-20a-5p and HOTAIR or Wilms' tumor 1 (WT1). RT-qPCR and Western blotting assays were performed to detect gene and protein expression. The results showed that curcumin suppressed the resistance to Adriamycin, inhibited the expression of HOTAIR and WT1, and promoted the expression of miR-20a-5p in human acute leukemia cells (HL-60) or Adriamycin-resistant HL-60 cells (HL-60/ADR). Furthermore, curcumin suppressed proliferation and promoted apoptosis of HL-60/ADR cells. Overexpression of HOTAIR reversed the regulatory effect of curcumin on apoptosis and migration and restored the effect of curcumin on inducing the expression of cleaved caspase3, Bax, and P27. In addition, HOTAIR upregulated WT1 expression by targeting miR-20a-5p, and inhibition of miR-20a-5p reversed the regulation of Adriamycin resistance by curcumin in AML cells. Finally, curcumin inhibited Adriamycin resistance by suppressing the HOTAIR/miR-20a-5p/WT1 pathway in vivo. In short, curcumin suppressed the proliferation and migration, blocked the cell cycle progression of AML cells, and sensitized AML cells to Adriamycin by regulating the HOTAIR/miR-20a-5p/WT1 axis. These findings suggest a potential role of curcumin and HOTAIR in AML treatment.

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INTRODUCTION

Acute myeloid leukemia (AML) has the highest prevalence and mortality rates of all the subtypes of leukemia and is characterized by the infiltration of abnormally and poorly differentiated cells into the bone marrow and blood [1–3]. Although substantial progress has been made in treating AML with chemotherapy, such as Adriamycin, a large proportion of patients still suffer drug resistance and disease recurrence [3, 4]. Targeted therapy has resulted in benefits for patients with several kinds of malignant diseases. Unfortunately, AML is an exception due to its drug resistance and heterogeneity [5]. Thus, there is still an urgent need to identify novel therapeutic targets and discover the mechanism underlying drug resistance in AML.

Curcumin is the main active flavonoid component of the traditional Chinese herb *Curcuma longa*, and curcumin possesses anti-oxidant [6], anti-inflammation [7], and anti-tumor properties [8]. Numerous studies suggest that curcumin induces apoptosis, inhibits proliferation, and blocks cell cycle progression in multiple cancer cell lines [7, 9]. Curcumin also reversed the resistance of breast cancer cells to Adriamycin by regulating miRNA expression [10]. Moreover, curcumin exerted cancer suppressive effects against multiple leukemia subtypes [11, 12]. However, the mechanisms underlying the effects of curcumin in suppressing cancer progression and drug resistance remain unclear.

Long non-coding RNAs (lncRNAs) perform indispensable functions in many diseases, especially malignancies [13]. lncRNAs are transcripts with lengths greater than 200 nucleotides that possess little or no ability to encode proteins. The biofunctions of lncRNAs include regulating the activities of gene promoters, participating in posttranslational modification, or targeting specific miRNAs [14]. The expression of several lncRNAs, including RP11-222K16.2, AC092580.4, and RP11-305O.6, was dysregulated in AML, which could influence AML pathogenesis and progression in different ways [15]. HOX transcript antisense intergenic RNA (HOTAIR) was overexpressed in several malignant diseases, such as breast cancer [16] and cervical cancer [17]. HOTAIR enhanced the resistance of gastric cancer cells to paclitaxel and Adriamycin by suppressing miR-217 expression [18]. Hao et al. suggested that HOTAIR was overexpressed in AML and was associated with poor prognosis [19]. However, the role of HOTAIR in AML pathogenesis is still not fully understood.

MicroRNAs (miRNAs) are also key components of the regulatory network of gene expression. miRNAs can bind to the 3' untranslated regions (3'UTRs) of target mRNAs and inhibit their translation. miR-20a-5p expression was found to be dysregulated in several malignant diseases, including breast cancer, pancreatic cancer, and nasopharyngeal cancer, and higher miR-20a-5p expression was often correlated with higher sensitivity to chemotherapeutic drugs [20–22]. Lei et al. reported that the upregulation of miR-20a-5p suppressed the growth of AML cells

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[23]. However, the role of miR-20a-5p in AML remains incompletely understood. The Wilms' tumor 1 (WT1) gene functions as an important transcription factor, it contributed to the pathogenesis of several malignant diseases, especially AML, and its high expression could indicate poor prognosis [24]. On the other hand, Yang et al. reported that silencing WT1 sensitized K562/A02 cells to Adriamycin (ADR) [25]. Through a bioinformatics analysis, we predicted that miR-20a-5p targets the 3'UTR of the WT1 transcript and that HOTAIR might bind miR-20a-5p.

In this study, we hypothesized that curcumin might exert cancer suppressive effects by targeting the HOTAIR/miR-20a-5p/WT1 axis. In a series of in vitro and in vivo experiments, we verified that curcumin inhibited the resistance of AML cells to Adriamycin and decreases the expression of HOTAIR, thus improving miR-20a-5p levels and suppressing WT1 expression. Curcumin suppresses cell proliferation and migration and promotes apoptosis in HL-60 or HL-60/ADR cells. These findings may help us to further understand the mechanisms underlying drug resistance of AML and develop new strategies for its treatment.

MATERIALS AND METHODS

Cell culture

Human acute leukemia cells (HL-60 cells) were obtained from Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China), and human Adriamycin-resistant acute leukemia cells (HL-60/ADR cells) were obtained from Mingzhou Biological Co., Ltd. (Ningbo, China). All the cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium supplemented with 10% foetal bovine serum (FBS; Gibco, USA) and incubated in an incubator containing 5% CO₂ at 37 °C.

MTT assay for cell viability

To assess the inhibitory effect of curcumin (Sigma, USA) on the resistance of HL-60 cells to Adriamycin, we treated HL-60 and HL-60/ADR cells with different doses of Adriamycin (0, 10, 25, 50, or 75 μM) and curcumin (0, 5, 10, 20, or 30 μM). Furthermore, the combination of curcumin and Adriamycin (0 + 0, 5 + 10, 10 + 25, 20 + 50, or 30 + 75 μM) was used to determine the effect of the combined use of the two drugs on cell proliferation. Cells were collected and seeded in 96-well plates (1 × 10³ cells/well) and incubated for 24 h. Then, the cells were treated according to the instructions described above for 24 h. After drug treatment, the medium was removed, and then, medium containing 0.5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each well. Then, the cells were incubated for another 4 h, the medium was gently removed, and 200 μL of dimethyl sulfoxide (DMSO) was added to the wells. The absorbance of the solution in each well was detected by a plate reader (Thermo, USA) at 490 nm.

