



## MiR-1299 is regulated by KCNQ1OT1 and inhibits cervical cancer progression

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### ABSTRACT

Cervical squamous cell carcinoma and endocervical adenocarcinoma (CESC) is the most common malignancy of the female genital tract. MiR-1299 serves as a tumor suppressor, while KCNQ1OT1 acts as an oncogene in multiple malignancies. This research was designed to investigate the impacts of miR-1299 and KCNQ1OT1 on CESC progression. The downstream target of miR-1299 and the underlying regulatory mechanism of KCNQ1OT1 action on miR-1299 were explored. RT-qPCR was applied for RNA expression detection in CESC tissues and cells. RNA immunoprecipitation, RNA pulldown and luciferase reporter assays were applied to evaluate the binding between molecules in CESC cells. Cell Counting Kit-8 and colony formation assays were used for the measurement of CESC cell viability and proliferation. Western blotting was utilized to measure levels of apoptosis-related in CESC cells. MiR-1299 was downregulated in CESC tissues and presented a negative correlation with KCNQ1OT1 expression. KCNQ1OT1 was directly bound to miR-1299 to negatively modulate miR-1299 expression in CESC cells. The proliferative ability of CESC cells was suppressed by miR-1299 overexpression and was facilitated by KCNQ1OT1 overexpression. CESC cells apoptosis was promoted by miR-1299 mimics and inhibited by KCNQ1OT1 overexpression. In addition, in *in vivo* studies, miR-1299 overexpression rescued the effects of KCNQ1OT1 overexpression on CESC xenograft tumor growth. Finally, KCNQ1OT1 was bound to miR-1299 to upregulate PDPK1 expression in CESC cells. Collectively, miR-1299 was regulated by KCNQ1OT1 and inhibited CESC progression *in vivo* and *in vitro*, suggesting the tumor-suppressor role of miR-1299 for CESC.

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### Introduction

Cervical squamous cell carcinoma and intracervical adenocarcinoma (CESC) belong to a type of cervical cancer and are the most common malignancies in the world, with over 570,000 new cases and 274,000 deaths each year (1). In recent years, long-time survival rates for CESC patients at the early stage have improved greatly, but the 5-year survival time for CESC patients at recurrent or metastatic stages is still poor (2). Therefore, there is an urgent need to find new biomarkers and prognostic indicators to guide the precise therapy for CESC.

A large proportion of the human genome is transcribed into noncoding RNAs, and only a minority of the genome is transcribed into messenger RNAs (mRNAs) (3). As highly conserved small noncoding RNAs, microRNAs (miRNAs) are 20-25 nucleotides at length (4). The dysregulation of miRNAs has been identified in CESC (5, 6). Although miRNAs lack protein-encoding ability (7), miRNAs can bind to target mRNAs 3' untranslated regions (3' UTRs) to modulate gene expression and thereby exert a tumor-suppressive or promotive effect by various cellular processes such as cell differentiation, proliferation, apoptosis, and autophagy (8).

Recently, miR-1299 has been revealed to exert an antioncogenic role in many cancers. MiR-1299 inhibits non-small-cell lung cancer cell motility and EMT process (9). Downregulation of miR-1299 rescues the repressive effects of RHPN1-AS1 silence on the proliferative ability and motility of ovarian cancer cells (10). MiR-1299 targets NEK2 to restrict the proliferative and migratory capacities of prostate cancer cells (11). MiR-1299 downregulates STAT3 expression to inhibit colon cancer cell growth (12). MiR-1299 restricts hepatocellular carcinoma cell proliferation and induces cell cycle arrest in G0/G1 phase by binding to CDK6 3'UTR (13). Moreover, based on GSE81137, miR-1299 exhibited downregulation in CESC tissues.

Recently, numerous reports have proposed a competitive endogenous RNA (ceRNA) mechanism in which long noncoding RNAs (lncRNAs) can sequester miRNAs to release downstream mRNAs (14). As an example, UICLM binds with miR-215 to enhance ZEB2 expression and promotes liver metastasis of colorectal cancer (15). SNHG1 is a negative regulator of miR-199a-3p to increase CDK7 expression, thus promoting prostate cancer cell proliferation (16). As a well-known oncogene, potassium voltage-gated

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channel subfamily Q member 1 opposite strand/antisense transcript 1 (KCNQ1OT1) binds to miR-15a to induce the upregulation of PD-L1, which is involved in the malignant progression of prostate cancer (17). KCNQ1OT1 silence inhibits the proliferative and migratory capacities of bladder cancer cells (18). KCNQ1OT1 upregulates CTNND1 by sponging miR-329-3p to facilitate the malignant phenotypes of colorectal cancer cells *in vitro* (19).

This study probed into the functions of miR-1299 and KCNQ1OT1 in malignant phenotypes of ME-180 and C33A cells and in mice xenograft tumors. In addition, the downstream target of miR-1299 was explored. This study may provide new insight into the understanding of the pathogenesis and treatment of CESC.

## Materials and Methods

### Tissue samples

CESC tissues (n=53) and adjacent normal tissues (n=53) were dissected from cancer patients at the Affiliated Suqian Hospital of Xuzhou Medical University. All tissue samples were preserved at -80°C. No patients had gotten anticancer treatments before they received the surgery. All patients had signed the informed consent for this research. The Ethics Committee of Affiliated Suqian Hospital of Xuzhou Medical University approved this exploration.

### Cell lines and cell culture

CESC cells ME-180 (#kl-xb-x0123, Ke Lei Biological Technology Co., Ltd) and C-33A (#CL-0155, Procell, Wuhan, China) were cultured in DMEM (Gibco, USA) under the standard conditions. The medium contained 10% FBS (Gibco) and was added to a penicillin-streptomycin solution (C0224, Beyotime).

