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ORIGINAL RESEARCH

RETRACTED ARTICLE: miR-188-5p Promotes Tumor Growth by Targeting CD2AP Through PI3K/AKT/mTOR Signaling in Children with Acute Promyelocytic Leukemia

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Dao Wang
Jiao Chen
Yanjie Ding
Huimin Kong
Hongliang You
Yanting Zhao
Huixia Wei
Yufeng Liu

Department of Pediatrics, The First Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, Henan, People's Republic of China

Purpose: Pediatric acute promyelocytic leukemia (APL) accounts for 10% of pediatric acute myelogenous leukemia (AML) cases and is accompanied by a tendency to hemorrhage. miR-188-5p plays an important role in adult AML. Therefore, the purpose of this study was to explore the effects of miR-188-5p on cell proliferation and apoptosis and tumor growth, and its mechanism in pediatric APL patients.

Materials and Methods: Survival-associated miRNAs or mRNAs from TCGA database associated with AML were identified via using the "survival R" package in R language. CCK8, clone formation, flow cytometry, RT-PCR, immunohistochemistry and Western blot assays were used to detect the viability, proliferation, apoptosis, cell cycle, and related gene expression in APL cell lines. The prognostic value of miR-188-5p was evaluated using a ROC curve. The tumorigenic ability of APL cell lines was determined using a nude mouse transplantation tumor experiment. Tumor cell apoptosis was determined by TUNEL assay in vivo. The target genes of miR-188-5p were predicted using the miRDB, miRTarBase, and TargetScan databases. A PPI network was constructed using STRING database and the hub gene was identified using the MCODE plug-in of the Cytoscape software. The DAVID database was used to perform GO and KEGG pathway enrichment analyses. A luciferase reporter assay was used to demonstrate the binding of miR-188-5p to CD2AP.

Results: miR-188-5p overexpression or CD2 associated protein (CD2AP) inhibition was significantly associated with poor survival in pediatric APL patients. Upregulation of miR-188-5p was identified in the blood of pediatric APL patients and cell lines. Increased expression of miR-188-5p also promoted the viability, proliferation, and cell cycle progression, and reduced the apoptosis of APL cells. Additionally, upregulation of miR-188-5p regulated the expressions of cyclinD1, p53, Bax, Bcl-2 and cleaved caspase-3. The area under the ROC curve (AUC) of miR-188-5p was 0.661. miR-188-5p overexpression increased the tumorigenic ability of APL and Ki67 expression, and reduced cell apoptosis in vivo. CD2AP was identified as the only overlapping gene from the list of miR-188-5p target genes and survival-related mRNAs of the TCGA database. It was mainly enriched in the "biological process (BP)" and "cellular component (CC)" terms, and was downregulated in the blood of pediatric APL patients and cell lines. The luciferase reporter, RT-PCR, and Western blot assays demonstrated that the binding of miR-188-5p to CD2AP. CD2AP inhibition promoted the proliferation and inhibited the apoptosis of APL cells. Rescue experiments showed that inhibition of miR-188-5p inhibited cell proliferation, activated the PI3K/AKT/mTOR signaling pathway, induced G0/G1 phase arrest, regulated gene expression, and promoted cell apoptosis, which were reversed by CD2AP inhibition.

Correspondence: Yufeng Liu
Department of Pediatrics, The First Affiliated Hospital of Zhengzhou University, No. 1, Constructive East Road, Zhengzhou 450052, Henan, People's Republic of China
Tel +86-0371-66295592
Email lyf6012@163.com

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Conclusion: miR-188-5p, an oncogene, promoted tumor growth and progression of pediatric APL in vitro and in vivo via targeting CD2AP and activating the PI3K/AKT/mTOR signaling pathway.

Keywords: miR-188-5p, tumor growth, APL, CD2AP, PI3K/AKT/mTOR, proliferation, apoptosis

Introduction

According to epidemiological statistics in China, leukemia ranks sixth in malignant tumors and ranks first among children and people under 35 years old. There are about 40,000 new leukemia patients every year, of which 50% are children, and most of them are 2–9 years old.[1,2] Leukemia has become one of the main causes of death in children, a serious threat to the lives and health of children. Acute myeloid leukemia (AML) is a type of malignant hematopoietic stem/progenitor cell proliferation disease, often accompanied by chromosomal translocation or gene mutation, which hinders the proliferation, differentiation and maturation of normal hematopoietic cells.[3] This leads to highly heterogeneous hematological diseases, accounting for 25−30% of childhood acute leukemia.[4] Acute promyelocytic leukemia (APL) is a special type of AML, which is characterized by the malignant proliferation of immature promyelocytic cells in the bone marrow.[5] The clinical manifestations of APL include anemia, bleeding, infection, hepatosplenomegaly, and lymph node enlargement, bone pain, and other common manifestations of leukemia, bleeding tendency is the main clinical feature. It has been reported that 10% to 20% of patients with early bleeding had died, and about 60% of patients develop diffuse intravascular coagulation (DIC).[6,7] Therefore, it is of great significance to identify a therapeutic target of pediatric APL patients.

MicroRNAs (miRNAs) are small non-coding RNAs, composed of 25 nucleotides. miRNA can regulate the protein expression of multiple target genes at the same time, and each target gene can be regulated by multiple miRNAs. miRNAs play a key role in many important biological processes, such as cell differentiation, apoptosis and proliferation.[8] It is worth noting that recent studies have found that miRNAs also play an important role in the maintenance of the self-renewal ability of hematopoietic stem/progenitor cells, the differentiation of hematopoietic cells and the regulation of the cell cycle.[9] Abnormal expression of miRNAs can often lead to hematopoietic diseases. For example, miR-181a can block the differentiation of myeloid cells by inhibiting the expression of adenylate cyclase 9 (AC9),[10] miR-29a and miR-142-3p participate in the regulation of granulocyte/monocyte differentiation,[11] and miR-125b1 can inhibit the phagocytosis and degradation of the promyelocytic leukemia and retinoic acid receptor alpha (PMLRARα) fusion protein via autophagy by regulating the expression of a series of autophagy-related genes, thus inhibiting the differentiation of leukemic cells.[12,13] These results strongly suggest that miRNA can participate in the differentiation of hematopoietic cells and are closely related to the occurrence and development of AML.

It had been reported that miR-188-5p, an oncogene, is highly expressed in bladder cancer, which is significantly related to the poor prognosis of patients, and promotes the proliferation and invasion of gastric cancer cells.[14,15] MiR-188-5p, a suppressor gene, inhibits cell proliferation and metastasis in colon cancer and breast cancer.[16,17] However, miR-188-5p was highly expressed in neutralizing cells of patients with chronic myeloid leukemia.[18] Overexpression of miR-188-5p significantly suppressed the proliferative ability and arrested cell cycle progression of osteosarcoma cells via targeting CCNT2.[19] miR-188-5p promoted epithelial–mesenchymal transition in retinoblastoma by downregulating ID4 expression.[20] To sum up, miR-188-5p plays different roles in different tumors. In addition, miR-188-5p was regarded as a serum marker in the placenta of patients with preeclampsia, narcolepsy patients, and focal cerebral ischemia.[21–23] miR-188-5p was a prognostic biomarker in cytogenetically normal AML and had been investigated in AML on a large scale of clinical samples.[24] It indicated that the abnormal expression of miR-188-5p in blood should be an important miRNA to affect the occurrence and progress of AML. However, current research on miR-188-5p focused on adult AML; there are only a few studies on pediatric APL. Therefore, the effects of miR-188-5p on the occurrence and progression of pediatric APL and the related underlying mechanism need to be explored using cell function experiments in vitro and tumor formation in nude mice in vivo.

Materials and Methods

Blood Specimens

Blood specimens of 57 children (24 males and 33 females, 2–12 years, 4.32 ± 2.56 years) were obtained from the First Affiliated Hospital of Zhengzhou University in

accordance with APL diagnostic standard between March 2017 and May 2018. These 57 children received bone marrow morphology, immunophenotyping, cytogenetics and molecular biology (MICM) test. The clinical characteristics of the 57 children with APL are listed in Table 1.

The adverse reaction (ADRs) from chemotherapy in the 57 children with APL included all-trans-retinoic acid (ATRA) syndrome, headache, and gastrointestinal reaction. Peripheral blood mononuclear cells (PBMNCs) from 23 healthy donors (12 males and 11 females, 6–12 years, 7.5 ± 2.3 years) were used as a comparison group. Written informed consent was obtained from all patients and healthy volunteers or their guardians, which was then analyzed anonymously. This study was performed in accordance with the Declaration of Helsinki. A parent or legal guardian provided the written informed consent for any patient under the age of 18 years. The inclusion criteria are as follows: (1) first time diagnosis; and (2) received no therapies before admission. Exclusion criteria: (1) complicated with other clinical disorders; (2) treatment before admission; and (3) history of previous malignancies. Venous blood samples (5–6 mL/person) were obtained from 57 children with APL and 23 healthy donors on an empty stomach and stored at 4°C. This research was approved by the Ethics Committees of the First Affiliated Hospital of Zhengzhou University for the use of blood samples.

TCGA Database and Data Analysis

Gene expression profiles of 188 AML patients were downloaded from The Cancer Genome Altas (TCGA) database (https://portal.gdc.cancer.gov/). Two hundred clinical data, including gender, age, and survival were also downloaded from the TCGA database. Survival-related miRNAs were identified using "survival R" package in R language. $P<0.05$ were identified as significant.

Construction of the Protein–Protein Interaction (PPI) Network and Module Analysis

The Search Tool for the Retrieval of Interacting Genes (STRING) database was used to construct PPI network with an interaction score > 0.4. Cytoscape software was used to perform the visualization of PPI network. Molecular Complex Detection (MCODE) plug-in of Cytoscape software was used to screen the significant modules in the PPI network in accordance with the screening criteria of node number ≥3 and MCODE score ≥3.

