

ORIGINAL ARTICLE

## Highly expressed microRNA-124 inhibits migration and promotes apoptosis of esophageal cancer cells by degrading PDCD6

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### Summary

**Purpose:** To investigate whether microRNA (miR)-124 could affect the proliferation, migration and apoptosis of esophageal cancer cells and participate in the occurrence of esophageal cancer through regulating PDCD6 expression.

**Methods:** The expression of miR-124 in cancer tissues and adjacent tissues of esophageal cancer patients were detected by quantitative real time-polymerase chain reaction (qRT-PCR). Esophageal cancer cells TE-1 and SKYSE30 were cultured. The effects of miR-124 on tumor size and metastasis were analyzed. The functions of miR-124 in cell proliferation, migration and apoptosis were detected by cell counting kit-8 (CCK-8), transwell and flow cytometry, respectively. Rescue experiments were performed to assess whether miR-124 could regulate the proliferation, migration and apoptosis of esophageal cancer cells by inhibiting PDCD6 expression.

**Results:** The expression of miR-124 in cancer tissues of esophageal cancer patients were lower than that of the control group. In addition, its expression in patients with stage

III-IV esophageal cancer was remarkably lower than that in patients with stage I-II. Based on the level of miR-124, we divided the patients into high and low expression group, and found significant differences in tumor size, tumor metastasis and lymph node metastasis between the two groups. Also, overexpression of miR-124 reduced the proliferation and migration of TE-1 and SKYSE30 cells and increased the apoptosis, and vice versa. Luciferase reporting assay results confirmed that PDCD6 was one of the target genes of miR-124. A further mechanism study demonstrated that overexpression of PDCD6 in TE-1 and SKYSE30 cells could partially reverse the effect of miR-124 on cell apoptosis.

**Conclusions:** The low expression of miR-124 can promote the proliferation as well as migration of esophageal cancer cells and inhibit cell apoptosis. The mechanism may be that miR-124 can regulate the expression of PDCD6.

**Key words:** apoptosis, esophageal cancer, microRNA-124, migration, PDCD6

### Introduction

Esophageal cancer is one of the ten most common malignant tumors in humans [1] with about 300,000 people worldwide dying of esophageal cancer each year. Due to the inconspicuous early symptoms and lack of effective treatment, most patients with esophageal cancer have been diagnosed

in middle or late stage. The 5-year survival rate after surgical resection treatment is only about 25% and the 5-year survival rate of all patients is less than 10%. Studies have confirmed that the process of esophageal cancer is a complex process, involving multiple genes mutations and epigenetic ab-

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normalities [2]. Therefore, the exploration for early specific markers of esophageal cancer will greatly improve the diagnosis and treatment of esophageal cancer.

MicroRNAs (miRs) are non-coding small RNAs of 21-25 nucleotides in length that regulate target genes expression by interfering with their translation [3]. Bioinformatics data suggest that miRs may involve almost all essential signaling pathways *in vivo*, including the pathways related to tumors [4]. Numerous studies have shown that dysregulated miR expression is closely related to tumorigenesis. A hundred and four cases of lung cancer and corresponding non-cancer tissues were compared by miR microarray technology, and it was found that the expression of 8 miRs was closely related to the prognosis of lung cancer [5]. Studies have illustrated that miR-143 and miR-145 expression levels were decreased in tumor and tumor-adjacent tissues during colon cancer formation [6]. He et al. [7] found that the miR-17-92 family was highly expressed in many malignant lymphomas and could promote c-MYC gene expression in a mouse model. It has also been found that miR-124 inhibited breast cancer cell proliferation, invasion and metastasis. Further analysis revealed that miR-124 expression was decreased in pathological specimens of breast cancer, and the decreased level was associated with the grade of tumor tissue [8]. In addition, the down-regulated miR-124 may be associated with the occurrence of tumors such as prostate cancer and gastric cancer [9,10], but the function of miR-124 in the pathogenesis of esophageal cancer has rarely been reported.

PDCD6, also known as apoptosis-associated gene 2 (ALG-2), is a calcium binding protein that is involved in the constitutive T cell receptors and FAS-glucocorticoid-induced programmed cell death [11,12]. However, studies have shown that the apoptosis of cells in PDCD6-deficient mice was not inhibited, suggesting that PDCD6 protein may also be involved in other biological processes [13].

