Downregulation of microRNA-494 inhibits cell proliferation in lung squamous cell carcinoma via the induction of PUMA-α-mediated apoptosis

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Abstract. Increased evidence has shown that abnormal microRNA (miRNA) plays pivotal roles in numerous types of cancer. However, their expression, function and mechanism in lung squamous cell carcinoma (LSCC) remains to be fully elucidated. The aim of the present study was to investigate the suppressive role of miR-494 in LSCC progression and elucidate its regulatory mechanism. By analyzing expression profiles of miRNAs in LSCC tissues using miRNA microarray, it was revealed that miR-494 was significantly upregulated in 22 pairs of LSCC tissues. Subsequently, reverse transcription-quantitative PCR was performed to determine the expression of miR-494 and p53-upregulated-modulator-of-apoptosis-α (PUMA-α). Western blot analysis was conducted to examine protein levels. Dual-luciferase reporter assay was used to confirm the binding between miR-494 and PUMA-α. Annexin V-fluoresceine isothiocyanate/propidium iodide staining and CCK-8 assays were employed to determine cell apoptosis and cell viability, respectively. It was also revealed that miR-494 was highly expressed in LSCC cell lines compared with that in 16HBE cells. Further experiments confirmed that knockdown of miR-494 reduced cell viability and induced LSCC apoptosis. Bioinformatics analysis predicted that miR-494 could potentially target PUMA-α; also known as Bcl-2-binding component 3, a pro-apoptotic factor, and an inverse correlation between the expression of miR-494 and PUMA-α mRNA levels in LSCC tissues was found. Furthermore, PUMA-α inhibition could reverse the promoting effect of miR-494 knockdown on apoptosis in LSCC cells. Taken together, these findings demonstrated that miR-494 functions as an oncogene by targeting PUMA-α in LSCC, and miR-494 may serve as a novel therapeutic target for treating LSCC.

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Introduction

Lung cancer remains one of the most common and serious types of cancer worldwide, and it has been estimated that at present there are 8.2 million mortalities annually (1). Lung cancer is divided into the classifications of small cell lung cancer (SCLC) and non-SCLC (NSCLC) (2). According to the pathophysiology and histological morphology, NSCLC, which comprises ~85% of lung cancer cases, is primarily split into lung adenocarcinoma and lung squamous cell carcinoma (LSCC) (3). Although significant advances have been made in the integration of targeted therapies in LSCC treatment, the overall 5-year survival rate remains low and the recurrence rate is <20% due to distal metastasis after operation (4). Therefore, it is of great importance to identify new molecular mechanisms underlying the process of LSCC and to discover molecular targets and novel drugs for improving survival.

MicroRNAs (miRNAs) are a class of short, highly conserved non-coding RNAs consisting of ~22 nucleotides that regulate gene expression by targeting the 3'-untranslated region (3'-UTR) of mRNAs (5,6). Accumulating evidence has shown that miRNAs play important roles in various biological processes of tumorigenesis, such as cell proliferation, apoptosis, migration and invasion (7,8). Functionally, several miRNAs have been described as tumor suppressors or oncogenes in LSCC (9,10). For example, Liu *et al* (11) showed that miR-155-5p, as an oncogene, negatively regulates fibroblast growth factor 9 expression to promote squamous cell carcinoma (SCC) occurrence and development in the lungs. Hu *et al* (12) demonstrated that overexpressed miR-497-5p markedly inhibits cancer progression by targeting cell division cycle associated protein 4. Shan *et al* (13) revealed that miR-448 targeting can regulate cell proliferation and inhibit apoptosis by targeting doublecortin-like kinase 1 in LSCC cells. These data suggest that miRNAs may be a new direction in LSCC diagnosis and treatment. Therefore, more extensive investigations on the identification of tumor-suppressive or oncogenic miRNAs are the first step in the construction of a new treatment strategy for the disease.

miR-494, a miRNA located on chromosome 14q32.31, participates in various stages of tumorigenesis (14). Extensive studies have shown that miR-494-3p is an oncogene that has a central role in numerous solid tumors. For example, Lin *et al* (15) demonstrated that miR-494 promotes human hepatocellular carcinoma progression by targeting the PTEN/PI3K/AKT pathway. Furthermore, Li *et al* (16) reported that miR-494-3p can promote glioma cell invasion and proliferation and inhibit its apoptosis through the suppression of PTEN expression. Another study performed by Zhang *et al* (17) indicated that miR-494 promotes cancer progression and targets adenomatous polyposis coli in colorectal cancer. However, the roles and potential mechanisms of miR-494 in NSCLC are still largely unknown.

In the present study, an miRNA dataset from the GEO database was analyzed to investigate the expression of miRNAs in LSCC tissues, and miR-494 was selected for further analysis. The effects of miR-494 on cell proliferation and apoptosis were then explored. The precise molecular mechanism of miR-494 in LSCC cells was further investigated, as well as the correlation between miR-494 and PUMA-α. The study aimed to determine whether miR-494 may be a potential target for LSCC treatment and may be important in the development of LSCC.

