MicroRNA-155-5p-dependent upregulation of PDCD4 is involved in oridonin-induced proliferation inhibition of leukemia cells

HUI DING[1,2*], MIN FANG[3*], QINGQING YANG[4*], CHAOZHE WANG[5], YUNHUA WU[4], XIDI WANG[6], GUOSHENG JIANG[4] and KEHONG BI[2]

[1]Department of Hematology, Children's Hospital Affiliated to Shandong University, Jinan, Shandong 250000, P.R. China; [2]Department of Hematology, Shandong Provincial Qianfoshan Hospital, Shandong University, Jinan, Shandong 250062, P.R. China; [3]Department of Hematology, Tengzhou Central People's Hospital, Zaozhuang, Shandong 277500, P.R. China; [4]Department of Immunology, School of Basic Medical Sciences, Binzhou Medical University, Yantai, Shandong 264003, P.R. China; [5]Department of Blood Transfusion, Yantaishan Hospital Affiliated to Binzhou Medical University, Yantai, Shandong 264008, P.R. China; [6]Department of Laboratory Medicine, Zhangqiu District People's Hospital, Jinan, Shandong 250200, P.R. China

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Abstract. As the antitumor mechanism of oridonin has not been clearly demonstrated, the effect of programmed cell death protein 4 (PDCD4) regulated by microRNA (miR)-155-5p on the proliferation of leukemia cells after exposure to oridonin was detected. The CCK-8 assay indicated that proliferation of the three kinds of leukemia cells was inhibited by oridonin, with the most significant inhibitory rate on HL60 cells compared with NB4 and U937 cells. Regarding the cell cycle profile, there was downregulation of the S phase, upregulation of the G_2/M phase and no significant change in the G_0/G_1 phase. The downregulation of miR-155-5p was demonstrated by the differentially gene chromatin immunoprecipitation and verified using RT-qPCR in HL60 cells treated with oridonin. Compared with the oridonin group, the expression of miR-155-5p was restored after co-transfection of miRNA-155-5p mimics with oridonin, and the miR-55-5p inhibitor significantly downregulated the expression of miR-155-5p compared with the control group. The proliferation of HL60 cells was restored after simultaneous transfection of miR-55-5p mimics and treatment with oridonin and the miR-155-5p inhibitor could significantly downregulate proliferation of HL60 cells. After intersection analysis based on StarBase and miRDB, PDCD4 was identified to be a possible downstream apoptosis-related target gene. After exposure to oridonin, the expression of PDCD4 of HL60 cells was significantly upregulated and it was significantly decreased after simultaneous transfection of miR-155-5p mimics plus treatment with oridonin compared with the mimics control group and the proliferation level was restored. The targeted regulation of miR-155-5p on PDCD4 was demonstrated using a luciferase assay and miR-155-5p was negatively associated with PDCD4. The expression of PDCD4 was significantly downregulated after treatment with si-PDCD4 transfection together with oridonin compared with oridonin, which was accompanied by proliferation restoration of HL60 cells. Furthermore, apoptosis of HL60 cells was induced by oridonin, which was associated with the decrease of miR-155-5p and increase of PDCD4 that was verified using miR-155-5p mimics and si-PDCD4 transfection, respectively. The upregulation of the Bax/Bcl-2 ratio resulted in upregulation of cleaved caspase-3 and -9 expression, which was identified using a si-PDCD4 transfection assay. In conclusion, The miR-155-5p dependent upregulation of PDCD4 expression resulted in the increase of Bax/Bcl-2 ratio and activation of caspase-3 and -9, which contributed to the apoptosis of HL60 cells induced by oridonin.

Correspondence to: Professor Guosheng Jiang, Department of Immunology, School of Basic Medical Sciences, Binzhou Medical University, 346 Guanhai Road, Yantai, Shandong 264003, P.R. China
E-mail: jiangguosh@163.com

Professor Kehong Bi, Department of Hematology, Shandong Provincial Qianfoshan Hospital, Shandong University, 16766 Jingshi Road, Jinan, Shandong 250062, P.R. China
E-mail: bikehong11@163.com

*Contributed equally

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Introduction

With an improved understanding of the pathobiology of acute myeloid leukemia (AML), several innovative drugs and targeted therapies have progressively been identified as successful treatments for leukemia. Currently, small-molecule targeted drugs, antibody-drug conjugates (ADCs), cell therapies and gene therapies are emerging as highly anticipated new treatment modalities. These cutting-edge therapeutic approaches can not only achieve personalized and precise tumor targeting but also provide patients with better treatment comfort and have the potential to delay disease progression (1,2). In recent years, understanding of the molecular

mechanisms of myeloid tumorigenesis has significantly improved, leading to the development of various effective small-molecule inhibitors targeting abnormal cell signaling. For example, receptor tyrosine kinase FLT3, tricarboxylic acid cycle enzymes IDH1 and IDH2, and transcription regulatory genes KMT2A and NPM1 (3). When used as monotherapy, these inhibitors exhibit moderate anti-leukemic activity. However, their clinical activity is enhanced when combined with drugs targeting a broader range of epigenetic and/or other oncogenic signaling pathways, or with conventional cytotoxic agents (4). The broad spectrum of AML immunotherapy targets includes endogenous and surface antigens of cancer cells as well as targets in the leukemia microenvironment; the main immunotherapy modalities include immune checkpoint inhibitors, antibody-drug conjugates, therapeutic vaccines, bispecific/trispecific antibodies, and chimeric antigen receptor (CAR)-T and CAR-NK cells (5). Furthermore, novel biomarkers based on molecular mechanisms have also provided insights for AML classification, risk stratification and targeted therapy (6).

Traditional treatments, such as chemotherapy, were used to induce remission from AML; however, it is toxic and has side effects such as hematopoiesis and immunosuppression. The aforementioned targeted drugs and immunotherapy also face issues such as drug resistance and recurrence. Therefore, it is necessary to explore novel effective drugs to improve the treatment of patients with AML (7,8). An important direction of current research is to discover drugs that induce differentiation and apoptosis of leukemia cells based on abnormal signaling pathways (9,10). Among them, medicinal plant extracts and plant growth regulators have demonstrated different effects on inducing leukemia cell differentiation or apoptosis, and natural plant active ingredients have become one of the key directions in the study of leukemia differentiation therapy (11-15). For example, oridonin is an ent-kaurane diterpenoid from *Rabdosia rubescens* (16). It has been reported that oridonin could modulate cell cycle profile, inhibit proliferation or induce apoptosis in certain solid tumor or leukemia cells (17,18). As an increasing number of studies have demonstrated that non-coding microRNAs (miRNAs/miRs) are closely associated with the proliferation, differentiation and apoptosis of tumor or leukemia cells (19,20), it is hypothesized that miRNAs may also be involved in proliferation inhibition of leukemia cells induced by oridonin. Changes in miRNAs, particularly apoptosis-related miRNAs, were first screened using high-throughput chromatin immunoprecipitation (ChIP) differential analysis with miRNA-155-5p identified as one of the miRNAs reported to be associated with proliferation and apoptosis of tumor cells. Furthermore, miRNAs often serve the role of promoting or inhibiting the proliferation of tumor cells by targeting the downstream oncogenes or tumor suppressor genes (21,22). According to literature, programmed cell death protein 4 (PDCD4) participates in the process of tumorigenesis and its development (23,24). Precisely due to the important roles of miRNA-155-5p and PDCD4 in the proliferation and apoptosis of tumor cells, the present study aimed to clarify whether oridonin induces apoptosis of leukemia cells contributing to the regulation of the miR-155-5p/PDCD4 signaling axis. The results could potentially provide novel biological markers or targets for the treatment of leukemia in the future.

Materials and methods

Reagents. Oridonin (1 mg; Sigma-Aldrich; Merck KGaA) was formulated into a stock of 1 mg/ml after addition of the appropriate amount of DMSO to assist dissolution. The following reagents and antibodies were used: 2X Taq MasterMix (cat. no. BC006M; Jiangsu Kangwei Century Biotechnology Co. Ltd.), fluorescent reverse transcription-quantitative PCR (RT-qPCR) reagents (cat. no. BCN043M; Jiangsu Kangwei Century Biotechnology Co. Ltd.), PDCD4 antibody (1:1,000; cat. no. 9535; Cell Signaling Technology, Inc.), Bcl-2 antibody (1:1,000; cat. no. 15071; Cell Signaling Technology, Inc.), Bax antibody (1:1,000; cat. no. 89477; Cell Signaling Technology, Inc.), caspase-3 (1:1,000; cat. no. 9662), caspase-9 (1:1,000; cat. no. 9502), cleaved caspase-3 (1:1,000; cat. no. 9661T), cleaved caspase-9 (1:1,000; cat. no. 7237T), β-actin antibody (1:2,000; cat. no. 20536-1-AP; Proteintech Group, Inc.) and HRP-labeled anti-rabbit IgG secondary antibody (1:20,000; cat. no. ZB-2301; OriGene Technologies, Inc.). The human acute monocytic leukemia cell line U937 (TCHu159) and the acute promyelocytic leukemia (APL) cell lines HL60 (SCSP-520) and NB4 (SCSP-5103) were purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences and maintained in subculture preservation. These cells were cultured in RPMI1640 medium (Gibco; Thermo Fisher Scientific, Inc.) with 10% FBS (HyClone™; Cytiva) and 1% penicillin and streptomycin mixture, under 37˚C, 5% CO_2 and saturated humidity.

Cell Counting Kit-8 (CCK-8) assay to assess cell proliferation of leukemia cells. Based on preliminary experiments on the proliferation of leukemia cells using oridonin, 5.0 μM was selected as the drug experimental concentration as it is close to the IC_{50}. In total, three types of AML cell lines were selected including the acute monocytic leukemia cell line U937 and the APL cell lines HL60 and NB4. Cultured until the logarithmic growth period, a total of $5x10^5$ leukemia cells/ml were seeded in 96-well cell culture plates and three replicates were set up for each experimental group. After 48 h culture at 37˚C, 5% CO_2 and saturated humidity, the CCK8 cell counting assay (Dojindo Laboratories, Inc.) was performed according to manufacturer's instructions. CCK-8 solution (10 μl) was added to each well and incubated at 37˚C, 5% CO_2 and saturated humidity for 1 h, the enzyme-labeled instrument was used to measure the optical density (OD) value (450 nm). Simultaneously, the routine trypan blue staining assay was used to detect cell viability; staining with a 0.2% final concentration of trypan blue for 5 min at room temperature and the dead cells were stained blue.