Based on the expression of miR-124 and PDCD6 in esophageal cancer tissues, we used *in vitro* cell experiments to further explore the mechanism of miR-124 and PDCD6 in the pathogenesis of esophageal cancer.

## Methods

### Participants and sample collection

Fresh esophageal cancer tissues and adjacent normal tissues were collected from 49 patients pathologically diagnosed with esophageal cancer and accepted surgical treatment from March 2013 to July 2017. No

patient had received any treatment before surgery and family history was clear. All patients voluntarily participated in this study and signed written informed consent. This study has been approved by the Ethics Committee of the Affiliated Yantai Yuhuangding Hospital of Qingdao University. All samples were stored in liquid nitrogen.

### Cell culture

Esophageal cancer cells TE-1 and SKYSE30 were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). The cells were cultured with Dulbecco's modified Eagle medium (DMEM) (Gibco, Rockville, MD, USA), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin + streptomycin (Gibco, Rockville, MD, USA). The cells were placed in incubator at 37°C, 5% CO<sub>2</sub> and cultured at constant temperature for 24 hrs. The medium was changed every other day.

### Cell transfection

The appropriate number of cells were seeded in culture plate or flask with DMEM medium. According to Lipofectamine 2000 instructions (Invitrogen, Carlsbad, CA, USA), miR-124 mimics, miR-124 inhibitors, pcDNA-PDCD6 and the corresponding negative controls were transfected into cells. MiR-124 mimics, inhibitors and pcDNA-PDCD6 were all designed and synthesized by GenePharma (Shanghai, China). The mixture was added to the culture plate or flask containing the culture solution and was gently mixed by shaking. Subsequent experiments were performed after 24-48 hrs transfection.

### RNA extraction

The cells and tissues required for the experiment were collected, and 1 mL of TRIzol (Invitrogen, Carlsbad, CA, USA) was added for lysing the samples. After 250 µL of chloroform was added to the cells, the mixture was shaken for 30 s and then centrifuged at 5000 RPM at 4°C. After centrifugation, the aqueous phase was aspirated and an equal volume of pre-cooled isopropanol was added. The precipitate was gently washed with 75% ethanol after centrifugation. The RNAs were dissolved in 20 µL of diethyl pyrocarbonate (DEPC) water, and then the RNA concentration was measured using a spectrophotometer and placed in a refrigerator at -80°C until use.

### Quantitative real time-polymerase chain reaction (qRT-PCR)

A reverse transcription reaction system was prepared on ice using the PrimeScript RT reagent Kit (TaKaRa, Tokyo, Japan) and complementary DNA (cDNA) was obtained when the reaction completed. The miR quantitative PCR procedure was performed according to the miScript SYBR Green PCR kit instructions (TaKaRa, Tokyo, Japan). PCR amplification conditions were: pre-denaturation at 94°C for 5 min, followed by 40 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 1.5 min. The primer sequences are as follows: microRNA-124 (F: GATACTCATAAGGCACGCGG, R: GTGCAGGGTC-CGAGGT), U6 (F: CTCGCTTCGGCAGCAGCACATATA, R: AAATATGGAACGCTTCACGA).

### Cell proliferation

Differently treated esophageal cancer cells were seeded in 96-well plates at  $1 \times 10^4$ /well and cultured for 6, 24, 48, 72, or 96 hrs. The optical density (OD) value was measured on a 450 nm using enzyme-linked immunosorbent instrument. Each group had 5 replicate wells.