Materials and methods

Collection of clinical samples. A total of 22 freshly frozen LSCC specimens and 22 adjacent non-tumor tissues (used as the normal control) (at least 5 cm away from the carcinoma) were obtained from patients who underwent pneumonectomy at the Department of Oncology, The First Affiliated Hospital of Xinxiang Medical University (Weihui, China) from January 2019 to July 2020. The inclusion criteria in present study were as follows: i) LSCC; ii) no radiotherapy or chemotherapy prior to surgery; iii) a patient age of ≥18 years; iv) weight loss of ≤10% in the 3 months before diagnosis; and v) Karnofsky performance status ≥70% (18). The exclusion criteria were as follows: i) Histology other than SCC; ii) metastatic lung cancer; iii) patients with serious medical or psychiatric illness, or a history of serious cardiac disease; iv) prior radiation therapy to the thorax or total surgical resection; v) prior systemic chemotherapy; and vi) an age of >80 years. The clinicopathological information of the patients is shown in Table I. The present study was approved by the Ethics Committee of The First Affiliated Hospital of Xinxiang Medical University (Weihui, China), and written informed consent was obtained from each patient.

miRNA microarray. The miRNA dataset (GSE74190) was searched and downloaded from the GEO database (https://www.ncbi.nlm.nih.gov/geo/). The GSE74190 dataset was based on the Agilent-019118 Human miRNA Microarray V1 G4470A platform (19). GEO2R (www.ncbi.nlm.nih.gov/geo/geo2r/), an interactive web tool, was applied to compare the samples in two different groups under the same experimental condition. Fold change ≥2 and P<0.05 served as basic screening parameters. Hierarchical clustering of differentially expressed miRNA was performed using the Multiple Experiment Viewer 4.7.1 software program (The Institute for Genomic Research, USA).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA of the cultured cells and tissues was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) as described previously (20). The TaqMan Reverse Transcription kit (Takara Biotechnology Co., Ltd.) was used to obtain cDNA for mRNA detection (42˚C for 1 h), while the TaqMan MicroRNA Reverse Transcription kit (Takara Biotechnology Co., Ltd.) was used for miRNA detection (42˚C for 1 h). qPCR was performed using SYBR Premix Ex Taq (Takara Bio, Inc.) on an ABI PRISM 7500 Sequence Detection System (Applied Biosystems; Thermo Fisher Scientific, Inc.). Primers for cDNA amplification were as follows: miR-494 forward, 5'-TGACCTGAAACATACACGGGA-3', and universal reverse primer, 5'-TATCGTTGTACTCCACTCCTTGAC-3'; U6 forward, 5'-GCTTCGGCAGCACATATACTAAAAT-3', and reverse, 5'-CGCTTCACGAATTTGCGTGTCAT-3'; PUMA-α forward, 5'-CGGCGGAGACAAGAGGAGC-3', and reverse, 5'-CAGGGTGTCAGGAGGTGGGAG-3'; and GAPDH forward, 5'-TCAACGACCCCTTCATTGACC-3', and reverse, 5'-CTTCCCGTTGATGACAAGCTTC-3'. The reaction mixtures were denatured at 95˚C for 3 min, followed by 40 cycles of 95˚C for 10 sec and 60˚C for 30 sec. The relative expression levels of miRNA and mRNA were normalized to that of U6 and GAPDH, respectively. The relative expression of each gene was calculated and normalized using the $2^{-\Delta\Delta Cq}$ method (21). All reactions were conducted in triplicate.

Cell culture. A total of four LSCC cell lines (NCI-H520, SW900, EBC-1 and SK-MES-1) were purchased from the American Type Culture Collection (ATCC) and cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Sigma-Aldrich; Merck KGaA), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C in a 5% CO_2 atmosphere. A human bronchial epithelial cell line (16HBE; ATCC) was used as the control. 16HBE cells were cultured in medium provided by Procell Life Science & Technology (cat. no. CM-0249) at 37˚C with 5% CO_2.

Cell transfection. miR-494 mimics (5'-UGAAACAUACACGGGAAACCUC-3'), negative control (NC) mimics (5'-CACGAUAAACAAUACGGGUACC-3'), inhibitor (5'-GAGGUUUCCCGUGUAUGUUUCA-3') and inhibitor NC (5'-UGUGUCGUUAACUAUGGGCUCU-3') were obtained from Shanghai GenePharma Co., Ltd. SW900 and EBC-1 cells were transfected with 20 nM miR-494 mimic/inhibitor and mimic NC/inhibitor NC using Lipofectamine™ RNAiMAX (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol at 37˚C in a 5% CO_2 incubator. Following 48 h of transfection, cells were collected for cell proliferation, RT-qPCR and western blot analysis. PUMA-α overexpression plasmid (pcDNA3.1-PUMA-α) and pcDNA vector, siRNAs against PUMA-α (si-PUMA-α) and the corresponding scrambled NC (si-NC) were designed and synthesized by Guangzhou RiboBio Co., Ltd. The sequences of si-PUMA-α and si-NC are as follows: si-PUMA-α sense, 5'-GGGUCCUGUACAAUCUCAUCAUGGG-3' and antisense, 5'-CCCAUGAUGAGAUUGUACAGGACCC-3'; and si-NC sense, 5'-GGGUGUCAACACUCUACUAUCUGGG-3' and antisense, 5'-CCCAGAUAGUAGAGUGUUGACACCC-3'. A total of 50 nM si-PUMA-α

Table I. Characteristics of the lung squamous cell carcinoma cases.

Characteristic	Subcategory	n (%)
Age, years	<60	14 (63.6)
	≥60	8 (36.4)
Sex	Male	17 (77.3)
	Female	5 (22.7)
Smoking history	<30 pack-years	4 (18.2)
	≥30 pack-years	11 (50.0)
	Never smoked	7 (31.8)
Stage	I	9 (40.9)
	II-IV	13 (59.1)
Lymphatic invasion	Absent	3 (13.6)
	Present	19 (86.4)
Differentiation	Well	4 (18.2)
	Moderately	16 (72.7)
	Poorly	2 (9.1)
Recurrence	Present	10 (45.5)
	Absent	12 (54.5)

and si-NC were co-transfected with 50 nM miR-494 inhibitor or inhibitor NC into SW900 and EBC-1 cells at 37˚C using Lipofectamine™ RNAiMAX according to the manufacturer's instructions. SW900 and EBC-1 cells were cultured at 37˚C for 24 h prior to transfection. After 48 h of transfection, cells were harvested and used for analysis. All the transfections were repeated >3 times independently.