Cell cycle profile analysis. Cell cycle was detected according to manufacturer's instructions about cell cycle detection Kit (Jiangsu KeyGen Biotech Corp., Ltd.; cat. no. KGA511). A total of $1x10^6$ HL60 cells were treated with oridonin for 48 h at 37˚C, 5% CO_2 and saturated humidity, ice-cold PBS (Shandong Keshuo Biotechnology Co., Ltd.) was taken to wash the cells twice and then they were fixed in cold 75% ethanol (Shandong Keshuo Biotechnology Co., Ltd.) at 4˚C overnight. Ethanol was removed by centrifugation (800 x g, 5 min) at 4˚C and cells were resuspended with 1X PBS and filter once

through a 400-mesh sieve, centrifuged at 800 x g for 5 min at room temperature and the PBS discarded. Then, 1 ml of PI staining solution (20 g/ml PI, 0.3 mM Na-citrate and 0.5 mg/ml RNase A) was added and incubate at 4˚C in the dark for 30 min and analyzed using CytoLEX® flow cytometry (Beckman Coulter, Inc.). The cell cycle was analyzed by ModFitLT 5.0, software (Verity Software House). The ratio of each phase of G_0/G_1, S and G_2/M was expressed as a percentage (%). Three replicate wells were set up for each experimental group.

Cell apoptosis detected by annexin V/PI double staining. Cell apoptosis was detected according to manufacturer's instructions about Annexin V-FITC/PI Apoptosis Detection Kit (Jiangsu KeyGen Biotech Corp., Ltd. cat. no. KGA1102). The key steps included: Following exposure to oridonin for 48 h at 37˚C with 5% CO_2 and saturated humidity, HL60 cells were collected from each indicated group, washed twice with 1X pre-cooled PBS, resuspended in 250 μl binding buffer and cell density was adjusted to $1x10^6$ cells/ml. A total of 100 μl cell suspension was taken, 5 μl annexin V/FITC and 1 μl of 100 μg/ml PI were added and cells were incubated in the dark for 20 min. The early apoptosis rate of cells was assessed using flow cytometry CytoFLEX® after filtering through a 300-mesh screen. Three replicate wells were set up for each group and the apoptosis level was expressed as mean ± standard deviation.

Differential miRNA screening and target gene prediction. HL60 cells were treated with 5 μg/ml oridonin for 48 h, at 37˚C, 5% CO_2 and saturated humidity, the cells were collected and total RNA was extracted by TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.; cat no. 15596026). Agilent miRNA Microarray Experiment was used to detect the miRNA expression profile. Labeling spike-in RNA and Hyb spike-in RNA were used as quality controls for the entire experimental process. The cleaned chips were scanned using an Agilent chip scanner (scanner G2565CA; Agilent Technologies, Inc.). The hybridization images were analyzed and data extracted using Ag Feature Extraction (v10.7) software (Agilent Technologies, Inc.). The Affymetrix IVT expression profile ChIP data analysis was performed to obtain the top 10 miRNAs associated with apoptosis. Further intersection analysis with the star cancer gene miRNA reported in the PubMed database (https://pubmed.ncbi.nlm.nih.gov/) was performed to determine the cancer gene miRNAs among the top 10 miRNAs. On the basis of determining the candidate miRNAs, the intersection analysis and the prediction of their downstream target genes were conducted using the miRDB (http://www.mirbase.org) and StarBase (https://rnasysu.com/encori/) databases. The complementary sequence of miR-155-5p and 3'-UTR region of PDCD4 were predicted using the TargetScan database (http://www.targetscan.org/vert_80/).

Western blotting. HL60 cells from the control group and from each indicated experimental group were collected. Pre-cooled PBS was used to wash cells, and $2x10^7$ cells were lysed and protein extraction reagent RIPA (Beijing Solarbio Science & Technology Co., Ltd. cat. no. R0010) was used to extract total cell protein. After the protein concentration was measured using the BCA method, 5X protein loading buffer was added and the protein was denatured at 90˚C for 10 min. A total of 20 μg protein of each sample was loaded for SDS-PAGE (10%) electrophoresis and the separated protein was transferred to a PVDF membrane, blocked with 5% skimmed milk for 90 min at room temperature and then incubated with the corresponding primary antibody including PDCD4, Bax, Bcl-2, cleaved caspase-3 (1:1,000), cleaved caspase-9 (1:1,000) and β-actin at 4˚C overnight. The membranes were washed for 30 min with 1X TBST washing buffer with 0.1% Tween, then the secondary antibody goat anti-rabbit IgG was added for incubation at room temperature for 90 min and then membranes were washed for 30 min again. Lastly, protein expression was detected using an ECL kit (arkjade ECL Super; cat. no. ED0015-B; Shandong SparkJade Scientific Instrument Co., Ltd.). ImageJ software (version 1.51j8; National Institutes of Health) was used for densitometry. Experiments were repeated three times for each sample.

RT-qPCR. A total of $2x10^5$ HL60 cells/ml were added to a 6-well cell culture plate followed by oridonin treatment and miR-155-5p transfection for 48 h at 37˚C. Next, the cells from the control and the different experimental groups were collected in 15-ml sterile and enzyme-free centrifuge tubes, washed once with pre-cooled PBS, mixed with PBS, transferred to an Eppendorf tube, washed once more with pre-cooled PBS and lastly any residual PBS was removed. TRIzol® (Thermo Fisher Scientific, Inc.) was used to extract total RNA, and an ultra-trace nucleic acid and protein analyzer was used to measure the concentration of total RNA. Complementary DNA (cDNA) was synthesized according to the PrimeScript RT kit with gDNA Eraser RT kit (Takara Bio Inc.) instructions. Samples were incubated at 42˚C for 30 min, 90˚C for 5 min and 5˚C for 5 min, respectively. Following RT, the cDNA was used as template for qPCR. The primers used were: miR-155-5p, forward (F) 5'-CAAGGTGGTTAA TGCTAATCGTGATA-3' and reverse (R) 5'-GTGCAGGGT CCGAGGT-3'; miRNA-21-3p, forward (F) 5'-TCCTATCGC CAACACCAGTC-3' and reverse (R) 5'-TATCCTTCTTCA CGAGTCCTACAC-3'.U6 F, 5'-CAGCACATATACTAAAAT TGGAACG-3' and R, 5'-ACGAATTTGCGTGTCATCC-3', synthesized by Shanghai GenePharma Co, Ltd., which was the miRNA-specific stem-loop primers with artificially-added stem-loop (Sangon Biotech, Co., Ltd.). PDCD4, F, 5'-AGGCAA AAAGGCGACTAAGGA-3' and R, 5'-TCCAGCAACCTT CCCTTTGG-3'; GAPDH F, 5'-CAACTTTGGTATCGTGGA AGG-3' and R, 5'-GCCATCACGCCACAGTTTC-3' synthesized by Shanghai BioSune Biotechnology (Shanghai) Co, Ltd. RT-qPCR was performed according to the instructions of TB Green® Premix Ex Taq (Tli RNase H Plus) kit (Takara Bio Inc.) and the qPCR conditions were as follows: 95˚C 10 sec, 40 cycles of 5 sec at 60˚C and 10 sec at 72˚C and 34 sec at 60˚C. Relative quantification analysis was performed using the $2^{-\Delta\Delta Cq}$ method (25). The experiment was repeated three times. HL60 cells were randomly selected at seven different time points after induction by oridonin. miR-155-5p and PDCD4 expression were assessed using RT-qPCR and Pearson's correlation coefficient was used to investigate correlation.

Bone marrow (5 ml) was extracted from three newly diagnosed patients with APL (47.67±13.65 years old, males), according to the 2022 WHO classification of leukemia (https://tumourclassification.iarc.who.int/), and 5 ml peripheral

blood from healthy subjects (51.00±9.54 years old, males) to obtain primary leukemic cells and normal peripheral blood mononuclear cells by Ficoll separation solution (800 x g, 20 min at room temperature, respectively. (Ethics Committee of Zhangqiu District People's Hospital of Jinan; approval no. 2024001; Jinan, China). Oral informed consent was obtained from both patients and healthy volunteers for participation. Under aseptic conditions, all specimens were collected between June and October 2024 and added to an anticoagulant tube. The bone marrow or peripheral blood was mixed with the PBS solution in equal proportions. The diluted bone marrow or blood was added to the upper layer of the cell separation solution, at a 3:2 ratio of diluted bone marrow or peripheral blood to cell separation solution. Centrifuged for 25 min at 800 x g at room temperature, the mononuclear cell layer of bone marrow or blood was carefully extracted and transferred to a 15 ml centrifuge tube, and an appropriate amount of PBS solution was added to wash once. miR-155-5p or PDCD4 expression levels were assessed using the aforementioned RT-qPCR method.

Luciferase reporter gene assay. The bioinformatics prediction website TargetScan (http://www.targetscan.org) was used to predict the binding sequence of PDCD4 and miR-155-5p. pmirGLO-PDCD4-wild type (wt) plasmid vector and pmirGLO-PDCD4-mutated (mut) plasmid vector were purchased from Boshang Biotechnology (Shanghai) Co., Ltd. and which were constructed by Shanghai BioSune Biotechnology (Shanghai) Co, Ltd. The wt-PDCD4 plasmid, mut-PDCD4 plasmid and miR-155-5p mimic were transfected using the jetPRIME transfection kit (Polyplus Co., Ltd.) for 48 h at 37˚C. After 48 h culture, as a normalization method, dual-luciferase reporter gene detection system (EnVision; PerkinElmer, Inc.) was used to measure luciferase activity (Firefly Luciferase/Renilla Luciferase as normalization). Three replicate wells were set up for each experimental group and the results were expressed as mean ± standard deviation.

