

## Original Article

# MicroRNA-9 functions as an oncogene and targets PDCD4 gene in cervical cancer

Hongmei Jin, Chuanli Wang

*Department of Oncology, Linyi Cancer Hospital, Linyi, Shandong, China*

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**Abstract:** Malignant proliferation and invasion of cervical cancer are the leading cause of women death worldwide. MicroRNAs are involved in cervical cancer-related processes. MicroRNA-9 has been reported ectopically expressed in cervical cancer tissues. However, little is known about its functions and regulatory mechanism in cervical carcinomas. In this study, we found overexpression of miR-9 caused profound promotion of cell proliferation, cell cycle transformation and suppression of apoptosis, while silencing miR-9 caused an opposite effect. We also provide direct evidence that PDCD4 is the target of microRNA-9. These results suggested that microRNA-9 may function as an oncogene in the cellular processes of cervical cancer and its regulatory roles were closely associated with down-regulation of PDCD4.

**Keywords:** microRNA-9, oncogene, PDCD4

## Introduction

Cervical cancer is one of the leading gynecological cancers which seriously threatens the health and life of women worldwide [1]. The initiation and development of cervical cancer is a complex pathological process accompanied with multiple biological changes. Tumorigenesis of cervical cancer was also considered relevant with chromosome mutation and single nucleotide polymorphisms, growth factors and their receptors etc. al, despite closely related to persistent infection of high-risk human papilloma virus [2]. Tumor invasion and metastasis is a dynamic intricate process regulated by various genes or proteins accurately. Numerous studies indicated that aberrant expression of some non-coding small RNA fragment microRNA were closely associated with tumorigenesis and progression of human tumors, besides, miRNAs possess discriminatory power as cancer biomarkers [3-5].

MicroRNAs (miRNAs) with approximately 18~25 nucleotides in length are endogenous, single stranded non-coding small RNA fragment, which typically regulate gene expression at post-transcription level by binding to 3' untrans-

lated regions (UTRs) of target mRNAs to participate regulation of cell proliferation, cell cycle, invasion and apoptosis [6, 7]. Liu et al. found that up-regulation of miR-21 expression profiles can significantly reduce expression of PDCD4, and that promoted proliferation of HeLa cells, whereas up-regulation of miR-143 can effectively inhibited proliferation and promoted apoptosis of HeLa cells [8]. Microarray assay and qPCR assay were used to investigate the differences miRNA expression pattern of cervical cancer tissue samples at II and III phase of cervical intraepithelial neoplasia (CIN), Wilting et al. reported that five miRNAs, including miR-9, miR-15B, miR-28, miR-10 and miR-125B, showed ectopically expression level in these two phases [9]. Using qPCR Lee et al. analyzed the distinctive miRNAs expression profile between 10 cases of Ib~IIa cervical cancer tissues and 10 cases of normal cervical tissue. It was found that 70 miRNAs showed aberrant expression level between them, and 68 miRNAs were up-regulated and 2 miRNAs were significantly down-regulated in expression. In these 68 miRNAs, 10 miRNAs (miR-199-s, miR-9, miR-199a\*, miR-199a, miR-199b, miR-145, miR-133a, miR-133b, miR-214, and miR-127) were significantly up-regulated. The two down-

regulated miRNAs were miR-149 and miR-203. Among these 70 miRNAs, little known is about their roles and mechanism in the initiation and pathological progression of cervical cancer [9-11].

Our study in this article aimed to elucidate the effect of miR-9 on the biological behavior of HeLa cells according to gain-of-function or loss-of function cell models by overexpression or silencing of miR-9 respectively. Combined with bioinformatics prediction and dual-luciferase assay to verify the possible target genes of miR-9, and investigate the effect of miR-9 on the proliferation, cycle and apoptosis of cervical cancer cells. The miR-9 expression pattern and corresponding mechanism may provide theoretical basis for the application of miR-9 in the diagnosis and treatment of cervical cancer.