Cell viability. Cell viability was measured using the Cell Counting Kit (CCK)-8 (Dojindo Laboratories, Inc.) assay. Briefly, SW900 and EBC-1 cells were seeded into a 96-well plate at a density of 3,000 cells/well and cultured at 37˚C for 24 h prior to transfection. Then, cells were transfected with corresponding oligonucleotides. After 24, 36 or 48 h, 10 μl CCK-8 solution was added into each well of the plate. The plates were incubated at 37˚C for 1 h, and the absorbance at 450 nm was measured. The experiment was repeated three times.

Apoptosis detection by flow cytometry. SW900 and EBC-1 cells were harvested 48 h after cell transfection, and then washed twice with phosphate-buffered saline. Next, the cells were stained with Annexin V (FITC) and propidium iodide reagent (Thermo Fisher Scientific, Inc.) at 37˚C for 10 min in the dark at room temperature according to the manufacturer's instructions. Cell apoptosis rates were detected via FACSAria flow cytometry (BD Biosciences) and analyzed using FlowJo 7.6 software (FlowJo, LLC).

Western blotting. For western blotting, SW900 and EBC-1 cells were lysed using RIPA lysis buffer (Beyotime Institute of Biotechnology). Protein concentrations were determined with the bicinchoninic acid assay (Beyotime Institute of Biotehnology). Cell lysate (40 μg total protein per lane) were separated on 15% gels using SDS-PAGE and transferred to polyvinylidene difluoride membranes (Roche Applied Science). The membranes were first blocked with 5% skimmed milk at room temperature for 30 min, after which the membranes were incubated with primary antibodies overnight at 4˚C. Membranes were incubated with primary antibodies against PUMA-α (1:1,000; cat. no. sc-377015; Santa Cruz Biotechnology, Inc.), cleaved caspase-3 (1:1,000; cat. no. ab2302; Abcam) and Bcl-2 (1:1,000; cat. no. ab32124; Abcam) overnight at 4˚C, followed by incubation with HRP-conjugated anti-rabbit secondary antibody (1:10,000; cat. no. ab205718; Abcam) for 1 h at room temperature. β-actin antibody (1:1,000; cat. no. ab8227; Abcam) was used as an internal control. The protein bands were developed using an ECL kit (GE Healthcare) and quantified using ImageJ Software (version 1.46; National Institutes of Health).

Luciferase reporter assays. The potential binding site between PUMA-α and miR-494 was searched in TargetScan (http://www.targetscan.org) and PicTar (https://pictar.mdc-berlin.de/). A whole fragment of 3'UTR PUMA-α mRNA and a mutant form were cloned into pGL-3-Luc (Promega Coporation). The 293T cells were seeded in 24-well plates at $5x10^4$ cells per well and co-transfected with the pGL-3-PUMA-α wild-type or mutant portion and TK100 *Renilla* combined with the aforementioned miR-494 mimic, miR-494 inhibitor, mimic NC or inhibitor NC using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Cells were harvested 48 h after transfection, and luciferase activity was measured using a Dual-Luciferase® Reporter System (Promega Corporation) according to the manufacturer's protocol. Relative firefly luciferase activity was normalized to *Renilla* luciferase activity. All the dual-luciferase reporter assays were performed in triplicate within each experiment.

Statistical analysis. Data are reported as the mean ± SD (unless otherwise presented). Statistical significance among different groups was determined using one-way ANOVA followed by Tukey's post-hoc test and the significance of the difference in means between two groups was evaluated using unpaired Student's t-test. The paired Student's t-test was used to compare data from cancerous vs. non-cancerous tissues. The correlation between PUMA-α and miR-494 expression was assessed using a two-tailed Pearson's correlation analysis. Statistical significance was analyzed using GraphPad Prism software (version 5.0; GraphPad Software, Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

miR-494 is upregulated in LSCC tissues and cell lines. To explore the role of miRNAs in LSCC, miRNA expression patterns were initially profiled using GSE74190 microarray data. Cluster analysis based on the miRNA expression pattern indicated a significant difference between LSCC tissues and adjacent non-cancerous tissues (Fig. 1A). Among the aberrantly expressed miRNAs, miR-494 was chosen for further study, which has been reported as an oncogenic miRNA in several types of human cancer (16,22-24). Moreover, several studies have revealed that miR-494 acts as an oncomir and is involved in tumor development, progression and metastasis,

