# Downregulation of microRNA-449a-5p promotes esophageal squamous cell carcinoma cell proliferation via cyclin D1 regulation

TAO JIANG[1], JUNFENG LIU[1] and JIXING MU[2]

[1]Department of Thoracic Surgery, Fourth Hospital of Hebei Medical University, Shijiazhuang, Hebei 050011; [2]First Hospital of Xingtai, Xingtai, Hebei 054000, P.R. China

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**Abstract.** Aberrant microRNA-449a (miR-449a-5p) expression has been demonstrated to be associated with the development of various cancer types. However, the effect of miR-449a-5p on esophageal squamous cell carcinoma (ESCC) cell proliferation remains unknown. The present study aimed to determine whether miR-449a-5p may regulate ESCC cell proliferation via negative regulation of cyclin D1. Reverse transcription quantitative-polymerase chain reaction was used to measure the expression of miR-449a-5p in ESCC tissues and cells. Western blot was performed to analyze the protein level of cyclin D1. The proliferation of ESCC cells was determined by MTT and clone formation assay. Paired ESCC and adjacent normal esophageal squamous tissues were collected from patients with ESCC. It was demonstrated that miR-449a-5p expression was reduced, whereas cyclin D1 expression was increased in ESCC tissues compared with adjacent normal tissues. Proliferation was investigated *in vivo* using the ESCC cell line Eca-190. miR-449a-5p inhibitor transfection facilitated the proliferation of Eca-109 cells. By contrast, transfection with miR-449a-5p mimics inhibited Eca-109 cell proliferation. Furthermore, it was confirmed that miR-449a-5p directly bound to the 3'-untranslated region of cyclin D1. Transfection with cyclin D1 small interfering RNA reversed the effects of the miR-449a-5p inhibitor on Eca-109 cell proliferation. In conclusion, miR-449a-5p may control ESCC proliferation through the negative regulation of cyclin D1 expression.

## Introduction

Esophageal carcinoma (EC) is a common type of cancer that is associated with millions of cases of mortality per year worldwide (1). The 5-year relative survival rate is ~40% for localized tumors and 4% for advanced distal metastatic tumors. Environmental and lifestyle factors, including smoking and alcohol consumption, are the risk factor for esophageal squamous cell carcinoma (ESCC) in Western countries, and the consumption of hot beverages is a major risk factor in East countries (2). The proliferation of cancer cells and metastasis are the common risk factor for ESCC development (3). ESCC can be treated with various techniques, including chemotherapy, radiotherapy and surgical resection. Surgery is suitable for early stage of EC. Chemotherapy and radiotherapy is the strategy for advanced stage of EC (4). In China, the histological subtype of most EC cases is ESCC (5). In recent years, EC treatments have advanced, but further improvement is required (6).

MicroRNAs (miRNAs/miRs) are highly conserved, small non-coding RNAs, which regulate the expression of several oncogenes and tumor suppressor genes (7). miRNAs bind the mRNA of their target genes in the 3'-untranslated region (UTR) to downregulate protein expression (8). It has been demonstrated that miRNAs have important roles in many biological cellular processes, including proliferation, differentiation, migration and apoptosis (9,10). In addition, aberrant miRNA expression is associated with the development of several types of cancer (11,12). It has been suggested that miR-449a-5p may suppress the proliferation of various cancer cells by negatively regulating the expression of several oncogenes (13). However, the effects of miR-449a-5p on ESCC remain to be elucidated. In the present study the aim was to determine the effects of miR-449a-5p and its target gene on proliferation of ESCC cells. The present study demonstrated that miR-449a-5p regulated ESCC cell proliferation via targeting cyclin D1.

## Materials and methods

*ESCC tissues and cell lines.* Paired ESCC tissues and adjacent normal esophageal squamous tissues were collected from patients with ESCC (n=7; age, 56-72 years; mean age, 68 years; sex: Males, 3 and females, 4) that under went esophagogastrostomy at the Fourth Hospital of Hebei Medical University (Shijiazhuang, China) between 2014/1-2015/10. Patients had not received any preoperative chemotherapy or radiotherapy, and all clinicopathological information was recorded. All tissue samples were flash-frozen in liquid nitrogen and stored at -80˚C. The present study was approved by the Ethical Review Committee of the Fourth Hospital of Hebei Medical

*Correspondence to:* Professor Jixing Mu, First Hospital of Xingtai, 376 Shunde Road, Xingtai, Hebei 054000, P.R. China
E-mail: 18531117986@163.com

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University (Shijiazhuang, China). Written informed consent was obtained from each patient.