Cell transfection and biological function rescue experiment. miR-155-5p mimics (2.5 μg), inhibitor (2.5 μg) and si-PDCD4 (100 pmol) synthesized by Shanghai GenePharma Co., Ltd., were transfected with Lipofectamine® 2000 Transfection Reagent Kit (Thermo Fisher Scientific, Inc.) into HL60 cells for 48 h at room temperature to detect the role of PDCD4 in modulating apoptosis or proliferation of HL60 cells. Validation of transfection efficiency was performed by RT-qPCR and was indicated by miRNA-155-5p or PDCD4 expression. The mimics negative control (NC) or si-NC were used as the control (by Shanghai GenePharma Co., Ltd.). The following sequences were used: si-PDCD4, sense (S), 5'-GCUGCUGAAAGAAUAUUUATT-3', antisense (AS), UAAAUAUUCUUUCAGCAGCTT; si-NC, S, 5'-UUCUCCGAACGUGUCACGU-3', AS, 5'-ACGUGACACGUUCGGAGAA-3'; miR-155-5p mimics, S, 5'-UUAAUGCUAAUCGUGAUAGGGGUU-3' and AS, 5'-CCCCUAUCACGAUUAGCAUUAAUU-3'; mimics NC, S, 5'-UUCUCCGAACGUGUCACGUTT-3' and AS, 5'-ACGUGACACGUUCGGAGAATT-3'; miR-155-5p inhibitor, 5'-AACCCCUAUCACGAUUAGCAUUAA-3' and inhibitor NC, 5'-CAGUACUUUUGUGUAGUACAA-3'. The CCK-8 assay was used to determine the changes at the cell proliferation level, western blotting was used to determine the changes of PDCD4 protein expression and flow cytometry was used to determine the changes in cell apoptosis.

Statistical analysis. SPSS (version 13.0; SPSS, Inc.) was used for statistical analysis and the results are expressed as mean ± standard deviation. The difference between two paired groups of data was compared using paired samples Student's t-test. One-way analysis of variance and the Bonferroni's correction were used to compare multiple groups and perform pairwise comparisons when differences were statistically significant. Pearson's correlation analysis was used to investigate correlations. Test level α=0.05 and P<0.05 were considered to indicate a statistically significant difference.

Results

Leukemia cell proliferation detection using the CCK-8 assay. Based on preliminary experiments on the IC_{50} of oridonin in leukemia cells, the IC_{50} concentration was 4.628 μM (Fig. 1A) and the concentration integer closest to the IC_{50}, 5.0 μM, was selected as the candidate drug concentration. After exposure to oridonin for 48 h, the proliferation of the three types of leukemia cells was measured using the CCK-8 assay. The results demonstrated that oridonin could inhibit proliferation of human HL60 cells (0.67±0.02 vs. 0.26±0.02; t=24.04; P<0.0001), U937 cells (0.68±0.01 vs. 0.35±0.01; t=41.93; P<0.0001) and NB4 cells (0.71±0.02 vs. 0.37±0.01; t=29.70; P<0.0001) compared with the control groups (Fig. 1B). The inhibitory effect on HL60 by oridonin was most notable compared with NB4 and U937 cells, indicated by the highest proliferation inhibition rate (both P<0.01; Fig. 1C). According to the results of the inhibition rate, HL60 cells with the greatest inhibitory effect were selected. The cell cycle in control group (Fig. 1D and E) and oridonin treatment group (Fig. 1F and G) was detected by FACS and the results indicated that there were no significant changes in the G_0/G_1 phase but there was a significant increase in percentage of cells in the G_2/M phase and a significant decrease in the S phase (P=0.1425, P=0.0263 and P=0.0046, respectively; Fig. 1H).

miR-155-5p expression of HL60 cells after exposure to oridonin. Based on the high expression of miR-155-5p in tumor cells reported in literature (26), Agilent miRNA Microarray was first used to observe the expression of miRNAs after oridonin treatment on HL60 cells, with the aim to detect downregulation of miR-155-5p in HL60 cells induced by oridonin. The differential analysis demonstrated that there were 598 differentially expressed miRNAs with a fold change ≥2.0 times (P≤0.05), and the expression of 1,128 miRNAs did not significantly change (P>0.05). Among them, 116 miRNAs were associated with apoptosis (Fig. 2A-C), including 33 significantly reduced miRNAs and 83 significantly increased miRNAs. The top 10 downregulated genes were used to intersect with the PubMed database (Fig. 2D). By contrast, leukemia-related oncogenic miRNAs were investigated using the PubMed database (Fig. 2E). Furthermore, the intersection analysis of the top 10 downregulated oncogenic miRNAs by ChIP and oncogenic miRNAs in the PubMed database was performed, and the intersection analysis of oncogenic miRNAs

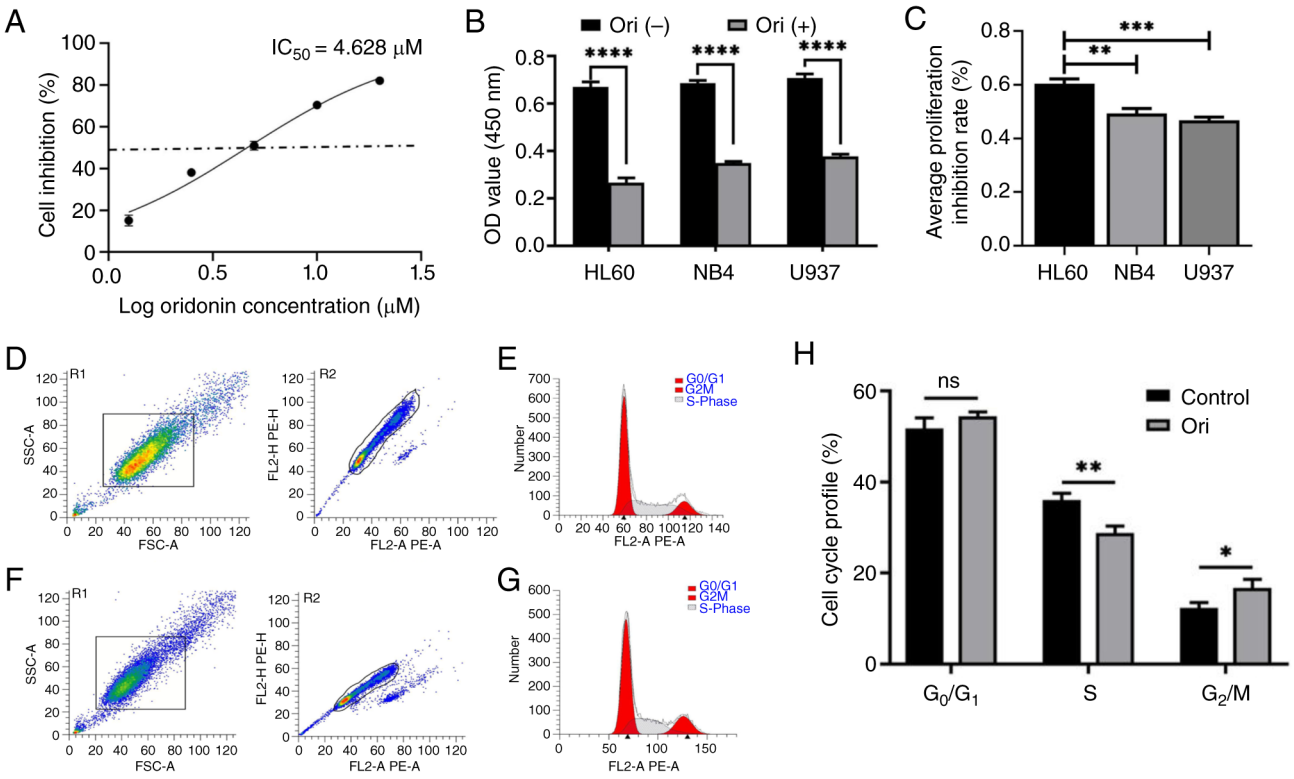


Figure 1. Effect of oridonin on proliferation and cell cycle profile. After the concentration of the candidate drug was determined according to the IC_{50}, HL60, NB4 and U937 cells were treated with oridonin *in vitro*, the proliferation was assessed using a CCK-8 cell counting assay and flow cytometry was used to detect the cell cycle profile. (A) IC_{50} detection and calculation results. (B) Proliferation of leukemia cells by CCK-8 cell counting assay. (C) Mean proliferation inhibition rate. (D) Removal of cell debris and cell aggregates by gating in control group. (E) Cell cycle profile of flow cytometry plot in control group. (F) Removal of cell debris and cell aggregates by gating in oridonin group. (G) Cell cycle profile of flow cytometry plot in oridonin group. (H) Cell cycle profile analysis by flow cytometry; n=3. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001. Ori, oridonin; ns, no significant; CCK-8, Cell Counting Kit-8.

identified miR-21-3p and miR-155-5p (Fig. 2F and G). miR-155-5p and miR-21-3p expression was assessed using RT-qPCR before and after oridonin treatment; miR-155-5p expression was downregulated (3.26±0.06 vs. 1.51±0.09; t=26.26; P<0.0001; Fig. 2H) and the miR-21-3p expression did not change significantly (Fig. 2I; P>0.05). The expression of miR-155-5p of primary APL cells was significantly higher than that of the PBMC group, which was isolated from the aforementioned patient or healthy control samples, respectively (1.04±0.12 vs. 0.4±0.03; t=8.67; P=0.001; Fig. 2J).

Effect of miR-155-5p on HL60 cell proliferation. To investigate the role of miR-155-5p on HL60 cell proliferation inhibition induced by oridonin, transfection experiments with miR-155-5p mimics or an miR-155-5p inhibitor were performed. The transfection efficiency of miR-155-5p was assessed using RT-qPCR assay, and the expression of miR-155-5p was notably upregulated in miR-155-5p mimics group as compared with the control group (P<0.0001) and there was no obvious difference between control group and mimics NC group (P=0.6521) (Fig. S1A). When miR-55-5p mimics were transfected simultaneously with oridonin, miR-155-5p expression was significantly increased compared with oridonin alone (0.44±0.03 vs. 1.74±0.06; t=32.02; P<0001; Fig. 3A).

The transfection efficiency of miR-155-5p inhibitor was evaluated using RT-qPCR and the expression of miR-155-5p was significantly downregulated compared with the control and inhibitor NC groups (P=0.5988 and P<0.0001, respectively; Fig. S1B). The miR-55-5p inhibitor significantly reduced miR-155-5p expression in HL60 cells compared with that of the control group (3.56±0.27 vs. 0.93±0.05; t=16.79; P<0.0001; Fig. 3B). Compared with oridonin alone, the proliferation of HL60 cells was significantly increased after simultaneous transfection of oridonin with miR-55-5p mimics (0.36±0.01 vs. 0.63±0.01; t=31.99; P<0.0001; Fig. 3C). Compared with the control group, the proliferation of HL60 cells was significantly decreased after transfection with the miR-155-5p inhibitor (0.73±0.01 vs. 0.52±0.01; t=2.80; P<0.0001; Fig. 3D).