Colony-formation assay

A soft agar colony-formation assay was used to evaluate the proliferation of HL-60 and HL-60/ADR cells after treatment with different concentrations of Adriamycin and curcumin. HL-60 and HL-60/ADR cells were evenly seeded in six-well plates pre-coated with soft agar (500 cells/well) and then treated with curcumin (0, 5, or 20 μM), Adriamycin (0, 25, or 50 μM) or their combination (0 + 0, 5 + 25, or 20 + 50 μM) for 24 h. Then, the plates were incubated in an incubator with 5% CO₂ at 37 °C for 3 weeks. During this period, the medium was changed every 4 d. Finally, the colonies were photographed with an optical camera and counted.

Cell apoptosis assay

The apoptosis of HL-60 and HL-60/ADR cells was evaluated using flow cytometry. After treatment with Adriamycin, curcumin, overexpression (OE)-HOTAIR, miR-20a-5p inhibitor, or miR-20a-5p mimics, the cells were collected and washed with PBS. Then, the cells were stained with Annexin V-FITC and PI (Thermo) for 20 min in the dark at room temperature, followed by detection and analysis by flow cytometry (Thermo). Finally, the data were analyzed by FlowJo VX10 software, and the apoptosis rates in each group are shown.

Cell migration assay

The migration capability of HL-60/ADR cells was detected via Transwell assays. After treatment with curcumin, OE-HOTAIR, miR-20a-5p inhibitor, or

miR-20a-5p mimics, the HL-60/ADR cells were collected and suspended in RPMI-1640 medium without FBS. Then, the cells were seeded into the upper chambers of Transwell plates (0.5 × 10⁵ cells/well), and RPMI-1640 medium containing 10% FBS was added into the lower chambers. The plates were incubated at 37 °C for 48 h. Then, the nonmigrated cells were gently removed with sterile cotton swabs, and the migrated cells were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet. Then, the migrated cells were observed with an Olympus microscope (Tokyo, Japan).

Cell transfection

We used OE-HOTAIR, mimics NC, miR-20a-5p mimics, inhibitor NC, miR-20a-5p inhibitor, sh-NC, sh-HOTAIR, and sh-WT1 from GenePharma Co., Ltd. (Suzhou, China). HL-60 and HL-60/ADR cells were plated in 24-well plates, incubated overnight with FBS-free medium, transfected with polynucleotides or plasmids using Lipofectamine 2000 reagent (Invitrogen; USA), and incubated for another 48 h. The transfection efficiencies were evaluated via RT-qPCR assay.

Protein extraction and Western blotting

After the different treatments, the cells were lysed with RIPA lysis buffer (Keygen, China) with 1% PMSF. Then, the protein concentrations were detected with a Bicinchoninic Acid (BCA) Protein Assay Kit (Keygen, China). Equal amounts of protein from the different samples were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the proteins were then transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, USA). Then, membranes were blocked with 5% skim milk for 1 h at room temperature, followed by incubation with different primary antibodies (anti-WT1, Cell Signaling Technology, Danvers, USA; anti-Bax, Proteintech, Chicago, USA; anti-Bcl-2, Proteintech, Chicago, USA; anti-cleaved caspase3, Cell Signaling Technology, Danvers, USA; anti-P27, Cell Signaling Technology, Danvers, USA; and anti-GAPDH, Proteintech, Chicago USA) overnight at 4 °C. After washing three times with TBST, the membranes were incubated with HRP-conjugated secondary antibodies for 0.5 h at 37 °C. Finally, the reactions on the membranes were visualized by chemiluminescence reagents (ECL) (Advasta, USA) using a Bio-Rad system (Bio-Rad, USA). The intensity of each band was analyzed with ImageJ software.

Reverse transcription-quantitative PCR (RT-qPCR)

HOTAIR, WT1, and miR-20a-5p expression levels were detected by RT-qPCR. Total RNA was extracted from cells and tumor tissues by a TRIzol Reagent Kit (Invitrogen, USA) and quantified by a Nanodrop 2000 instrument before reverse transcription. Then, cDNA was synthesized with a miRNA First-Strand cDNA Synthesis SuperMix Kit (TransGen Biotech) or a RT Reagent Kit (TransGen Biotech). Then, RT-qPCR was performed with SYBR[®] Green Premix Ex Taq[™] (Takara, Japan) using an ABI Detection System (Thermo, USA). The thermocycling conditions were as follows: 30 s at 95 °C for 1 cycle; 5 s at 95 °C and 30 s at 60 °C for 40 cycles. Finally, the RNA expression levels were analyzed by the 2^{-ΔΔCt} method. HOTAIR and WT1 expression was normalized to GAPDH expression, and miR-20a-5p expression was normalized to U6 expression. All the primer sequences were as follows:

HOTAIR Forward: 5'-CAAACAGAGTCCGTTTCAGTGTCA-3'
 HOTAIR Reverse: 5'-GGTGGATTCCTGGGTGGGT-3'
 miR-20a-5p Forward: 5'-TTGTTACAGGAAGTCCCTTGCC-3'
 miR-20a-5p Reverse: 5'-ATGCTATCACCTCCCTGTGTG-3'
 WT1 Forward: 5'-CAGAACTGGACCCCGTTACC-3'
 WT1 Reverse: 5'-ATGCATTCACCTCAGCCATG-3'
 U6 Forward: 5'-CGCTTCGGCAGCACATATACTA-3'
 U6 Reverse: 5'-CGCTTCAGCAATTTGCTGTCA-3'
 GAPDH Forward: 5'-AGGTCGGTGTGAACGGATTG-3'
 GAPDH Reverse: 5'-GGGGTCGTTGATGGCAACA-3'.

Tumor xenografts in nude mice

Male four-week-old BALB/c nude mice were obtained from Liaoning Changsheng Biotechnology Co., Ltd. (Liaoning, China). All the mice were housed in a suitable environment with a relative humidity of 40–60% and a temperature of 20 ± 2 °C. The study was approved by the Animal Ethics Committee of the People's Hospital of Longhua District. After being acclimated for one week and given free access to food and water, the mice were randomly divided into nine groups: the control, vector, OE-HOTAIR, vector + curcumin (cur), vector + Adriamycin (Adr), vector + cur + Adr, OE-HOTAIR + cur, OE-HOTAIR + Adr, and OE-HOTAIR + Adr + cur groups. Then, 1 × 10⁶ HL-60 cells/200 μL (the HL-60 cells had been transfected with

