MicroRNA-199a-5p functions as a tumor suppressor in oral squamous cell carcinoma via targeting the IKKβ/NF-κB signaling pathway

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Abstract. MicroRNAs (miRNAs) have been shown to have a significant role in the progression of several types of cancer, including oral squamous cell carcinoma (OSCC). However, the biological function and regulatory mechanisms of miRNAs in OSCC remain to be fully elucidated. The aim of the present study was to investigate the role of miRNAs in OSCC and the relevant mechanism. Using a microarray, it was found that miRNA (miR)-199a-5p was one of the most downregulated miRNAs in OSCC tissues. A low expression of miR-199a-5p was closely associated with tumor differentiation, lymph node metastasis, tumor-node-metastasis stage, and overall survival rate. Functionally, the overexpression of miR-199a-5p suppressed cell proliferation, induced G0/G1 cell cycle arrest, and promoted the apoptosis of Tca8113 and SCC-4 cells. Subsequently, inhibitor of nuclear factor-κB (NF-κB) kinase β (IKKβ), an important regulator of NF-κB activation, was identified as a direct target of miR-199-5p. An inverse correlation was found between miR-199a-5p and IKKβ in tumor tissues. Further investigations revealed that the overexpression of IKKβ efficiently abrogated the influences caused by the overexpression of miR-199a-5p. It was also found that the miR-199a-5p-mediated anticancer effects were dependent on the inhibition of NF-κB activation. These findings indicate that miR-199a-5p functions as a tumor suppressor through regulation of the NF-κB pathway by targeting IKKβ in OSCC.

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Introduction

Oral squamous cell carcinoma (OSCC) is the sixth most common malignancy worldwide (1,2). According to statistics, >300,000 new cases are reported annually in the US (3). Although progress has been made in OSCC therapy, including surgery, and chemo- or radiotherapy, the 5-year-survival rate of OSCC remains <60% due to local recurrence and nodal metastasis (4). Therefore, there is an urgent requirement to identify potential molecular therapeutic targets for the treatment of OSCC.

Inhibitor of nuclear factor (NF)-κB (IκB) kinase β (IKKβ), a key catalytic subunit of the IKK complex, is crucial in the activation of NF-κB, which activates cellular programs critical for cell survival and proliferation (5,6). IKKβ can activate the NF-κB dimers by means of IKK-mediated, phosphorylation-induced degradation of the IκB inhibitor, which enables the NF-κB dimers to enter the nucleus and activate specific target gene expression (7). In OSCC, the activation of NF-κB induces epithelial-mesenchymal transition, and the elevated expression of NF-κB is correlated with enhanced invasion and metastasis (8-11). The constitutive activation of NF-κB in OSCC has also been reported (12). It has been reported that inhibiting IKKβ can markedly reduce the cellular invasiveness of SCC-25 cells and may be useful for OSCC therapy (13). However, the molecular regulatory mechanism of the IKKβ/NF-κB signaling pathway in OSCC remains to be fully elucidated.

MicroRNAs (miRNAs) are a class of small non-coding RNA, which negatively regulate gene expression at the post-transcriptional level by binding to the 3'-untranslated region (3'-UTR) of the mRNAs of target genes (14,15). Alterations in miRNA expression have been implicated in the pathogenesis of a variety of human diseases, notably cancer (16-18). A number of miRNAs have been identified to contribute to the development of OSCC. For example, Zhang *et al* demonstrated that miRNA (miR)-375 was significantly lower in OSCC tissues, and investigated the prognostic value of miR-375 in patients with OSCC (19). Feng *et al* showed that miR-22 suppressed cell proliferation, migration and invasion in OSCC by targeting NLR family pyrin domain

containing 3 (20). However, whether there are other miRNAs involved, and the specific mechanisms require further investigation.

In the present study, an miRNA microarray was performed to investigate the expression of miRNAs in OSCC tissues and the most downregulated of these, miR-199a-5p, was selected for further analysis. *In vitro* experiments were performed to investigate the functional role of miR-199a-5p in OSCC cells and to examine the underlying mechanisms. The findings of these experiments suggested that miR-199a-5p may be a potential target for OSCC treatment and may be important in the development of OSCC.

Materials and methods

Clinical specimens. Samples of 60 pairs of tumor tissues and matched tumor-adjacent tissues were obtained from patients with OSCC with pathologically diagnostic criteria between January 2014 and July 2016 in the Department of Oral and Maxillofacial Surgery, the First Affiliated Hospital of Xinxiang Medical University (Weihui, China). The clinicopathological data are shown in Table I. Written consent for tissue donation for research purposes was obtained from each patient prior to tissue collection. The protocol was approved by the Ethics Committee of the First Affiliated Hospital of Xinxiang Medical University. All the tissue samples were collected, immediately snap-frozen in liquid nitrogen and stored at -80˚C until RNA was extracted.

Microarray analysis. Total RNA was extracted from the OSCC tissues using an miRNeasy Mini kit (Qiagen, Inc., Valencia, CA, USA). The purity and quantity of the total RNA were evaluated via NanoDrop ND-1000 spectrophotometry (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and the Agilent 2100 Bioanalyzer. Total RNA (200 ng) was labeled with fluorescence dye hy3 or hy5 using the miRCURY Hy3/Hy5 Power Labeling kit and hybridized on the miRCURY™ LNA Array (v.16.0), both obtained from Exiqon; Qiagen, Inc., according to the manufacturer's protocol. Following washing with PBS, the Axon GenePix 4000B microarray scanner (Axon Instruments; Molecular Devices, LLC, Sunnyvale, CA, USA) was used to scan the fluorescence intensity of the microarray. The scanned images were then imported into the GenePix Pro 6.0 program (Axon Instruments; Molecular Devices, LLC) for grid alignment and data extraction. Finally, the heat map of the 57 miRNAs with the most marked differences was created using a method of hierarchical clustering in GeneSpring GX software, version 7.3 (Agilent Technologies, Inc., Santa Clara, CA, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. miRNA was prepared using the miRNeasy Mini kit (Qiagen, Inc.) according to the manufacturer's protocol. The concentration and quality of total RNA was determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc.). For miRNA reverse transcription, cDNA was synthesized using the PrimeScript RT reagent kit (Takara Bio, Inc., Tokyo, Japan). For mRNA, total RNA was isolated using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol and reverse transcribed with the SuperScript III First-Strand Synthesis System (Thermo Fisher Scientific, Inc.). qPCR analyses for miRNA and mRNA were performed on an ABI PRISM 7300 sequence detection system in an SYBR-Green I Real-Time PCR kit (Applied Biosystems; Thermo Fisher Scientific, Inc.). First, 20-μl PCRs included 2 μl cDNA, 10 μl of 2X qPCR mix, 250 nM concentrations of forward (1 μl) and reverse primers (1 μl), and 6 μl ddH_2O. The reaction mixtures were denatured at 95˚C for 3 min, followed by 40 two-step cycles of 95˚C for 10 sec and 60˚C for 30 sec. The primers for RT-qPCR analysis were as follows: miR-199a-5p forward, 5'-TCCCAGTGTTCA GACTACC-3' and miR-199a-5p reverse, 5'-TTTGGCACT AGCACATT-3'; IKKβ forward, 5'-ACTTGGCGCCCAATG ACCT-3' and IKKβ reverse, 5'-CTCTGTTCTCCTTGCTGC A-3'; GAPDH forward, 5'-GAAGATGGTGATGGGATTTC-3' and GAPDH reverse, 5'-GAAGGTGAAGGTCGGAGT-3'; U6 forward, 5'-TGCGGGTGCTCGCTTCGCAGC-3' and U6 reverse, 5'-CCAGTGCAGGGTCCGAGGT-3'. The expression of miR-199a-5p and IKKβ in the tissues was normalized to the expression of U6 and GAPDH, respectively. The RT-qPCR assays were performed in triplicate and the change in expression level was calculated using the $2^{-\Delta\Delta Cq}$ method (21).

