RESEARCH ARTICLE

DOI: 10.4274/tjh.galenos.2023.2022.0484
Turk J Hematol 2023;40:154-161

# Circ_0001946 Promotes the Development of Acute Myeloid Leukemia by Upregulating PDL1

## Circ_0001946 PDL1'in Düzenlenmesini Artırarak Akut Myeloid Lösemi Gelişimini Teşvik Eder

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## Abstract

**Objective:** Circ_0001946 has been identified as an oncogenic factor, and the aim of this study was to explore the detailed roles and putative targets of circ_0001946 in acute myeloid leukemia (AML).

**Materials and Methods:** Levels of circ_0001946 were examined in AML tissues and cells. Furthermore, the regulatory functions of circ_0001946 in AML were explored. The expression of circ_0001946 was evaluated in AML samples and a matched para-carcinoma control, as well as in AML cell lines and a human bone marrow stromal cell line using reverse transcription-quantitative polymerase chain reaction. Cell proliferation was examined using a CCK-8 kit, and migration/invasion was measured by transwell assay. Furthermore, interactions between associated molecules were assessed using RNA pulldown, and the mRNA stability of the relevant gene was examined by mRNA stability assay.

**Results:** Our data indicated that circ_0001946 was upregulated in AML specimens/cells. Additionally, overexpression of circ_0001946 promoted the proliferation, migration, and invasion of AML cells and, vice versa, these biological processes were suppressed by knockdown of circ_0001946. Furthermore, PDL1 is a potential downstream molecule of circ_0001946 in AML and its stability was improved by circ_0001946. The expression of PDL1 was increased in AML specimens and positively correlated with circ_0001946 expression. Moreover, biological behavioral alterations in AML cells induced by oe-circ_0001946 were abrogated by sh-PDL1 and the effects of sh-circ_0001946 were enhanced by treatment with sh-PDL1.

**Conclusion:** Taken together, these data suggest that levels of circ_0001946 are elevated in AML and that circ_0001946 could promote the growth of AML cells. Furthermore, PDL1 is a novel downstream molecule of circ_0001946 in AML. Circ_0001946/PDL1 signaling may play crucial roles in tumor progression in AML and could be a novel candidate for targeted treatments for AML patients.

**Keywords:** Circ_0001946, PDL1, Acute myeloid leukemia

## Öz

**Amaç:** Circ_0001946 onkojenik bir faktör olarak tanımlanmıştır ve bu çalışmanın amacı circ_0001946'nın akut myeloid lösemideki (AML) ayrıntılı rollerini ve varsayılan hedeflerini araştırmaktır.

**Gereç ve Yöntemler:** AML doku ve hücrelerinde circ_0001946 düzeyleri incelenmiştir. Ayrıca, circ_0001946'nın AML'deki düzenleyici işlevleri araştırılmıştır. Circ_0001946 ekspresyonu AML örneklerinde ve eşleşen bir para-karsinom kontrolünde, ayrıca AML hücre hatlarında ve bir insan kemik iliği stromal hücre hattında ters transkripsiyon-kantitatif polimeraz zincir reaksiyonu kullanılarak değerlendirilmiştir. Hücre proliferasyonu CCK-8 kiti kullanılarak incelenmiş ve migrasyon/invazyon trans kuyu göç analizi ile ölçülmüştür. Ayrıca, ilişkili moleküller arasındaki etkileşimler RNA çekme analizi yapılarak değerlendirilmiş ve ilgili genin mRNA stabilitesi mRNA stabilite yöntemi ile incelenmiştir.

**Bulgular:** Verilerimiz circ_0001946'nın AML örneklerinde/hücrelerinde düzenlenmesinde artış olduğunu göstermiştir. Ek olarak, circ_0001946'nın aşırı ifadesi AML hücrelerinin proliferasyonunu, göçünü ve invazyonunu sağlarken bunun tersi olarak, bu biyolojik süreçler circ_0001946'nın susturulması ile baskılanmıştır. Ayrıca, PDL1 AML'de circ_0001946'nın potansiyel bir aşağı akış molekülüdür ve stabilitesi circ_0001946 tarafından geliştirilmiştir. PDL1 ifadesi AML örneklerinde artmış ve circ_0001946 ifadesi ile pozitif korelasyon göstermiştir. Ayrıca, oe-circ_0001946 tarafından indüklenen AML hücrelerindeki biyolojik davranış değişiklikleri sh-PDL1 ile ortadan kaldırılmış ve sh-circ_0001946'nın etkileri sh-PDL1 ile tedavi edilerek artırılmıştır.