### Cell transfection

MiR-1299 inhibitor and miR-1299 mimics were used to reduce and enhance the functions of miR-1299, respectively. NC inhibitors or NC mimics were the negative controls. shRNA targeting KCNQ1OT1 (sh-KCNQ1OT1) was performed to inhibit KCNQ1OT1 expression, and sh-NC was the negative control. pcDNA3.1/KCNQ1OT1 or pcDNA3.1/3-PDPK1 vectors were used to overexpress KCNQ1OT1 or PDPK1, and an empty pcDNA3.1 vector (pcDNA<sup>TM</sup>3.1/Hygro(+), catalogue number: V87020; Thermo Fisher) served as a control. sh-KCNQ1OT1, miR-1299 mimics/inhibitor, pcDNA3.1/KCNQ1OT1 and pcDNA3.1/PDPK1 vectors were commercially provided by Genepharma Company (Shanghai, China). Lipofectamine 2000 was used for the transfection of shRNAs and pcDNA3.1 vectors into ME-180 and C33A cells (Invitrogen, USA). Transfection was performed at room temperature for 48 h. MiRNA inhibitors/mimics were transfected into ME-180 and C33A cells at the concentration of 50 nM with Lipofectamine 3000 (Invitrogen) for 48 h.

### Reverse-transcription quantitative polymerase chain reaction (RT-qPCR)

TRIzol reagent (Invitrogen) was used for extracting total RNA from CESC tissues and cells. The concentration of the isolated RNA was measured using a Nanodrop ND-1000 spectrophotometer (Agilent Technologies, USA). Based on the manufacturer's instructions, 10 ng of miR-1299 was reverse transcribed to generate cDNA using a

microRNA Reverse Transcription Kit (EZB-miRT2). LncRNA and mRNAs were reversely transcribed into cDNA with a PrimeScript RT Reagent Kit (Invitrogen, USA). The generated cDNA was diluted in a 1:3 ratio. Expression of KCNQ1OT1, miR-1299 or mRNAs was assessed using a SYBR Premix Ex Taq II kit (Takara). snRNA U6 was a loading control for miR-1299. GAPDH was an internal control for KCNQ1OT1 and mRNAs. The criteria used to choose control genes for RT-qPCR were as follows: genes with too high or too low expression were excluded; the expression level was not affected by any exogenous factors; the genes couldn't be pseudogenes. The  $2^{-\Delta\Delta CT}$  method was used for RNA quantification.

### Hematoxylin and eosin (H&E) staining

H&E staining assays were implemented as described before (20). Briefly, cervical tissues were immobilized with 4% formaldehyde in phosphate buffer brine and then stained with hematoxylin and eosin. Standard microscopy (Olympus) was used to photograph the images.

### Cell viability and proliferation detection

For the CCK-8 assay, ME-180 and C33A cells underwent transfection of indicated vectors and were cultured in 96-well cell culture plates. The viability of ME-180 and C33A cells was evaluated with a CCK-8 kit (Dojindo) by measurement of optical density at a wavelength of 450 nm on a microplate reader (BD company) according to the manufacturer's instructions. For the colony formation assay, ME-180 and C33A cells were plated in a six-well cell culture plate. The colonies formed by ME-180 and C33A cells after cultivation for 7 days were stained with crystal violet. The colony number was counted using microscopy.

### Bioinformatics analysis

LncBase predicted v.2 (category: cancer/malignant) (<https://dianalab.e-ce.uth.gr/html/diana/web/index.php?r=lncbasev2%2Findex-predicted>) (21) was applied to identify the lncRNAs which can bind with miR-1299. Targetscan ([https://www.targetscan.org/vert\\_72/](https://www.targetscan.org/vert_72/)) (22) was utilized to reveal target mRNAs of miR-1299.

### Luciferase reporter assay

ME-180 and C33A cells were cultured in 12-well plates. The pmirGLO vectors carrying either wild-type or mutated KCNQ1OT1 were cotransfected with miR-1299 mimics/inhibitors. To confirm the role of KCNQ1OT1 and miR-1299 in regulating PDPK1, the wild-type or mutant PDPK1 3'UTR was inserted into pmirGLO vectors. Subsequently, the pmirGLO-PDPK1 3'UTR-WT/Mut was cotransfected with the indicated vectors. All vectors were purchased from GenePharma. A dual-luciferase reporter assay (Promega) was used to detect luciferase activities 48 h after transfection. Relative luciferase activity was defined as firefly luciferase activity/Renilla luciferase activity.

### RNA immunoprecipitation (RIP) assay

RIP experiments were performed with a commercial kit (#17-701, Millipore, USA). Lysates (ME-180) were cocultured with RIPA buffer that contained magnetic beads conjugated with human anti-Ago2 antibody (ab32381, Abcam) or controls anti-IgG (401455-M, Millipore). Purified RNA expression was detected by RT-qPCR as above mentioned.

## RNA pull-down assay

Cells were treated with 50 nM of biotinylated miR-1299 (Bio-miR-1299-Wt) or biotinylated miR-1299 mutated at the binding sequences complementary to KCNQ1OT1 or PDPK1 (Bio-miR-1299-Mut). Forty-eight hours later, cells were harvested, washed with PBS, and lysed in lysis buffer for 10 min. Magnetic beads were pre-coated with M-280 streptavidin. The lysate was incubated with the magnetic bead at 4°C for 3 h. Next, the beads were washed with pre-cooled lysis buffer twice, low salt buffer three times, and high salt buffer once. The bound RNAs were purified by TRIzol. RT-qPCR was used to determine the expression of KCNQ1OT1 or PDPK1 as abovementioned.