Cell lines and culture. The SCC-25, CAL-27, Tca8113, SCC-4 OSCC and 293 cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), 100 U/ml penicillin and 100 μg/ml streptomycin at 37˚C in a 5% CO_2 atmosphere. The normal oral mucosa cell line (Human Oral Keratinocyte; HOK, Invitrogen; Thermo Fisher Scientific, Inc.) was used as control and was maintained in oral keratinocyte media, supplemented with 1% keratinocyte growth factor plus epithelial growth factor mixture (Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C in a 5% CO_2 atmosphere.

Cell transfection. The miR-199a-5p mimics, mimics negative control (mimics NC), miR199a-5p inhibitor, and inhibitor NC were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). The detailed information regarding miR-199a-5p mimics, miR-199a-5p inhibitor and their controls are as follows: i) miR-199a-5p mimic sense, 5'-CCCAGUGUU CAGACUACCUGUUC-3'; mimics NC sense, 5'-CGGTGU GUUCAGACUACCUGUUC-3'; ii) miR-199a-5p inhibitor, 5'-AACAGGTAGTCTGAACACT-3'; inhibitor NC, 5'-TAA CACGTCTATACGCCCA-3'. To induce the overexpression of IKKβ, the coding domain sequences of IKKβ mRNA were amplified by PCR, and inserted into the pcDNA 3.0 vector to enhance its expression (Invitrogen; Thermo Fisher Scientific, Inc.), named pcDNA-IKKβ. The empty pcDNA3.1 was used as a negative control (NC). The Tca8113 and SCC-4 cells ($1.0x10^6$/well) were seeded and grown overnight in six-well plates. The following day, Lipofectamine 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was used for transient transfection of the cells with miR-199a-5p mimics (50 nM), miR-199a-5p inhibitor (100 nM) or NC (100 nM), whereas Oligofectamine transfection reagent (Thermo Fisher Scientific, Inc.) was used for transfection with miR-199a-5p mimics (50 nM) + 2 μg pcDNA-IKKβ for 48 h, following

Table I. Correlation between miR-199a-5p and clinicopathological features in patients with oral squamous cell carcinoma.

Clinical parameter	Cases (n=60)	miR-199-5p expression		P-value
		High (22)	Low (38)	
Sex				0.512
Male	36	12	24	
Female	24	10	14	
Age (years)				0.242
≥50	41	13	28	
<50	19	9	10	
Site				0.381
Buccal mucosa	31	13	18	
Non-buccal mucosa	29	9	20	
Alcohol use				0.694
Yes	28	11	17	
No	32	11	21	
Smoking habit				0.801
Yes	26	10	16	
No	34	12	22	
Tumor size (cm)				0.024[a]
≥2	22	4	18	
<2	38	18	20	
Differentiation				0.015[a]
Well and moderate	37	18	19	
Poor	23	4	19	
Lymph node metastasis				0.009[b]
Present	35	8	27	
Absent	25	14	11	
cTNM stage				0.030[a]
I+II	15	9	6	
III+IV	45	13	32	

miR, microRNA; TNM, tumor-node-metastasis. [a]P<0.05, [b]P<0.01.

the manufacturer's protocol. The transfection efficiency was confirmed by analyzing the expression levels of miR-199a-5p using RT-qPCR at 24 post-transfection. At 48 h post-transfection, the cells were harvested for western blot or RT-qPCR analyses, or other experiments at the indicated times.

Cell proliferation. The Tca8113 and SCC-4 cells ($5x10^3$/well) were seeded in 96-well plates overnight. At 24, 48 and 72 h post-transfection, Cell Counting Kit-8 solution (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was added to cells and incubated at 37˚C for an additional 2 h. The absorbance rates were then measured at 450 nm using a microplate reader (Infinite M200; Tecan Group, Ltd., Mannedorf, Austria). All experiments were performed in triplicate.

Cell apoptosis. Apoptosis was measured using an Annexin V-FITC Apoptosis Detection kit (Abcam, Cambridge, UK) according to the manufacturer's protocol. At 48 h post-transfection, the Tca8113 and SCC-4 cells were harvested and washed twice with PBS, and were stained with Annexin V and propidium iodide (PI). Following incubation at room temperature in the dark for 15 min, cell apoptosis was analyzed on a FACScan flow cytometer (Beckman Coulter, Inc., Brea, CA, USA).

Cell cycle analysis. Cell cycle distribution was determined using flow cytometry (22). Briefly, at 48 h post-transfection, the Tca8113 and SCC-4 cells were harvested by trypsinization and plated in 6-cm dishes at a density of $1.0x10^5$ cells/dish. The cells were then washed with PBS and fixed in 70% ethanol overnight at 4˚C, following which they were then stained with 40 μg/ml PI, and incubated at 4˚C for 30 min in the dark. The cells were analyzed by flow cytometry using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA).

Immunohistochemistry (IHC). Two paired OSCC tissues and matched tumor-adjacent tissues were embedded in paraffin and sliced. The thickness of the tissue sections were 4-5 mm.

Immunohistochemical staining were performed as described previously (23), IKKβ was detected using anti-IKKβ antibody (cat. no. sc8014; 1:200; Santa Cruz Biotechnology, Inc., Dallas, TX, USA).

Bioinformatics analysis and luciferase reporter assay. miRNA target prediction tools, including PicTar version 2007 (https://pictar.mdc-berlin.de/) and TargetScan Release 7.0 (http://targetscan.org/) were used to search for the putative targets of miR-199a-5p. The 3'-UTR of IKKβ and the mutated sequence were inserted into the pGL3 control vector (Promega Corporation, Madison, WI, USA) to construct the wild-type (wt) IKKβ-3'-UTR vector and mutant IKKβ-3'-UTR vector, respectively. For the luciferase reporter assay, 293 cells were transfected with the corresponding vectors; at 48 h post-transfection, the dual-luciferase reporter assay system (Promega Corporation) was used to measure the luciferase activity. All experiments were performed in triplicate.