**Sonuç:** Birlikte ele alındığında, bu veriler AML'de circ_0001946 seviyelerinin yükseldiğini ve circ_0001946'nın AML hücrelerinin büyümesini destekleyebileceğini göstermektedir. Ayrıca PDL1, AML'de circ_0001946'nın yeni bir aşağı akış molekülüdür. Circ_0001946/PDL1 sinyali AML'de tümör ilerlemesinde önemli roller oynayabilir ve AML hastaları için hedefe yönelik tedavilerde yeni bir aday olabilir.

**Anahtar Sözcükler:** Circ_0001946, PDL1, Akut myeloid lösemi

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**Received/Geliş tarihi:** November 10, 2022
**Accepted/Kabul tarihi:** June 26, 2023

## Introduction

Acute myeloid leukemia (AML) is a type of cancer of the myeloid line of blood cells [1]. It progresses rapidly and is typically fatal within weeks or months if left untreated [2]. Diagnosis is generally based on specific blood tests and bone marrow aspiration [3]. The main treatment for AML is chemotherapy, with the aim of inducing remission. Patients may also receive additional chemotherapy, radiation therapy, or stem cell transplantations [4]. New therapies including hypomethylating agents and allogeneic hematopoietic cell transplantation are promising, too. AML resulted in 147,000 deaths worldwide in 2015 [4]. It commonly occurs in older adults, and men are affected more often than women [5]. The prognosis of AML patients varies greatly, being dependent on various factors such as age and physical fitness, whether the disease is of low or high risk, and whether the cancer is primary or secondary [5]. Therefore, it is necessary to identify novel biomarkers for AML, and the present study was conducted with the aim of developing targeted treatment approaches for AML patients.

CircRNAs are a type of non-coding RNA containing a continuous loop; thus, they are more stable compared to linear RNAs [6]. Due to the absence of free 5′ and 3′ overhangs on their structures, circRNAs cannot be degraded by exonuclease [6,7]. Recent studies have focused on the biological functions of circRNAs and their underlying mechanisms. CircRNAs are able to exert functions as novel gene regulators, but the detailed molecular mechanisms are still unclear [6,7,8,9]. A previous study revealed the novel roles of circRNAs as "sponges" of miRNA, competitively suppressing the activity of target miRNAs [10]. CircRNAs serve essential roles in the onset and development of numerous diseases including cancer [11,12,13,14]. For instance, circRNA ACVR2A was able to inhibit bladder cancer cell growth by targeting miR-626/EYA4 signaling [15]. Additionally, circSATB2 enhanced the progression of non-small cell lung cancer cells in vitro [16]. CircERBB2 may promote the development of gallbladder cancer by targeting PA2G4-dependent rDNA transcription [17], while circ0005276 induces the growth of prostate cancer cells by interfering with FUS and activating XIAP at the transcription level [18]. Furthermore, circPICALM could sponge miR-1265 and suppress bladder cancer metastasis, consequently affecting the phosphorylation of FAK [19]. Among these circRNAs, circ_0001946 is particularly well studied and it functions as an oncogenic regulator; the dysregulation of circ_0001946 was detected in numerous types of cancer, associated with tumor progression through its effect of cancer cell growth [20,21,22, 23,24,25,26,27,28,29]. However, the full roles and downstream signaling of most circRNAs including circ_0001946 in AML are not completely understood. PDL1 is a novel oncogenic factor and it is well established that PDL1 is a downstream molecule of circ_0001946 [30]; inhibition of PDL1 is considered a promising therapy option for cancer treatment [31,32,33].

In this study, circ_0001946 was identified as an oncogenic factor and the aim was to explore the detailed roles and putative targets of circ_0001946 in AML. Upregulation of circ_0001946 was detected in AML specimens/cells. Additionally, the proliferation, migration, and invasion of AML cells were promoted by transfection with oe-circ_0001946 and inhibited by sh-circ_0001946, respectively. Moreover, PDL1 is a promising downstream molecule of circ_0001946 and it is involved in circ_0001946-modulated biological behavioral changes in AML cells. Circ_0001946/PDL1 signaling is associated with tumor progression via its regulation of the growth of AML cells, and this novel pathway could be a putative target for the development of AML treatments.

## Materials and Methods

### Clinical Samples

Fifty AML patients and 50 healthy controls were enrolled in this study. Bone marrow samples were collected from Shaanxi Provincial Cancer Hospital from August 2010 to September 2015. All healthy controls had no history of AML and their bone marrow aspiration results were normal. All enrolled patients had no previous history of AML or other malignancies. Patients with other clinical disorders including metabolic disorders, chronic diseases, and severe infections were excluded. Our study was approved by the hospital's ethics committee (approval no. 2010-R006), and all patients and controls provided written informed consent.