## Western blot

Protein samples from ME-180 and C33A cells or tumor tissues were acquired using RIPA lysis buffer (Beyotime) and separated by 10% SDS-PAGE. After transferring the proteins onto PVDF, 5% nonfat milk was added. Later, samples were treated with primary antibodies for Caspase-3 (ab2302, 1/50), Bcl-2 (ab32124, 1/1000), Bax (ab32503, 1/1000), Cyclin A1 (ab53699, 1/500), PCNA (ab18197, 1 µg/ml), PDPK1 (ab52893, 1/1000), CDK2 (ab32147, 1/1000), β-actin (ab6276, 1/5000) and Tubulin-α (ab7291, 1/5000), which were all obtained from Abcam (Cambridge, UK). Subsequently, a secondary antibody (ab205718, 1/2000, Abcam) was added and incubated with the membranes in the dark for 1 h. Finally, proteins were evaluated by a chemiluminescence detection system (Thermo Fisher Scientific, USA). All results were derived from three independent replicates.

## Animal assay

Female BALB/c-nu mice (n=25) at the age of 5-6 weeks old and weighing 18-20 g were purchased from Kay Biological Technology Co., Ltd. (Shanghai, China). Mice were divided into 5 groups with 5 mice in each group. All experimental procedures on mice were conducted following the institutional animal care and use committee of the Affiliated Suqian Hospital of Xuzhou Medical University. ME-180 cells were first infected with miR-1299-overexpressing lentiviral vector (lenti-miR-1299 mimics), with the lenti-NC mimic vector as a control. The lentiviral vectors GV159 (H1-MCS-CMV-EGFP, Genechem, China) were used for miR-1299 mimics transfection. After 48 h of transfection, ME-180 transfected with lenti-miR-1299 mimics were sampled. Suspensions of ME-180 cells ( $1 \times 10^7$  cells/ml) transfected with the indicated vectors were injected subcutaneously into the flanks of each mouse. Lenti-NC mimics served as the control for lenti-miR-1299 mimics; pcDNA3.1 served as the control for pcDNA3.1/KCNQ1OT1. Moreover, xenograft tumor volume was detected with vernier calipers and recorded every 2 days (from the 3<sup>rd</sup> day to the 15<sup>th</sup> day) to evaluate tumor growth. Fifteen days later, all mice were sacrificed by cervical dislocation. Xenograft tumors were immediately dissected, weighed, and lysed for western blot analysis. Tumor volume = (length × width<sup>2</sup>)/2. The maximum diameter of the tumor was 18 mm.

## Statistical analysis

Values are exhibited as the means ± standard deviation from three dependent repeats. Statistical analysis was conducted using SPSS 19.0 and graphs were generated

using the GraphPad Prism 5 software. For different comparisons of the mean values between the 2 groups, the Student's t-test was used. For that among groups, analysis of variance (ANOVA) followed by Tukey's *post hoc* test was used. Pearson correlation analysis was applied to analyze the relationship between miR-1299 and KCNQ1OT1 expression in CESC tissues. Kaplan-Meier analysis using log-rank test was applied for survival analysis.  $p < 0.05$  was considered statistically significant.

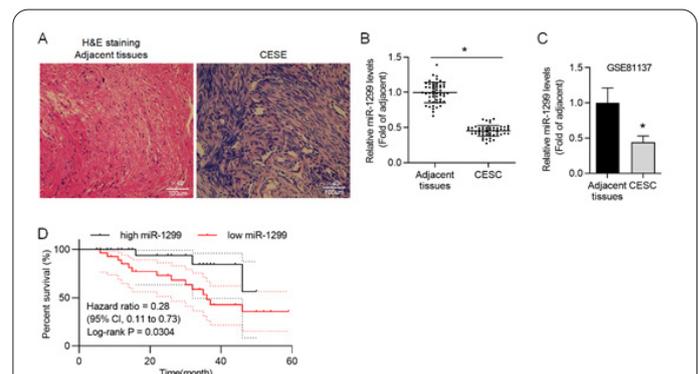
## Results

### MiR-1299 was downregulated in CESC tissues

Clinical samples, both tumor and adjacent nontumor tissues, were used in the H&E staining assay. As Figure 1A revealed, CESC tissues showed nuclear enlargement, pleomorphism, and frequent multinucleation. MiR-1299 was underexpressed in CESC tissues (Figure 1B). Additionally, the result from the GEO2R analysis of NCBI data (GSE81137) showed that the miR-1299 level was lower in CESC tissues compared to adjacent normal tissues (Figure 1C). Low expression of miR-1299 predicted an unfavorable prognosis for CESC patients (Figure 1D). Collectively, miR-1299 was lowly expressed in CESC tissues and correlated with the prognosis of CESC patients.

### MiR-1299 was negatively regulated by KCNQ1OT1

Based on prediction from LncBase, we screened the first 10 lncRNAs with the highest binding scores with miR-1299 (Supplementary Figure 1A) and detected their expression in CESC tissues. RT-qPCR results in Figure 2A indicated that CESC tissues presented higher KCNQ1OT1 expression than adjacent normal tissues. The expression of the other 9 lncRNAs exhibited no significant changes between CESC tissues and adjacent normal tissues (Supplementary Figure 1B). Additionally, KCNQ1OT1 is a well-known oncogene in multiple tumors through ceRNA