Western blot analysis. Total protein was extracted using radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China) supplemented with protease inhibitors (Roche Diagnostics, Guangzhou, China). The concentrations of total cellular protein were determined using a BCA assay kit (Pierce; Thermo Fisher Scientific, Inc.). The extraction and isolation of nuclear and cytoplasmic proteins were performed according to the Nuclear and Cytoplasmic Protein Extraction kit (Beyotime Institute of Biotechnology). Briefly, the cells were collected following washing with 1 ml ice-cold PBS and then centrifuged for 5 min at 400 x g at 4˚C, and the pellet was dissolved with cytoplasmic protein extraction agent A supplemented with PMSF. The tubes were vortexed for 4 sec and incubated for 10-15 min on ice to promote lysis. Cytoplasmic protein extraction agent B was then added, vortexed for 5 sec and incubated on ice for 5 sec. The samples were then centrifuged for 5 min at 14,000 x g at 4˚C and the supernatant, containing the cytoplasmic fraction, was immediately frozen for further analysis. The pellet was re-suspended in nuclear protein extraction agent supplemented with PMSF. Following vortexing 15-20 times for 30 min and centrifuging for 10 min at 14,000 x g at 4˚C, the supernatants containing the nuclear extracts were obtained. The nuclear and cytoplasmic proteins were quantified with the BCA kit according to the manufacturer's protocol. The protein samples (40 μg/lane) were analyzed on an 8% SDS-PAGE gel and transferred onto polyvinylidene difluoride membranes (GE Healthcare, Freiburg, Germany) by electroblotting. The membranes were blocked for 1 h with 5% non-fat milk at room temperature and then incubated with primary antibodies against IKKβ (cat no. 8943; 1:1,000 dilution; Cell Signaling Technology, Inc., Danvers, MA, USA), total p65 (cat. no. 8242; 1:1,000 dilution; Cell Signaling Technology, Inc.), nuclear phosphorylated (p-)p65 (cat. no. 3033; 1:1,000 dilution; Cell Signaling Technology, Inc.), inhibitor of NF-κB (IκB)-α (cat. no. sc-52900; 1:1,000 dilution; Santa Cruz Biotechnology, Inc.), p-IκB-α (cat. no. 2859; 1:1,000 dilution; Cell Signaling Technology, Inc.), Histone H3 (cat. no. 9728; 1:1,000 dilution; Cell Signaling Technology, Inc.), β-actin (cat. no. 4970; 1:2,000 dilution; Cell Signaling Technology, Inc.) and α-tubulin (cat. no. ab7291; 1:2,000; Abcam) at 4˚C overnight. Following incubation with the corresponding horseradish peroxidase-conjugated goat anti-rabbit or goat anti-rat secondary antibodies (cat. nos. ab6721 and 6785; 1:2,000; Abcam) for 1 h at room temperature, the bands were detected using an enhanced chemiluminescence kit (GE Healthcare). The intensities of the bands of interest were analyzed using ImageJ software (version 1.46; National Institutes of Health, Bethesda, MD, USA). β-actin and α-tubulin proteins were used as the inner controls of the cytoplasmic proteins; Histone H3 protein was used as the inner control of the nuclear proteins. Each experiment was run in triplicate.

NF-κB activity assay. The Tca8113 and SCC-4 cells were plated in 6-well plates at a concentration of $5x10^4$ cells/well. The cells were allowed to attach overnight and then co-transfected with 20 ng of the pGL4.32 [luc2P/NF-κB-RE/Hygro] vector and 5 ng of the pRL-TK vector in each well (Promega Corporation). After 6 h, the cells were washed with PBS and then transfected with miR-199a-5p mimics and pcDNA-IKKβ for 24 h. The cells were washed in PBS and harvested in 500 μl 1X passive lysis buffer. Luciferase activity was quantified using the Promega luciferase assay kit on a luminometer.

Statistical analysis. Data are presented as the mean ± standard deviation. SPSS 19.0 statistical software (IBM Corp., Armonk, NY, USA) was used to perform all statistical analyses. When only two groups were compared, Student's t-test was conducted. One-way analysis of variance followed by Tukey's post hoc test was applied to compare differences between multiple groups. Pearson's or Spearman's analyses were used for correlation analysis. $P \leq 0.05$ was considered to indicate a statistically significant difference.

Results

Expression of miR-199a-5p is downregulated in OSCC and correlates with clinicopathologic parameters. To examine the potential involvement of miRNAs in the development of OSCC, miRNA microarray profiling was performed in three pairs of OSCC tissues and matched tumor-adjacent tissues. The miRNA microarray identified 39 miRNAs that were upregulated in OSCC and 18 miRNAs that were downregulated in OSCC tissues (Fig. 1A). Of the downregulated miRNAs, miR-199a-5p was identified as one of the most markedly downregulated, which is consistent with a previous study (24). Of note, miR-199a-5p has previously been reported to function as a tumor suppressor in a several types of human cancers (25-29). However, its role in the tumorigenesis of OSCC remains to be fully elucidated. Therefore, the present study focused on miR-199a-5p in OSCC for molecular and clinical analyses, to clarify the previously unknown role of miR-199a-5p.

To further verify the dysregulation of miR-199a-5p, RT-qPCR analysis was performed based on 60 paired tumor tissues and matched tumor-adjacent tissues. The results showed that miR-199a-5p was downregulated in the OSCC tissues (Fig. 1B). In addition, to further elucidate whether the altered expression of miR-199a-5p occurred in OSCC cells, RT-qPCR was performed to detect miR-199a-5p in four OSCC cell lines (SCC-25, CAL-27, Tca8113 and SCC-4) and

