

Overexpression of long noncoding RNA HOXA-AS2 predicts an adverse prognosis and promotes tumorigenesis via SOX4/PI3K/AKT pathway in acute myeloid leukemia

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Abstract

Long noncoding RNAs (lncRNAs) play important roles in diverse cellular processes and carcinogenesis. Homeobox A cluster antisense RNA 2 (HOXA-AS2) is a 1,048-basepairs lncRNA located between human HOXA3 and HOXA4 genes, whose overactivation was previously found to promote the proliferation and invasion of solid tumors. However, its clinical and biological roles in acute myeloid leukemia (AML) remain unclear. This study showed that HOXA-AS2 was overexpressed in AML patients. In addition, the increased HOXA-AS2 expression was correlated with higher white blood cell and bone marrow blast counts, unfavorable karyotype classification, more measurable residual disease positivity, and earlier death. There was also a tendency toward inferior survival in patients with high HOXA-AS2 expression, and HOXA-AS2 was an independent prognostic factor among the normal-karyotype AMLs. Furthermore, the results of in vitro study showed that silencing HOXA-AS2 significantly inhibited the growth of leukemic cells by inducing G1/G0-phase arrest and apoptosis. Further analysis demonstrated that silencing HOXA-AS2 suppressed the phosphorylation level of PI3K and AKT, which thereafter promoted the expression of P21 and P27. Moreover, it was suggested that the sex-determining region Y-box 4 (SOX4), which is closely involved in the PI3K/AKT pathway, might be one of the major downstream targets of HOXA-AS2. Silencing HOXA-AS2 decreased the expression of SOX4, whereas the upregulation of SOX4 partially abrogated the inhibitory effect of silencing HOXA-AS2 on leukemic cells. In conclusion, these findings suggest that HOXA-AS2 probably functions as an oncogene via SOX4/PI3K/AKT pathway and might be a useful biomarker for the prognostic prediction in AML patients, providing a potential therapeutic target for AML.

KEY WORDS

acute myeloid leukemia, HOXA-AS2, lncRNA, PI3K/AKT, SOX4

Abbreviations: AML, acute myeloid leukemia; BM, bone marrow; EdU, 5-ethynyl-2'-deoxyuridine; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; HOX, homeobox; HOXA-AS2, homeobox A cluster antisense RNA 2; lncRNAs, long noncoding RNAs; MRD, measurable residual disease; OS, overall survival; PBS, phosphate-buffered saline; PCNA, proliferating cell nuclear antigen; PI, propidium iodide; qRT-PCR, quantitative real-time polymerase chain reaction assay; RFS, relapse-free survival; SOX4, sex-determining region Y-box 4; WBC, white blood cell.

1 | INTRODUCTION

Acute myeloid leukemia (AML), a highly heterogeneous malignancy, originates from hematopoietic stem cells. It is characterized by aberrant myeloid blast proliferation and severe differentiation blockage, leading to hematopoietic insufficiency (Dohner, Weisdorf, & Bloomfield, 2015). It has previously been identified that various genetic abnormalities, such as chromosomal alterations and somatic mutations, contribute to the pathogenesis of AML (Ley et al., 2013; Li et al., 2016). They are also critical factors determining the survival of AML patients (Papaemmanuil et al., 2016). Despite significant advances in the molecular targeting therapy of AML, the overall survival still needs improvement and the accurate biomarkers associated with the prognosis demand more solid data. Therefore, the key factors for the pathogenesis and clinical relevance of AML need further investigation.

Long noncoding RNAs (lncRNAs) are a kind of RNA transcripts longer than 200 nucleotides with limited or absent protein-coding capacity. They play important roles in cellular function and gene regulation (Chen, Satpathy, & Chang, 2017; Guttman & Rinn, 2012). Aberrant expression of lncRNAs has been observed in a wide range of human diseases. Importantly, accumulating evidence suggests that lncRNAs can act as oncogenes or tumor repressors. They are closely involved in the initiation, development, and progression of certain cancers, including leukemia (Garzon et al., 2014; Gu et al., 2015; Kondo, Shinjo, & Katsushima, 2017; Xiao et al., 2017). For instance, HOTAIR promoted the growth and inhibited apoptosis of leukemic cells by modulating c-Kit (Xing et al., 2015), whereas lncRNA CCAT1 repressed monocytic differentiation and promoted cell growth by sponging microRNA-155 (miR-155; Chen, Wang, Cao, Li, & Wang, 2016). Furthermore, MALAT-1 is closely related to the poor prognosis of patients with the M5 subtype (Huang et al., 2017). It is reasonable to suggest that lncRNAs might be ideal research candidates for better understanding the pathogenesis and potential therapeutic targets in AML.

The homeobox (HOX) genes are a group of highly conserved homeodomain-containing transcription factors expressed in hematopoietic cells in the specific lineage and in a differentiation stage-restricted pattern (Alharbi, Pettengell, Pandha, & Morgan, 2013). HOX genes are crucial for hematopoiesis and leukemogenesis. Their expression strongly correlates with disease status and outcomes of AML patients (Alharbi et al., 2013; de Braekeleer et al., 2014; Spencer et al., 2015). HOXA cluster antisense RNA 2 (HOXA-AS2) is a 1,048-basepairs lncRNA located in the HOXA genomic cluster on chromosome 7p15.12 between and antisense to HOXA3 and HOXA4 genes. Several studies had revealed that HOXA-AS2 was aberrantly expressed in some types of solid cancers, such as colorectal cancer, gastric cancer, pancreatic cancer, hepatocellular carcinoma, gallbladder carcinoma, breast cancer, and glioma. It was associated with the clinical characteristics and affects the proliferation, invasion, or migration of tumor cells (Ding et al., 2017; Fang et al., 2017; Gao et al., 2018; Li, Dai, Wang, & Hou, 2016; Lian et al., 2017; Wang et al., 2016; Xie et al., 2015; Zhang et al., 2017). However, the role of HOXA-AS2 in leukemia remains unclear.

The present study revealed the clinical relevance of HOXA-AS2 in AML patients and explored its potential role in the growth of leukemic cells. The results suggested that HOXA-AS2 probably functions as an oncogene by modulating the SOX4/PI3K/AKT pathway and might be a valuable biomarker for the prognostic prediction of AML as well as a potential therapeutic target for leukemia.

2 | MATERIALS AND METHODS

2.1 | Patients and samples

A total of 108 patients with AML (50 males and 58 females) in the First Affiliated Hospital of China Medical University from January 2014 to June 2016 were enrolled in the present study. Bone marrow (BM) specimens were available at the time of diagnosis from the remaining samples used for clinical BM test. The patients were diagnosed according to the French–America–British (FAB) and World Health Organization criteria (Bennett et al., 1985; Wandt, Haferlach, Thiede, & Ehninger, 2010). Patients diagnosed as a nonmalignant hematologic disease were defined as controls (36 patients; 20 males and 16 females). CD34 positive cord blood cells isolated from six individuals were used as controls for AML patients or leukemic cell lines by using the CD34 MicroBead Kit (Miltenyi Biotech, Bergisch Gladbach, Germany). The average age of participants was 48.5-years (ranging from 14 to 84 years) for AML patients and 41-years (ranging from 18 to 72 years) for the control populations. The karyotype risk assessment was classified as reported previously (Grimwade et al., 2010). All patients provided written informed consent for the use of their BM samples. This study was approved by the Ethics Committee of the First Affiliated Hospital of China Medical University (No. AF-SOP-07-1.0-01; 2016-8-2).

2.2 | Cell lines and culture

HEK-293T and the human leukemic cell lines HL-60, U937, THP-1, OCI-AML3, NB4, Kasumi-1, and K562 were kind gifts from the Shanghai Institute of Hematology (Shanghai, China). All these cells were grown at 37°C in a humidified atmosphere with 5% CO₂. The leukemic cells were cultured in Rosewell Park Memorial Institute-1640 medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT) and 1% penicillin/streptomycin (Hyclone). HEK-293T cells were cultured in Dulbecco's modified Eagle's medium (Hyclone) supplemented with 10% FBS (Hyclone) and 1% penicillin/streptomycin.

2.3 | Lentivirus construction and production

pshRNA-U6-GFP plasmid (Wanleibio, Shenyang, China) containing a cytomegalovirus-driven green fluorescent protein (GFP) reporter and a U6 promoter upstream of the *Hpa*I and *Xba*I restriction sites was

used in this study. On the basis of the sequence of HOXA-AS2 (GenBank accession No. NR_122069.1) and Ambion's short hairpin RNA (shRNA) design online tool, three specific HOXA-AS2 shRNAs (HOXA-AS2 shRNA-1, HOXA-AS2 shRNA-2, HOXA-AS2 shRNA-3) and one scramble shRNA (shRNA control) were synthesized, and their sequences are listed in Table S1.

To produce lentivirus, HEK-293T cells were cotransfected with the Packaging Plasmid Mix (Wanleibio) and the pshRNA-U6-GFP by using the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The supernatant containing lentivirus was harvested at 48 hr after transfection, centrifuged, filtered through a 0.45- μ m filter, and then stored at -80°C. The lentivirus titers produced were approximately 10⁸ transducing units/ml.

2.4 | Cell infection and transfection

The leukemic cells were seeded into 24-well plates at a density of 2 × 10⁴ cells per well and grown to 50% confluence prior to infection. Thereafter, they were infected with recombinant lentivirus at a multiplicity of infection (MOI) of 30. The medium was replaced by a fresh medium 24 hr later. The SOX4 overexpression plasmid and the empty plasmid (GeneScript, Nanjing, China) were transfected into leukemic cells using Attractene Transfection Reagent (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The silencing and overexpression efficiencies were determined by quantitative real-time polymerase chain reaction (qRT-PCR) assay and western blot assays.