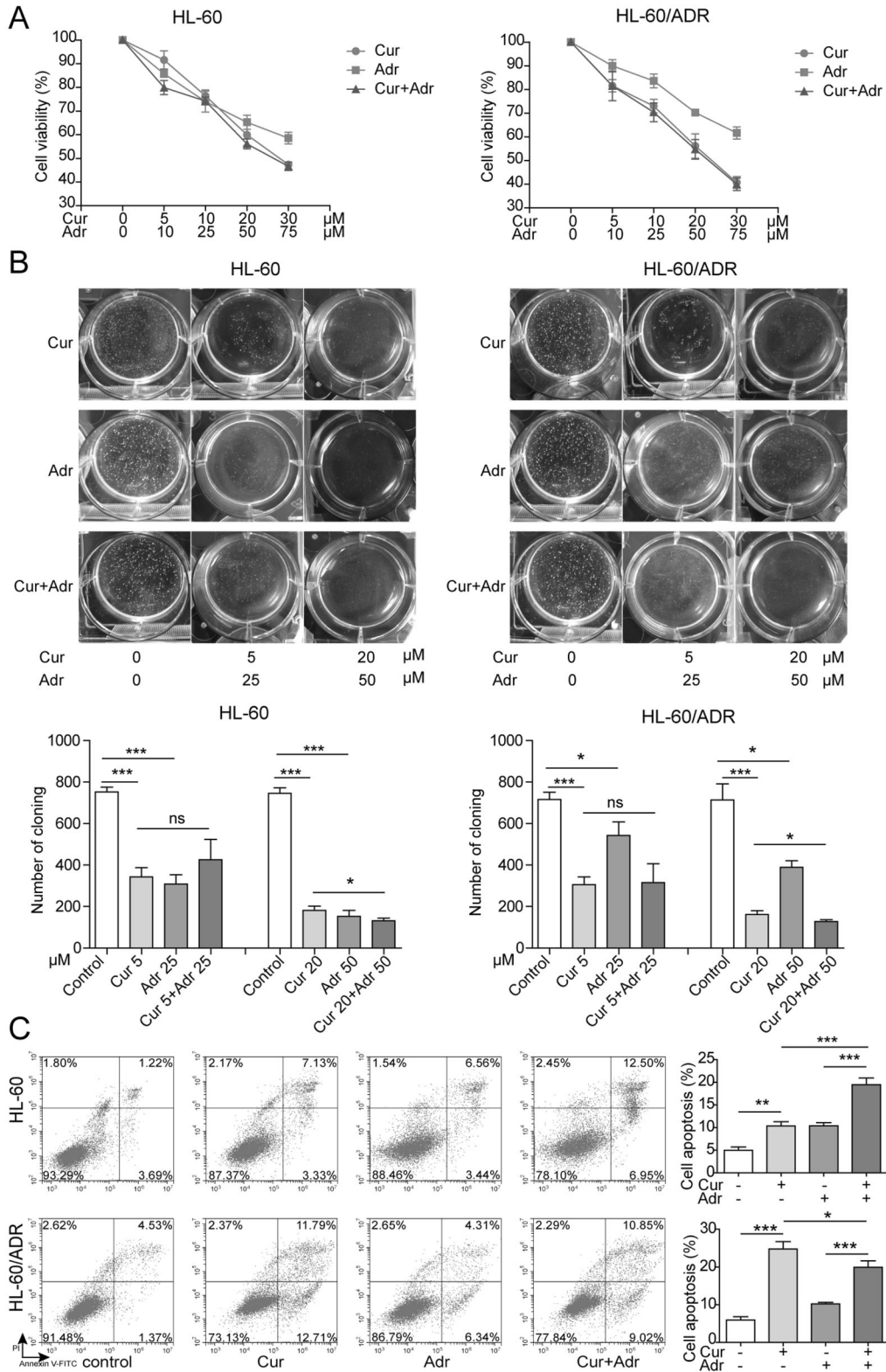


Fig. 1 Curcumin suppresses the resistance of HL-60 cells to Adriamycin. **A** An MTT assay was used to detect the viability of HL-60 or HL-60/ADR cells treated with curcumin and Adriamycin. **B** Colony-formation assay was used to assess the proliferation of HL-60 or HL-60/ADR cells treated with curcumin and Adriamycin. **C** Flow cytometry was used to measure the apoptosis of HL-60 or HL-60/ADR cells treated with curcumin and Adriamycin. The data are presented as the mean ± SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

vector or OE-HOTAIR) was injected into the dorsal skin of the mice. Twelve days after injection, the animals were injected with 20 mg/kg curcumin, 10 mg/kg Adriamycin, and a combination of curcumin and Adriamycin. The tumor size was measured once a week for 24 d. After 31 d, the mice were sacrificed by injection of 5% chloral hydrate, and the tumors were removed and collected for further analysis.

Statistical analysis

All the data are expressed as the mean \pm standard deviation (SD). The Student's *t*-test or one-way ANOVA was used to analyze all the data, $P < 0.05$ was considered to indicate statistically significant differences. All the experiments were repeated at least three times. All the statistical analyses were performed using SPSS13.0 software.

RESULTS

Curcumin suppresses the resistance of HL-60 cells to Adriamycin

In order to investigate the positive effect of curcumin in improving the sensitivity of HL-60 cells to Adriamycin, HL-60 and HL-60/ADR cells were treated with various doses of curcumin. As shown in Fig. 1A, the results of the MTT assay show that the combination of curcumin and Adriamycin effectively reduced the viability of both HL-60 and HL-60/ADR cells. Furthermore, compared with the effect of Adriamycin on HL-60 cells, the effect of Adriamycin on HL-60/ADR cells was limited. In addition, a colony-formation assay was used to further verify the effect of curcumin on cell proliferation. In both the curcumin-treated group and the Adriamycin-treated group, the number of clones decreased with increasing drug concentrations. The inhibition rate of Adriamycin on the HL-60/ADR cells was weaker than that on the HL-60 cells. Furthermore, the combination of curcumin and Adriamycin was more effective than either drug alone in inhibiting colony-formation in both HL-60 and HL-60/ADR cells (Fig. 1B). Moreover, the flow cytometry results also demonstrated that the cell apoptosis rate was increased in the combination group compared with the curcumin-treated group or the Adriamycin-treated group, and cur + Adr exerted a greater tumor-suppressive effect than cur alone in both the HL-60 and HL-60/ADR cell lines (Fig. 1C). The

present data indicate that curcumin enhanced the sensitivity of HL-60 cells to Adriamycin.

