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RESEARCH PAPER

Abnormal regulation of miR-29b-ID1 signaling is involved in the process of decitabine resistance in leukemia cells

Jichun Ma[a,b,c*], Xiangmei Wen[a,b,c*], Zijun Xu[a,b,c*], Peihui Xia[a,b,c], Ye Jin[b,c,d], Jiang Lin[a,b,c] and Jun Qian[b,c,d]

[a]Department of central lab, Affiliated People's Hospital of Jiangsu University, Zhenjiang, Jiangsu, China; [b]Zhenjiang Clinical Research Center of Hematology, Affiliated People's Hospital of Jiangsu University, Zhenjiang, Jiangsu, China; [c]The Key Lab of Precision Diagnosis and Treatment in Hematologic Malignancies of Zhenjiang City, Affiliated People's Hospital of Jiangsu University, Zhenjiang, Jiangsu, China; [d]Department of Hematology, Affiliated People's Hospital of Jiangsu University, Zhenjiang, Jiangsu, China

ABSTRACT

Decitabine (DAC) is an inhibitor of DNA methyltransferase used to treat leukemia, but primary or secondary resistance to DAC may develop during therapy. The mechanisms related to DAC resistance remain poorly understood. In this study, we find that miR-29b expression was decreased in various leukemia cell lines and AML patients and was associated with poor prognosis. In DAC-sensitive cells, miR-29b inhibited cell growth, promoted apoptosis, and increased the sensitivity to DAC. Similarly, it exerted anti-leukemic effects in DAC-resistant cells. When the miR-29b promoter in DAC-resistant cells was demethylated, its expression was not up-regulated. Furthermore, the expression of ID1, one of the target genes of miR-29b, was down-regulated in miR-29b transfected leukemic cells. ID1 promoted cell growth, inhibited cell apoptosis, and decreased DAC sensitivity in leukemic cells in vitro and in vivo. ID1 was down-regulated in DAC-sensitive cells treated with DAC, while it was up-regulated in DAC-resistant cells. Interestingly, the ID1 promoter region was completely unmethylated in both DAC-resistant cells and sensitive cells before DAC treatment. The growth inhibition, increased DAC sensitivity, and apoptosis induced by miR-29b can be eliminated by increasing ID1 expression. These results suggested that DAC regulates ID1 expression by acting on miR-29b. Abnormal ID1 expression of ID1 that is methylation independent and induced by miR-29b may be involved in the process of leukemia cells acquiring DAC resistance.

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Decitabine; drug resistance; MiR-29b; ID1; acute myeloid leukemia

1. Introduction

Acute myeloid leukemia (AML) is a group of malignant clonal diseases of hematopoietic system and the most common hematological malignant tumor [1,2]. Leukemia cells excessively proliferate in hematopoietic tissues such as bone marrow, which inhibits normal hematopoiesis [2,3]. AML pathogenesis involves many abnormal changes such as cell differentiation, proliferation, apoptosis, and chromosome and genome instability [4–8]. In addition, epigenetic modifications such as microRNA (miRNA) and DNA methylation expression play an important role in AML pathogenesis and progression [9–13]. Disease development is characterized by numerous hypermethylation changes of tumor suppressor genes, which are related to DNA methyltransferases (DNMTs) [14–16]. The DNMT inhibitors 5-azadeoxycytidine and 5-aza-2-deoxycytidine (decitabine, DAC) can replace cytosine to covalently bind to and inactivate DNMTs, which reactivates tumor suppressor genes silenced by methylation, and eventually exert an anti-tumor role by restoring tumor cells to normal terminal differentiation, aging, or apoptosis [17,18].

Decitabine has become one of the important drugs for patients with AML and myelodysplastic syndrome [19–22]. However, some patients develop primary or secondary resistance during treatment, which leads to treatment failure and disease progression. Reducing or delaying the occurrence of drug resistance is a challenge in the clinical treatment of leukemia that has become an important area of research [23–25]. However, the mechanism of DAC resistance is

CONTACT Jun Qian qianjun0007@hotmail.com Department of Hematology, Affiliated People's Hospital of Jiangsu University, Dianli RD. 8, Zhenjiang 212002, China; Jiang Lin 2651329493@qq.com Department of central lab, Affiliated People's Hospital of Jiangsu University, Dianli RD. 8, Zhenjiang, Jiangsu 212002, China
*These authors contributed equally to this work.

not clear. Jia Yu reported decreased of TRAF6 blocked decitabine-induced DNMT degradation, leading to decitabine resistance in triple-negative breast cancer [26]. Knockdown of TBC1D16 decreased cell proliferation and ERK phosphorylation levels, as well as increased sensitivity to decitabine in AML cells [27]. SAMHD1 expression inversely correlates with response to decitabine in leukemic cells and SAMHD1 ablation could increase the anti-leukemic activity of decitabine [28].

We previously established a DAC-resistant leukemic cell line (K562/DAC) and characterized its expression profile [29]. The results showed some genes and miRNAs with significantly different expressions compared with DAC-sensitive cells; miR-29b was down-regulated, and inhibitor of DNA binding 1 (ID1) expression was up-regulated in K562/DAC cells. MiR-29b plays a role as an anti-oncogene in many types of tumors including leukemia [30–33]. ID1 is a target gene of miR-29b [34]; a negative regulator of the helix-loop-helix (HLH) transcription factor; and plays an oncogenic role in promoting cell cycle, proliferation, migration and inhibiting apoptosis [35–38]. This evidence led us to assess the role of miR-29b-ID1 signaling in DAC drug resistance. In this study, we investigated miR-29b expression in AML patients, evaluated the clinical significance of miR-29b expression, and explored the role of miR-29b-ID1 in DAC resistance from an epigenetic perspective.

2. Materials and methods

2.1. Samples

The Ethics Committee of Affiliated People's Hospital of Jiangsu University approved this study, including the use of samples from de novo AML patients and healthy controls, approved this study. 25 healthy controls and 102 AML patients came from the sample bank of our hospital and all patients signed the informed consent form. The diagnosis of AML was made according to World Health Organization criteria and the French-American-British classification. Bone marrow (BM) mononuclear cells were extracted from BM specimens used gradient centrifugation (TBD Sciences, China).

2.2. BALB/C nude mice

The Experimental Animal Management and Use Committee of Jiangsu University approved animal studies. Twenty 6-week-old female specific pathogen-free female BALB/c nude mice weighing (20 ± 2) g were purchased from Shanghai Slack Experimental Animal Co., Ltd. with license number of SCXK (Shanghai, China) 2017–0005. All mice keep in specific pathogen free. Cells injected subcutaneously on the back of mice. The weight of mice and the volume of tumors measured every three days. Sacrifice of mice abides by the ethical principles of welfare of experimental animals. Volume = $0.5 \times \text{length} \times \text{width}^2$

2.3. Cell culture and transfection

Human leukemia cell lines K562, THP-1, HL60, and U937 were purchased from the American Type Culture Collection (ATCC). The K562/DAC line was constructed in our laboratory [29]. Cells were cultured in 10% fetal calf serum (ExCell Bio, Shanghai, China) and 100 U/ml penicillin/streptomycin (Wisent, Nanjing, China) in RPMI 1640 medium (Wisent, Nanjing, China) at 37°C in a 5% CO_2 humidified atmosphere. Lentiviruses over-expressing ID1 or silencing ID1, lentivirus over-expressing miR-29b, and inhibitor of miR-29b were purchased from Shanghai Jikai Biological Co., Ltd. (Shanghai, China). Cell transfection was performed according to the manufacturer's instructions. In brief, 5×10^5 cells were cultured in 12-well plates containing 10% serum and 10 μl lentivirus was added to each well. The supernatant was removed by centrifugation, and a new culture medium containing 10% serum was added after 24 hours. The puromycin was added after 48 hours of transfection. After sorting by flow cytometry, the sorted cells were used in subsequent experiments.

2.4. Cell growth assays

Cells (3×10^3) were distributed equally into 96-well plates. After culture for 0, 24, 48, and 72 h, 10 μl CCK-8 (Dojindo, Kumamoto, Japan) reagent was added to each well. The optical density (OD) value was measured at 450 nm of the absorbance using a microplate reader to analyze cell growth.

2.5. Half maximal inhibitory concentration (IC50) detection

Cells (1×10^4) cells were seeded onto a 12-well plate containing complete culture solution and different concentrations of DAC (0, 0.125, 0.25, 0.5, 1, and 2 μM). DAC was added every 24 hours for 3 days, and the number of viable cells was counted by trypan blue staining on day 4.

2.6. Cell apoptosis assays

Cells (5×10^5) cells were seeded in 6-well plates containing complete 1640 culture solution without fetal bovine serum for 48 h. The apoptosis rate was detected with an apoptosis detection kit (Annexin V PE/7-AAD, 559763, BD Biosciences, Franklin Lakes, NJ, USA), and then analyzed by flow cytometry on a FACSCalibur platform (Becton Dickinson, San Jose, CA, USA).

2.7. Western blot analysis

Western blotting was conducted as previously described [39]. The antibodies used in this investigation were anti-ID1 (Abcam, Cambridge, UK), anti-GAPDH (BOSTER, Wuhan, China), and anti-β-actin (Fcmacs Biotech Co., Ltd., Nanjing, China).

2.8. RNA extraction, reverse transcription, and real-time quantitative polymerase chain reaction (qRT-PCR)

RNA extraction and reverse transcription were conducted based on the miScript kits instructions (Qiagen, Tilden, Germany). Reverse transcription and qRT-PCR was conducted as previously reported [40]. The forward primer for miR-29b was 5”- TAGCACCATTTGAAATCAGTGTT –3‘ and the reverse universal primer was provided by the manufacturer. The primers for ID1 were 5’-CTCCATCATGCCAAGTTCTGC-3‘ (forward) and 5’-GAAGGC AGGCAAGACTCGAA-3‘ (reverse), respectively. The primers used as controls were ABL and sequences were 5’-TCCTCCAGCTGTTATCTGGAAGA –3‘(forward) and 5’- TCCAACGAGCGGCTTCAC –3” (reverse).

2.9. Bisulfite sequencing PCR (BSP)

Genomic DNA was isolated and modified according to the genomic DNA Purification Kit manufacturer’s instructions (Gentra Systems Inc., Minneapolis, MN, USA). The primers for the methylated miR-29b promoter were 5”-TAGTAG TGG TTG TTT GTT TTT TTG A-3‘ (forward) and 5’-CCA CTC TAC TAA AAA CTC CAT CTC C-3” (reverse). BSP was conducted as previously reported [37].