### Materials and methods

#### *Tissue samples and cell lines*

A total of 20 samples were collected from cervical cancer patients before they received therapy in department of oncology, Linyi Cancer Hospital, these patients had complete clinical information. The samples included cervical cancer tissues and adjacent non-malignant tissues. Cervical cancer cell lines HeLa, ME-180, c4-1 and c-33a were purchased from China Center for Type Culture Collection (CCTCC), primary human cervical epithelial cell was obtained from Jiangyin CHI Biological Technology Co., Ltd. Cells were cultured in RPMI 1640 medium (Gibco, USA) supplement with 10% fetal calf serum (Gibco, USA), 100 U/mL and 100 mg/mL streptomycin. All the cells were incubated in a humidified atmosphere containing 5% CO<sub>2</sub> at 37°C.

#### *Cell transfection*

MiR-9 mimics and anti-miR-9 were synthesized by Guangzhou Ribo Biology Technology Co., Ltd. Transfection were performed according to Lipofectamine™ 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) manufactures' instructions. 1×10<sup>5</sup>/mL cells in logarithmic growth phase were plated in each well of 6-well plate. Scramble nucleotides (termed as negative control, 100 nmol/L) were included as negative control. MiR-9 mimics (100 nmol/L) or miR-9 inhibitor was added into HeLa cells to

investigate the effect of miR-9 on cell biological behaviors. After transfection, HeLa cells were incubated in RPMI-1640 supplemented with 10% fetal bovine serum in a humidified atmosphere with 5% CO<sub>2</sub> at 37°C, and then collected cells at different time points for detection. SiRNA-PDCD4 (Guangzhou Ribo Biology Technology Co., Ltd) was applied for silencing the expression of PDCD4. Oligonucleotides synthesized by Guangzhou Ribo Biology Technology Co., Ltd was adopted as a negative control. Transfections of oligonucleotide (100 nM) and siRNA (100 nM) were performed according to Lipofectamine™ 2000 reagent (Invitrogen, Carlsbad, CA, USA) manufacturer's protocol. The transfection efficiency of each group was validated by qPCR assay.

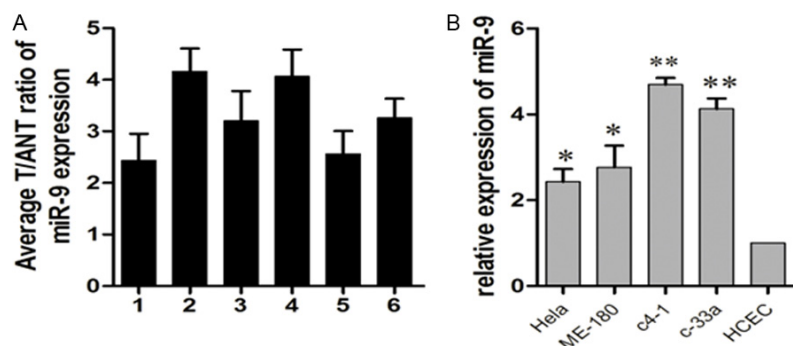
#### *qPCR*

Total RNA was extracted by TRIzol reagent (Invitrogen, Carlsbad, CA), the concentration and purity of RNA were determined by spectrophotometer. RNA quality was confirmed by Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Primers were designed using the Primer 5 software, and cDNA was prepared using the TransScript First-strand cDNA SynthesisMix Kit (Invitrogen, Carlsbad, CA). Operation process was carried out strictly according to the kit instructions of SYBR Green PCR Kit (Qiagen). MiR-9 primer: forward 5'-TGCGCTCTTTGGTTATCTAGCTG-3', reverse 5'-CCAGTGCAGGGTCCGAGGTATT-3', the U6 gene was used as an internal control. PDCD4 primer: forward 5'-GTGCCAACCA GTCCAA-3', reverse 5'-TTCCCTCCAATGCTA-3'. The amount of target gene mRNA relative to the internal control was calculated using the cycle threshold ( $\Delta\Delta CT$ ) method as follows: relative expression =  $2^{-\Delta\Delta CT}$ ,  $\Delta\Delta CT = \Delta CT$  (test)  $\Delta CT$  (calibrator).

