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Original Article

MicroRNA-330-3p functions as an oncogene in human esophageal cancer by targeting programmed cell death 4

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Abstract: MicroRNAs comprise a family of small non-coding RNA molecules that have emerged as key post-transcriptional regulators of gene expression. Aberrant miRNA expression has been linked to various human tumors. This study was aimed to identify novel miRNAs involved in the carcinogenesis of esophageal squamous cell carcinoma (ESCC) and their potential functions. We performed miRNA microarray and found that miR-330-3p was highly expressed in ESCC tumor tissues. qRT-PCR further confirmed the result in other 35 pairs of ESCC tumor tissues and ESCC cell lines. Ectopic expression of miR-330-3p significantly promoted ESCC cell proliferation, survival, migration, invasion *in vitro* and stimulated tumor formation in nude mice. Knockdown of miR-330-3p leaded to the opposite effects. The luciferase assay confirmed that miR-330-3p directly interacted with the PDCD4 mRNA 3' un-translated region (UTR). Moreover, expression of PDCD4 was inversely associated with miR-330-3p in ESCC tissues. Silencing of PDCD4 significantly promoted cell growth, cell migration, invasion and inhibited cisplatin-induced apoptosis in ESCC cells. This study suggested that miR-330-3p might play an oncogenic role in the development of ESCC partially via suppression of PDCD4 expression.

Keywords: Esophageal squamous cell carcinoma, miR-330-3p, cell proliferation, cell invasion, PDCD4

Introduction

Esophageal cancer is the eighth most common cancer worldwide and esophageal squamous cell carcinoma (ESCC) is the predominant subtype in eastern Asia and southern Africa. The incidence of ESCC is increasing recently, and the overall five-year survival rate is only 20-30%, ESCC has been the fourth largest cause of cancer mortality in China [1, 2]. Thus, understanding the mechanism of ESCC development is essential for improving disease diagnosis, treatment and prevention.

MicroRNAs (miRNAs) are small non-coding RNAs which regulate gene expression at post-transcriptional level. They function in various processes such as cell proliferation, apoptosis, and differentiation [3]. Altered expression of miRNAs have been reported in various types of cancer, and their expression profiles can be used as hallmarks for diagnosis, classification and prognosis of human malignancies [4]. Recent datas have provided evidence that miRNAs also involve in ESCC initiation and development. Several miRNAs have been found participating in ESCC cell growth and invasion, such as miR-21 [5], miR-10b [6], miR-92 [7], miR-375 [8]. Our group previously found that CpG island methylation status and polymorphisms in 3'UTR binding region of miRNA target genes could affect miRNA function in ESCC tissues [9, 10].

Here, using miRNA microarray, we found that miR-330-3p was one of the most upregulated genes in ESCC tissues (GSE61047). MiR-330-3p was a recently recognized miRNA whose role on oncogenesis was not clearly understood. Different researches reported contradictory role in different cancer carcinogenesis [11-14]. Nevertheless, its expression and function in ESCC remained largely unknown. Thus, the

present study was performed to investigate the effects of miR-330-3p on ESCC carcinogenesis and provided evidence that a novel downstream target gene programmed cell death 4 (PDCD4) was negatively regulated by miR-330-3p in ESCC.

Materials and methods

Cell lines, cell culture and tumor tissues

Two esophageal cancer cell lines (EC109 and KYSE150) were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and Cancer Institute and Hospital, Chinese Academy of Medical Sciences (Beijing, China), respectively. A human esophageal epithelial cell (HET-1A) was purchased from the Company of Guangzhou Jenniobio Biotechnology (Guangzhou, China). The cells were cultured in RPMI-1640 (Hyclone, Logan, UT, USA) medium with 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA) at 37°C with 5% CO_2. Human esophageal tissue specimens were kindly provided by the Department of Thoracic and Cardiac Surgery, Southwest Hospital (Chongqing, China). All patients were given their informed consent and the study was approved by the Ethics Committee of Third Military Medical University.

miRNAs, RNA interference and transfection

miRNA mimics, inhibitor, miRNA UP™ agomir and their negative controls, small interfering RNA against human PDCD4 were commercially synthesized from GenePharma (Shanghai, China). miRNA and siRNA transfection were performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocols. The sequence is listed in Supplementary Table 1.

MicroRNA array

Total RNA was isolated using TRIzol (Invitrogen) and miRNeasy mini kit (QIAGEN, Germany) according to manufacturer's instructions. RNA quality and quantity was measured by using nanodrop spectrophotometer (ND-1000, Nanodrop Technologies) and RNA Integrity was determined by gel electrophoresis. MiRNA microarray analysis was performed by the 7th generation of miRCURY™ LNA Array (v.18.0) (Exiqon) supplied by KangChen Corporation (Shanghai, China).

RNA extraction and real-time PCR

Total RNA was extracted from cells or tissues, and then 200 ng of total RNA was reversely transcribed to cDNA. Expression of miR-330-3p was quantified by SYBR-Green quantitative real-time PCR using an All-in-One miRNA qRT-PCR Kit (GeneCopoeia, USA). For PDCD4 and other genes detection, 200 ng of total RNA was converted to cDNA using oligo-dT primer (Takara, Japan) and the cDNA was amplified using SYBR Premix ExTaq (Takara, Japan). The expression of miR-330-3p and PDCD4 were normalized against the relative expression of U6 and GAPDH, respectively. The relative expression level was calculated using the $2^{-\Delta Ct}$ method. All primers are provided in Supplementary Table 1.

Cell viability assay

The esophageal cancer cells were planted in 96 well plates with 8×10^3 cells per well one day prior to transfection. When the cells reached 50-70% confluence, miRNA or siRNA was transfected. After incubation for 24 h, 48 h, 72 h and 96 h, 100 ul fresh medium and 10 ul Cell Counting Kit-8 (Dojindo, Japan) were together incubated for 2 h and then detected by absorbance at 450 nm. The cell viability assay was performed for four times.

Cell proliferation

5-ethynyl-2'-deoxyuridine (Edu) assay was used for detecting the ability of cell proliferation using Cell-Light Edu Apollo DNA in vitro Kit (RiboBio, Guangzhou, China). Cells were seeded into 96 well plates at density of 5000 cells. After transfected for 48 h, cells were incubated with 25 μM Edu for 4 h at 37°C. Cells were then fixed with PBS containing 4% paraformaldehyde for 30 min, followed by permeabilization with 0.5% TritonX-100 for 10 min at room temperature. After washed three times, cells were incubated for 30 min with 40 μl Apollo reaction. The DNA contents were stained with 40 μl of Hoechst 33342 for 30 min. Images were taken using a fluorescent microscope. Assays were performed three times with triplicate wells.

Cell cycle and apoptosis

Cell Cycle and Apoptosis Analysis Kit (Beyotime, China) and flow cytometry were used for analy-