PDCD4 as a downstream target gene of miR-155-5p. miRNAs serve their inhibitory role in regulating expression of target genes by targeting their 3'-UTR. Studies identified through the PubMed database search and the preliminary experiments indicated that PDCD4 was associated with apoptosis or proliferation of numerous types of tumor cells. Target gene database prediction and database intersection analysis were further performed to identify the possible role of PDCD4 as a target gene of miR-155-5p. The results of the StarBase and miRDB databases intersection analysis indicated that PDCD4 was one of the possible downstream apoptosis-related target genes of miR-155-5p (Fig. 4A and B). After the PubMed database retrieval, PDCD4 was demonstrated to be a tumor suppressor gene closely associated with leukemia cell differentiation, apoptosis and proliferation (Fig. 4C). The results of RT-qPCR demonstrated that the PDCD4 expression in

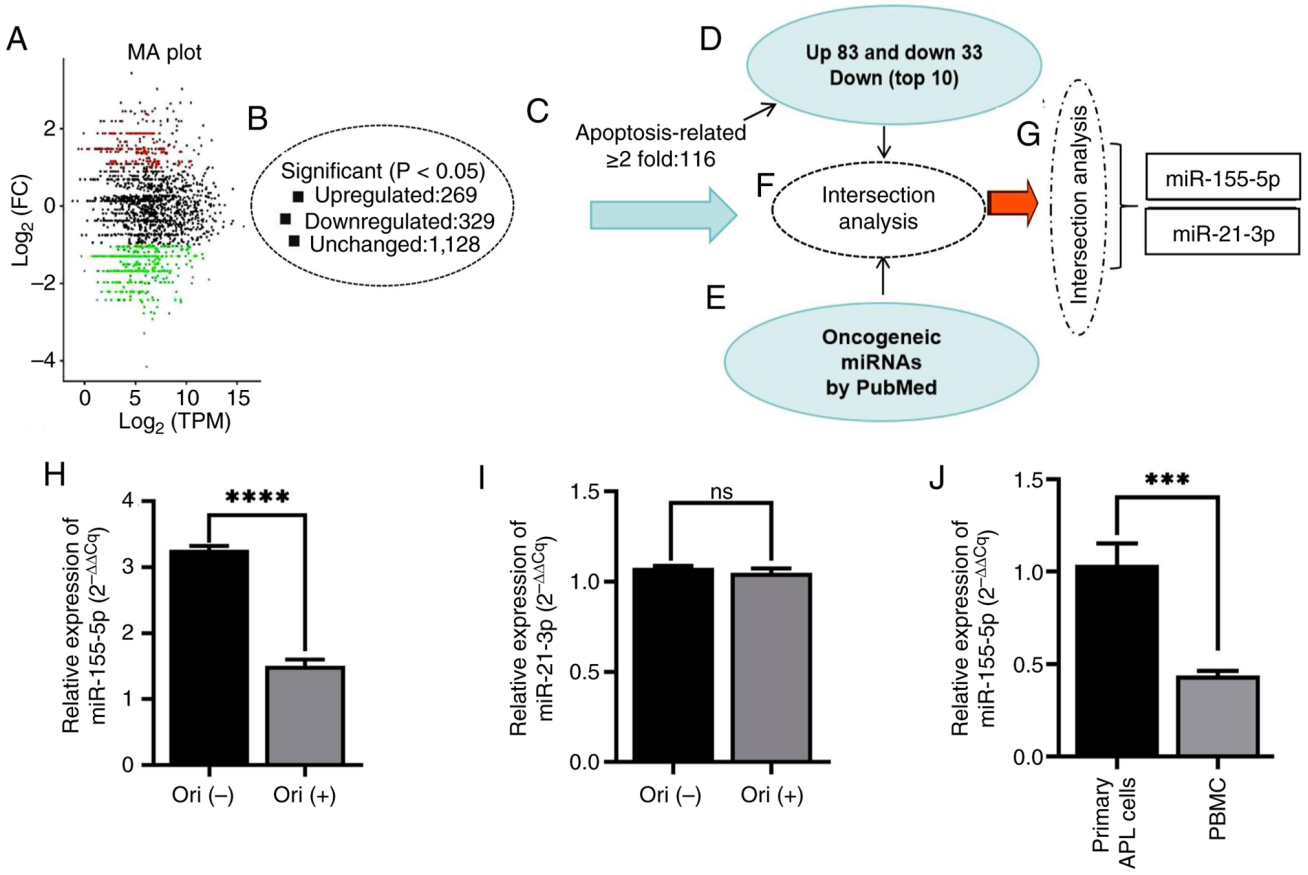


Figure 2. Analysis of differentially expressed miRNAs using ChIP. miRNA expression was detected using a ChIP assay before and after exposure to oridonin *in vitro*, and apoptosis-related and downregulated oncogenic miRNAs were identified using ChIP and intersection analysis with leukemia-related oncogenic miRNAs in PubMed. (A) MA plot of miRNA expression detected by ChIP assay. (B) A total of 598 differential miRNAs with a fold ≥2.0 by MA plot. (C) A total of 116 differential miRNAs associated with apoptosis. (D) A total of 83 upregulated and 33 downregulated miRNAs (fold change ≥2.0), top 10 regulated miRNAs were used to conduct intersection with PubMed. (E) Oncogenic miRNAs by PubMed database research. (F) Intersection analysis of downregulated top 10 oncogenic miRNAs by ChIP and oncogenic miRNAs in PubMed. (G) Intersection analysis of oncogenic miRNAs with miR-155-5p and miR-21-3p. (H) Relative expression of miR-155-5p before and after oridonin treatment. (I) Elative expression of miR-21-3p before and after oridonin treatment. (J) Expression level of miR-155-5p in primary APL cells and PBMC cells. ***P<0.001 and ****P<0.0001. Ori, oridonin; FC, fold-change; TPM, transcript per million; ns, no significant; miRNA/miR, microRNA; ChIP, chromatin immunoprecipitation; APL, acute promyelocytic leukemia; PBMC, peripheral blood mononuclear cells; MA, M-versus-A.

primary APL cells was significantly lower compared with that in PBMCs (0.76±0.05 vs. 3.32±0.04; t=69.68; P<0.0001; Fig. 4D). Following induction by oridonin, the expression level of PDCD4 was significantly increased in HL60 cells (0.69±0.04 vs. 2.20±0.23; t=12.81; P=0.0002; Fig. 4E).

miR-155-5p regulates the expression of PDCD4. After exposure to oridonin *in vitro*, the PDCD4 protein expression of HL60 cells was significantly upregulated (0.57±0.12 vs. 1.15±0.12; t=6.027; P=0.0038; Fig. 5A and B). Compared with the oridonin group, PDCD4 expression was significantly downregulated after simultaneous transfection with miR-155-5p mimics (1.04±0.15 vs. 0.54±0.15; t=4.12; P<0.05); Fig. 5C and D). There was no obvious difference in proliferation between the oridonin group and mimics NC group (P>0.05), however, the proliferation of HL60 cells in oridonin + miR-155-5p mimics group was upregulated as compared with the oridonin group (0.429±0.003 vs. 0.616±0.005; t=61.21; P<0.0001; Fig. 5E). PDCD4 was predicted as a target gene of miR-155-5p by The Cancer Genome Atlas (TCGA) database (https://portal.gdc.cancer.gov/) and the binding region and sequence of the 3'-UTR were predicted (Fig. 5F). Furthermore, luciferase activity was significantly higher after co-transfection of miR-155-5p mimics and luciferase-PDCD4-wt. The luciferase activity did not change significantly in the luciferase-PDCD4-mut experimental group. The results also indicated that miR155-5p served a regulatory role through binding on the 3'-UTR of PDCD4 (Fig. 5G). Synchronous detection of miR-155-5p and PDCD4 was attempted *in vitro* at seven timepoints after exposure to oridonin; the correlation analysis indicated that they were negatively correlated (r=-0.9446; P=0.0053; Fig. 5H). The transfection efficiency of si-PDCD4 was assessed using RT-qPCR assay. The expression of PDCD4 was markedly downregulated by transfection with si-PDCD4 as compared with control group (t=43.00, P<0.0001; Fig. S1C). To further confirm the effect of PDCD4 expression on the proliferation of HL60 cells, si-PDCD4 was transfected into HL60 cells after exposure to oridonin and the PDCD4 expression was significantly downregulated (1.13±0.08 vs. 0.33±0.11; t=10.13; P=0.0005; Fig. 5I and J), accompanied by an increase in proliferation of HL60 cells (0.42±0.01 vs. 0.64±0.01; t=29.65; P<0.0001; Fig. 5K).