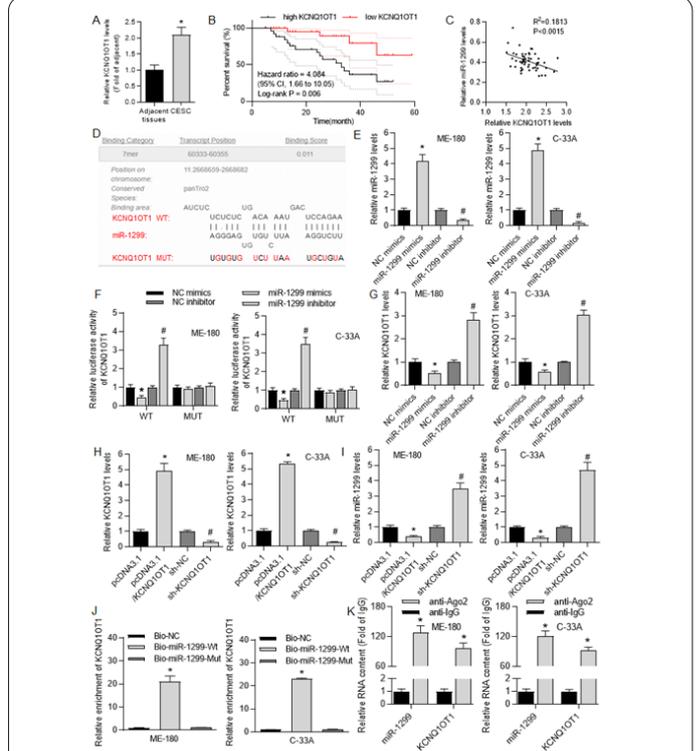


**Figure 1.** MiR-1299 was downregulated in CESC tissues. (A) H&E staining assays (magnification:  $\times 40$ ) were used to observe the morphology of CESC tissues and adjacent nontumor tissues. (B) RT-qPCR analysis was performed to measure miR-1299 levels in CESC tissues and adjacent nontumor tissues. Student t-tests were performed, and  $*p < 0.05$  compared with adjacent tissues. N=53. (C) MiR-1299 levels in CESC tissues and adjacent nontumor tissues were analyzed by GEO2R analysis according to NCBI data (GSE81137). Confidence limits: 95%. (D) Survival analysis of patients with high (N=25) or low (N=28) expression of miR-1299 based on the average value of miR-1299 expression in CESC patients. CESC: cervical squamous cell carcinoma and endocervical adenocarcinoma; H&E: hematoxylin and eosin; RT-qPCR: reverse-transcription quantitative polymerase chain reaction. NCBI: National Center for Biotechnology Information.

mechanisms to regulate miRNAs (23). Thus, we focused on KCNQ1OT1 in subsequent assays. Patients with lower KCNQ1OT1 expression had a significantly longer survival time (Figure 2B). Moreover, Pearson analysis illustrated a negative correlation between miR-1299 and KCNQ1OT1 expression in CESC tissues (Figure 2C). Binding sequences shared by KCNQ1OT1 and miR-1299 were obtained from LncBase v.2 (Figure 2D). Afterward, miR-1299 levels were successfully overexpressed by miR-1299 mimics and knocked down by miR-1299 inhibitors (Figure 2E). Additionally, the luciferase activity of pmirGLO-KCNQ1OT1-WT was weakened by miR-1299 mimics and increased by miR-1299 inhibitor (Figure 2F). KCNQ1OT1 levels were negatively regulated by miR-1299 in ME-180 and C33A cells (Figure 2G). Afterward, RT-qPCR analysis confirmed the overexpression and knockdown efficacy of KCNQ1OT1 (Figure 2H). As shown in Figure 2I, relative miR-1299 expression was negatively regulated by KCNQ1OT1 in ME-180 and C33A cells. RNA pull-down assay revealed that, compared to bio-NC, KCNQ1OT1 could be significantly pulled down by biotin-labeled miR-1299-Wt, but was not affected by biotin-labeled miR-1299-Mut (Figure 2J). Finally, both KCNQ1OT1 and miR-1299 were enriched in RNA-induced silencing complexes (RISCs) immunoprecipitated by Ago2 antibodies, implying that KCNQ1OT1 and miR-1299 coexisted in the RISCs, which indicated the binding of KCNQ1OT1 and miR-1299 (Figure 2K). In a word, miR-1299 bound to KCNQ1OT1, and their expressions were negatively correlated in CESC cells.

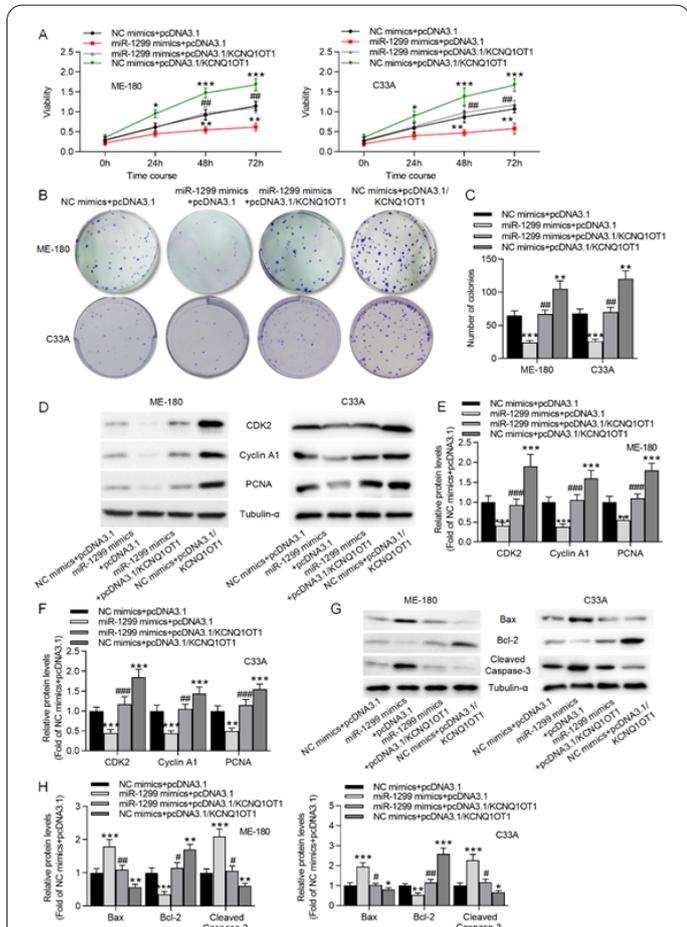
**KCNQ1OT1 modulated ME-180 and C33A cells proliferation and apoptosis by regulating miR-1299**