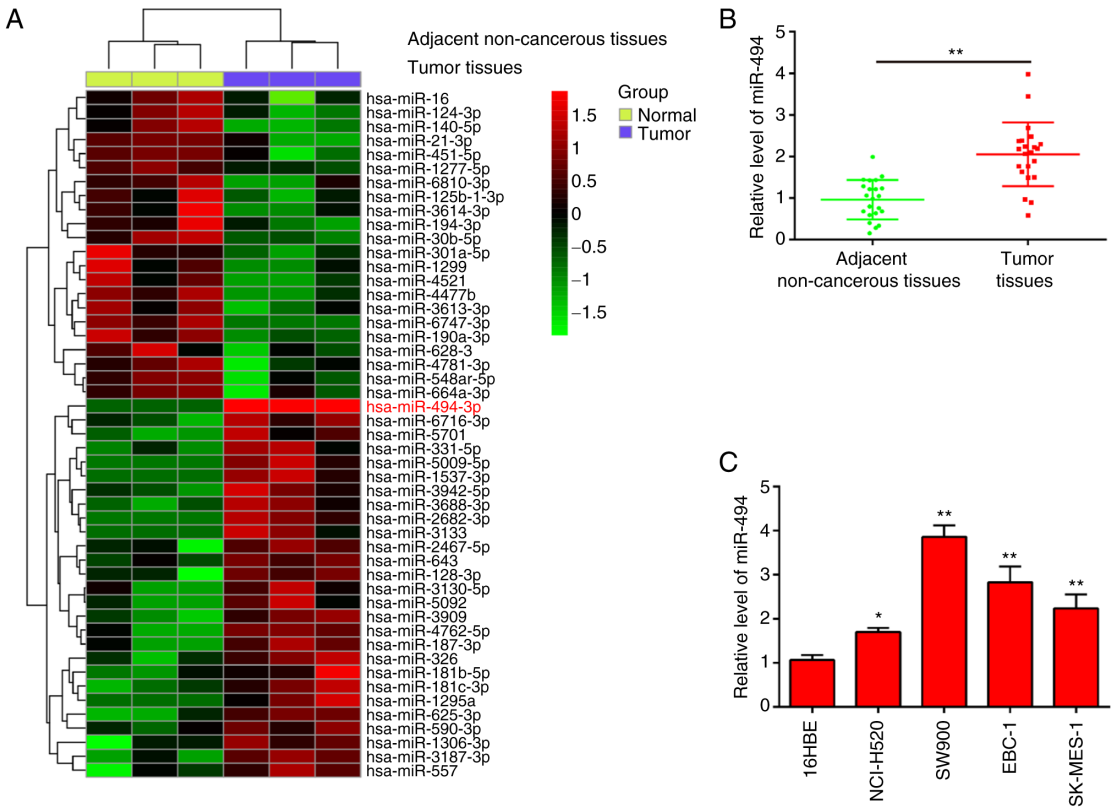

Figure 1. miR-494 is significantly upregulated in LSCC tissues and cell lines. (A) Hierarchical clustering of miRNAs differentially expressed in LSCC tissues compared with adjacent non-cancerous tissues. (B) miR-494 level was increased in 22 pairs of LSCC tissues. Data are shown as median and interquartile range. **P<0.01 vs. adjacent non-cancerous tissues. (C) Reverse transcription-quantitative PCR was performed to determine miR-494 expression in four LSCC cell lines (NCI-H520, SW900, EBC-1 and SK-MES-1) and a human bronchial epithelial cell line (16HBE). U6 was used as the endogenous control. Data are shown as the mean ± SD of three separate experiments. *P<0.05 and **P<0.01 vs. 16HBE cells. LSCC, lung squamous cell carcinoma; miR/miRNA, microRNA.

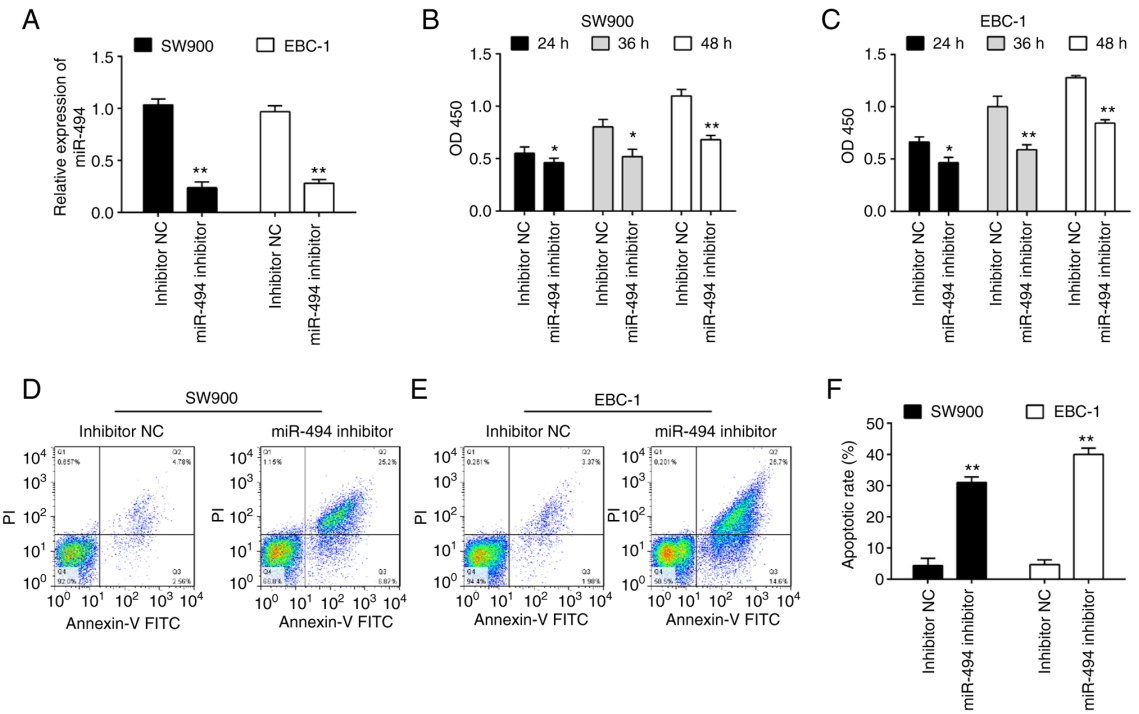

Figure 2. Knockdown of miR-494 inhibits cell proliferation and induces apoptosis. SW900 and EBC-1 cells were transfected with miR-494 inhibitor and inhibitor-NC. (A) SW900 and EBC-1 cells were harvested 48 h after transfection, and the expression of miR-494 was assessed using reverse transcription-quantitative PCR. After 24, 36 and 48 h of transfection, the viability of (B) SW900 and (C) EBC-1 cells were determined using Cell Counting Kit-8 assays. (D) SW900 and (E) EBC-1 cells were harvested 48 h after transfection, and apoptosis was assessed using flow cytometry and (F) quantified. Data are presented as the mean ± SD of three separate experiments. *P<0.05 and **P<0.01 vs. inhibitor NC. NC, negative control; miR, microRNA; OD, optical density.