2.5 | RNA extraction and qRT-PCR

Total RNA was extracted from BM mononuclear cells with TRIzol reagent (Invitrogen). Then, RNA was transcribed into complementary DNA using a PrimeScript Real-time Reagent Kit with a genomic DNA Eraser (TaKaRa, Shiga, Japan) according to the manufacturer's protocol. The qRT-PCR analysis was performed with SYBR Premix Ex Taq II (TaKaRa) on an ABI 7500 Real-time PCR System (Applied Biosystems, Foster City, CA) using glyceraldehyde-3-phosphate dehydrogenase or β -actin as the endogenous control. The specific primers used are listed in Table 1. The conditions for thermal cycling

were as follows: 94°C for 5 min; 40 cycles of 94°C for 10 s, 60°C for 20 s, and 72°C for 30 s; then 72°C for 2.5 min and 40°C for 1.5 min; melting from 60°C to 94°C, 1°C/s; and incubation at 25°C for 2 min. The expression of HOXA-AS2 and SOX4 were normalized to the endogenous control to obtain the relative threshold cycle (ΔC_t) value. For clinical samples, the levels of HOXA-AS2 and SOX4 were calculated using the 2^{- $\Delta\Delta C_t$} method. For cells cultured in vitro, fold changes in HOXA-AS2 and SOX4 expression were calculated using 2^{- $\Delta\Delta C_t$} method.

2.6 | Western blot assay

Cells in each group were collected and lysed with radioimmunoprecipitation lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 1 mM Na₃VO₄, 2 mM sodium pyrophosphate, 1 mM ethylenediaminetetraacetic acid, 1 mM β -glycerophosphate, and 1 μ g/ml leupeptin) containing 1 mM phenylmethane sulfonyl fluoride (Beyotime, Shanghai, China). After centrifugation at 10,000g for 10 min at 4°C, the protein concentration of the supernatant was measured with a bicinchoninic acid protein assay kit (Beyotime). Thereafter, 40 μ g of protein in each group was separated using 10–15% SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes (Millipore, MA). The membranes were blocked with 5% non-fat milk at room temperature for 1 hr and subsequently incubated with primary antibodies overnight at 4°C. The primary antibodies used were as follows: proliferating cell nuclear antigen (PCNA; 1:1,000; Proteintech, Wuhan, China), cyclin D1 (1:1,000; Cell Signaling Technology, MA), BCL2 (1:1,000; Sangon Biotech, Shanghai, China), BAX (1:1,000; Sangon Biotech), cleaved caspase 3 (1:1,000; Abcam, Cambridge, UK), cleaved caspase 9 (1:1,000; Cell Signaling Technology), cleaved PARP (1:1,000; Abcam), PI3K (1:500; Wanleibio), p-PI3K (1:1,000; Abcam), AKT (1:1,000; Wanleibio), p-AKT (1:1,000; Wanleibio), P21 (1:500; Wanleibio), P27 (1:500; Wanleibio), SOX4 (1:500; Abcam), and β -actin (1:500; Santa Cruz Biotechnology, Inc., TX). After washing with Tris-buffered saline with Tween 20, the blots were incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (1:5,000; Proteintech) at room temperature for 1 hr, detected using enhanced chemiluminescence (Millipore), and visualized with Image Quant LAS-4000 image acquisition system (FujiFilm, Wuhan, China).

TABLE 1 Sequences of qRT-PCR primer

Name	Forward primer (5'-3')	Reverse primer (5'-3')
HOXA-AS2	CCCGTAGGAAGAA-CCGATGA	TTTAGGCCTCGCAGACAGC
SOX4	CAAGATCATGGAGCAGTCG	TTGTAGTCGGGTAGTCAG
β -Actin	CACTGTGCCATCTACGAGG	TAATGTCACGCACGATTCC
GAPDH	CGGATTGGTCGTATTGGG	CTGGAAGATGGTGATGGGATT

Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HOXA-AS2, homeobox A cluster antisense RNA 2; qRT-PCR, quantitative real-time polymerase chain reaction assay; SOX4, sex-determining region Y-box 4.

2.7 | Proliferation assay

Cell proliferation was assessed using the cell counting kit-8 (CCK-8) assay. Cells were seeded into 96-well plates at a density of 4×10^3 cells/well per 100 μ l and incubated at 37°C for 12, 24, 48, and 72 hr. Subsequently, 10 μ l CCK-8 solution was added into each well at the indicated time. After additional 2-hr incubation, the absorbance at 450 nm was measured with a microplate reader (Biotek, Winooski, VT).

2.8 | Flow cytometric analysis

For cell cycle assay, 1×10^6 cells from each group were fixed using ice-cold 70% ethanol at 4°C for 2 hr. Following washing with phosphate-buffered saline (PBS) twice, cells were re-suspended in 500 μ l staining buffer and incubated with 25 μ l propidium iodide (PI), and 10 μ l RNase A at 37°C for 30 min in the dark. Cell apoptosis assay was performed using an annexin V-fluorescein isothiocyanate (FITC)/PI double-staining cell apoptosis kit (KeyGen, Nanjing, China) according to the manufacturer's protocol. Briefly, cells were washed with PBS twice and resuspended in 100 μ l 1× binding buffer. Then, they were double-stained with 5 μ l Annexin V-FITC and 10 μ l PI for 20 min at room temperature in the dark. Cell cycle and apoptosis were analyzed using flow cytometry (BD Biosciences, Franklin Lakes, NJ). Data were processed using matched Accuri C6 Flow Plus software (version 1.0.1727; BD).

2.9 | 5-Ethynyl-2'-deoxyuridine (EdU) incorporation assay

EdU incorporation assay was performed using an EdU assay kit (KeyGen) according to the manufacturer's protocol. Briefly, leukemic cells were placed into 96-well plates at a density of 5×10^3 cells per well and incubated with 10 μ M EdU for 2 hr at 37°C, and then the cells were fixed with 4% paraformaldehyde and washed with PBS containing 3% bovine serum albumin for 20 min at room temperature. After permeabilization with 100 μ l 0.5% Triton X-100 for 20 min, the cells were stained with click-IT (KeyGen) for 30 min in the dark. Subsequently, the cells' nuclei were stained with Hoechst 33342 (Beyotime) for 15 min. Finally, samples were visualized with a fluorescent microscope (Olympus, Tokyo, Japan) under a $\times 400$ magnification.

2.10 | Hoechst staining assay

For Hoechst staining, the cultured cells were washed with PBS twice and fixed in 4% paraformaldehyde for 20 min at room temperature. Then, 0.5 ml Hoechst 33258 solution (Beyotime) was applied to stain cell nuclei for 10 min at room temperature. Subsequently, the coverslips were re-washed twice with PBS and air-dried. The anti-quenching agent was added, while cells were mounted onto glass slides. The apoptotic nuclei were observed with a fluorescence microscope under $\times 400$ magnification.

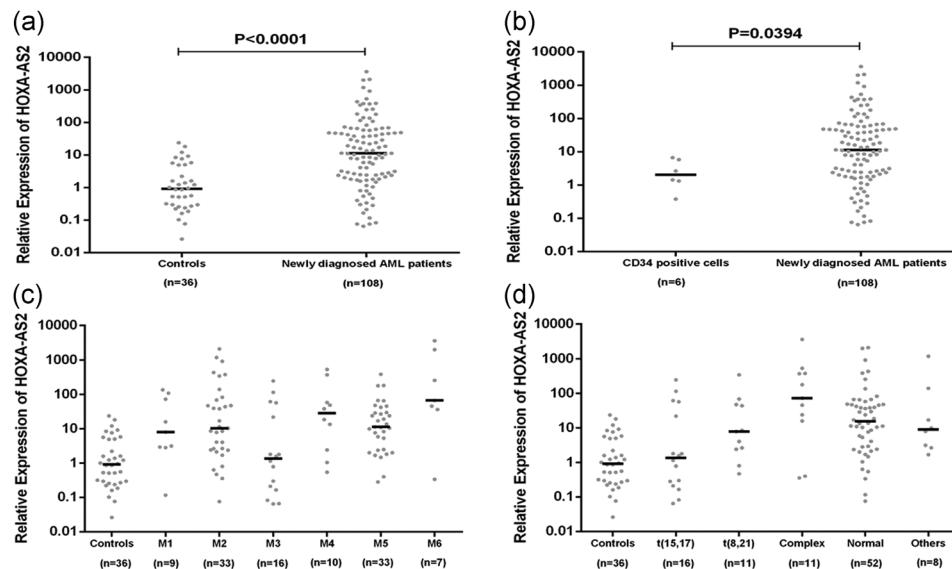


FIGURE 1 HOXA-AS2 expression was upregulated in patients with AML. (a) Quantitative analysis of HOXA-AS2 in AML patients and controls. (b) Quantitative analysis of HOXA-AS2 in AML patients and CD34 positive cells. (c) Expression of HOXA-AS2 in AML patients categorized by karyotype classification. (d) Expression of HOXA-AS2 in AML patients categorized by karyotype classification. Data were presented as median with interquartile range or frequency. AML, acute myeloid leukemia; HOXA-AS2, homeobox A cluster antisense RNA 2

TABLE 2 Correlation between HOXA-AS2 level and clinical/laboratory characteristics of patients with newly diagnosed AML