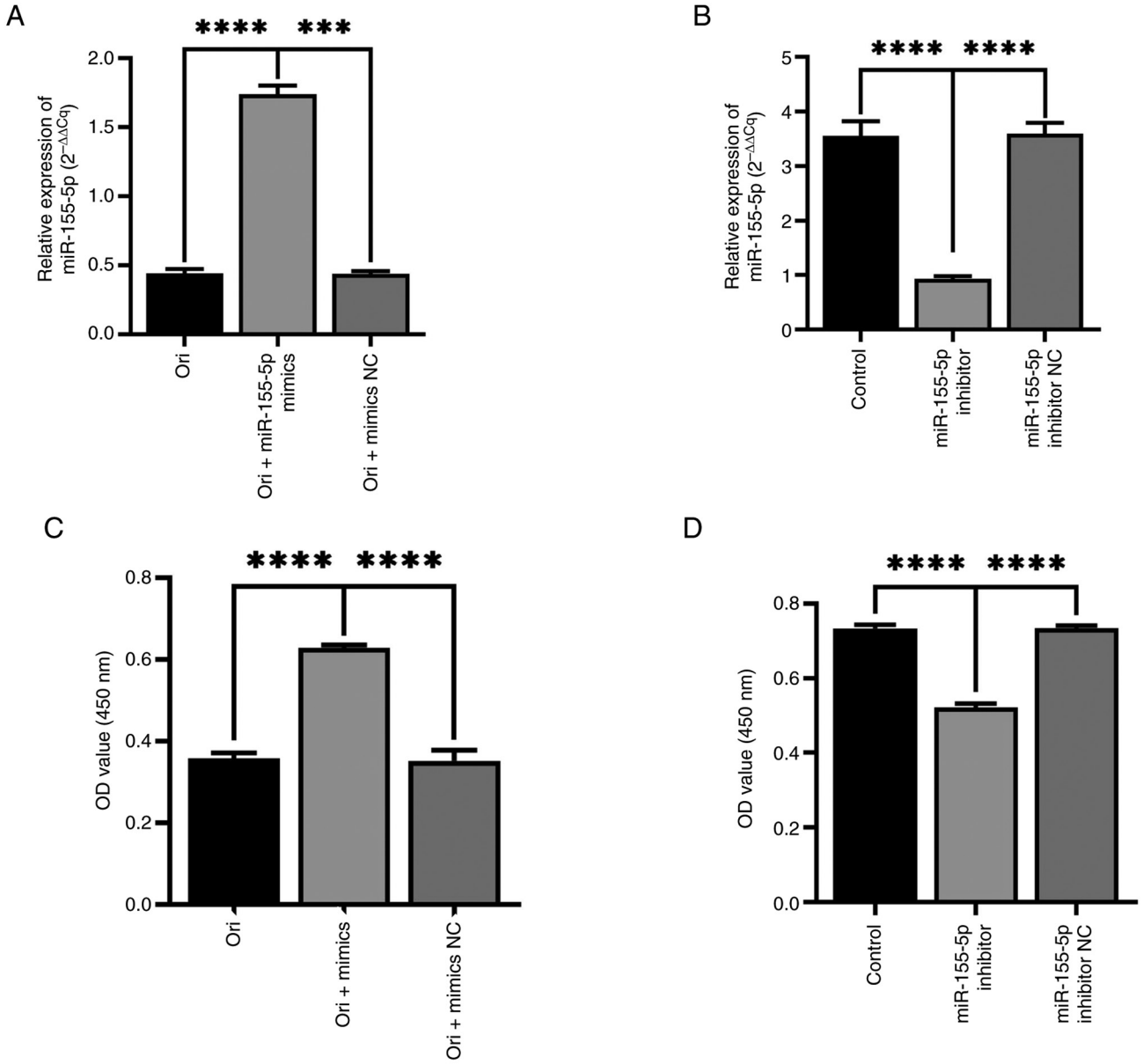


Figure 3. Effects of miR-155-5p on HL60 cell proliferation. miR-155-5p mimics or inhibitor were transfected into HL60 cells at the same time as treatment with oridonin, and the Cell Counting Kit-8 assay was used to assess their role in modulating proliferation of HL60 cells. (A) Expression of miR-55-5p after simultaneous transfection of miR-155-5p mimics together with oridonin. (B) Effect of miR-155-5p inhibitor on expression of miR-155-5p in HL60 cells. (C) Reversal of inhibitory effect of oridonin on proliferation of HL60 cells by miR-155-5p mimics. (D) Influence of miR-155-5p inhibitor on HL60 cells. ***P<0.001 and ****P<0.0001. Ori, oridonin; ns, no significant; miRNA/miR, microRNA; NC, negative control.

Effect of PDCD4 expression on proliferation and apoptosis of HL60 cells. Firstly, a trypan blue exclusion test was used to rule out cytotoxic effects and the results indicated that there was no significant difference between the control group and oridonin group (t=0.000, P>0.9999; Fig. 6A). On the basis of the aforementioned experimental results which demonstrated that miR-155-5p and PDCD4 were involved in the regulation of proliferation of leukemia cells induced by oridonin The apoptosis of HL60 cells induced by oridonin was also detected and the results demonstrated that oridonin induced significant apoptosis of HL60 cells compared with the control group (4.783±2.66 vs. 22.98±2.69; t=8.329; P=0.0011. Fig. 6B and C). When miR-155-5p mimics were transfected simultaneously with oridonin, the apoptosis of HL60 cells was significantly decreased compared with the mimics NC group (17.44±2.29 vs. 7.76±0.91; t=6.77; P<0.01; Fig. 6D and E). Compared with the si-NC group, when si-PDCD4 was transfected simultaneously with oridonin, the apoptosis of HL60 cells was significantly decreased (15.53±1.64 vs. 860±0.37; t=7.166; P<0.01; Fig. 6F and G).

Effect of PDCD4 on apoptosis proteins in HL60 cells. Based on the determination of oridonin-induced apoptosis in leukemia cells, its molecular mechanism of inducing apoptosis was explored. As compared with the control group, the expression of Bax protein was significantly upregulated (0.44±0.13 vs. 1.03±0.10; t=4.728; P=0.0091; Fig. 7A and B) and Bcl-2 protein expression was significantly downregulated (1.06±0.10 vs. 0.39±0.19; t=5.416; P=0.0056; Fig. 7C and D) in HL60 cells after exposure to oridonin. There was no

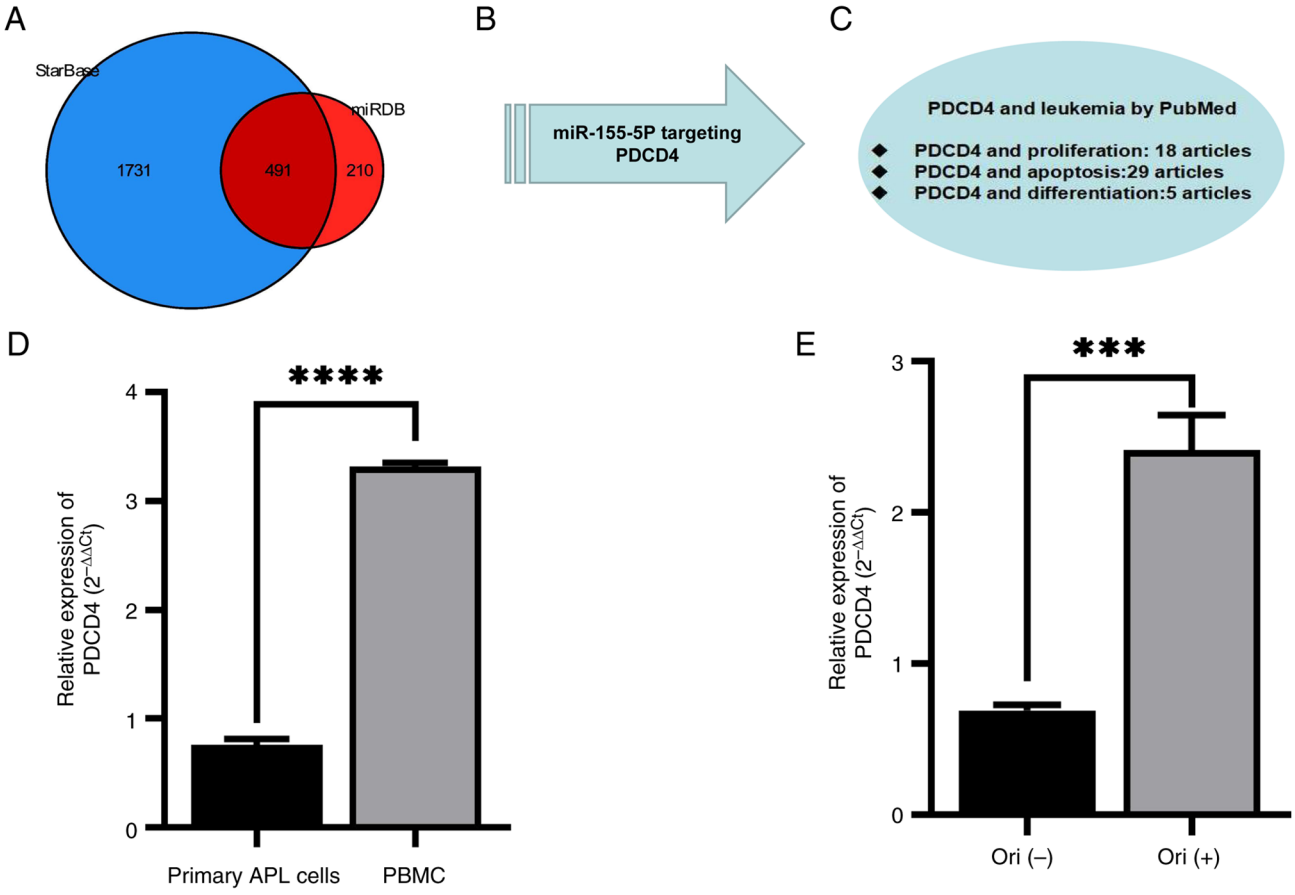


Figure 4. Predicted analysis of the downstream target gene PDCD4 of miR-155-5p. Downstream gene PDCD4 of miR-155-5p was evaluated by StarBase and miRDB database intersection analysis and literature research by PubMed. (A) Intersection analysis results by StarBase and miRDB databases. (B) PDCD4 was the candidate target gene of miR-155-5p. (C) Literature retrieved by PubMed on PDCD4 and the proliferation, differentiation and apoptosis of leukemia cells. (D) Expression of PDCD4 in primary APL cells and PBMC. (E) Expression of PDCD4 in HL60 cells before and after the action of oridonin. ***P<0.001 and ****P<0.0001. Ori, oridonin; PDCD4, programmed cell death protein 4; miRNA/miR, microRNA; APL, acute promyelocytic leukemia; PBMC, peripheral blood mononuclear cells; miRDB, MicroRNA Target Prediction Database.

notable change in caspase-3 expression (t=1.116; P=0.3268; Fig. 7E and F), a significant increase in the expression of cleaved caspase-3 (0.24±0.18 vs. 0.95±0.11; t=5766; P=0.0045; Fig. 7E and G) and a significant upregulation of ratio of cleaved caspase-3/caspase-3, (t=4.555, P=0.045; Fig. 7E and H). There was no significant variation of caspase-9 expression (t=0.6397; P=0.5572; Fig. 7E and I), a significant upregulation of cleaved caspase-9 expression (0.47±0.91 vs. 1.05±0.02; t=10.68; P<0.01; Fig. 7E and J) and a significant upregulation of ratio of cleaved caspase-9/caspase-9 (t=15.75, P=0.004; Fig. 7E and K). Moreover, the inversion of the Bax/Bcl-2 expression ratio was accompanied by the significant increase in the expression of cleaved caspase-3 and cleaved caspase-9. To verify whether the aforementioned apoptosis-related proteins Bax and Bcl-2 were associated with the change in PDCD4 expression, si-PDCD4 was transfected simultaneously with oridonin treatment in HL60 cells. The results demonstrated that compared with the si-NC control group, si-PDCD4 significantly attenuated the upregulation of PDCD4 expression induced by oridonin (0.46±0.17 vs. 1.13±0.12; t=5.482; P=0.0054; Fig. 7L and M). Compared with the si-NC group, the oridonin plus si-PDCD4 group resulted in the significant downregulation of Bax expression (1.20±0.14 vs. 0.7±0.03; t=5.809; P=0.0044; Fig. 7N and O) and the significant upregulation of Bcl-2 expression (0.473±0.14 vs. 1.04±0.05; t=6.588; P=0.0027; Fig. 7P and Q).