#### *MTT assay*

Twenty-four hours after transfection, 1.0×10<sup>5</sup> cells were seeded into 96-well microtiter plate for 24 h, 48 h and 72 h, respectively. Then, the cells were incubated with 20  $\mu$ L of MTT (5 mg/ml, PH = 7.4) for 4 h at 37°C and 150  $\mu$ L of dimethyl sulfide was added to solubilize the crystals for 10 min at room temperature. The absorbance at 570 nm was detected using a  $\mu$ Quant Universal Microplate Spectrophotometer (Bio-Rad, Hercules, CA). Experiments were carried out at least three times.

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**Figure 1.** Relative expression level of miR-9 in cervical cancer tissues and cervical cell line. A. Relative expression level of miR-9 in six cervical cancer tissues and related ANT by qPCR. B. Expression level of miR-9 in HeLa, ME-180, C4-1 and C-33a by qPCR in comparison with that of normal cervical epithelial cell HCEC. Parallel experiments were carried out for over three times. \* $P < 0.05$ , \*\* $P < 0.01$ .

Cells were collected 48 h after transfected with miR-9 mimics or anti-miR-9 or NC, and then stained with Annexin V and 7-AAD according to the manufacturer's instruction. The combination of Annexin V and 7-AAD staining distinguished early apoptotic cells (Annexin V+, 7-AAD-) and late apoptotic cells (Annexin V+, 7-AAD+). Experiments were conducted in triplicate. Apoptosis ration was analyzed by FACSCalibur flow cytometry (BD, Bedford, MA).

### Western blot

Cells were lysed in RIPA lysis buffer at 72 h post-transfection. Protein concentration was measured using BCA protein assay reagent kit (Beyotime, China). Equal quantities whole protein were loaded on 5% SDS-PAGE gels and transferred onto PVDF membrane. After blocking with 5% non-fat dry milk, the membranes were incubated with rabbit-anti-human PDCD4 antibody (dilution ratio 1:1000) at room temperature for 2 h, goat-anti-human PDCD4 antibody (1:2000) was used as a secondary antibody, and  $\beta$ -Actin as an internal reference. Immunodetection was performed using the ECL system (Amersham Pharmacia Biotech).

### Cell cycle analysis by flow cytometry

Cell were collected 48 h after transfected with miR-9 mimics or anti-miR-9 or NC,  $1.0 \times 10^6$  cells were fixed in 75% ethanol overnight at  $4^\circ\text{C}$ . After washing with PBS, cells were treated with  $50 \mu\text{g}/\text{mL}$  Rnase A (Sigma-Aldrich, USA) for 1 h at  $37^\circ\text{C}$ , and then stained with  $20 \mu\text{g}/\text{mL}$  propidium iodide (Sigma-Aldrich, USA) for 30 min at room temperature. The cell cycle profiles were analyzed using an FACS Calibur flow cytometer (BD, Bedford, MA) and distribution of cell-cycle phases was determined using Modfit Software (BD Biosciences).

### Cell apoptosis analysis by flow cytometry

Apoptosis was determined by dual staining with Annexin V and 7-Aminoactinomycin D (7-AAD) (BD Biosciences, Erembodegem, Belgium).

### Hoechst33258 staining

Hela cells were inoculated on cell slides with a concentration of  $5 \times 10^4/\text{mL}$ , and transfected with miR-9 mimics or anti-miR-9 or NC by Lipofectamine reagent 2000 after cell adhesion. After cell transfection, cells were fixed with 0.5 mL fixative for 10 min, then stained with 0.5 mL Hoechst33258 (Sigma, USA) for 5 min at room temperature and photographed under fluorescent microscope.