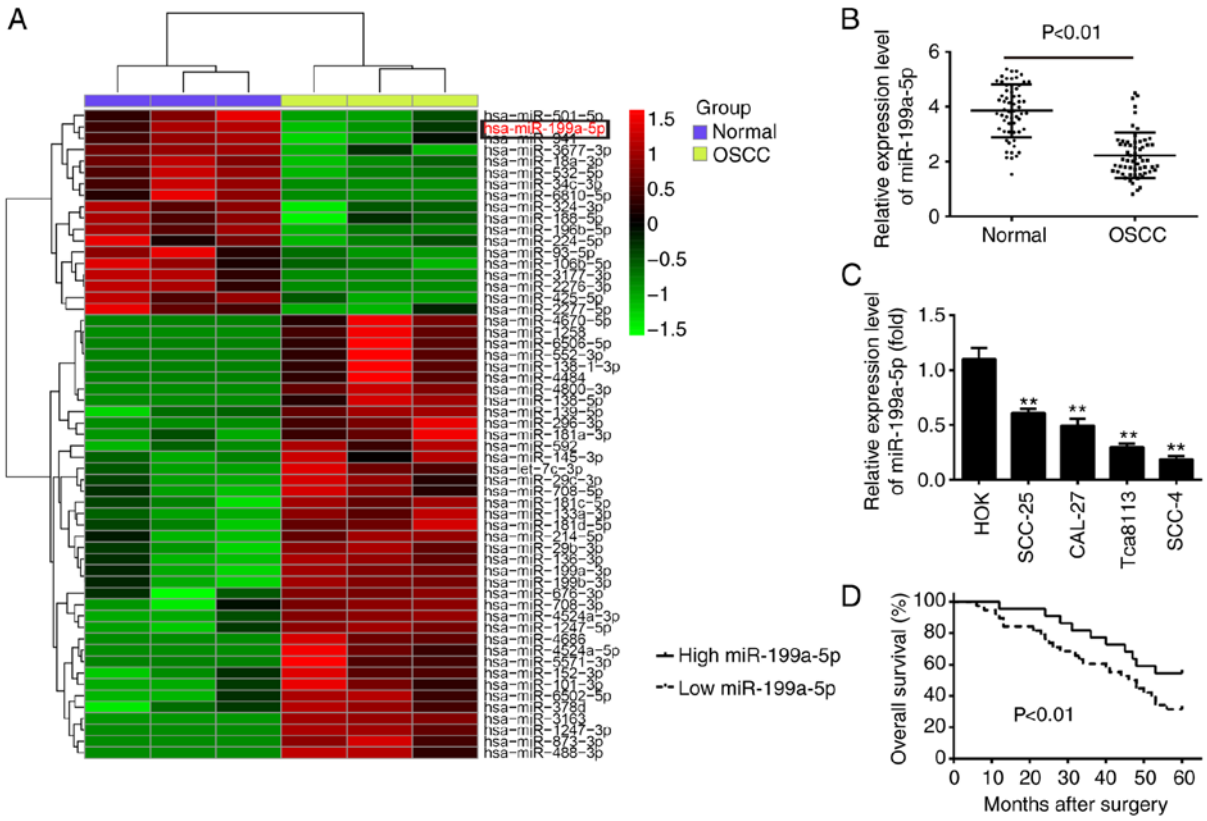

Figure 1. miR-199a-5p is downregulated in OSCC tissues and cell lines. (A) Heat map of miRNA profiles representing the significantly up/downregulated miRNAs. The color code in the heat maps is linear, with green as the lowest and red as the highest. The miRNAs that were upregulated are shown in green to red, whereas the miRNAs that were downregulated are shown from red to green. (B) Expression of miR-199a-5p was validated by RT-qPCR analysis in OSCC tissues and matched tumor-adjacent tissues (n=60). (C) Expression of miR-199a-5p was measured in four OSCC cell lines (SCC-25, CAL-27, Tca8113 and SCC-4), and the HOK normal oral mucosa cell line, used as a control, by RT-qPCR analysis. Data are presented as the mean ± standard deviation of three independent experiments. **P<0.01 vs. HOK cells. (D) Kaplan-Meier survival curves of patients with OSCC according to the expression of miR-199a-5p. Patients with a low tumor expression of miR-199a-5p showed significantly shorter survival rates than those with a high tumor expression of miR-199a-5p (P<0.01; log-rank test). OSCC, oral squamous cell carcinoma; miR, microRNA; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; HOK, Human Oral Keratinocyte.

a normal oral mucosa cell line (HOK) used as a control. As shown in Fig. 1C, consistent with the results in the OSCC tissues, miR-199a-5p was significantly decreased in the four OSCC cell lines compared with the HOK cells, suggesting that alteration in the expression of miR-199a-5p may contribute, at least in part, to the carcinogenesis of OSCC.

To determine the clinical values of miR-199a-5p, the mean expression level of miR-199a-5p was used as a cut-off value to divide 60 patients with OSCC into two groups: miR-199a-5p high expression group and miR-199a-5p low expression group. The associations between the expression of miR-199a-5p and clinicopathological features are summarized in Table I. It was found that a low expression of miR-199a-5p was associated with tumor size, tumor differentiation, lymph node metastasis and tumor-node-metastasis (TNM) stage. In this cohort, compared with the patients in the high miR-199a-5p expression group, patients in the low miR-199a-5p expression group had a lower 5-year overall survival (OS) rate (Fig. 1D). These result indicated that miR-199a-5p may serve as an effective biomarker for the prognosis of patients with OSCC.

Overexpression of miR-199a-5p suppresses cell viability, inhibits cell cycle and promotes cell apoptosis. Previous evidence shows that miR-199a-5p has a tumor suppressive function in several types of cancer, including ovarian cancer and hepatocellular carcinoma (26,28). As the findings in the present study showed that this miRNA was downregulated in OSCC, it was hypothesized that miR-199a-5p may serve as a tumor-suppressive miRNA in OSCC. To confirm this hypothesis, Tca8113 and SCC-4 cells, which showed the lowest level of miR-199a-5p among the four OSCC cell lines, were transfected with the miR-199a-5p mimics or mimics-NC to investigate the biological function of miR-199a-5p in OSCC. The RT-qPCR assays revealed that the expression of miR-199a-5p was significantly upregulated following transfection with the miR-199a-5p mimics, compared with that in the NC control group in the Tca8113 and SCC-4 cells (Fig. 2A). The CCK-8 assay revealed that transfection with miR-199a-5p mimics significantly inhibited cell viability compared with that in the mimics NC-transfected cells (Fig. 2B and C), and a significant induction of apoptosis in the Tca8113 and SCC-4 cells was observed (Fig. 2D). The results of the flow cytometric analysis revealed that the overexpression of miR-199a-5p led to G0/G1 phase arrest by elevating the percentage of cells at the G0/G1 phase in Tca8113 and SCC-4 cells (Fig. 2E and F), suggesting that miR-199a-5p decreased viability partially through inducing cell apoptosis and cell cycle arrest. These results support the