HOXA-AS2 expression (n, %)					
Variables	No. of patients (%)	High	Low	p Value ^a	
Gender					
Female	58 (53.70)	31 (57.41)	27 (50)	.4402	
Male	50 (46.30)	23 (42.59)	27 (50)		
Age (years)					
<60	85 (78.70)	41 (75.93)	44 (81.48)	.4807	
≥60	23 (21.30)	13 (24.07)	10 (18.52)		
WBC ($\times 10^9/L$)					
<30	56 (51.85)	20 (37.04)	36 (66.67)	.0021	
≥30	52 (48.15)	34 (62.96)	18 (33.33)		
HGB, g/L					
<80	45 (41.67)	27 (50)	18 (33.33)	.0790	
≥80	63 (58.33)	27 (50)	36 (66.67)		
PLT ($\times 10^9/L$)					
<50	65 (60.19)	37 (68.52)	28 (51.85)	.0769	
≥50	43 (39.81)	17 (31.48)	26 (48.15)		
Blast in BM, %					
<70	45 (41.67)	15 (27.78)	30 (55.56)	.0060	
≥70	63 (58.33)	39 (72.22)	24 (44.44)		
Extramedullary disease					
Absent	9 (18.42)	45 (83.33)	46 (85.19)	.7916	
Present FAB subtype	17 (15.74)	9 (16.67)	8 (14.81)		
M1/M2	42 (38.89)	20 (37.04)	22 (40.74)		
M3	16 (14.81)	5 (9.26)	11 (20.37)	.1056	
M4/M5	43 (39.81)	23 (42.59)	20 (37.04)		
M6	7 (6.49)	6 (11.11)	1 (1.85)		
Karyotype classification ^b					
Favorable	30 (27.77)	10 (18.51)	20 (37.04)		
Intermediate	56 (51.85)	31 (57.41)	25 (46.30)	.0311	
Unfavorable	12 (11.11)	9 (16.67)	3 (5.56)		
Karyotypes					
Normal	52 (48.15)	29 (53.70)	23 (42.59)		
t(15;17)	16 (14.81)	5 (9.26)	11 (20.37)		
t(8;21)	11 (10.185)	4 (7.41)	7 (12.96)	.1046	
Complex	11 (10.19)	9 (16.67)	2 (3.71)		
Others	8 (7.41)	3 (5.56)	5 (9.26)		
No data	10 (9.26)	4 (7.41)	6 (11.11)		
MRD					
Positive	34 (31.48)	20 (37.04)	14 (28.40)	.0434	
Negative	47 (43.52)	17 (31.48)	30 (44.44)		
Gene mutation					
NPM1					
Positive	20 (18.52)	8 (14.81)	12 (25.93)	.3357	
Negative	65 (60.19)	34 (62.96)	31 (57.41)		

(Continues)

TABLE 2 (Continued)

HOXA-AS2 expression (n, %)					
Variables	No. of patients (%)	High	Low	p Value ^a	
DNMT3A					
Positive	14 (12.96)	10 (18.52)	4 (7.41)	.0714	
Negative	71 (65.74)	32 (59.26)	39 (48.15)		
FLT3-ITD/TKD					
Positive	17 (15.74)	12 (22.22)	5 (9.26)	.0509	
Negative	68 (62.96)	30 (55.56)	38 (70.37)		
C/EBPA					
Positive	13 (12.04)	4 (7.41)	9 (16.67)	.1441	
Negative	72 (69.23)	38 (70.37)	34 (62.96)		
Complete remission					
Yes	84 (77.78)	38 (70.37)	46 (85.19)	.0641	
No	24 (22.22)	16 (29.63)	8 (14.81)		
Early death ^c					
Yes	10 (9.26)	9 (16.67)	1 (1.85)	.0161	
No	98 (90.74)	45 (83.33)	53 (98.15)		
Relapse					
Yes	37 (44.05)	21 (55.26)	16 (34.78)	.0783	
No	47 (55.95)	17 (44.74)	30 (65.22)		

The bolded values emphasize the statistical difference of the related clinical parameters.

Abbreviations: AML, acute myeloid leukemia; BM, bone marrow; HGB, hemoglobin; HOXA-AS2, homeobox A cluster antisense RNA 2; MRD, measurable residual disease; PLT, blood platelet; WBC, White blood cell.

^aPearson's χ^2 analysis.^bFavorable, inv(16), or t(16;16); t(8;21); t(15;17). Intermediate, normal karyotype; +8 alone; t(9;11); other undefined. Unfavorable, complex (≥ 3 clonal chromosomal abnormalities); monosomal karyotype, -5, 5q-, -7, 7q-; 11q23-non-t(9;11); inv(3), t(3;3); t(6;9); t(9;22).^cEarly death was defined as death within 30 days.

2.11 | Statistical analysis

GraphPad Prism 6.0 (GraphPad Software, CA) and SPSS 22.0 (IBM Corp, Armonk, NY) statistical software were used for data analysis. Normally distributed continuous variables were presented as mean \pm standard deviation and analyzed using Student's t test, one- or two-way analysis of variance, whereas non-normal distributed parameters were presented as median with interquartile range or frequency using the Wilcoxon rank-sum test for the difference analysis. Pearson's χ^2 analysis or Fisher's exact test was used to compare the difference in categorical variables. Survival probabilities were estimated using the Kaplan-Meier method, and differences in patient survival were compared using the log-rank test. Univariate and multivariate analysis of variables affecting survival were determined by Cox proportional hazards regression model. All tests were two-tailed, and $p < .05$ was considered statistically significant.

3 | RESULTS

3.1 | Expression of HOXA-AS2 was higher in AML patients

The expression of HOXA-AS2 was evaluated in a cohort of 108 patients with newly diagnosed AML and in control populations. The expression level of HOXA-AS2 in AML patients was higher than that in controls (Figure 1a) or in CD34⁺ cord blood cells (Figure 1b). Subsequently, the expression of HOXA-AS2 was compared according to the FAB subtypes. No significant difference was found among the FAB subtypes, whereas there was a tendency toward lower expression in the patients with the M3 subtype (Figure 1c). The expression was also compared based on karyotypes and the result showed that no significant correlation was found between HOXA-AS2 expression and specific karyotypes (Figure 1d). HOXA-AS2 expression seemed to be relatively higher in the patients with a complex or normal karyotype, whereas relatively lower in t(15;17) or t(8;21) ones.

Consistently, analysis of The Cancer Genome Atlas (TCGA) dataset indicated that M3 AML patients showed a lower level of HOXA-AS2 than the other FAB subtypes (Figure S1a). Moreover, AML patients with complex or normal karyotypes expressed a higher level of HOXA-AS2 than ones with t(15;17) or t(8;21) in the TCGA cohort (Figure S1b).

3.2 | A higher HOXA-AS2 expression was associated with aggressive clinical characteristics and tended to reflect adverse prognosis in AML patients

AML patients ($n = 108$) were divided into a HOXA-AS2-high group ($n = 54$) and a HOXA-AS2-low group ($n = 54$) according to the median values of HOXA-AS2 expression to identify whether the expression of HOXA-AS2 was related to the clinical features and outcomes in patients with AML. The clinical and laboratory characteristics of patients in these two groups are summarized in Table 2. The results of Pearson χ^2 analysis showed that patients with increased levels of HOXA-AS2

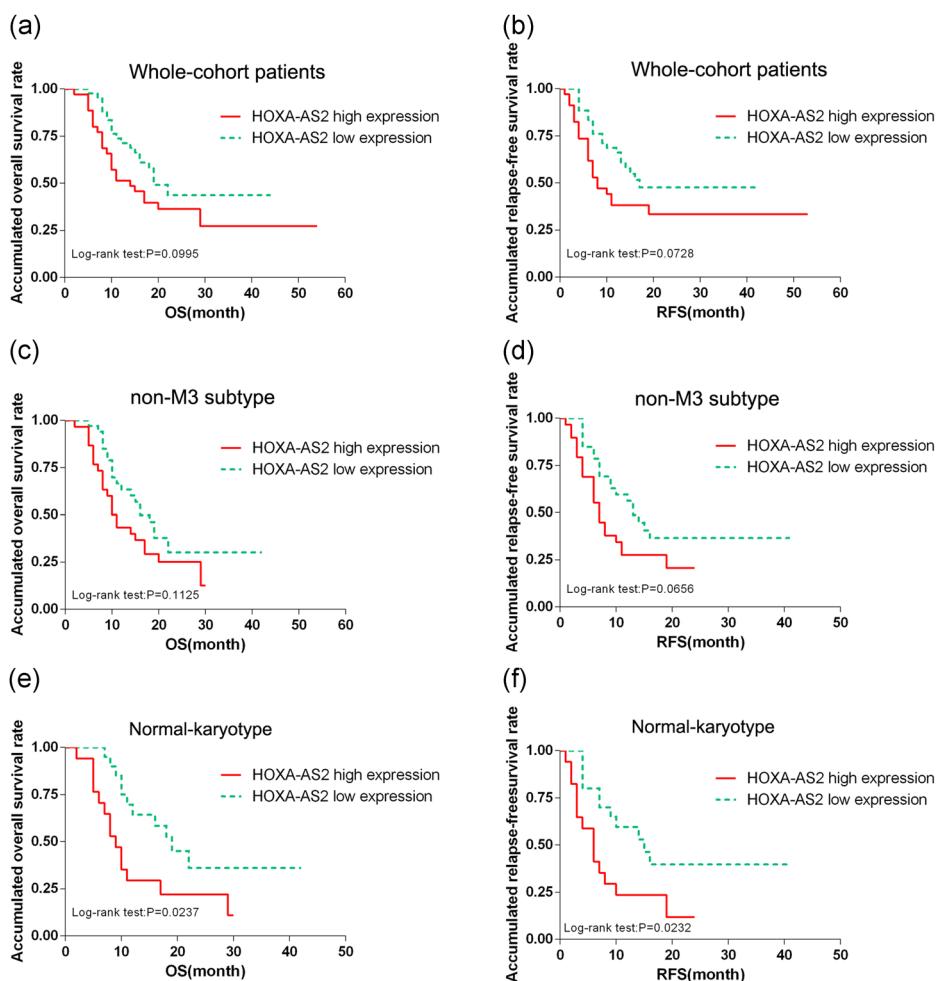


FIGURE 2 Accumulated OS and RFS rates in different subtypes of AML patients stratified according to HOXA-AS2 expression level. (a,b) Accumulated OS and RFS rates in whole-cohort patients with AML. (c,d) Accumulated OS and RFS rates in non-M3 subtype patients with AML. (e,f) Accumulated OS and RFS rates in patients with normal-karyotype AML. AML, acute myeloid leukemia; HOXA-AS2, homeobox A cluster antisense RNA 2; OS, overall survival; RFS, relapse-free survival

TABLE 3 Univariate and multivariate analyses of variables affecting OS in normal-karyotype AML patients

Variables	Univariate Cox's regression				Multivariate Cox's regression			
	<i>p</i> Value	HR	95% CI		<i>p</i> Value	HR	95% CI	
			Lower	Higher			Lower	Higher
High HOXA-AS2 expression	.024	2.353	1.171	6.052	.033	0.988	0.561	3.141
WBC ($\geq 30 \times 10^9/L$)	.014	0.364	0.154	0.860	.008	4.406	1.473	13.184
BM blast ($\geq 70\%$)	.403	1.418	0.626	3.216				
Elderly age (≥ 60 years)	.736	1.185	0.442	3.181				
Gender (male)	.676	1.187	0.531	2.654				
MRD positivity	.024	2.595	1.133	5.946	.017	2.058	1.551	8.977
NPM1 mutation	.075	0.319	0.091	1.121				
DNMT3A mutation	.093	2.240	0.873	5.744				
FLT3-ITD/TKD mutation	.079	2.318	0.908	5.917				
C/EBPA mutation	.225	0.404	0.093	1.747				

The bolded values emphasize the statistical difference of the related clinical parameters.

