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Down-regulation of miR-29a facilitates apoptosis of colorectal carcinoma cell SW480 and suppresses its Paclitaxel resistance

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Abstract. – OBJECTIVE: PTEN can suppress PI3K/AKT activity, and regulate cell proliferation, apoptosis, and drug resistance. The previous study showed that up-regulation of miR-29a played an essential role in the occurrence of colorectal carcinoma. This study aimed to investigate the role and related mechanism of miR-29a in mediating Paclitaxel sensitivity of colorectal carcinoma cells.

MATERIALS AND METHODS: Bioinformatics analysis was performed to study the existence of binding sites between miR-29a and targeting gene mRNA. Dual luciferase reporter assay was conducted to validate the targeted regulation. The expressions were compared between CCD841 CoN cells, colorectal carcinoma cell line SW480, and drug-resistant cell line SW480/Paclitaxel. Cell apoptosis and proliferation were measured by flow cytometry. *In vitro* cultured SW480/Paclitaxel cells were transfected with miR-29a or pcDNA3.1-PTEN. MiR-29a and PTEN expressions were measured by quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR) and Western blot, followed by flow cytometry on the detection of cell apoptosis as well as proliferation assay.

RESULTS: A targeted regulatory relationship existed between miR-29a and PTEN. Comparing to CCD841 CoN cells, high level of miR-29a and decreasing expression of PTEN were found in SW480 cells. Moreover, further higher miR-29a and lower PTEN expressions were observed in SW480/Paclitaxel cells. Paclitaxel remarkably inhibited proliferation and facilitated apoptosis of SW480 cells but not SW480/Paclitaxel cells. Transfection of miR-29a inhibitor or pcDNA3.1-PTEN remarkably elevated PTEN expression, suppressed *p*-AKT expression, weakened proliferation, and enhanced apoptosis of SW480/Paclitaxel cells.

CONCLUSIONS: Our data demonstrated that suppression of miR-29a enhanced PTEN expression, inhibited cancer cell proliferation, facilitated apoptosis, and weakened drug resistance, which provides academic basis for the treatment of colorectal cancer.

Key Words
MicroRNA-29a, PTEN, PI3K/AKT, Paclitaxel, Colorectal carcinoma, Drug resistance.

Introduction

Colorectal carcinoma (CRC) is a type of common malignant tumor in the digestive tract and ranks as the third popular malignant tumor[1]. CRC frequently occurred in the population aged between 40 and 50 years old, with the ratio of male to female at around 2:1-3:1. In recent years, increasing numbers of people in China are diagnosed with CRC, which thus causes severe threaten for the public health[2]. CRC is featured as an insidious onset, high frequency of metastasis, and unfavorable prognosis. Therefore, the investigation of abnormal mechanistic change in CRC pathogenesis is of critical importance for early diagnosis and individualized treatment.

Phosphatase and tensin homologue deleted on chromosome ten (PTEN)/phosphatidylinositol-3 kinase (PI3K)/AKT/protein kinase B (PKB) signal pathway exerts as a tumor suppressor towards multiple tumors[3]. Previous studies showed that the down-regulation of PTEN is involved in occurrence, progression[4], invasion[5], and drug resistance[6-8] of CRC, indicating the tumor suppressor role of PTEN in CRC. MicroRNA (miR) is a newly discovered type of non-coding single-stranded RNA with 22-25 nucleotides, and

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can negatively regulate more than one-third of human gene expressions via targeted degradation or suppression of mRNA translation of target genes. It participates in the regulation of multiple biological processes including tissue/organ development, cell proliferation, apoptosis, and differentiation. The role of abnormal expression or dysfunction of miRNAs in tumor pathogenesis, progression, and drug resistance has drawn increasing amounts of research interests[9]. Based on differential target genes, miR-29a may play dual roles for induction or suppression of tumor. For example, aberrant elevation of miR-29a in breast cancer is correlated with tumor's distal metastasis and prognosis[10] and can enhance drug resistance of breast cancer[11]. In gastric cancer, however, miR-29a played a significant anti-tumor role via abnormal upregulation[12,13]. This study aimed to determine the effect of miR-29a in proliferation, apoptosis, and drug resistance of CRC cells.