Curcumin decreases the expression of HOTAIR and WT1 and increases the expression of miR-20a-5p

RT-qPCR and Western blotting were used to assess the effects of curcumin and Adriamycin on the expression of HOTAIR, WT1, and miR-20a-5p. HOTAIR and WT1 expression was upregulated while miR-20a-5p expression was downregulated in HL-60/ADR cells compared to HL-60 cells (Fig. 2A). Compared with the control, curcumin effectively suppressed the HOTAIR and WT1 expression levels and promoted the miR-20a-5p expression levels in HL-60 cells or HL-60/ADR cells (Fig. 2B, C). The combination of curcumin and Adriamycin further decreased the HOTAIR and WT1 levels and increased the miR-20a-5p levels (Fig. 2B, C). However, the effect of Adriamycin on the HL-60/ADR cells was limited (Fig. 2C). The WT1 protein level is shown in Fig. 2D, and the Western blotting results were consistent with the RT-qPCR results. The above results suggested that curcumin suppressed HOTAIR and WT1 expression and enhanced miR-20a-5p expression.

Overexpression of HOTAIR reverses the suppression of Adriamycin resistance by curcumin via miR-20a-5p

To further reveal the role of HOTAIR in the mechanisms underlying the effects of curcumin, an RT-qPCR assay was performed to evaluate HOTAIR expression. The RT-qPCR results showed that knockdown of HOTAIR downregulated the HOTAIR level in HL-60/ADR cells (Fig. S1A). In addition, knockdown of HOTAIR inhibited cell migration and promoted apoptosis, increased Bax, cleaved caspase3 and P27 expressions, and decreased Bcl-2 expression (Fig. S1B–D). Overexpression of HOTAIR reversed the inhibitory effect of curcumin on HOTAIR and WT1 expression and eliminated the effect of curcumin in promoting miR-20a-5p expression. However, when OE-HOTAIR and miR-20a-5p mimics were cotransfected into cells, the expression levels of miR-20a-5p and WT1 exhibited opposite trends, showing that the transfection efficiencies were high (Fig. 3A). Next, a Transwell assay was performed to assess the effect of HOTAIR on cell migration. As shown in Fig. 3B, the number of migrated cells was

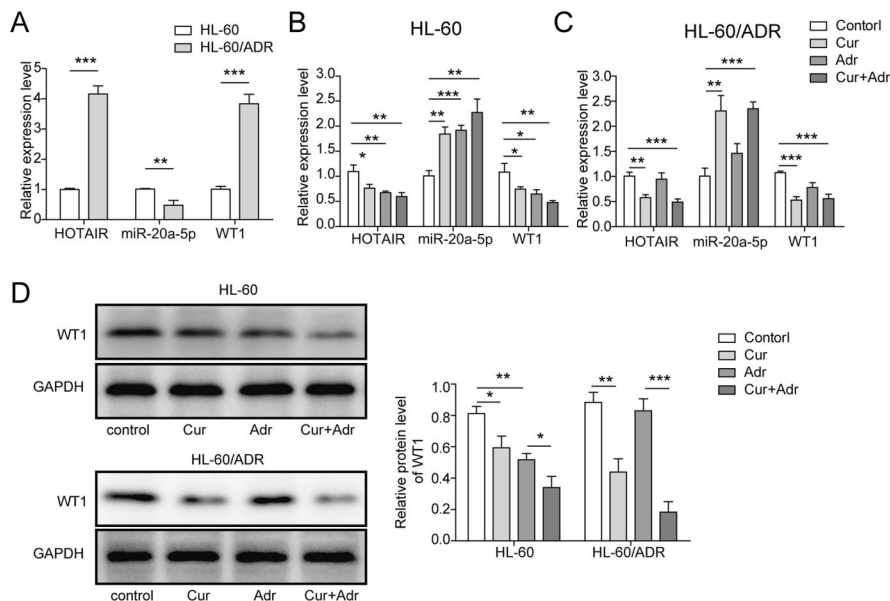


Fig. 2 Curcumin suppresses the expression of HOTAIR and WT1 and promotes the expression of miR-20a-5p. **A** RT-qPCR assay was used to determine the expression levels of HOTAIR, WT1, and miR-20a-5p in HL-60/ADR cells. **B, C** RT-qPCR assay was used to detect the HOTAIR, miR-20a-5p, and WT1 expression levels in HL-60 or HL-60/ADR cells treated with or without curcumin and Adriamycin. **D** Western blotting was used to assess WT1 expression at the protein level. The data are presented as the mean \pm SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

decreased in the curcumin group but increased in OE-HOTAIR + cur group; however, cell migration was reduced in the OE-HOTAIR + cur + miR-20a-5p mimics group. The flow cytometry results demonstrated that curcumin promoted the apoptosis of HL-60/ADR cells. Furthermore, overexpression of HOTAIR reversed the apoptosis-inducing effect of curcumin, but overexpression of miR-20a-5p reversed this effect (Fig. 3C). Western blotting was used to detect the expression levels of apoptosis- and cell cycle-related proteins. The Bax, cleaved caspase3, and P27 levels were increased in curcumin-treated cells, while the Bcl-2 levels were decreased (Fig. 3D). In addition, these alterations were reversed after overexpression of HOTAIR, but miR-20a-5p mimics restored the effect of HOTAIR overexpression (Fig. 3D). These results suggested that HOTAIR reversed the effect of curcumin on HL-60/ADR cells by targeting miR-20a-5p.

HOTAIR positively regulates the expression of WT1 by targeting miR-20a-5p

By bioinformatics analysis, we predicted the relationship among HOTAIR, miR-20a-5p, and WT1. As shown in Fig. 4A, both HOTAIR transcripts bind to miR-20a-5p, and WT1 was a target gene of miR-20a-5p. Furthermore, we further performed a dual-luciferase reporter assay to verify this hypothesis. The co-transfection of WT-HOTAIR or WT-WT1 with miR-20a-5p mimics significantly

decreased the luciferase activities, while no significant alteration was observed after the co-transfection of HOTAIR-MUT or WT1-MUT with miR-20a-5p mimics (Fig. 4B). To further verify this theory, we performed RT-qPCR to investigate the regulatory relationship among HOTAIR, WT1, and miR-20a-5p. As shown in Fig. 4C, overexpression of HOTAIR inhibited miR-20a-5p expression, and the level of WT1 was downregulated in the cells transfected with the miR-20a-5p mimics but upregulated in the cells transfected with the miR-20a-5p inhibitor (Fig. 4C). Western blotting was used to further assess WT1 expression at the protein level. As shown in Fig. 4D, WT1 expression was decreased when miR-20a-5p was upregulated but increased when miR-20a-5p was downregulated, which was consistent with the RT-qPCR results. Based on the results described above, we verified that HOTAIR bound to miR-20a-5p and thus increased WT1 expression.