### Statistical analysis

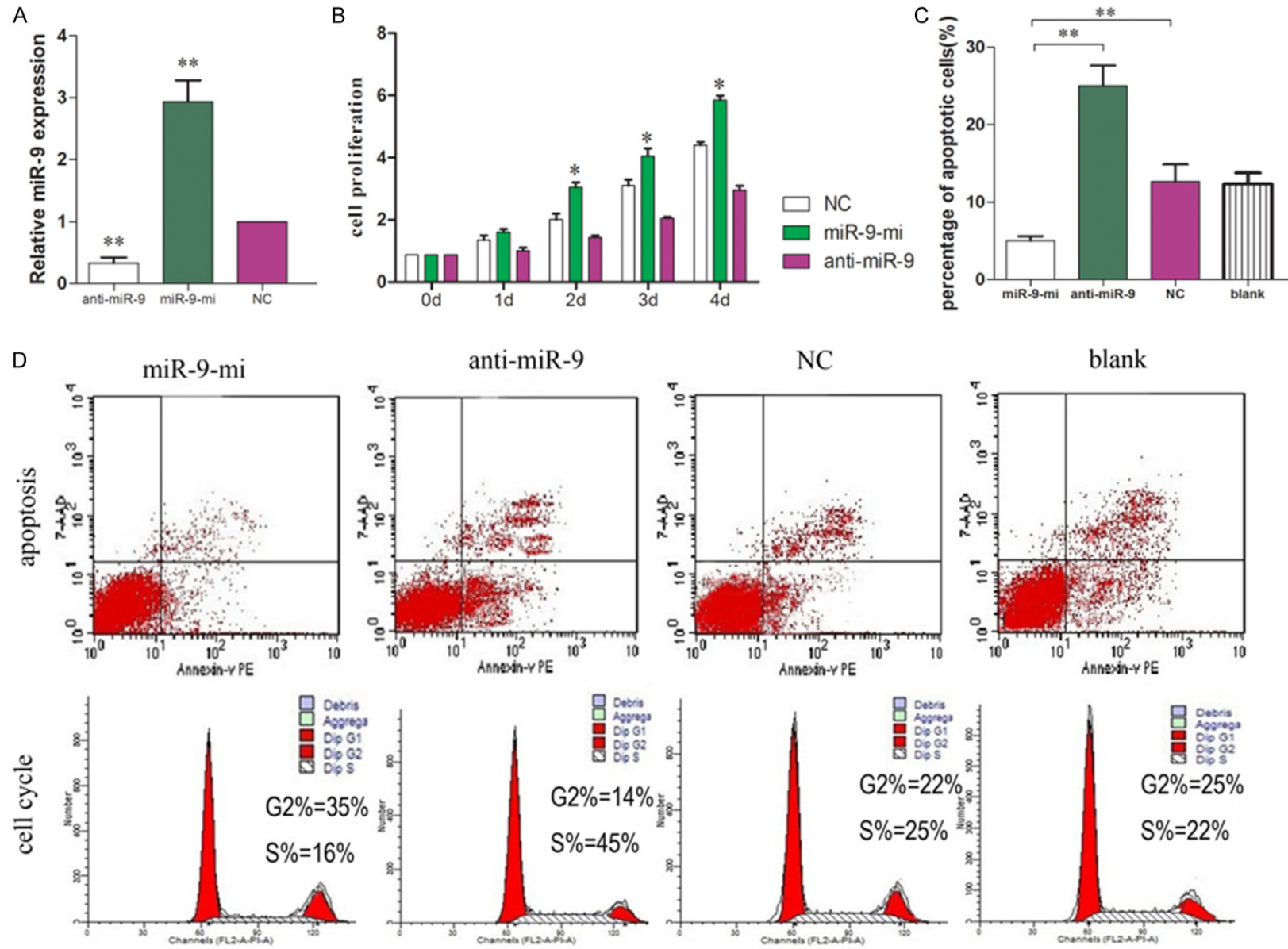
Data were expressed as mean  $\pm$  SD for the indicated number of independent performed experiments. The SPSS 20.0 was applied to complete data processing. Differences between two samples were determined by Student's t-test, while comparisons in different groups were analyzed by a one-way analysis of variance (ANOVA).  $P < 0.05$  was considered as statistically significant.