Materials and Methods

Reagent and Equipment

Human normal colorectal epithelial cell line CCD841 CoN (CRL-1790) and CRC cell line SW480 were purchased from ATCC. HEK293T cell was kept in our own lab. Roswell Park Memorial Institute-1640 (RPMI-1640) culture medium and Opti-MEM were bought from Gibco (Thermo Fisher Scientific, Waltham, MA, USA). Penicillin-streptomycin was collected from CellGro (Thermo Fisher Scientific, Waltham, MA, USA). Fetal bovine serum (FBS) was obtained from Hyclone (Thermo Fisher Scientific, Waltham, MA, USA). TRIzol and Lip 2000 were acquired from Invitrogen (Thermo Fisher Scientific, Waltham, MA, USA). PrimerScript™ RT reagent kit was purchased from TaKaRa (Kusatsu, Otsu, Shiga, Japan). Annexin V/PI apoptosis kit was got from BioLegend (San Diego, CA, USA). Luciferase activity assay kit Dual-Luciferase Reporter Assay System was from Promega (Madison, WI, USA). Luciferase reporter plasmid pMIR-REPORT vector was provided from Ambion (Thermo Fisher Scientific, Waltham, MA, USA). Rabbit polyclonal antibody against PTEN and beta-actin was offered from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse monoclonal antibody against AKT and p-AKT was purchased from CST (Beverly, MA, USA). MiR-29a mimic and miR-29a inhibitor and miR-NC were synthesized by Gimma Bio (Shanghai, China). EdU cell proliferation assay kit on flow cytometry was obtained from Molecular Probes (Thermo Fisher Scientific, Waltham, MA, USA). Radioimmunoprecipitation assay (RIPA) lysis buffer, bicinchoninic acid (BCA) protein quantification kit, and horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG (H+L), and goat anti-mouse IgG (H+L) were collected from Beyotime (Beijing, China). Paclitaxel was purchased from Sangon (Shanghai, China).

Cell Culture

CCD 841 CoN and SW480 cells were cultured in RPMI-1640 medium containing 10% FBS and 1% penicillin-streptomycin, in a 37°C chamber with 5% CO_2. Culture medium was changed every 2-3 days. Cells were passed when reaching 70%-80% confluence at 1:3-1:4 ratio.

Generation of SW480/Paclitaxel Cell Model and Determination of Drug Resistant Index

SW480/Paclitaxel cell model was generated by treatment with gradient concentrations of Paclitaxel. In brief, cells at log-growth phase were firstly treated with 0.1 μM Paclitaxel for 24 h. PBS was then used to remove fresh medium containing Paclitaxel for continuous culture. After cells were passed for three times, concentration of Paclitaxel was gradually elevated 0.2 M, 0.5 μM, 1.0 μM, and 2.0 μM, until SW480 cells could maintain stable growth at 2.0 μM Paclitaxel to establish drug resistant cell strain SW480/Paclitaxel.

SW480/Paclitaxel and SW480 cells were treated with 0, 0.25, 0.5, 1, 2.5, 5, 10, and 20 μM Paclitaxel. Six replicated wells were allocated for each concentration. The CCK-8 solution was added after 48 h culture. After 4 h continuous culture, absorbance values (A450) from each well were measured. Inhibition rate = (1-A450 of drug treatment group)/A450 of control group X 100%. Excel software was used to calculate the drug concentration for inhibiting 50% cell growth (IC_{50}). Resistance index (RI) = IC_{50} of SW480/Paclitaxel/IC_{50} of SW480 cells.

Luciferase Reporter Gene Assay

HEK293T cells were lysed by TRIzol for extracting mRNA. Using HEK293T mRNA as the template, 3'-UTR of PTEN gene containing targeted binding sites or mutant fragment was amplified. PCR products were extracted from agarose gel, and were digested by enzymes to ligate into pMIR-REPORT plasmid for transforming DH5α competent cells. Positive clones of bacteria colony were selected