Curcumin inhibits the resistance of leukemia cells to Adriamycin by regulating the miR-20a-5p/WT1 axis

To investigate the effect of curcumin on Adriamycin resistance through the miR-20a-5p/WT1 axis, RT-qPCR was performed. The results showed that WT1 expression was suppressed in HL-60/ADR cells treated with sh-WT1 (Fig. 5A). Furthermore, overexpression of miR-20a-5p or knockdown of WT1, inhibited cell migration and induced cell apoptosis, while promoting the expressions of Bax,

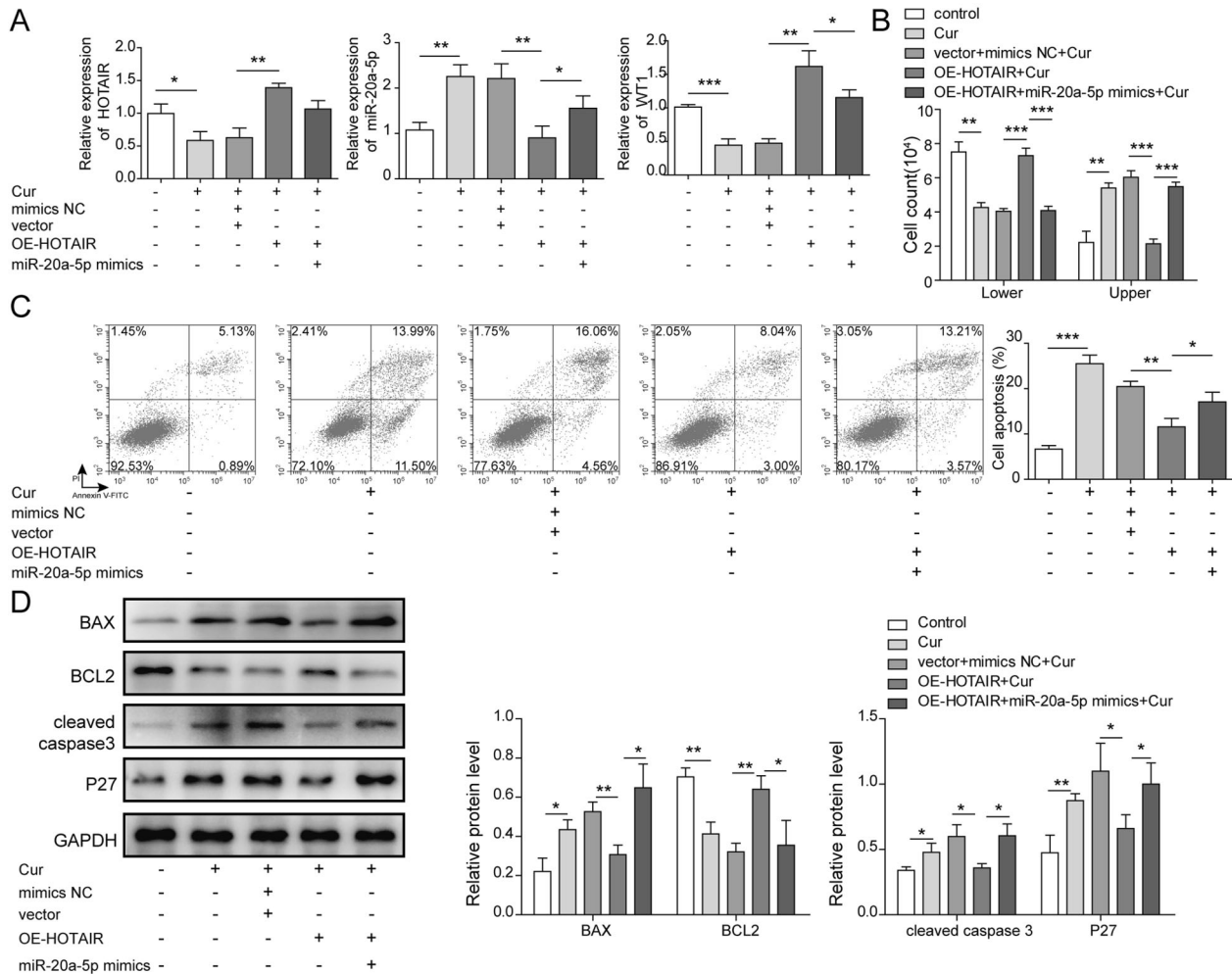


Fig. 3 Overexpression of HOTAIR reverses the suppression of Adriamycin resistance by curcumin by targeting miR-20a-5p. HL-60/ADR cells were treated with curcumin. Then, the cells were divided into four groups: the cur, cur+NC, OE-HOTAIR+cur, and OE-HOTAIR+cur+miR-20a-5p mimics groups. **A** RT-qPCR was used to detect the HOTAIR, miR-20a-5p, and WT1 expression levels. **B** Transwell assay was used to assess cells migration. **C** Flow cytometry was used to measure cell apoptosis. **D** Western blotting was used to analyze the expression level of apoptosis-related proteins. The data are presented as the mean ± SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