The human ESCC cell line Eca-109 was purchased from American Type Culture Collection (Manassas, VA, USA). Eca-109 cells were cultured in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 80 U/ml penicillin and 100 $\mu$g/ml streptomycin at 37˚C in a humidified atmosphere containing 5% $CO_2$.

*Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)*. Total RNA of ESCC cells and tissues was extracted with TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. cDNA was reverse transcribed from 1 $\mu$g total RNA sample mixing with 1 $\mu$l (500 nM) miRNA-specific loop RT-primers and 2 $\mu$l dNTP (10 mM; Takara Biotechnology Co., Ltd., Dalian, China), then added RNase-free water to 10 $\mu$l, mixed well. 70˚C for 10 min, then placed on ice for 5 min. Then 0.5 $\mu$l Recombinant RNase inhibitor (40 U/$\mu$l; Takara Biotechnology Co., Ltd., Dalian, China), 0.5 $\mu$l MMLV Reverse Transcriptase (200 U/$\mu$l; New England BioLabs, Inc., Ipswich, MA, USA), 2 $\mu$l 10X Transcriptase Buffer, added RNase-free water to 10 $\mu$l, mix well. The thermocycling conditions were: 42˚ for 60 min, 95˚C for 5 min, 4˚C forever. The primer sequences used for reverse transcription were as follows (5'-3'): miR-449a-5p, 5'-GTCGTA TCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACG ACGCTTTG-3' and U6, 5'-GTCGTATCCAGTGCAGGG TCCGAGGTATTCGCACTGGATACGACAAAAATATG-3'. Stem-loop RT-qPCR was performed to analyze miR-449a-5p levels. PCR was performed with the SYBR Green master mix (Takara Biotechnology Co., Ltd., Dalian, China) and iQ5 system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The procedures of the PCR are described as follows: 95˚C for 30 sec, followed by 40 cycles at 95˚C for 5 sec and 60˚C for 20 sec. The relative expression levels of miR-449a-5p were normalized to U6 and were calculated with the $2^{-\Delta\Delta Cq}$ method (14). All reactions were performed in triplicate. The primer sequences used for PCR were as follows: miR-449a-5p forward, 5'-ATAGTG GCAGTGTATTGTTAG-3'; U6 forward, 5'-GCGCGTCGT GAAGCGTTC-3'; universal reverse primer, 5'-GTGCAGGGT CCGAGGT-3'.

*Transfection*. miR-449a-5p mimic (449AM), miR-449a-5p inhibitor (449AI), microRNA mimic negative control (NC) and microRNA inhibitor negative control (NCI) were obtained from Shanghai GenePharma Co., Ltd., (Shanghai, China). The day prior to transfection, $8x10^5$ Eca-$10^9$ cells per well were cultured in 6-well plate. NC; sense 5'-UUCUCCGAACGUGUC ACGU-3' and antisense 5'-ACGUGACACGUUCGGAGAA; 449AM, sense 5'-UGGCAGUGUAUUGUUAGCUGGU-3' and antisense 5'-CAGCUAACAAUACACUGCCAUU-3', NCI, sense 5'-CAGUACUUUUGUGUAGUACAA-3'; 449AI, sequence 5'-ACCAGCUAACAAUACACUGCCA-3') were transfected into Eca-109 cells using HiPerFect transfection reagent (Qiagen China Co., Ltd., Shanghai, China). A total of 150 ng miRNA oligonucleotides and 3 $\mu$l HiPerFect transfection reagent were mixed in 100 $\mu$l RPMI-1640 and the mixture was added to the cell culture medium. At 48 h post-transfection, cells were harvested for miR-449a-5p detection.

Cyclin D1-specific siRNA transfection was performed by using HiPerFect transfection**.** Cyclin D1-specific siRNA (SI) and negative control siRNA (siNC) were obtained from Shanghai GenePharma Co., Ltd. The sequences were described as following: Cyclin D1-specific siRNA (sense 5'-CCUCGGUGUCCUACUUCAAAUGUGU-3' and anti-sense ACACAUUUGAAGUAGGACACCGAGG-3'); and negative control siRNA (sense 5'-UUCUCCGAACGU GUCACGU-3' and antisense 5'-ACGUGACACGUUCGG AGAA). The day before transfection, $8x10^5$ Eca-109 cells per well were cultured in 6-well plate. A total of 150 ng miRNA oligonucleotides and 3 $\mu$l HiPerFect transfection reagent were mixed in 100 $\mu$l RPMI-1640 and the mixture was added to the cell culture medium. At 48 h post-transfection, cells were harvested for detecting cyclin D1 protein and mRNA levels.