Functional assays were carried out for exploring the biological roles of KCNQ1OT1 and miR-1299 in ME-180 and C33A cells. First, miR-1299 expression was elevated by transfection with miR-1299 mimics and reduced by treatment of pcDNA3.1/KCNQ1OT1, while transfection of miR-1299 mimics + pcDNA3.1/KCNQ1OT1 neutralized miR-1299 expression in ME-180 and C33A cells. KCNQ1OT1 expression was reduced by transfection with miR-1299 mimics and increased by treatment of pcDNA3.1/KCNQ1OT1, while co-transfection of miR-1299 mimics+pcDNA3.1/KCNQ1OT1 neutralized KCNQ1OT1 expression in ME-180 and C33A cells (Supplementary Figure 1C). The viability of ME-180 and C33A cells was decreased by miR-1299 mimics and increased by pcDNA3.1/KCNQ1OT1 treatment. MiR-1299 mimics + pcDNA3.1/KCNQ1OT1 reversed the effects of miR-1299 mimics or pcDNA3.1/KCNQ1OT1 on cell viability (Figure 3A). Likewise, the number of colonies was reduced by miR-1299 overexpression and increased by KCNQ1OT1 overexpression in comparison with that in the NC mimics + pcDNA3.1 group. Co-transfection of miR-1299 mimics + pcDNA3.1/KCNQ1OT1 reversed the effects of miR-1299 overexpression or KCNQ1OT1 overexpression on ME-180 and C33A cell proliferation (Figure 3B-C). Subsequently, transfection with miR-1299 mimics decreased levels of CDK2, Cyclin A1 and PCNA in ME-180 and C33A cells. In contrast, their levels were increased by KCNQ1OT1 overexpression. All these effects were reversed by co-overexpression of KCNQ1OT1 and miR-1299 (Figure 3D-F). Finally, Bax and cleaved caspase-3 mRNA and protein levels in ME-180 and C33A cells were eleva-



**Figure 2.** MiR-1299 was negatively regulated by KCNQ1OT1. (A) Relative KCNQ1OT1 levels in CESC tissues and normal tissues were detected by RT-qPCR. Student t-tests were performed, and  $*p < 0.05$  compared with adjacent tissues. N=53. (B) Survival analysis of patients with high (N=26) or low (N=27) expression of KCNQ1OT1 based on the average value of KCNQ1OT1 expression in CESC patients. (C) The correlation between KCNQ1OT1 and miR-1299 expression in CESC tissues was determined by Pearson correlation analysis. N=53. (D) LncBase predicted v.2 was performed to predict the binding sequences between KCNQ1OT1 and miR-1299. (E) The overexpression and knockdown efficacy of miR-1299 in ME-180 and C33A cells was evaluated by RT-qPCR. Student's t-tests were performed;  $*p < 0.05$  compared with NC mimics, and  $\#p < 0.05$  compared with NC inhibitor. N=3. (F) The binding capacity between KCNQ1OT1 and miR-1299 was tested by luciferase reporter assay in ME-180 and C33A cells. Student's t-tests were performed;  $*p < 0.05$  compared with NC mimics, and  $\#p < 0.05$  compared with NC inhibitor. N=3. (G) Relative expression of KCNQ1OT1 under the influence of miR-1299 overexpression or inhibition in ME-180 and C33A cells was detected by RT-qPCR analysis. Student's t-tests were performed;  $*p < 0.05$  compared with NC mimics, and  $\#p < 0.05$  compared with NC inhibitor. N=3. (H) The overexpression and knockdown efficiency of KCNQ1OT1 in ME-180 and C33A cells were verified through RT-qPCR analysis. Student's t-tests were performed;  $*p < 0.05$  compared with pcDNA3.1,  $\#p < 0.05$  compared with sh-NC. N=3. (I) The effects of KCNQ1OT1 overexpression and knockdown on miR-1299 expression in ME-180 and C33A cells were measured through RT-qPCR analysis. Student's t-tests were performed;  $*p < 0.05$  compared with pcDNA3.1, and  $\#p < 0.05$  compared with sh-NC. N=3. (J) RIP assay was conducted to assess the binding capacity between KCNQ1OT1 and miR-1299 in ME-180 and C33A cells. Student's t-tests were performed, and  $*p < 0.05$  compared with anti-IgG. N=3. (K) An RNA pull-down assay was conducted to reveal the relative enrichment of KCNQ1OT1 pulled down by bio-miR-1299-Wt and bio-miR-1299-Mut.  $*p < 0.05$  compared with Bio-NC. N=3. KCNQ1OT1: potassium voltage-gated channel subfamily Q member 1 opposite strand/antisense transcript 1; CESC: cervical squamous cell carcinoma and endocervical adenocarcinoma; RT-qPCR: reverse-transcription quantitative polymerase chain reaction; NC: negative control; RIP: RNA immunoprecipitation; IgG: immunoglobulin G.