## Results

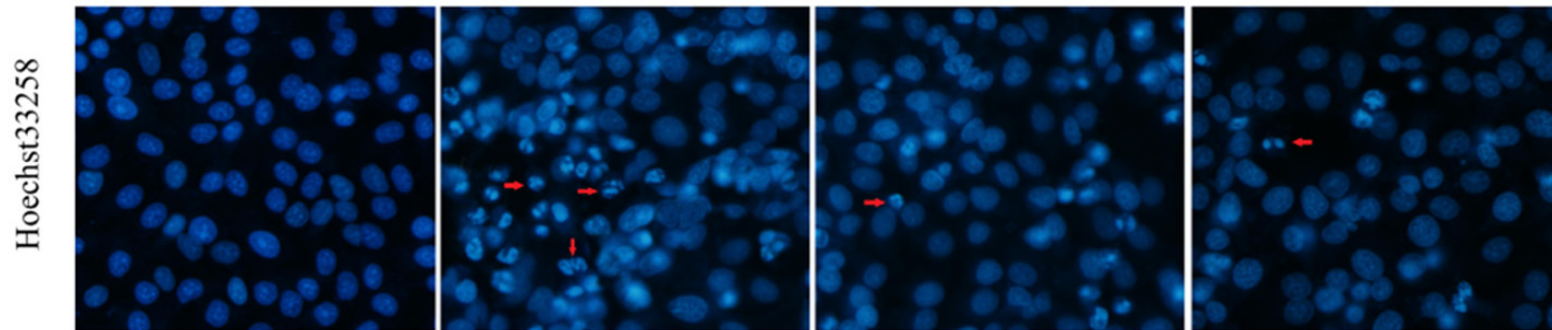
### Over-expression of miR-9 in cervical cancer tissue and cervical cancer cell line

Using qPCR, relative expression of miR-9 in cervical cancer tissues relative to adjacent normal tissue (ANT) was detected. Additionally, the expression level of miR-9 in four epidermal cervical cells relative to normal cervical cancer cell lines were detected in the same way. As shown in **Figure 1A**, compared with ANT, relative expression level of miR-9 in cervical cancer tissue is higher. Similarly, in HeLa, ME-180, C4-1

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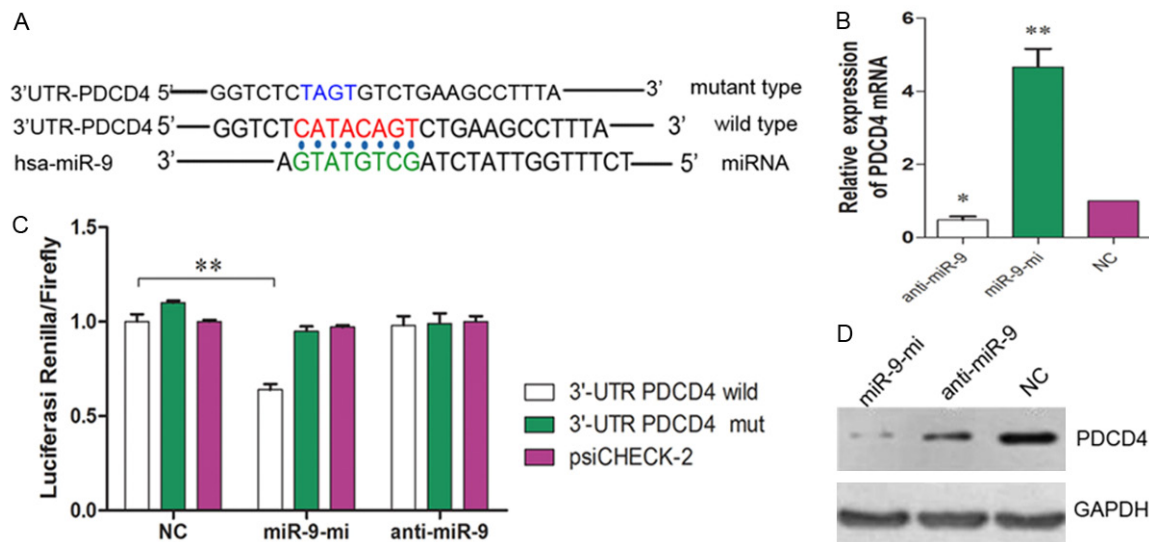


