

Original Article

miR-219 inhibits the growth and metastasis of TSCC cells by targeting PRKCI

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Abstract: Tongue squamous cells carcinoma (TSCC) is the most common type in oral cancers. Recently, accumulating evidence suggests that microRNAs (miRNAs) play critical roles in tumorigenesis. Here, we demonstrated that miR-219 was significantly downregulated in TSCC tissues and cell lines. miR-219 overexpression remarkably suppressed cell proliferation, colony formation, migration and invasion of TSCC cells. In addition, protein kinase C δ (PRKCI) was identified as a target of miR-219, and overexpression of PRKCI could significantly attenuated the tumor suppressive effects of miR-219. Furthermore, PRKCI inversely correlates with miR-219 in TSCC tissues. Taken together, miR-219 inhibited growth and metastasis by targeting PRKCI and might be used as a potential target for the treatment of TSCC.

Keywords: TSCC, miR-219, PRKCI, growth, metastasis

Introduction

Oral squamous cell carcinoma (OSCC) represents the tenth most frequent solid cancer around the world [1], while the tongue squamous cells carcinoma (TSCC) is the most common type in OSCC [1, 2]. Despite the advancement of multimodal diagnosis and therapies for tongue cancer including radical surgery, chemotherapy plus radiotherapy, the 5-year survival rate of TSCC patients remains poor during the past decades, mainly due to regional recurrence and lymph node metastasis [3]. Therefore, it is vital to elucidate the etiology of TSCC and explore diagnostic and prognostic markers and therapeutic strategies. Accumulating studies have suggested that TSCC arises as a result of the oncogenes activation or tumor suppressor genes inactivation, but the detailed molecular mechanisms of TSCC remain elusive.

microRNAs (miRNAs) are a class of small non-coding RNAs approximately 22 nucleotides in length, which post-transcriptionally suppress target gene expression by binding to the complementary sequence in the 3'-untranslated

regions (3'-UTRs) of target mRNAs, leading to mRNA degradation or translation inhibition. Aberrant miRNA expression has also been frequently reported to correlate with various cancers and can act as tumor suppressors or oncogenes [4-6]. Many miRNAs have also been found to dysregulated and play important roles in the development and progression of TSCC [7, 8], miR-219 was downregulated in some cancers and functioned as a tumor suppressor [9-11]. However, the detailed role of miR-219 in TSCC carcinogenesis is also unclear.

In this study, we demonstrated that the expression of miR-219 was significantly decreased in both TSCC samples and cell lines, and overexpression of miR-219 suppressed growth, migration, and invasion of TSCC cells. Moreover, Protein kinase C δ (PRKCI) was identified as a direct target of miR-219 in TSCC cells, and restoration of PRKCI remarkably attenuated the tumor-suppressive effects of miR-219, suggesting that miR-219 acted as a tumor suppressor in TSCC partially by targeting PRKCI. Furthermore, PRKCI was inversely correlated with miR-219 in TSCC tissues.

The inhibitory roles of miR-219 in TSCC

Table 1. Primers and oligonucleotides

Name	Sequence (5'-3')
pre-miR-219-s	AATTCCTCCGCCCCGGGCGCGGCTCCTGATTGTCCAAACGCAATTCTCGAGTCTATGGCTCCGGCCGAGAGTT-GAGTCTGGACGTCCCGAGCCGCCGCCCAACCTCGAGCGGGA
pre-miR-219-a	AGCTTCCCGCTCGAGTTTGGGGGCGGCGGCTCGGGACGTCCAGACTCAACTCTCGGCCGGAGC-CATAGACTCGAGAATTGCGTTTGGACAATCAGGAGCCGCGGCCCGGGGCGGG
PRKCI-WT-S	CTACTACTAGCTTCCAGACAATCAC
PRKCI-WT-A	TCGAGTGATTGTCTGGAAGCTAGTAGTAGAGCT
PRKCI-MT-S	CTACTACTAGCTTCCAGACATAGAC
PRKCI-MT-A	TCGAGTCTATGTCTGGAAGCTAGTAGTAGAGCT
miR-219-RT	GTCGTATCCAGTGCCTGTCTGTGGAGTCCGCAATTGCACTGGATACGAC AGAATTG
miR-219-F	ATCCAGTGCCTGTCTGT
miR-219-R	TGCTTGATTGTCCAAACG
PRKCI-F	CGTTCTCCGAAATGTTGATTG
PRKCI-R	TCCCCAGAAATATTTGGTTTAAAGG
U6-RT	GTCGTATCCAGTGCAGGGTCCGAGGTGCACTGGATACGACAAAATATGG
U6-F	TGCGGGTGTCTCGCTTCGGCAGC
U6-R	CCAGTGCAGGGTCCGAGGT
β -actin-F	AGTGTGACGTGGACATCCGCAAAG
β -actin-R	ATCCACATCTGCTGGAAGGTGGAC