### Cell migration

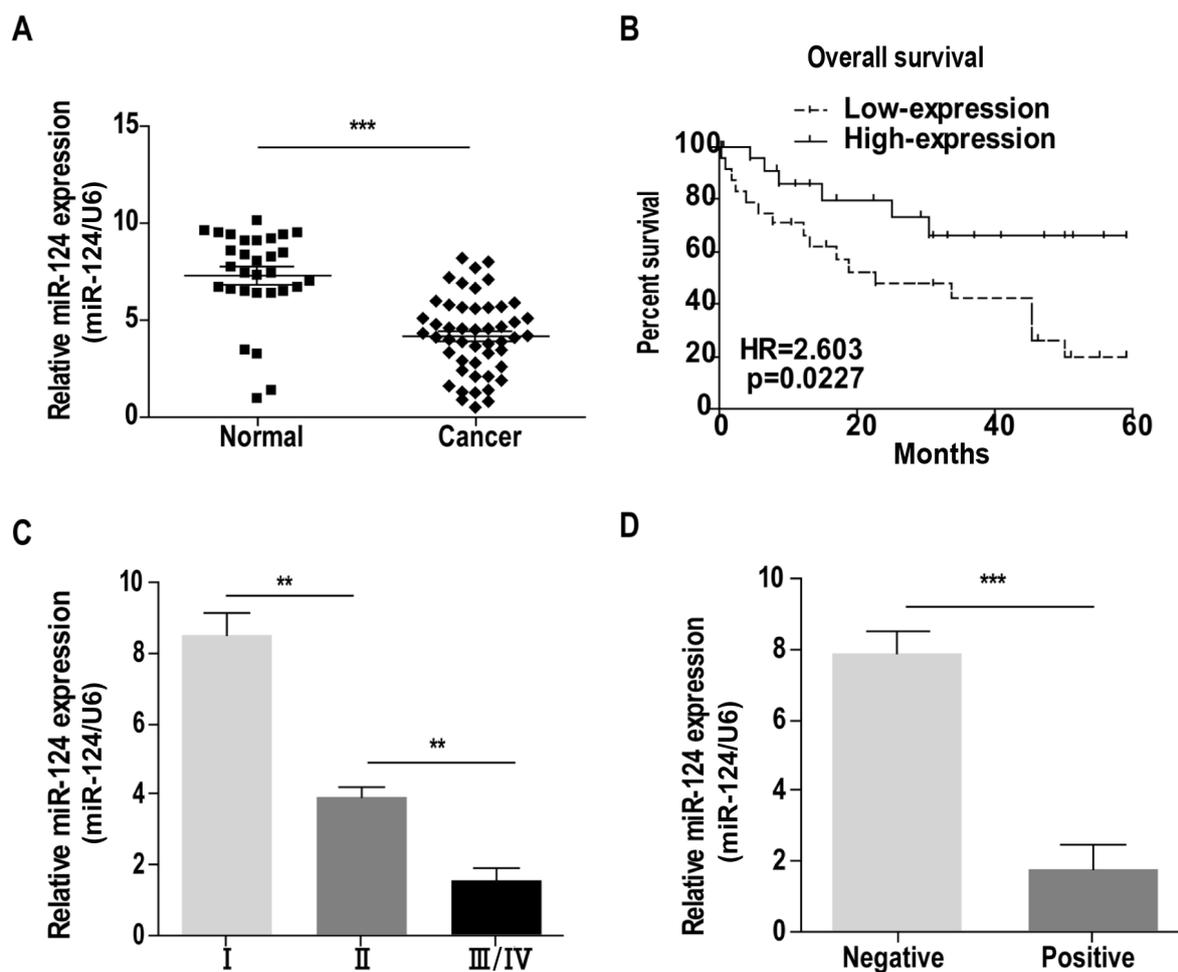
Transwell chambers were placed in 24-well plates. Transfected esophageal cancer cells were collected, digested with trypsin and suspended in serum-free medium. The cells were then diluted with culture medium and the density was adjusted to  $1 \times 10^5$ /mL. The medium containing 10% FBS was added to the cells and then cultured in the incubator. After 48 hrs, the culture fluid was removed and the cells were washed twice with phosphate buffered saline (PBS). After fixed with 4% paraformaldehyde for 30 min, the cells were stained with 0.5% crystal violet for 10-15 min. PBS was then used to wash the excess dye. The cells were counted by a microscope.

### Luciferase assay

The 3' UTR sequence of PDCD6 gene was cloned into the vector pGL3 containing the Luciferase reporter gene, namely the Wt-3' UTR. Using the Site-Directed Mutagenesis kit (Thermo Fisher Scientific, Waltham, MA, USA), the core binding region of miR on the 3' UTR was mutated to an ineffective binding region to construct the control plasmid Mut-3' UTR. Full-length vectors and mutant vectors were transfected into cells by Lipofectamine 2000. After 36 hrs, the culture medium was discarded and the cells were rinsed 3 times with PBS. Then the lysate was added and allowed to stand at room temperature for 10 min. A Dual Luciferase Reporter Assay System was subsequently used to detect fluorescence intensity.