*Prediction of target genes*. Target Scan (http://www.targetscan.org/),miRanda(http://34.236.212.39/microrna/home.do) and PicTar (http://pictar.mdc-berlin.de/) were used to predict the target genes of miR-449a-5p. Cyclin D1 was predicted as putative target gene.

*Luciferase assay*. To confirm cyclin D1 as a target of miR-449a-5p, a luciferase assay was performed. The binding region of miR-449a-5p in the 3'-UTR of cyclin D1 was cloned using the following primers; the restriction sites are underlined: Cyclin D1-F-*Sac*I, TCGAGCTCCTGTTTGGCG TTTCCCAGAG; cyclin D1-R-*Xba*I, GCTCTAGAACTACTA TGATGCTACGCCCC. Eca-109 cell genomic DNA was used as a template. PCR was performed by using Q5 DNA polymerase (New England BioLabs, Inc.). The PCR thermo cycling conditions were used: 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec, 55˚C for 45 sec and 72˚C for 30 sec. The PCR product was digested by the endonucleases *Sac*I and *Xba*I, and inserted into the pmiRGLO vector (Promega Corporation, Madison, WI, USA). For the luciferase reporter assay, Eca-109 cells were cultured in a 96-well plate at 5,000 cells/well in 100 $\mu$l culture medium. Subsequently, the recombinant luciferase vector (0.1 mg) and miR-449a-5p mimic or inhibitor (5 ng) were transfected into Eca-109 cells with Effectene reagent (Qiagen China Co., Ltd.) for 48 h. A dual-luciferase reporter assay system (Promega Corporation) was subsequently used to detect the luciferase activity of cells. Luciferase activity was normalized to Renilla luciferase activity. A total of six samples were measured for each group. The experiment was repeated three times.

*Cell proliferation assay*. AMTT assay was performed to analyze cell proliferation. Eca-109 cells were seeded into a 96-well plate ($1x10^4$ cells/well) for 24, 48 and 72 h. MTT (20 $\mu$l; 10 mg/ml) was subsequently added to each well and incubated for 4 h at 37˚C. The supernatant was removed and 150 $\mu$l dimethyl sulfoxide was added for 15-20 min. Absorbance was measured at a wavelength of 450 nm. All experiments were performed in sextuplicate.

*Colony formation assay*. A day following transfection, ~300 Eca109 cells were cultured in each well of 6-well plate and incubated at 37˚C for 2 weeks. The culture medium

was removed once a week and replaced with fresh medium containing the miR-449a-5p mimic or inhibitor transfection mixture (150 ng miRNA oligonucleotides and 3 $\mu$l HiPerFect transfection reagent). On day 14, the cells were washed three times with PBS, fixed with 4% polymerized formaldeyde (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) for 30 min at room temperature and stained with 2.5% crystal violet staining solution (Beijing Solarbio Science & Technology Co., Ltd.) for 30 min at room temperature. The 6-well plates were washed with PBS three times and air-dried. The colonies that contained >50 cells were counted with the naked eye in from each well. The relative colony numbers were calculated as the ratio of 449aM to NC or 449aI to NCI. Experiments were carried out in triplicate each time and repeated three times.

*Western blot analysis*. A total of 48 h following transfection, the cells were washed by ice PBS and harvested by centrifuging at 200 x g for 10 min at 4˚C. Cell protein was extracted by Lysis Buffer (CST Biological Reagents Co., Ltd., Danvers, MA, USA). Protein concentration was determined with a bicinchoninic acid protein assay kit. Protein samples (15 $\mu$g/lane) were separated by 10% SDS-PAGE and electrotransferred to polyvinylidene fluoride membranes (EMD Millipore, Billerica, MD, USA). Membranes were subsequently blocked with 5% non-fat milk for 2 h at room temperature and incubated with cyclin D1 (1:1,000; cat. no. 2978; CST Biological Reagents Co., Ltd.) and GAPDH (1:1,000; cat. no. 5174; CST Biological Reagents Co., Ltd.) primary antibodies overnight at 4˚C. Blots were washed five times in Tris-buffered saline (TBS) with 0.1% Tween-20 (TBST) followed by incubation with horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (1:5,000; cat. no. 7074; CST Biological Reagents Co., Ltd.) for 1 h at room temperature. Membranes were washed in TBST for 10 min three times and bands were visualized using an enhanced chemiluminescence kit (EMD Millipore). Relative protein levels were calculated as the ratio of cyclin D1 band intensity to that of GAPDH using ImageJ version 1.42 (National Institutes of Health, Bethesda, MD, USA). Experiments were carried out in triplicate each time and repeated three times.