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Enrichment Analyses

The Database for Annotation, Visualization and Integrated Discovery (DAVID) 6.8 (https://david.ncifcrf.gov/) was employed for GO and KEGG enrichment analyses.

Table 1 Association Between miR-188-5p Expression and Clinical Characteristics of Children with APL

		miR-188-5p Expression			
Clinical Characteristics	**Cases(n=57)**	**High (n=26)**	**Low (n=31)**	**χ^2/t**	**P-value**
Sex				0.260	0.610
Male	24	10	14		
Female	33	16	17		
Age (years)		4.58±2.78	4.10±2.37	−0.703	0.485
WBC (C_{cell}/×10^9 L^{-1})		28.17±4.88	4.04±1.12	−26.742	0.000*
PLT (C_{cell}/×109 L^{-1})		25.19±2.76	64.99±24.51	8.125	0.000*
Hb (/×109 L^{-1})		64.44±2.89	79.11±4.62	14.051	0.000*
DIC				2.57	0.109
Yes	20	12	8		
No	37	14	23		
ADR				4.800	0.028*
Yes	16	11	5		
No	41	15	26		

Note: *$P<0.05$.

Abbreviations: WBC, white blood cell; PLT, platelet; Hb, hemoglobin; DIC, disseminated vascular coagulation; ADR, adverse reaction; APL, pediatric acute promyelocytic leukemia.

A difference of P <0.05 indicated statistical significance. GO analysis was involved in the terms of cellular component (CC), biological process (BP), as well as molecular function (MF).

Cell Lines

APL cell lines (NB4 and HL-60) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). All cell lines were maintained at 37°C in the RPMI-1640 (Gibco Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen Life Technologies, Carlsbad, CA, USA).

Cell Proliferation Analysis

APL cells (2×10^4) were seeded in 96-well plates overnight. Then, 10 μL Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) solution was added to each well, incubated at 37°C for 0, 12, 24, 48, and 72 h. The optical density (OD) values were measured at 450 nm using a scanning multi-well spectrophotometer (Bio-Rad Model 550; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Flow Cytometry Analysis

Cells were collected and fixed at 4°C with cold ethanol overnight. After two washes in phosphate-buffered saline (PBS), the cells were re-suspended in 200 μL binding buffer, followed by staining with 40 μL PI (BestBio) for 30 min in the dark. Next, the cell cycle distribution was analyzed using a flow cytometry with FlowJo software (BD Bioscience).

To assess cell apoptosis, cells were collected, re-suspended and stained with Annexin V-FITC and PI (BestBio) for 20 min in the dark at 37°C. The numbers of early (Annexin V^+/PI^-), late (Annexin V^+/PI^+) and total apoptotic cells were detected using a flow cytometer equipped with CellQuest Pro software (BD Bioscience).

Cell Transfection

Negative control miRNA (mimics/inhibitors NC) and miR-188-5p mimics/inhibitors were synthesized by GenePharma (Shanghai, China). Forty-five nM miRNAs were transfected into APL cells via using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Subsequent experiments were performed at 48 h after transfections.

Luciferase Reporter Assay

TargetScan database (www.targetscan.org/vert_72) was used to predict the putative target genes associated with miR-188-5p. For the luciferase reporter assay, the wild-type (WT) or mutant (MUT) 3′-untranslated region (3′-UTR) of CD2AP was cloned into the pmirGLO dual-luciferase reporter vectors (Promega) using RIBOBIO. Then, they were transfected into HEK293T cells with miR-188-5p mimics/mimics NC or miR-188-5p inhibitors/inhibitors NC using Lipofectamine 2000 (Invitrogen). Cells were harvested after 48 h transfection and relative luciferase activities were determined using the Dual-Luciferase Reporter Assay System (Promega).

Prediction of the Target Genes of miR-188-5p

miRDB (http://mirdb.org/download.html), miRTarBase (http://mirtarbase.mbc.nctu.edu.tw/php/download.php), and TargetScan databases were used to predict the target genes of miR-188-5p.

Small Interfering RNA (siRNA)

siRNA duplexes targeting CD2AP (siRNA: 5′-GCUGACCUUACGGCCUAAACTT-3′) and a negative control (NC) siRNA duplex (forward: 5′-TTCTGTGTCTTCCAC GGAACT-3′; reverse: 5′-GGAGTTACACGTGAATC CGT-3′) were chemically synthesized by Shanghai GenePharma Co. Ltd. (Shanghai, China). Transfection was performed using Lipofectamine RNAiMAX (Invitrogen; Carlsbad, CA) according to the manufacturer's instructions.

Cell Grouping

This experiment was divided into nine groups: 1) NB4 or HL60 cells transfected with mimics NC was regarded as mimics NC group; 2) NB4 or HL60 cells transfected with miR-188-5p mimics were regarded as mimics group; 3) NB4 or HL60 cells transfected with inhibitors NC were regarded as inhibitors NC group; 4) NB4 or HL60 cells transfected with miR-188-5p inhibitors was regarded as inhibitors group; 5) NB4 or HL60 cells transfected with negative control siRNA were regarded as siNC group; 6) NB4 or HL60 cells transfected with siRNA targeting CD2AP were regarded as siCD2AP group; 7) NB4 cells co-transfected with inhibitors NC and siNC were regarded as Ctrl+siNC group; 8) NB4 cells co-transfected with miR-188-5p inhibitors and siNC were regarded as inhibitors+siNC group; and 9) NB4 cells co-transfected with miR-188-5p inhibitors

and siRNA targeting CD2AP was regarded as inhibitors +siCD2AP group.

Colony Formation Assay

A total of 500 cells infected with miR-194-expressing recombinant lentivirus (Hanbio, Shanghai, China) were seeded in each well of a 6-well plate. After 14 days of culture, the colonies were fixed in methanol for 10 min and then stained with a 1% crystal violet solution (Beyotime Institute of Biotechnology) for 20 min for imaging.

Tumor Growth in vivo

Male athymic nude mice (BALB/c-nu, 4 weeks, 18–22 g) were bred at the animal facility of the Center of Experimental Animals, Sun Yat-sen University (China). In brief, each nude mouse was implanted subcutaneously under the right armpit with 5×10^6 relative cells (NB4 cell, or K562 cell). Each cell line included two groups, and each group contained four mice. Ten days later, the mice with each cell line xenograft were injected with the following reagents: (1) mimics/or inhibitors NC, and (2) miR-188-5p mimics/inhibitors. The injection was twice a week, and the tumor size was measured with [illegible] calipers before injection. Two weeks later, the mice were sacrificed, and tumors were collected for further measurement. Five monitoring points for tumor volume of mice were collected. The mean of tumor volumes was calculated using the following formula: volume = length × width^2 × 0.5. All animal studies were conducted with the approval of the Medical Experimental Animal Care Commission of Zhengzhou University. Animal experiments were performed according to the Institutional Animal Care and Use Committee (IACUC) protocol and approved by the Medical Experimental Animal Care Commission of Zhengzhou University.

Immunohistochemistry (IHC) Assay

Ki67 expression was determined using IHC assay. Paraffin sections were dewaxed and hydrated. Antigens were retrieved by citrate buffer and blocked with 3% H_2O_2 at room temperature for 15 mins. Goat serum was used to block nonspecific binding sites for 15 mins. Slides were incubated with diluted primary antibody (Ki67; 1:600; ab16667; Abcam) at 4°C overnight followed by the secondary antibody for 20 mins at room temperature, and then incubated in streptavidin-horseradish peroxidase (SA-HRP) solution for 20 mins. The slides were stained by diaminobenzidine (DAB) (ZSGB-BIO, China) and hematoxylin and then mounted by neutral balata. Pictures were taken by 200 × magnification light microscope (Olympus, Japan). The results were analyzed using imagepro plus software. The positively stained samples were scored as follows: 1, ≤25% of positively stained cells; 2, >25%-≤50% of positively stained cells; 3, >50%-≤75% of positively stained cells; 4, >75% of positively stained cells. The intensity of staining was scored according to the following standard: 0, negative staining; 1, weak staining; 2, moderate staining; and 3, strong staining. The final score was calculated by multiplying the percentage score by the staining intensity score. The scores were independently determined by two pathologists.

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End Labeling (TUNEL) Assay

Tumor sections were treated with formalin for 48 hrs at RC, then with 4% formaldehyde in phosphate-buffered saline (PBS) for 25 mins at 4°C, and finally immersed in 0.2% Triton X-100 in PBS for 5 mins. Equilibration Buffer (100 µL) was added to the slides at room temperature for 5–10 mins, and staining was performed using the TUNEL assay kit (Promega Systems, Madison, WI, USA). Apoptotic cells that stained pale brown were visualized at × 100 or × 200 magnification under light microscopy (Olympus Optical Co., Tokyo, Japan) equipped with the Moticam 5000 C camera (Richmond, BC, Canada). Five randomly selected fields were used to count apoptotic cells, and image analysis was performed using Motic Med 6.0 software (Xiamen Motic Software Engineering Co., Ltd., Xiamen, China). The apoptosis index was calculated as the number of apoptotic cells/total number of cells × 100%. All other chemicals including xylene and ethanol were purchased from Sigma (St. Louis, MO, USA).