Abbreviations: AML, acute myeloid leukemia; BM, bone marrow; CI, confidence interval; HOXA-AS2, homeobox A cluster antisense RNA 2; HR, hazard ratio; OS, overall survival; MRD, measurable residual disease; WBC, white blood cells.

had higher white blood cell (WBC) and BM blast ratios ($p = .021$ and $.0060$, respectively), more immunophenotypic measurable residual disease (MRD) positivity ($p = .0434$), and much earlier death ($p = .0161$). Moreover, based on the karyotype risk assessment, the patients with AML were divided into favorable, intermediate, and unfavorable groups. High expression of HOXA-AS2 was more frequently seen in patients with unfavorable karyotype groups, whereas a

low level of HOXA-AS2 was observed in patients with favorable risk ($p = .0331$). However, other clinical parameters, such as age, gender, hemoglobin level, platelet count, extramedullary diseases, specific gene mutations, complete remission, and relapse occurrence, were not directly associated with the expression of HOXA-AS2.

In addition, the follow-up data was obtained in 80 cases of AML patients. As shown in Figure 2a,b, in whole-cohort AML patients, the

TABLE 4 Univariate and multivariate analysis of variables affecting RFS in normal-karyotype AML patients

Variables	Univariate Cox's regression				Multivariate Cox's regression			
	<i>p</i> Value	HR	95% CI		<i>p</i> Value	HR	95% CI	
			Lower	Higher			Lower	Higher
High HOXA-AS2 expression	.023	2.347	1.182	6.108	.021	2.751	1.062	4.544
WBC ($\geq 30 \times 10^9/L$)	.021	0.363	0.153	0.860	.005	3.632	1.466	9.000
BM blast ($\geq 70\%$)	.472	0.741	0.328	1.677				
Elderly age (≥ 60 years)	.808	0.885	0.329	2.375				
Gender (male)	.722	0.864	0.387	1.931				
MRD positivity	.029	0.421	0.184	0.959	.011	3.412	1.303	6.567
NPM1 mutation	.100	0.353	0.102	1.219				
DNMT3A mutation	.097	0.448	0.173	1.158				
FLT3-ITD/TKD mutation	.192	0.537	0.211	1.365				
C/EBPA mutation	.244	2.381	0.552	10.264				

The bolded values emphasize the statistical difference of the related clinical parameters.

Abbreviations: AML, acute myeloid leukemia; BM, bone marrow; CI, confidence interval; HR, hazard ratio; MRD, measurable residual disease; RFS, relapse-free survival; WBC, white blood cells.

overall survival (OS) and relapse-free survival (RFS) rates of patients in the HOXA-AS2-high group seemed to be relatively lower than those of patients in the HOXA-AS2-low group. Similar results were obtained in non-M3 AML patients (Figure 2c,d). Intriguingly, among normal-karyotype AML patients, it showed that the patients with higher HOXA-AS2 expression had shorter OS and RFS ($p = .0237$ and $.0232$, respectively; Figure 2e,f). Thus, univariate and multivariate analyses were subsequently performed to assess the prognostic value of HOXA-AS2 in patients with normal karyotype. As shown in Tables 3 and 4, the univariate analysis indicated that higher expression of HOXA-AS2 showed a prognostic significance for predicting inferior OS ($p = .024$) and RFS ($p = .023$). In the multivariate analysis, higher expression of HOXA-AS2 was further confirmed as an independent factor for unfavorable OS ($p = .033$) and RFS ($p = .021$), along with several well-known prognostic factors including higher WBC counts and immunophenotypic MRD positivity.

These data indicated that HOXA-AS2 could be a useful predictor for the prognosis of AML patients, especially in the normal-karyotype groups. As higher expression of HOXA-AS2 is associated with more aggressive disease and inferior prognosis, we proposed that HOXA-AS2 might play a role in the leukemogenesis.

3.3 | Silencing HOXA-AS2 attenuated the proliferation of leukemic cells in vitro

To investigate the biological function of HOXA-AS2 in leukemia, the expression of HOXA-AS2 was first examined in different myeloid leukemia cell lines. The results showed that HOXA-AS2 expression was upregulated in all myeloid leukemia cell lines compared with the control (Figure 3a). K562 and HL-60 cells with relatively higher HOXA-AS2 expression were chosen for further investigation. As previous results showed that HOXA-AS2 probably had positive effect on the pathogenesis of AML, thus we downregulated HOXA-AS2 expression by shRNAs for further analysis. The expression of HOXA-AS2 was decreased by all of the three shRNAs (Figure S2a). HOXA-AS2 shRNA-1 and shRNA-2 were used in subsequent experiments due to a higher interference efficiency. After silencing HOXA-AS2, the proliferation of leukemic cells was assessed by using the CCK-8 assay. Leukemic cells with downregulated HOXA-AS2 showed a slower proliferation compared with respective controls (Figure 3b). Consistently, the protein level of PCNA was also decreased upon HOXA-AS2 downregulation (Figure 3c). These findings indicated that silencing of HOXA-AS2 inhibited the proliferation of leukemic cells.

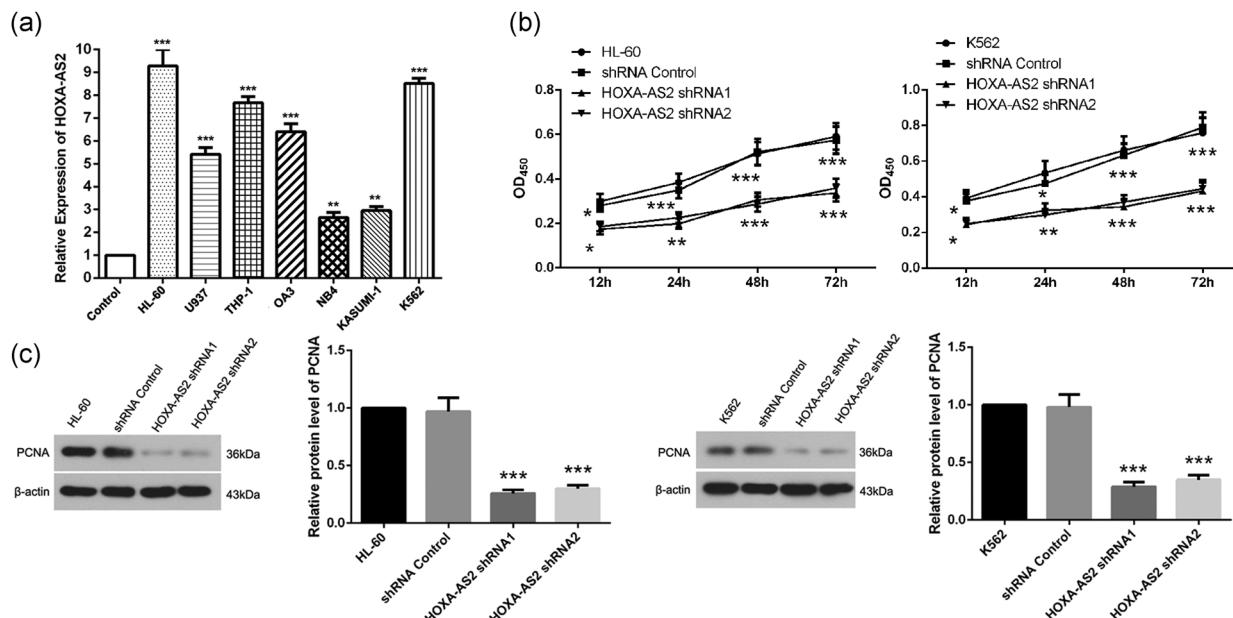


FIGURE 3 HOXA-AS2 silencing attenuated leukemic cell proliferation in vitro. (a) qRT-PCR was performed to measure the expression level of HOXA-AS2 in different myeloid leukemia cell lines in comparison with the control. (b) CCK-8 assay was performed to assess the proliferation of HL-60 and K562 cells after infection of HOXA-AS2 shRNAs. (c) The protein levels of PCNA were detected using western blot assay. Data were presented as mean \pm SD of three independent experiments. CCK-8, cell counting kit-8; HOXA-AS2, homeobox A cluster antisense RNA 2; PCNA, proliferating cell nuclear antigen; qRT-PCR, quantitative real-time polymerase chain reaction; SD, standard deviation; shRNA, short hairpin RNA. * $p < .05$, ** $p < .01$, and *** $p < .001$ compared with the shRNA control

3.4 | Downregulation of HOXA-AS2 arrested cell cycle of leukemic cells

The effect of silencing HOXA-AS2 on cell cycle was then detected using flow cytometry. The results showed that silencing HOXA-AS2 upregulated the G0/G1 phase percentage and downregulated the S phase percentage of leukemic cells compared with the control groups (Figure 4a). Consistently, the results of EdU incorporation assay also indicated that silencing of HOXA-AS2 decreased the EdU-positive cell population, an important hallmark of DNA synthesis in S phase (Figure 4b). In addition, the results of western blot assay showed that the protein level of cyclin D1 also declined upon HOXA-AS2 knockdown (Figure 4c). These results demonstrated that silencing HOXA-AS2 arrested the cell cycle of leukemic cells in the G0/G1 phase.