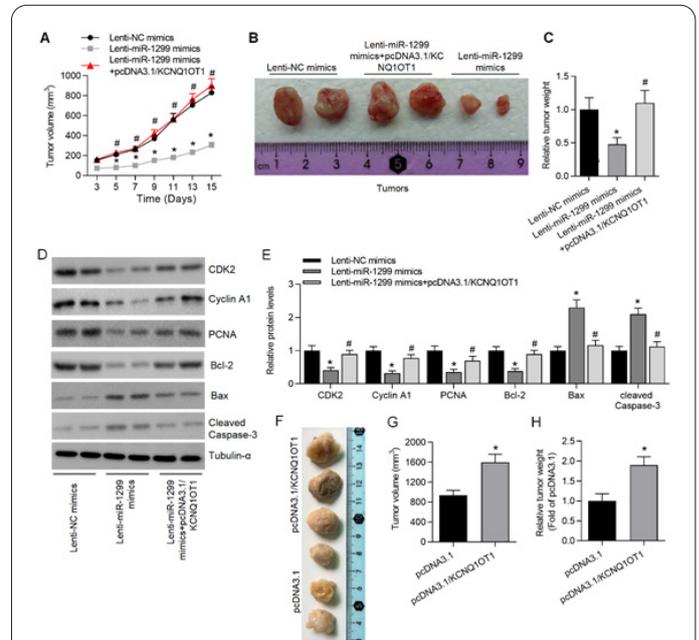
ted by miR-1299 mimics and repressed by transfection of pcDNA3.1/KCNQ1OT1, and Bcl-2 expression indicated an opposite pattern. All these effects caused by miR-1299 mimics or pcDNA3.1/KCNQ1OT1 were rescued by co-expression of KCNQ1OT1 and miR-1299 (Figure 3G-H). In conclusion, KCNQ1OT1 was bound to miR-1299 to modulate ME-180 and C33A cells proliferation, apoptosis, and cell cycle progression.



**Figure 3.** KCNQ1OT1 modulated ME-180 and C33A cell proliferation and apoptosis by regulating miR-1299. (A-C) CCK-8 and colony formation assays were performed to test ME-180 and C33A cell viability and proliferation. ANOVA was performed: \*\* $p < 0.01$ , \*\*\* $p < 0.001$  compared with NC mimics+pcDNA3.1; # $p < 0.01$  compared with miR-1299 mimics+pcDNA3.1 or NC mimics+pcDNA3.1/KCNQ1OT1. N=3. (D-F) The protein levels of CDK2, Cyclin A1 and PCNA in ME-180 and C33A cells were measured by western blot analysis with Tubulin- $\alpha$  as the loading control. ANOVA was performed: \*\* $p < 0.01$ , \*\*\* $p < 0.001$  compared with NC mimics+pcDNA3.1; ## $p < 0.01$ , ### $p < 0.001$  compared with miR-1299 mimics+pcDNA3.1 or NC mimics+pcDNA3.1/KCNQ1OT1. N=3. (G-H) The protein levels of Bax, Bcl-2, cleaved Caspase-3 in ME-180 and C33A cells were measured by western blot analysis with Tubulin- $\alpha$  as the loading control. ANOVA was performed: \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  compared with NC mimics+pcDNA3.1; # $p < 0.05$ , ## $p < 0.01$  compared with miR-1299 mimics+pcDNA3.1 or NC mimics+pcDNA3.1/KCNQ1OT1. N=3. KCNQ1OT1: potassium voltage-gated channel subfamily Q member 1 opposite strand/antisense transcript 1; CCK-8: Cell Counting Kit-8; CDK2: cyclin-dependent kinase 2; PCNA: proliferating cell nuclear antigen; BAX: B-cell lymphoma-2 Associated X; Bcl-2: B-cell lymphoma-2; Caspase 3: cleaved cysteinyl aspartate specific proteinase 3; ANOVA: analysis of variance; NC: negative control.

### KCNQ1OT1 interacted with miR-1299 to regulate tumor growth of CESC *in vivo*

To determine whether lncRNA KCNQ1OT1 and miR-1299 regulate tumor growth of CESC *in vivo*, animal assays were conducted. As revealed in Supplementary Figure 1D, stable ME-180 cells overexpressing miR-1299 were successfully established by infection with lenti-miR-1299 mimics. Additionally, miR-1299 expression was elevated in xenograft tumors by injection of ME-180 cells overexpressing miR-1299, and was decreased by injection of ME-180 cells overexpressing KCNQ1OT1; co-overexpression of miR-1299 and KCNQ1OT1 reversed these effects. KCNQ1OT1 expression was reduced in xenograft tumors by injection of ME-180 cells overexpressing miR-1299, and was increased by injection of ME-180 cells overexpressing KCNQ1OT1; co-overexpression of miR-1299 and KCNQ1OT1 rescued these effects (Supplementary Figure 1E). According to the experimental results, compared with the tumors formed by ME-180 cells in the lenti-NC mimic group, tumors formed by ME-180 cells in the lenti-