for sequencing. Plasmids were renamed as pMIR-PTEN-wt or pMIR-PTEN-mut. Lipo 2000 was used to co-transfection 100 ng pMIR-PTEN-wt (or pMIR-PTEN-mut), 50 pmol miR-29a mimic (or miR-NC, miR-29a inhibitor), and 50 ng pRL-null renilla luciferase into HEK293T cells. After 48 h incubation, the Dual-Glo Luciferase Assay System kit was used to measure relative luciferase activity. Oligonucleotide sequences were: miR-29a mimic sense, 5'-UAGCA CCAUC UGAAA UCGGU UA-3'; miR-29a antisense, 5'-ACCGA UUUCA GAUGG UGCUA UU-3'; miR-29a inhibitor, 5'-UAACC GAUUU CAGAU GGUGC UA-3'; miR-NC sense, 5'-UUCUC CGAAC GUGUC ACGUT T-3'; miR-NC antisense, 5'-ACGUG ACACG UUCGG AGAAT T-3'.

Construction of PTEN Over-Expression Plasmid

Using whole genomic sequence of SW480 cells as the template, CDS fragment of PTEN gene was amplified using specific primers (Forward: 5'-AAGGA TCCCC AGACA TGACA GCCAT CATC-3'; Reverse: 5'-CACAA CTCGA GTCAG ACTTT TGTAA TTTGT GTATG C-3'). The length of targeted fragment was determined by gel electrophoresis, and was digested by Xho I and BamH I enzymes, and was ligated into pcDNA3.1 vector. The recombinant plasmid was used to transform competent cell JM109. Positive clones were sorted out by ampicillin resistance. Correct insertion of PTEN gene targeted sequence was determined by sequencing and was renamed as pcDNA3.1-PTEN. Blank plasmid pcDNA3.1-Blank was used as the control group.

Transfection of Experimental Cells and Grouping

In vitro cultured SW480/Paclitaxel cells were divided into four groups: miR-NC group, miR-29a inhibitor group, pcDNA3.1-Blank group, pcDNA3.1-PTEN group. All those oligonucleotide fragments and Lipo2000 were diluted in Opti-MEM for 5 min at room temperature. Those oligonucleotide fragments were mixed with Lipo2000 for gentle mixture and incubated for 30 min at room temperature. The original medium was changed, and cells were rinsed twice in PBS to remove the serum. Opti-MEM serum-free culture medium was then used. The transfection mixture was added into the culture medium for the mixture, and cells were cultured in the chamber for 6 h. RPMI-1640 medium containing 10% FBS and 1% penicillin-streptomycin was used for 72 h continuous incubation. Cells were then digested by trypsin and were collected for assays.

Table I. Components of reverse transcription system.

Component	Volume
oligdT Primer (50 μM)	0.5 μL
Random 6 mers (100 μM)	0.5 μL
PrimeScript RT Enzyme Mix	0.5 μL
RNA	1.0 μg
5×PrimeScript Buffer	2 μL
RNase Free dH2O	Up to 10.0 μL

Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

PrimerScript™ RT reagent kit was used to perform qRT-PCR after RNA was extracted by TRIzol as the template for measuring relative expression of genes. Reverse transcription system was shown in Table I, and qPCR reaction system was shown in Table II.

Reaction conditions: 95°C pre-denature for 10 min, followed by 40 cycles each containing 95°C denature for 10 s, 60°C annealing for 2, and 72°C elongation for 15 s. PCR was performed on a Bio-Rad CFX96 Real-Time PCR Detection System. Primer sequences were designed by Primer Premier 5.0. Primer sequences were: miR-29aP_F: 5'-TAGCA CCATC TGAAA TCGGT TA-3'; miR-29aP_R: 5'-GCGAG CACAG AATTA ATACG AC-3'; U6P_F: 5'-ATTGG AACGA TACAG AGAAG ATT-3'; U6P_R: 5'-GGAAC GCTTC ACGAA TTTG-3'; PTENP_F: 5'-GCATG TATTC GGGTT AGG-3'; PTENP_R: 5'-TGAAT GAAAC TGACA AGG-3'; β-actinP_F: 5'-GAACC CTAAG GCCAA C-3'; β-actinP_R: 5'-TGTCA CGCAC GATTT CC-3'.

Western Blot

Cells were lysed by RIPA lysis buffer on ice for 15 min incubation. The cell lysate was centrifuged at 4°C under 10000 g for 10 min. Protein in the supernatant was transferred to a new pre-cold tube. Protein concentration was measured by BCA approach. 40 μg samples were loaded

Table II. Components of reverse transcription system.