3.5 | HOXA-AS2 silencing induced apoptosis in leukemic cells

To further explore the effects of silencing HOXA-AS2 on the leukemic cells, apoptotic analysis was performed. The results of flow cytometry analysis demonstrated that the cells infected with HOXA-AS2 shRNAs showed more apoptosis compared with the shRNA control (Figure 5a). This finding was also confirmed by Hoechst staining, showing that more apoptotic bodies in HOXA-AS2 silencing group (Figure 5b). Furthermore, the results of western blot assay showed that the levels of proapoptotic protein, such as cleaved caspase 3, cleaved caspase 9, cleaved PARP, and BAX were upregulated upon HOXA-AS2 knockdown (Figure 6a-d), whereas the level of antiapoptotic protein BCL2, was consequentially decreased (Figure 6e). These findings suggested that HOXA-AS2 had an oncogenic role in leukemic cell proliferation by affecting apoptosis.

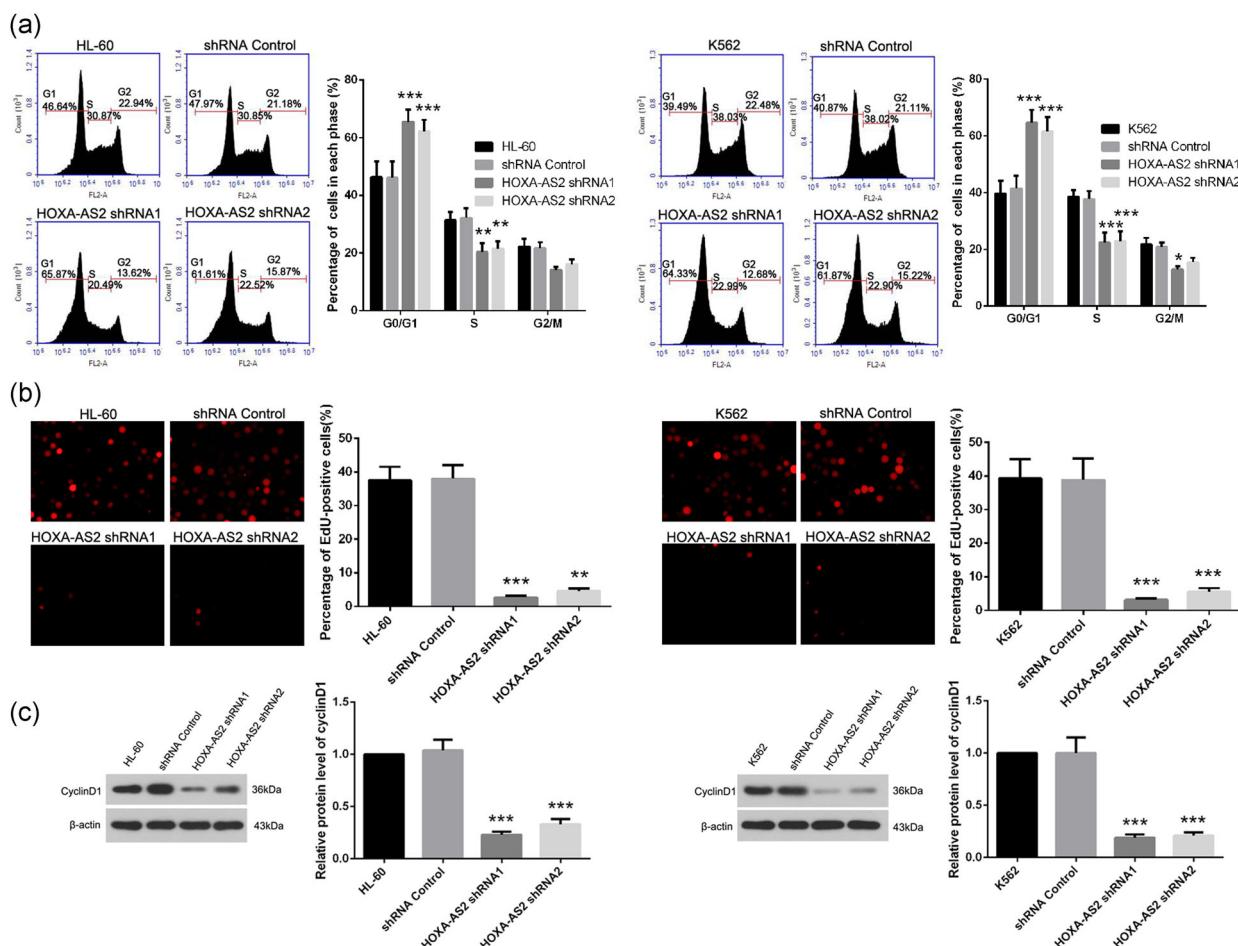


FIGURE 4 HOXA-AS2 silencing arrested the cell cycle of leukemic cells. (a) After being infected with HOXA-AS2 shRNAs, HL-60 and K562 cells were analyzed for cell cycle by flow cytometry. (b) The cell cycle analysis for HL-60 and K562 with HOXA-AS2 knockdown was performed by using EdU incorporation assay. (c) The protein level of cyclin D1 was detected by western blot assay. Data were presented as mean \pm SD from three independent experiments. EdU, 5-ethynyl-2'-deoxyuridine; HOXA-AS2, homeobox A cluster antisense RNA 2; SD, standard deviation; shRNA, short hairpin RNA. * $p < .05$, ** $p < .01$, and *** $p < .001$ compared with the shRNA control

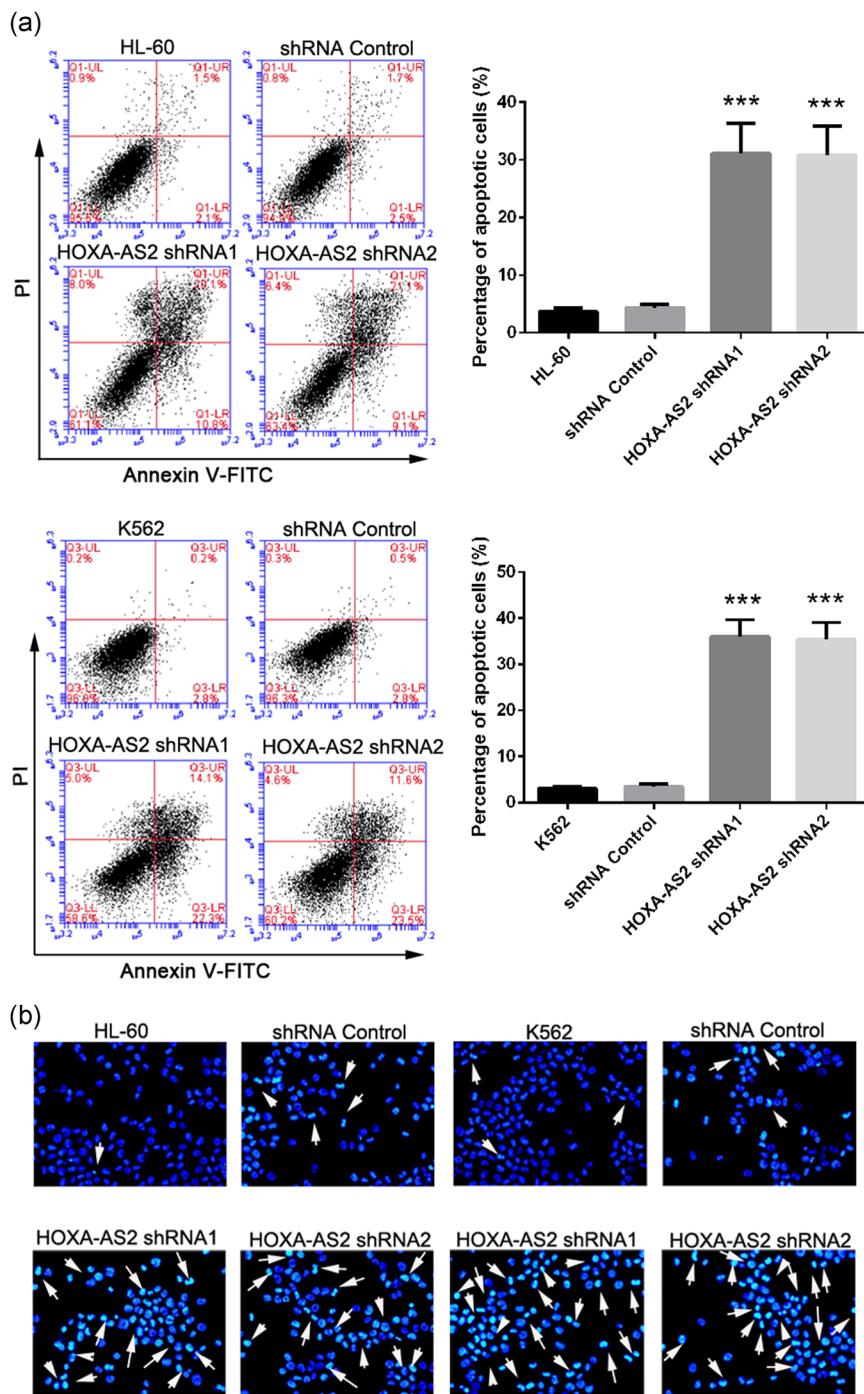


FIGURE 5 HOXA-AS2 silencing induced apoptosis in leukemic cells. (a) The apoptosis of HL-60 and K562 cells with HOXA-AS2 knockdown was assessed by flow cytometry. (b) Apoptosis of HL-60 and K562 cells was evaluated using Hoechst assay. Arrows indicated chromatin condensation. Data were presented as mean \pm SD of three independent experiments. FITC, fluorescein isothiocyanate; HOXA-AS2, homeobox A cluster antisense RNA 2; SD, standard deviation; shRNA, short hairpin RNA. *** $p < .001$ compared with the shRNA control

3.6 | HOXA-AS2 silencing inhibited the SOX4/PI3K/AKT pathway

Recently, the PI3K/AKT signaling pathway is found to be aberrantly activated in AML and contributes to leukemogenesis. Previous studies have shown that SOX4, a putative driver of PI3K/AKT signaling pathway, acted as the critical oncogene in acute leukemia, particularly in the myeloid lineage. So, here we tried to investigate whether HOXA-AS2 regulated the leukemic

cell growth by the SOX4/PI3K/AKT pathway. Western blot and qRT-PCR analyses were applied to detect the changes in SOX4 expression upon HOXA-AS2 knockdown. Both messenger RNA and protein levels of SOX4 were obviously reduced upon HOXA-AS2 silencing (Figure 7a). Interestingly, SOX4 expression was increased in AML patients and cell lines (Figure 7b), which was significantly positively correlated with HOXA-AS2 level (Figure 7c). Furthermore, silencing HOXA-AS2 resulted in an obvious decreasing phosphorylated PI3K and AKT, without