Western Blotting

Cells were washed with ice-cold PBS and lysed with ice-cold lysis buffer (1% Triton X-100, 50 mmol/l HEPES, 50 mmol/l sodium pyrophosphate, 100 mmol/l sodium fluoride, 10 mmol/l EDTA, 10 mmol/l sodium vanadate) containing protease inhibitors cocktail on ice. After centrifugation at 15,000g for 15 min at 4°C, the supernatant was analyzed for protein content using BCA protein assay kit (Beyotime, Shanghai, China). The protein was

heated at 100°C for 5 min, and a total of 60 μg protein were separated on 8–15% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gels, then transferred onto a PVDF membrane (Millipore, USA). The membranes were blocked with 5% skim milk in TBST buffer at room temperature for 1 h and were incubated with the antibodies against Ki67 (ab16667, 1:500; Abcam), cyclinD1 (ab134175, 1:800; Abcam), Bax (ab32503, 1:1000; Abcam), Bcl-2 (ab32124, 1:800; Abcam), cleaved caspase-3 (ab2302, 1:800; Abcam), anti-phospho-PI3K (CST4228s, 1:1000; CST, USA), anti-PI3K (AF1549, 1:1000; Beyotime, China), anti-phospho-AKT(AF2680, 1:800; Beyotime, China), anti-AKT (AF1777, 1:1000; Beyotime, China), anti-mTOR (AF1648, 1:800; Bey otime, China), anti-phospho-mTOR (AF5869, 1:1000; Beyotime, China) and anti-GAPDH (AF5009, 1:2000; Beyotime, China) at 4°C overnight. After the membranes were washed three times with TBST buffer, they were incubated with a corresponding secondary antibody in TBST buffer for 1 hr at room temperature, followed by washing three times with TBST. The protein-antibody bound bands were visualized using ECL reagents (Beyotime) and the signal strength of each protein was normalized against the corresponding control.

RNA Extraction and Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-PCR) Assay

The qRT-PCR for miRNA and mRNA was performed after the concentration of total RNA from prepared cells or serum extracted with TRIzol (Invitrogen) was calculated by measuring the absorbance at A260/280. Twenty μL RNA was reverse transcribed into cDNA using miScript reverse transcription kit (Bio-Rad Laboratories, Hercules, CA, USA). For miRNA quantification, each RT reaction was performed in a final volume of 20 μL consisting of 0.5 μg total RNA, 2.0 μL 5× RT buffer containing dNTPs (Takara Bio, Otsu, Japan), 0.2 μL 10 μmol/L stem-loop RT primer (Invitrogen), 0.2 μL RNase inhibitor protein (Takara Bio), and 0.8 μL reverse transcriptase (Takara Bio), and incubated at 42°C for 60 min and at 85°C for 5 min. RT-PCR was performed in triplicate using an Applied Biosystems 7500H system (Foster City, CA, USA) using SYBR Premix Ex Taq (Takara Bio). Cycling conditions were 1 cycle at 95°C for 30 sec and 40 cycles at 95°C for 5 sec and 60°C for 30 sec. Total complementary DNA (cDNA) was synthesized using a TaKaRa RT kit (Takara Bio). RT-PCR was performed using SYBR Premix Ex Taq (Takara Bio). Relative mRNA expression was determined by normalizing the expression of each threshold cycle (Ct) value to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or U6 Ct value, and data were analyzed according to the comparative Ct method ($2^{-\Delta\Delta Ct}$). The following primer sequences were used: miRNA-188-5p forward, 5′-GTTGGTATCGTAGTTG AGCTCAA-3′ and reverse, 5′-GTACCACAGGTCCTGT CAATTAT-3′; U6 forward, 5′-CCCGCCGCCAGGCGCCC CC-3′ and reverse, 5′-TTCATACATTTGCGAAGAACG-3′; CD2AP forward, 5′-CTGGAGCTGAAAGTGGGAGA-3′ and reverse, 5′-TTCTGAATCGTCCTGGGCTT-3′; GAPDH forward, 5′-ACCCAGAAGACTGTGGATGG-3′ and reverse, 5′-TCAGCTCAGGGATGACCTTC-3′.

Statistical Analysis

We defined relative miR-188-5p expression >3.34 (the average value of miR-188-5p expression) as high expression and relative CD2AP expression >0.98 (the average value of CD2AP expression) as high expression. Data are expressed as the means ± SD of at least three independent experiments. The prognostic value of miR-188-5p, WBC. PLT, Hb, or disseminated vascular coagulation (DIC) were evaluated using a receiver operating characteristic (ROC) curve. All statistical analyses were performed using SPSS 22.0 software. The OS rate was calculated using the Kaplan–Meier method, and the difference in survival curves was evaluated using the Log-rank test. The Student's *t*-test was used to analyze differences between two groups, and one-way analysis of variance was used to determine the significance of differences among multiple groups. *P*-values <0.05 were considered statistically significant.

Results

miR-188-5p Was Upregulated in APL Patients and Cell Lines and Was Closely Related to a Poor Survival of Pediatric APL Patients

First, gene expression profiles of 188 AML patients, and 200 clinical data, including gender, age, and survival were downloaded from the TCGA database. Survival-related miRNAs are listed in Figure 1A; The *P*-value of miR-188-5p was the smallest (P=0.0005421). In addition, high expression of miR-188-5p was significantly associated with poor survival in AML patients (Figure 1B). Next, our results showed that the survival rate of pediatric APL patients with high expression of miR-188-5p was lower than that of pediatric APL

patients with low expression of miR-188-5p in children (Figure 1C). miR-188-5p was significantly upregulated in pediatric APL patients (n=57, Figure 1D). The miR-188-5p mRNA level in 31 APL patients was lower than 3.34 (low expression group), and in 26 pediatric APL patients, it was higher than 3.34 (high expression group) (Figure 1E). In addition, miR-188-5p expression was significantly related to the concentration of white blood cell (WBC), platelet (PLT), and hemoglobin (Hb), as well as adverse reaction (ADR) in the 57 children with APL (Table 1). There was no significant difference in sex, age and disseminated vascular coagulation (DIC) between the high miR-188-5p expression group and the low miR-188-5p expression group. ROC curve analysis showed that the AUC values of miR-188-5p, WBC, PLT, Hb, and DIC were 0.661, 0.601, 0.425, 0.319, and 0.734, respectively (Figure 1F). These results suggested that miR-188-5p expression could predict poor prognosis in pediatric APL patients. Furthermore, miR-188-5p mRNA levels were significantly upregulated in APL cell lines (NB4 and HL60), compared to the PBMNC group (Figure 1G).

High Expression of miR-188-5p Promoted the Proliferation and the Cell Cycle, Inhibited Apoptosis, and Regulated Protein Expression in NB4 and HL60 Cells

To confirm the effects of miR-188-5p on APL cells, miR-188-5p mimics were transfected into NB4 and HL60 cells. RT-PCR assay results showed that miR-188-5p was significantly upregulated in NB4 and HL60 cells (Figure 2A and B). Both the CCK8 assay and clone formation assays were used to assess the effects of miR-188-5p overexpression on the proliferation of NB4 and HL60 cells. Our results showed that miR-188-5p overexpression promoted cell proliferation (Figure 2B and C). Flow cytometry analysis indicated that miR-188-5p mimics inhibited G1-phase and increased G2 phase in the cell cycle and inhibited the apoptosis of NB4 and HL60 cells (Figure 2D and E). Next, high expression of miR-188-5p significantly inhibited the protein expressions of cyclin D1, Bax and cleaved caspase-3, and increased the protein expressions of Ki67 and Bcl-2 in NB4 and HL60 cells (Figure 2F and G).

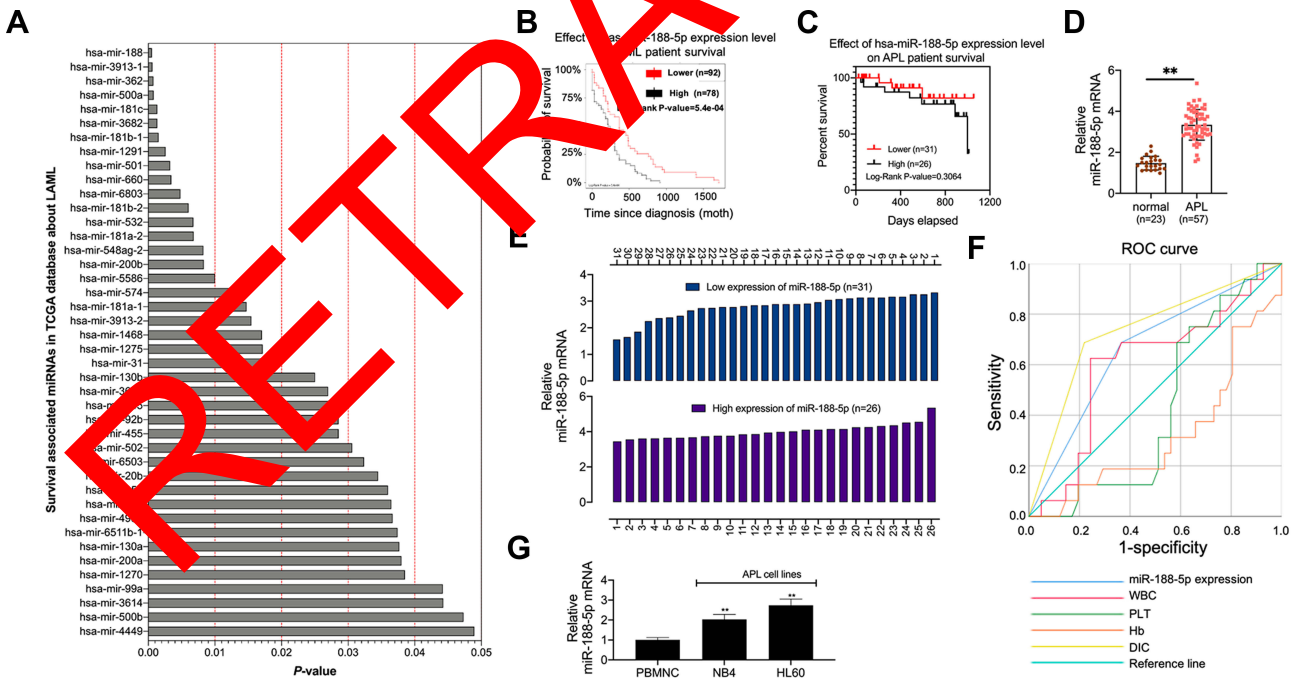

Figure 1 miR-188-5p was upregulated in APL patients and cell lines and was closely related to a poor survival of pediatric APL patients. (**A**) Survival-related miRNAs are shown based on TCGA database; (**B**) Kaplan-Meier survival analysis was performed to investigate the relationship between miR-188-5p low expression and miR-188-5p high expression based on TCGA database; (**C**) Kaplan-Meier survival analysis was performed to investigate the relationship between miR-188-5p low expression and miR-188-5p high expression in 57 pediatric APL patients; RT-PCR assay was used to detect miR-188-5p mRNA level (**D**) between normal donors and 57 APL patients, and (**E**) in 57 APL patients; (**F**) ROC curve analysis showed that the AUC values of miR-188-5p, WBC, PLT, Hb, and DIC in 57 pediatric APL patients; (**G**) RT-PCR assay was used to detect miR-188-5p mRNA level in NB4 and HL60 cells. U6 was used as a load control. Each experiment was carried out in triplicate. Data are presented as the mean ± standard deviation. ***P*<0.01 versus Normal group/PBMNC group.