Molecular mechanism of oridonin inducing apoptosis in leukemia cells by regulating miR-155-5p/PDCD4. After comprehensive analysis of the experimental results of the present study, it was preliminarily hypothesized that proliferation of AML cells could be inhibited and simultaneously could induce apoptosis of this type of leukemia cells. Oridonin first downregulated the expression of miR-155-5p, the latter then resulted in the upregulation of PDCD4 expression by targeting the 3'-UTR of PDCD4. The increase in the expression of PDCD4 led to the inversion of the Bax/Bcl-2 ratio, which may upregulate the levels of cleaved caspase-3 and cleaved caspase-9, ultimately leading to apoptosis of HL60 cells (Fig. 8).

Discussion

With the continuous application of innovative drugs and the continuous optimization of treatment schemes, patients with hematological tumors including leukemia have received effective treatment, which is demonstrated by the improvement of the remission rate and prolonged survival period (27-30).

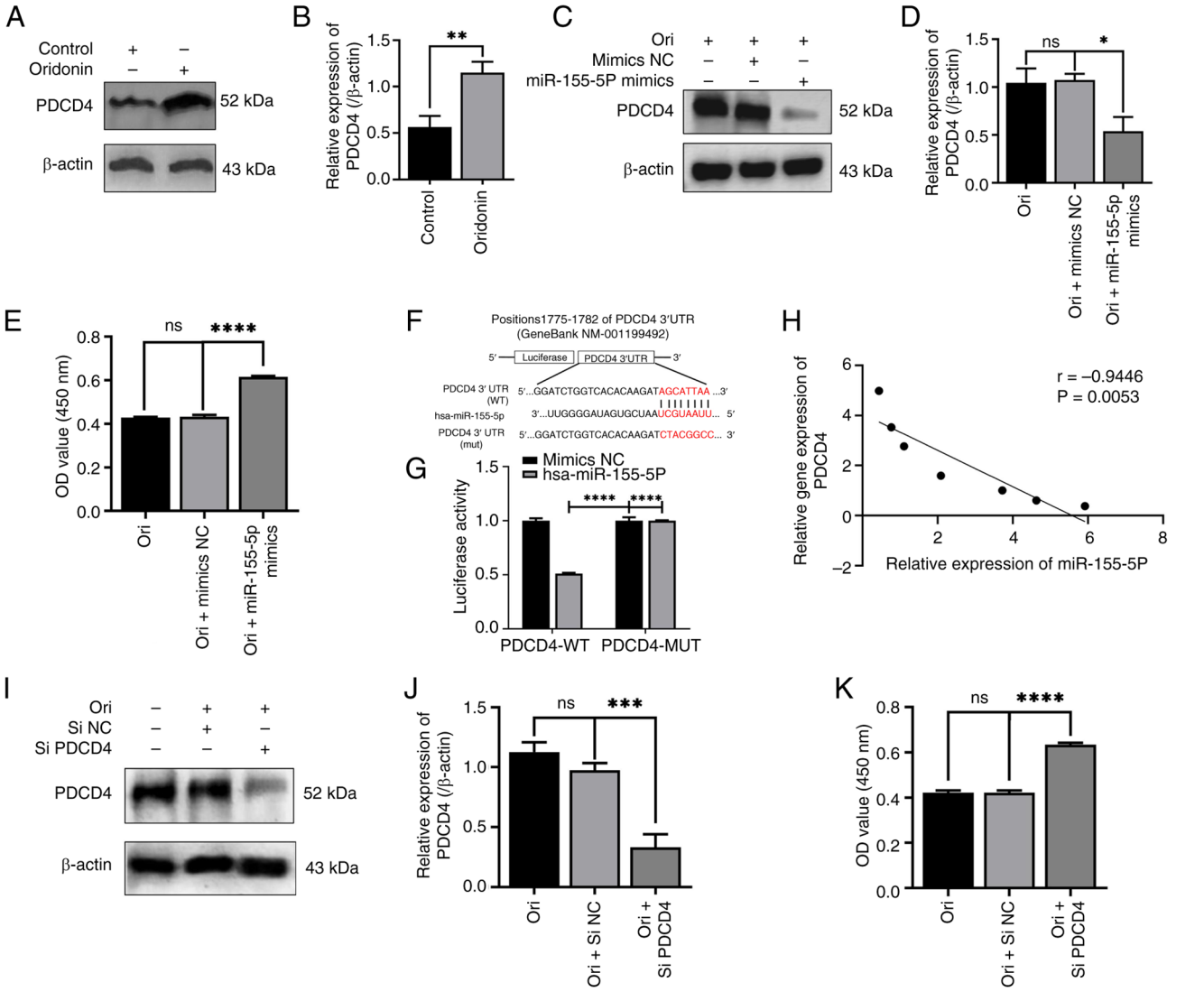


Figure 5. Regulatory effect of miR-155-5p on the expression of PDCD4. To assess the role of miR-155-5p in modulating expression of PDCD4, the miR-155-5p mimics or si-PDCD4 were transfected into HL 60 cells, and compared with the treatment of oridonin alone. (A) Western blotting results of PDCD4 protein before and after treatment with oridonin. (B) Average relative expression of PDCD4 protein before and after treatment with oridonin. (C) Western blotting results of PDCD4 protein before and after the transfection of miR-155-5p mimics. (D) Average relative expression of PDCD4 protein before and after transfection of miR-155-5p mimics with oridonin. (E) Proliferation of HL60 cells before and after the transfection of miR-155-5p mimics with oridonin. (F) Target sequence of miR-155-5p to bind the 3'-UTR of PDCD4. (G) Results of luciferase activity detection. (H) Correlation analysis between miR-155-5p and PDCD4 expression. (I) Expression of PDCD4 protein by western blotting after the simultaneous transfection of si-PDCD4 with oridonin treatment. (J) Average relative expression of PDCD4 protein after the simultaneous transfection of si-PDCD4 with oridonin treatment. (K) Proliferation of HL60 cells after transfection of si-PDCD4. n=3. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001. Ori, oridonin; PDCD4, programmed cell death protein 4; miRNA/miR, microRNA; NC, negative control; ns, not significant; si, small interfering RNA; wt, wild-type; mut, mutated; hsa, *Homo sapiens*.

However, phenomena such as drug resistance and relapse after remission are still key factors affecting the prognosis of patients with leukemia (31,32). Therefore, the development of novel low-toxic and highly efficient antitumor drugs is urgent. The identification of active ingredients from natural plants or traditional Chinese medicine could aid in the development of novel anti-hematological tumor drugs. For example, oridonin is a tetracyclic diterpenoid compound extracted from a medicinal herb. Since 1976, increasing research has reported that oridonin can exert pharmacological effects through different mechanisms, for example anti-bacterial, anti-inflammatory, immunomodulatory and anticancer effects.

Although oridonin exhibits promising anticancer activity in hematological malignancies, the exact targeted molecules and signaling pathways regulated by it remain to be elucidated. Therefore, further investigation of its molecular mechanisms is required to further understand its anticancer activity. At present, the anticancer effects of oridonin are mostly focused on the regulation of apoptotic signaling pathways (33,34). For example, oridonin can modulate the expression of mitogen-activated protein kinase or Bcl-2/Bax pathways and the caspase family. In addition, oridonin can also induce apoptosis by regulating the progression of the cell cycle, phagocytosis of macrophages or the promotion of autophagy (35). Furthermore, certain researchers have also demonstrated that oridonin is able to inactivate Akt and ERK and activate p38 MAPK and JNK signaling pathways in osteosarcoma cells causing the suppression of proliferation