### Cell apoptosis

The cells were digested with trypsin containing no EDTA, and no less than  $1 \times 10^5$  cells/mL of suspended cells were collected. A hundred  $\mu$ L of  $1 \times$  Annexin buffer



**Figure 1.** Low expression of miR-124 in esophageal cancer tissues. **A:** miR-124 expression was significantly lower in esophageal cancer tissues in 49 patients than in esophageal normal tissues. **B:** The overall survival rate of esophageal cancer patients with high miR-124 expression was significantly higher than that of miR-124 low expression group. **C:** miR-124 expression in patients with stage III-IV esophageal cancer was significantly lower than in patients with stage I-II. **D:** The expression of miR-124 in tumor tissues of metastatic patients was significantly lower than that of patients without metastasis. \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

was added to suspend the cells. Five  $\mu\text{L}$  of Annexin V and 1  $\mu\text{L}$  of propidium iodide (PI) staining were added into cells, then mixed and incubated for approximately 15 min at room temperature in the dark. Four hundred  $\mu\text{L}$  of  $1\times$  buffer were added to the flow cytometer before testing. Each group had 3 replicates and the experiment was repeated 3 times.

#### Statistics

SPSS 22.0 statistical software package (IBM, Armonk, NY, USA) was used for statistical analyses. Quantitative data were expressed as mean $\pm$ standard deviation. Two-sample t-test was used for comparison between groups.  $P<0.05$  was considered statistically significant. GraphPad Prism 7 software (La Jolla, CA, USA) was used to perform related statistical analyses charting.

## Results

### *MiR-124 was lowly expressed in esophageal cancer tissues*

First, we collected clinical information from 49 patients with esophageal cancer and detected their miR-124 expression. We found that miR-124 was strikingly lower in esophageal cancer tissues than in normal tissues (Figure 1A). Survival analysis demonstrated that the overall survival rate of esophageal cancer patients with high expression of miR-124 was strikingly higher than those with miR-124 low expression (Figure 1B). We then examined the expression of miR-124 in tissues of esophageal cancer patients at different stages. The results illustrated that miR-124 expression was lower in esophageal cancer patients in III-IV stage than

those in I-II stage (Figure 1C). In addition, we found that the expression of miR-124 in the tumor tissues of metastatic patients was significantly lower than that in patients without metastasis (Figure 1D). Based on miR-124 expression, we divided the tumor tissues into high and low expression group and found that high expression of miR-124 was closely related to tumor size, tumor metastasis and lymph node metastasis (Table 1).

### *Highly expressed miR-124 suppressed the proliferation of esophageal cancer cells and promoted apoptosis*

To further explore the possible regulation role of miR-124 in the development of esophageal cancer, we examined the effect of miR-124 on cell proliferation after overexpressing or knocking down miR-124 in TE-1 and SKYSE30 cell lines. CCK-8 experiments indicated that overexpression of miR-124 inhibited the viability of TE-1 cells and *vice versa* (Figure 2A, 2B). Transwell results demonstrated that overexpressed miR-124 inhibited the migration of TE-1 and SKYSE30 cells, while inhibition of miR-124 enhanced the migration ability of cells (Figure 2C,2D). And last, flow cytometry assay showed that overexpression of miR-124 could promote the apoptosis of TE-1 and SKYSE30 cells, while knockdown of miR-124 cancelled apoptosis (Figure 2E, 2F).

### *miR-124 regulated PDCD6 expression*

To further assess the function of miR-124 in esophageal cancer cells, the targets of miR-124 were predicted using bioinformatics analy-