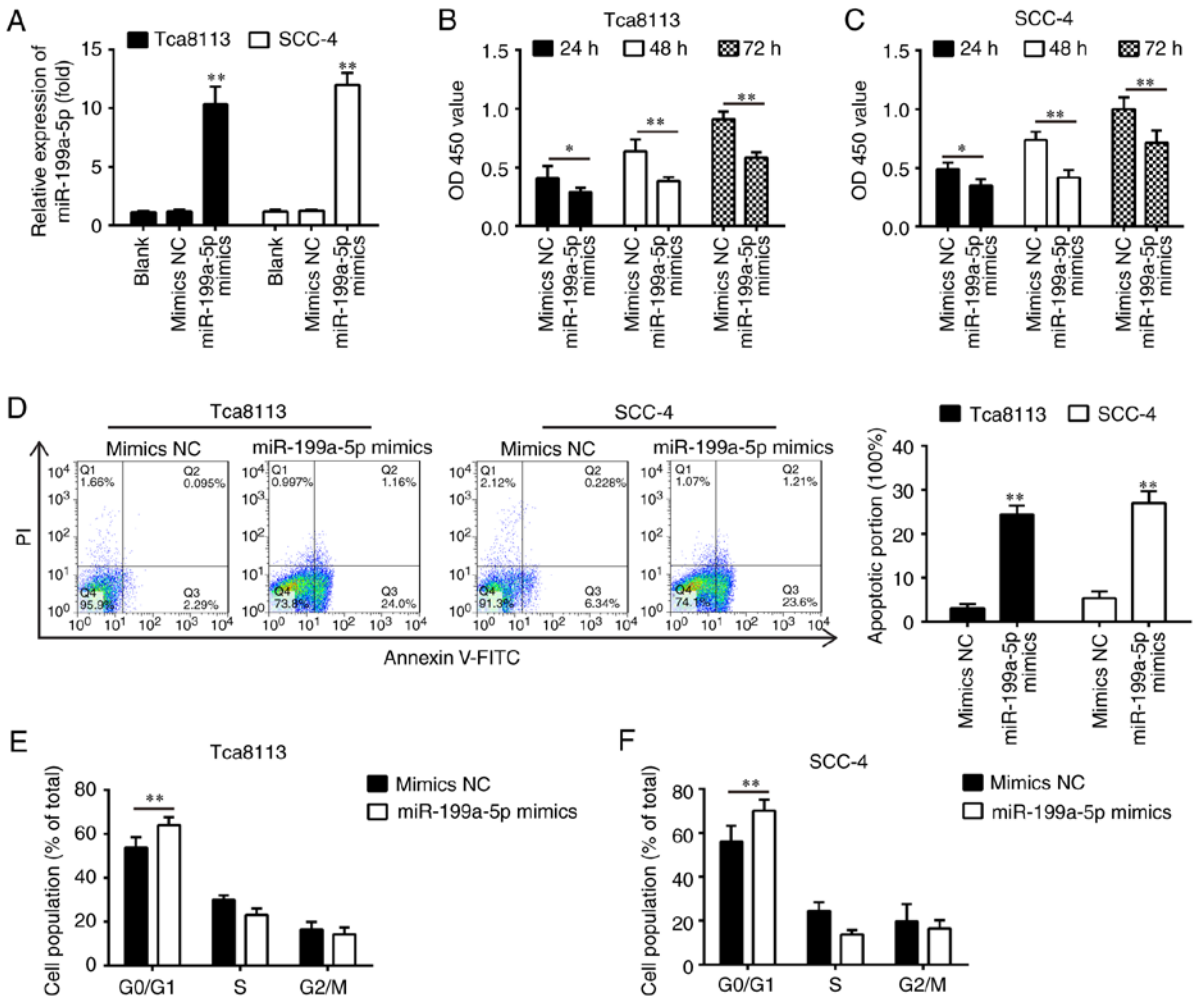

Figure 2. Overexpression of miR-199a-5p suppresses cell proliferation, causes cell cycle arrest and promotes cell apoptosis. Tca8113 and SCC-4 cells were transfected with the miR-199a-5p mimics or mimics-NC for 48 h, and the cells were used for analysis. (A) Transfection efficiency was assessed by reverse transcription-quantitative polymerase chain reaction analysis. Cell proliferation was measured using a Cell Counting Kit-8 assay in (B) Tca8113 and (C) SCC-4 cells at the indicated times. (D) Apoptosis was detected by flow cytometry. The cell cycle stages of the (E) Tca8113 and (F) SCC-4 cells were detected by flow cytometry. Data are presented as the mean ± standard deviation of three independent experiments. *P<0.05, **P<0.01 vs. mimics NC. miR, microRNA; NC, negative control; PI, propidium iodide.

hypothesis that miR-199a-5p functions as a tumor suppressor in the development of OSCC.

IKKβ is a direct target of miR-199a-5p. To examine the molecular mechanism by which miR-199a-5p functions in OSCC, candidate target genes of miR-199a-5p were computationally screened using the TargetScan and PicTar algorithms. Among several predicted target genes, IKKβ was noted for its high scores in both algorithms. The bioinformatics analysis showed that miR-199a-5p directly targeted the IKKβ gene and that the target sequences were highly conserved among species (Fig. 3A). In our previous study, it was shown that IKKβ was a direct target of miR-199a-5p in ovarian cancer cells (30). However, the association between miR-199a-5p and IKKβ in OSCC has not been clarified. To verify whether IKKβ is a direct target of miR-199a-5p, a dual-luciferase reporter assay was performed by integrating sequences of the IKKβ 3'-UTR containing the binding sites for miR-199a-5p or corresponding mutated sequences into 293T cells (Fig. 3B). The luciferase reporter gene assay showed that the overexpression of miR-199a-5p markedly repressed the luciferase activity, whereas the knockdown of miR-199a-5p increased the relative luciferase activity of constructs containing the wt IKKβ 3'-UTR. However, the luciferase activity of the reporter containing the mutant binding site showed no significant change (Fig. 3C). Subsequently, the effect of miR-199a-5p on the expression of IKKβ was measured at the protein level in OSCC cells by western blot analysis. As shown in Fig. 3D, the expression of IKKβ at the protein level was significantly downregulated following the overexpression of miR-199a-5p, but was upregulated following knockdown of miR-199a-5p in the Tca8113 and SCC-4 cells. As miR-199a-5p was downregulated in the OSCC tumor samples, the expression levels of IKKβ were also detected in OSCC tumor tissues and adjacent tissues by IHC. As shown in Fig. 3E, the protein expression level of IKKβ was markedly upregulated in OSCC tissues compared with matched tumor-adjacent tissues. In addition, the expression of IKKβ was examined in cell lines and clinical tissue samples. As shown in Fig. 3F and G, the expression of IKKβ was also upregulated in cell lines and clinical tissue samples. In addition, there was an inverse correlation between the miR-199a-5p and IKKβ in tumor tissues (Fig. 3H). Taken