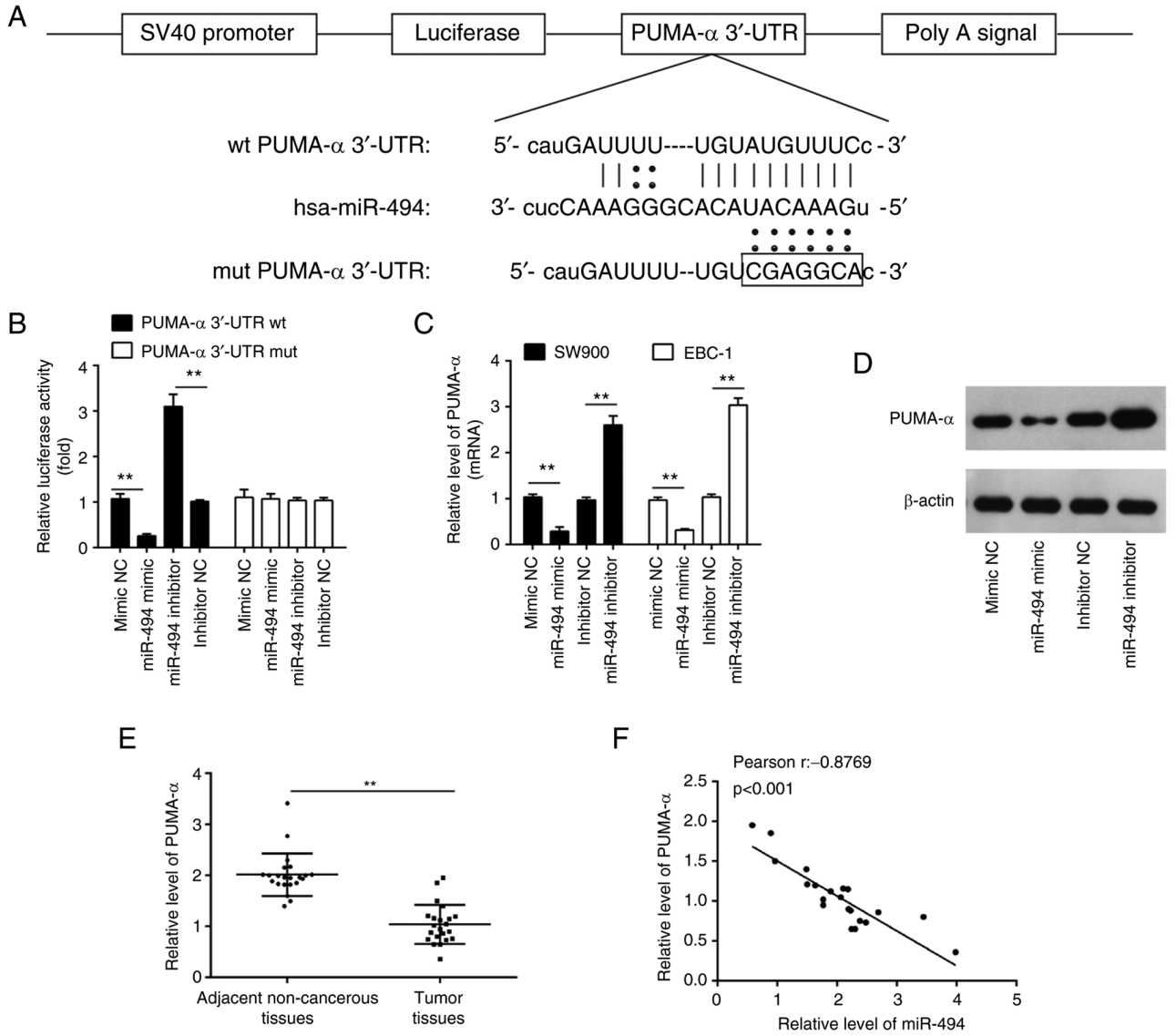

Figure 3. miR-494 directly targets PUMA-α. (A) miR-494 binds to the predicted site of the 3'-UTR of PUMA-α. (B) Luciferase activities; 293T cells were co-transfected with firefly luciferase constructs containing PUMA-α wt or mut 3'-UTRs and miR-494 mimic, mimic NC, miR-494 inhibitor or inhibitor NC. PUMA-α (C) mRNA and (D) protein levels in the indicated cells transfected with 20 nM miR-494 mimic, mimic NC, miR-494 inhibitor or inhibitor NC were measured using RT-qPCR and western blotting, respectively. (E) Expression levels of PUMA-α in 22 pairs of LSCC tissues and adjacent non-cancerous tissues were determined using RT-qPCR. (F) Analysis of the correlation between levels of PUMA-α and miR-494 expression in LSCC tissues (r=-0.8769; P<0.001). Data are shown as the mean ± SD of three separate experiments. **P<0.01. PUMA-α, p53 upregulated modulator of apoptosis-α; UTR, untranslated region; NC, negative control; RT-qPCR, reverse transcription-quantitative PCR; LSCC, lung squamous cell carcinoma; wt, wild-type; mut, mutated; miR, microRNA.

and confers resistance to chemotherapeutic drugs by targeting a number of molecules in several types of human cancer, such as endometrial cancer and hepatocellular carcinoma (25,26). However, the function and underlying molecular mechanism of miR-494 in LSCC has not been fully elucidated. To validate the expression trend of miR-494 in the LSCC tissues, RT-qPCR was performed to detect miR-494 expression in 22 pairs of LSCC tissues and adjacent non-cancerous tissues. As presented in Fig. 1B, the expression level of miR-494 in LSCC tissues was significantly higher compared with that in adjacent non-cancerous tissues. In addition, miR-494 levels were assessed in four LSCC cell lines (NCI-H520, SW900, EBC-1 and SK-MES-1) and in 16HBE, which acted as a normal control. miR-494 expression in all LSCC cells was upregulated compared with 16HBE, especially in SW900 and EBC-1 cells (Fig. 1C). These findings suggest that upregulation of miR-494 could be involved in the progression of LSCC.

miR-494 knockdown suppresses cell viability and promotes apoptosis. Given the upregulation of miR-494 in LSCC tissues, it was predicted that miR-494 may function as an oncogene. To verify this hypothesis, miR-494 inhibitor was transfected into two LSCC cell lines, namely SW900 and EBC-1. The miR-494 level was significantly downregulated in both SW900 and EBC-1 cells after miR-494 inhibitor transfection (Fig. 2A). To evaluate whether miR-494 could affect cell proliferation, a CCK-8 assay was performed, and it was revealed that knockdown of miR-494 significantly suppressed the viability of SW900 and EBC-1 cells compared with inhibitor NC transfected cells (Fig. 2B and C). The effects of miR-494 knockdown on cell apoptosis were further examined.