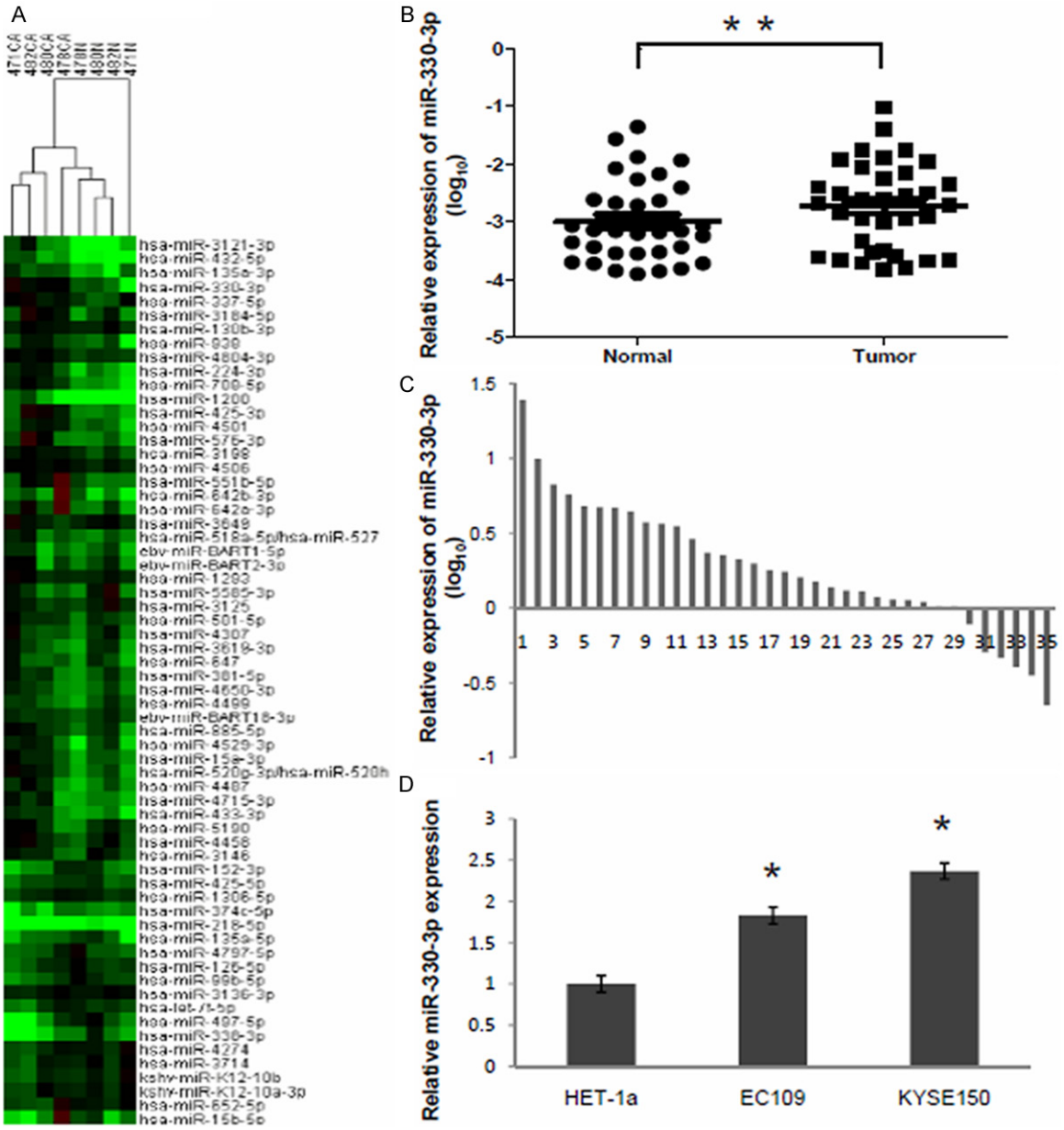

Figure 1. Expression of miR-330-3p in ESCC specimens. A. Differentially upregulated miRNAs selected from the miRNA array data. miRNA profiles from 4 paired patients tissues were clustered using Cluster 3.0. B. Expression of miR-330-3p was determined by qRT-PCR and normalized against U6. Data were analyzed using a $2^{-\Delta CT}$ method and expressed as $\log_{10}$ fold change. n = 35, **p < 0.005. C. miR-330-3p expression was frequently upregulated in tumor tissues. Normal tissues are defined as 1. n = 35. D. Expression of miR-330-3p in two ESCC cell lines (EC109 and KYSE150) and an immortalized esophageal squamous cell line (HET-1A). *p < 0.05.

sis of cell cycle. EC109 and KYSE150 cells were seeded into 6 well plates at density of 3.0×10^5 cells. The cells were then transfected with miR-330-3p oligonucleotide or PDCD4 siRNA, respectively. 48 hours after being transfected, cells were fixed in 70% ethanol and stored at 4°C overnight, then were incubated in RNase (1 μg/ml) at 37°C for 30 min, followed by staining of DNA with propidium iodide (50 μg/ml) for 30 min in the dark. Then samples were analyzed using Flow Cytometry. Cells apoptosis were performed with flow cytometry analysis by Annexin V-FITC Apoptosis Detection Kit (Beyotime, China). Cells were treated as before. Cells were stained with 5 μl Annexin V-FITC and 10 μl propidium iodide for 15 min and then ana-

lyzed by flow cytometry. Three repeat experiments were set up.

Cell migration and invasion assay

The 8 μm pore size (corning, USA) for migration or ECM gel-coated (sigma, USA) for invasion were placed into the wells of 24-well culture plates. After transfection with miRNA or siRNA, cells were incubated in serum-free medium for starvation and then 5×10^4 cells per well were seeded into the upper chamber. Then 600 μl 1640 medium with 20% FBS were added into the lower chambers. After incubation for 48 h at 37°C with 5% CO_2, cells on the top side were removed with a cotton swab and cells on the undersurface of the membrane were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. The migratory and invasive cells were counted from five randomly selected fields.

Luciferase reporter assay

PDCD4 3'-untranslated region was amplified by PCR and cloned at the SacI and MluI sites into pMIR-REPORT vector (Promega, USA). EC109 cells were co-transfected with a reporter construct (pmiR-PDCD4-WT plasmid or pmiR-PDCD4-MUT plasmid) and miR-330-3p mimics or negative control. Twenty-four hours later, luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega, USA). All constructs were confirmed by sequencing (BGI).

Western blotting

Total protein from cells or tissues was extracted in T-PER Tissue Protein Extraction Reagent (Pierce, USA) with protease inhibitors Phenylmethanesulfonyl fluoride. Protein were subjected to SDS-PAGE and then electrophoretically transferred to a PVDF membrane (Millipore, USA). After blocking with 5% nonfat dry milk, the membranes were incubated with primary monoclonal antibody against p21 (2947, Cell Signaling Technology), p27 (3686, Cell Signaling Technology), PDCD4 (9535, Cell Signaling Technology), CDK6 (3136, Cell Signaling Technology), cyclinA (4656, Cell Signaling Technology), GAPDH (KC5G5, Kangchen Bio-Tech). And then the membranes incubated with a horseradish peroxidase-conjugated secondary antibody. The immunoreactive proteins were detected by ECL Detection Systems (Thermo Scientific).

In vivo tumorigenicity

1×10^7 EC109 cells transfected with miR-330-3p UP™ agomir or agomir control were injected subcutaneously into the 4 weeks old female nude BALB/c mice. Tumor size was measured every few days after one week of injection. The tumor volume was calculated as length × $width^2/2$. All mice were killed in day 25, and the average tumor size and weight were estimated.

Statistical analysis

Statistical analysis was carried out using SPSS 16.0 (SPSS Inc., Chicago, IL, USA). Student's t test was used to analyze the results expressed as mean ± SD. A p value of < 0.05 was considered to be statistically significant. All experiments were performed in triplicate.

Results

MiR-330-3p is frequently up-regulated in human esophageal cancer tissues and cancer cell lines

In order to identify aberrant expressed miRNAs in human esophageal squamous cell carcinoma, miRNA microarray analysis with 4 ESCC tissues and adjacent non-tumor tissues was performed. Of all differentially expressed miRNAs, miR-330-3p was one of the up-regulated expression genes in tumor tissues (Fold change = 2.0) (**Figure 1A**). To validate microarray data, miR-330-3p levels were detected using SYBR-Green qRT-PCR in 35 pairs of ESCC tissues and adjacent normal tissues. As shown in **Figure 1B**, miR-330-3p expressions were significantly higher in ESCC tissues than that in the adjacent normal tissues ($P < 0.005$) and up-regulation of miR-330-3p ($\log_{10}$ fold change > 0) were observed in 29 (82.8%) cases (**Figure 1C**). This was consistent with the result of microarray. However, we found that miR-330-3p expression had no significant correlation with tumor clinical stage (Supplementary Figure 1). Then we detected the expression of miR-330-3p in human esophageal cancer cell lines. Compared with the immortalized human esophageal epithelial cell HET-1A, miR-330-3p expressions were upregulated in two ESCC cell lines (EC109