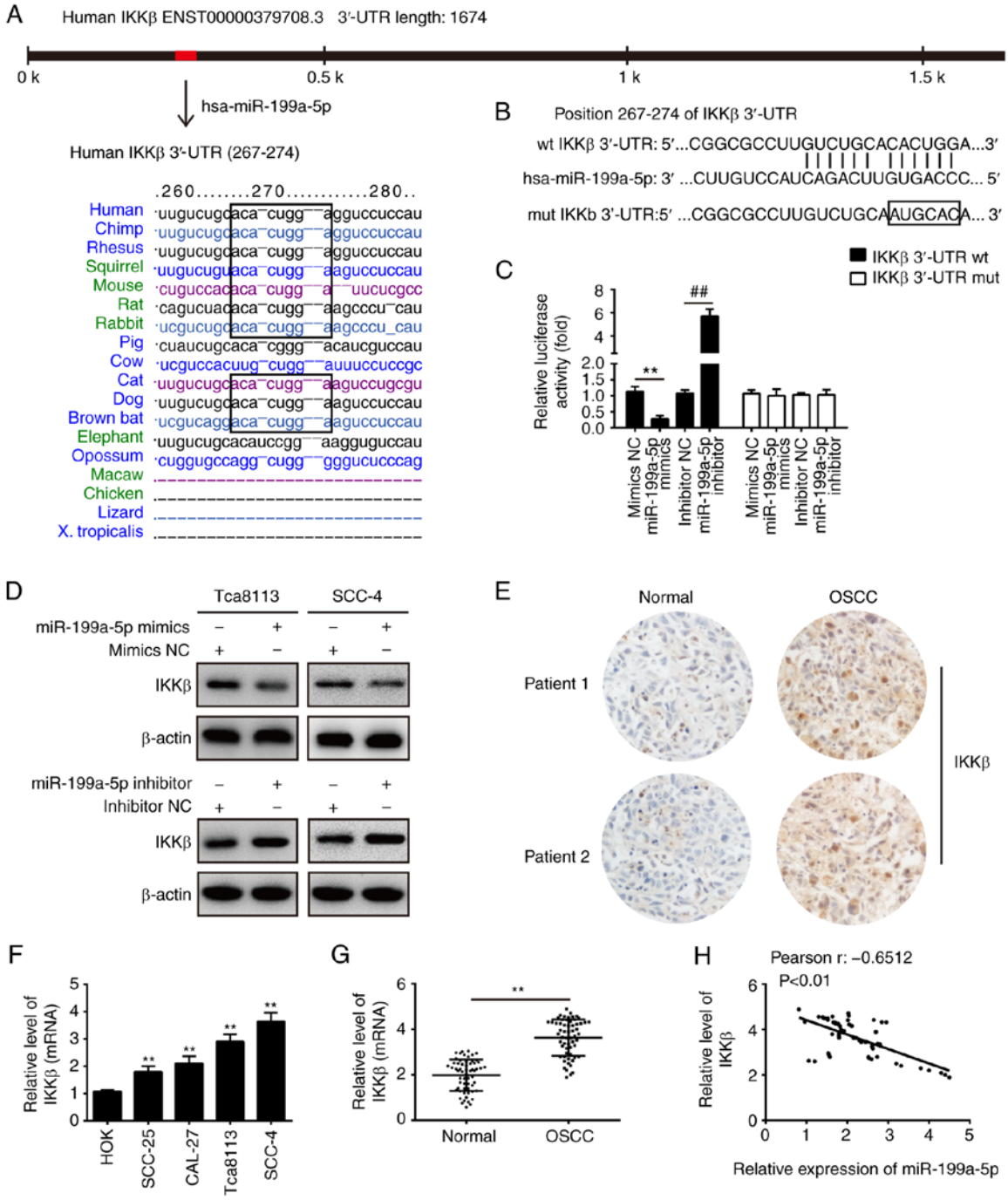

Figure 3. IKKβ is a direct target of miR-199a-5p. (A) miR-199a-5p sequence is shown to be highly conserved among species. (B) Putative binding sites of miR-199a-5p and IKKβ. (C) Luciferase assay of 293T cells co-transfected with firefly luciferase constructs containing the IKKβ wt or mut 3'-UTRs and miR-199a-5p mimics, mimics NC, miR-199a-5p inhibitor or inhibitor NC, as indicated (n=3). Data are presented as the mean ± standard deviation of three independent experiments. **P<0.01 vs. mimics NC, $^{\#\#}$P<0.01 vs. inhibitor NC. (D) Protein expression of IKKβ following transfection with miR-199a-5p mimics or miR-199a-5p inhibitor was measured by western blot analysis. **P<0.01 vs. mimics NC, $^{\#\#}$P<0.01 vs. inhibitor NC. (E) Expression of IKKβ was assessed by immunohistochemistry in OSCC tissues and matched tumor-adjacent tissues (magnification, x200). (F) Expression was of IKKβ examined in four OSCC cell lines (SCC-25, CAL-27, Tca8113 and SCC-4) and HOK cells, used as a control, by RT-qPCR analysis. Data are presented as the mean ± standard deviation of three independent experiments. **P<0.01 vs. HOK cells. (G) Expression of IKKβ was measured by RT-qPCR analysis in OSCC tissues and matched tumor-adjacent tissues (n=60). **P<0.01 vs. Normal group. (H) Spearman's rank correlation analysis revealed a negative correlation between the expression of IKKβ and miR-199a-5p (r=-0.6512, P<0.01). miR, microRNA; 3'-UTR, 3'-untranslated region; wt, wild-type; mut, mutant; NC, negative control; IKKβ, inhibitor of nuclear factor-κB kinase β; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; HOK, Human Oral Keratinocyte.

together, these data indicate that miR-199a-5p may function as a tumor suppressor by targeting IKKβ.

miR-199a-5p inhibits cell viability and induces cell apoptosis by targeting IKKβ. To investigate whether IKKβ mediated the inhibitory effects of miR-199a-5p on cell viability and cell apoptosis, rescue experiments were performed by transfecting pcDNA-IKKβ into Tca8113 and SCC-4 cells with higher expression of miR-199a-5p. It was found that the protein expression of IKKβ was significantly increased in the

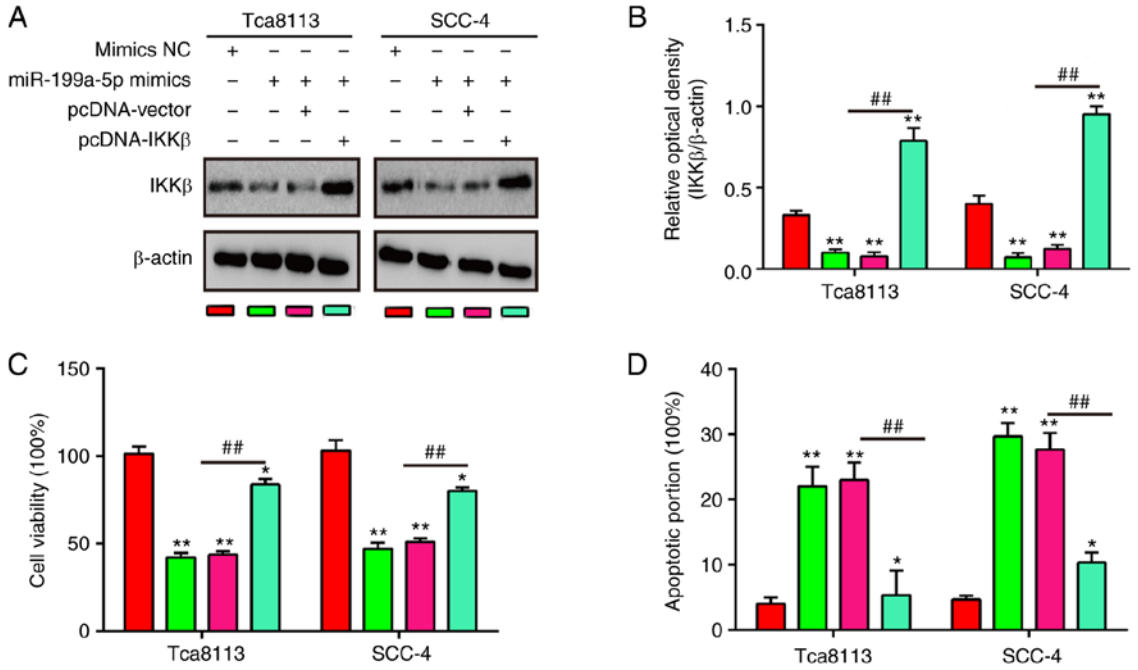

Figure 4. Overexpression of IKKβ abrogates the inhibitory effects of miR-199a-5p mimics on cell proliferation and apoptosis. Tca8113 and SCC-4 cells were co-transfected with miR-199a-5p mimics, mimics NC, pcDNA-IKKβ and pcDNA-vector for 48 h, and the cells were used for further analysis. (A) Protein levels of IKKβ were detected by western blot analysis. (B) Protein bands were analyzed semi-quantitatively using ImageJ software, normalized to β-actin density. (C) Cell viability was measured using a Cell Counting Kit-8 assay. (D) Apoptosis was detected by flow cytometry. Data are presented as the mean ± standard deviation of three independent experiments. *P<0.05 and **P<0.01 vs. mimics NC group, $^{\#\#}$P<0.01 vs. miR-199a-5p + pcDNA-vector group. miR, microRNA; NC, negative control; IKKβ, inhibitor of nuclear factor-κB kinase β.

miR-199a-5p-overexpressing Tca8113 and SCC-4 cells when the pcDNA-IKKβ plasmid was transfected (Fig. 4A and B). Cell viability and cell apoptosis were then examined using a CCK-8 assay and flow cytometry. As shown in Fig. 4C, the decreased cell viability induced by miR-199a-5p mimics was rescued by the overexpression of IKKβ. Furthermore, the flow cytometry data indicated that the promotion of apoptosis caused by miR-199a-5p mimics was attenuated when IKKβ was overexpressed (Fig. 4D). These findings demonstrated that IKKβ is involved in the tumor-suppressive functions of miR-199a-5p in OSCC cells.