2.10. Statistical analysis

Data analysis was performed using SPSS 20.0 software (IBM Corp., Armonk, NY, USA) and GraphPad Prism 5 software (GraphPad Inc., San Diego, CA, USA). Relative levels of miR-29b expression were calculated with the $2^{-\Delta\Delta CT}$ method. Categorical variables were analyzed using chi square tests and/or Fisher’s exact tests. The diagnostic value of gene expression was analyzed using receiver operating characteristic (ROC) curves and areas under the curve (AUCs). Survival was analyzed by Kaplan-Meier. Univariate and multivariate were analyzed by Cox regression analyses. The experiments were repeated at least three times. The measurement date are presented as mean ± SD. Differences in continuous variables between two groups were compared by Student’s t-tests. IC50 values were calculated by Probit regression analysis. Differences were considered statistically significant for two-tailed $P < 0.05$.

3. Results

3.1. miR-29b expression in AML and its clinical significance

The median levels of miR-29b expression in 25 healthy controls and 102 AML patients were 0.1995 and 0.0667, respectively. miR-29b expression was significantly lower in subjects with AML compared with controls ($P < 0.001$, Figure 1a). Moreover, ROC curve analysis suggested that miR-29b might be a potential biomarker for distinguishing AML ($n = 102$) and cytogenetically normal AML (CN-AML, $n = 45$) from subjects with controls (Figure 1b, c).

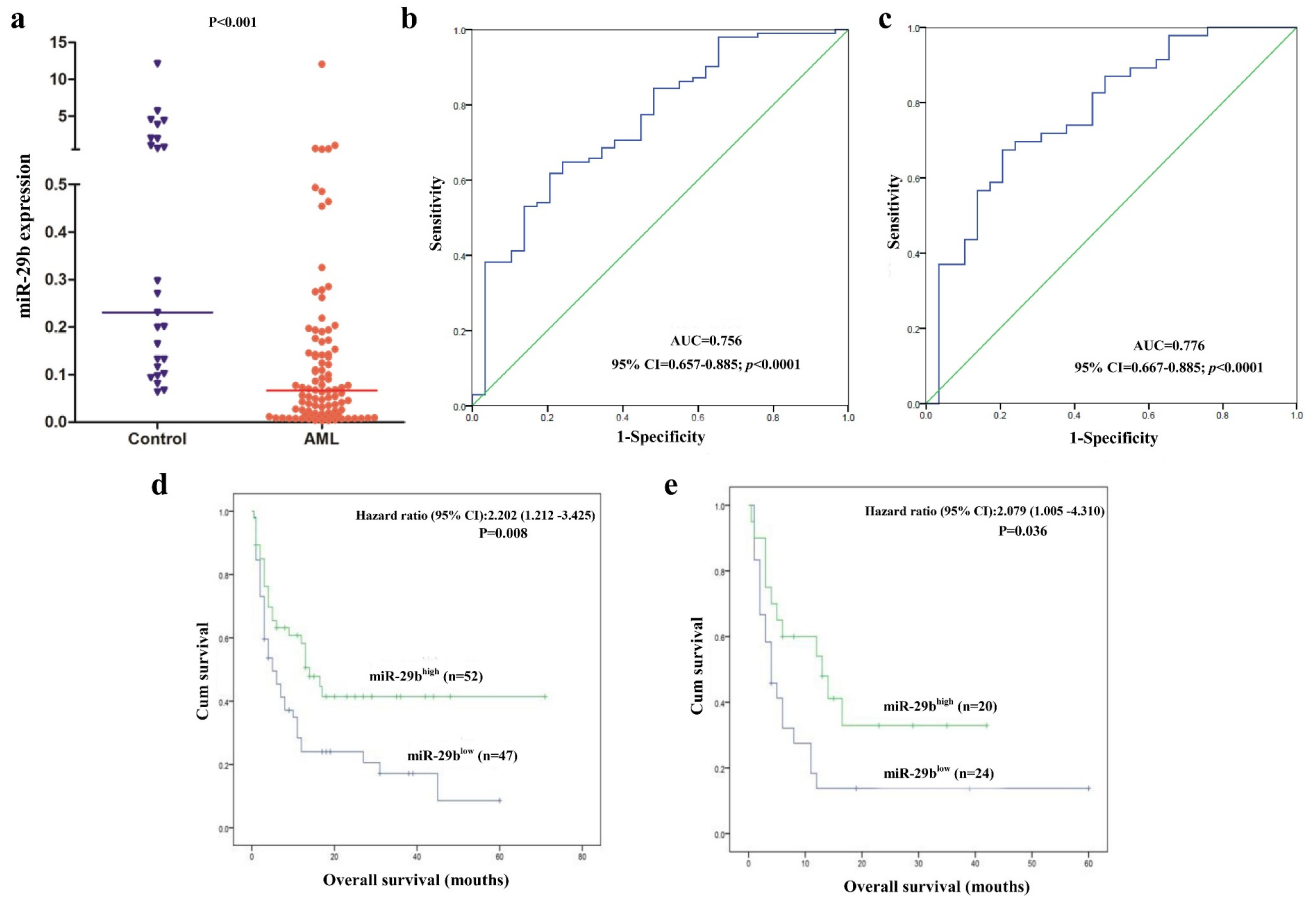

Figure 1. MiR-29b expression in AML patients and its impact on OS. (a) miR-29b expression levels in controls and AML patients were detected by qRT-PCR. (b) Discriminative capacity of miR-29b expression in AML patients by ROC curve analysis. (c) Discriminative capacity of miR-29b expression in CN-AML patients by ROC curve analysis. (d) Prognostic value of miR-29b expression in all AML patients. (e) Prognostic value of miR-29b expression in CN-AML patients.

In order to analyze the associations of miR-29b expression with clinical characteristics of AML, patients were divided into miR-29b ^{high} and miR-29b^{low} groups, according to miR-29b expression at the cutoff value of 0.0084 (sensitivity is 60% and specificity is 80%) based on the ROC curve. The clinical parameters of both two groups are shown in Table 1. White blood cell (WBC) counts in peripheral blood samples from the miR-29b^{high} group were significantly higher than that of miR-29b^{low} group ($P = 0.022$). No significant differences were observed in other parameters ($P > 0.05$).

The survival analysis of 99 patients with available data showed that there was no significant difference in complete remission (CR) rates between these two groups ($P = 0.533$). However, the overall survival (OS) of miR-29b^{low} patients (median 4.5 months, range 1.0–60.0 months) was significantly shorter than that of miR-29b^{high} patients (median 12.0 months, range 1.0–71.0 months) ($P = 0.008$, Figure 1d). The OS of miR-29b^{low} patients (median 4.0 months, range 1.0–60.0 months) was also significantly shorter than that of miR-29b^{high} patients (median 12.0 months, range 1.0–42.0 months) in CN-AML ($P = 0.036$, Figure 1e).

Variables in the univariate analysis with $P < 0.2$ (age, sex, WBC, hemoglobin, gene mutations, and miR-29b expression) were included in the multivariate analysis, which showed that miR-29b expression might be an independent prognostic event affecting patients' survival in all AML ($P = 0.042$) and CN-AML ($P = 0.037$, Tables 2, 3).

Table 1. Correlation between miR-29b expression and patients parameters.

	Status of miR-29b expression		
Patient's parameters	Low ($n = 54$)	High ($n = 48$)	*P* value
Sex, male/female	34/20	26/22	0.841
Age, median (range), yearsΔ	56 (18–93)	57 (19–87)	0.483
WBC, median (range), $\times 10^9$/LΔ	16.3 (1.1–528.0)	6.8(0.3–185.4)	0.022
Hemoglobin, median (range), g/LΔ	75 (34–138)	74 (32–113)	0.355
Platelets, median (range),$\times 10^9$/LΔ	36 (3–447)	40(4–399)	0.564
BM blasts, median (range), %Δ	49.5 (6.5–97.5)	39 (1.0–92)	0.172
FAB classification			0.134
M0	1	0	
M1	4	5	
M2	27	20	
M3	4	11	
M4	11	9	
M5	7	2	
M6	0	1	
WHO classification			0.195
t(8;21)	8	4	
t(15;17)	4	10	
AML without maturation	4	4	
AML with maturation	20	16	
Acute myelomonocytic leukemia	12	10	
Acute monoblastic and monocytic leukemia	6	1	
Acute erythroid leukemia	0	1	
No data	0	2	
Risk classification			0.596
Low	12	16	
Intermediate	35	23	
High	7	5	
No data	0	4	
Karyotypes			0.183
normal	25	20	
t(8;21)	8	6	
t(15;17)	4	10	
11q23	1	0	
complex	6	5	
others	10	3	
No data	0	4	
Gene mutations *			
C-KIT (±)	1/51	2/43	0.595
FLT3 (±)	7/45	6/39	1.000
NPM1(±)	6/46	4/41	0.748
C/EBPA (±)	7/45	5/40	0.767
N/K-RAS (±)	3/45	4/40	0.706
IDH1/2 (±)	2/46	4/40	0.421
DNMT3A (±)	7/41	2/42	0.162
U2AF1 (±)	1/47	4/40	0.189
CR(±)	34/19	25/19	0.533

WBC, white blood cells; FAB, French-American-British classification; AML, acute myeloid leukemia ; CR, complete remission.

3.2. Expression and methylation of miR-29b in leukemia cells

As DAC is a demethylation drug, we detected miR-29b expression and methylation with qRT-PCR and BSP. Expression of miR-29b was gradually increased in DAC-sensitive K562 cells after treatment with increasing concentrations of DAC (Figure 2a). The methylation densities of the miR-29b promoter region in K562 cells treated with DAC 0, 0.1, 1, and 10 μM were 96.92%, 80%, 70.77%, and 49.23% respectively [41].

qRT-PCR showed no significant difference in the expression of miR-29b in K562/DAC cells compared with K562 cells. A DAC concentration of 1 μM is routinely used to maintain cellular drug resistance, so that treatment was applied to K562

Table 2. Univariate and multivariate analyses of prognostic factors for overall survival in whole-cohort AML patients.