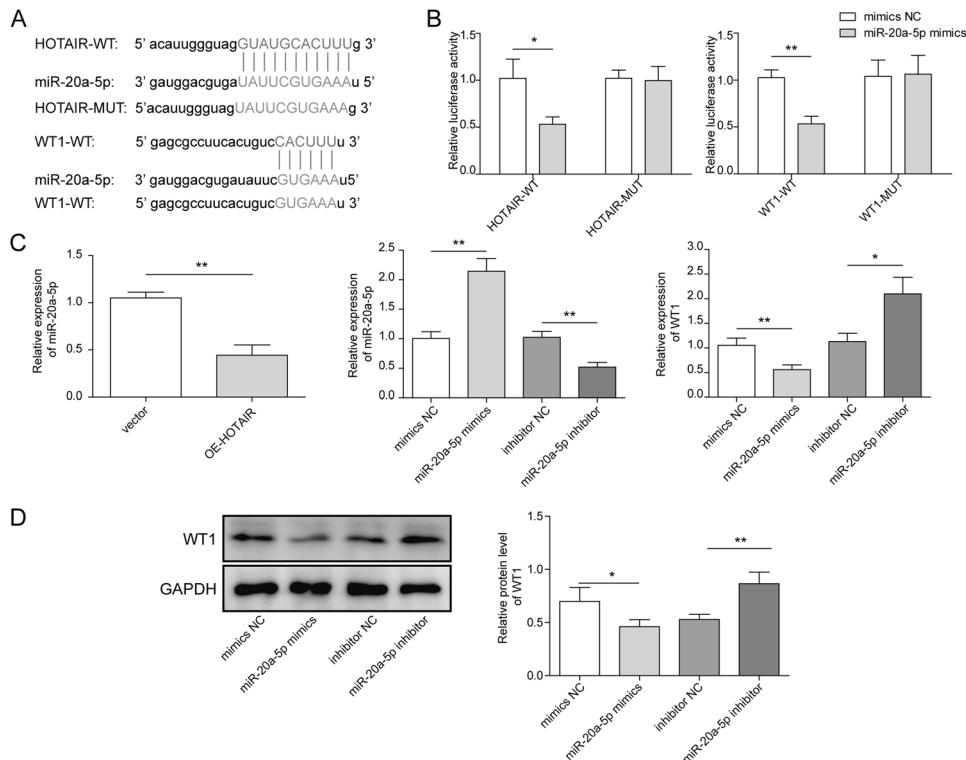


Fig. 4 HOTAIR positively regulates the expression of WT1 by targeting miR-20a-5p. **A** Bioinformatics analysis was used to predict binding sites between miR-20a-5p and HOTAIR or WT1. **B** A luciferase reporter assay was used to detect the relationship between miR-20a-5p and HOTAIR or WT1. **C** RT-qPCR assay was used to determine the expression of miR-20a-5p and WT1. **D** Western blotting was used to analyze WT1 expression. The data are presented as the mean \pm SD. * $P < 0.05$, ** $P < 0.01$.

cleaved caspase3, and P27 and inhibiting the expression of Bcl-2 (Figs. S2, S3). Compared with the curcumin-treated group, the group treated with the miR-20a-5p inhibitor and curcumin exhibited decreased miR-20a-5p expression, while WT1 expression increased (Fig. 5B). Then, Transwell assays were used to detect the effects of curcumin and miR-20a-5p treatment on cell migration. As shown in Fig. 5C, curcumin significantly suppressed the migration of HL-60/ADR cells, while downregulated miR-20a-5p expression reversed this effect, and the inhibitory effect of curcumin was also observed in the miR-20a-5p inhibitor + sh-WT1 + cur group. The flow cytometry results suggested that curcumin promoted cell apoptosis, but the cell apoptosis rate was decreased in the miR-20a-5p inhibitor + cur group but was increased in the miR-20a-5p inhibitor + sh-WT1 + cur group (Fig. 5D). Finally, the Western blotting results indicated that curcumin promoted the expression of Bax, cleaved caspase3, and P27 but suppressed the expression of Bcl-2; however, knockdown of miR-20a-5p restored the promoting effect of curcumin (Fig. 5E). The results described above indicated that curcumin inhibited the resistance of leukemia cells to Adriamycin by regulating the miR-20a-5p/WT1 axis.

Curcumin suppresses the resistance of leukemia cells to Adriamycin by inhibiting the HOTAIR/miR-20a-5p/WT1 axis in vivo

To further investigate the biofunction of curcumin and HOTAIR in vivo, we performed a tumor formation experiment in nude mice. As shown in Fig. 6A–C, overexpression of HOTAIR increased the growth rate and size of the tumors. Either curcumin or Adriamycin alone inhibited the tumor growth rate, and cur + Adr exerted the greatest suppressive effect on tumor growth. Moreover, overexpression of HOTAIR reversed the tumor-suppressive effects of curcumin and Adriamycin. The expression of HOTAIR,

miR-20a-5p, and WT1 in the tumors was assessed by RT-qPCR. As shown in Fig. 6D, transfection with the empty vector did not affect HOTAIR, miR-20a-5p, or WT1 expression. However, curcumin significantly decreased the expression of HOTAIR and WT1 and increased the expression of miR-20a-5p. HOTAIR overexpression combined with curcumin or Adriamycin treatment promoted HOTAIR and WT1 expression but decreased miR-20a-5p expression. The above results indicated that curcumin inhibited the Adriamycin resistance of leukemia cells in vivo by inhibiting the HOTAIR/miR-20a-5p/WT1 axis.

DISCUSSION

The current mainstream methods for treating AML are chemotherapy and haematopoietic stem cell transplantation. The prognosis of AML is not satisfactory, and the 5-year survival rates are 35–40% and 5–15% in patients under and over 60 years of age, respectively [5, 26]. Current AML treatments can also cause several side effects due to the use of high-dose cytarabine, which markedly impairs patients' quality of life [27]. Due to the frequent occurrence of drug resistance and tumor heterogeneity, targeted drugs, such as imatinib, cannot exhibit good efficacy in AML patients [28]. Therefore, novel and potent treatments for AML are still urgently required. In addition, the HL-60 cell line was derived from a patient with acute promyelocytic leukemia. Compared with other leukemia cells (K562), HL-60 cells can continuously replicate in a university culture medium. Moreover, a study showed that HL-60 cells can form localized tumors in nude mice, indicating that HL-60 cells are carcinogenic in vivo [29, 30]. In our study, we found that curcumin decreased the HOTAIR and WT1 levels in HL-60 cells and increased the miR-20a-5p levels; thus, curcumin inhibited Adriamycin resistance in AML by suppressing the HOTAIR/miR-20a-5p/WT1 pathway in vitro and in vivo (Fig. 7).