### Cell Culture

Bone marrow samples were collected from all patients and healthy controls by biopsy and used to isolate bone marrow-derived mononuclear cells (BMMNCs) using lymphocyte separation medium (TBD Science, Tian Jin Hao Yang Biological Manufacture Co., Ltd., China). Briefly, 2 mL of bone marrow mixed with the aforementioned medium was centrifuged at 400xg for 15 min. The second layer of the supernatant, containing the lymphocytes, was then used for cell culture. The Beckman MoFlo Astrios high-performance live-cell sorting system (Beckman Coulter, Inc., USA) was used to isolate BMMNC subpopulations as previously described [34]. PE-conjugated mouse anti-human CXCR4 (Cat. No. 60089PE.1; Stemcell Technologies, Inc., Canada) and APC-conjugated rat anti-human CD45 (Cat. No. 28145-1; Signalway Antibody LLC, USA) were used to isolate the BMMNCs [34]. The cells were resuspended in minimal essential medium with Earle's salts (Gibco, Thermo Fisher Scientific, Inc., USA) containing 10% fetal calf serum and 1% pen/strep (Gibco, Thermo Fisher Scientific, Inc., USA), and $1x10^7$ resuspended bone marrow cells were seeded into 100-mm cell culture dishes and incubated at 37 °C in a humidified incubator with 5% $CO_2$. Human AML cells (KG-1a, THP-1, K562, and U937) and human bone marrow stromal cell line HS-5 were obtained from the

American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI-1640 medium supplemented with 10% FBS, 100 µg/mL streptomycin, and 100 U/mL penicillin (all purchased from GE Healthcare Life Sciences, USA). Cells were maintained in a humid incubator supplied with 5% $CO_2$ and held at 37 °C.

### Cell Transfection

In order to produce the knockdown model of circ_0001946 and *PDL1*, shRNAs against circ_0001946 (sh-circ_0001946), PDL1 (sh-PDL1), and a negative control (sh-NC) were obtained from Genepharm Co. Ltd. (China). An annealed shRNA segment was inserted into the pU6-Luc-Puro lentivirus vector (Genepharm Co. Ltd., Greece). To generate the cell model with circ_0001946 overexpression, a wild-type (oe-circ_0001946)/mutant (oe-NC) sequence was amplified by PCR and subsequently cloned into the PLCDH-cir vector (Invitrogen, Thermo Fisher Scientific, Inc., USA). Up- and downregulation of the associated genes were evaluated by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Cell transfection was carried out with Lipofectamine®2000 (Invitrogen, Thermo Fisher Scientific, Inc., USA). After 12 h, culture media were replenished with DMEM containing 10% FBS.

### RT-qPCR

RNA was extracted from cells using TRIzol reagent (Invitrogen, Thermo Fisher Scientific, Inc., USA). Isolated RNA was subjected to reverse transcription using a PrimeScript RT kit (Takara Biotechnology Co., Ltd., China). Real-time PCR was then performed using SYBR Green PCR Master Mix (Takara Biotechnology Co., Ltd., China), and PCR reactions were carried out on an ABI 7500 system (Thermo Fisher Scientific, Inc., USA). The expression of GAPDH was used as a control for normalization. The forward and reverse primer pairs for real-time PCR were as follows: circ_0001946, 5′-CCACGTCTTCCCAACAATCC-3′ and 5′-GACCTGGAGGCCATTGGAAG-3′; *PDL1*, 5′-AAACAATTAGACCT GGCTG-3′ and 5′-TCTTACCACTCAGGAC TTG-3′; *GAPDH*, 5′-CATGGCCTTCCGTGTTCCTA-3′ and 5′-TACTTGGCAGGTTTCTCC AGG-3′. The program used for qPCR was as follows: 95 °C for 5 min, and 45 cycles of 95 °C for 15 s, 60 °C for 20 s, and 72 °C for 10 s.

### Immunofluorescence Staining

Cells were fixed using ice-cold acetone (Sigma Aldrich, UK) for 15 min. Following fixation, cells were rinsed with PBS and subsequently incubated with blocking solution for 30 min and then with primary Ki67 antibody (1:200; Cat. No. 9129, Cell Signaling Technology, USA) overnight in a cold room. The next day, cells were rinsed and incubated with Alexa-Fluor 568-labeled secondary antibody (1:1000; Molecular Probes, USA) for 1 h in the dark. The secondary antibody alone was used as a negative control. Cell nuclei were stained using DAPI reagent (Vector Laboratories, UK). Subsequently, cells were rinsed and mounted onto glass slides using Mowiol reagent with 10% Mowiol D488 (Calbiochem, UK) and stored in a freezer. Staining was visualized with a Leica DMLB microscope. Cell images were acquired using a CCD camera (Cool-SNAP-Pro, Media Cybernetics, USA) with Image-Pro Plus software version 6.0 (Media Cybernetics, USA).