	Univariate		Multivariate	
Prognostic factors	Hazard ratio (95% CI)	*P* value	Hazard ratio (95% CI)	*P* value
Age (>60 vs ≤60 years)	2.840 (1.708–4.723)	<0.001	1.381(0.553–3.449)	0.489
Sex (Male vs Female)	1.447 (0.976–2.146)	0.066	1.345 (0.645–0.975)	0.712
WBC (>30 vs ≤ 30×10^9/L)	2.111 (1.424–3.127)	<0.001	1.572 (0.420–4.132)	0.381
Hb(>110 vs ≤ 110×10^9/L)	1.009 (0.564–1.805)	0.997	—	—
Risk classification (Low vs Intermediate vs High)	1.932 (1.567–2.383)	<0.001	1.967(1.375–2.841)	0.001
MiR-29b expression (high/low)	2.202 (1.212–3.425)	0.007	1.879 (1.021–2.863)	0.042
K/N-RAS mutations (±)	1.622 (0.840–3.131)	0.150	1.115(0.147–8.467)	0.916
U2AF1 mutations (±)	1.814 (0.732–4.500)	0.199	1.014(0.492–5.806)	0.986
IDH1/2 mutations (±)	1.191 (0.578–2.454)	0.636	—	—
CKIT mutations (±)	1.387 (0.439–4.367)	0.577	—	—
NPM1 mutations (±)	1.096 (0.569–2.110)	0.784	—	—
FLT3 mutations (±)	1.109 (0.577–2.132)	0.757	—	—
DNMT3A mutations (±)	1.189 (0.551–2.566)	0.660	—	—
CEBPA mutations (±)	1.185 (0.554–2.538)	0.661	—	—

Table 3. Univariate and multivariate analyses of prognostic factors for overall survival in CN-AML patients.

	Univariate		Multivariate	
Prognostic factors	Hazard ratio (95% CI)	*P* value	Hazard ratio (95% CI)	*P* value
Age (>60 vs ≤60 years)	2.080 (1.017–4.255)	0.045	1.1251(0.262–4.825)	0.874
Sex (Male vs Female)	1.473 (0.824–2.625)	0.191	1.118 (0.620–2.015)	0.712
WBC (>30 vs ≤ 30×10^9/L)	1.522 (0.852–2.718)	0.049	1.174 (0.305–4.505)	0.816
Hb(>110 vs ≤ 110×10^9/L)	1.242 (0.555–2.783)	0.598	—	—
MiR-29b expression (high/low)	2.079 (1.005–4.310)	0.049	4.187(1.320–24.341)	0.037
IDH1/2 mutations (±)	1.042 (0.441–2.463)	0.926	—	—
K/N-RAS mutations (±)	1.395 (0.548–3.550)	0.485	—	—
CKIT mutations (±)	2.584 (0.355–18.868)	0.387	—	—
NPM1 mutations (±)	1.182 (0.526–2.653)	0.686	—	—
FLT3 mutations (±)	1.185 (0.503–2.793)	0.697	—	—
DNMT3A mutations (±)	1.525 (0.769–3.024)	0.227	—	—
U2AF1 mutations (±)	1.181 (0.574–2.452)	0.651	—	—
CEBPA mutations (±)	1.189 (0.551–1.556)	0.665	—	—

+: positive; -: negative; *, +: bi-allelic mutation; -: mono-allelic mutation or wild type.

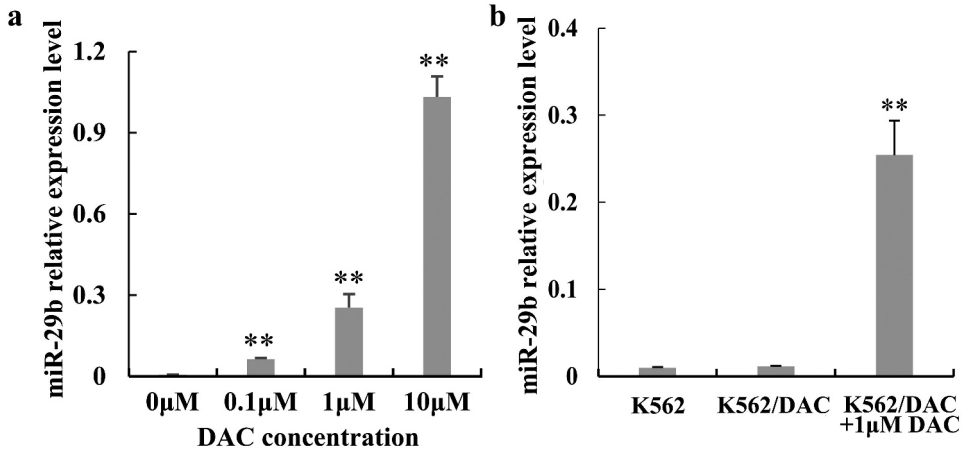

Figure 2. MiR-29b expression and methylation levels in DAC-sensitive and DAC-resistant cells. (A) miR-29b expression in K562 cells under different concentrations of DAC was detected by qRT-PCR. (B) miR-29b expression in DAC-resistant cells was detected by qRT-PCR. $^{**}P < 0.01$ compared with 0 group or K562.

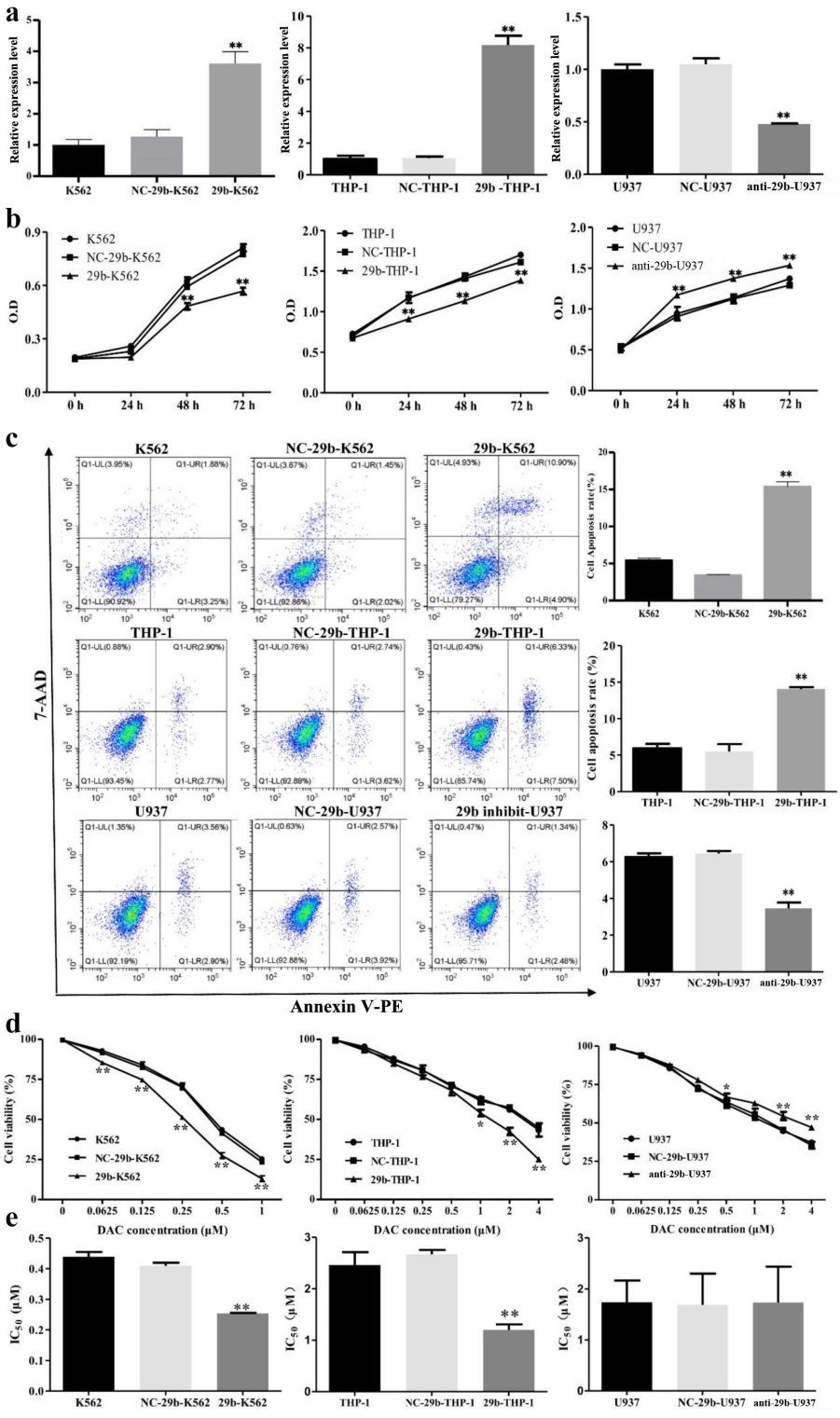

Figure 3. MiR-29b inhibited cell growth, promoted cell apoptosis, and increased sensitivity to DAC in K562, HL60, and U937 cells. (a) miR-29b expression detected by qRT-PCR. (b) CCK-8 analysis showing the effect of miR-29b expression on cell growth. (c) Effect of miR-29b expression on cell apoptosis detected by flow cytometry. (d) miR-29b increased sensitivity to DAC, and miR-29b silencing decreased sensitivity to DAC. (e) IC_{50} calculated from the data in panel D *$P < 0.05$ compared with the NC group, ** $P < 0.01$ compared with the NC group.

cells. miR-29b expression in K562 cells treated with 1 μM DAC was significantly up-regulated compared with K562 and K562/DAC cells ($P < 0.01$, Figure 2b).