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**Figure 2.** Influence of over-expression or down-regulation of miR-9 on proliferation, apoptosis and cell cycle of Hela cell. A. Detection of transfection efficiency of miR-9 using qPCR. B. Influence of miR-9 over-expression on proliferation detected by MTT assay. Cell viability detected after transfection of 0 d, 1 d, 2 d, 3 d, 4 d. Scramble nucleotides were used as negative control. \* $P < 0.05$ . C. Using FCM assay we detected the effect of miR-9 on cell cycle after miR-9-mi and anti-miR-9 were transfected into Hela cells. \*\* $P < 0.01$ . D. Upper part shows representative result of apoptosis detected by FCM. Apoptosis cell is shown in second and fourth quadrants. The middle part displays the results of cell cycle distribution detected by FCM. Ration of different phases is marked in corresponding picture. The lower part indicates the result of cell nucleus stained by Hoechst33258. Apoptosis body is shown by the white arrow.

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**Figure 3.** MiR-9 directly target 3'-UTR mRNA of PDCD4 and decrease its expression. A. Predicted miR-9 target sequence in the 3'-UTR of PDCD4 (psi-CHECK2-PDCD4) and of three mutant sites in the psi-CHECK2-PDCD4-mut. Mutation site showed in blue letter, while red and green letters means miR-9 seed sequence and target sites of mRNA respectively. B. Relative expression level of PDCD4 at mRNA level after miR-9-mi and anti-miR-9 transfection. C. Luciferase reporter assay of the indicated cells transfected with the miR-9 or anti-miR-9. Relative enzymatic activities = renilla luciferase/firefly luciferase. D. Western blotting analysis of PDCD4 expression at protein level. GAPDH served as the internal control.

and C-33a, the expression levels of miR-9 are apparently higher than that in normal cervical epithelial cell HCEC. In summary, expression of miR-9 in cervical cancer tissues and cervical cancer cell is remarkably up-regulated.