### Western Blotting

Proteins were extracted with RIPA lysis buffer (Beyotime Institute of Biotechnology, China). Protein concentrations were measured using a BCA kit (Beyotime Institute of Biotechnology, China). The same amounts of protein samples (~30 µg) were subjected to 12% SDS-PAGE and samples were transferred to PVDF membranes (EMD Millipore, USA). Subsequently, the membranes were blocked in PBS supplemented with 5% skimmed milk for 1 h and incubated with primary PDL1 antibody (1:2000; Cat. No. 13684, Cell Signaling, USA) or GAPDH antibody (1:1,000; Cat. No. sc-32233, Santa Cruz Biotechnology Inc., USA) at 4 °C overnight. The following day, membranes were incubated with HRP-labeled anti-mouse (1:5,000; Cat. No. 7076, Cell Signaling, USA) or anti-rabbit IgG (1:5,000; Cat. No. 7074, Cell Signaling, USA) for 1 h. Blots were visualized with an ECL kit (Pierce Biotechnology, Thermo Fisher Scientific, Inc., USA). Protein bands were analyzed with ImageJ software (National Institutes of Health, USA).

### CCK-8 Assay

Cells were inoculated on 96-well plates at a density of $5x10^4$ cells/well. Cell proliferation was then examined on days 1, 2, 3, and 4. In brief, 10 µL of CCK-8 reagent (Dojindo Molecular Technologies, Inc., Japan) was added to the cells. Cells were incubated for another 2 h and then absorbance at 450 nm was measured using a microplate reader (Bio-Rad Laboratories, Inc., USA).

### Transwell Migration/Invasion Assay

Migration and invasion of the cells were examined using a transwell assay. For cell migration, $3x10^5$ cells were resuspended in culture media without FBS and seeded in an upper chamber with pore size of 8 µm (BD Biosciences, USA). For cell invasion, cells were inoculated onto Matrigel (Sigma-Aldrich, USA) in a precoated upper chamber and 500 µL of DMEM with 10% FBS was added to the lower chamber. After 2 days of incubation, non-migratory/non-invasive cells were scratched with cotton swabs. Cells in the lower chamber were fixed with methanol for 10 min and stained using crystal violet (0.5%). Cells were then counted in five randomly selected fields under an inverted light microscope (magnification $100^x$; Olympus Corporation, Japan).

### RNA Pulldown

The biotin-labeled probe of PDL1 and the negative control were obtained from GenePharma (China). Cell lysates were labeled using Dynabeads M-280 Streptavidin (Thermo Fisher Scientific,

Inc., USA). Beads with immobilized PDL1 were then treated with 10 mM ethylenediaminetetraacetic acid. Bound RNAs were extracted using TRIzol reagent and amplified by RT-qPCR.

### Measurement of mRNA Stability

Cells were inoculated on 6-well plates and left in the incubator overnight. In order to suppress further synthesis of RNA, cells were treated using actinomycin D (5 μg/mL; MedChemExpress, USA) and then further treated at different time intervals in the presence of actinomycin D. The RNA was extracted and subjected to RT-qPCR. The remaining RNA level of *PDL1* was normalized at every time point according to the level at the beginning.

### Immunostaining of PDL1

Staining of PDL1 was performed on paraffin-embedded tissues. Samples were dewaxed in xylene and rehydrated using graded ethanol/water series. Antigen retrieval was conducted by microwave treatment using sodium citrate (10 mM) for 5 min three times. Subsequently, biopsy samples were incubated using FBS for 30 min followed by primary PDL1 antibody (1:200; Cat. No. 13684, Cell Signaling, USA) in a humid chamber in a cold room overnight. The next day, samples were washed using PBS and incubated in biotin-labeled secondary antibody (1:100; Dako, UK). The antigen was then detected with a streptavidin-biotin-peroxidase system (ABC Kit; Dako, UK). The intensity of staining was analyzed with ImageJ software version 1.48 (National Institutes of Health, USA).

### Statistical Analysis

Data were presented as means ± standard deviations and data analysis was performed using IBM SPSS Statistics 25.0 (IBM Corp., USA). The significance of differences was evaluated by Student's t-test or one-way analysis of variance (ANOVA). The post hoc Tukey test was performed after ANOVA. Associations within RNA expressions were studied by Pearson's correlation analysis. Values of $p<0.05$ were accepted as indicating statistically significant differences. Receiver operating characteristic (ROC) curve analysis was conducted to evaluate the power of circ_0001946 levels in differentiating AML versus non-cancerous samples and patients with relapse (RE) versus those with complete remission (CR).