3.3. Effect of miR-29b on DAC resistance

In order to explore the effect of miR-29b on the sensitivity of leukemia cells to DAC, miR-29b was overexpressed or inhibited in DAC-sensitive cells, including K562, THP-1 and U937 cell lines. The growth rates of K562 and THP-1 cells were inhibited by miR-29b transfection, while that of U937 cells was significantly promoted by the miR-29b inhibitor (Figure 3a, b). Moreover, the apoptosis rates of 29b-K562 and 29b-THP-1 cells were significantly higher than in NC-29b-K562 and NC-29b-THP-1 ($P < 0.01$). The apoptosis rate of anti-29b-U937 cells was significantly lower than NC-29b-U937 ($P < 0.01$, Figure 3c). After treatment with different concentrations of DAC for 72 h, cell viability decreased in 29b-K562 and 29b-THP-1 cells compared with the NC-29b-K562 and NC-29b-THP-1. The IC50 was decreased in K562 and THP-1 cell lines after miR-29b overexpression ($P < 0.01$, Figure 3d, e). Conversely, U937 cell viability was increased following incubation with 2 and 4 μM of DAC. However, there was no significant difference in IC50 values between the anti-29b-U937 and NC-29b-U937 groups (Figure 3d, e).

miR-29b was overexpressed to explore its effect on DAC resistance in K562/DAC cells. The growth rate of K562/DAC cells was inhibited by miR-29b transfection (Figure 4a, b). The apoptosis rate of 29b-K562/DAC cells was significantly higher than

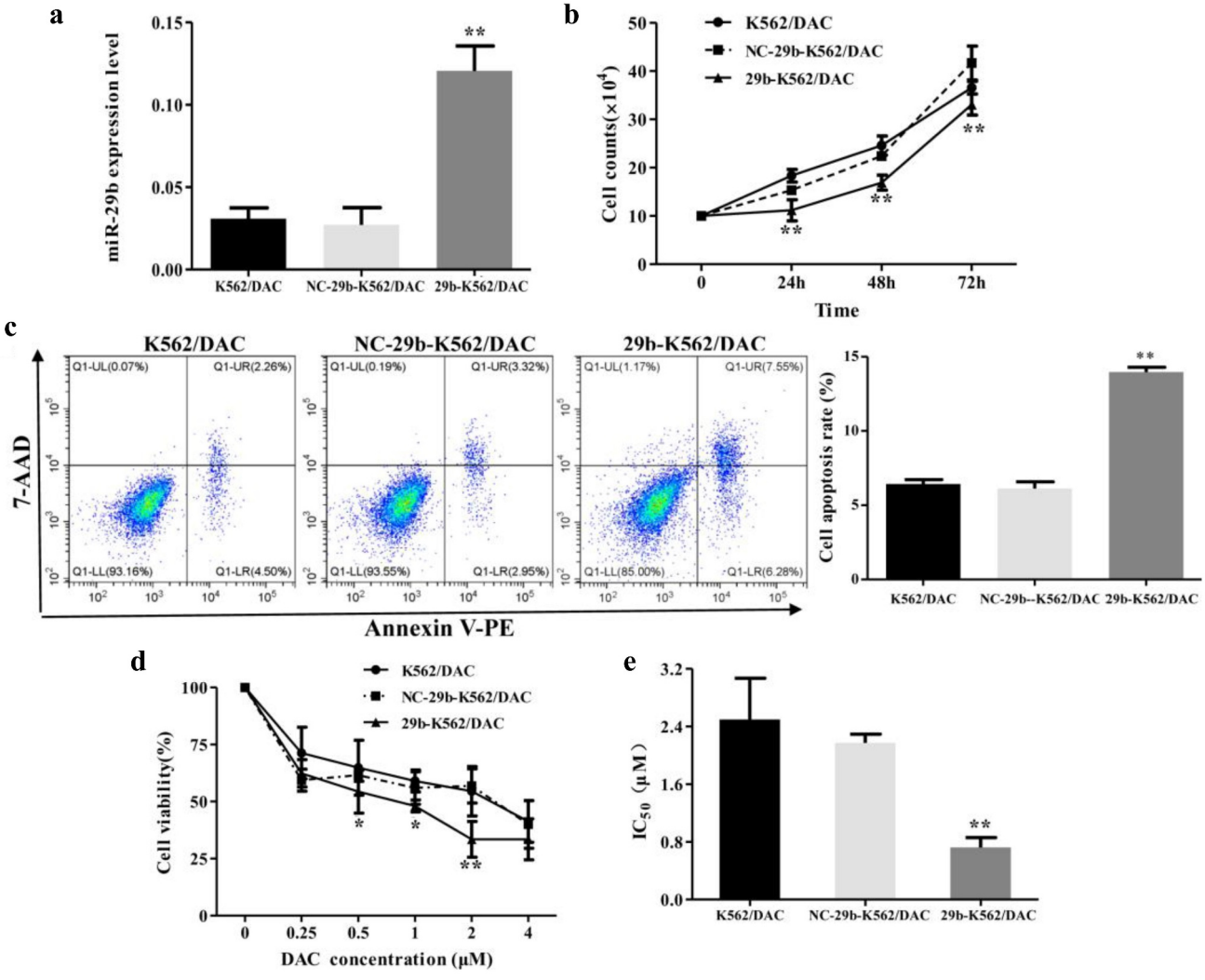

Figure 4. MiR-29b inhibited cell growth, promoted cell apoptosis, and increased the sensitivity to DAC in K562/DAC cells. (a) miR-29b expression detected by qRT-PCR. (b) CCK-8 analysis of the effect of miR-29b expression on K562/DAC cell growth. (c) Effect of miR-29b expression on cell apoptosis in K562/DAC cells detected by flow cytometry. (d) miR-29b overexpression increased sensitivity to DAC; (e) IC_{50} calculated from the data in panel D *$P < 0.05$ and **$P < 0.01$ compared with the NC group.

NC-29b-K562/DAC ($P < 0.01$, Figure 4c). Cell viability was decreased in 29b-K562/DAC cells, compared with NC-29b- 562/DAC. The IC50 was also decreased in K562/DAC cell lines after miR-29b overexpression ($P < 0.01$; Figure 4d, e).

3.4. MiR-29b may participate in DAC resistance through ID1

ID1 was reported as a target gene of miR-29b [30]. Compared with the NC group, expression of ID was down-regulated in 29b-K562 and 29b-THP-1 cells and up-regulated in anti-29b-U937 cells (Figure 5a, b). Expression levels of ID1 mRNA and protein in 29b-K562/DAC cells were decreased compared with the control group (Figure 5c, d).

To analyze the relationship between DAC resistance and ID1 expression, miR-29b expression and methylation were detected by qRT-PCR and BSP, respectively. The results showed that ID1 expression decreased in DAC-treated K562 cells compared to untreated K562 cells ($P < 0.01$, Figure 6a). The promoter region of ID1 in K562 cells was completely unmethylated, while ID1 in K562 cells treated with DAC was highly unmethylated (Figure 6c). We also detected the expression of ID1 in K562/DAC by qRT-PCR, which showed increased expression of ID1 in K562/DAC compared with K562 and K562 treated with 1 μM DAC ($P < 0.01$, Figure 6b).

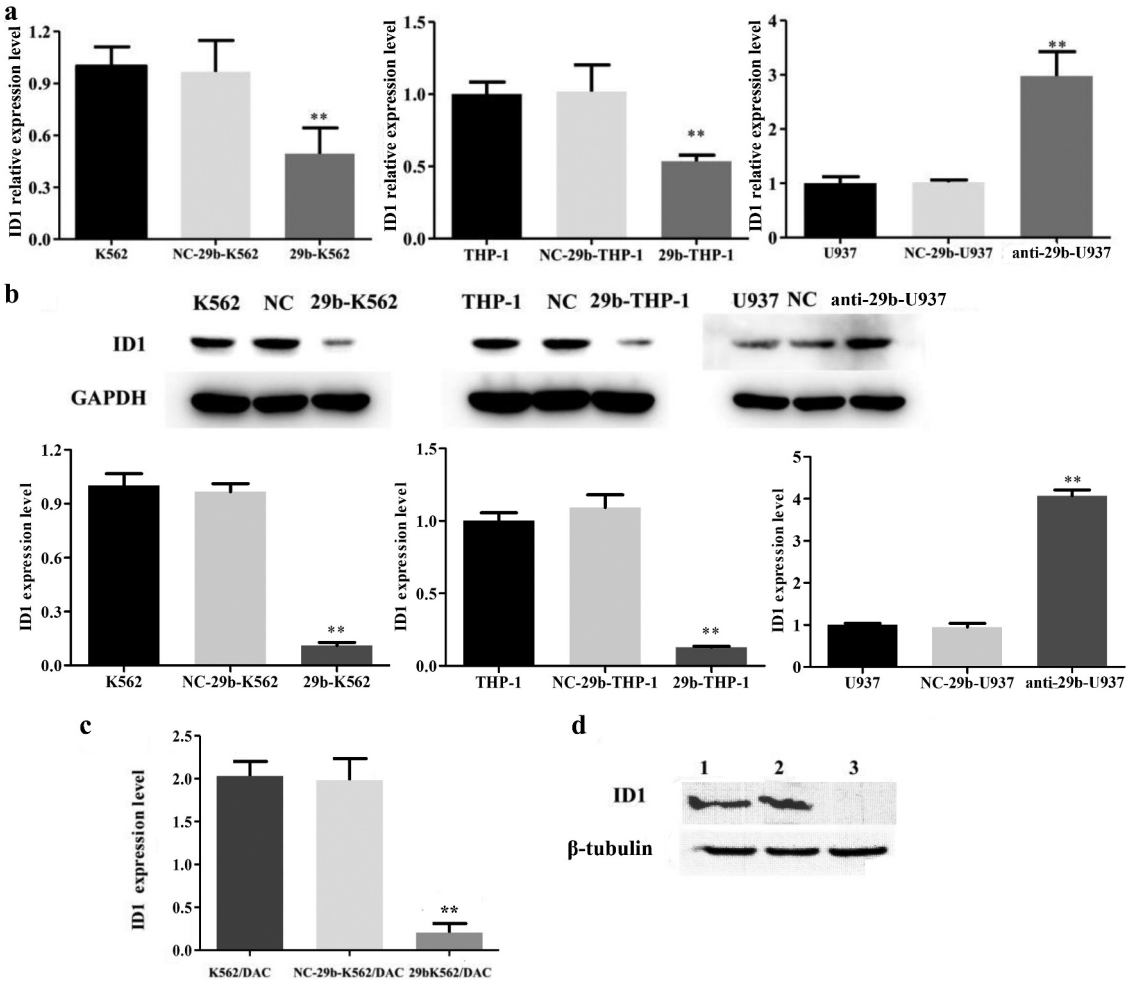

Figure 5. ID1 expression in miR-29b transfected cells. (a) ID1 expression in 29b-K562, 29b-THP-1, and anti-29b-U937 cells detected by qRT-PCR. (b) ID1 expression in 29b-K562, 29b-THP-1, and anti-29b-U937 cells detected by western blot. (c) ID1 expression in 29b-K562/DAC cells detected by qRT-PCR. (d) ID1 expression in 29b-K562/DAC cells detected by western blot; lane 1: K562/DAC, lane 2: NC-29b-K562/DAC, lane 3: 29b-K562/DAC. $^{**}P < 0.01$ compared with the NC group.