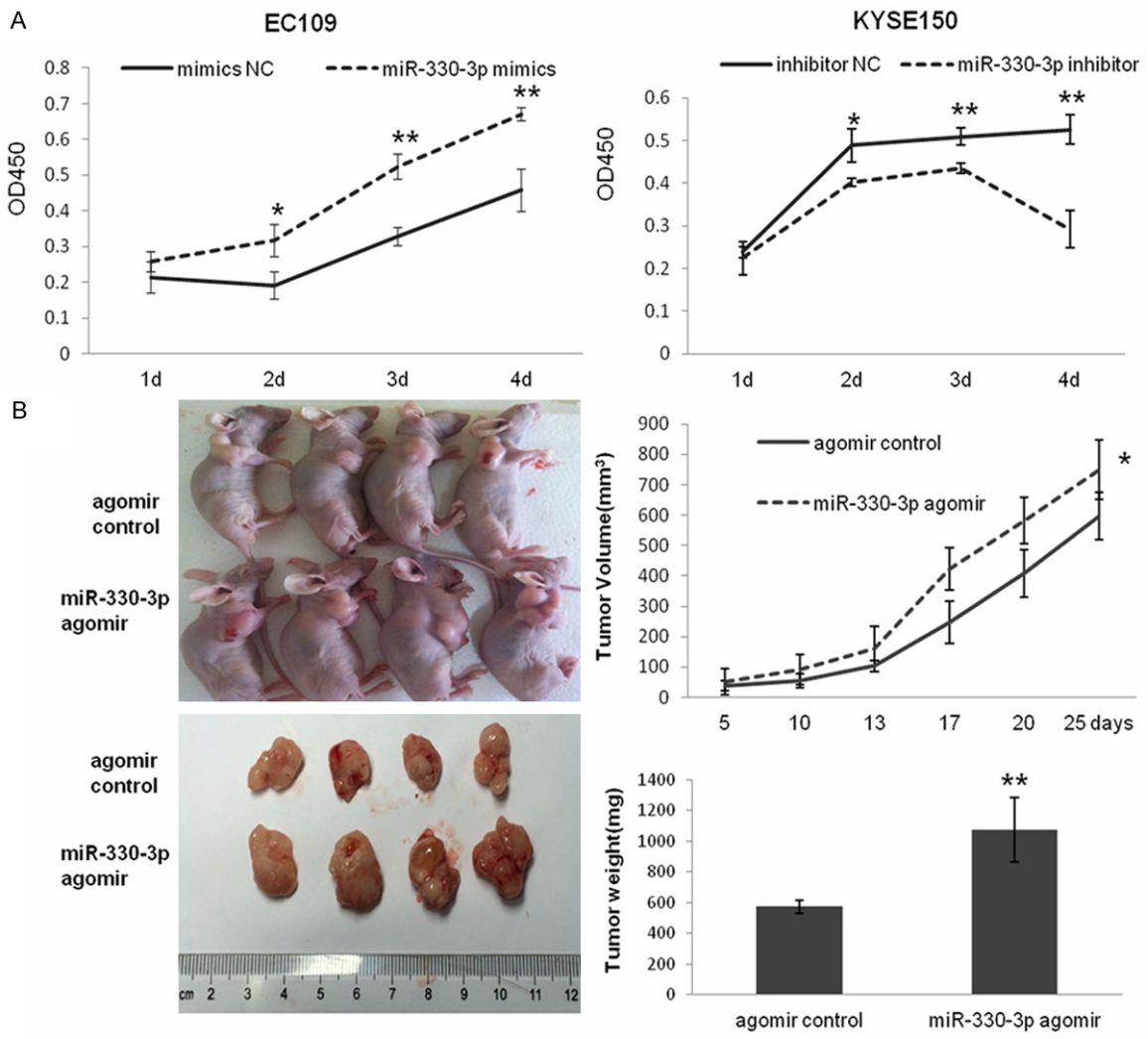

Figure 2. miR-330-3p promotes ESCC cell growth *in vitro* and *in vivo*. A. EC109 or KYSE150 cells transfected with miR-330-3p mimics, miR-330-3p inhibitor or their negative control were analyzed by CCK-8 assay. n = 4, error bars indicate SD. B. miR-330-3p promoted ESCC cell growth *in vivo*. Angomir-330-3p-transfected or angomir-control-transfected EC109 cells were injected into nude mice (left). Tumor growth curves (top right) and tumor weight (bottom right) in nude mice were shown. Data were mean ± SD, n = 4/group. *p < 0.05 **p < 0.01.

and KYSE150) (P < 0.05) (**Figure 1D**). Taken together, these results suggested that up-regulation of miR-330-3p was a frequent event in ESCC.

MiR-330-3p promotes esophageal cancer cell growth in vitro and in vivo

Due to the abnormal expression of miR-330-3p in ESCC tissues, we speculated that miR-330-3p might have some potential roles in ESCC development. In this regard, we examined the effect of miR-330-3p on the pathological process of ESCC cells. Noticing that miR-330-3p expression level was higher in KYSE150 than that in EC109 cells (**Figure 1D**), we increased the expression of miR-330-3p in EC109 cells with miR-330-3p mimics and decreased its expression in KYSE150 cells with miR-330-3p inhibitor (Supplementary Figure 2). Firstly, CCK8 cell viability assays were performed to determine the impact of miR-330-3p on cell growth *in vitro*. We found that cell population was greatly increased when EC109 cells were transfected with miR-330-3p mimics, whereas decreased by transfecting miR-330-3p inhibitor into KYSE150 cells (**Figure 2A**). To further investigate whether miR-330-3p could promote tumorigenicity *in vivo*, we conducted animal experiments. EC109 cells transfected with