miR-199a-5p inhibits the IKKβ-mediated activation of the NF-κB pathway. IKKβ has been shown to activate the NF-κB pathway (31-33), and IKKβ/NF-κB is a classic signaling pathway that is important in tumorigenesis (34,35). To investigate whether miR-199a-5p influences the activity of the NF-κB signaling pathway, NF-κB reporter luciferase activity and the expression levels of downstream proteins in the NF-κB signaling pathway, namely total-p65, cytoplasm-p-p65, nuclear p-p65, p-IκB-α and IκB-α, were evaluated. In addition, the phosphorylation of IκB-α (levels of p-IκB-α) were quantified in Tca8113 and SCC-4 cells. The results showed that the miR-199a-5p mimics suppressed the levels of p-IκB-α, whereas the overexpression of IKKβ reversed these inhibitory effects of the miR-199a-5p mimics in Tca8113 and SCC-4 cells (Fig. 5A and B). To confirm the possibility that miR-199a-5p can suppress the NF-κB signaling pathway, the effect of miR-199a-5p on the expression of cytoplasmic p-p65 and nuclear p-p65 was examined. As shown in Fig. 5A, C and D, the expression of cytoplasmic p-p65 was significantly increased in the miR-199a-5p-overexpressing Tca8113 and SCC-4 cells, whereas the reverse changes were observed in the expression level of nuclear p-p65, compared with the mimics NC group. By contrast, the overexpression of IKKβ reversed the effects of miR-199a-5p on the expression levels of cytoplasmic p-p65 and nuclear p-p65. Furthermore, the overexpression of miR-199a-5p led to a significant decrease in NF-κB reporter luciferase activity, and this reduction was reversed by the overexpression of IKKβ (Fig. 5E). Overall, these results demonstrate that miR-199a-5p inhibited the activation of the NF-κB pathway through the downregulation of IKKβ.

Discussion

In the present study, miR-199a-5p was found to be downregulated in OSCC tissues and cell lines, and a low expression of miR-199a-5p was associated with tumor differentiation, lymph node metastasis and TNM stage. The overexpression of miR-199a-5p also suppressed cell viability, caused cell cycle arrest and promoted cell apoptosis. Additionally, IKKβ was confirmed as a functional target of miR-199a-5p and miR-199a-5p inhibited the IKKβ-mediated activation of the NF-κB pathway, and thus inhibited malignancy in OSCC cells. These findings indicate that miR-199a-5p, as a potential biomarker for the clinical diagnosis and prognosis of OSCC, may be an effective anticancer target for the treatment of OSCC.

Increasing evidence has demonstrated that miRNAs function as either oncogenic or tumor-suppressing genes in OSCC. For example, Lu *et al* found that miR-654-5p was upregulated

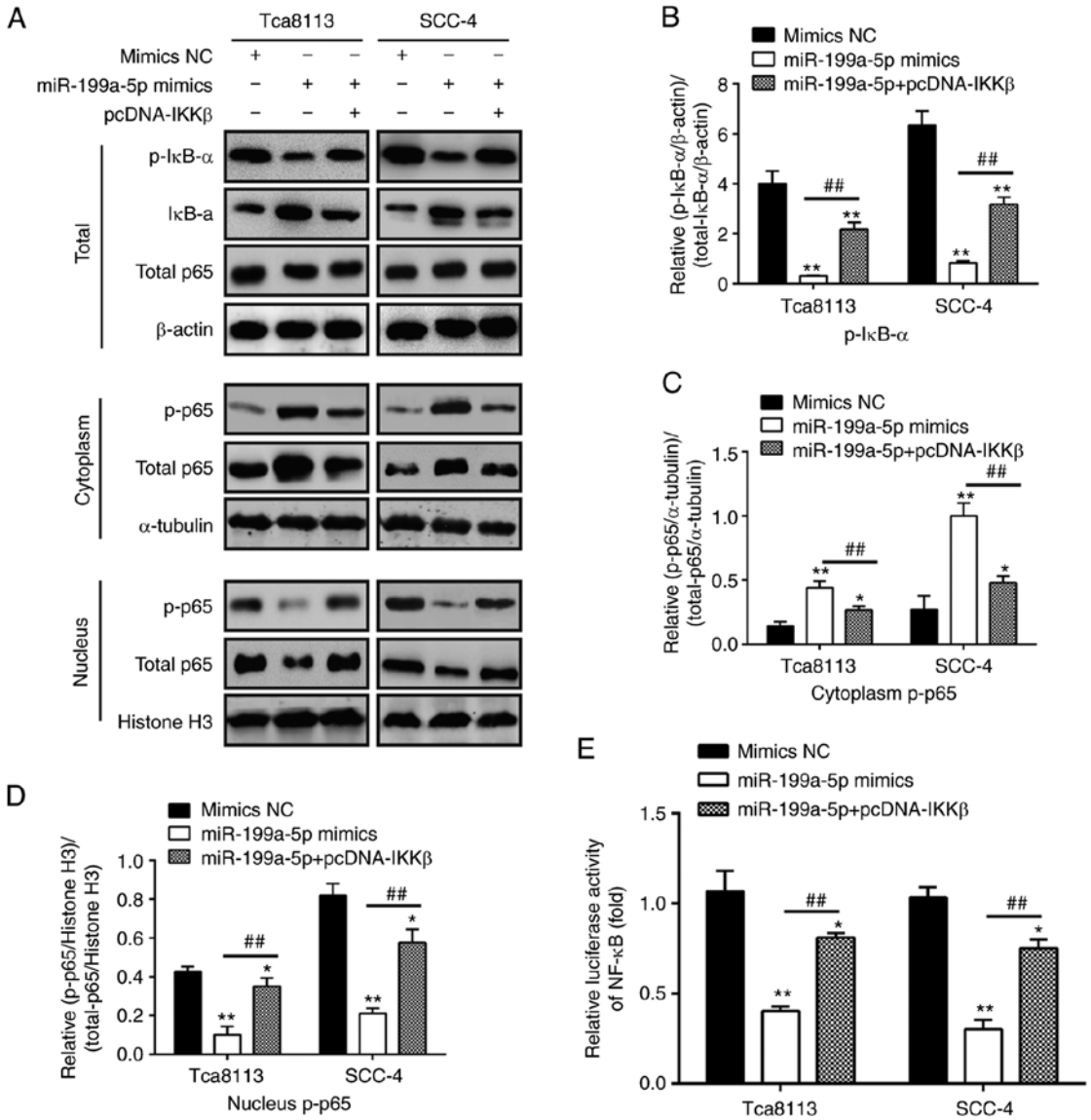

Figure 5. miR-199a-5p inhibits the IKKβ-mediated activation of the NF-κB pathway. Tca8113 and SCC-4 cells were transfected with the miR-199a-5p mimics or mimics-NC for 48 h, and were used for western blot and NF-κB activity assays. (A) Levels of nuclear p-p65, cytoplasm-p-p65, total p65, p-IκB-α and IκB-α were measured by western blot analysis in the whole cell lysate (upper), cytoplasm (middle) and nuclei (lower). β-actin protein was used as the inner control of total proteins; α-tubulin and Histone H3 protein was used as the inner control of the cytoplasmic and nuclear proteins, respectively. (B) Phosphorylation levels of IκB-α were quantified as (p-IκB-α/control)/(total IκB-α/control). Expression levels of p-p65 in the (C) cytoplasm and (D) nucleus were quantified. α-tubulin protein was used as the inner control of the cytoplasmic proteins; Histone H3 protein was used as the inner control of the nuclear proteins. (E) NF-κB activity was quantified using a Promega luciferase assay kit. Data are presented as the mean ± standard deviation of three independent experiments. *P<0.05 and **P<0.01 vs. mimics NC group; $^{\#\#}$P<0.01 vs. miR-199a-5p mimics group. miR, microRNA; NC, negative control; NF-κB, nuclear factor-κB; IκB-α, inhibitor of NF-κB-α; IKKβ, inhibitor of NF-κB kinase β; p-, phosphorylated.