## Results

### Upregulation of Circ_0001946 in AML Samples

The levels of circ_0001946 were determined in 50 AML specimens and healthy samples by RT-qPCR. The expression of circ_0001946 was notably increased in AML samples compared to the control group (Figure 1A). Moreover, the expression of circ_0001946 was remarkably elevated in AML cases with relapse in comparison to individuals with CR (Figure 1B). Furthermore, analyses of ROC curves indicated that circ_0001946 expression had high values of area under the curve in differentiating between AML and non-cancerous samples and between patients with relapse and those with CR (Figures 1C and 1D). The clinical features of the patients are summarized in Table 1. The expression of circ_0001946 showed no significant differences among different ages, genders, and AML subtypes. Overall, the expression of circ_0001946 was increased in AML tissues, which may lead to tumor progression in AML.

Overexpression of circ_0001946 enhanced the proliferation, migration, and invasion of AML in vitro while these biological processes were suppressed by knockdown of circ_0001946. Furthermore, upregulation of circ_0001946 was also detected in AML cells in comparison with normal HS-5 cells (Figure 2A). In KG-1a and U397 cells, circ_0001946 was respectively least and most upregulated. Therefore, these two cell lines were used for further function studies. For that purpose, cell models with circ_0001946 overexpression and knockdown were generated. Transfection efficiencies were confirmed by RT-qPCR (Figures 2B and 2C). Data from CCK-8 assays revealed that the proliferation of AML cells was enhanced following treatment with oe-circ_0001946 and was suppressed by sh-circ_0001946, respectively (Figures 2D and 2E). In addition, the expression levels of cell proliferation marker Ki67 were increased in cells transfected with oe-circ_0001946 and reduced in cells treated with sh-circ_0001946 (Figures 2F-2I). Transwell assays further indicated that AML cell migration and invasion were enhanced by treatment with oe-circ_0001946 and suppressed by sh-circ_0001946, respectively (Figure 3). These findings suggest

**Table 1. Clinical features of the patients.**

| Parameter | Number of patients | Low circ_0001946 expression (n=25) | High circ_0001946 expression (n=25) | p |
|---|---|---|---|---|
| Age (years) | | | | |
| ≤40 | 24 | 13 | 11 | 0.571 |
| >40 | 26 | 12 | 14 | |
| Gender | | | | |
| Female | 25 | 12 | 13 | 0.777 |
| Male | 25 | 13 | 12 | |
| FAB subtype | | | | |
| M1 | 8 | 4 | 4 | 0.780 |
| M2 | 8 | 3 | 5 | |
| M3 | 16 | 9 | 7 | |
| M4 | 10 | 6 | 4 | |
| M5 | 8 | 3 | 5 | |
| Prognosis | | | | |
| Complete remission | 18 | 13 | 5 | 0.018 |
| Relapse | 32 | 12 | 20 | |

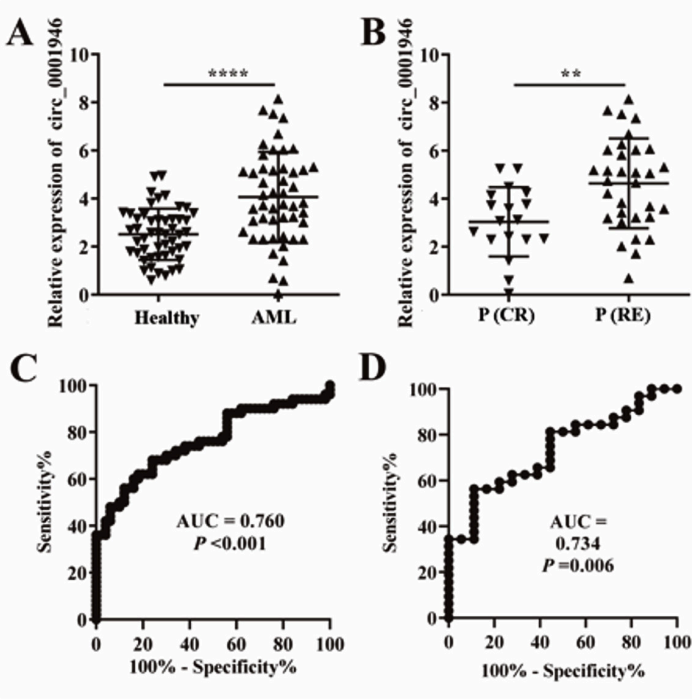


**Figure 1.** Levels of circ_0001946 were elevated in acute myeloid leukemia (AML) specimens: A) The expression of circ_0001946 was evaluated in 50 AML samples and paired healthy specimens. B) Circ_0001946 expression was examined in patients (P) with complete remission (CR) and those with relapse (RE). C, D) Receiver operating characteristic curves were used for analysis and the results indicated that the expression profile of circ_0001946 exhibited high values of area under the curve for distinguishing between AML and normal specimens and between AML cases with relapse and those with complete remission. **: p<0.01; ****: p<0.0001.

that overexpressed circ_0001946 could promote the growth of AML cells, which may be suppressed by the knockdown of circ_0001946.