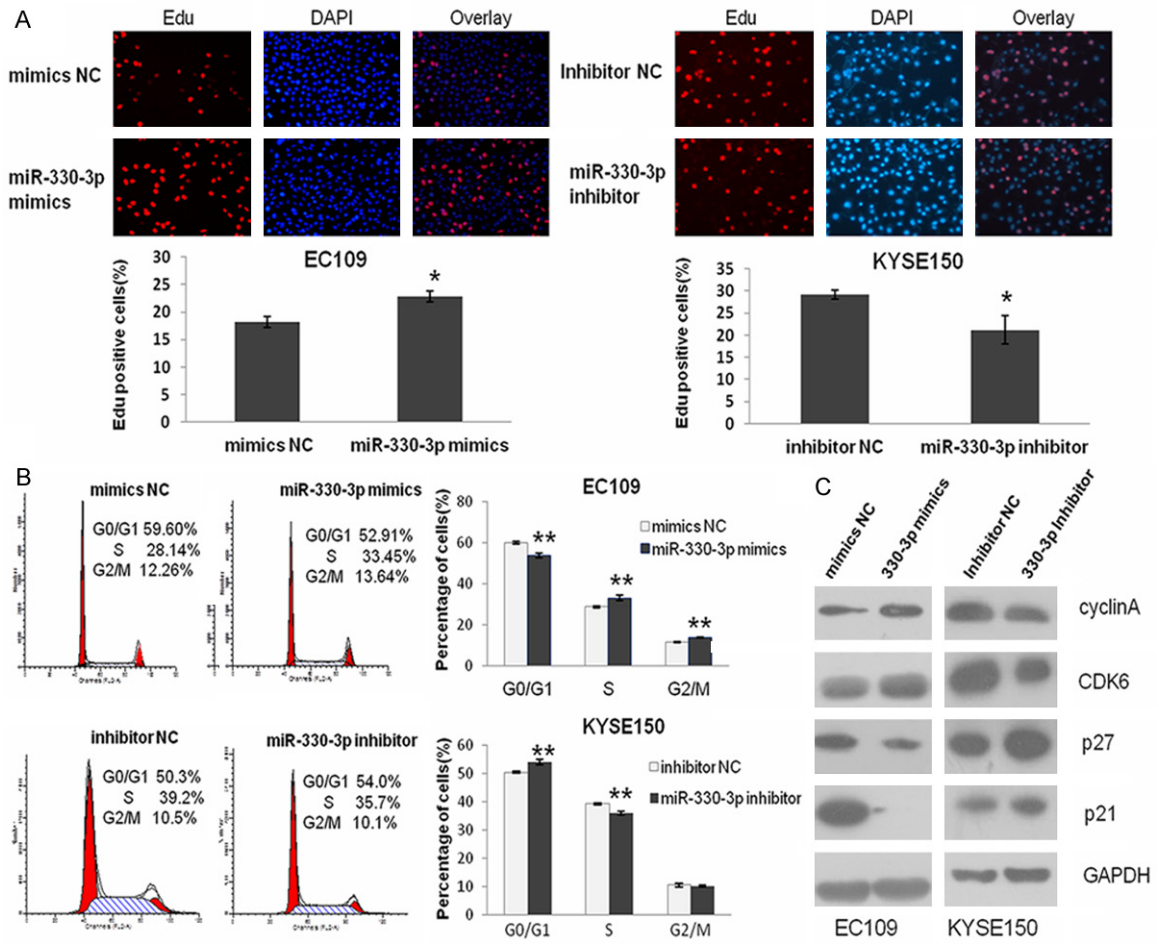

Figure 3. miR-330-3p regulates ESCC cell proliferation. A. Edu assay was used to determine cell proliferation of EC109 and KYSE150 cells after transfected with miR-330-3p mimics or inhibitor. B. Cell cycle distribution of EC109 and KYSE150 cells transfected with miR-330-3p mimics or inhibitor and their corresponding negative control. The percentage of cells in the different cell cycle phases was plotted, and the results represent the mean ± SD. *P < 0.05, **p < 0.01. C. Cell cycle-related gene protein expression including $p21^{Waf1/Cip1}$, $p27^{Kip1}$, CDK6 and cyclinA were determined by western blot in EC109 and KYSE150 cells.

miR-330-3p UP™ agomir or agomir control were injected subcutaneously into the nude mice. After 25 days, the mice were sacrificed and tumors were weighed. As observed in **Figure 2B**, tumors derived from miR-330-3p group were significantly larger than those from control group, whatever the volume or weight (p = 0.025 and 0.003, respectively). Thus, *in vitro* and *in vivo* study both suggested that miR-330-3p had the ability to promote ESCC cell growth.

MiR-330-3p can promote cell cycle progression of ESCC cells

Given that miR-330-3p obviously promoting ESCC cell growth *in vitro* and *in vivo*, we next determine whether miR-330-3p has any impact on cell proliferation of ESCC cells. As indicated in Edu assay, overexpressing miR-330-3p in EC109 cells could promote cell proliferation and KYSE150 cells with downregulated miR-330-3p expression showed a lower proliferation rate (**Figure 3A**). To further explore the contribution of miR-330-3p to cell cycle, miR-330-3p mimics or inhibitor were transfected in ESCC cells and then Flow Cytometry analyses were performed. Consistent with the promotion of cell proliferation, ectopic expression of miR-330-3p in EC109 cells decreased G1 phase cells and increased S/G2/M phase cells, while knocking-down miR-330-3p expression in KYSE150 cells leaded to G1 phase arrest (**Figure 3B**). We next examined the alteration of cell cycle-related genes after miR-330-3p mimics or inhibitor transfection. As expected, we found that overexpressioned miR-330-3p

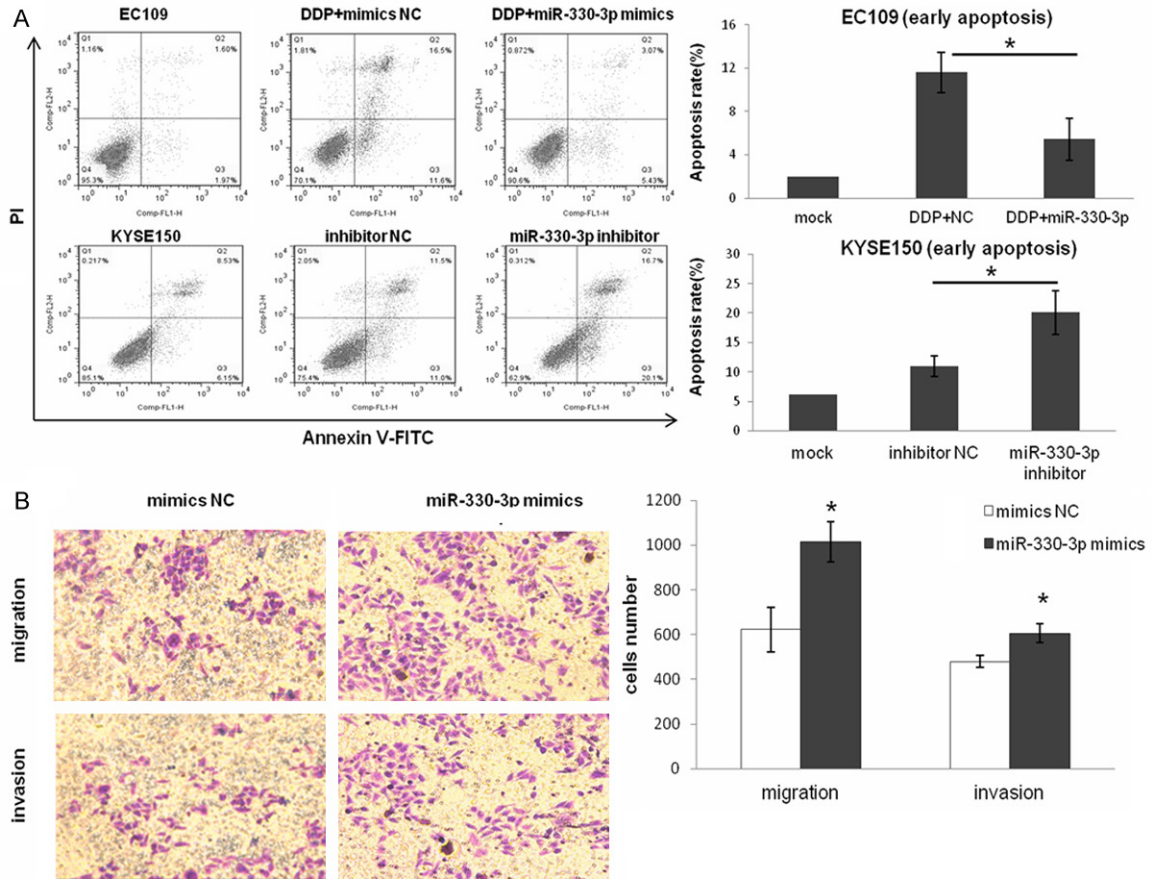

Figure 4. miR-330-3p inhibits cell apoptosis and stimulates cell migration and invasion of ESCC cells. A. Cell apoptosis was performed by flow cytometry using Annexin V-FITC staining. After incubation with 50ug/ml cisplatin, miR-330-3p overexpression resulted in a decrease in apoptotic rate of EC109 cells (top), while KYSE150 cells transfected with miR-330-3p inhibitor showed an increase of apoptosis (bottom). B. Transwell assay was used to determine cell migration and invasion ability of EC109 cells after transfected with miR-330-3p mimics. The data represent the mean ± SD. *P < 0.05, **p < 0.01.

caused downregulation of cyclin-dependent kinase inhibitors $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ and upregulation of S-phase protein CDK6 and cyclinA in EC109 cells (**Figure 3C**). Concomitant with these findings, over-expression of miR-330-3p significantly upregulated several cell cycle regulatory genes mRNA expression, including CCND1, CCND2, CDK4 and C-myc (Supplementary Figure 3). And an opposite result was observed in KYSE150 cells transfected with miR-330-3p inhibitor (**Figure 3C** and (Supplementary Figure 3)). Taken together, we speculated that miR-330-3p stimulated ESCC cell proliferation possibly through promoting G1/S transition.