**Figure 4.** KCNQ1OT1 interacted with miR-1299 to regulate tumor growth of CESC *in vivo*. (A) Tumor growth curves were made according to the change in tumor volume on different days. ANOVA was performed: \* $p < 0.05$  compared with lenti-NC mimics; # $p < 0.05$  compared with lenti-miR-1299 mimics. N=5. (B) Xenografts from mice were photographed. N=5. (C) The xenograft weight was quantified. ANOVA was performed: \* $p < 0.05$  compared with lenti-NC mimics; # $p < 0.05$  compared with lenti-miR-1299 mimics. N=5. (D-E) Protein levels of CDK2, Bax, cleaved caspase-3, Bcl-2, Cyclin A1 and PCNA in xenografts were investigated by western blot analysis with Tubulin- $\alpha$  as the loading control. ANOVA was performed: \* $p < 0.05$  compared with lenti-NC mimics; # $p < 0.05$  compared with lenti-miR-1299 mimics. N=5. (F-H) The tumor size, volume and weight were detected in the pcDNA3.1 group and pcDNA3.1/KCNQ1OT1 group. Student's t-tests were performed, and \* $p < 0.05$  compared with pcDNA3.1. N=5. KCNQ1OT1: potassium voltage-gated channel subfamily Q member 1 opposite strand/antisense transcript 1; CESC: cervical squamous cell carcinoma and endocervical adenocarcinoma; ANOVA: analysis of variance; CDK2: cyclin-dependent kinase 2; BAX: B-cell lymphoma-2 Associated X; Caspase 3: cleaved cysteinyl aspartate specific proteinase 3; Bcl-2: B-cell lymphoma-2; PCNA: proliferating cell nuclear antigen; NC: negative control.

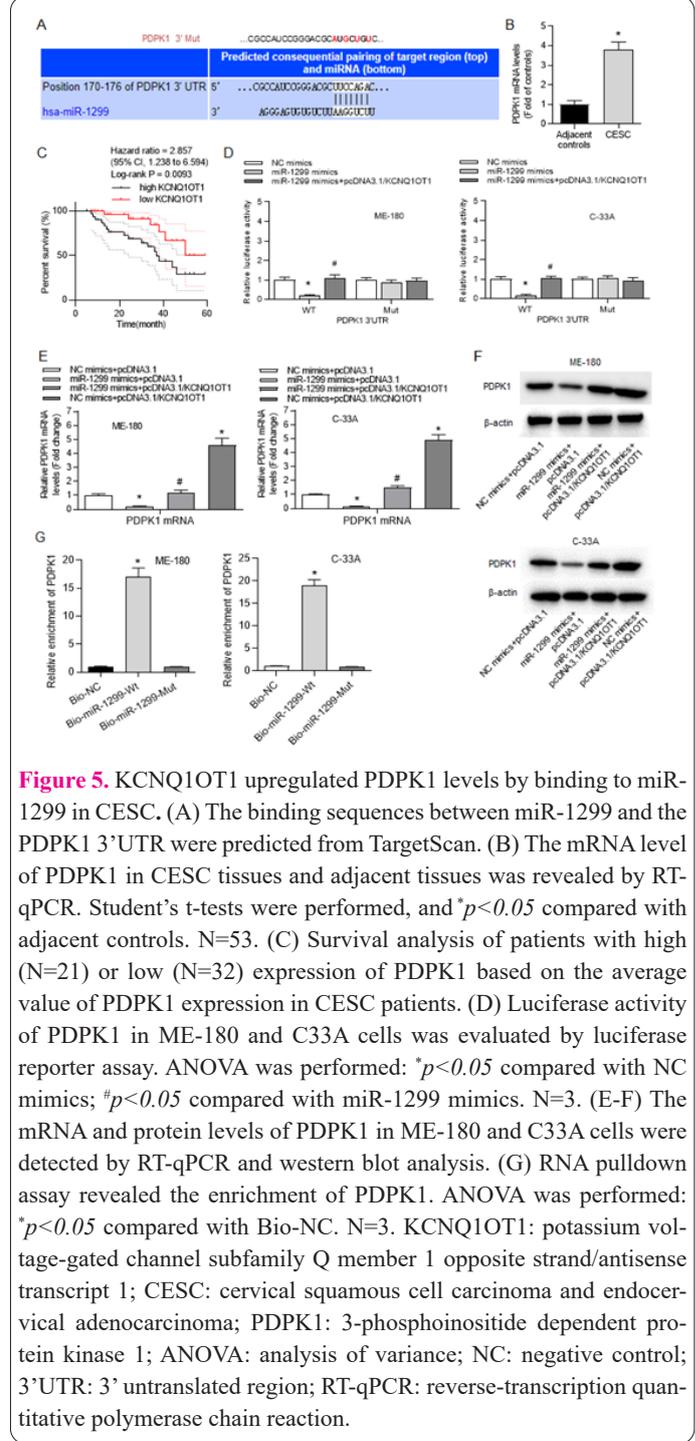
miR-1299 mimic group had smaller tumor volume and size and reduced weight. However, simultaneous KCNQ1OT1 overexpression reversed the impacts of lenti-miR-1299 mimics on tumor volume, size, and weight (Figure 4A-C). Furthermore, lenti-miR-1299 mimics mediated the reduction in CDK2, Cyclin A1, PCNA, and Bcl-2 protein levels, as well as promoted Bax and Cleaved Caspase-3 levels. However, simultaneous KCNQ1OT1 elevation rescued this phenomenon (Figure 4D-E). In addition, KCNQ1OT1 overexpression increased tumor size, volume, and weight (Figure 4F-H). Collectively, KCNQ1OT1 interacted with miR-1299 to affect tumor growth of CESC in vivo.