### Over-expression of miR-9 can significantly influence biological behavior of Hela cell

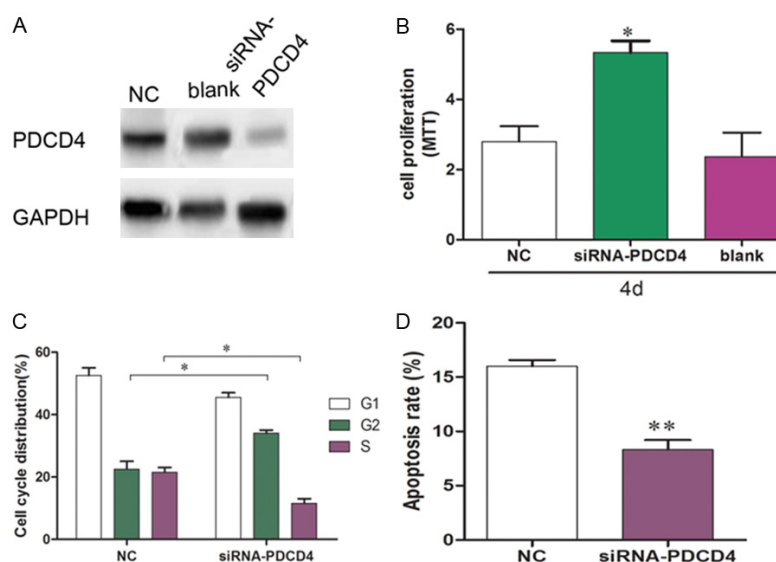
Next, to explore the effect of miR-9 overexpression on cervical cancer cell lines, Hela cell was transfected by miR-9 mimic and anti-miR-9 respectively. Transfection efficiency was validated by qPCR (**Figure 2A**). MTT assay indicated that over-expression of miR-9 can promote cell proliferation, while anti-miR-9 showed opposite effects. Flow cytometry assay was applied to detect the influence of over-expression or down-regulation of miR-9 on apoptosis and cell cycle. As shown in **Figure 2C** and **2D**, our data indicated that apoptosis rate decreased dramatically when Hela cells were transfected with miR-9-mi compared with both NC control group and anti-miR-9 group. In miR-9-mi transfection group, the proportion of cells stayed in S phase is remarkably lower than that of control group, while the ration of G2-phase cells was higher than that in other group. Detected by fluorescence microscope, normal

cell nucleus stained by Hoechst 33258 displayed uniform light blue [12]. The bottom part of **Figure 2D** showed more apoptosis bodies of nucleus in anti-miR-9-transfected group than that of others under fluorescence microscope, and that higher rate always mean higher apoptosis level. Collectively, our results suggest that miR-9 over-expression can facilitate proliferation of Hela cells, promotes the transition of cell cycle from S period to G2 period, and inhibits apoptosis at the same time.

### Target gene of miR-9 is PDCD4

Potential target genes of miR-9 were predicted by Targetscan and RNAhybrid 22 software. PDCD4 was selected as the target for further analysis. **Figure 3A** displayed binding sites in 3'UTR mRNA of PDCD4. After psi-CHECK2-PDCD4 and psi-CHECK2-PDCD4-mut plasmids were constructed, dual-luciferase assay showed that co-transfection of miR-9 remarkably inhibited the activity of psi-CHECK2-PDCD4 but fail to influence the luciferase activity of psi-CHECK2-PDCD4-mut in Hela cells, and no obvious differences of luciferase activity was found between NC control group and mutant reporter. To further verify the target role of miR-9 on PDCD4, western blot and qPCR

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**Figure 4.** PDCD4 down-regulation is required for miR-9-induced changes of biological behaviors of HeLa cells. A. Silencing PDCD4 expression after siRNA transfection verified by western blotting. At least three parallel experiments were carried out. B. MTT assay was carried out to detect cell viability between siRNA-PDCD4 group and NC control group. C. FCM assay was used to verify cell cycle distribution of HeLa cells after siRNA-PDCD4 transfection. D. Apoptosis rate was detected after siRNA-PDCD4 transfection using FCM. \* $P < 0.05$ , \*\* $P < 0.01$ .

were used to investigate the effect of miR-9 overexpression, as shown in **Figure 2B** and **2D**, on PDCD4 expression ( $P < 0.05$ ) at protein level and mRNA level respectively. In summary, our data indicated that miR-9 could directly decrease the expression of PDCD4 by targeting of its mRNA 3'-UTR in cervical cancer cell lines.

### *miR-9 plays a regulatory role through targeting PDCD4 gene expression*

To further investigate whether the influence of miR-9 on HeLa cell phenotype is induced by down-regulation of PDCD4 gene expression or not, MTT and FCM were applied to detect cell biological behaviors after silencing PDCD4. As shown in **Figure 4**, results of MTT and FCM revealed that treatment with siRNA-PDCD4 was able to promote cell proliferation, inhibit apoptosis of HeLa cells and promote cell cycle into G2 phase from S phase, suggesting that miR-9 influences biological behavior by suppressing PDCD4 expression in cervical cells.