Low Expression of miR-188-5p Inhibited the Proliferation, Induced G1-Phase Arrest, Promoted Apoptosis, and Regulated Protein Expression in NB4 and HL60 Cells

Inhibition of miR-188-5p was detected in miR-188-5p mimics transfected NB4 and HL60 cells using RT-PCR assay (Figure 3A). Both the CCK8 assay and clone formation assay were used to assess the effects of miR-188-5p inhibition on the proliferation of NB4 and HL60 cells. Our results showed that inhibition of miR-188-5p inhibited cell proliferation (Figure 3B and C). Flow cytometry analysis indicated that inhibition of miR-188-5p induced G1-phase arrest in the cell cycle and promoted the apoptosis of NB4 and HL60 cells (Figure 3D and E). Next, low expression of miR-188-5p significantly upregulated the protein expression of cyclinD1, Bax and cleaved caspase-3, and inhibited the protein expression of Ki67 and Bcl-2 in NB4 and HL60 cells (Figure 3F and G).

Overexpression of miR-188-5p Dramatically Promoted Tumor Growth of APL in vivo

The nude mouse tumorigenicity assay was used to evaluate the effect of miR-188-5p on tumor growth. After tmiR-188-5p mimics transfected NB4 cells were injected into the nude mouse for 14 days (Figure 4A), tumor weight and tumor volume were markedly upregulated (Figure 4B and C). Ki67 expression was significantly downregulated in tumor tissues using IHC assay (Figure 4D and E). Meanwhile, tumor cell apoptosis was inhibited by miR-188-5p mimics via using TUNEL assay (Figure 4F).

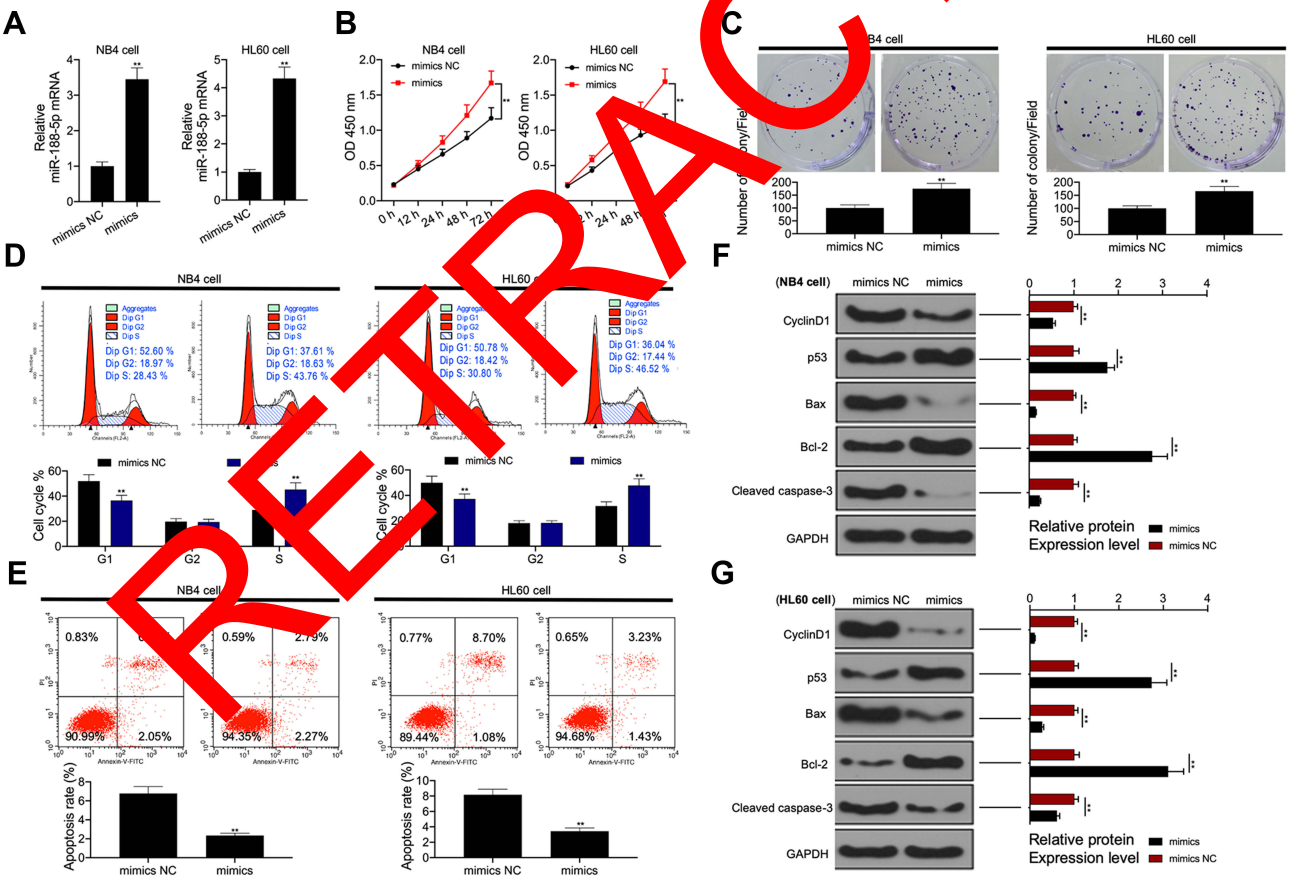

Figure 2 miR-188-5p overexpression regulated the viability, proliferation, cell cycle, apoptosis and gene expression in NB4 and HL60 cells. After miR-188-5p mimics was transfected into NB4 and HL60 cells, (**A**) RT-PCR assay was used to detect miR-188-5p expression, (**B**) CCK8 assay was used to detect cell viability, (**C**) colon formation assay was to detect cell proliferation, (**D** and **E**) flow cytometry assay was used to detect the change of cell cycle and cell apoptosis, and (**F** and **G**) Western blot was used to detect the expression of cyclinD1, Ki67, Bax, Bcl-2 and cleaved caspase-3. GAPDH was used as a load control. Each experiment was carried out in triplicate. Data are presented as the mean ± standard deviation. $^{**}P<0.01$ versus mimics NC group.