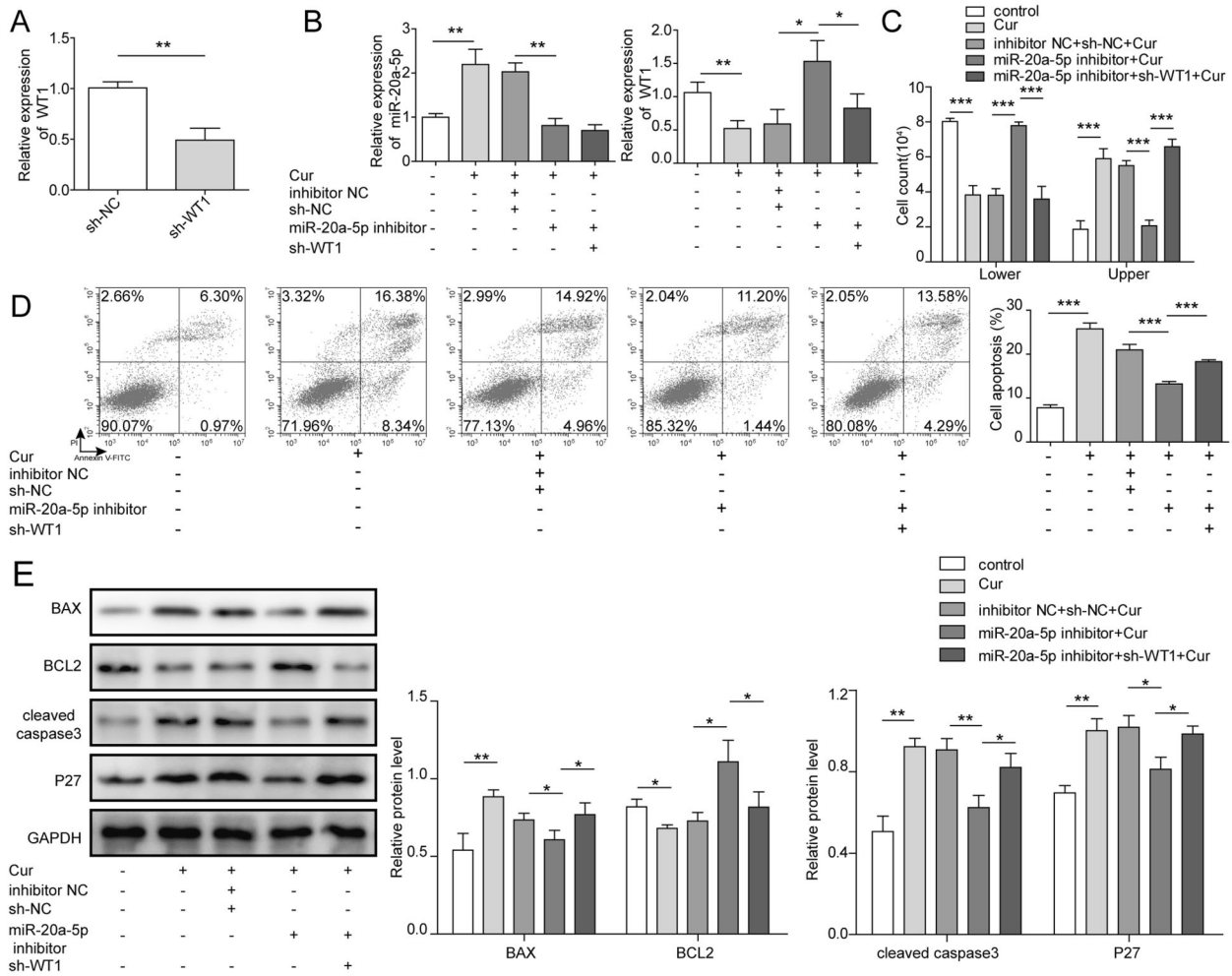


Fig. 5 Curcumin inhibits the resistance of leukemia cells to Adriamycin by regulating the miR-20a-5p/WT1 axis. HL-60/ADR cells were treated with curcumin and then divided into four groups: the cur, cur+NC, cur+miR-20a-5p inhibitor, and cur+miR-20a-5p inhibitor+sh-WT1 groups. **A** The expression of WT1 was detected by RT-qPCR. **B** RT-qPCR assay was used to determine the expression of miR-20a-5p and WT1. **C** Transwell assay was used to assess cell migration. **D** Flow cytometry was used to measure cell apoptosis. **E** Western blotting was used to detect the expression of Bax, cleaved caspase3, P27, and bcl-2. The data are presented as the mean \pm SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Traditional Chinese herbs have been widely studied for the treatment of several diseases, especially cancers [31, 32]. Curcumin, the active flavonoid component of the traditional Chinese herb *Curcuma longa*, has been reported to exert anti-tumor effects and to decrease drug resistance in multiple malignancies [33]. Here, we found that curcumin suppressed malignant phenotypes, including the inhibition of cell proliferation and the promotion of apoptosis, and especially attenuated the resistance of HL-60 cells to Adriamycin. Previous studies proposed that curcumin suppresses cancer progression via various mechanisms; for instance, Pei et al. reported that curcumin suppressed HOTAIR expression in renal cell carcinoma cells [34]. In our study, we found that curcumin suppressed the expression of HOTAIR in AML cells, which led us to consider whether HOTAIR is involved in the bioactivities by which curcumin inhibits drug resistance.

In recent years, lncRNAs have been found to play crucial roles in the pathogenesis of several malignant diseases [35, 36]. HOTAIR has been widely studied in several malignancies. In AML, HOTAIR was upregulated and associated with poor prognosis [19]. In addition, HOTAIR exerts pro-cancer effects via multiple mechanisms. Knockdown of HOTAIR could inhibit cell proliferation and promote cell apoptosis by decreasing DNA methyltransferase 3b (Dnmt3b) expression, thus leading to the demethylation of HOXA5

[37]. A study found that knockdown of HOTAIR inhibited the proliferation of HL-60 cells [19]. A previous study reported that knockdown of HOTAIR significantly increased the sensitivity of NCI-H1299/DDP cells by regulating the Wnt pathway [38]. Similar to the present results, our study found that overexpression of HOTAIR promoted cell proliferation and reduced cell apoptosis, suggesting that overexpression of HOTAIR reversed the inhibitory effect of curcumin on Adriamycin resistance in AML and that HOTAIR induced the development of AML.

MiRNAs are involved in the occurrence and development of various diseases, such as cancer. Overexpression of miR-582-3p inhibited cell proliferation and blocked cell cycle progression in AML [39]. Furthermore, it was reported that miR-20a-5p acted as a tumor suppressor to regulate cell progression [23]. In addition, miR-20a increased the chemosensitivity of osteosarcoma to multiple drugs by targeting SDC2 [40]. RNA absorption is the mechanism by which lncRNAs regulate gene expression. In the present study, we verified that HOTAIR could bind miR-20a-5p and that miR-20a-5p targeted the 3'UTR of WT1. Moreover, we found that the miR-20a-5p/WT1 axis played a crucial role in the mechanism underlying drug resistance in AML.

The WT1 gene has been reported to be upregulated by over 1000-fold in leukemia cells compared to normal cells, and a large