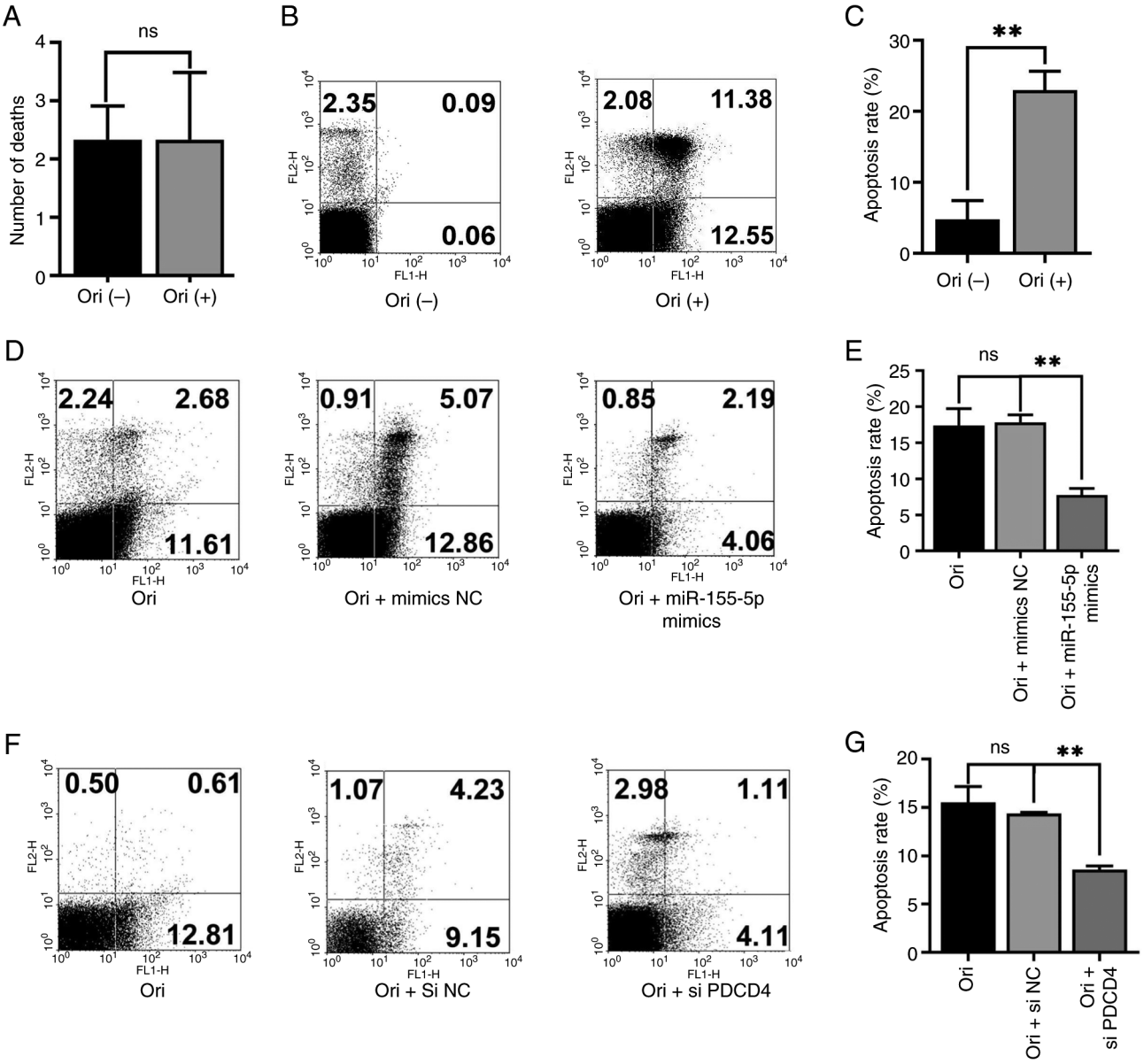


Figure 6. Effect of oridonin on the apoptosis of leukemia cells. To detect the mechanism of oridonin to inhibit proliferation of HL60 cells, the cell viability was detected by trypan blue exclusion test, the apoptosis of HL60 cells was detected using a flow cytometry assay with PI/annexin V double staining method. (A) Cell death percentage (%). (B) Flow cytometry detection of apoptosis in HL60 cells before exposure to oridonin. (C) Average apoptotic percentage of HL60 cells before and after exposure to oridonin. (D) Flow cytometry detection of apoptosis in the oridonin, oridonin mimics NC and oridonin plus miR-155-5p mimics groups. (E) Average apoptotic percentage of the oridonin, oridonin mimics NC, and oridonin plus miR-155-5p mimics groups. (F) Flow cytometry detection of apoptosis in the oridonin, oridonin si-NC, and oridonin plus si-PDCD groups. (G) Average apoptotic percentage of the oridonin, oridonin plus si-NC, and oridonin plus si-PDCD4 groups. n=3. **P<0.01. Ori, oridonin; PDCD4, programmed cell death protein 4; miRNA/miR, microRNA; NC, negative control; ns, no significant; si, small interfering RNA.

and induction of mitochondria- and caspase-dependent apoptosis (36). Zhao and Chen (37) systematically reviewed the research progress on the antitumor mechanism of oridonin. The results suggested that oridonin could serve an anti-hematological tumor role through a variety of mechanisms such as regulating the apoptosis signaling pathway, cancer proteins, chaperone proteins and miRNA expression or reactive oxygen species activity.

The literature has indicated that oridonin exerts multiple effects through the regulatory mechanisms such as inhibiting tumor growth, inducing apoptosis, promoting cell differentiation and reversing chemotherapy resistance (37,38). However, research on the mechanisms still has limitations and novel molecular mechanisms still remain to be explored in depth. Recent studies have reported that miRNAs could regulate the expression of numerous downstream genes and their dysregulation has been implicated in the pathogenesis of AML. Due to the dual role of miRNAs as either oncogenes or tumor suppressor genes, they are not only potential targets for drug action, but also potential anticancer drugs, and several treatments involving RNA inhibition and replacement have been developed. miRNA-based therapies have also become increasingly notable therapeutic strategies for patients with cancer (39-41). Therefore, the role of anticancer drugs including oridonin in the regulation of the expression of miRNAs has subsequently gained extensive

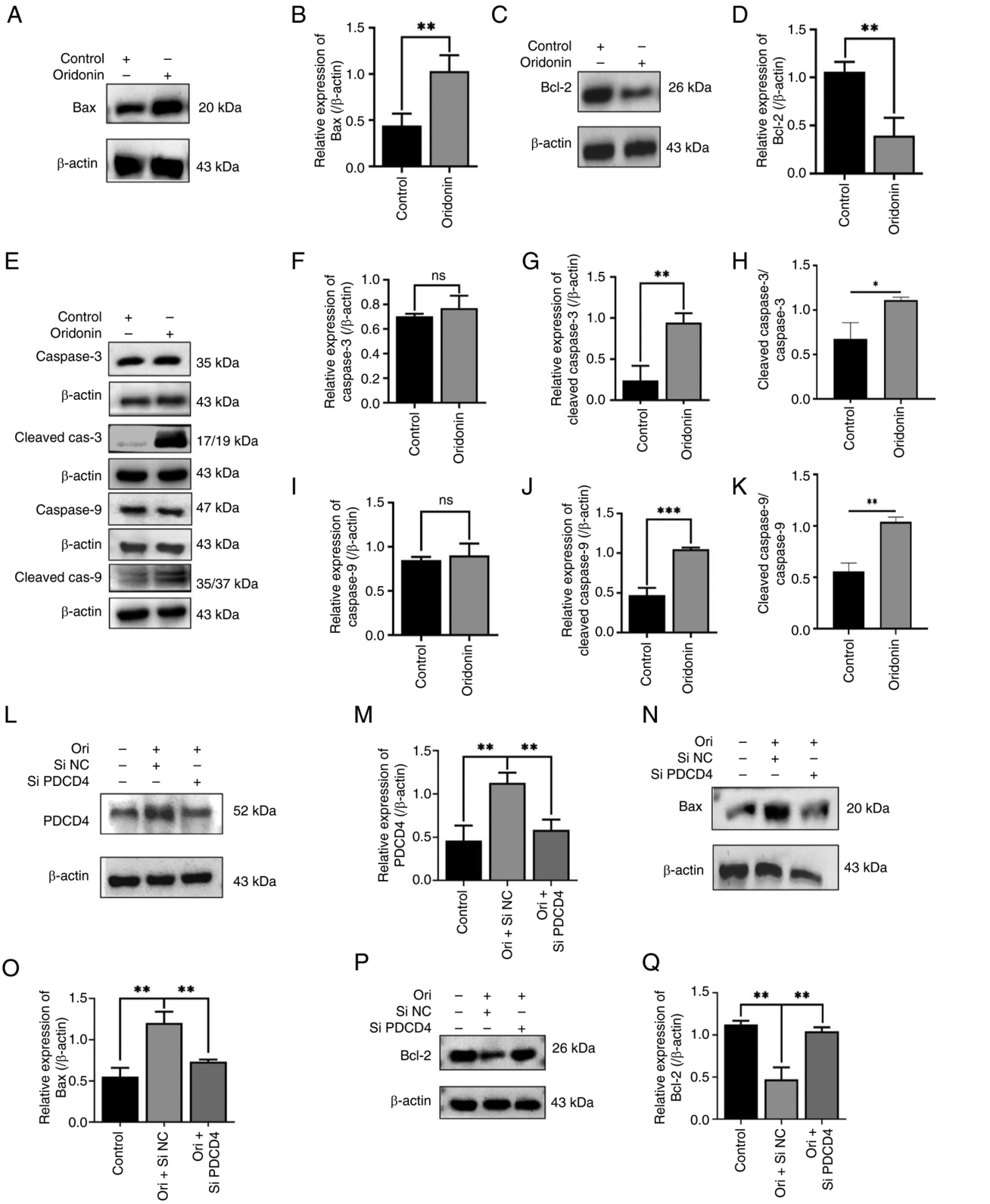


Figure 7. Expression of apoptosis-related proteins in HL60 cells before and after treatment with oridonin. To assess whether upregulation of PDCD4 by oridonin contributes to apoptosis by regulating apoptosis-related proteins, the expression of Bax, Bcl-2, cleaved caspase-3 or -9 were detected using western blotting assay in HL60 cells treated with oridonin or together with si-PDCD4 transfection. (A) Western blotting results and (B) average relative expression of Bax protein in HL60 cells before and after exposure to oridonin. (C) Western blotting results and (D) average relative expression of Bcl-2 protein in HL60 cells before after exposure to oridonin. (E) Western blotting results of caspase-3 or cleaved caspase-3 and caspase-9 or cleaved caspase-9 proteins in HL60 cells before and after exposure to oridonin. (F) Average relative expression of caspase-3. (G) Average relative expression of cleaved caspase-3 and (H) ratio of cleaved caspase-3/caspase-3. (I) Average relative expression of caspase-9 protein, (J) average relative expression of cleaved caspase-9 protein and (K) ratio of cleaved caspase-9/caspase-9 in HL60 cells before and after exposure to oridonin. (L) Western blotting results and (M) average relative expression of PDCD4 protein in HL60 cells before and after si-PDCD4 transfection. (N) Western blotting results and (O) average relative expression of Bax protein in HL60 cells before and after si-PDCD4 transfection. (P) Western blotting results and (Q) average relative expression of Bcl-2 protein in HL60 cells before and after si-PDCD4 transfection. n=3. *P<0.05 and **P<0.01 and ***P<0.001. ns, no significant; Ori, oridonin; PDCD4, programmed cell death protein 4; miRNA/miR, microRNA; NC, negative control; si, small interfering RNA; cas, caspase.