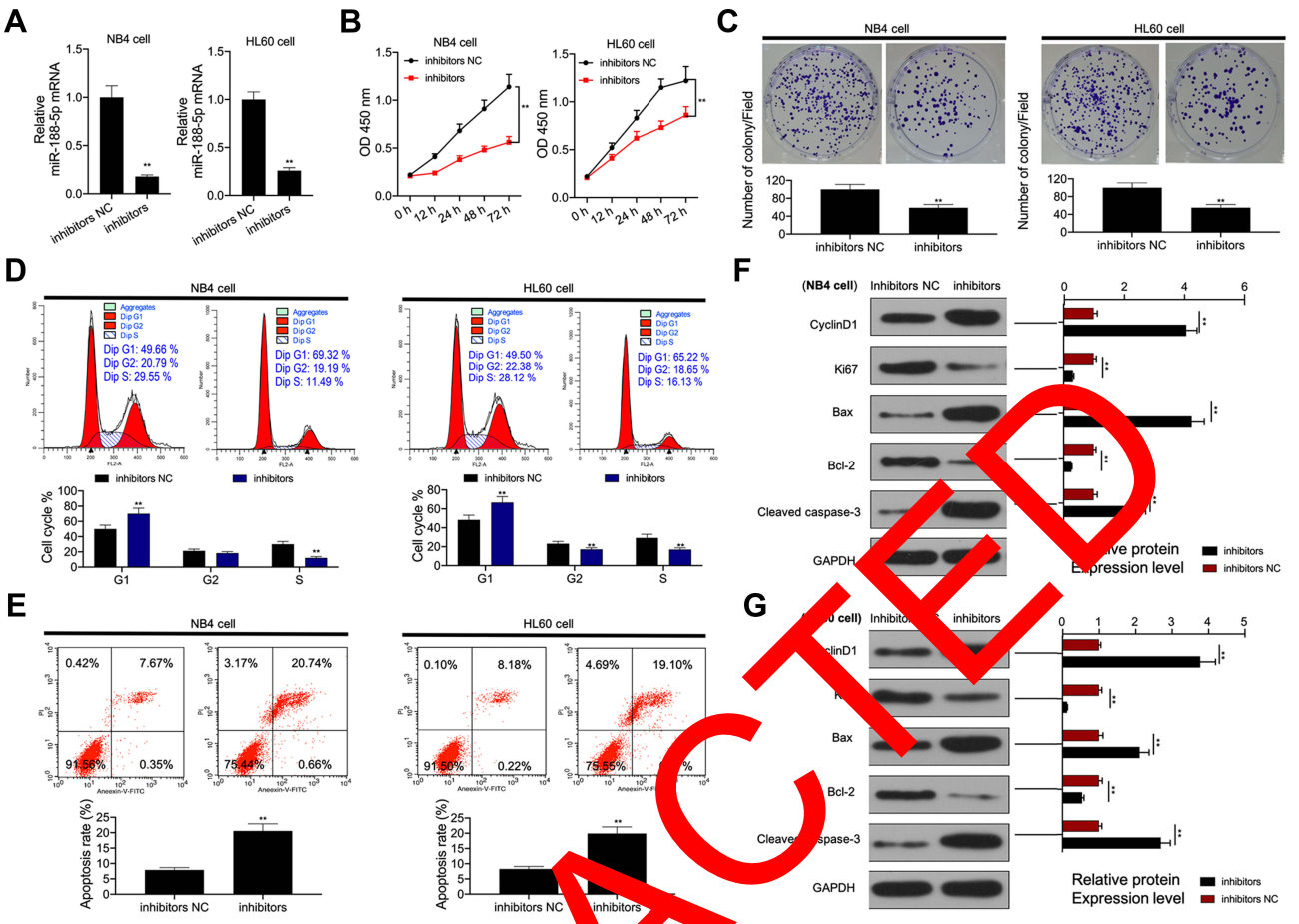

Figure 3 miR-188-5p inhibition regulated the viability, prolife[illegible]cell cycle, [illegible]ptosis and related genes expression in NB4 and HL60 cells. After miR-188-5p inhibitors were transfected into NB4 and HL60 cells, (**A**) RT-PCR [illegible]ay was [illegible]ed to de[illegible]t tmiR-188-5p expression, (**B**) CCK8 assay was used to detect cell viability, (**C**) colon formation assay was to detect cell proliferation, (**D** and [illegible] flow cyto[illegible]try assay [illegible] used to detect the change of cell cycle and cell apoptosis, and (**F** and **G**) Western blot was used to detect the expression of cyclinD1, Ki67, [illegible] Bcl-2 a[illegible] [illegible]aspase-3. GAPDH was used as a load control. Each experiment was carried out in triplicate. Data are presented as the mean ± standard devi[illegible]on. *[illegible]0[illegible]ersus inhibitors NC group.

Bioinformatics Analysis of the Target Genes of miR-188-5p

miRDB, miRTarBase and TargetScan databases were used to predict the target genes of miR-188-5p (Figure 5A). A total of 33 overlapping genes (Table 2) were used to construct PPI network, Module analysis and GO and KEGG pathway enrichment analyses [illegible] STRING database, Cytoscape software and DAVID database. Our results showed that CD2AP was a hub gene of the most significant module from PPI network (Figure 5B and C). We found that CD2AP mainly enriched in BP terms, including GO:0019941, GO:0043632, GO:0051603, GO:0044257, GO:0030163, GO:0044265, GO:0009057, and GO:0048259, and CC term, including GO:0031981, GO:0070013, GO:0043233, and GO:0031974 (Figure 5D–F). Next, 33 overlapping genes were only related to 'hsa04120:Ubiquitin mediated proteolysis'.

CD2AP Was the Target Gene of miR-188-5p and Was Downregulated in Pediatric APL Patients and Cell Lines

CD2AP was the only overlapping gene identified from the miRDB, miRTarBase, and TargetScan databases, as well as survival-associated mRNAs from the TCGA database about AML patients (Figure 6A). TCGA database analysis indicated that low expression of CD2AP was significantly associated with poor survival in AML patients (Figure 6B). Furthermore, CD2AP was significantly downregulated in children with APL and cell lines (Figure 6C and D). The CD2AP mRNA level in 36 children with APL was lower than 0.98 (low expression group), and in 21 children with APL, it was higher than 0.98 (high expression group) (Figure 6E). In addition, the mRNA levels of CD2AP and miR-188-5p were negatively correlated using Spearman

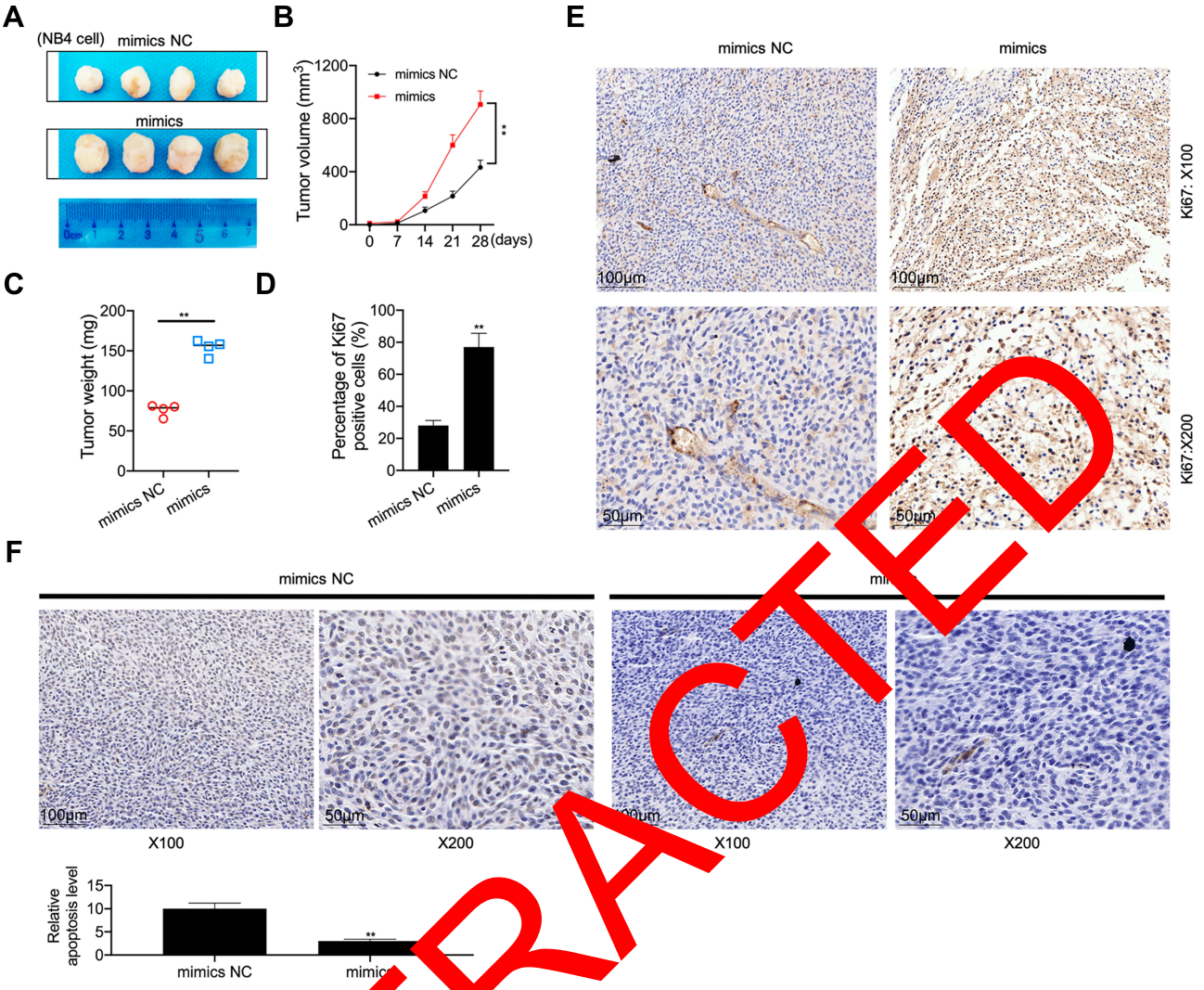

Figure 4 miR-188-5p affected tumor growth of A[illegible] vivo. NB4 cells [illegible]sfected with miR-188-5p mimics or mimics NC were subcutaneously injected into the nude mice, (**A**) The nude mice were sacrificed and the tumors [illegible]e col[illegible]d after 28 days; [illegible]the volume of the tumors were determined; (**C**) the weight of the tumors were determined; (**D** and **E**) Ki67 expression in the tumors collected from different g[illegible]ps were determined using immunohistochemistry assays; (**F**) Cell apoptosis in tumor tissues was determined using TUNEL assay. Each experiment was carr[illegible]out in triplicate. [illegible] are presented as the mean ± standard deviation. ***P*<0.01 versus mimics NC group.

correlation analysis ([illegible]ure 6F). TargetScan database was used to identify the poten[illegible]binding [illegible]e between CD2AP and miR-188-[illegible]. A [illegible]tative [illegible]te[illegible]ction between CD2AP and miR-1[illegible]-5p was [illegible]ound, and binding sites of wild type (CD2AP wt) a[illegible]i[illegible] mutant type (CD2AP mut) are shown in Figure 6I. Lucife[illegible]e reporter assay demonstrated that co-transfection with CD[illegible]AP wt and miR-188-5p mimics significantly reduced the luciferase activity; meanwhile, co-transfection with CD2AP mut and miR-188-5p mimics failed to affect the luciferase activity (Figure 6G). In addition, luciferase reporter assay also demonstrated that co-transfection with CD2AP wt and miR-188-5p inhibitors significantly increased the luciferase activity; meanwhile, co-transfection with CD2AP mut and miR-188-5p inhibitors failed to affect the luciferase activity (Figure 6H). The results of RT-PCR and Western blot assays showed transfection with miR-188-5p mimics significantly inhibited CD2AP mRNA and protein levels in NB4 and HL60 cells (Figure 6J) while transfection with miR-188-5p inhibitors significantly increased CD2AP mRNA and protein levels in NB4 and HL60 cells (Figure 6L).