### *PDL1*, Novel Downstream Molecule of Circ_0001946 in AML, Is Stabilized by Circ_0001946

In order to discover the potential downstream targets of circ_0001946 in AML, further function experiments were carried out. The interaction between circ_0001946 and *PDL1* in AML cells was revealed by RNA pulldown assay (Figure 4A). In addition, the mRNA stability of *PDL1* was increased in AML cells treated with oe-circ_0001946, but it was reduced following transfection with sh-circ_0001946 (Figures 4B and 4C). RT-qPCR further indicated that the levels of *PDL1* were elevated in AML specimens (Figure 4D). A positive correlation between circ_0001946 and *PDL1* was revealed by Pearson's correlation test (Figure 4E). Protein levels of PDL1 were upregulated or downregulated in AML cells treated with oe-circ_0001946 or sh-circ_0001946, respectively (Figures 4F and 4G). Our findings indicated that circ_0001946 could stabilize the mRNA of *PDL1* in AML.

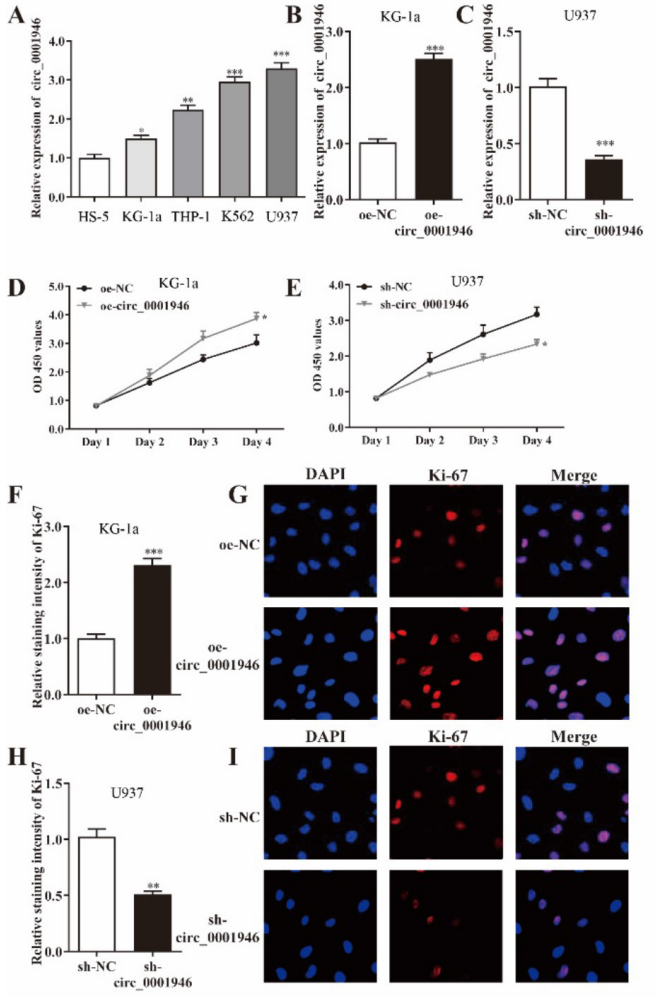


**Figure 2.** Overexpressed circ_0001946 promoted the growth of acute myeloid leukemia (AML) cells, which was inhibited by silenced circ_0001946: A) Upregulation of circ_0001946 was revealed in AML cells. B, C) Transfection efficiencies of o/e-circ_0001946 and sh-circ_0001946 were confirmed using RT-qPCR. D, E) Cell proliferation was determined after treatment with oe-circ_0001946 and sh-circ_0001946. F-I) Expression levels of Ki67 were examined in AML cells treated with oe-circ_0001946 and sh-circ_0001946. *: p<0.05; **: p<0.01; ***:p<0.001; NC: negative control.

### Biological Behavioral Changes in AML Cells Caused by oe-circ_0001946 or sh-circ_0001946 Respectively Reversed or Strengthened by sh-PDL1

The proliferation of AML cells was enhanced following treatment with oe-circ_0001946 and abolished by sh-PDL1 (Figure 5A). On the other hand, proliferation was suppressed in cells transfected with sh-circ_0001946, which was reversed by sh-PDL1 (Figure 5B). Migration and invasion of AML cells were promoted after transfection with oe-circ_0001946, and these effects were abrogated by sh-PDL1 (Figures 5C and 5E). On the contrary, AML cell migration and invasion were inhibited following transfection with sh-circ_0001946, and they were rescued by sh-PDL1 (Figures 5D and 5F).