MiR-330-3p inhibits cisplatin-induced apoptosis in ESCC cells

Cisplatin (DDP) is a well known chemotherapeutic agent for the treatment of cancers, and it can be applied to induce cell apoptosis by several signal transduction pathways [15]. Cisplatin can induce apoptosis of esophageal cancer cells, which has been confirmed in our work (data not shown). Meanwhile, overexpression of miR-330-3p antagonized cisplatin-induced apoptosis in EC109 cells, and down-regulation of miR-330-3p in KYSE150 cells resulted in a higher apoptosis rate than the control cells (**Figure 4A**). All together, we speculated that miR-330-3p could augment the resistance to cisplatin-induced apoptosis in ESCC cells.

MiR-330-3p stimulates ESCC cells migration and invasion

We next examined the effect of miR-330-3p on ESCC cell migration and invasion, transwell migration and matrigel invasion assay were performed. As shown in **Figure 4B**, significant

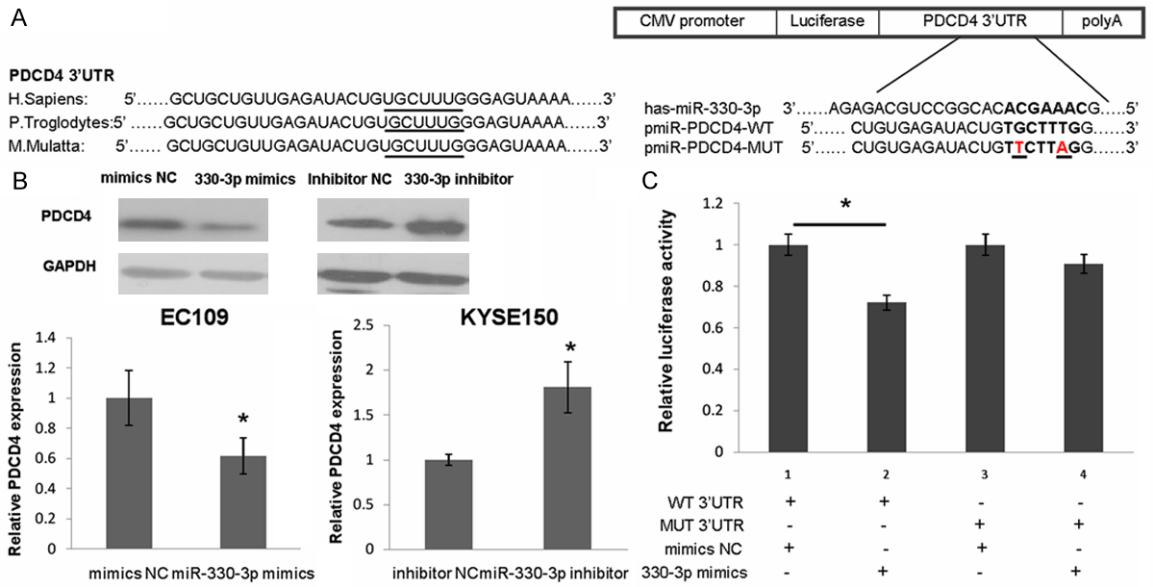

Figure 5. miR-330-3p directly target PDCD4 by binding to the PDCD4 3'UTR. A. Sequence conservation of target region of PDCD4 by in different species (left). Human PDCD4 gene 3'UTR region containing the wild-type or mutant miR-330-3p binding site was cloned into luciferase reporter vectors (right). B. Overexpression or downexpression of miR-330-3p in ESCC cells affected PDCD4 protein and mRNA expression. C. miR-330-3p repressed PDCD4 luciferase activity by targeted its wild-type but not mutant 3'UTR. *P < 0.05.

increases of migration and invasion cells were observed in EC109 cells transfected with miR-330-3p mimics compared to control cells.

MiR-330-3p targets PDCD4 via binding to its 3'UTR

MiRNAs usually play important roles in regulation of gene expression by binding to 3'UTR of target mRNAs. *In silico* analysis (TargetScan, miRanda and PicTar), we found that the 3'UTR of PDCD4 mRNA contained a highly conserved binding site from position 433 to 439 for miR-330-3p (**Figure 5A**). To determine if miR-330-3p could regulate PDCD4 expression in esophageal cancer cells, we detected PDCD4 mRNA and protein level by RT-PCR and western blot. In EC109 cells, the protein and mRNA expression of PDCD4 were significantly suppressed by miR-330-3p overexpression (**Figure 5B**). Conversely, the expression level of PDCD4 was markedly excited by miR-330-3p inhibition in KYSE150 cells (**Figure 5B**). To further determine the relationship between PDCD4 and miR-330-3p, we cloned the wild-type and mutant 3'UTR of PDCD4 mRNA that included the binding site of miR-330-3p into pMIR-REPORT vector, named pmiR-PDCD4-WT and pmiR-PDCD4-MUT respectively. And then these constructs were co-transfected with miR-330-3p mimics or mimics control into EC109 cells. Compared with the NC, luciferase activities were decreased in cells cotransfected with pmiR-PDCD4-WT vectors and miR-330-3p mimics. However, when the binding site was mutated, this inhibition was attenuated (**Figure 5C**). Collectively, these data showed that miR-330-3p could regulate PDCD4 expression via direct targeting its 3'UTR. We next tested the expression of PDCD4 in ESCC and normal esophageal tissues. As indicated in **Figure 6A**, **6B**, the expression of PDCD4 protein and mRNA were both down-regulated in ESCC tissues. In addition, the Pearson correlation analysis showed that PDCD4 mRNA expression was inversely correlated with miR-330-3p expression ($r = -0.5421$, $p = 0.0008$) (**Figure 6C**). These results suggested that PDCD4 might be a direct downstream target for miR-330-3p in ESCC cells.

Effect of PDCD4 loss-of-function on cell growth and invasion in ESCC cell lines

Given that miR-330-3p could promote cell proliferation, invasion and migration as well as inhibit the expression of PDCD4 in ESCC cells, we predicted that miR-330-3p might affect the biological behavior of ESCC cells by specifically regulating PDCD4. To determine the effects of PDCD4 on ESCC cells, we performed loss-of-

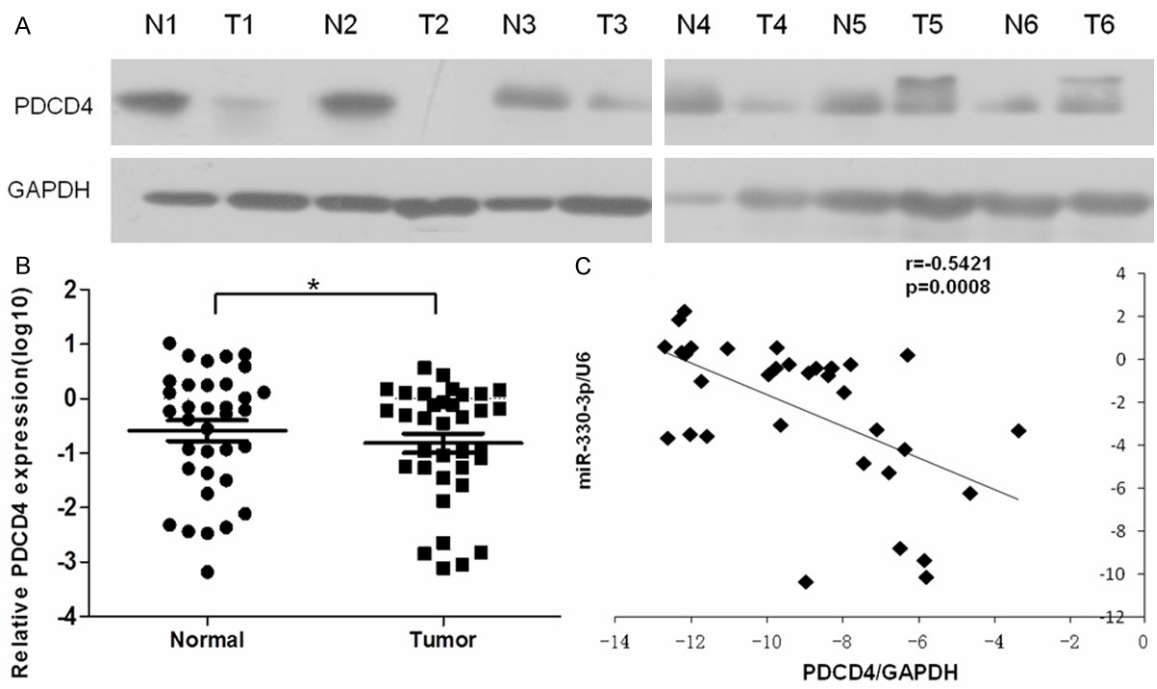

Figure 6. miR-330-3p and PDCD4 are inversely correlated in ESCC tissues. PDCD4 expression was frequently down-regulated in cancer tissues as showed by western blot (A) and qPCR (B). Data were analyzed using a $2^{-\Delta CT}$ method and expressed as $\log_{10}$ fold change. n = 35, **p < 0.01. (C) Scatter plots showed the inverse association between miR-330-3p and PDCD4 expression (r = -0.5421, p = 0.0008).