Low Expression of CD2AP Promoted the Proliferation and Cell Cycle Progression, Inhibited Apoptosis, and Regulated Protein Expression in NB4 and HL60 Cells

Inhibition of CD2AP was detected in siRNA targeting CD2AP transfected NB4 and HL60 cells using RT-PCR

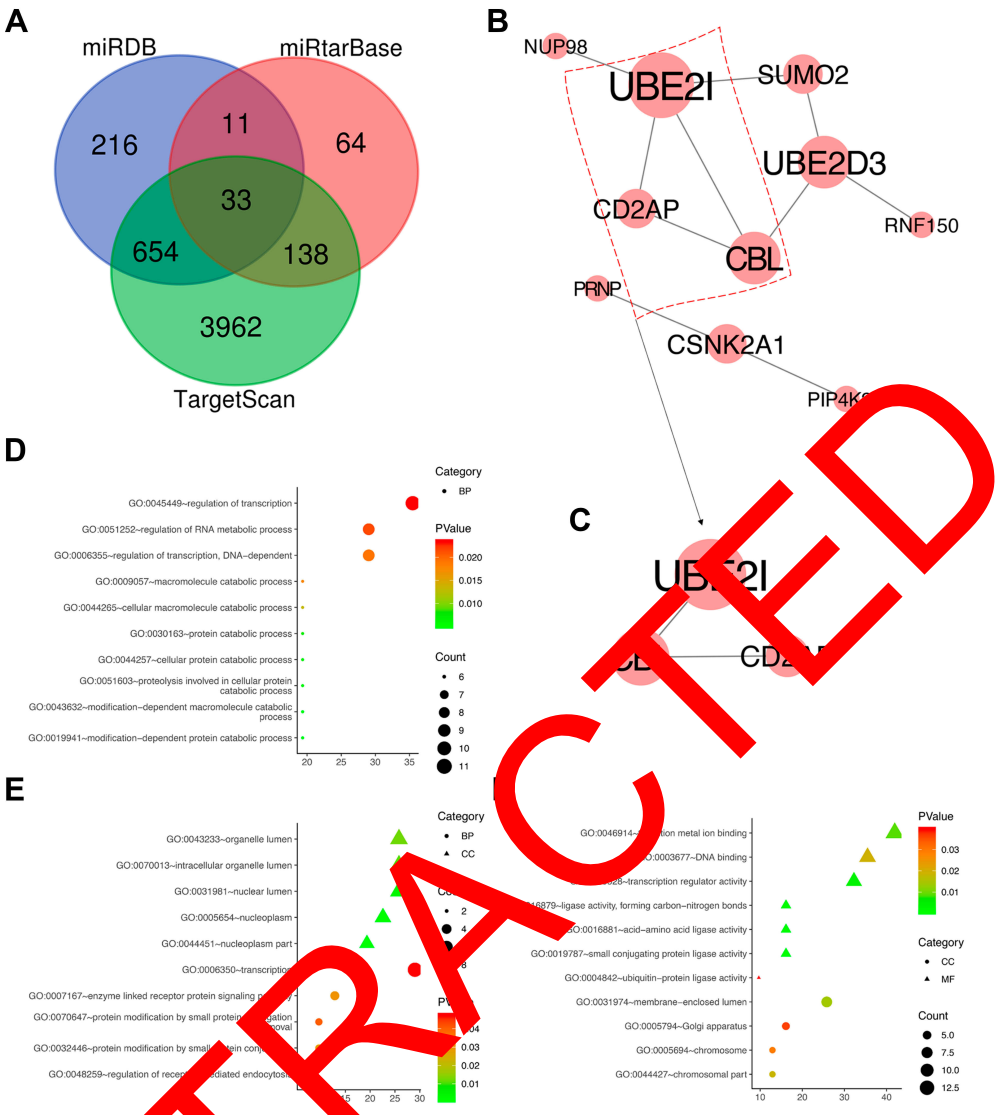

Figure 5 Bioinformatics analysis of the target genes of miR-188-5p. (**A**) Venn diagram of the target genes of miR-188-5p based on miRDB, miRTarBase and TargetScan databases; A total of 33 overlapping genes were used for (**B**) PPI network, (**C**) Module analysis, (**D**–**F**) GO enrichment analyses using STRING database, Cytoscape software and DAVID database.

assay (Figure 7A). Both the CCK8 assay and clone formation assay were used to assess the effects of CD2AP inhibition on the proliferation of NB4 and HL60 cells. Our results showed that inhibition of CD2AP promoted the cell proliferation (Figure 7B–D). Flow cytometry analysis indicated that inhibition of CD2AP inhibited G1-phase and increased G2-phase in cell cycle and inhibited the apoptosis of NB4 and HL60 cells (Figure 7E). Next, inhibition of CD2AP significantly inhibited the protein expressions of cyclinD1, Bax and cleaved caspase-3, and increased the protein expressions of Ki67 and Bcl-2 in NB4 and HL60 cells (Figure 7G and H).

miR-188-5p Promoted the Proliferation and Inhibited the Apoptosis of APL Cells by Targeting CD2AP and Activating PI3K/AKT/mTOR Signaling Pathway

Our results showed that co-transfection with miR-188-5p inhibitors and siRNA targeting CD2AP significantly reversed the effects of miR-188-5p inhibitors that inhibited the proliferation of NB4 cells using clone formation assay (Figure 8A). Furthermore, CD2AP inhibition abolished G1-phase arrest that was induced by miR-188-5p inhibitors (Figure 8B). Co-transfection with miR-188-5p inhibitors and siRNA targeting CD2AP significantly reversed

Table 2 The Target Genes of miR-188-5p from miRDB, miRTarBase, and TargetScan Databases

ID	Gene Symbol	Gene Name
1	ARHGEF26	Rho guanine nucleotide exchange factor 26
2	FOXN2	Forkhead box N2
3	GALNT10	Polypeptide N-acetylgalactosaminyltransferase 10
4	LCLAT1	Lysocardiolipin acyltransferase 1
5	TCF20	Transcription factor 20
6	CSNK2A1	Casein kinase 2 alpha 1
7	NUFIP2	Nuclear FMR1 interacting protein 2
8	ABL2	ABL proto-oncogene 2, non-receptor tyrosine kinase
9	UBE2I	Ubiquitin conjugating enzyme E2 I
10	SCD	Stearoyl-CoA desaturase
11	ZBTB6	Zinc finger and BTB domain containing 6
12	PRNP	Prion protein
13	ZNF732	Zinc finger protein 732
14	GCC1	GRIP and coiled-coil domain containing 1
15	SUMO2	Small ubiquitin like modifier 2
16	STK38	Serine/threonine kinase 38
17	KRBOX4	KRAB box domain containing 4
18	E2F3	E2F transcription factor 3
19	ZNF711	zinc finger protein 711
20	UBE2D3	Ubiquitin conjugating enzyme E2 D3
21	WBP11	WW domain binding protein 11
22	RNF150	Ring finger protein 150
23	TMED3	Transmembrane p24 trafficking protein 3
24	FGF5	Fibroblast growth factor 5
25	ATXN1	Ataxin 1
26	ARID5B	AT-rich interaction domain 5B
27	CD2AP	CD2 associated protei
28	NUP98	Nucleoporin 98 an precursor
29	IKZF2	IKAROS family ic finge 2
30	CBL	Cbl proto- ogene
31	ZNF367	Zinc fin protein 367
32	PIP4K2C	Pho atidylino l-5-phosphate 4-kinase type 2 gam
33	UBR7	iquitin tein liga E3 component n-r ognin 7

miR-188-5p inhi or-induced cell apoptosis in NB4 cells, as determined by f w cytometry analysis (Figure 8C). Likewise, the upregulation of cyclinD1, Bax, and cleaved caspase-3 and the downregulation of Ki67 and Bcl-2 by miR-188-5p inhibitors could be abolished by siRNA targeting CD2AP (Figure 8D). In addition, transfection with miR-188-5p inhibitors transfection significantly inhibited the activation of the PI3K/AKT/mTOR signaling pathway via inhibiting the phosphorylation levels of the PI3K, AKT, and mTOR proteins (Figure 8E). However, co-transfection with miR-188-5p inhibitors and siRNA targeting CD2AP co-transfection observably reversed the effects of miR-188-5p inhibition on the PI3K/AKT/mTOR signaling pathway.