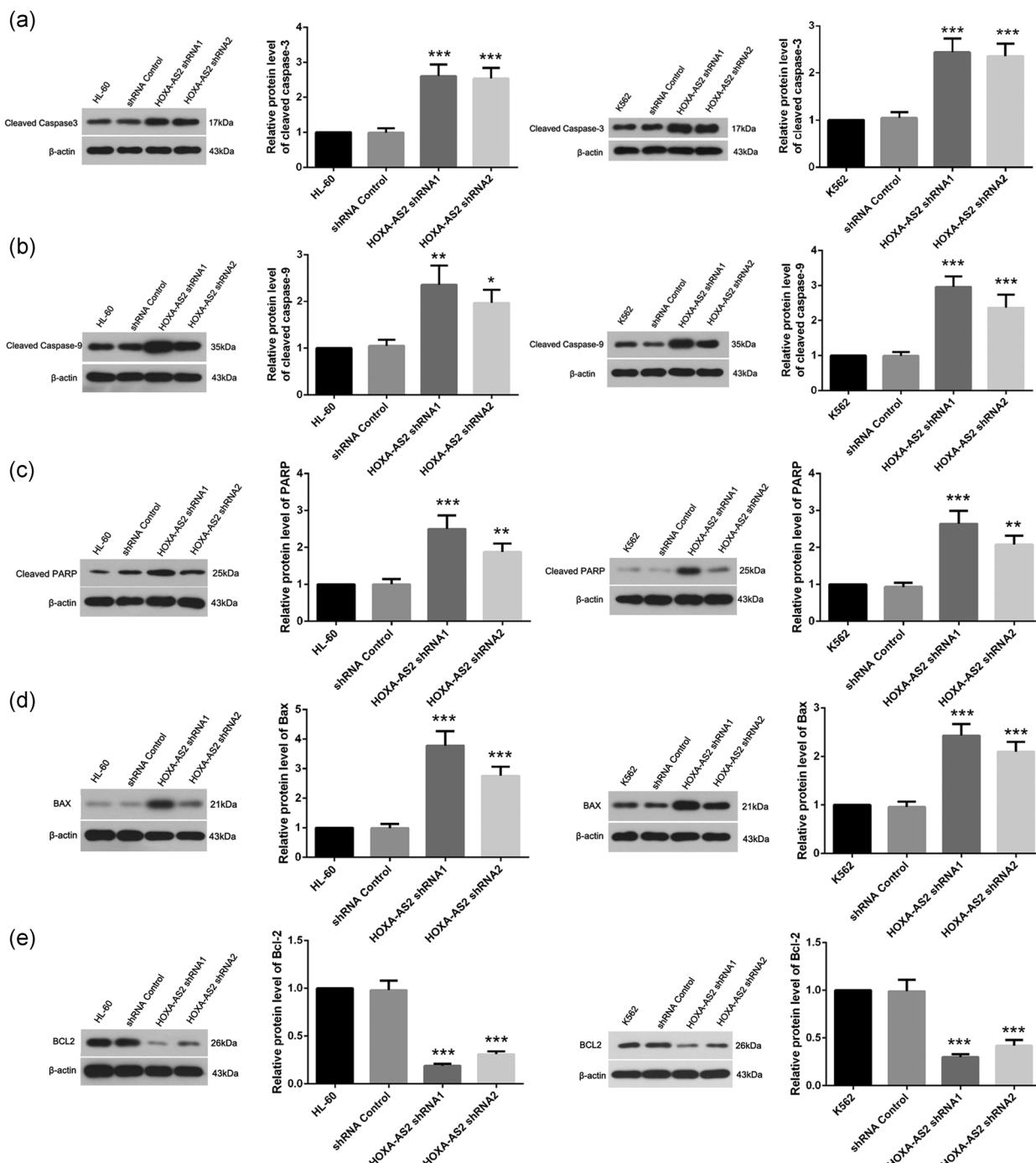


FIGURE 6 HOXA-AS2 modulated the expression of the apoptosis-related proteins. The protein level of cleaved caspase 3 (a), cleaved caspase 9 (b), cleaved PARP (c), BAX (d), and BCL2 (e) in different groups were detected using the western blot assay. Data were presented as mean \pm SD of three independent experiments. HOXA-AS2, homeobox A cluster antisense RNA 2; SD, standard deviation; shRNA, short hairpin RNA. * $p < .05$, ** $p < .01$, and *** $p < .001$ compared with the shRNA control

alterations in total PI3K and AKT (Figure 7d,e). Additionally, the PI3K/AKT downstream proteins, P21 and P27, were remarkably increased after HOXA-AS2 silencing (Figure 7f,g). These findings indicated that SOX4 might be a downstream target of HOXA-AS2 and HOXA-AS2 silencing might inhibit the SOX4/PI3K/AKT pathway.

3.7 | Upregulation of SOX4 partially abrogated the inhibitory effect of HOXA-AS2 knockdown on the leukemic cells

Following the determination of SOX4 as a downstream target of HOXA-AS2, the subsequent study was explored to investigate

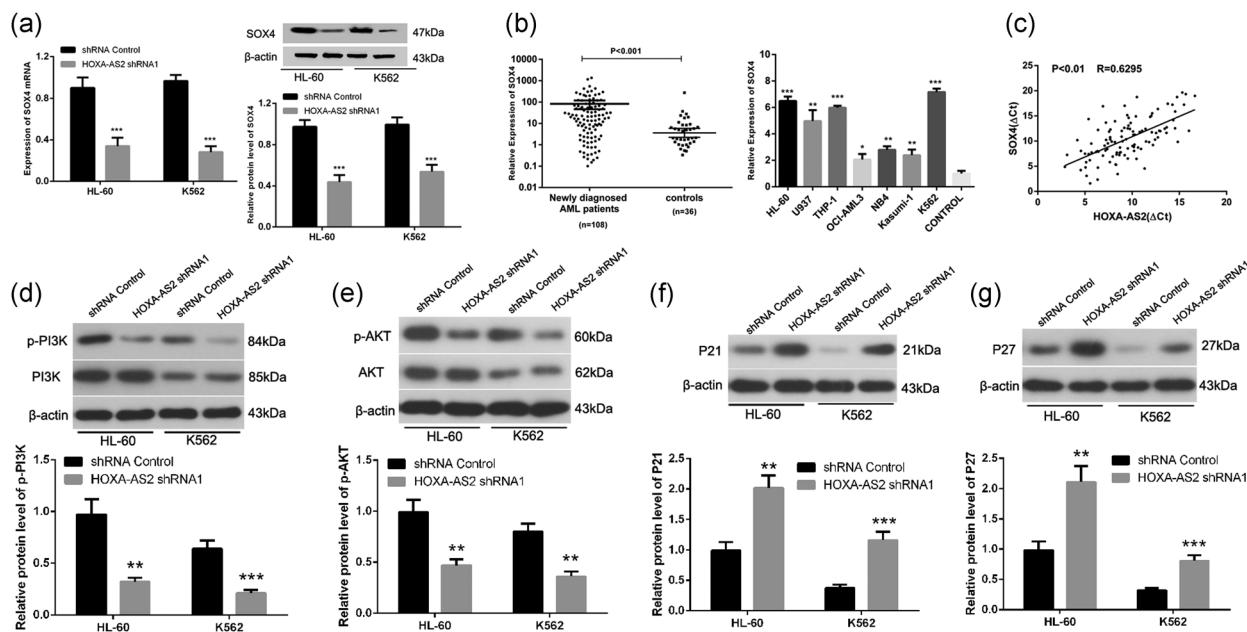


FIGURE 7 HOXA-AS2 silencing inhibited the SOX4/PI3K/AKT pathway. (b) The expression of SOX4 were measured by qRT-PCR and western blot assay in HL-60 and K562 cells infected with HOXA-AS2 shRNA or shRNA control. Data were presented as mean \pm SD of three independent experiments. *** $p < .001$ compared with the shRNA control. (b) The expression of SOX4 was investigated in AML patients and cell lines by qRT-PCR. Clinical data were presented as median with interquartile range or frequency; experimental data of AML cell lines were presented as mean \pm SD of three independent experiments. * $p < .05$, ** $p < .01$, and *** $p < .001$ compared with the control. (c) The correlation between SOX4 and HOXA-AS2 expression was proved by Pearson's correlation method. (d-g) The protein level of total PI3K, p-PI3K, total AKT, p-AKT, P21, and P27 in HL-60 and K562 cells infected with HOXA-AS2 shRNA and shRNA control was analyzed by western blot assay. Data were presented as mean \pm SD of three independent experiments. AML, acute myeloid leukemia; HOXA-AS2, homeobox A cluster antisense RNA 2; qRT-PCR, quantitative real-time polymerase chain reaction assay; SD, standard deviation; shRNA, short hairpin RNA; SOX4, sex-determining region Y-box 4. ** $p < .01$ and *** $p < .001$ compared with the shRNA control

whether SOX4 mediated the inhibitory effect of HOXA-AS2 silencing on the leukemic cell growth. SOX4 overexpression plasmid (SOX4 OE) and the empty plasmid (Vector) were employed in our study (Figure S2B,S2C). After cotransfection with the SOX4 OE plasmid and HOXA-AS2 shRNA, relevant cell function assays were performed to detect the proliferation, cell cycle, and apoptosis of the cotransfected leukemic cells. As shown in Figure 8a, the results of western blot assay showed that the decreased protein levels of SOX4 caused by HOXA-AS2 knockdown were recovered by SOX4 upregulation. Results of the subsequent CCK-8 assay indicated that overexpression of SOX4 partially abrogated the inhibition of the cell proliferation induced by HOXA-AS2 silencing (Figure 8b). Similarly, silencing HOXA-AS2 arrested the cell cycle of leukemic cells in the G0/G1 phase while overexpression of SOX4 partially reversed this trend (Figure 8c). Moreover, flow cytometric analysis also revealed that the increased cell apoptosis caused by downregulated HOXA-AS2 was decreased again by SOX4 overexpression (Figure 8d). All these findings demonstrated that SOX4 upregulation partially abrogated the inhibition of the malignant behaviors mediated by HOXA-AS2 knockdown on leukemic cells, which further suggested that SOX4 might be one of the major downstream targets regulated by HOXA-AS2.