function study using PDCD4-specific siRNA. Two siRNAs were synthesized, and the one with better silence effect was chosen to use in the subsequent experiments (Supplementary Figure 4). CCK8 and Edu assay revealed that inhibition of PDCD4 promoted cell growth and proliferation in ESCC cells (**Figure 7A**, **7B**). Furthermore, Flow cytometric analysis showed that knockdown of PDCD4 could promote G1/S transition (**Figure 7C**) and inhibit cisplatin-induced apoptosis in both two ESCC cell lines (**Figure 7D**). In addition, transwell assay indicated that knockdown of PDCD4 promoted migration and invasion of EC109 cells (**Figure 7E**). Therefore, down-regulation of PDCD4 played a facilitative role on ESCC cell proliferation and invasion. These results are similar to those obtained with over-expression of miR-330-3p, which indicated that miR-330-3p might play an oncogenic role in the development of ESCC partially via downregulating PDCD4 expression.

Discussion

MiRNAs are the most important gene-expression regulator found so far. Aberrant expression of miRNAs were reported to function in cancer initiation and progression [16]. In this study, we first identified dysregulated miRNAs in human ESCC tumor tissues and their adjacent normal tissues using miRNA microarray. Specially, miR-330-3p was one of the up-regulated miRNAs in ESCC tissues. This result was further confirmed by qRT-PCR in other 35 paired tissues and esophageal squamous cancer cell lines. Unfortunately, there was no significant relationship between miR-330-3p expression and clinical stage. This might be due to small population size, and further investigation of a larger sample was necessary to study its clinical significance and prognostic evaluation in ESCC. In consistent to our results, Lee KH's miRNA expression profiling data also showed up-regulation of miR-330 in ESCC tissues [17]. Expressions of miR-330-3p in other types of cancer were controversial, previous studies showed that miR-330-3p was up-regulated in glioblastoma and metastatic brain cancer [12, 18], while down-regulated in prostate cancer [11]. Different expression status suggested that miR-330-3p may exert diverse effects in different cancers.

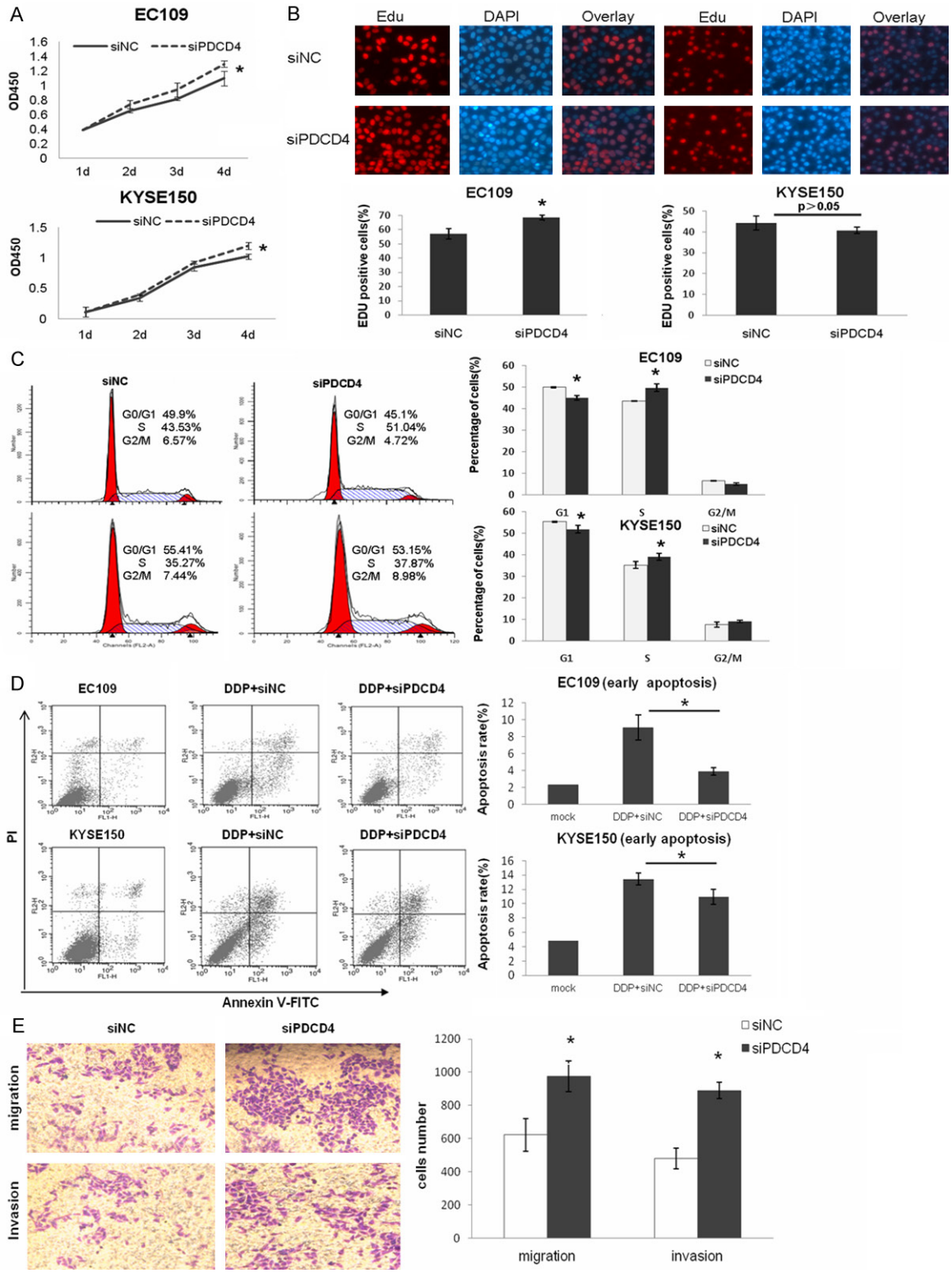

Figure 7. PDCD4 downregulation promotes ESCC cell proliferation and invasion *in vitro.* (A) Down-regulation of PDCD4 promoted cell growth in both EC109 and KYSE150 cells. *p < 0.05. Knockdown of PDCD4 accelerated cell proliferation (B), G1/S transition (C) and inhibited cisplatin-induced cell apoptosis (D). *p < 0.05. (E) EC109 cells transfected with si-PDCD4 exerted an increase in cell migration and invasion ability.