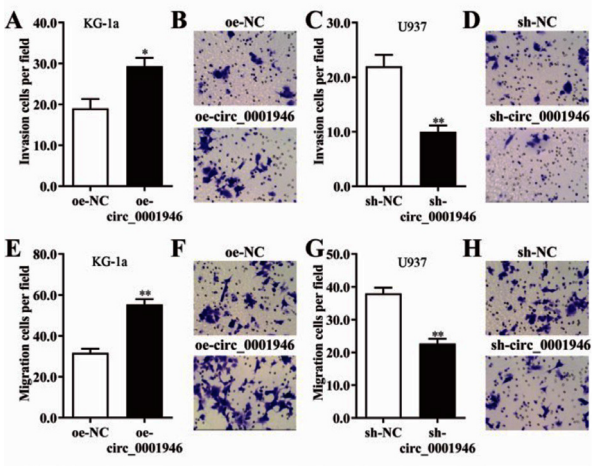


**Figure 3.** Acute myeloid leukemia (AML) cell migration and invasion were promoted by oe-circ_0001946 and suppressed by sh-circ_0001946, respectively: A-D) The invasion of AML cells was enhanced following treatment with oe-circ_0001946 and inhibited by sh-circ_0001946. E-H) The migration of AML cells was promoted by oe-circ_0001946 and suppressed by sh-circ_0001946, respectively. *: p<0.05; **: p<0.01; ***: p<0.001; NC: negative control.

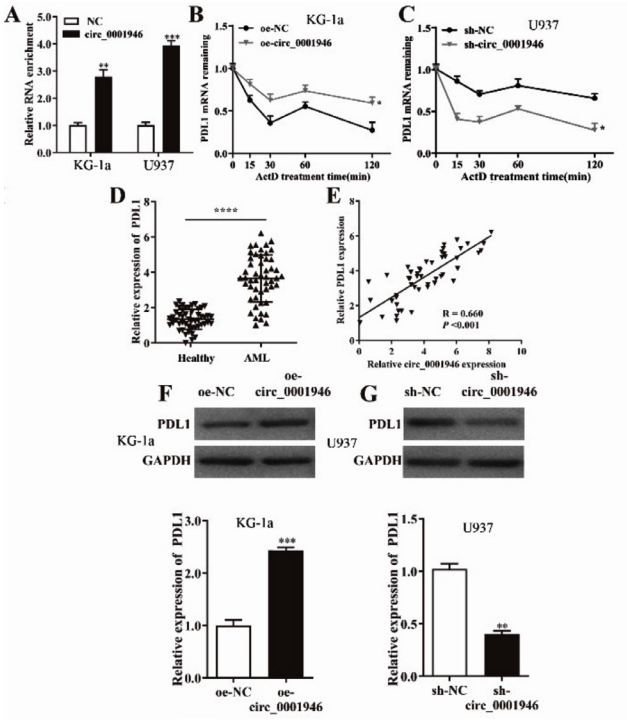


**Figure 4.** *PDL1* is a putative downstream molecule of circ_0001946 and is upregulated in acute myeloid leukemia (AML): A) The interaction between circ_0001946 and *PDL1* in AML cells was revealed by RNA pulldown assay. B, C) The stability of *PDL1* mRNA was enhanced by oe-circ_0001946 but decreased by sh-circ_0001946. D) *PDL1* was upregulated in AML samples compared to the normal control. E) The expressions of circ_0001946 and PDL1 were positively correlated in AML samples. F, G) Protein levels of *PDL1* were examined in AML cells treated with oe-circ_0001946 or sh-circ_0001946. *: p<0.05; **: p<0.01; ***: p<0.001; ****: p<0.0001; NC: negative control.