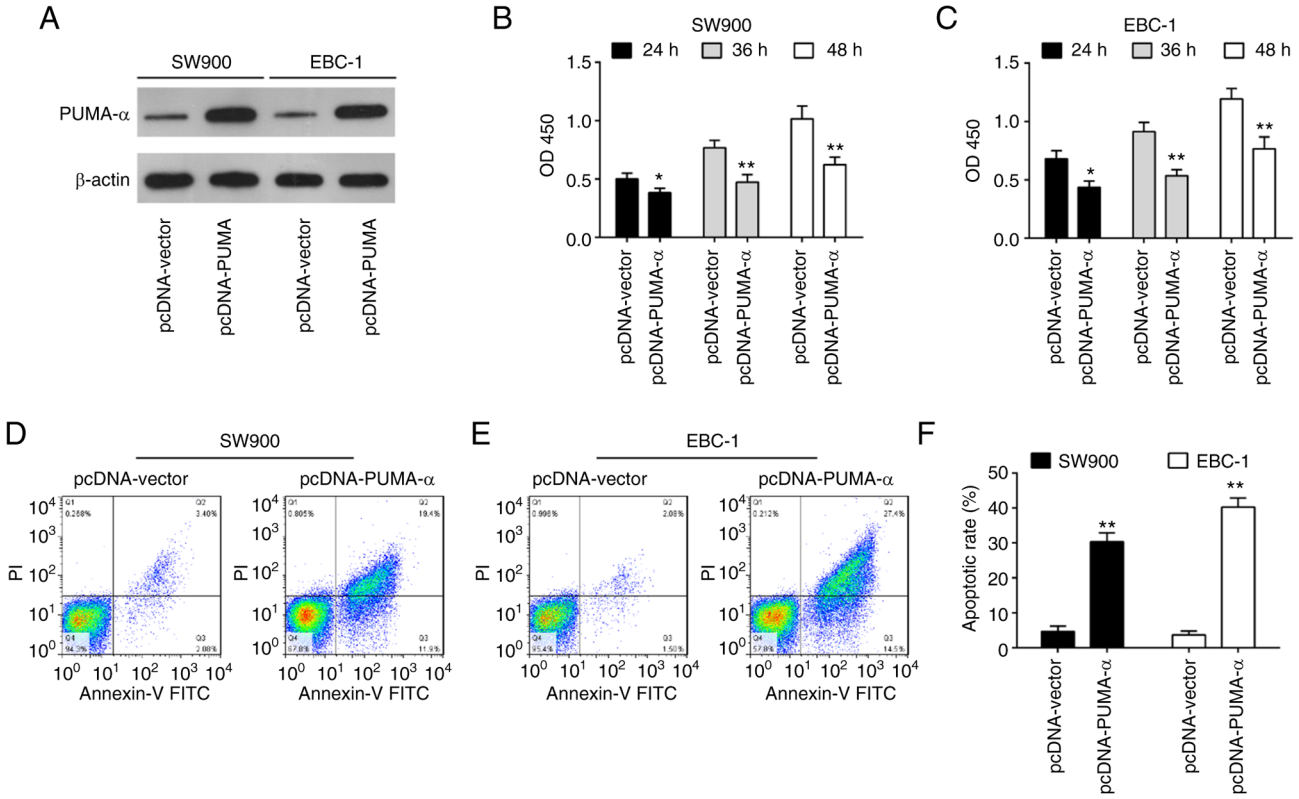

Figure 4. Overexpression of PUMA-α inhibits cell proliferation and promotes apoptosis. (A) Western blotting was performed to evaluate the overexpression efficiency of pcDNA-PUMA-α. β-actin was used as an internal control. (B and C) After 24, 36 and 48 h of transfection, the viabilities of SW900 cells and EBC-1 cells were determined using Cell Counting Kit-8 assays. At 48 h after transfection, the apoptosis rate was assessed by flow cytometry in (D) SW900 cells and (E) EBC-1 cells, and (F) quantified. Data are presented as the mean ± SD of three separate experiments. *P<0.05 and **P<0.01 vs. pcDNA vector. PUMA-α, p53 upregulated modulator of apoptosis; OD, optical density.

As shown in Fig. 2D-F, knockdown of miR-494 promoted apoptosis in both SW900 and EBC-1 cells compared with the inhibitor NC. Taken together, these data suggest that miR-494 may function as an oncogene in LSCC.

PUMA-α is a direct target of miR-494. To uncover the potential molecular mechanisms involved in the suppressive role of miR-494 inhibition in LSCC cells, the potential downstream targets of miR-494 were searched for using publicly available databases, including TargetScan and PicTar. According to bioinformatics analysis, PUMA-α was selected for further study due to its promotive role in apoptosis (27). The binding sites between miR-494 and PUMA-α are illustrated in Fig. 3A. To examine whether PUMA-α is a direct target of miR-494, a luciferase activity assay was conducted. As shown in Fig. 3B, miR-494 overexpression reduced the luciferase reporter activity of the PUMA-α 3'UTR, while inhibition of miR-494 had the opposite effect. Additionally, PUMA-α 3'UTR luciferase reporter activity was unaffected by point mutations in the miR-494-binding seed region. Moreover, it was observed that miR-494 overexpression inhibited the mRNA and protein expression levels of PUMA-α, whereas miR-494 knockdown enhanced the expression levels of PUMA-α (Fig. 3C and D). These results suggested that miR-494 suppressed the expression of PUMA-α at the transcriptional level.