*Statistical analysis*. Statistical analysis was performed with the SPSS 13.0 statistical software package (SPSS, Inc., Chicago, IL, USA). Data are expressed as the means ± standard error of the mean. The non-parametric Spearman's rank-order correlation was used to determine the correlation between miR-449a and cyclin D1 in ESCC tissues. The non-parametric Mann-Whitney U test was used to compare two groups, and one-way analysis of variance followed by a Tukey's post-hoc test was used to compare three or more groups. P<0.05 was considered to indicate a statistically significant difference.

## Results

*miR-449a-5p expression is reduced in ESCC tissues*. Changes in miR-449a-5p expression were analyzed in ESCC (n=7) and adjacent normal tissues (n=7) by RT-qPCR. The results revealed that the expression levels of miR-449a-5p were significantly reduced in the ESCC tissues (P<0.01; Fig. 1) compared with in the adjacent normal tissues. These findings suggested that decreased miR-449a-5p may be associated with ESCC.

*miR-449a-5p regulates ESCC cell proliferation*. In order to investigate the effects of miR-449a-5p on ESCC cell proliferation, 499AM and 499AI were transfected into Eca-109 cells. After 48 h, the expression levels of miR-449a-5p were increased by ~50-fold in Eca-109 cells transfected with 499AM, whereas miR-449a-5p expression was reduced to 40% of that in the NCI group (P<0.01; Fig. 2A and B), indicating successful transfection. Colony number was significantly decreased in cells transfected with 499AM, whereas 499AI significantly increased colony number (P<0.05; Fig. 2C and D). Additionally, 499AM inhibited the proliferation of Eca-109 cells, whereas 499AI increased cell proliferation (P<0.05; Fig. 2C and D). These results suggested that miR-449 may regulate Eca-109 cell proliferation.

*miR-449a-5p negatively regulates cyclin D1 expression by binding to its 3'-UTR*. miRanda, TargetScan and PicTar were used to predict the potential target genes of miR-449a-5p. Cyclin D1was identified as a potential target gene; a miR-449a-5p binding site was revealed to be present at nucleotides 2021-2080 in the cyclin D1 3'-UTR (Fig. 3A). This region of the cyclin D1 3'-UTR was subsequently cloned and inserted into a pmiRGLO vector. Transfection with 499AM significantly decreased the luciferase activity of Eca-109 cells (Fig. 3B). However, 499AI transfection did not significantly alter luciferase activity (Fig. 3B). Additionally, cyclin D1 protein levels were reduced in Eca-109 cells transfected with 499AM (Fig. 3C). By contrast, 499AI upregulated cyclin D1 protein levels (Fig. 3D). These results suggested that miR-449a-5p may negatively regulate cyclin D1 expression by binding to its 3'-UTR.

*miR-449a-5p regulates Eca-109 cell proliferation via cyclin D1 targeting*. Cyclin D1 protein expression was increased in ESCC tissues (Fig. 4A). To further determine the role of cyclin D1 in 499AI-induced Eca-109 cell proliferation, small interfering siRNA targeting cyclin D1 mRNA and 499AI were co-transfected into Eca-109 cells. Cyclin D1 expression was decreased by ~50% compared with in cells transfected with siNC (Fig. 4B). Downregulation of cyclin D1 expression rescued the effects of 449AI on Eca-109 proliferation (Fig. 4C) and colony formation (Fig. 4D). The non-parametric Spearman's correlation test was used to determine if a correlation existed between the levels of miR-449a-5p and cyclin D1. The results revealed that in ESSC tissues miR-449a levels were not correlated with cyclin D1 (r=-0.406; P=0.425; data not shown); however, this may be due to the small sample size. Taken together, these results indicated that miR-449a-5p may regulate the proliferation of Eca-109 cells by targeting cyclin D1.