**KCNQ1OT1 upregulated PDPK1 expression levels by binding to miR-1299 in ME-180 and C33A cells**

Furthermore, the downstream targets of miR-1299 were identified. PDPK1 has been reported to promote cervical cancer progression (24, 25). Based on Targetscan prediction, PDPK1 was a target downstream of miR-1299. We hypothesized that PDPK1 was involved in a ceRNA network consisting of KCNQ1OT1 and miR-1299. The binding sites of miR-1299 on PDPK1 were predicted by Targetscan (Figure 5A). PDPK1 mRNA levels were upregulated in CESC tissues (Figure 5B). Patients with lower PDPK1 expression had a significantly longer survival time (Figure 5C). The luciferase activity of wild-type PDPK1 3'UTR was decreased by miR-1299 overexpression, and such effect was reversed by co-transfection of miR-1299 mimics and pcDNA3.1/KCNQ1OT1 in ME-180 and C33A cells. However, the luciferase activity of the pmirGLO-PDPK1 3'UTR-Mut exhibited no significant changes in ME-180 and C33A cells after transfection of miR-1299 mimics or miR-1299 mimics + pcDNA3.1/KCNQ1OT1 (Figure 5D). Finally, PDPK1 mRNA and protein expression was increased by KCNQ1OT1 overexpression and were reduced by miR-1299 overexpression in ME-180 and C33A cells, and these impacts were counteracted by co-overexpression of KCNQ1OT1 and miR-1299 (Figure 5E-F). RNA pulldown assay revealed that, compared to bio-NC, PDPK1 was significantly enriched in biotin-labeled miR-1299-Wt groups rather than biotin-labeled miR-1299-Mut groups (Figure 5G). In a word, KCNQ1OT1 positively regulated PDPK1 expression via sponging miR-1299 in CESC cells.

**Discussion**

CESC, a common gynecologic cancer, has become a major health threat to women (26). The typical symptoms of CESC are vaginal drainage or bleeding (27). With the development of advanced therapeutic methods (including surgical resection, radiotherapy and chemotherapy), the quality of life and survival time of CESC patients have been improved to some extent (27). However, CESC patients still possess a low survival rate over 5 years with approximately 260,000 deaths every year (27, 28). Numerous miRNAs, including miR-3647-5p and miR-432, have been identified as potential markers for predicting the outcomes of CESC patients (29, 30). Recently, miR-1299 is expressed at a low level in esophageal squamous cell carcinoma and breast cancer (31, 32). In our research, miR-1299 was discovered to be downregulated in CESC tissues and inhibited CESC progression by inhibiting cell proliferation and facilitating apoptosis, which implied the



tumor suppressor role of miR-1299 in CESC. In line with our results, previous literatures have suggested that miR-1299 acts as a tumor suppressor to reduce the proliferation and metastasis of prostate cancer (33).

Mechanistically, lncRNAs can interact with miRNAs to regulate mRNA expression levels in a ceRNA regulatory network (14). For example, lncRNA TMPO-AS1 interacts with miR-199a-5p by serving as a ceRNA to facilitate osteosarcoma tumorigenesis by modulating WNT7B (34). The OSER1-AS1/miR-372-3p axis regulates tumorigenesis of hepatocellular carcinoma by modulating Rab23 levels (35). MiR-1299 has been reported to target CDK6 in osteosarcoma (36), to inhibit ETS1 expression in gastric cancer (37) and to negatively regulate IRF7 expression in renal cell carcinoma (38). Moreover, it has been documented that RHPN1-AS1 interacts with miR-1299 to accelerate the deterioration of gastric cancer (37). Similarly, with the assistance of bioinformatics analysis, miR-

1299 was confirmed to bind with KCNQ1OT1. MiR-1299 expression had a negative correlation with KCNQ1OT1 expression in CESC tissues. Additionally, KCNQ1OT1 negatively regulated miR-1299 levels in CESC cells. KCNQ1OT1 is a vital regulator of tumor development. For example, KCNQ1OT1 contributes to hepatocellular carcinoma growth by competitively binding with miR-504 (39). Knockdown of KCNQ1OT1 inhibits cell malignancy via the miR-370/CCNE2 axis in glioma (40). In the same way, our study revealed that KCNQ1OT1 promoted ME-180 and C33A cells proliferation and inhibited apoptosis by downregulating miR-1299. Furthermore, KCNQ1OT1 facilitated CESC xenograft tumor growth via downregulation of miR-1299 *in vivo*.

PDPK1, also known as PDK1 or PDK2, is an oncogene in CESC. For instance, the PI3K/PDK1/AKT pathway is involved in the proliferative, migratory, invasive, and apoptotic abilities of cervical cancer cells (24). MiR-155-5p is downregulated in cervical cancer cells and activates the PDK1/mTOR pathway to promote C33A cell autophagy (41). NPP4B reduces p-AKT, p-SGK3, p-mTOR, and PDK1 proteins in the PI3K/AKT/SGK3 pathway to inhibit cell proliferation and invasion in cervical cancer (42). This work demonstrated that miR-1299 bound to the 3'UTR of PDPK1 to inhibit PDPK1 mRNA expression and thus suppressed the protein levels of PDPK1. KCNQ1OT1 was bound to miR-1299 to antagonize the suppressive effects of miR-1299 on PDPK1, thus upregulating PDPK1 expression in CESC cells.

There are several limitations to our study. First, the up-regulation mechanism of KCNQ1OT1 in CESC is not investigated. Besides, many studies have revealed that PDPK1 regulates tumor progression via activation of signaling pathways (43-45). However, whether PDPK1 is involved in regulating signaling pathways in CESC is unclear. Thus further studies should be carried out in the future.

In summary, the current study highlighted the KCNQ1OT1/miR-1299/PDPK1 axis in CESC cells, and we discovered that the KCNQ1OT1/miR-1299 axis regulated CESC progression by targeting PDPK1. This study may provide a possible therapeutic target for CESC patients.

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Conflict of interest exists when an author (or the author's institution) has financial or personal relationships that inappropriately influence (bias) his or her actions (such relationships are also known as dual commitments, competing interests, or competing loyalties).

### Consent for publications

The author read and proved the final manuscript for publication.

### Availability of data and material

All data generated during this study are included in this published article

### Authors' Contribution

All authors had equal roles in study design, work, statistical analysis and manuscript writing.

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All experimental procedures on mice were conducted following the institutional animal care and use committee of Affiliated Suqian Hospital of Xuzhou Medical University.

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