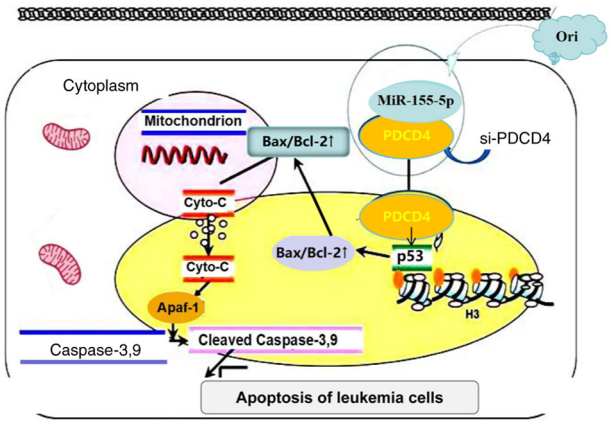


Figure 8. Schematic diagram of the molecular mechanism of oridonin targeted regulation of miR-155-5p/PDCD4 in apoptosis of HL60 cells. After comprehensive analysis of the experimental results of the present study, it was preliminarily demonstrated that the regulation of miR-155-5p/PDCD4 signaling axis contributed to the role of oridonin in inhibiting proliferation and inducing apoptosis of HL60 cells. Ori, oridonin; miRNA/miR, microRNA; PDCD4, programmed cell death protein 4; si, small interfering RNA; Apaf-1, apoptotic protease activating factor-1.

attention. For example, inhibition of the miR-200b-3p/zinc finger E-box-binding homeobox 1 axis by oridonin contributed to proliferation inhibition of human pancreatic cancer (42). Apoptosis induction of colorectal carcinoma cells by oridonin was associated with the downregulation of miR-32 (43). Oridonin inhibited the proliferation of human pancreatic cancer cells by modulating the profiles of certain miRNAs; for example, 105 miRNAs were significantly altered by oridonin treatment. Among them, both miR-424-5p and miR-17-5p were found to be downregulated by oridonin in the microarray data, implying that oridonin may inhibit pancreatic cancer cell proliferation, migration and invasion, and induce apoptosis by downregulating miR-424-5p and miR-17-5p (44). It also induced apoptosis of leukemia K562 cells by inhibiting the expression of miR-17 and miR-20a (45). Furthermore, oridonin exerts protective effects against hydrogen peroxide-induced damage by altering microRNA expression profiles in human dermal fibroblasts (46) and it could protect HaCaT keratinocytes against hydrogen peroxide-induced oxidative stress by altering miRNA expression (47). Whether the antioxidant stress effect is associated with the regulation of proliferation and apoptosis of tumor cells requires further investigation.

Generally, when using databases to predict the downstream genes of a certain miRNA, a large number of target genes are often obtained. Therefore, it is necessary to perform intersection analysis of different databases, or intersect them with experimentally detected differentially expressed genes in order to narrow down the selection range of candidate target genes. In addition, the target miRNA can also be selected directly based on the results reported in the literature such as the PubMed database. In the present study, 116 apoptosis-related miRNAs with significant expression changes were identified, including 83 upregulated and 33 downregulated miRNAs after exposure to oridonin in leukemia cells. According to the general principle of narrowing the range, the focus was on the top 10 miRNAs on the magnitude of the change. To further narrow down the range of candidate miRNAs, known oncogenic miRNAs reported on PubMed were investigated. Relatively consistent summary results of oncogenic miRNAs can be identified in multiple review articles. Mardani *et al* (48) or Wiśnik *et al* (49) reported that certain miRNAs promoted leukemogenesis or were oncogenic miRNAs, such as miR-223, miR-196b, miR-143-3, miR-125b, miR-93, miR-21, miR-17-92 and miR-15. In addition to these miRNAs, miR-155, miR-2, miR-10, miR-222 and miR-181 have also been reported as key oncogenic miRNAs (50). Lastly, based on the focus on oncogenic miRNAs, an intersection analysis between the top 10 microarray-screened miRNAs and the known leukemia-associated oncogenic miRNAs reported in existing literature was performed and the overlap analysis results indicated that miR-155-5p and miR-21-3p are preliminary candidate oncogenic miRNAs. Further verification was performed using RT-qPCR and the results demonstrated that only miR-155-5p expression in HL60 cells induced by oridonin was markedly reduced as compared with the control group. Therefore, miR-155-5p was selected as the candidate miRNA and miR-21-3p was excluded. In order to further explore the role of miR-155-5p in the inhibition of proliferation of leukemia cells by oridonin, HL60 cells were treated with oridonin *in vitro*. The results indicated that miR-155-5p expression was higher in primary APL cells compared with the normal PBMC group and the expression of miR-155-5p was significantly downregulated after treatment with oridonin. Compared with the mimics NC, the miR-155-5p inhibitor could promote apoptosis of HL60 cells treated with oridonin. At present, there is no study on the downregulation of miR-155-5p to induce apoptosis of leukemia cells by oridonin.

As miRNAs often serve physiological or pathological roles by targeting and regulating downstream target genes, after identification of miR-155-5p, it was also key to determine the downstream gene. A common approach is to analyze the database and predict the targets; however, it often leads to a large number of candidate target genes. The second approach is to identify the candidate gene through a literature review. In the present study, since miRNAs often negatively regulate the expression of downstream target genes miR-155-5p expression was decreased after treatment with oridonin, a tumor suppressor gene that is upregulated and could inhibit the proliferation of leukemia cells was investigated. The PubMed database literature review indicated that miR-155 could regulate PDCD4 expression in certain tumors. StarBase, miRDB and other database intersection analysis were further used and it was predicted that PDCD4 is a target of miR-155-5p. The TargetScan database predicted that there is a complementary sequence of miR-155-5p to bind the 3'-UTR region of PDCD4. Therefore, it was hypothesized that PDCD4 might be a possible a target gene of miR-155-5p.

PDCD4 is a tumor suppressor gene of concern in previous years (24,51,52). It has been demonstrated that PDCD4 could be modulated by certain miRNAs, for example miR-21 in breast cancer, oral cancer, hepatocellular carcinoma (HCC) and other types of cancer (53), miR-96 in glioma cells (54) and miR-21 or miR-499 in oropharyngeal cancer cells (55). Previously, it has been reported that miR-155 may also serve a role in regulating other target genes. Thomsen *et al* (56) revealed a downregulation of PDCD4 in miR-155-overexpressing

tumors, validated by immunohistochemistry, and that miR-155 directly downregulates PDCD4 expression in lung cancer cells; however, whether it directly targets the 3'-UTR of PDCD4 to exert its effects warrants further evidence (57). Zargar *et al* (58) demonstrated that miR-155 could directly target PDCD4 and modulate cell proliferation of tongue cancer cells. Xia and Zhao (59) reported that miR-155 expression was upregulated in patients with polycystic ovary syndrome, which promoted proliferation of human ovarian granulosa cells by targeting PDCD4 through activation of JNK or PI3K/AKT. Zadeh *et al* (60) confirmed that the anti-proliferation activity of the flavonoid compound silymarin against human breast cancer MCF-7 cells may be partially attributed to the downregulation of miR-21 and miR-155, and upregulation of their downstream gene PDCD4. This relationship between miR-155 and PDCD4 was also demonstrated in patients with HCC (61) and in natural killer lymphoma cells (62).

The results of the present study indicated that the expression of PDCD4 protein was significantly increased after treatment with oridonin. Compared with the mimics NC, miR-155-5p mimics could significantly reduce the expression of PDCD4. Next, TCGA database was used to predict the region and 3'-UTR sequence of PDCD4 for miR-155-5p to bind. The results of the luciferase assay demonstrated that miR-155-5p could target on the 3'-UTR of PDCD4. By contrast, during oridonin treatment, the transfection of miR-155-5p mimics could reduce the increase in oridonin-induced PDCD4 expression in HL60 cells and si-PDCD4 could reduce the upregulation of PDCD4 induced by oridonin compared with si-NC. These results first indicated that downregulation of miR-155-5p by oridonin could inhibit the proliferation of HL60 cells by upregulating the expression of PDCD4. Furthermore, it was reported that after treatment with oridonin, Bax and Bcl-2 expression levels were significantly upregulated and downregulated, respectively, and cleaved caspase-3 and -9 expression levels were significantly increased at the same time. Furthermore, compared with the si-NC group, when HL60 cells were transfected with si-PDCD4 after treatment with oridonin, PDCD4 and Bax expression was significantly downregulated, while Bcl-2 expression was significantly upregulated.

In conclusion, the expression level of miR-15-5p decreased when HL60 cells were induced by oridonin, accompanied by a decrease in the levels of cell proliferation. Second, the proliferation inhibitory effect oridonin on HL60 cells could be reversed with miR-155-5p overexpression, indicating that miR-155-5p mediates the proliferation inhibitory effect of oridonin on HL60 cells. Third, the upregulation of PDCD4 expression of HL60 cells induced by oridonin could be significantly increased by simultaneous transfection of miR-155-5p mimics and the proliferation level was restored. The targeted regulation of PDCD4 by miR-155-5p was demonstrated by luciferase assay and miR-155-5p was negatively associated with PDCD4, and compared with the oridonin experimental group, PDCD4 expression was significantly downregulated after simultaneous transfection of miR-155-5p mimics. Furthermore, apoptosis of HL60 cells induced by oridonin was associated with a decrease in miR-155-5p and increase in PDCD4 that was verified by miR-155-5p mimics and si-PDCD4 transfection, respectively. Besides, oridonin induced downregulation of miR-155-5p, resulting in an upregulation of PDCD4 expression, which led to the inversion of the Bcl-2/Bax ratio, which then activated cleaved caspase-3 and -9, and lastly led to the occurrence of apoptosis in HL60 cells. The clinical biological marker role of the miR-155-5p/PDCD4 signaling axis and its potential for targeted intervention therapy requires further research in the future. However, the present study was merely an *in vitro* one. The next step requires *in vivo* animal experiments to confirm the role of this signaling axis. Meanwhile, retrospective and prospective clinical studies can be conducted to observe the potential of this signaling axis as a biological indicator for clinical prognosis assessment.

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Availability of data and materials

The data generated in the present study may be found in the National Genomics Data Center under accession number OMIX017071 or at the following URL: https://ngdc.cncb.ac.cn/omix/release/OMIX017071.

Authors' contributions

KB, GJ and HD made substantial contributions to the conception and design and also critically reviewed the present study. MF, QY, CW, XW and YW performed the experiments. CW and YW analyzed the data and wrote the manuscript. KB and GJ confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Zhangqiu District People's Hospital of Jinan (approval no. 2024001; Jinan, China). Oral informed consent was obtained from both patients and healthy volunteers for participation.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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