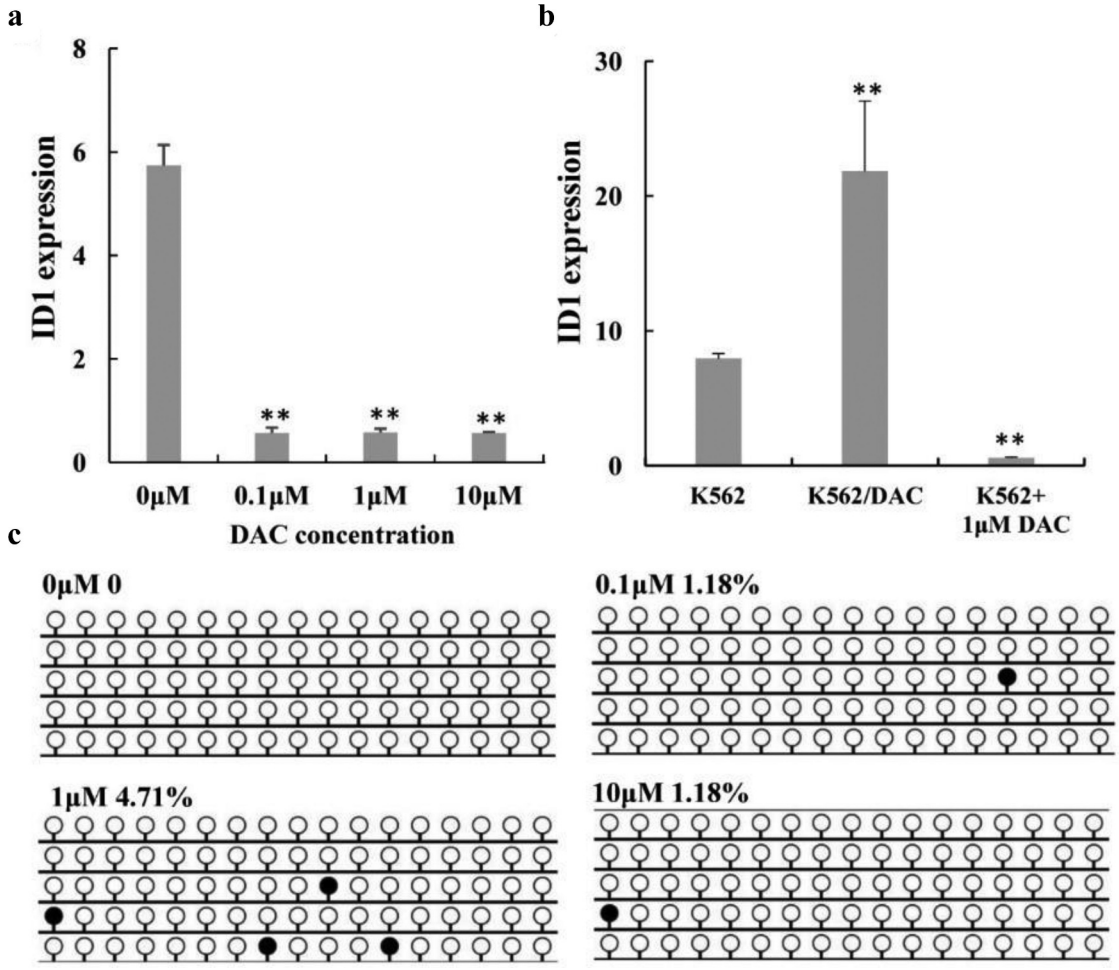

Figure 6. Level of ID1 expression and methylation in K562 and K562/DAC cells. (a) Level of ID1 expression in K562 under different DAC concentrations detected by qRT-PCR. (b) ID1 expression in K562/DAC detected by qRT-PCR. (c) ID1 methylation was detected by BSP. $^{**}P < 0.01$ compared with 0 group or K562.

To study the effect of ID1 on leukemia cell sensitivity to DAC, we over-expressed ID1 in K562 and HL60 cells (Figure 7a). The growth rates of K562 and HL60 cells increased following ID1 transfection (Figure 7b). The apoptosis rates of ID1-K562 and ID1-HL60 cells were significantly decreased compared with the control group ($P < 0.01$, Figure 7c, d). Cell viability increased in ID1-K562 and ID1-HL60 cells compared with the NC-K562 and NC-HL60 groups. The IC50 was increased in K562 and HL60 cells after ID1 overexpression ($P < 0.01$, Figure 7e, f).

To further confirm the role of ID1 on DAC resistance in vivo, NC-K562 and ID1-K562 cells were subcutaneously injected into BALB/c nude mice. We detected the weight of mice and volume of tumor every 3 days. The weights of mice were not significantly different among the groups (Figure 8a). The tumor volume of the ID1-K562-injected NS group was increased compared with the NC-K562-injected NS group and the tumor volume of the ID1-K562 injected DAC group increased compared with the NC-K562-injected DAC group after injected 12 days (Figure 8b). The mice were sacrificed at 15 days after tumor inoculation. The tumors were separated and weighed. The resulted showed that the tumor volume increased in the ID1-K562-injected NS group, compared with the NC-K562 injected NS group. The NC-K562 injection DAC group had a smaller tumor volume than the ID1-K562 injection DAC group (Figure 8c). ID1 expression levels in each group of tumors were detected by qRT-PCR and western blot. It showed that ID1 expression was higher in the ID1-K562 group compared to the NC-K562 group (Figure 8d, e). The results confirmed that ID1 overexpression promoted tumor growth and reduced sensitivity to DAC in vivo.

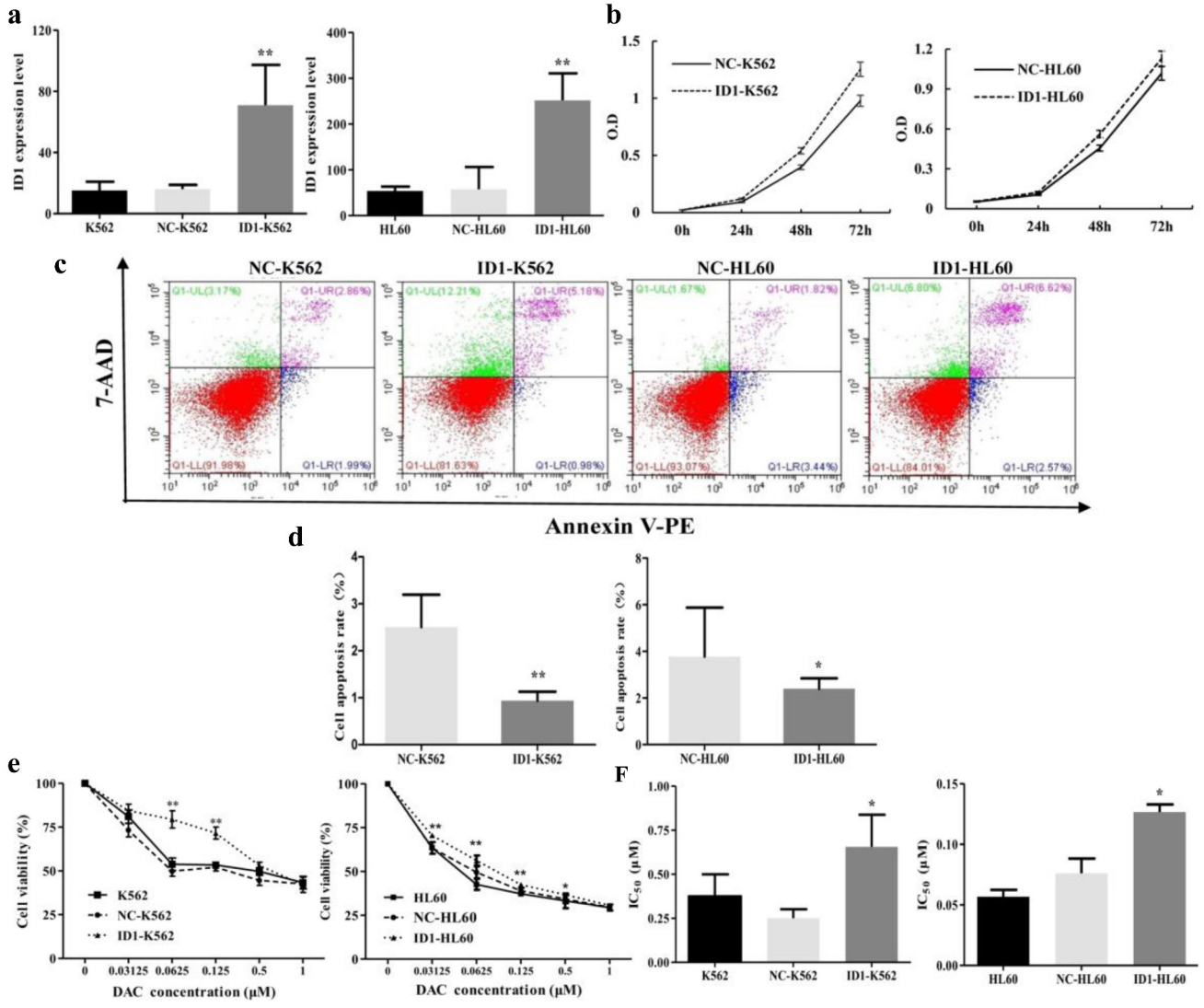

Figure 7. ID1 promoted growth, inhibited apoptosis, and decreased sensitivity to DAC in K562 and HL60 cells. (a) ID1 expression in transfected cells detected by qRT-PCR. (b) ID1 promoted K562 and HL60 cell growth. (c,d) ID1 inhibited apoptosis in K562 and HL60 cells. (e) ID1 increased sensitivity to DAC in K562 and HL60 cells; (f) IC_{50} calculated from the data in panel e data. *$P < 0.05$ compared with the NC group, **$P < 0.01$ compared with the NC group.

To further analyze the effect of ID1 on DAC resistance, ID1 expression was silenced in K562/DAC cells (Figure 9a, b). The growth rate of cells in the ID1 silence group was lower than in the control group ($P < 0.01$, Figure 9c). The IC50 of ID1-silenced K562/DAC cells were also significantly lower than control group ($P < 0.01$, Figure 9d). In summary, silencing ID1 expression increased cell sensitivity to DAC.