Component	Volume
SYBR Fast qPCR Mix	10.0 μL
Forward Primer (10 μM)	0.8 μL
Reverse Primer (10 μM)	0.8 μL
cDNA	2.0 μL
RNase Free dH2O	6.4 μL

and were separated by 8%-10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) separating gel and 5% condensing gel (40 V, 200 min). Proteins were then transferred to polyvinylidene difluoride (PVDF) membrane (300 mA current, 100 min), which was blocked in 5% defatted milk powder for 60 min under room temperature. Primary antibody (PTEN at 1:2000, AKT at 1:3000, p-AKT at 1:1000, and β-actin at 1:5000) was added for 4°C overnight incubation. The membrane was then washed three times in PBST, and HRP conjugated secondary antibody (1:5000 dilution) was added for 60 min incubation at room temperature. The membrane was then rinsed three times in PBST. ECL approach was then used to measure protein expression level.

CCK-8 Cell Proliferation Assay

All transfected cells were re-suspended in complete medium. After incubation in 10 μM EdU for 2 h, cells were inoculated into 6-well plate for 48 h continuous incubation. Cells were then digested by trypsin and were collected for 20 min fixation at room temperature. With centrifugation washing in wash buffer, cells were processed at room temperature for 15 min. 500 μL reaction buffer was added for 30 min dark incubation at room temperature. After centrifugation rinsing in wash buffer, cells were re-suspended in 500 μL wash buffer. Cell proliferation was measured by Beckman FC500 MCL flow cytometry.

Flow Cytometry for Detecting Cell Apoptosis

Cells were collected by centrifugation, and were washed twice. 100 μL binding buffer was added for the mixture. 5 μL Annexin V-FTC and 5 μ PI were added for 10 min dark incubation. 400 μL binding buffer was added to resuspend cells, which were loaded into Beckman FC500 MCL flow cytometry for analysis.

Statistical Analysis

SPSS 18.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Measure data were presented as mean ±standard deviation (SD). Comparison of measurement data between groups was performed by the Student *t*-test. A statistical significance was defined when $p<0.05$.

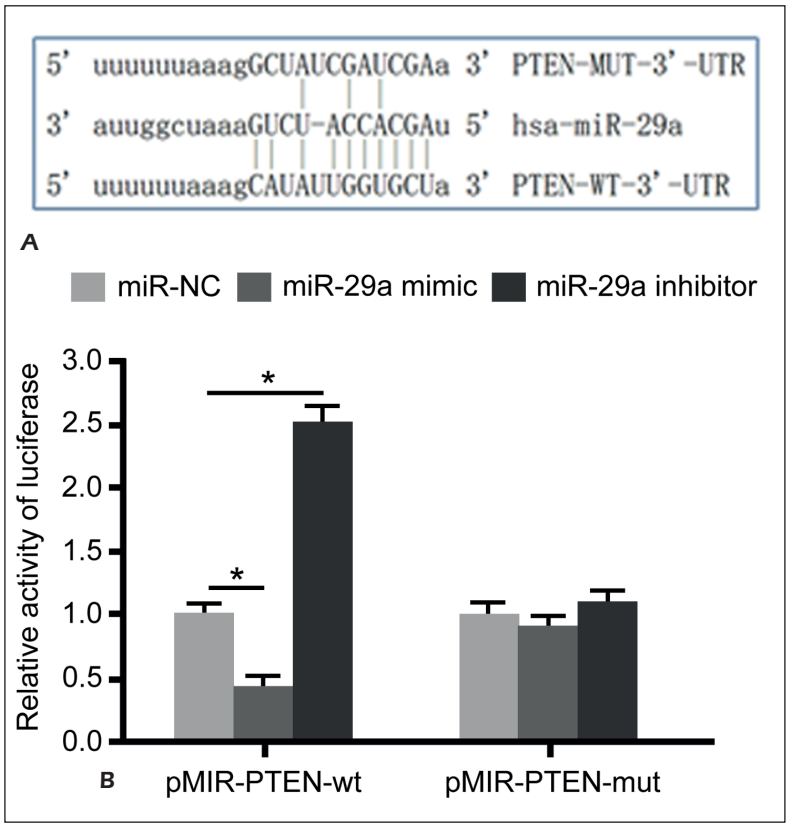

Figure 1. Targeted regulation between miR-29a and PTEN. **A**, Binding sites between miR-29a and 3'-UTR of PTEN mRNA; **B**, Dual-luciferase gene reporter assay. *, $p<0.05$ comparing between two groups.