## Discussion

Although the treatment of ESCC has advanced, disease prognosis remains poor, as most patients are diagnosed at an advanced stage; consequently, the 5-year survival rate of patients is <30% post-surgery (15). In addition, ~10% of patients were diagnosed at an advanced and the tumor

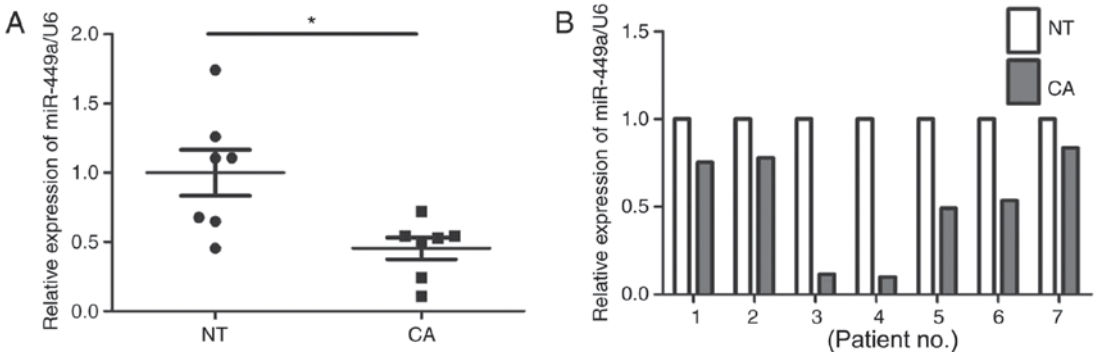


Figure 1. miR-449a-5p is downregulated in esophageal squamous cell carcinoma. (A) miR-449a-5p expression in CA and adjacent NT was analyzed by reverse transcription-quantitative polymerase chain reaction. (B) miR-449a-5p expression levels in each patient. Compared with in the adjacent NT, the expression levels of miR-449a-5p were significantly decreased in CA. n=7, $^{*}$P<0.05. CA, cancerous tissue; miR-449a-5p, microRNA-449a-5p; NT, normal tissue.