According to the abnormal expression of miR-330-3p in ESCC tissues, we speculated that miR-330-3p might contribute to ESCC carcinogenesis and progression. CCK8 assay indicated miR-330-3p could promote ESCC cell growth *in vitro*. Also in nude mice, tumors with overexpressed miR-330-3p emerged a significantly faster growth rate than the control. These indicated miR-330-3p had the ability of stimulating ESCC cell growth. For cell growth was affected by both cell proliferation and apoptosis, we wondered which was responsible to the effect elicited by miR-330-3p or both. Edu assay indicated miR-330-3p had the ability to stimulate ESCC cell proliferation. FACS studies showed that ESCC cells with high miR-330-3p levels had a lower proportion of G0/G1 cells. Overexpression and knockdown experiments showed that miR-330-3p levels were positively related to CDK6 and cyclinA levels and negatively associated with $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ levels. CDK6 and cyclinA appeared at G1 phase before DNA synthesis and it was required for S phase entry and passage through G2/M phase [19]. $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ were members of the cyclin-dependent kinase inhibitors which could block cell cycle progression [20]. Therefore, the above results suggested that miR-330-3p could promote ESCC cell proliferation through facilitating G1/S transition. In addition, we found that miR-330-3p could protect ESCC cells against cisplatin-induced cell apoptosis. Thus, we suggested that growth stimulation effect caused by miR-330-3p may be mediated both by accelerating cell cycle progression and resisting apoptosis. Except for impact on cell growth, we also studied whether miR-330-3p could affect ESCC cell motility and found that ectopic expression of miR-330-3p promoted migration and invasion in ESCC cells. Taken together, our study first demonstrated that miR-330-3p played an oncogenic role in ESCC initiation and progression through promoting cell cycle, cell survival, migration and invasion.

Like the contradictory expressions of miR-330-3p, complicated roles were reported in different cancers. Similar to our results, transfection of breast cancer cells with miR-330 decreased G1 population and promoted cell survival [21]. Over-expression of miR-330 enhanced cell proliferation, migration and invasion, and inhibit apoptosis in glioblastoma cells [12]. On the contrary, ectopic expression of miR-330 were reported to inhibit cell growth in prostate [11] and colorectal cancer [13], and also inhibit cell motility in prostate cancer cells [14]. With in-depth understanding of miRNA, more and more research indicated that some miRNAs may function in tissue-dependent or context-dependent manners. Besides miR-330-3p, function of some other miRNAs was also found varied in different cancer types. MiR-9 inhibited ovarian cancer cell growth while promoted gastric cancer cell proliferation [22, 23]. Moreover, epithelial-mesenchymal transition was suppressed by inhibiting E-cadherin via miR-9 in breast cancer and colon cancer [24, 25], and miR-9 also promoted tumor metastasis by activating β-catenin pathway and inducing EMT via targeting E-cadherin [26]. However, miR-9 targeted matrix metalloproteinase 14 to inhibit invasion, metastasis, and angiogenesis of neuroblastoma cells [27]. miR-10a, miR-155 and miR-200 exhibited different function in different cancers too [28]. As we all know, miRNAs silenced gene expression by targeting 3'UTR of mRNAs, single miRNA could target different genes and one mRNA could be regulated by several miRNAs. Varied effects of the same miRNA might be due to regulating different downstream genes in different environment. E2F1 [11], sp1 [14] were target genes responsible for tumor suppressor role of miR-330-3p. Qu S demonstrated miR-330-3p played an oncogenic role in glioblastoma cells by regulating SH3GL2 gene [12]. We next identified the potential downstream targets of miR-330-3p in ESCC tissue.

Bioinformatical analysis suggested several mRNAs had binding sites for miR-330-3p, among which tumor suppressor gene PDCD4 drew our attention. PDCD4 was originally identified to inhibit transformation in a mouse model [29, 30]. Recent studies have pointed to that downregulation of PDCD4 could facilitate cell survival and invasion in a variety of cancers, such as breast cancer [31], colorectal cancer [32, 33], ovarian cancer [34] and hepatocellular carcinoma [35]. Coincidently, Fassan M's work showed that PDCD4 was downregulated in esophageal cancer and patient with higher level of PDCD4 had a longer disease survival rate [36]. These evidence suggested PDCD4 as a potential downstream target for miR-330-3p in ESCC tissues. So we investigated whether miR-330-3p could regulate PDCD4 expression in ESCC tissue. Ectopic expression of miR-330-3p decreased PDCD4 mRNA and protein levels

in ESCC cells, while downregulation leaded to the opposite effects. Luciferase reporter assay indicated that miR-330-3p could repress expression by targeting 3'UTR of PDCD4. Moreover, we found that PDCD4 was frequently downregulated in our ESCC tissues as Fassan M's work described and its expression was inversely correlated with miR-330-3p level. In addition, we also showed that knockdown of PDCD4 significantly promoted ESCC cell growth, invasion and migration, and inhibited apoptosis induced by cisplatin, indicating PDCD4 function as a tumor suppressor in ESCC cancer. Combination the negative regulation between miR-330-3p and PDCD4 and their opposite roles in ESCC, we now demonstrated that miR-330-3p might play the tumor stimulation role in ESCC through negatively regulating PDCD4 expression.

Inactivation of tumor suppressors was a key rate-limiting step in early stage of oncogenesis. PDCD4 was an important tumor suppressor function in either the initial or progression stage. Downregulation of PDCD4 expression was frequently found in a variety of cancer. Unlike other tumor suppressor genes, PDCD4 was not mutationally inactivated in human cancer [37]. Colburn NH who first discovered PDCD4's transformation suppressor role revealed that PDCD4 downregulation in cancer was attributable to increased proteasomal degradation mediated by protein kinase B (Akt) or 70-kDa ribosomal protein S6 kinase 1 (p70S6K1) [38]. Except for that, abnormal expressions of miRNAs which were recently-founded post-transcriptional gene silencers might be another prominent contributor to the decrease of PDCD4. Allgayer H found miR-21 down-regulated PDCD4 expression in colorectal cancer in 2008 [39], after then, PDCD4 expressions in many other types of cancer were revealed to be downregulated by miR-21 [40-42], including ESCC [43]. Our work revealed that miR-330-3p overexpression might be another novel mechanism leaded to downregulation of PDCD4 in ESCC.

In conclusion, for the first time, we demonstrated that miR-330-3p was frequently overexpressed in human esophageal squamous cell carcinoma. MiR-330-3p functioned as an oncogene in ESCC involving in promotion of cell growth, cell survival and invasion. PDCD4 was a novel target for miR-330-3p and contributing to ESCC carcinogenesis. These findings may provide a strategy for targeting miR-330-3p/PDCD4 as new evidence to ESCC diagnosis and therapy.

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Disclosure of conflict of interest

None.

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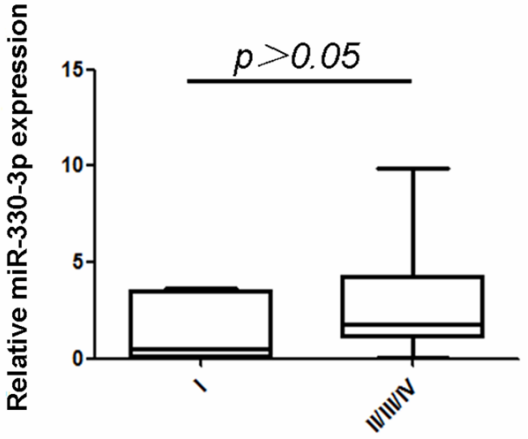
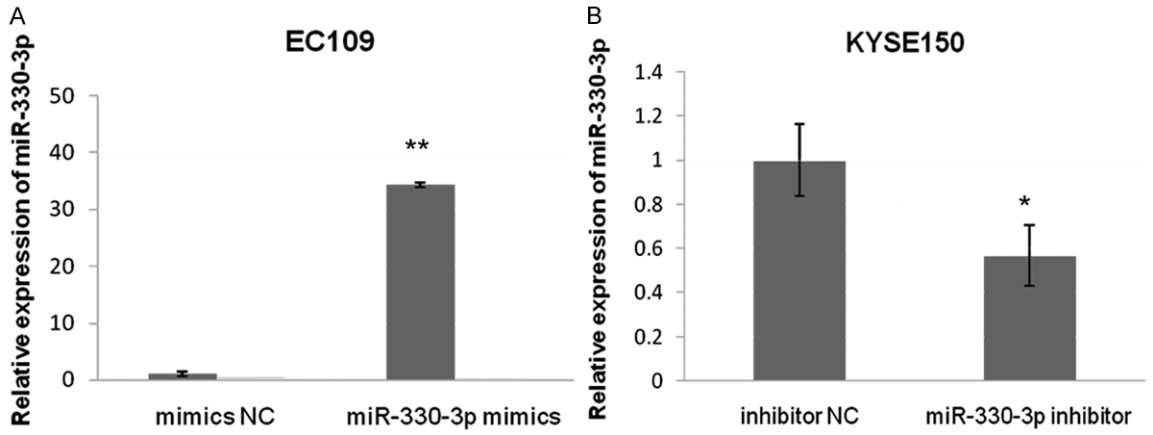
Supplementary Table 1. All primers and sequences are provided in the study