### Discussion

Metastasis and malignant proliferation of cervical cancer cell are the major causes of deaths

for cervical cancer [13]. Anomaly expression of miRNAs is closely related to tumorigenesis and different miRNAs expression profiles have been sequentially reported in multiple cancers such as breast cancer, prostate cancer and liver cancer et al. [14, 15]. Abnormal expression of miRNAs participates in many processes including malignant transformation, apoptosis, invasion and metastasis of cervical cancer [1, 16]. Lee et al. reported that miR-9 has an up-regulated expression in cervical cancer tissues, but its effect on cervical cancer and its regulatory mechanism have not been reported yet. Relevant studies demonstrated that miR-9 can affect the proliferation ability of gastric cancer cell by

regulating specific target genes [17]. In our study, we analyzed the role of miR-9 in cell proliferation, apoptosis via construction of gain-of-function and loss-of-function cell model after transfecting HeLa cells with miR-9-mi and anti-miR-9 respectively; Our results indicate that overexpression of miR-9 could promote proliferation of HeLa cell and reduce the apoptosis rate of HeLa cells, and it could also promote HeLa cell turning into G2 phase from S phase; Combined with dual luciferase report assay and bioinformatics prediction, we verified PDCD4 is targeted directly by miR-9. In order to further confirm that overexpression of miR-9 is the major cause of cell phenotypic changes, using MTT assay, flow cytometry assay we specifically transfected shRNA, for silencing PDCD4 expression, into HeLa cells to validate whether biological behavior changes caused by overexpression of miR-9 would consistent with that after silencing of PDCD4 expression. Suggestive results demonstrated that miR-9 indeed change cell biology behavior through directly downregulating PDCD4 gene expression.

Numerous experiments suggested that PDCD4 is a novel tumor suppressor gene, 83% absence rate of PDCD4 in primary lung cancer was

closely related to degree of malignant tumor. Patients lacking PDCD4 always have a relatively high malignant level and these patients belong to G3 grade adenocarcinoma according to the TNM classification [18]. Postoperative follow-up studies indicated that survival rate of patients with insufficient PDCD4 expression was significantly lower than that having normal expression level, it means that lacking of PDCD4 always lead to low degree level of tumor differentiation and poor prognosis [19]. High frequency deficiency or down-regulation of PDCD4 not only exists in lung cancer, but also can be detected in other malignant tumors including colon cancer, glioma, pancreatic cancer, breast cancer. These data demonstrated that PDCD4 plays an important role in the pathological process of tumor.

MiRNAs widely participate in the regulation of different pathological processes of carcinogenesis according to down-regulating target gene expression, meanwhile, they also can be modulated by upstream regulators, such as transcription factors or epigenetic growth factors. One kind of miRNA may target various genes and involved multiple distinctive signaling pathways. Moreover, various miRNAs can also target a certain gene. Different miRNAs and mRNAs interact at corresponding posttranscriptional level and form an intricate regulatory network [7]. For example, miR-21 could post-transcriptionally decrease some tumor suppressor including PDCD4, TIMPK MARCK5, PTEN, etc [20-23]; C-Myc is the target of miR-185-3p and feedback the expression of this miRNA [22]. Validation of miRNA target is important for understanding the mechanism of this regulatory role [24]. The present study showed that miR-9 was upregulated in cervical tissues and cervical cell lines, and its ectopic expression promoted cell proliferation and invasion and inhibited cells apoptosis, probably by down-regulating the expression of the PDCD4 gene. Together, our data demonstrated that miR-9 is a key regulator and function as an oncogene in cervical cancer and may be a potential therapeutic target for cervical treatment.

### Disclosure of conflict of interest

None.

**Address correspondence to:** Dr. Chuanli Wang, Department of Oncology, Linyi Cancer Hospital, 7

Linyuandong, Linyi 276000, Shandong, China.  
E-mail: refusionwang@sina.com

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