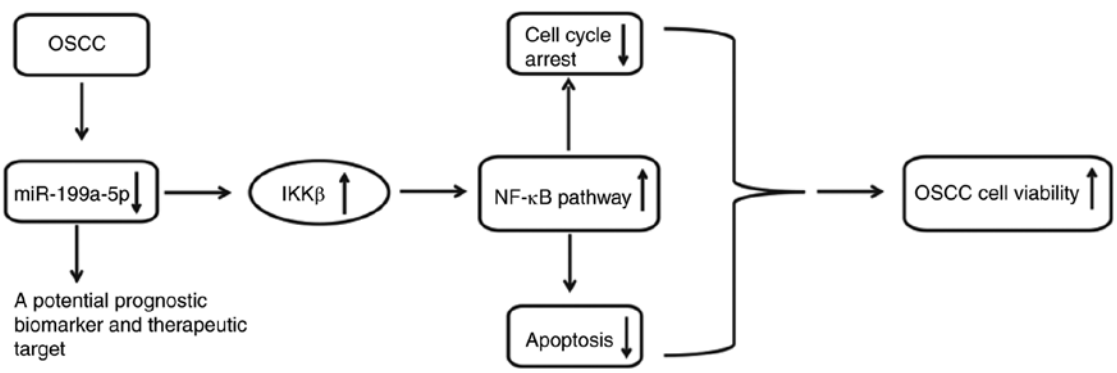

Figure 6. Schematic diagrams showing that miR-199a-5p is downregulated in OSCC tissues and cell lines, and miR-199a-5p acts as tumor suppressor that inhibits NF-κB signaling pathways by targeting IKKβ, thereby inhibiting the progression of OSCC. miR, microRNA; OSCC, oral squamous cell carcinoma; NF-κB, nuclear factor-κB; IKKβ, inhibitor of NF-κB kinase β.

in late-stage OSCC tissues, and promoted the proliferation and metastasis of OSCC *in vitro* and *in vivo* (36). Shiah *et al* demonstrated that miR-329 and miR-410 promoted the proliferation and invasiveness of OSCC cells by targeting Wnt-7b (37). Wang *et al* showed that miR-139-5p was downregulated in OSCC tissues, and that the overexpression of miR-139-5p inhibited the proliferation, invasion and migration ability of OSCC cells by targeting homeobox A9 (38). Understanding the role of miRNAs that are aberrantly expressed in OSCC can assist in understanding the underlying mechanisms of OSCC and improve therapeutic approaches for OSCC. In the present study, a large set of miRNAs were found to be significantly deregulated in OSCC tissues using an miRNA microarray, and miR-199a-5p was one of the most markedly downregulated miRNAs. Its lower expression was further confirmed by RT-qPCR analysis. It was also observed that a low expression of miR-199a-5p was closely associated with tumor differentiation, lymph node metastasis, TNM stage, and a poor OS rate. Taken together, these findings suggest that miR-199a-5p may be important in OSCC carcinogenesis.

A large number of studies have investigated the expression of miR-199a-5p in human cancer and have reported it to be downregulated in several types of cancer (39,40). Several studies have identified the tumor suppressor functions of miR-199a-5p (41-43). For example, Cheng *et al* showed that miR-199a suppressed the proliferation of ovarian cancer-initiating cells *in vitro* and *in vivo* by targeting targets cluster of differentiation-44 (26). In addition, it was shown that the re-expression of miR-199a suppressed renal cancer cell proliferation and survival by targeting glycogen synthase kinase-3-β (GSK-β) (27). However, whether miR-199a-5p was involved in OSCC remained to be elucidated. In the present study, the experiments showed that the enforced expression of miR-199a-5p inhibited cell proliferation, inhibited cell cycle and induced the apoptosis of Tca8113 and SCC-4 cells, indicating that miR-199a-5p also serves as a tumor suppressor in OSCC.

miR-199a-5p has been reported to downregulate the expression of several target genes in different types of tumor, including CD44 (25), GSK-3β (27) and connective tissue growth factor (44). IKKβ, one of the catalytic subunits of the IKK complex, is an inhibitor of the NF-κB signaling pathway (5). Several studies have shown that IKKβ functions as an oncogene in different types of carcinoma (45-47). For example, Greten *et al* found that IKKβ contributed to tumor promotion by suppressing apoptosis through the mitochondrial pathway in epithelial cells (48). Zhang *et al* demonstrated that IKKβ promoted proliferation and migration, and inhibited apoptosis in prostate cancer cells (49). In addition IKKβ acts as downstream molecule of certain miRNAs to mediate the role of the miRNAs in different tumor types, including miR-429 (50) and miR-497 (51). In the present study, IKKβ was predicted to be a target of miR-199a-5p using online informatics tools, and this hypothesis was tested and confirmed by a luciferase reporter assay. In addition, it was found that the levels of IKKβ in cell lines and OSCC tissues were significantly higher than those in HOK cells and matched adjacent tissues, respectively. It was also found that there was an inverse correlation between miR-199a-5p and IKKβ in tumor tissues. Furthermore, the overexpression of IKKβ reversed the suppressive effects induced by the enhanced expression of miR-199a-5p in OSCC cells. Taken together, these data indicate that miR-199a-5p exerts its antitumor effects by targeting IKKβ.

Activation of canonical NF-κB has been found to be important in the pathogenesis of several types of tumor in humans (45,52). In OSCC, NF-κB is constitutively active and is involved in promoting the invasion of OSCC cells, which suggests that inhibiting the activity of NF-κB may constitute a promising therapeutic approach to treat the invasiveness of OSCC (13). A previous study showed that the upregulation of miR-92b accelerates tumor growth and that this effect may be associated with activation of the NF-κB signaling pathway in OSCC (53). In the present study, the results showed that the upregulation of miR-199a-5p reduced the levels of key NF-κB pathway proteins by suppressing IKKβ. The data suggested that miR-199a-5p suppressed IKKβ to inhibit NF-κB activity and therefore inhibit the malignancy of OSCC cells.

In conclusion, the results of the present study indicated that miR-199a-5p was markedly downregulated in OSCC tissues and cell lines, and the decreased level of miR-199a-5p was relative to tumor differentiation, metastatic lymph nodes and advanced TNM stage in patients with OSCC. It was also demonstrated that miR-199a-5p functions as a tumor suppressor via the suppression of IKKβ, which inhibits activation of the NF-κB pathway (Fig. 6). These findings indicate that miR-199a-5p may be a potential target for prognostic prediction and therapeutic strategies.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

DW, BS, WW, YZ, XY, GL and JY performed the experiments, contributed to data analysis and wrote the manuscript. DW, BS, WW, YZ, XY, GL and JY analyzed the data. YS conceptualized the study design, and contributed to data analysis and experimental materials. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All individuals provided informed consent for the use of human specimens for clinical research. The present study was approved by the First Affiliated Hospital of Xinxiang Medical University Ethics Committees.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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