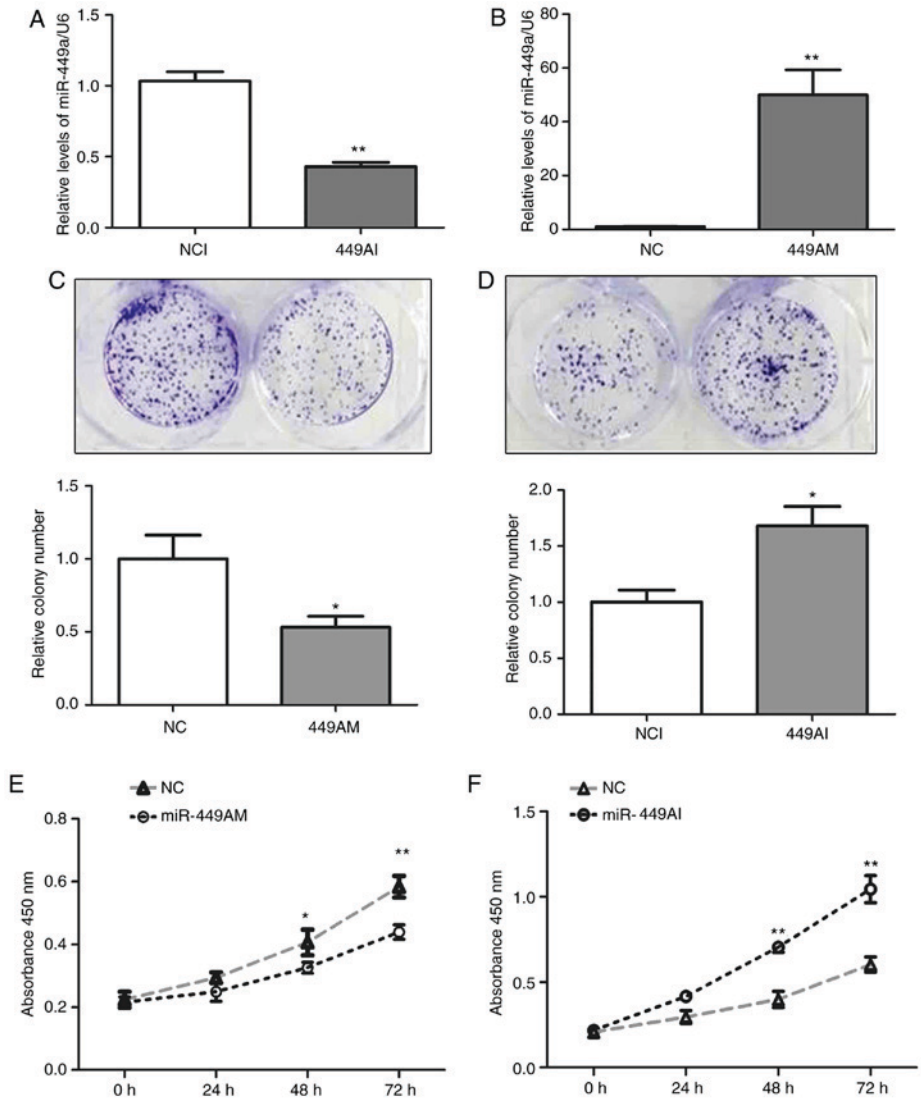


Figure 2. Downregulation of miR-449a-5p promotes Eca-109 cell proliferation. (A) miR-449a-5p expression was effectively decreased in Eca-109 cells transfected with 499AI. (B) miR-449a-5p expression was effectively increased in Eca-109 cells transfected with 499AM. Cell colony formation assays were performed with Eca-109 cells transfected with (C) 499AM or (D) 499AI. MTT assays were used to determine proliferation of Eca-109 cells transfected with (E) 499AM or (F) 499AI. $^{*}$P<0.05, $^{**}$P<0.01 vs. NC or NCI. 499AI, miR-499a-5p inhibitor; 499AM, miR-499a-5p mimic; miR-449a-5p, microRNA-499a-5p; NC, negative control; NCI, negative control inhibitor.

had spread to other organs. So, the patients do not have the opportunity to undergo surgery (16,17). Molecular targeted therapy improves the 5-year survival rate of patients with ESCC (18).