Results

Targeted Regulatory Relationship Between miR-29a and PTEN

Bioinformatics analysis showed the existence of complementary binding sites between miR-29a and PTEN mRNA (Figure 1A). Furthermore, dual luciferase gene reporter assay showed that transfection of miR-29a mimic significantly depressed relative luciferase activity inside HEK293T cells, and transfection of miR-29a inhibitor remarkably elevated relative luciferase activity of HEK293T cells (Figure 1B). These data showed the targeted regulatory role of miR-29a on the inhibition of 3'-UTR of PTEN mRNA.

MiR-29a Down-Regulation Was Correlated With Paclitaxel Resistance of SW480 Cells

The CCK-8 assay showed that different concentrations of Paclitaxel significantly inhibited proliferation activity of SW480 cells (IC_{50} = 0.26 μM), but presented little effect on proliferation of SW480/Paclitaxel cells (IC_{50} of 2.94 μM). The RI of SW480/Paclitaxel cell was 11.31 (Figure 2A). qRT-PCR results showed that comparing to normal colorectal cell CCD 841 CoN, remarkably high miR-29a and low PTEN mRNA expressions were found in CRC cell line SW480 (Figure 2B). In drug resistant SW480/Paclitaxel cells, the manifestation of miR-29a up-regula-

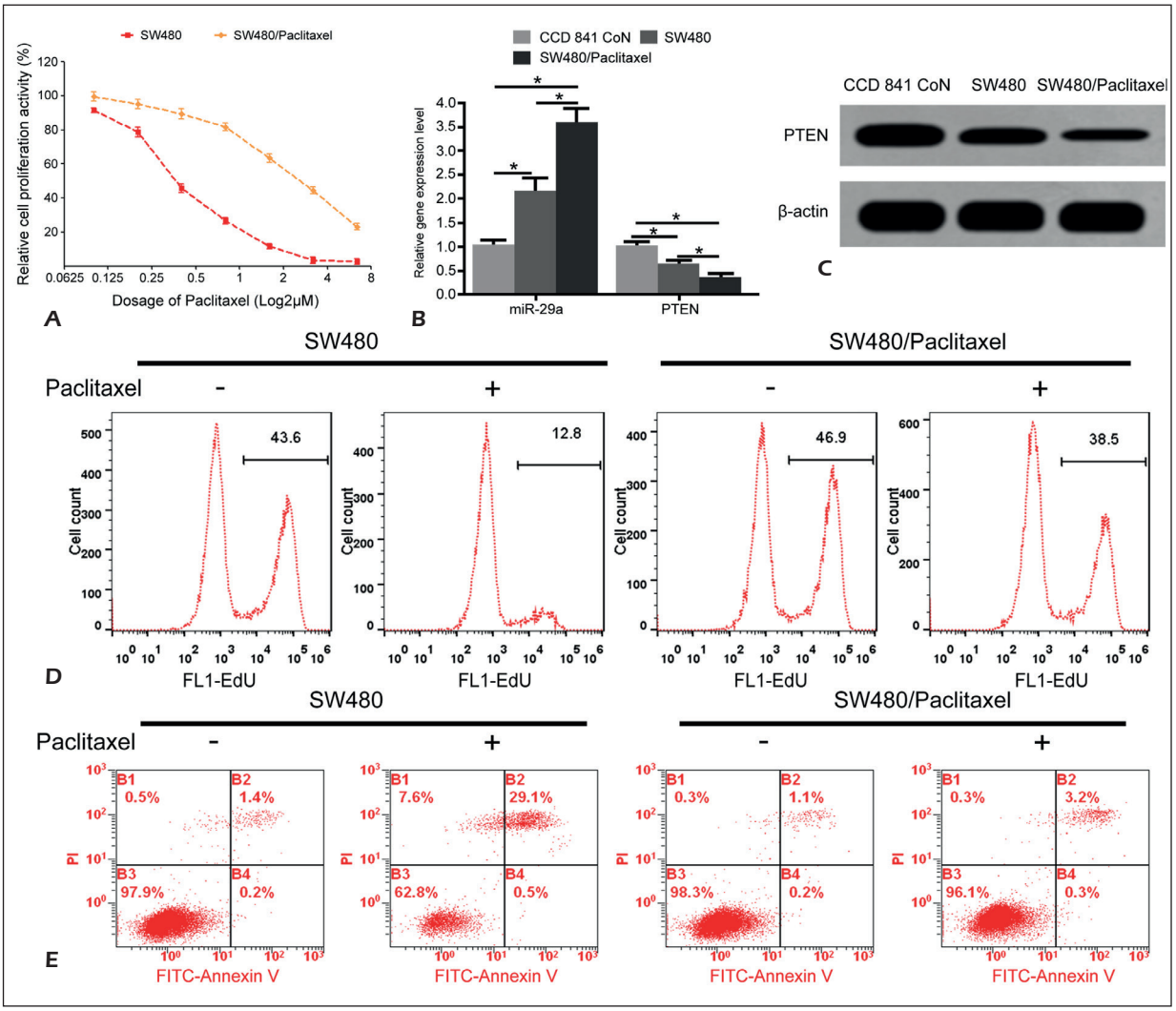