3.5. Re-expression of ID1 eliminated the effects induced by miR-29b

ID1 expression in miR-29b-K562/DAC cells was down-regulated, so ID1 was over-expressed with lentivirus transfection. ID1 mRNA and protein levels were higher in the miR-29b-K562/DAC transfected ID1 group compared to the ID1 control plasmid group (Figure 10 a, b). Compared with miR-29b-K562/DAC-ID-NC group, the growth rate of miR-29b-K562/DAC-ID group cells were increased ($P < 0.01$). Compared with the NC-miR-29b-K562/DAC-ID1 group, the growth rate of miR-29b-K562/DAC-ID1 group cells was decreased ($P < 0.01$). There was no significant difference in growth among the K562/DAC, NC-miR-29b-K562/DAC-ID-NC, and miR-29b-K562/DAC-ID groups (Figure 10c). Compared with the miR-29b-K562/DAC-ID-NC

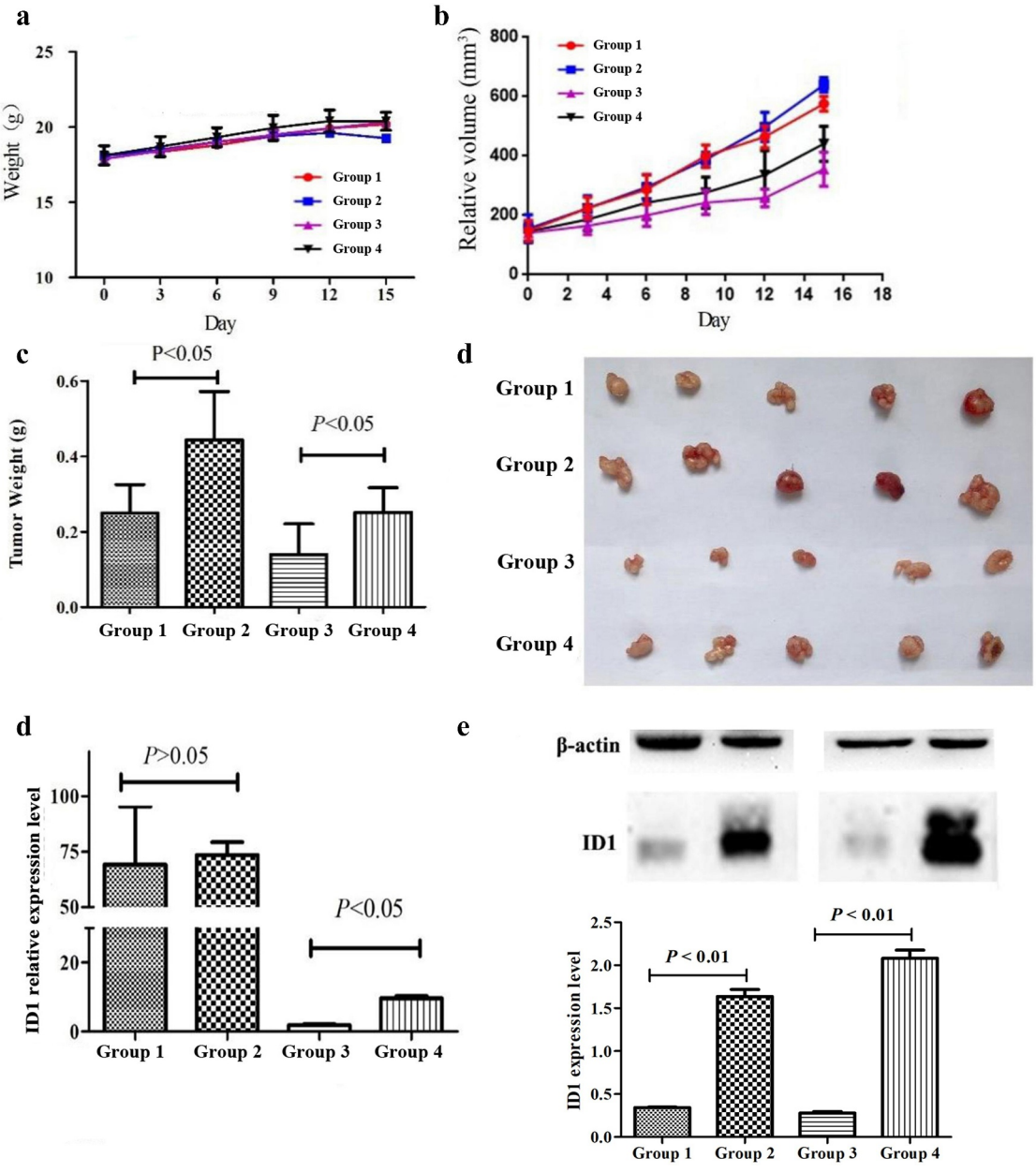

Figure 8. ID1 promotes tumor growth and reduce the sensitivity to DAC in vivo. (a) Weight curves. (b) Tumor volume curves. (c) Subcutaneous tumor weights. (d) Images of subcutaneous tumors. (e) ID1 expression in tumor tissue detected by qRT-PCR. (F) ID1 expression in tumor tissue detected by western blot. Group 1: subcutaneous injection of NC-K562 cells and NS treatment group; Group 2: subcutaneous injection of ID1-K562 cells and NS treatment group; Group 3: subcutaneous injection of NC-K562 cells and DAC treatment group; Group 4: subcutaneous injection of ID1-K562 cells and DAC treatment group.

group, the apoptosis rate was lower in miR-29b-K562/DAC-ID cells ($P < 0.01$). Compared with the NC-miR-29b-K562/DAC-ID1-NC group, the apoptosis rate was increased in the miR-29b-K562/DAC-ID-NC group ($P < 0.01$). Compared with the NC-miR-29b-K562/DAC-ID1-NC group, cell apoptosis increased in the miR-29b-K562 /DAC-ID1-NC group ($P < 0.01$, Figure 10d). Cell viability was increased in the miR-29b-K562 /DAC-ID group compared with the miR-29b-K562/DAC-ID-NC group ($P < 0.01$). Cell viability was decreased in the miR-29b-K562/DAC-ID-NC group compared with the NC-miR-29b-K562 /DAC-ID1-NC group ($P < 0.01$). Cell viability was decreased in miR-29b-K562/DAC-ID-NC group compared to the NC-miR-29b-K562/DAC-ID1-

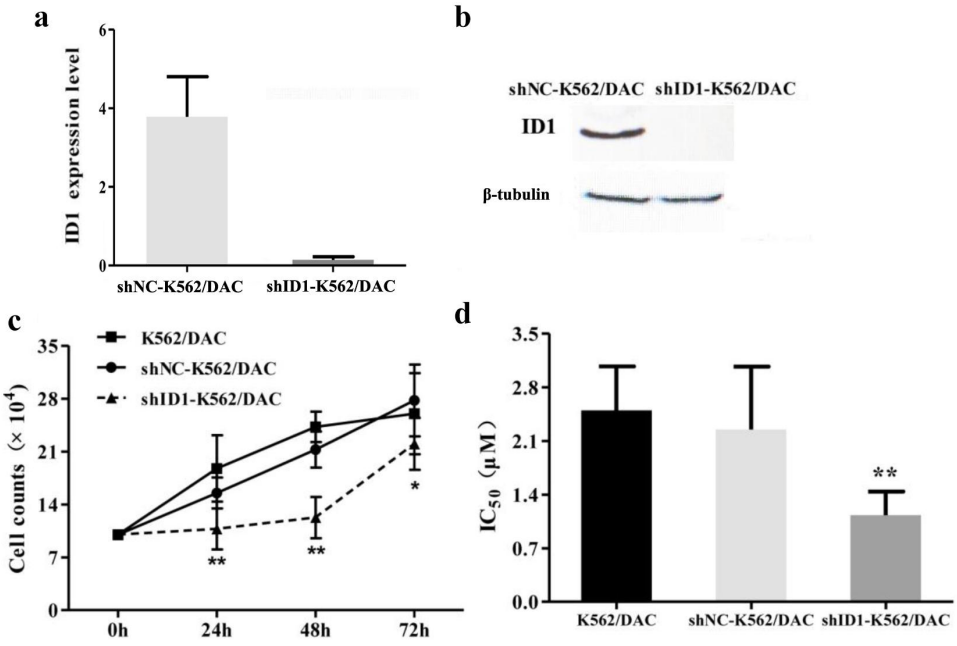

Figure 9. Effects of ID1 silencing on the growth and DAC resistance in K562/DAC cells. (a,b) ID1 expression in transfected cells was detected by qRT-PCR and western blot. (c) Silencing ID1 inhibited K562/DAC cell growth. (d) Silencing ID1 reduced the IC_{50} of K562/DAC to DAC. * $P < 0.05$ and ** $P < 0.01$ compared with the NC group.

NC group ($P < 0.01$; Figure 10e, f). Collectively, these results indicate that miR-29b's effects of growth inhibition, increased DAC sensitivity, and apoptosis are attenuated by increasing ID1 expression, further suggesting that miR-29b is the upstream regulatory molecule of ID1.