4 | DISCUSSION

Several previous studies had reported the overexpression of HOXA-AS2 in multiple solid tumors, which was significantly associated with the advanced clinical parameters, such as increased tumor size, advanced TNM stage, and more lymphatic and distal metastases (Ding et al., 2017, Fang et al., 2017, Gao et al., 2018, Xie et al., 2015). However, the clinical relevance of HOXA-AS2 expression in AML patients remains obscure. In this study, HOXA-AS2 was found to be significantly upregulated in patients with AML compared with the control ones, which was consistent with the studies on solid tumors. Interestingly, here, we showed that the elevated expression of HOXA-AS2 was associated with the aggressive clinical characteristics, including higher white blood counts, more BM blasts, unfavorable karyotypes, and increased early death. In line with the clinical characteristics, the survival of AML patients with higher HOXA-AS2 was inferior. Furthermore, the patients with higher expression of HOXA-AS2 had more possibility of residual disease (MRD positive). All these findings indicated that HOXA-AS2 expression positively correlated with the tumor burden in patients with AML and reflected their aggressive disease status. It suggests that HOXA-AS2 might serve as a potential biomarker for prognosis and MRD monitoring in AML patients.

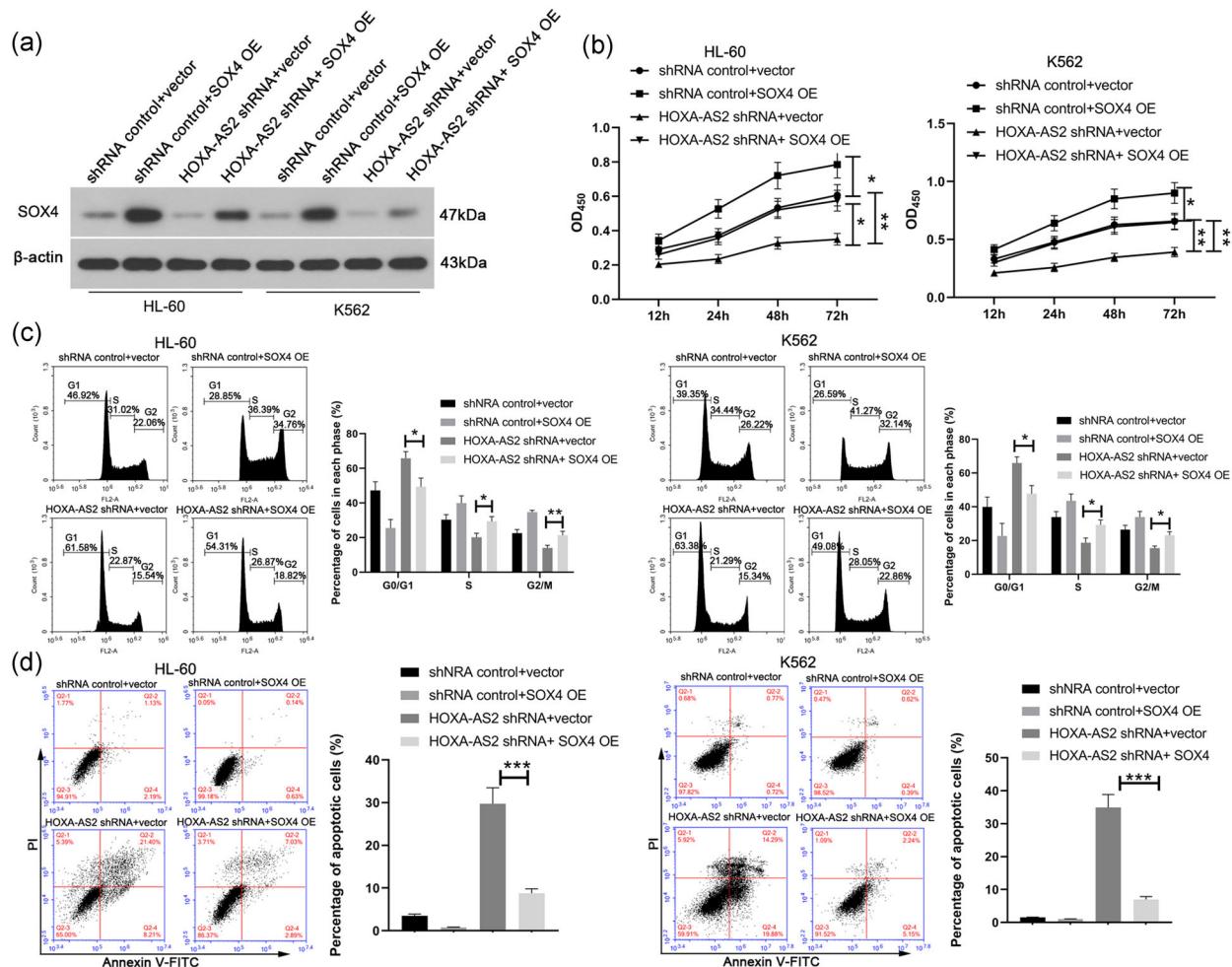


FIGURE 8 Upregulation of SOX4 partially abrogated the inhibitory effect of HOXA-AS2 knockdown on the leukemic cells. (a) Western blot assay was used to detect the protein level of SOX4 in the leukemic cells cotransfected with SOX4 OE plasmid and HOXA-AS2 shRNA. (b) After being cotransfected with SOX4 OE plasmid and HOXA-AS2 shRNA, the proliferation of transfected leukemic cells was assessed by CCK-8 assay. (c) After being cotransfected with SOX4 OE plasmid and HOXA-AS2 shRNA, flow cytometry was performed to detected the cell cycle. (d) Flow cytometry was employed to assess the apoptosis of the leukemic cells cotransfected with SOX4 OE plasmid and HOXA-AS2 shRNA. Data were presented as mean \pm SD of three independent experiments. HOXA-AS2, homeobox A cluster antisense RNA 2; OE, overexpression; SD, standard deviation; shRNA, short hairpin RNA; SOX4, sex-determining region Y-box 4; Vector, empty plasmid. *p < .05, **p < .01, and ***p < .001.

Despite rapid development in understanding the biology of AML and use of risk-directed treatment, the prognosis of patients are still not good. The patients with almost the same cytogenetic and molecular characteristics even do not have identical outcomes (Dombret & Gardin, 2016; Garzon et al., 2014; Mendler et al., 2012). It is necessary to explore the novel genetic alterations with clinical relevance to improve the accuracy of risk stratification. Several studies had demonstrated that lncRNAs could be used as biomarkers for outcome prediction in AML (Diaz-Beyaa et al., 2015; Huang et al., 2017; Mer et al., 2018). MALAT-1 had been reported to serve as an independent candidate indicator for evaluating the prognosis of patients with M5 subtype (Huang et al., 2017). The HOTAIR M1 level at diagnosis provided relevant information on worse prognosis in the intermediate-risk AML subgroup; it was strengthened on combining with miR-196b expression (Diaz-Beyaa et al., 2015). A lncRNA-lncRNA coexpression network constructed

using TCGA dataset displayed a significant correlation with patient survival (Mer et al., 2018). Here, our results revealed that there was a tendency toward shorter OS and RFS in AML patients with high expression of HOXA-AS2 compared with those with low expression. In particular, the prognostic value of HOXA-AS2 in the normal-karyotype subgroup appeared to be more significant. Notably, both univariate and multivariate Cox regression analysis further showed that the elevated HOXA-AS2 level was independently associated with lower OS and RFS in the normal-karyotype AML patients. Therefore, it would be conceivable to develop HOXA-AS2 expression as a potential biomarker for predicting prognosis of AML patients, especially in refining the risk stratification of patients with normal karyotype.

On the basis of the clinical findings, it is speculated that HOXA-AS2 might have an oncogenic role on leukemic pathogenesis. To confirm this hypothesis, the effect of HOXA-AS2 silencing on

leukemic cell growth was analyzed. Downregulation of HOXA-AS2 in HL-60 and K562 cells markedly attenuated cell proliferation, induced G1/G0-phase arrest, and promoted cell apoptosis. Consistently, it was reported that HOXA-AS2 negatively regulated ATRA-induced TRAIL-mediated apoptosis in NB4 cells (Zhao, Zhang, Frazao, Condino-Neto, & Newburger, 2013). These findings suggested that HOXA-AS2 was involved in AML pathology, providing a potential novel therapeutic target for AML.

Aberrant activation of the PI3K/AKT pathway is widespread in hematological malignancies including AML and plays an important role in mediating cell differentiation, proliferation, metastasis, cell cycle, and apoptosis (Liao, Zhang, Zhao, & Liu, 2018; Manning & Toker, 2017). SOX4 is identified as a novel mediator of PI3K/AKT signaling and is essential for triggering leukemogenesis. It had been revealed that SOX4 bound directly to the promoters of genes involved in PI3K/AKT signaling, such as PIK3R3 in Ph+ acute lymphoblastic leukemia (Ramezani-Rad et al., 2013). In this study, downregulation of HOXA-AS2 obviously decreased SOX4 expression in leukemic cells. Also, the association between HOXA-AS2 and the SOX4 gene expressions was positive in AML samples and cell lines. Importantly, upregulation of SOX4 partially reversed the inhibition mediated by HOXA-AS2 silencing on the malignant behavior of leukemic cells. This is suggestive of HOXA-AS2 exerting its oncogenic roles in AML by targeting SOX4. Consequently, HOXA-AS2 was further verified to modulate the growth of AML through the PI3K/AKT pathway. However, the exact regulatory correlation between HOXA-AS2 and SOX4 is still unclear. Whether HOXA-AS2 upregulates SOX4 by sponging certain miRNA or epigenetic modification, needs further research.