Name	Sequence
miRNAs and siRNAs	
hsa-miR-330-3p mimics sence	GCAAAGCACACGGCCUGCAGAGA
hsa-miR-330-3p mimics antisence	UCUGCAGGCCGUGUGCUUUGCUU
mimics control sence	UUCUCCGAACGUGUCACGUTT
mimics control antisence	ACGUGACACGUUCGGAGAATT
hsa-miR-330-3p inhibitor	UCUCUGCAGGCCGUGUGCUUUGC
inhibitor control	CAGUACUUUUGUGUAGUACAA
siPDCD4-1	GUGUUGGCAGUAUCCUUAG
siPDCD4-2	CAUUCAUACUCUGUGCUGG
hsa-miR-330-3pUP^{TM} agomir sence	GCAAAGCACACGGCCUGCAGAGA
hsa-miR-330-3pUP^{TM} agomir antisence	UCUGCAGGCCGUGUGCUUUGCUU
agomiR control sence	UUCUCCGAACGUGUCACGUTT
agomiR control antisence	ACGUGACACGUUCGGAGA ATT
For RT-PCR	
PDCD4 Forward	AGTGACGCCCTTAGAAGTGG
PDCD4 Reverse	TCATATCCACCTCCTCCACA
CCND1 Forward	TCCTCTCCAAAATGCCAGAG
CCND1 Reverse	GGCGGATTGGAAATGAACTT
CCND2 Forward	GCTGGCTAAGATCACCAACACA
CCND2 Reverse	CCTCAATCTGCTCCTGGCAA
CDK4 Forward	AAATCTTTGACCTGATTGGG
CDK4 Reverse	CCTTATGTAGATAAGAGTGCTG
C-MYC Forward	TGCTCCATGAGGAGACACC
C-MYC Reverse	CTTTTCCACAGAAACAACATCG
For Reporter Assay	
WT-PDCD4 3'UTR Forward	CGAGCTCTCTGACTGCCACTCCTTT
WT-PDCD4 3'UTR Reverse	CGACGCGTGATGTTCCAGCCACCTTT
MUT-PDCD4 3'UTR Forward	ATACTGTTCTTAGGGAGTAAAA
MUT-PDCD4 3'UTR Reverse	ACTCCCTAAGAACAGTATCTCA

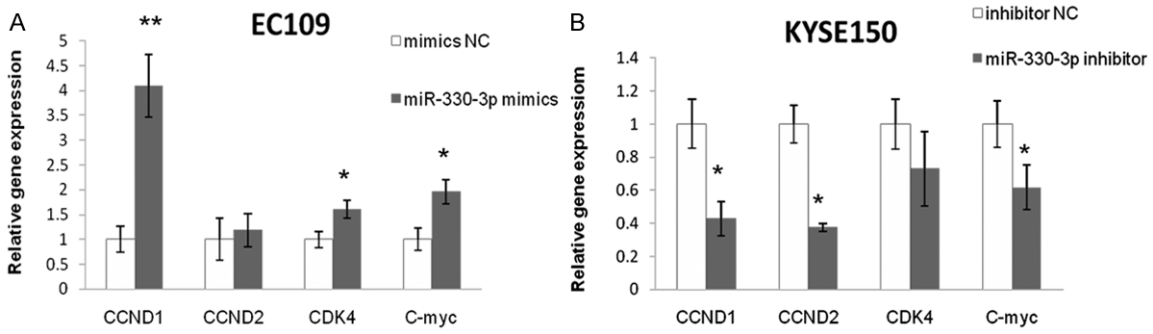
Restriction sites are underlined.



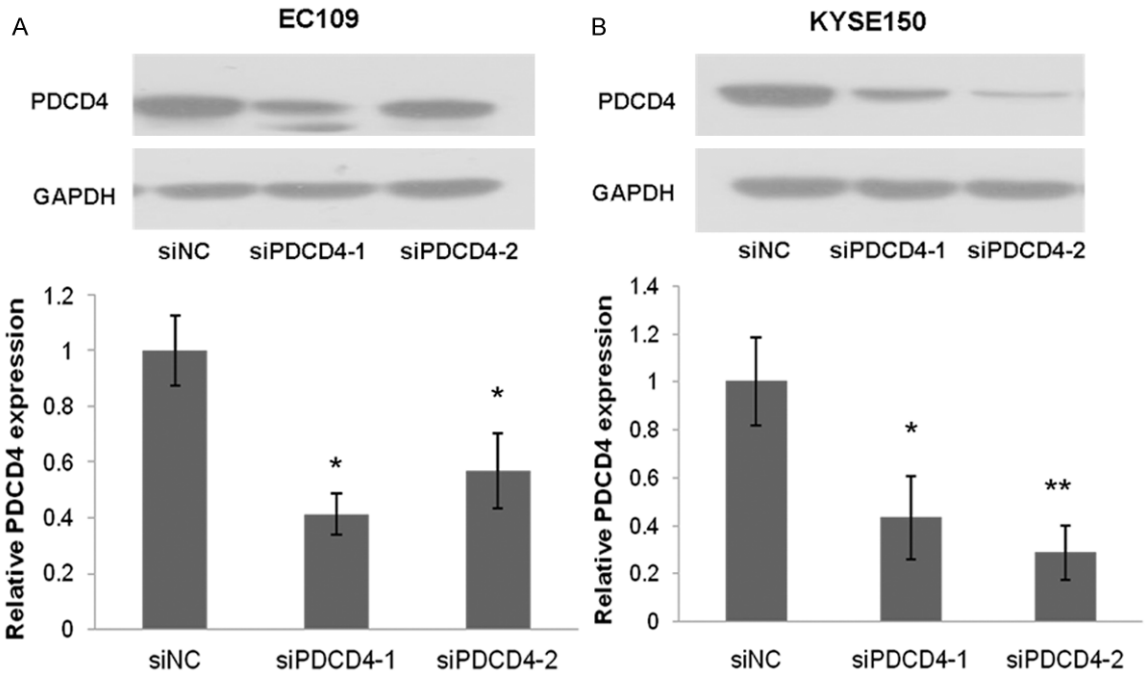
Supplementary Figure 1. Expression levels of miR-330-3p in different clinical stages of ESCC patients. MiR-330-3p expression had no significant correlation with high-grade ESCC tissues (II/III/IV) compared with low-grade tissues (I). $p > 0.05$.



Supplementary Figure 2. Expression of miR-330-3p in ESCC cell lines EC109 and KYSE150 after transfection of miR-330-3p mimics or inhibitor. Analysis of miR-330-3p expression following treatment of EC109 cells with miR-330-3p mimics or mimics NC (A) and treatment of KYSE150 cells with miR-330-3p inhibitor or inhibitor NC (B). *p < 0.05, **p < 0.01.



Supplementary Figure 3. Several cell cycle regulatory genes mRNA expression caused by overexpression or downexpression of miR-330-3p. MiR-330-3p overexpression significantly upregulated the mRNA levels of CCND1, CCND2, CDK4 and C-myc (A), and downregulated their expression by transfected miR-330-3p inhibitor (B). *p < 0.05, **p < 0.01.



Supplementary Figure 4. Analysis of PDCD4 protein and mRNA expression are reduced by small interfering RNA. PDCD4 ablations in EC109 (A) and KYSE150 (B) cells were confirmed by western blot and qPCR. *$p < 0.05$, **$p < 0.01$.