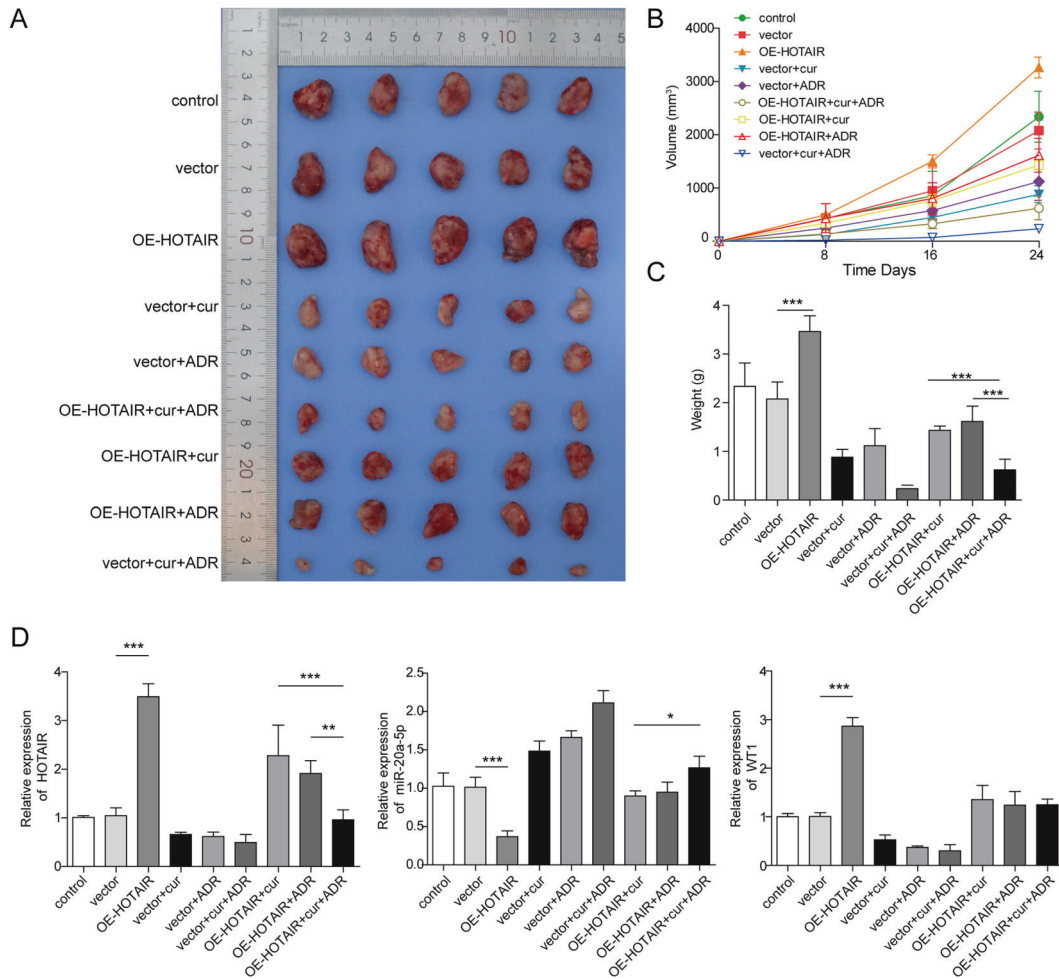


Fig. 6 Curcumin suppresses the resistance of leukemia cells to Adriamycin by inhibiting the HOTAIR/miR-20a-5p/WT1 axis in vivo. **A** Representative images after tumor transplantation are shown. **B** Tumor size was measured every week. **C** Tumor weight was detected in every group. **D** RT-qPCR was used to detect the expression of HOTAIR, miR-20a-5p, and WT1. The data are presented as the mean ± SD. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

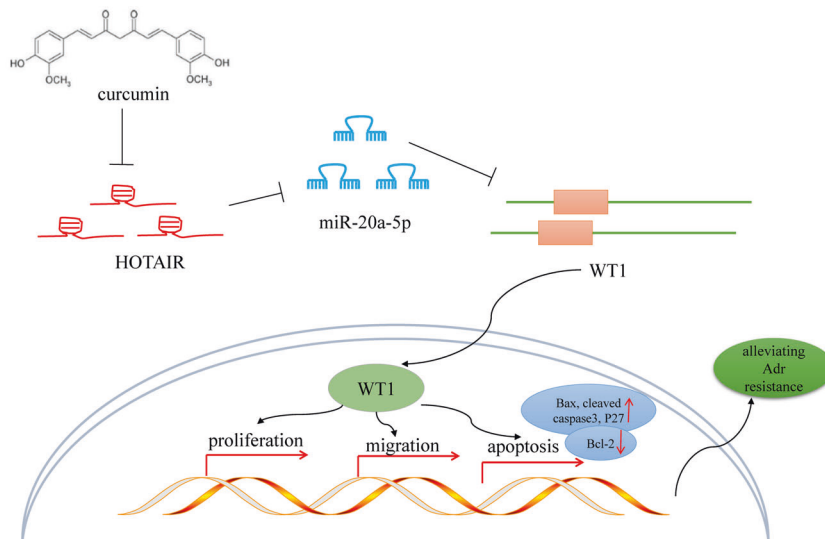


Fig. 7 The schematic diagram of this study. Curcumin attenuates the resistance of Adriamycin in acute myeloid leukemia by inhibiting cell proliferation and promoting apoptosis by mediating HOTAIR/miR-20a-5p/WT1 axis.

number of studies suggested that its high expression was associated with faster disease progression and shorter survival [41–43]. Glienke et al. suggested that silencing WT1 significantly decreased the proliferation of leukemia cells, which was consistent with our results [44]. A study found that WT1 was involved in the resistance of non-small-cell lung cancer to cisplatin [45]. Moreover, WT1 enhanced the resistance of AML to imitinib [46]. We also observed this phenomenon in AML cells. Moreover, curcumin has been found to decrease WT1 expression, but the underlying mechanism remains unclear [44, 47]. miR-15a/16-1 played an important role in the curcumin-mediated inhibition of HL-60 cell growth by mediating the downregulation of WT1 expression [48]. In our study, we proposed a potential regulatory network among HOTAIR, miR-20a-5p, and WT1. A series of phenotypic and molecular experiments have supported this theory. In the present study, we found that curcumin inhibited the WT1 levels in HL-60 or HL-60/ADR cells and that inhibition of miR-20a-5p by increased WT1 expression attenuated the effect of curcumin on the resistance of leukemia cells to Adriamycin, indicating that curcumin inhibited the resistance of tumor cells to Adriamycin by inhibiting the HOTAIR/miR-20a-5p/WT1 axis.

In conclusion, our present study suggested that curcumin enhanced the sensitivity of AML cells to Adriamycin, can suppress proliferation and migration, and block cell cycle progression. However, overexpression of HOTAIR or inhibition of miR-20a-5p expression attenuated the inhibitory effect of curcumin on Adriamycin resistance in AML. The effect of curcumin on the HOTAIR/miR-20a-5p/WT1 axis contributes to its tumor-suppressive effects. Therefore, curcumin is a potential treatment for AML, and HOTAIR may also be a therapeutic target.

DATA AVAILABILITY

All data generated or analyzed during this study are included in this article.

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AUTHOR CONTRIBUTIONS

Guarantor of integrity of the entire study: J-ML, study concepts: J-ML, study design: J-ML, definition of intellectual content: J-ML, literature research: WL, experimental studies: ML, data acquisition: ML, data analysis: J-ML, ML, statistical analysis: J-ML, paper preparation: J-ML, paper editing: J-ML, paper review: H-BS.

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COMPETING INTERESTS

The authors declare no competing interests.

ETHICAL APPROVAL

The study was approved by the Animal Ethics Committee of the People's Hospital of Longhua District.

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