Figure 2. Correlation between miR-29a down-regulation and Paclitaxel resistance of SW480 cells. **A**, CCK-8 assay for the effect of different concentrations of Paclitaxel on cell proliferation; **B**, qRT-PCR for gene expression; **C**, Western blot for protein expression; **D**, EdU flow cytometry for cell proliferation; **E**, Flow cytometry for cell apoptosis. *, $p<0.05$ comparing between two groups.

tion and PTEN mRNA down-regulation became more significant. Western blot results also showed significantly decreasing PTEN protein expression in SW480/Paclitaxel cells compared to SW480 cells, with lower PTEN protein expression than that in CCD 841 CoN cells (Figure 2C). Flow cytometry for cell proliferation and apoptosis assay indicated that 0.26 μM Paclitaxel significantly suppressed proliferation of SW480/Paclitaxel cells and remarkably facilitated cell apoptosis. However, the same concentration of Paclitaxel had no evident effect of proliferation or apoptosis of SW480/Paclitaxel cells (Figure 2D and 2E).

Suppression of MiR-29a or Elevation of PTEN Expression Weakened Drug Resistance of SW480/Paclitaxel Cells

In this group, Paclitaxel concentration was set at 2.94 μM, and the growth of SW480/Paclitaxel cells was effectively inhibited. Of note, the results of miR-29a inhibition or PTEN up-regulation by transfection of miR-29a inhibitor or pcDNA3.1-PTEN significantly depressed p-AKT expression in SW480/Paclitaxel cells (Figure 3A and 3B), and remarkably weakened proliferation activity under 2.94 μM Paclitaxel treatment (Figure 3C), which also facilitated cell apoptosis (Figure 3D).