Discussion

In recent years, with the development of bioinformatics, an increasing number of public databases have been used by researchers as the data basis of experiments. Through the analysis of the data associated with AML in the TCGA database, our team found that miR-188-5p was the most significant miRNA correlated with poor sur al in adult AML. Several common microR A signatures indicating favorable outcomes in a pr ious st y includ low miR-188-5p expression.[25] Ou results showe th the survival rate of the low miR-1 5p g up in adult AML patients and pediatric API patients as highe han that of the high miR-188-5p g ROC curv ar lysis showed that miR-188-5p expression c ld predict poor prognosis in pediatric API ients (AUC alue=0.661). It has been reported that iR-188-5p is involved in cell proliferation and tumor gro th in a vari y of cancers.[10,14–16] The results of cell funct al expe ments showed that high expression of miR-188- omoted the viability, cell cycle progression, pr tion, and tumor growth, and inhibited apoptosis in PL cells. In contrast, the effect of miR-188-5p silencing n the proliferation, apoptosis, and tumor growth was pposite to that of the highly expressed miR-188-5p phenotype in APL cells. This indicated that miR-188-5p played an oncogenic role in APL cells and might represent a potential marker for pediatric APL. CyclinD1, a cell cycle-related protein, has been used to reflect the change in the G1 phase.[26] Ki67, Bax/Bcl-2 and caspase-3, as proliferation and apoptosis-related proteins, have been used to reflect the change in proliferation and apoptosis at the molecular level.[27] miR-188-5p mimics inhibited the expression of cyclinD1, Bax, and cleaved caspase-3, and promoted the expression of Ki67 and Bcl-2. Together, these findings together demonstrated a tumor-stimulating role of miR-188-5p in APL cell proliferation and tumor growth. CD2AP was a target gene of miR-188-5p using miRDB, miRTarBase and TargetScan databases, and CD2AP was closely associated with poor survival of children with APL using TCGA database. CD2AP was first discovered in 1998 as a new ligand interacting with a cluster of differentiation 2 (CD2), is located on chromosome 6 (6p12.3) and contains 18 exons.[28] As a key promoter of T cell adhesion to antigen-presenting cells,

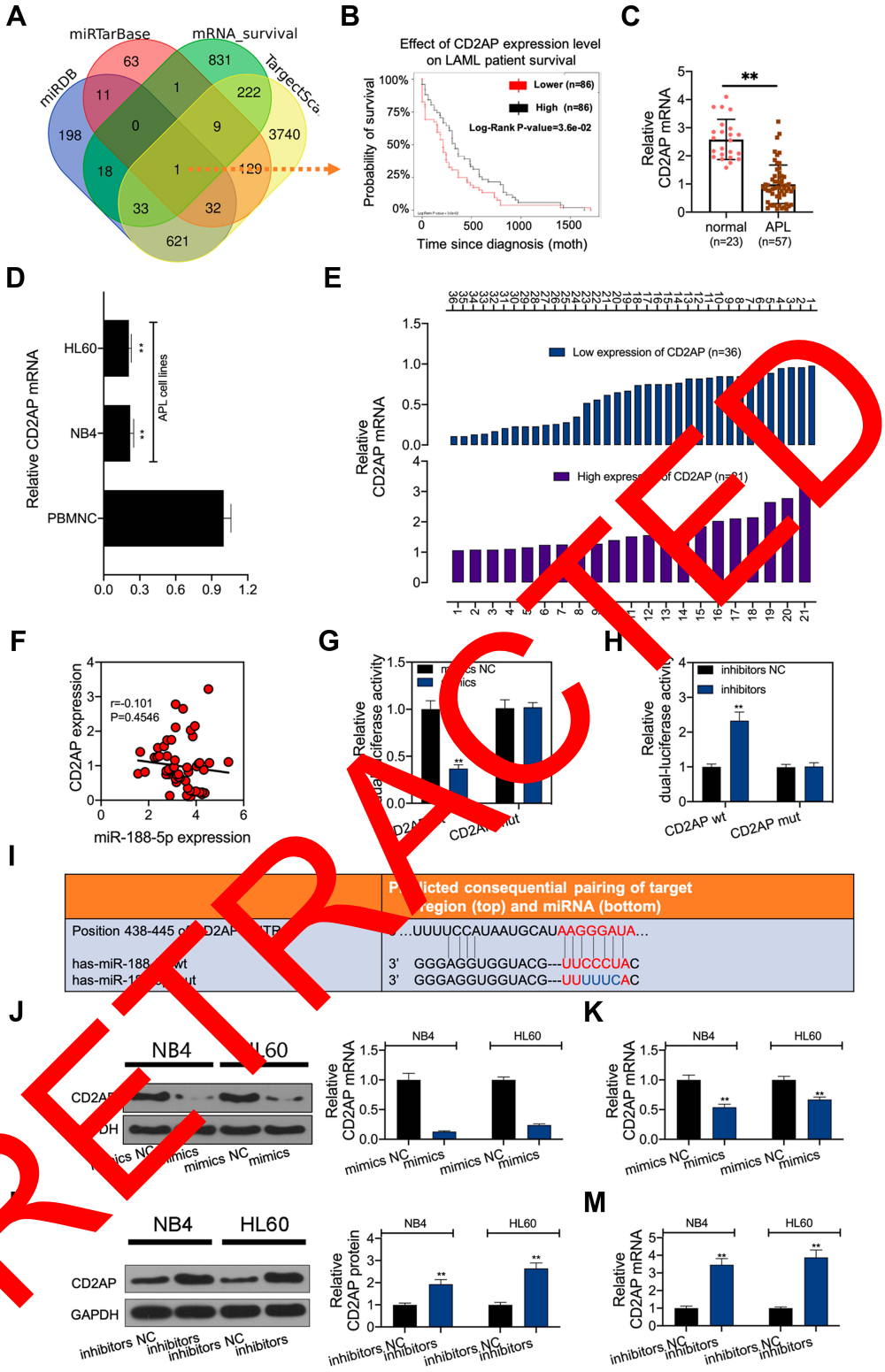

Figure 6 CD2AP was the target gene of miR-188-5p. (**A**) Venn diagram of overlapping genes from miRDB, miRTarBase, TargetScan and TCGA databases; (**B**) Kaplan-Meier survival analysis was performed to investigate the relationship between CD2AP low expression and CD2AP high expression based on TCGA database; RT-PCR assay was used to detect CD2AP mRNA level (**C**) between normal donors and 57 APL patients, (**D**) 57 APL patients and (**E**) APL cell lines; (**F**) The correlation analysis between miR-188-5p expression and CD2AP expression in APL patients (n = 57) was performed by Spearman's rank correlation analysis; (**G** and **H**) Luciferase reporter assays were used to prove CD2AP can target miR-188-5p; (**I**) Putative miR-188-5p binding sequence and mutation sequence of CD2AP mRNA were as shown; (**J** and **K**) Western blot and RT-PCR assays were used to detect CD2AP expression in miR-188-5p mimics/mimics NC transfected NB4 and HL60 cells; (**L** and **M**) Western blot and RT-PCR assays were used to detect CD2AP expression in miR-188-5p inhibitors/inhibitors NC transfected NB4 and HL60 cells. GAPDH was used as a load control. Each experiment was carried out in triplicate. Data are presented as the mean ± standard deviation. ***P*<0.01 versus mimics NC/inhibitors NC group.

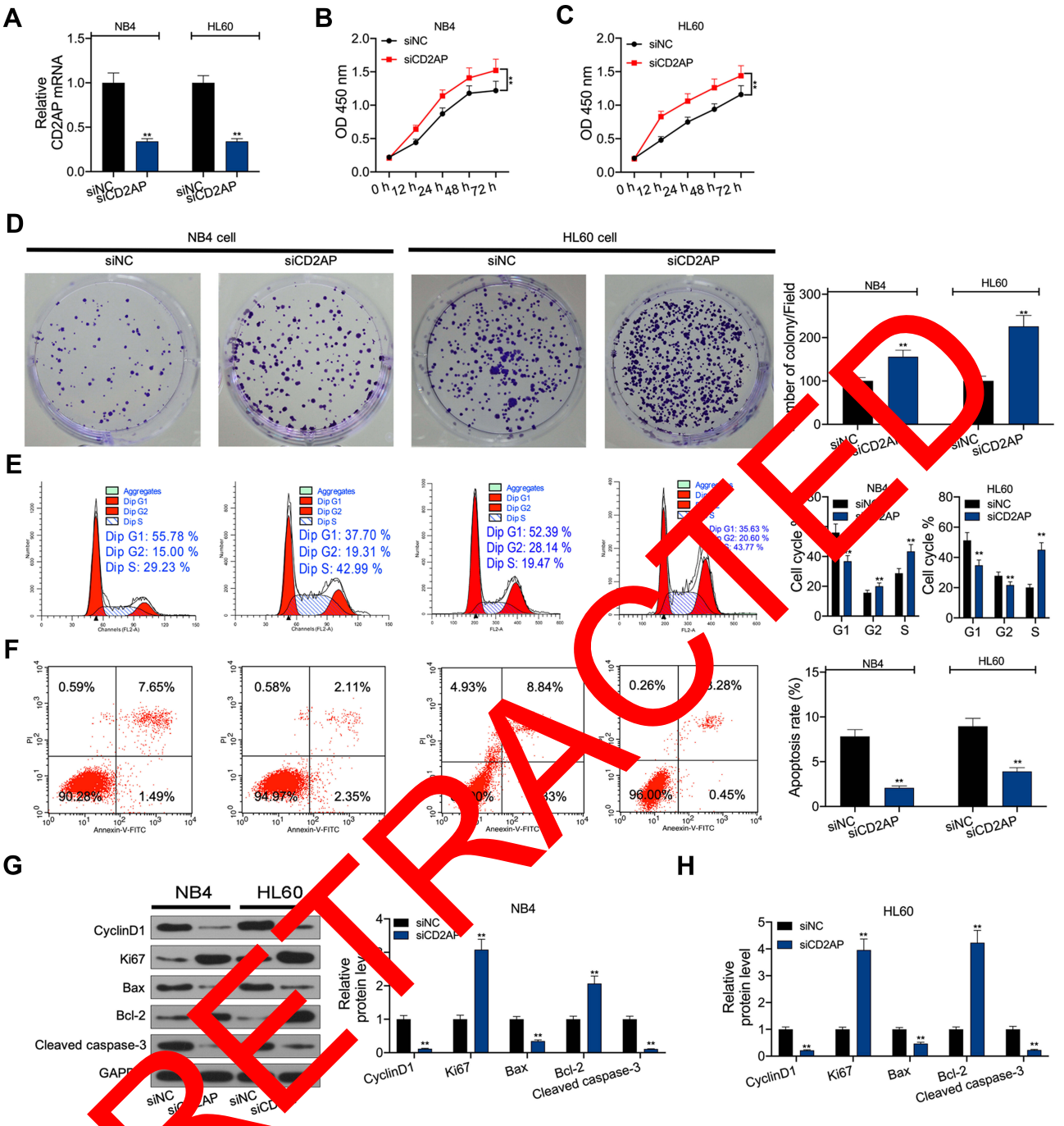

Figure 7 CD2AP in[illegible]on regulated the viability, proliferation, cell cycle, apoptosis and gene expression in NB4 and HL60 cells. After siRNA targeting CD2AP was transfected into NB4 an[illegible]L60 cells, (**A**) RT-PCR assay was used to detect CD2AP expression, (**B** and **C**) CCK8 assay was used to detect cell viability, (**D**) colon formation assay was to detect cell pro[illegible]ation, (**E** and **F**) flow cytometry assay was used to detect the change of cell cycle and cell apoptosis, and (**G** and **H**) Western blot was used to detect the expression of cyclinD1, Ki67, Bax, Bcl-2 and cleaved caspase-3. GAPDH was used as a load control. Each experiment was carried out in triplicate. Data are presented as the mean ± standard deviation. ***P*<0.01 versus siNC group.