In addition, RT-qPCR was used to determine the PUMA-α expression levels in 22 pairs of LSCC tissues and adjacent non-tumor tissues. It was demonstrated that the PUMA-α expression levels were significantly decreased in LSCC tissues compared with the adjacent non-tumor tissues (Fig. 3E). Correlation analysis revealed a strong negative correlation between the expression levels of miR-494 and PUMA-α mRNA levels in LSCC tissues (Fig. 3F). These data indicated that PUMA-α is a functional target of miR-494.

Overexpression of PUMA-α inhibits cell viability and promotes apoptosis. To investigate the function of PUMA-α in SW900 and EBC-1 cells, SW900 and EBC-1 cells were transiently transfected with pcDNA-PUMA-α for 24 h. As shown in Fig. 4A, PUMA-α expression levels were increased in SW900 and EBC-1 cells after pcDNA-PUMA-α transfection compared with the pcDNA-vector group. CCK-8 assay demonstrated that overexpression of PUMA-α significantly inhibited the proliferation of SW900 and EBC-1 cells compared with the pcDNA-vector group (Fig. 4B and C). Additionally, a significant increase in the percentage of apoptotic cells was also observed in the pcDNA-PUMA-α-transfected cells compared with the pcDNA-vector transfected cells (Fig. 4D-F). These findings suggest that overexpression of PUMA-α has a similar role to miR-494 inhibition in SW900 and EBC-1 cells.

Knockdown of PUMA-α attenuates the inhibitory effects of miR-494 inhibition on the SW900 and EBC-1 cells. Since PUMA-α was identified as the target gene of miR-494 in LSCC cells, whether PUMA-α is involved in miR-494-mediated