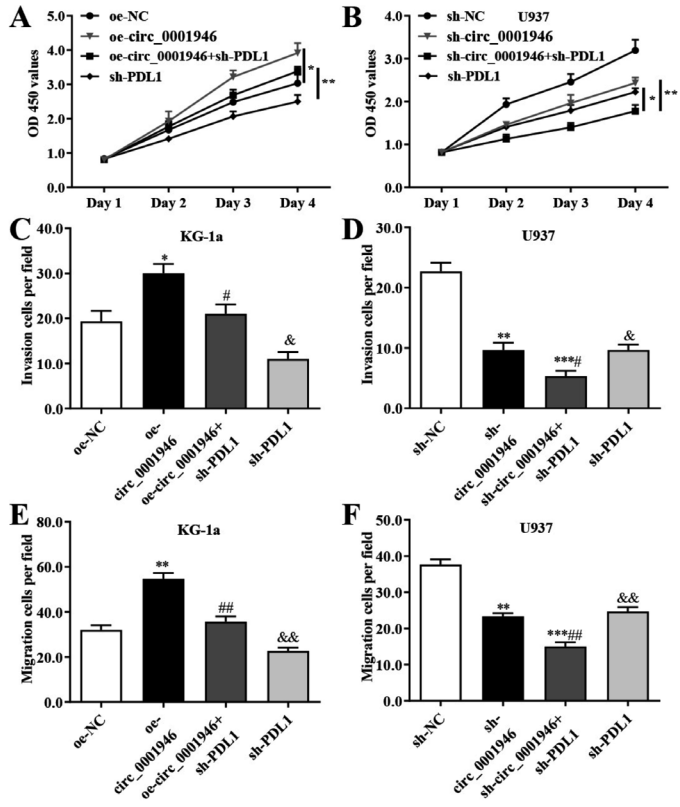


**Figure 5.** Acute myeloid leukemia (AML) cell proliferation, migration, and invasion were regulated by the circ_0001946/PDL1 axis: A, B) Cell proliferation was determined after transfection with oe-circ_0001946/sh-circ_0001946 or co-transfection with oe-circ_0001946+sh-PDL1/sh-circ_0001946+sh-PDL1. C, D) Invasion of AML cells was examined in cells treated with oe-circ_0001946/sh-circ_0001946 or co-treated with oe-circ_0001946+sh-PDL1/sh-circ_0001946+sh-PDL1. E, F) Cell migration was evaluated following treatment with oe-circ_0001946/sh-circ_0001946 or co-transfection with oe-circ_0001946+sh-PDL1/sh-circ_0001946+sh-PDL1. *: p<0.05; **: p<0.01; ***: p<0.001 vs. oe-NC/sh-NC; #: p<0.05; ##: p<0.01 vs. oe-circ_0001946/sh-circ_0001946; NC: negative control.

## Discussion

Upregulation of circ_0001946 has been found in numerous types of cancer including glioma and lung cancer. Thus, circ_0001946 is a novel oncogenic factor in tumor progression [20,21,22,23, 24,25,26,27,28,29]. Our data, consistent with previous findings, revealed the upregulation of circ_0001946 in AML cells. Furthermore, the overexpression of circ_0001946 enhanced AML cell growth in vitro while the proliferation, migration, and invasion of AML cells were inhibited by silenced circ_0001946. Similarly, recent studies have suggested that circRNAs such as circSATB2, circERBB2, circ0005276, and circPICALM could promote tumor progression in numerous types of malignancies [16,17,18,19].

In our study, RNA pulldown assays suggested interaction between circ_0001946 and *PDL1*, which is a novel downstream molecule of circ_0001946. *PDL1* is oncogenic factor associated with tumor progression [31,32,33]. In addition, our data indicated that the mRNA stability of *PDL1* was also enhanced by circ_0001946. Previous studies emphasized the importance of mRNA stability in the pathogenesis of cancer [35,36,37]. For instance, calreticulin is able to modulate the mRNA stability of vascular endothelial growth factor-A in gastric cancer cells [36]. Moreover, the expression of *PDL1* was increased in AML specimens, where the levels of circ_0001946 and *PDL1* were positively correlated. *PDL1* is also involved in circ_0001946-regulated biological behavioral changes of AML cells. Thus, overall, circ_0001946/PDL1 signaling is involved in tumor progression by modulating the growth of AML cells and this novel pathway may be a promising target for the treatment of AML. However, there are some limitations to the present study. For example, the expression of EMT-associated genes were not examined; this should be considered in future work. Future in vivo studies should also be performed to confirm the present findings.

## Conclusion

This study has provided essential evidence on the functions of circ_0001946 in tumor development in AML. Our findings revealed the upregulation of circ_0001946 in AML, and circ_0001946 serves as a putative oncogenic factor and could enhance the proliferation, migration, and invasion of AML cells, consequently leading to tumor progression. Furthermore, PDL1 is a novel downstream molecule of circ_0001946, which is a key regulator in circ_0001946-modulated AML cell growth. More importantly, as the circ_0001946/PDL1 axis could regulate the development of tumors in cases of AML, it is a promising candidate for the targeted treatment of this disease.

### Ethics

**Ethics Committee Approval:** Our study was approved by the Ethics Committee of Shaanxi Provincial Cancer Hospital (approval no: 2010-R006).

**Informed Consent:** All patients and controls provided written informed consent.

### Authorship Contributions

Data Collection or Processing- G.L., C.Z.,D.Q.; Writing- G.L., C.Z., D.Q., R.C.

**Conflict of Interest:** No conflict of interest was declared by the authors.

**Financial Disclosure:** The authors declared that this study received no financial support.

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