CD2AP can enhance the clustering and anchoring of CD2T cells.[29] Previous studies have focused on the protective effect of CD2AP on the kidney.[30] In addition, CD2AP is a scaffold molecule that regulates signal transduction and cytoskeleton molecules and is involved in the pathogenesis of Alzheimer's disease.[31] However, the role and mechanism of CD2AP in pediatric APL have not been reported. Our results showed that CD2AP was significantly downregulated in the blood of pediatric APL and cell lines. There was no significant correlation between miR-188-5p and CD2AP; this may be due to the low sample size. Luciferase reporter assay demonstrated that

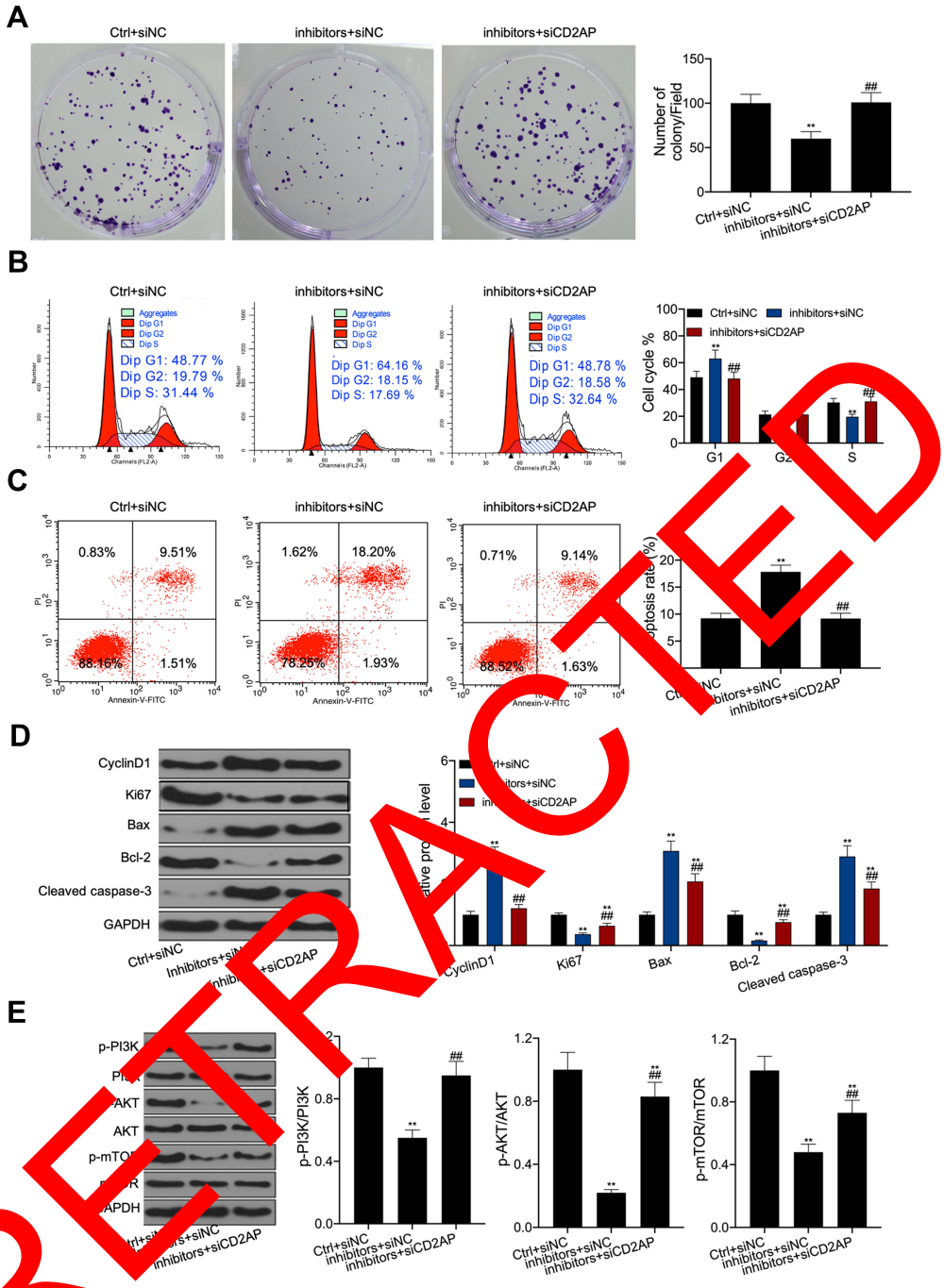

Figure 8 miR-188-5p in APL regulated cell viability, proliferation, cell cycle, apoptosis and related genes expression via targeting CD2AP and thus affecting the activation of PI3K/AKT/mTOR signaling pathway. After miR-188-5p inhibitors and siRNA targeting CD2AP were co-transfected into NB4 cells, (**A**) colon formation assay was to detect cell proliferation, (**B** and **C**) flow cytometry assay was used to detect the change of cell cycle and cell apoptosis, (**D**) Western blot was used to detect the expression of cyclinD1, Ki67, Bax, Bcl-2 and cleaved caspase-3, and (**E**) Western blot was used to detect the phosphorylation levels of PI3K, AKT and mTOR proteins. GAPDH was used as a load control. Each experiment was carried out in triplicate. Data are presented as the mean ± standard deviation. $^{**}P<0.01$ versus Ctrl + siNC group and $^{\#\#}P<0.01$ versus inhibitors + siNC group.

co-transfection with CD2AP wt and miR-188-5p mimics significantly decreased luciferase activity, and co-transfection with CD2AP wt and miR-188-5p significantly increased luciferase activity. CD2AP expression was inhibited by miR-188-5p mimics and was increased by miR-188-5p inhibitors. These results showed that there was targeted binding between CD2AP and miR-188-5p. We further verified the effect of CD2AP on APL cells by performing cell functional experiments. CD2AP silencing could significantly promote the viability, proliferation, and

cell cycle progression of APL cells, and inhibit cell viability. The regulatory effect of CD2AP on cyclinD1, Ki67, Bax/Bcl-2, and caspase-3 was consistent with that of miR-188-5p overexpression on these same proteins. These data suggested that miR-188-5p inhibited CD2AP expression by binding CD2AP. Thus, the functions of miR188-5p and CD2AP on APL cells were opposite to that of tumor growth.

The PI3K/AKT/mTOR signaling pathway is involved in cell proliferation and apoptosis in many types of malignant tumors.[32,33] It has been reported that miR-188-5p in Keloids inhibits cell proliferation and invasion via PI3K/Akt/MMP-2/9 signaling[34] and in chronic myeloid leukemia-induced cell apoptosis by repressing the PI3K/AKT signaling.[16] The above results suggest that miR188-5p was involved in the activation of the PI3K/AKT/mTOR signaling pathway. CD2AP links actin to PI3K kinase activity to extend epithelial cell height and constrain cell area.[29] This indicated that CD2AP inhibited cell proliferation by binding to PI3K. Together, these findings together demonstrated that both miR-188-5p and CD2AP were involved in the activation of the PI3K/AKT/mTOR signaling pathway. Our results showed that CD2AP inhibition abolished G1-phase arrest, promoted cell apoptosis, and inhibited cell proliferation that was induced by miR-188-5p inhibitors. Likewise, the upregulation of cyclinD1, Bax and cleaved caspase-3 and the downregulation of Ki67 and Bcl-2 by miR-188-5p inhibitors could be reversed by CD2AP inhibition. These data showed that in APL, miR-188-5p in APL regulated cell proliferation and apoptosis by targeting CD2AP. We found that CD2AP inhibition abolished the inhibition of the activation of the PI3K/AKT/mTOR signaling pathway that was induced by inhibition of miR-188-5p. The results of this study inversely verified that miR-188-5p activated the PI3K/AKT/mTOR signaling pathway by inhibiting the expression of CD2AP. This was consistent with the literature report.

In conclusion, miR-188-5p was significantly upregulated in pediatric APL patients and closely related to a poor survival. miR-188-5p overexpression promoted cell viability, cell proliferation, cell cycle progression and tumor growth, and inhibited cell apoptosis via its target CD2AP. miR-188-5p in pediatric APL exerted significant pro-cancer effects in pediatric APL by regulating CD2AP expression, thus activating the PI3K/AKT/mTOR signaling pathway; thus, miR-188-5p might be a potential prognostic marker and therapeutic target for pediatric APL.

Data Sharing Statement

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

Ethics and Consent Statement

This research was approved by the Ethics Committees of the First Affiliated Hospital of Zhengzhou University for the use of blood samples. All of the samples were obtained with written informed consent was obtained for each sample, which was then anonymously analyzed. This work has been performed following the Declaration of Helsinki. Animal experiments were performed according to the Institutional Animal Care and Use Committee (IACUC) protocol and approved by the Medical Experimental Animal Care Commission of Zhengzhou University.

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Disclosure

The authors declare that they have no competing interests.

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