4. Discussion

DNMT inhibitors including DAC have achieved good curative effects in clinical application. However, some patients cured with DAC develop primary or secondary drug resistance, which reduces their survival rate [23,24]. A growing number of studies are exploring the mechanism of DAC resistance. Most existing reports describe changes in drug metabolism-related enzymes such as deoxycytidine kinase (DCK) and cytidine deaminase (CDA). Qin and colleagues reported that DCK gene mutation or decreased transcription dampens the phosphorylation ability of DAC and results in a significant decrease in the concentration of the intracellular metabolic active product 5-aza-2-deoxycytidine monophosphate after contact with DAC in leukemia HL60 cells [42]. As a result, the 5-aza-2-deoxycytidine triphosphate incorporated into DNA double strands is decreased, and the 5-aza-2-deoxycytidine triphosphate is removed. Enhanced CDA activity deaminates DAC into 5-aza-2-deoxyuracil, which also reduces the demethylation effect [43]. However, the existing research also confirmed that there was no mutation of DCK gene and no significant change in CDA and DCK activities in patients with secondary drug resistance, which suggested that other molecular mechanisms are involved in the resistance of DNMT inhibitors. We previously demonstrated that DAC could restore the activity of tumor suppressor genes silenced by hypermethylation in tumor cells, but it also induced extensive demethylation and lacked targeting specificity. In addition to up-regulating tumor suppressor gene expression, can also activate the expression of some oncogenes. This may counteract the anti-tumor effect and make tumor cells resistant to DAC [44, 45]. It is therefore very important to understand the role of these oncogenes and tumor suppressor genes in the process of DAC resistance and reverse their detrimental effects.

miR-29b serves as a tumor suppressor gene in many kinds of malignancies and participates in the

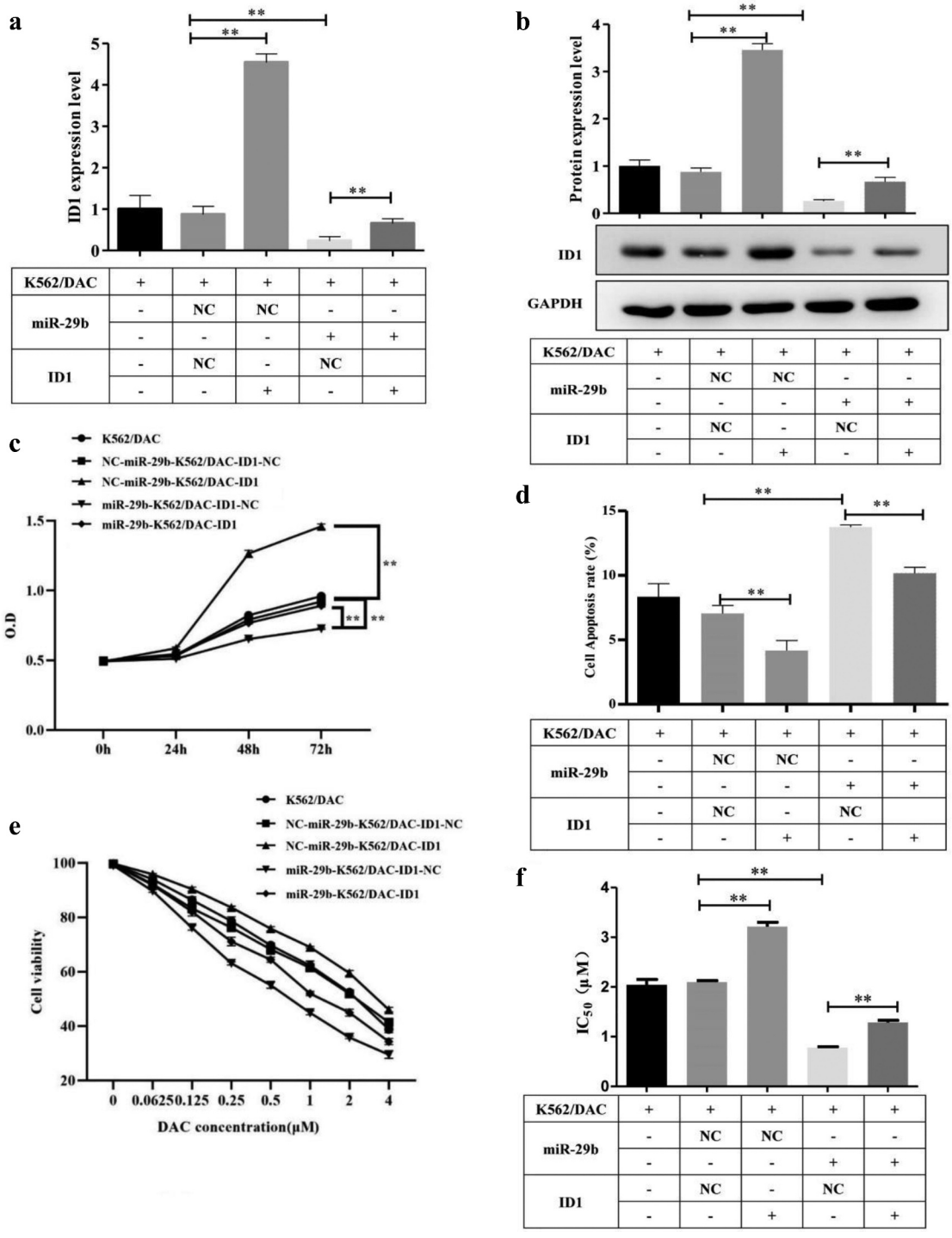

Figure 10. ID1 re-expression eliminated the effect of miR-29b on growth and DAC sensitivity in K562/DAC cells. (a) ID1 expression in transfected cells detected by qRT-PCR. (b) ID1 expression in transfected cells detected by western blot. (c) ID1 re-expression eliminated miR-29b-induced growth inhibition in K562/DAC cells. (d) ID1 re-expression eliminated inhibition of DAC sensitivity inhibition induced by miR-29b in K562/DAC cells. (e) ID1 re-expression eliminated miR-29b-induced apoptosis promotion in K562/DAC cells. * $P < 0.05$ and ** $P < 0.01$ compared with the NC group.

regulation of many important signaling pathways [46, 47]. It was reported that miR-29b expression is obviously down-regulated in AML cell lines, and over-expression of miR-29b can obviously promote the apoptosis of AML cells by down-regulating myeloid cell leukemia-1 expression [48]. This proposal was supported by our results. We found significantly reduced miR-29b

expression in AML patients; miR-29b might be an independent prognostic molecule affecting survival in all AML patients and CN-AML patients. miR-29b inhibited cell growth, promoted apoptosis, and increase DAC sensitivity in leukemia cells.

ID1 is a negative regulator of the HLH transcription factor that plays the role of an oncogene in promoting cell cycle, proliferation, migration and inhibiting apoptosis [49–54]. Wang et al reported that ID1 can promote the occurrence and development of AML by regulating AKT signaling [49]. We previously reported that ID1 expression was up-regulated in AML patients, and patients with high expression had poor prognoses [51]. Bioinformatics predicted that miR-29b could combine with the 3"-untranslated region (UTR) region of ID1, and one group reported that miR-29b directly bound to the ID1 3"-UTR region [34]. These results suggest that the miR-29b-ID1 pathway may play an important role in AML pathogenesis.

In our studies, the demethylation density of the miR-29b promoter region was obviously decreased in DAC-sensitive cells under DAC treatment, and its expression was up-regulated, suggesting that miR-29b can be regulated by methylation, and demethylation drug DAC could up-regulate miR-29b expression by demethylating its promoter [41]. The ID1 promoter was completely unmethylated before and after DAC treatment, but its expression was down-regulated under DAC treatment, suggesting that ID1 cannot be regulated by methylation and DAC might play a role in AML treatment through the miR-29b-ID1 pathway. However, we found that the promoter regions of miR-29b were demethylated in DAC-resistant cells, but their expression was not up-regulated and remained low [41], while ID1 expression was significantly increased. We therefore speculate that DAC regulates ID1 by acting on miR-29b, and the abnormal expression of ID1—which is methylation independent and induced by miR-29b – may be involved in the process of DAC resistance.

The DNA methylation is an important pathological mechanism in the development of leukemia. DAC, demethylation drugs, DNMT inhibitors, have achieved good clinical results. It has become an important treatment for elderly acute myeloid leukemia patients and myelodysplastic syndrome patients [55, 56]. It is important to explore the mechanism of primary and secondary of DAC resistance and then develop new therapy strategy [57, 58]. In this study, we analyzed the role of miR-29b-ID1 in DAC resistance, revealed the epigenetic regulation mechanism of miR-29b-ID1 in leukemia treatment. It provides guidance for its clinical application, prognosis evaluation and disease outcome, provides way for exploring new diagnostic markers and targeted treatment markers, and provides foundation for improving the antileukemia efficacy of DAC.

5. Conclusions

Taken together, our results indicate that down-regulation of miR-29b is a frequent event and predicts poor prognosis in de novo AML patients. miR-29b overexpression can increase leukemia cell sensitivity to DAC and provides possible guidance for clinical DAC resistance. DAC regulates the expression of ID1 by acting on miR-29b. The abnormal expression of ID1 which is methylation-independent induced by miR-29b may be involved in the process of acquiring DAC resistance.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Author contributions

Jichun Ma, Xiangmei Wen, Zijun X performed the experiments. Peihui Xia and Ye Jin collected the data, performed data analyses. Jiang Lin provided the resources. Jun Qian reviewed and edited the manuscript.

Institutional review board statement

Ethical approvals for the study were obtained from the Ethics Committee of Affiliated People's Hospital of Jiangsu University (K-20190020-Y). The Experimental Animal Management and Use Committee of Jiangsu University approved animal studies.

Informed consent statement

Written informed consent has been obtained from the patient(s) to publish this paper.

References

[1] Shlush LI, Zandi S, Mitchell A, et al. Identification of pre-leukaemic haematopoietic stem cells in acute leukaemia. Nature. 2014;506(7488):328–333.

[2] Khwaja A, Bjorkholm M, Gale RE, et al. Acute myeloid leukaemia. Nat Rev Dis Primers. 2016;2(1):16010.

[3] Akinduro O, Weber TS, Ang H, et al. Proliferation dynamics of acute myeloid leukaemia and haematopoietic progenitors competing for bone marrow space. Nat Commun. 2018;9(1):519.

[4] Wang E, Zhou H, Nadorp B, et al. Surface antigen-guided CRISPR screens identify regulators of myeloid leukemia differentiation. Cell Stem Cell. 2021;28(4):718–731 e716.

[5] Sachs K, Sarver AL, Noble-Orcutt KE, et al. Single-cell gene expression analyses reveal distinct self-renewing and proliferating subsets in the leukemia stem cell compartment in acute myeloid leukemia. Cancer Res. 2020;80(3):458–470.

[6] Wang C, He H, Liu G, et al. DT-13 induced apoptosis and promoted differentiation of acute myeloid leukemia cells by activating AMPK-KLF2 pathway. Pharmacol Res. 2020;158:104864.

[7] Duployez N, Marceau-Renaut A, Boissel N, et al. Comprehensive mutational profiling of core binding factor acute myeloid leukemia. Blood. 2016;127(20):2451–2459.

[8] Tyner JW, Tognon CE, Bottomly D, et al. Functional genomic landscape of acute myeloid leukaemia. Nature. 2018;562(7728):526–531.

[9] Crunkhorn S. Epigenetic therapy prevents AML. Nat Rev Drug Discov. 2020;19(3):168.