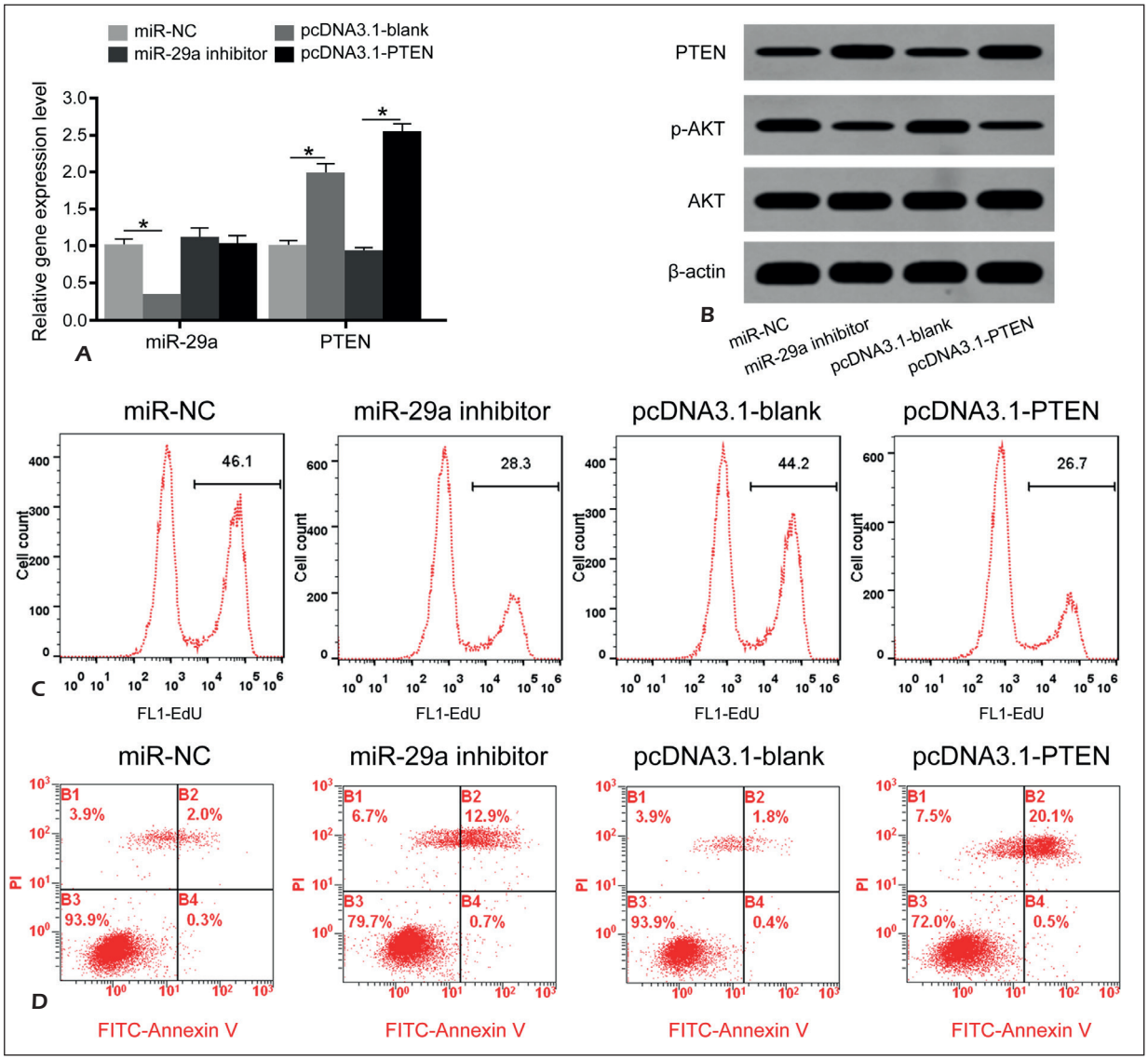

Figure 3. Inhibition of miR-29a or PTEN up-regulation weakened drug resistance of SW480/Paclitaxel cells. **A**, qRT-PCR for gene expression; **B**, Western blot for protein expression; **C**, EdU flow cytometry for cell proliferation; **D**, Flow cytometry for cell apoptosis. *, $p<0.05$ comparing between two groups.

Discussion

Phosphatidylinositol-3 kinase (PI3K)/protein kinase B (PKB) represents an important signal regulatory pathway that is widely distributed in multiple types of tissues and cells, and plays important roles in regulating cell growth, survival, proliferation, apoptosis, glycogen synthesis, and glucose transition. The over-activation of PI3K/AKT signal pathway can lead to abnormal proliferation of cells, and is closely correlated with tumor pathogenesis, progression, metastasis, and drug resistance. PI3K is mainly composed of catalytic subunit P110 and regulatory subunit P95. When PI3K/AKT signal pathway is under active status, PI3K can be recruited to the region adjacent to the plasma membrane. It catalyzes phosphorylation of substrate phosphatidylinositol 4,5-diphosphate (PIP2) to generate phosphatidylinositol 3,4,5-trisphosphate (PIP3). PIP3 can recruit AKT from plasma to the membrane. Under the effect of phosphoinositide-dependent protein kinase (PDK), it can phosphorylate Ser/Thr protein kinase AKT at Ser473 and Thr308 sites. Such phosphorylated and activated AKT further transmits signals toward downstream targets for regulating life activities of proliferation, cycle, and apoptosis. PTEN gene locates on chromosome 10q23.3, and produces a transcript with 515kb mRNA. PTEN has been recognized as the most tumor suppressor gene after p53. The most important substrate of PTEN is PIP3, and PTEN can dephosphorylate PIP3. PTEN then impedes the activation of AKT signal pathway and downstream targets via suppressing PI3K for its phosphorylation by PIP3[3]. Expression of PTEN may be related with drug resistance of multiple tumors including breast cancer[14], esophagus carcinoma[15], and pancreatic cancer[16]. PTEN has also been reported to be related with occurrence and drug resistance of CRC[6-8]. Previous studies revealed the abnormally elevating level of miR-29a in CRC, suggesting its potential tumor facilitating roles[13].