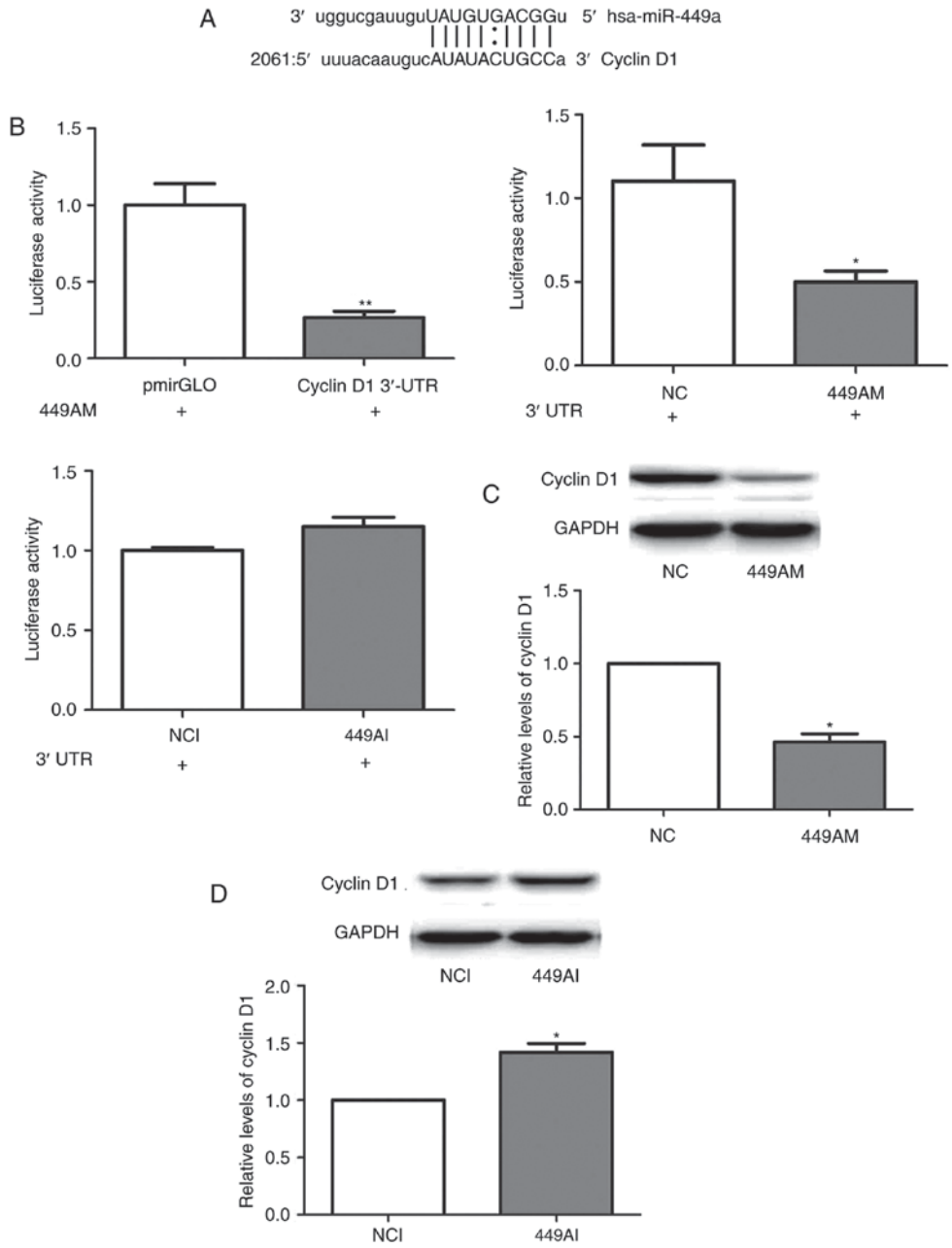


Figure 3. miR-449a-5p regulates cyclin D1 expression by directly binding to its 3'-UTR. (A) A miR-449a-5p binding site was identified in the 3'-UTR of cyclin D1. (B) Luciferase activity was significantly decreased in Eca-109 cells transfected with a luciferase reporter vector containing the cyclin D1 3'-UTR and 499AM. 499AI had no significant effect on luciferase activity. (C) 499AM transfection decreased cyclin D1 protein expression and (D) 499AI transfection increased cyclin D1 protein expression. **P<0.05 vs. pmiRGLO; *P<0.05 vs. NC or NCI. 3'-UTR, 3'-untranslated region; 499AI, miR-499a-5p inhibitor; 499AM, miR-499a-5p mimic; miR-449a-5p, microRNA-499a-5p; NC, negative control; NCI, negative control inhibitor.

miRNAs are a class of small non-coding RNAs, which negatively regulate gene expression via binding to the 3'-UTR of target mRNAs. It has been demonstrated that miRNAs participate in the pathogenesis of various types of cancer by targeting numerous oncogenes, and aberrant expression of miRNAs may contribute to carcinogenesis (19-22). It has been verified that ~50% of miRNAs are involved in the development of human cancer (23); miRNAs can regulate the development of various human cancers by acting as both oncogenes and tumor suppressors (24-26). miR-449a-5p expression is reduced in various cancer cells, including prostate (27), gastric (28), bladder (29) and lung cancer (30). Furthermore, miR-449a-5p is involved in $G_1$ cell cycle arrest, apoptosis and senescence via the regulation of key factors in cell cycle and apoptosis regulation, including histone deacetylase 1 (30), cyclin-dependent kinase 6 (31-33), cell division cycle 25A (31,33), cyclin D1 (34) and nicotinamide adenine dinucleotide-dependent protein deacetylase sirtuin-1 (35).

In the present study, it was demonstrated that miR-449a-5p expression was significantly reduced in ESCC tissues compared within adjacent normal esophageal squamous tissues. The effects of miR-449a-5p on Eca-109 cell proliferation were subsequently determined *in vitro*. miRNAs can post-transcriptionally negatively regulate their target genes (35), by binding to the 3'-UTR of target mRNA (36).The present study confirmed that cyclin D1 was a target gene of miR-449a-5p