[10] Benetatos L, Vartholomatos G. Enhancer DNA methylation in acute myeloid leukemia and myelodysplastic syndromes. Cell Mol Life Sci. 2018;75(11):1999–2009.

[11] Schoofs T, Muller-Tidow C. DNA methylation as a pathogenic event and as a therapeutic target in AML. Cancer Treat Rev. 2011;37(*Suppl* 1):S13–18.

[12] Bhayadia R, Krowiorz K, Haetscher N, et al. Endogenous tumor suppressor microRNA-193b: therapeutic and prognostic value in acute myeloid leukemia. J Clin Oncol. 2018;36(10):1007–1016.

[13] Liu Y, Cheng Z, Pang Y, et al. Role of microRnas, circRnas and long noncoding RNAs in acute myeloid leukemia. J Hematol Oncol. 2019;12(1):51.

[14] Man CH, Fung TK, Wan H, et al. Suppression of SOX7 by DNA methylation and its tumor suppressor function in acute myeloid leukemia. Blood. 2015;125(25):3928–3936.

[15] Zhou L, Fu L, Lv N, et al. Methylation-associated silencing of BASP1 contributes to leukemogenesis in t(8;21) acute myeloid leukemia. Exp Mol Med. 2018;50(12):1–8.

[16] Lakshmikuttyamma A, Scott SA, DeCoteau JF, et al. Reexpression of epigenetically silenced AML tumor suppressor genes by SUV39H1 inhibition. Oncogene. 2010;29(4):576–588.

[17] Nunes SP, Henrique R, Jeronimo C, et al. DNA methylation as a therapeutic target for bladder cancer. Cells. 2020;9(8):1850.

[18] Cai Y, Geutjes EJ, de Lint K, et al. The NuRD complex cooperates with DNMTs to maintain silencing of key colorectal tumor suppressor genes. Oncogene. 2014;33(17):2157–2168.

[19] Das M. Venetoclax with decitabine or azacitidine for AML. Lancet Oncol. 2018;19(12):e672.

[20] Welch JS, Petti AA, Miller CA, et al. TP53 and decitabine in acute myeloid leukemia and myelodysplastic syndromes. N Engl J Med. 2016;375(21):2023–2036.

[21] Steensma DP. Myelodysplastic syndromes current treatment algorithm 2018. Blood Cancer J. 2018;8(5):47.

[22] Lund K, Cole JJ, VanderKraats ND, et al. DNMT inhibitors reverse a specific signature of aberrant promoter DNA methylation and associated gene silencing in AML. Genome Biol. 2014;15(7):406.

[23] Long L, Assaraf YG, Lei ZN, et al. Genetic biomarkers of drug resistance: a compass of prognosis and targeted therapy in acute myeloid leukemia. Drug Resist Updat. 2020;52:100703.

[24] Huang X, Xiao F, Li Y, et al. Bypassing drug resistance by triggering necroptosis: recent advances in mechanisms and its therapeutic exploitation in leukemia. J Exp Clin Cancer Res. 2018;37(1):310.

[25] Kim S, Shin DY, Kim D, et al. Gene expression profiles identify biomarkers of resistance to decitabine in myelodysplastic syndromes. Cells. 2021;10(12):3494.

[26] Yu J, Qin B, Moyer AM, et al. DNA methyltransferase expression in triple-negative breast cancer predicts sensitivity to decitabine. J Clin Invest. 2018;128(6):2376–2388.

[27] Liu H, Chen P, Yang YL, et al. TBC1D16 predicts chemosensitivity and prognosis in adult acute myeloid leukemia (AML) patients. Eur J Pharmacol. 2021;895:173894.

[28] Oellerich T, Schneider C, Thomas D, et al. Selective inactivation of hypomethylating agents by SAMHD1 provides a rationale for therapeutic stratification in AML. Nat Commun. 2019;10(1):3475.

[29] Wen XM, Zhang TJ, Ma JC, et al. Establishment and molecular characterization of decitabine-resistant K562 cells. J Cell Mol Med. 2019;23(5):3317–3324.
[30] Muluhngwi P, Krishna A, Vittitow SL, et al. Tamoxifen differentially regulates miR-29b-1 and miR-29a expression depending on endocrine-sensitivity in breast cancer cells. Cancer Lett. 2017;388:230–238.
[31] Yin H, Wang Y, Wu Y, et al. EZH2-mediated epigenetic silencing of miR-29/miR-30 targets LOXL4 and contributes to tumorigenesis, metastasis, and immune microenvironment remodeling in breast cancer. Theranostics. 2020;10(19):8494–8512.
[32] Sharma S, Pavlasova GM, Seda V, et al. MiR-29 modulates CD40 signaling in chronic lymphocytic leukemia by targeting TRAF4: an axis affected by BCR inhibitors. Blood. 2021;137(18):2481–2494.
[33] Sampath D, Liu C, Vasan K, et al. Histone deacetylases mediate the silencing of miR-15a, miR-16, and miR-29b in chronic lymphocytic leukemia. Blood. 2012;119(5):1162–1172.
[34] Rothschild SI, Tschan MP, Federzoni EA, et al. MicroRNA-29b is involved in the Src-ID1 signaling pathway and is dysregulated in human lung adenocarcinoma. Oncogene. 2012;31(38):4221–4232.
[35] Hasskarl J, Duensing S, Manuel E, et al. The helix–loop–helix protein ID1 localizes to centrosomes and rapidly induces abnormal centrosome numbers. Oncogene. 2004;23(10):1930–1938.
[36] Cook PJ, Thomas R, Kingsley PJ, et al. Cox-2-derived PGE2 induces Id1-dependent radiation resistance and self-renewal in experimental glioblastoma. Neuro Oncol. 2016;18(10):1379–1389.
[37] Yin X, Tang B, Li JH, et al. ID1 promotes hepatocellular carcinoma proliferation and confers chemoresistance to oxaliplatin by activating pentose phosphate pathway. J Exp Clin Cancer Res. 2017;36(1):166.
[38] Valanti EK, Dalakoura-Karagkouni K, Fotakis P, et al. Reconstituted HDL-apoE3 promotes endothelial cell migration through ID1 and its downstream kinases ERK1/2, AKT and p38 MAPK. Metabolism. 2022;127:154954.
[39] Lin J, Ma JC, Yang J, et al. Arresting of miR-186 and releasing of H19 by DDX43 facilitate tumorigenesis and CML progression. Oncogene. 2018;37(18):2432–2443.
[40] Ma J, Lin J, Qian J, et al. MiR-378 promotes the migration of liver cancer cells by down-regulating Fus expression. Cell Physiol Biochem. 2014;34(6):2266–2274.
[41] Tang LJ, Sun GK, Zhang TJ, et al. Down-regulation of miR-29c is a prognostic biomarker in acute myeloid leukemia and can reduce the sensitivity of leukemic cells to decitabine. Cancer Cell Int. 2019;19(1):177.
[42] Qin T, Jelinek J, Si J, et al. Mechanisms of resistance to 5-aza-2′-deoxycytidine in human cancer cell lines. Blood. 2009;113(3):659–667.
[43] Qin T, Castoro R, El Ahdab S, et al. Mechanisms of resistance to decitabine in the myelodysplastic syndrome. PLoS ONE. 2011;6(8):e23372.
[44] Zhang TJ, Xu ZJ, Gu Y, et al. Identification and validation of prognosis-related DLX5 methylation as an epigenetic driver in myeloid neoplasms. Clin Transl Med. 2020;10(2):e29.
[45] Lin J, Chen Q, Yang J, et al. DDX43 promoter is frequently hypomethylated and may predict a favorable outcome in acute myeloid leukemia. Leuk Res. 2014;38(5):601–607.
[46] Hou K, Li G, Zhao J, et al. RETRACTED ARTICLE: bone mesenchymal stem cell-derived exosomal microRNA-29b-3p prevents hypoxic-ischemic injury in rat brain by activating the PTEN-mediated Akt signaling pathway. J Neuroinflammation. 2020;17(1):46.
[47] Li YY, Xu QW, Xu PY, et al. MSC-derived exosomal miR-34a/c-5p and miR-29b-3p improve intestinal barrier function by targeting the Snail/Claudins signaling pathway. Life Sci. 2020;257:118017.
[48] Garzon R, Heaphy CE, Havelange V, et al. MicroRNA 29b functions in acute myeloid leukemia. Blood. 2009;114(26):5331–5341.
[49] Wang L, Man N, Sun XJ, et al. Regulation of AKT signaling by Id1 controls t(8;21) leukemia initiation and progression. Blood. 2015;126(5):640–650.
[50] Man N, Sun XJ, Tan Y, et al. Differential role of Id1 in MLL-AF9–driven leukemia based on cell of origin. Blood. 2016;127(19):2322–2326.
[51] Zhou JD, Yang L, Zhu XW, et al. Clinical significance of up-regulated ID1 expression in Chinese de novo acute myeloid leukemia. Int J Clin Exp Pathol. 2015;8(5):5336–5344.
[52] He Y, Wei T, Ye Z, et al. A noncanonical AR addiction drives enzalutamide resistance in prostate cancer. Nat Commun. 2021;12(1):1521.
[53] Liu K, Chen X, Wu L, et al. ID1 mediates resistance to osimertinib in EGFR T790M-positive non-small cell lung cancer through epithelial–mesenchymal transition. BMC Pulm Med. 2021;21(1):163.
[54] Sachdeva R, Wu M, Smiljanic S, et al. ID1 is critical for tumorigenesis and regulates chemoresistance in glioblastoma. Cancer Res. 2019;79(16):4057–4071.
[55] Chang E, Ganguly S, Rajkhowa T, et al. The combination of FLT3 and DNA methyltransferase inhibition is synergistically cytotoxic to FLT3/ITD acute myeloid leukemia cells. Leukemia. 2016;30(5):1025–1032.
[56] Montalban-Bravo G, Garcia-Manero G. Myelodysplastic syndromes: 2018 update on diagnosis, risk-stratification and management. Am J Hematol. 2018;93(1):129–147.
[57] Kadia TM, Jabbour E, Kantarjian H. Failure of hypomethylating agent–based therapy in myelodysplastic syndromes. Semin Oncol. 2011;38(5):682–692.
[58] Sasca D, Huntly BJ. Independence of epigenetic and genetic diversity in AML. Nat Med. 2016;22(7):708–709.