**Table 1.** Relationship between miR-124 expression and clinicopathologic features of esophageal cancer

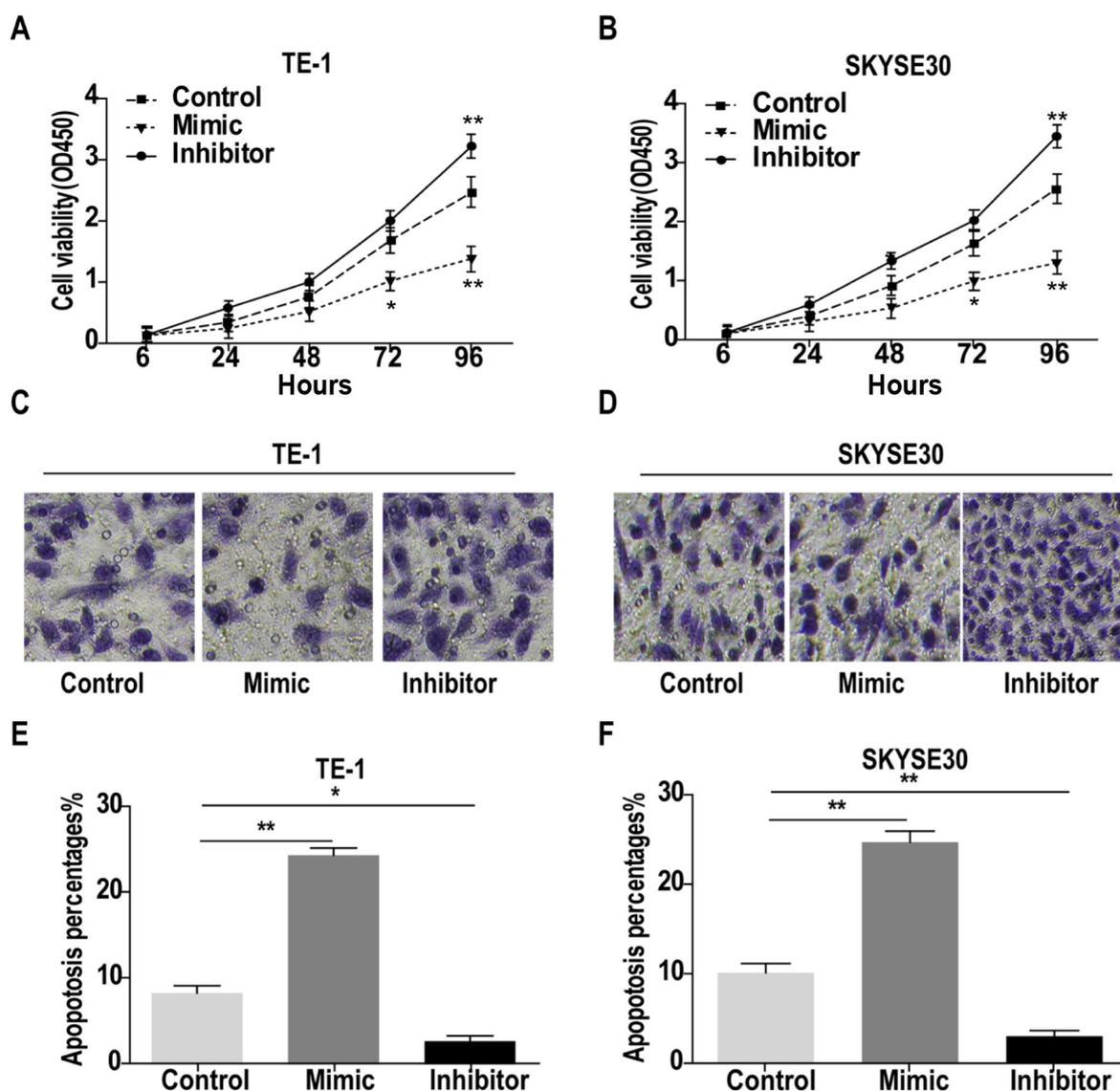
Clinicopathologic features	Number of cases	miR-124 expression		p value
		Low (n=25)	High (n=24)	
Age (years)				0.6559
$\leq 60$	22	12	10	
$> 60$	27	13	14	
Gender				0.4767
Male	24	11	13	
Female	25	14	11	
Tumor size, cm				0.0065
$\leq 4$	21	6	15	
$> 4$	28	19	9	
Metastasis				0.0067
Yes	23	7	16	
No	26	18	8	
Lymph node metastasis				0.0301
Yes	27	10	17	
No	22	15	7	

sis, and we found that PDCD6 may be a potential target of miR-124. The predicted binding sites are shown in Figure 3A. To further verify the binding of PDCD6 to miR-124, luciferase assay was performed and it was found that miR-124 could indeed regulate PDCD6 level (Figure 3B). In addition, flow cytometry showed that overexpression of miR-124 enhanced the apoptosis of TE-1 and SKYSE30 cells (Figures 3C, 3D), and overexpression of PDCD6 also restored the apoptosis of TE-1 and SKYSE30 (Figure 3C, 3D). These results