Combined with bioinformatics analysis, dual luciferase gene reporter assay showed that, compared to miR-NC group, the transfection of miR-29a mimic towards HEK293T cells significantly decreased relative luciferase activity. However, miR-29a mimic had no major effect on the relative luciferase activity in HEK293T cells transfected with pMIR-PTEN-mut, indicating targeted regulatory relationship between miR-29a and PTEN[17]. Notably, we found miR-29a expression was significantly elevated in SW480 cells, a type of cancer cell line, with decrease of PTEN expression. Moreover, in drug resistant cell line, more significant change of miR-29a and PTEN levels were shown, indicating that abnormal increase of miR-29a might be the reason causing PTEN down-regulation and enhanced Paclitaxel resistance of CRC cells. Brunet Vega et al[18] showed that compared to tumor adjacent tissues, miR-29a expression in CRC tumor tissues was remarkably elevated. Also, higher miR-29a level in peripheral blood samples was detected compared to healthy control people. Fu et al[17] reported that compared to normal colorectal tissues, miR-29a expression was elevated in CRC tumor tissues. Huang et al[19] found that, compared to healthy control tissues, high level of miR-29a was observed in patients with precancerous lesion of colorectal adenoma in serum, and high expression of miR-29a was also found in CRC patients compared to healthy control people. Moreover, abnormally higher miR-29a presented clinical value for differential diagnosis between CRC or colorectal adenoma or healthy control, with the area under the ROC curve (AUC) at 0.844 and 0.769. In addition, miR-29a expression was correlated with patient survival and prognosis, as shown by significantly lower overall survival rate in patients with high level of miR-29a compared to those patients with low miR-29a expression.

This study found that transfection of miR-29a inhibitor or pcDNA3.1-PTEN all remarkably decreased drug resistance of SW480/Paclitaxel cells, as shown by weakened proliferation activity plus a higher number of apoptotic cells, which demonstrates that miR-29a could mediate CRC cell drug resistance via targeting PTEN, whilst drug sensitivity was markedly improved by antagonizing miR-29a. Similarly, Tang et al[13] found that miR-29a up-regulation remarkably enhanced invasion potency of CRC cells lines HCT-116 and LoVo, and facilitated its hepatic metastasis. All these regulatory mechanisms are achieved via modulating the expression of target gene KLF4. Yuan et al[20] performed a human study and indicated that abnormally growing miR-29a expression could enhance the risk of CRC recurrence (HR=2.61, 95% CI = 1.34-5.07). Our study found that the reduction of miR-29a expression could remarkably weaken malignant biological features of CRC cells, as similar to Tang et al[13]. Bai et al[21] showed microRNAs play a significant role in suppression of CRC progression. Furthermore, our data also revealed the role of miR-29a in mediating CRC cell drug sensitivity, which was in agreement with the previous study[20]. Fang et al[6] demonstrated

that PTEN down-regulation was correlated with enhanced migration, invasion, and chemotherapy drug resistance of CRC cells, and over-expression of PTEN significantly weakened drug resistance of CRC cells. Wang et al[8] illustrated that the inability of PTEN was involved in potentiated drug resistance of Cetuximab of CRC. Interestingly, in contrast with Fang et al[6] and Wang et al[8], this study presented the inhibition of miR-29a expression could elevate PTEN expression and weaken the resistance of CRC cells. All these findings have not been reported previously.

Conclusions

Our data validated that the decrease of miR-29a could elevate PTEN expression, suppress CRC cell proliferation, facilitate cell apoptosis, and decrease drug resistance, which provides insights for the further therapy of colorectal carcinoma.

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Conflict of Interests:

The Authors declare that they have no conflict of interests.

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