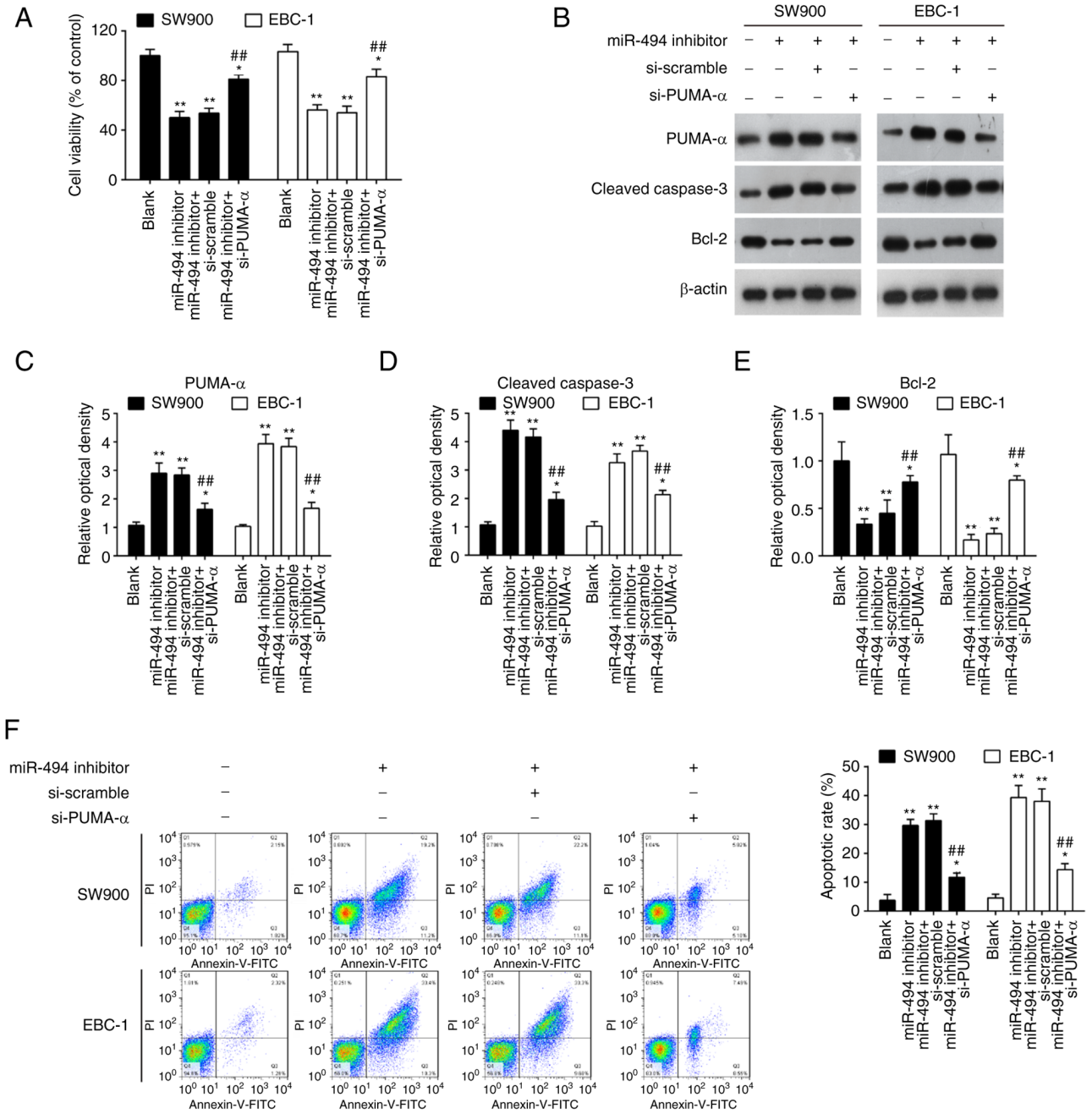

Figure 5. Knockdown of PUMA-α attenuates the inhibitory effects of the miR-494 inhibitor on SW900 and EBC-1 cells. SW900 and EBC-1 cells were transfected with miR-494 inhibitor, miR-494 inhibitor + si-scramble or miR-494 inhibitor + si-PUMA-α. Non-transfected cells were used as the 'Blank' control. (A) After 48 h transfection, the viabilities of SW900 and EBC-1 cells were determined using Cell Counting Kit-8 assays. (B) Using western blotting, the expression levels of (C) PUMA-α, (D) cleaved caspase-3 and (E) Bcl-2 were measured. β-actin was used as an internal control. (F) The apoptotic rate was assessed by flow cytometry. *P<0.05, **P<0.01 vs. Blank; $^{\#\#}$P<0.05 vs. miR-494 inhibitor + si-scramble. Data are shown as the mean ± SD of three separate experiments. PUMA-α, p53 upregulated modulator of apoptosis; si, short interfering.

roles in LSCC cells was determined. As shown in Fig. 5A, PUMA-α knockdown could rescue the suppression effect of miR-494 inhibitor on SW900 and EBC-1 cell viability. Furthermore, the data showed that miR-494 inhibition significantly increased the expression levels of pro-apoptotic protein PUMA-α and cleaved caspase-3, and also decreased the expression levels of anti-apoptotic protein Bcl-2 in the SW900 and EBC-1 cells compared with the Blank group. However, the effects of the miR-494 inhibitor were partially attenuated by the knockdown of PUMA-α (Fig. 5B-E). Subsequently, it was confirmed that the promotion of apoptosis mediated by miR-494 inhibition was also attenuated by the knockdown of PUMA-α (Fig. 5F). These results indicated that the knockdown of miR-494 induced apoptosis in LSCC cells by targeting PUMA-α.

Discussion

The present study revealed that miR-494 was upregulated in LSCC tissues and cell lines, and that knockdown of miR-494 suppressed cell proliferation and induced apoptosis. PUMA-α was verified as the target of miR-494 in LSCC cells. It was

shown that knockdown of PUMA-α reversed the inhibitory effects of the miR-494 inhibitor on LSCC cells. These results provide new insights into LSCC research and therapeutic strategies.

Mounting evidence has indicated that miRNAs play an important role in the initiation and progression of human cancer (28,29). Thus, identification of tumor-associated miRNAs and their target genes is critical for understanding the roles of miRNAs in tumorigenesis (29). Up to now, several miRNAs have been identified in LSCC and demonstrated to regulate cell migration, invasion, proliferation and apoptosis (30-32). For example, miR-218 is frequently downregulated in LSCC clinical specimens and appears to function in anti-migration and anti-invasion roles through targeting tumor protein D52 (33). In the present study, using GSE74190 microarray data, it was found that large numbers of miRNAs were significantly upregulated; in particular, miR-494 was identified as the most upregulated miRNA in human LSCC tissues, which is consistent with a previous study that showed that miR-494 expression is increased in LSCC tissues (22), thus indicating that increased miR-494 may be involved in LSCC carcinogenesis.

In previous studies, miR-494 has been implicated in several types of human cancer, such as endometrial cancer and prostate cancer (25,34). It has been reported that miR-494 inhibition can suppress cell proliferation, migration and invasion, as well as induce apoptosis by targeting downstream genes, including p190B and mutated in colorectal cancer (35,36). A previous report demonstrated that miR-494 promotes cell proliferation in hepatocellular carcinoma by targeting PTEN (22). However, its biological roles in LSCC remain largely unknown. In the present study, it was found that miR-494 knockdown inhibited LSCC cell proliferation and induced apoptosis, suggesting that miR-494 functioned as an oncogene in LSCC. However, the underlying molecular mechanisms involved in miR-494 inhibition-mediated proliferation and apoptosis suppression have not been completely clarified.

The Bcl-2 family of proteins, which includes the protein known as PUMA-α, regulates the mitochondrial apoptotic pathway to maintain the integrity of the outer membrane of the mitochondria (37). According to reports, PUMA-α is important for the apoptosis of certain cancer cells (27,38). For example, Yang *et al* (39) showed that, in colon cancer cells, PUMA-α mediates the pro-apoptotic impact of idelalisib through the mitochondrial route. In colorectal and lung cancer cells, overexpression of PUMA-α has been linked to miR-203-induced cell death, according to a previous study (40). However, whether PUMA-α participates in the antitumor roles of miR-494 inhibition in LSCC remains unknown. In the present study, PUMA-α was shown to be a target of miR-494. Moreover, it was demonstrated that overexpression of PUMA-α has similar effects to miR-494 inhibition on SW900 and EBC-1 cells, whereas knockdown of PUMA-α reversed the inhibitory effects of miR-494 inhibition on LSCC cells. Collectively, these results suggest that miR-494 inhibition upregulates the expression of PUMA-α, resulting in the promotion of the apoptosis of LSCC cells.

In conclusion, the present study confirmed that miR-494 knockdown suppressed LSCC cell proliferation and promoted apoptosis by targeting PUMA-α. These findings provide a theoretical basis for the prevention and treatment of LSCC, and implicate miR-494 as a potential prognostic biomarker and therapeutic target of LSCC.

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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

XG, XYa and FH performed all the experiments and collected the data. XYu conceived and designed the study. XYa, XL and DL wrote the main manuscript and analyzed the data. XG and XYu confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of The First Affiliated Hospital of Xinxiang Medical University (Weihui, China; approval no. XXMC2019-0112) and written informed consent was obtained from each patient.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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