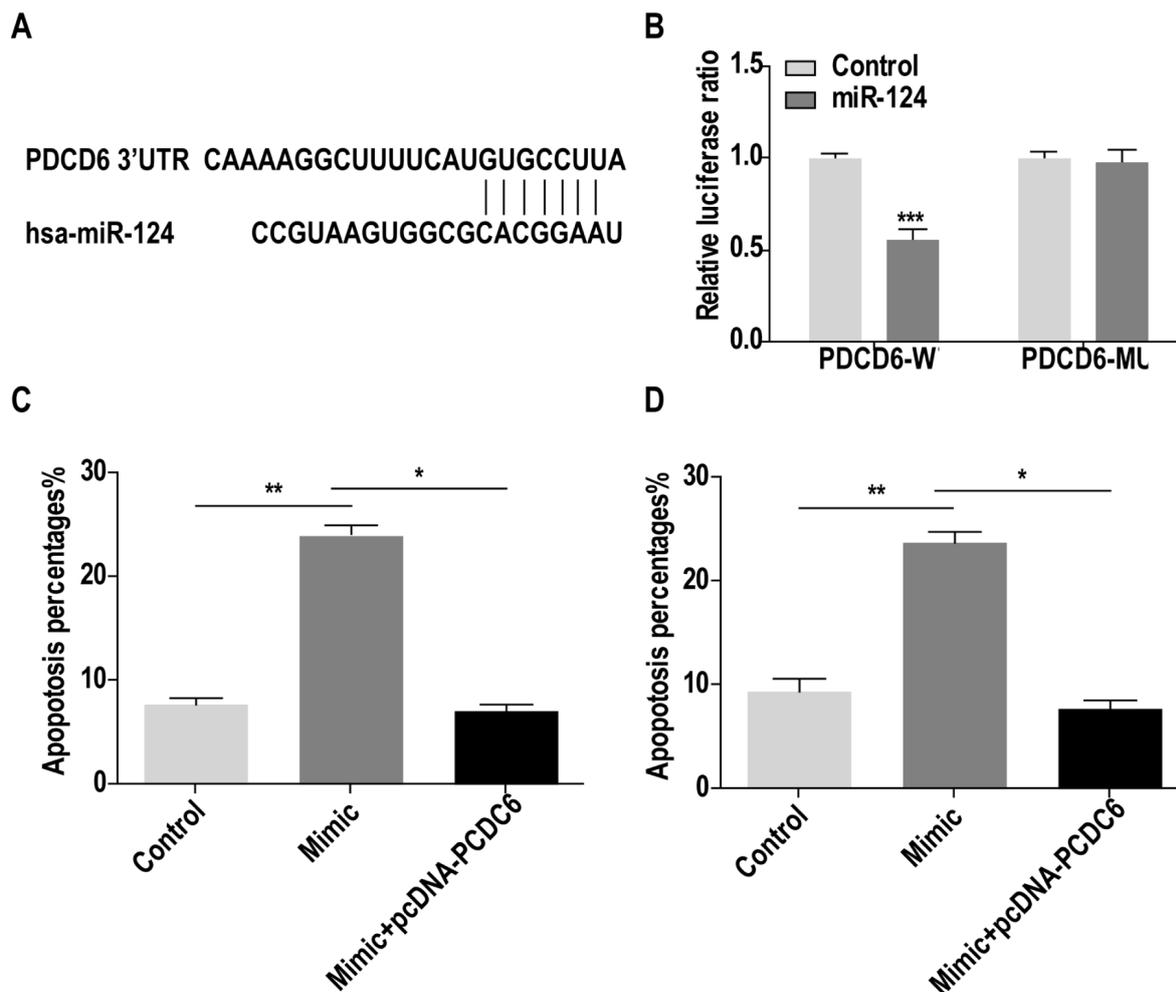
suggested that miR-124 may exert its biological function through selectively combining with PDCD6.