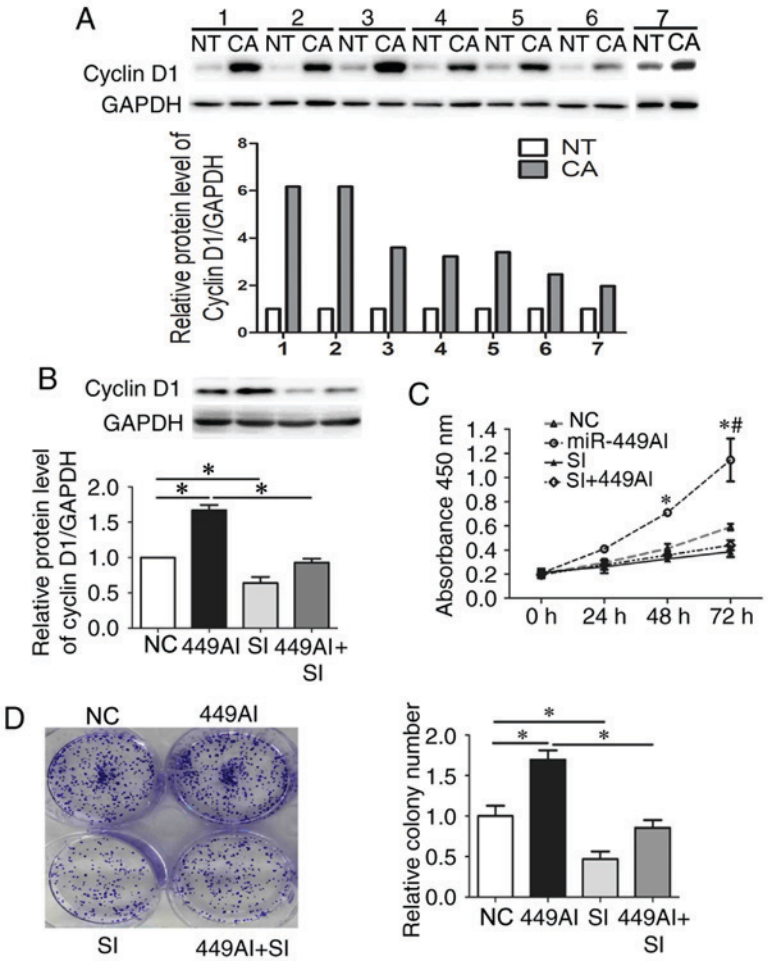


Figure 4. miR-449a-5p regulates the proliferation of Eca-109 via targeting cyclin D1. (A) Cyclin D1 expression was markedly increased in CA. (B) Transfection of cyclin D1-specific SI reversed the effects of miR-449a-5p inhibitor (499AI) on cyclin D1 protein expression. *P<0.05. (C) A MTT assay was used to determine the proliferation of Eca-109 cells transfected with 499AI and cyclin D1 SI. *P<0.05 vs. NCI; #P<0.05 vs. SI. (D) Cell colony formation assays were performed in Eca-109 cells transfected with 499AI and cyclin D1 SI. *P<0.05. NC, negative control, 499AI, miR-499a-5p inhibitor; CA, carcinoma tissue; miR-449a-5p, microRNA-499a-5p; NT, normal tissue; SI, small interfering RNA.

using a luciferase assay. Transfection with 499AM decreased the luciferase activity of Eca-109 cells. However, 499AI transfection did not alter luciferase activity Cyclin D1. The level of miR-449a-5p was increased by ~50 fold in cells transfected with miR-449a-5p mimics. However, in cells transfected with the miR-449a-5p inhibitor the level of miR-449a-5p was reduced to 40% of NCI group. Cyclin D1is involved in the growth progression of various cells, and is considered a proto-oncogene that is overexpressed in several types of cancer. The results of the current study revealed that cyclinD1 was downregulated in ESCC cells transfected with 499AM, whereas cyclin D1 was upregulated in ESCC cells transfected with 499AI. The results of the luciferase assay confirmed that the 3'-UTR of cyclin D1 mRNA contained a miR-449a-5p binding site. Inhibition of cyclin D1 reversed the effects of 499AI on the proliferation of ESCC cells. However, the results of the Spearman's rank correlation test did not demonstrate a correlation between miR-449a and cyclin D1 expression; this is likely due to the small sample size used in the present study. In future experiments, we aim to collect more ESCC tissue samples.

In conclusion, miR-449a-5p expression was significantly reduced in ESCC tissues compared with in the adjacent normal tissues. In addition, inhibition of miR-449a-5p was able to promote the proliferation of ESCC cells by upregulating cyclin D1 expression. Therefore, the findings of the present study indicated that miR-449a-5p may be an effective biomarker and therapeutic target for ESCC in the future.

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**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### Authors' contributions

TJ and JM planned the experiments and wrote the paper; TJ and JL performed the experiments; TJ analyzed data.

### Ethics approval and consent to participate

The present study was approved by the Ethical Review Committee of the Fourth Hospital of Hebei Medical University (Shijiazhuang, China). Written informed consent was obtained from each patient.

### Consent for publication

Written informed consent was obtained from each patient.

### Competing interests

The authors declare that they have no competing interests.

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