5 | CONCLUSIONS

In summary, lncRNA HOXA-AS2 functioned as an oncogene in AML. It was associated with the aggressive disease status and adverse prognosis in patients. HOXA-AS2 promoted the proliferation of leukemic cells and repressed cell apoptosis via the SOX4/PI3K/AKT signaling pathway. These findings strengthen our better understanding of the role of lncRNAs in leukemogenesis, providing a promising prognostic biomarker and a potential therapeutic target for AML.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

REFERENCES

- Alharbi, R. A., Pettengell, R., Pandha, H. S., & Morgan, R. (2013). The role of HOX genes in normal hematopoiesis and acute leukemia. *Leukemia*, 27, 1000–1008.
- Bennett, J. M., Catovsky, D., Daniel, M. T., Flandrin, G., Galton, D. A., Gralnick, H. R., & Sultan, C. (1985). Proposed revised criteria for the classification of acute myeloid leukemia. A report of the French-American-British Cooperative Group. *Annals of Internal Medicine*, 103, 620–625.
- Chen, L., Wang, W., Cao, L., Li, Z., & Wang, X. (2016). Long non-coding RNA CCAT1 acts as a competing endogenous RNA to regulate cell growth and differentiation in acute myeloid leukemia. *Molecules and Cells*, 39, 330–336.
- Chen, Y. G., Satpathy, A. T., & Chang, H. Y. (2017). Gene regulation in the immune system by long noncoding RNAs. *Nature Immunology*, 18, 962–972.
- de Braekeleer, E., Douet-Guilbert, N., Basinko, A., Le Bris, M. J., Morel, F., & de Braekeleer, M. (2014). Hox gene dysregulation in acute myeloid leukemia. *Future Oncology*, 10, 475–495.
- Diaz-Beyaa, M., Brunet, S., Nomdedeu, J., Pratcorona, M., Cordeiro, A., Gallardo, D., ... Esteve, J. (2015). The lncRNA HOTAIR1, located in the HOXA genomic region, is expressed in acute myeloid leukemia, impacts prognosis in patients in the intermediate-risk cytogenetic category, and is associated with a distinctive microRNA signature. *Oncotarget*, 6, 31613–31627.
- Ding, J., Xie, M., Lian, Y., Zhu, Y., Peng, P., Wang, J., ... Wang, K. (2017). Long noncoding RNA HOXA-AS2 represses P21 and KLF2 expression transcription by binding with EZH2, LSD1 in colorectal cancer. *Oncogenesis*, 6, e288.
- Dohner, H., Weisdorf, D. J., & Bloomfield, C. D. (2015). Acute myeloid leukemia. *New England Journal of Medicine*, 373, 1136–1152.
- Dombret, H., & Gardin, C. (2016). An update of current treatments for adult acute myeloid leukemia. *Blood*, 127, 53–61.
- Fang, Y., Wang, J., Wu, F., Song, Y., Zhao, S., & Zhang, Q. (2017). Long non-coding RNA HOXA-AS2 promotes proliferation and invasion of breast cancer by acting as a miR-520c-3p sponge. *Oncotarget*, 8, 46090–46103.
- Gao, Y., Yu, H., Liu, Y., Liu, X., Zheng, J., Ma, J., ... Xue, Y. (2018). Long non-coding RNA HOXA-AS2 regulates malignant glioma behaviors and vasculogenic mimicry formation via the MiR-373/EGFR axis. *Cellular Physiology and Biochemistry*, 45, 131–147.
- Garzon, R., Volinia, S., Papaioannou, D., Nicolet, D., Kohlschmidt, J., Yan, P. S., ... Bloomfield, C. D. (2014). Expression and prognostic impact of lncRNAs in acute myeloid leukemia. *Proceedings of the National Academy of Sciences of the United States of America*, 111, 18679–18684.
- Grimwade, D., Hills, R. K., Moorman, A. V., Walker, H., Chatters, S., Goldstone, A. H., ... Burnett, A. K. (2010). Refinement of cytogenetic classification in acute myeloid leukemia: determination of prognostic significance of rare recurring chromosomal abnormalities among 5876 younger adult patients treated in the United Kingdom Medical Research Council trials. *Blood*, 116, 354–365.
- Gu, Y., Chen, T., Li, G., Yu, X., Lu, Y., Wang, H., & Teng, L. (2015). LncRNAs: Emerging biomarkers in gastric cancer. *Future Oncology*, 11, 2427–2441.
- Guttman, M., & Rinn, J. L. (2012). Modular regulatory principles of large non-coding RNAs. *Nature*, 482, 339–346.
- Huang, J. L., Liu, W., Tian, L. H., Chai, T. T., Liu, Y., Zhang, F., ... Shen, J. Z. (2017). Upregulation of long non-coding RNA MALAT-1 confers poor prognosis and influences cell proliferation and apoptosis in acute monocytic leukemia. *Oncology Reports*, 38, 1353–1362.
- Kondo, Y., Shinjo, K., & Katsushima, K. (2017). Long non-coding RNAs as an epigenetic regulator in human cancers. *Cancer Prevention Research*, 108, 1927–1933.
- Ley, T. J., Miller, C., Ding, L., Raphael, B. J., Mungall, A. J., Robertson, A., ... Eley, G. (2013). Genomic and epigenomic landscapes of adult de novo acute myeloid leukemia. *New England Journal of Medicine*, 368, 2059–2074.
- Li, Q., Dai, Y., Wang, F., & Hou, S. (2016). Differentially expressed long non-coding RNAs and the prognostic potential in colorectal cancer. *Neoplasma*, 63, 977–983.

- Li, S., Garrett-Bakelman, F. E., Chung, S. S., Sanders, M. A., Hricik, T., Rapaport, F., ... Mason, C. E. (2016). Distinct evolution and dynamics of epigenetic and genetic heterogeneity in acute myeloid leukemia. *Nature Medicine* (New York, NY), 22, 792–799.
- Lian, Y., Li, Z., Fan, Y., Huang, Q., Chen, J., Liu, W., ... Xu, H. (2017). The lncRNA-HOXA-AS2/EZH2/LSD1 oncogene complex promotes cell proliferation in pancreatic cancer. *American Journal of Translational Research*, 9, 5496–5506.
- Liao, Y. X., Zhang, Z. P., Zhao, J., & Liu, J. P. (2018). Effects of fibronectin 1 on cell proliferation, senescence and apoptosis of human glioma cells through the PI3K/AKT signaling pathway. *Cellular Physiology and Biochemistry*, 48, 1382–96.
- Manning, B. D., & Toker, A. (2017). AKT/PKB signaling: Navigating the network. *Cell*, 169, 381–405.
- Mendler, J. H., Maharry, K., Radmacher, M. D., Mrozek, K., Becker, H., Metzeler, K. H., ... Bloomfield, C. D. (2012). RUNX1 mutations are associated with poor outcome in younger and older patients with cytogenetically normal acute myeloid leukemia and with distinct gene and microRNA expression signatures. *Journal of Clinical Oncology*, 30, 3109–3118.
- Mer, A. S., Lindberg, J., Nilsson, C., Klevebring, D., Wang, M., Gronberg, H., ... Rantalaisten, M. (2018). Expression levels of long non-coding RNAs are prognostic for AML outcome. *Journal of Hematology & Oncology*, 11, 52.
- Papaemmanuil, E., Gerstung, M., Bullinger, L., Gaidzik, V. I., Paschka, P., Roberts, N. D., ... Campbell, P. J. (2016). Genomic classification and prognosis in acute myeloid leukemia. *New England Journal of Medicine*, 374, 2209–21.
- Ramezani-Rad, P., Geng, H., Hurtz, C., Chan, L. N., Chen, Z., Jumaa, H., ... Muschen, M. (2013). SOX4 enables oncogenic survival signals in acute lymphoblastic leukemia. *Blood*, 121, 148–155.
- Spencer, D. H., Young, M. A., Lamprecht, T. L., Helton, N. M., Fulton, R., O'Laughlin, M., ... Ley, T. J. (2015). Epigenomic analysis of the HOX gene loci reveals mechanisms that may control canonical expression patterns in AML and normal hematopoietic cells. *Leukemia*, 29, 1279–1289.
- Wandt, H., Haferlach, T., Thiede, C., & Ehninger, G. (2010). WHO classification of myeloid neoplasms and leukemia. *Blood*, 115, 748–749.
- Wang, F., Yang, H., Deng, Z., Su, Y., Fang, Q., & Yin, Z. (2016). HOX antisense lncRNA HOXA-AS2 promotes tumorigenesis of hepatocellular carcinoma. *Cellular Physiology and Biochemistry*, 40, 287–296.
- Xiao, Z. D., Han, L., Lee, H., Zhuang, L., Zhang, Y., Baddour, J., ... Gan, B. (2017). Energy stress-induced lncRNA FILNC1 represses c-Myc-mediated energy metabolism and inhibits renal tumor development. *Nature Communications*, 8, 783.
- Xie, M., Sun, M., Zhu, Y. N., Xia, R., Liu, Y. W., Ding, J., ... De, W. (2015). Long noncoding RNA HOXA-AS2 promotes gastric cancer proliferation by epigenetically silencing P21/PLK3/DDIT3 expression. *Oncotarget*, 6, 33587–33601.
- Xing, C. Y., Hu, X. Q., Xie, F. Y., Yu, Z. J., Li, H. Y., Bin, Z., ... Gao, S. M. (2015). Long non-coding RNA HOTAIR modulates c-KIT expression through sponging miR-193a in acute myeloid leukemia. *FEBS Letters*, 589, 1981–1987.
- Zhang, P., Cao, P., Zhu, X., Pan, M., Zhong, K., He, R., ... Gao, Y. (2017). Upregulation of long non-coding RNA HOXA-AS2 promotes proliferation and induces epithelial-mesenchymal transition in gallbladder carcinoma. *Oncotarget*, 8, 33137–43.
- Zhao, H., Zhang, X., Frazao, J. B., Condino-Neto, A., & Newburger, P. E. (2013). HOX antisense lncRNA HOXA-AS2 is an apoptosis repressor in all trans retinoic acid treated NB4 promyelocytic leukemia cells. *Journal of Cellular Biochemistry*, 114, 2375–2383.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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