## Discussion

At present, the treatment of malignant tumors, such as esophageal cancer, is still limited to surgery, chemotherapy and radiotherapy. Although some progress has been made in individual treatment and comprehensive treatment in recent years,



**Figure 2.** Highly expressed miR-124 inhibited proliferation of esophageal cancer cells and promoted apoptosis. **A:** Overexpression of miR-124 inhibited TE-1 cell viability. After knockdown of miR-124 expression, the results were the opposite. **B:** Overexpression of miR-124 inhibited SKYSE30 cell viability. After knockdown of miR-124 expression, the results were the opposite. **C:** Overexpression of miR-124 inhibited cell migration of TE-1 cells. After knockdown of miR-124 expression, the opposite was true. **D:** Overexpression of miR-124 inhibited cell migration in SKYSE30 cells. After knockdown of miR-124 expression, the opposite was true. **E:** Overexpression of knockdown of miR-124 promoted the apoptosis of TE-1 cells. After knockdown of the expression of miR-124, the results were reversed. **F:** Overexpression of miR-124 promoted the apoptosis of SKYSE30 cells. After inhibiting the expression of miR-124, the results were reversed. \* $p < 0.05$ , \*\* $p < 0.01$ .



**Figure 3.** miR-124 can target PDCD6. **A:** The binding site of miR-124 on PDCD6. **B:** Luciferase assay showed that miR-124 can bind to PDCD6. **C:** Overexpression of miR-124 promoted the apoptosis of TE-1 cells. However, after overexpression of PDCD6, cell apoptosis was reduced. **D:** Overexpression of miR-124 promoted apoptosis in SKYSE30 cells. After overexpression of PDCD6, cell apoptosis was reduced. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

the overall survival rate of patients has not been significantly improved. Therefore, studies on the pathogenesis of malignant tumors such as esophageal cancer can provide a new direction for the further search of molecular therapeutic targets.

MiRs are stable in human serum and plasma and are tissue-specific. They are relatively stable, reproducible and consistent among different individuals in the same species [14]. In tumors, miRs can exert functions similar to oncogenes or tumor suppressor genes, and are closely related to tumor development, invasion and metastasis. A large number of studies in esophageal cancer has demonstrated that miRs also exert important functions and can participate in cell proliferation regulation. Studies found that the prediction rate of miRs in patients with esophageal adenocarcinoma reached 77%, while in patients with esophageal squamous cell carcinoma reached 82% [15]. Compared with normal esophageal squamous epi-

thelium, the specific miR expression in low-grade dysplasia, high-grade dysplasia and esophageal adenocarcinoma were predicted to reach 60, 90 and 100%, respectively [16]. On the other hand, there are many regulatory sites between miR and esophageal cancer. MiRs can regulate proliferation, invasion, metastasis and apoptosis of esophageal cancer cells through different mechanisms. Zhu et al. found that miR-16 can downregulate SOX6 and RECK to promote esophageal cancer cells proliferation and inhibit apoptosis [17]. Another study found that increased miR-208 expression can downregulate SOX6 level, which led to decreased p21 expression and upregulation of cyclin D1, promoting cell proliferation, cell cycle progression and tumor formation [18]. Studies also reported that miR-203 can act on BMI-1 and LASP1 respectively, thereby reducing the formation of esophageal cancer cell colonies and inhibiting invasion and migration of cancer cells [19,20].

Programmed cell death (PDCD) is a costimulatory molecule belonging to the CD28/CTLA-4 family. In recent years, several studies have demonstrated that the PDCD family was closely related to the occurrence and development of various diseases. PDCD4 is a newly discovered tumor suppressor gene associated with cell cycle and apoptosis. The expression of PDCD4 was found significantly decreased in lung cancer tissues and was associated with the grade of malignancy and poor prognosis [21]. In gastric cancer, the expression of PDCD4 was lower than that in paracancer and normal tissues, suggesting that PDCD4 may participate in the development of gastric cancer [22]. Other studies have shown that PDCD6 was upregulated in lung and liver cancer tissues compared with normal tissues, indicating that PDCD6 play a crucial role in promoting the survival of tumor cells in addition to the previously known apoptosis-inducing function [23]. In this study, we found that miR-124 and its target gene PDCD6 were abnormally expressed

in esophageal cancer tissues. Further functional verification by overexpressing and knocking down miR-124 revealed that high expression of miR-124 inhibited the proliferation and migration of esophageal cancer cells and promoted cell apoptosis by inhibiting PDCD6.

## Conclusions

In summary, this study found that miR-124 was lowly expressed in esophageal cancer tissues, and the low expression of miR-124 could promote the proliferation as well as the migration of esophageal cancer cells and reduce cell apoptosis. In addition, miR-124 may be involved in the development of esophageal cancer through targeted regulation of PDCD6 expression.

## Conflict of interests

The authors declare no conflict of interests.

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