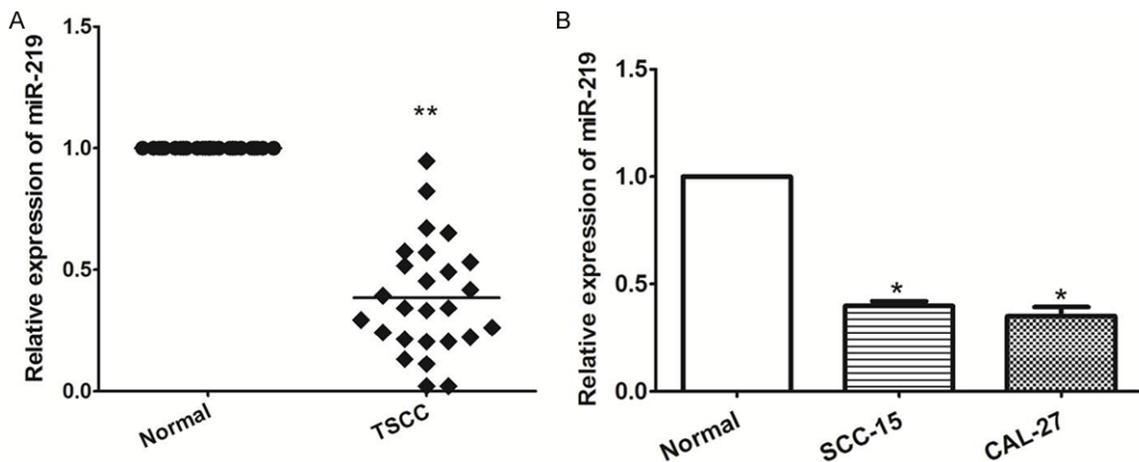


Figure 1. miR-219 was downregulated in TSCC tissues and cell lines. A. Expression level of miR-219 between TSCC tissue and the matched adjacent tissue samples detected by qRT-PCR. B. Expression level of miR-219 between TSCC cells and normal oral mucosa cells. * $P < 0.05$, ** $P < 0.001$ compared with the control group.

Materials and methods

Tissue samples, cell lines and transfection

Twenty-six paired TSCC and adjacent non-tumor normal tissues were obtained in our hospital. Tissues were immediately snap frozen and stored at -80°C . No patients received chemotherapy or radiotherapy before operation and all the histological diagnoses for TSCC were confirmed by two independent pathologists.

This study was approved by the institutional ethics committee and informed consents were obtained from each patient.

Two human tongue squamous carcinoma cell lines (SCC-15 and CAL-27) were obtained from American Type Culture Collection (ATCC) and HEK293 cells were cultured in Dulbecco's MEM. All the media were supplemented with 10% fetal bovine serum (FBS), 50 U/ml penicillin, and 50 U/ml streptomycin and all cell lines

The inhibitory roles of miR-219 in TSCC

were incubated at 37°C under 5% CO₂. Transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction.

RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA, USA) to isolate both miRNA and mRNA. All reagents for qRT-PCR were purchased from Takara (Dalian, China). The cDNA for mRNA was generated using the PrimeScript® RT reagent Kit according to the manufacturer's protocol, and the mature miRNA was reverse transcribed using miRNA-specific primers for quantification of miR-219. qRT-PCR was performed using SYBR reagent on the IQ5 Optical System real-time PCR machine. β -actin and U6 were used to normalize mRNA and miRNA respectively. The primers used were showed in **Table 1**. Relative quantitation was calculated using the $2^{-\Delta\Delta Ct}$ method. All experiments were carried out in triplicate.

Plasmid construction

The complimentary site in 3'-UTR of PRKCI (PRKCI-WT) and mutated 3'-UTR sequence of PRKCI (PRKCI-MT) for miR-219 were synthesized (SangonBiotech, Shanghai, China) and cloned into pmirGLO vector at Sac I and Xho I sites (Promega). The ORF region of the PRKCI cDNA was synthesized and then subcloned into the GV230 vector (GeneChem, Shanghai, China). The sequences of constructed plasmids were confirmed by qRT-PCR or western blot in TSCC cells. The sequences of constructed plasmids were showed in **Table 1**.

Cell proliferation

Cells were plated in 96-well plates at 3000 cells/well and incubated at 37°C until the cells reached 30-40% confluence. At 24, 48 and 72 h after transfection, the cell proliferation was assessed at using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. 20 μ l (5 mg/ml) MTT solution (Sigma, USA) was added in each well and incubated for 4 h at 37°C, then the supernatant was discarded and replaced with 150 μ l Dimethyl sulfoxide (DMSO). The OD at 492 nm was read by a microplate spectrophotometer.

Colony formation assay

The cells were seeded in 12-well plates at 2000 cells/well after transfection and grown for 14 days. The colonies were fixed with ethyl alcohol, stained with 0.1% crystal violet for 30 min, and washed thrice. The number of clones in 4 random fields was counted under a light microscope.

Migration and invasion assays

Migration and invasion were examined using transwell chambers (Millipore, Billerica, MA, USA). Cell migration and invasion assays were then performed with uncoated (for migration) or coated Matrigel (for invasion). 1×10^5 transfected cells were seeded into the upper chamber, and 500 μ l complete medium was added to the lower chamber. After 24 h incubation at 37°C, cells remaining on the upper surface of membrane were removed using cotton swabs and the membranes were stained with 0.1% crystal violet. Four random fields were counted for each chamber.

Luciferase activity assay

HEK-293 cells were cultured and co-transfected with miR-219 or control plasmid (miR-ctrl) and WT or MT of PRKCI using Lipofectamine 2000. Twenty-four hours after transfection, cells were harvested and luciferase activity was assayed using Dual-Luciferase Reporter Assay System (Promega, USA).

Western blot

Twenty-four hours after transfection, cells were lysed in RIPA buffer. Proteins were loaded and separated by 10% SDS-PAGE and transferred to PVDF membranes (Millipore, Bedford, MA, USA). The membranes were incubated with primary antibodies overnight at 4°C, followed by incubation with secondary antibodies labeled with HRP for 2 h at 37°C. The signal was developed with ECL, β -actin was used as a loading control.

Statistical analysis

Data were expressed as mean \pm SEM. Statistical differences were determined by Student *t* test or ANOVA with SPSS 13.0. $P < 0.05$ was considered statistically significant.

The inhibitory roles of miR-219 in TSCC

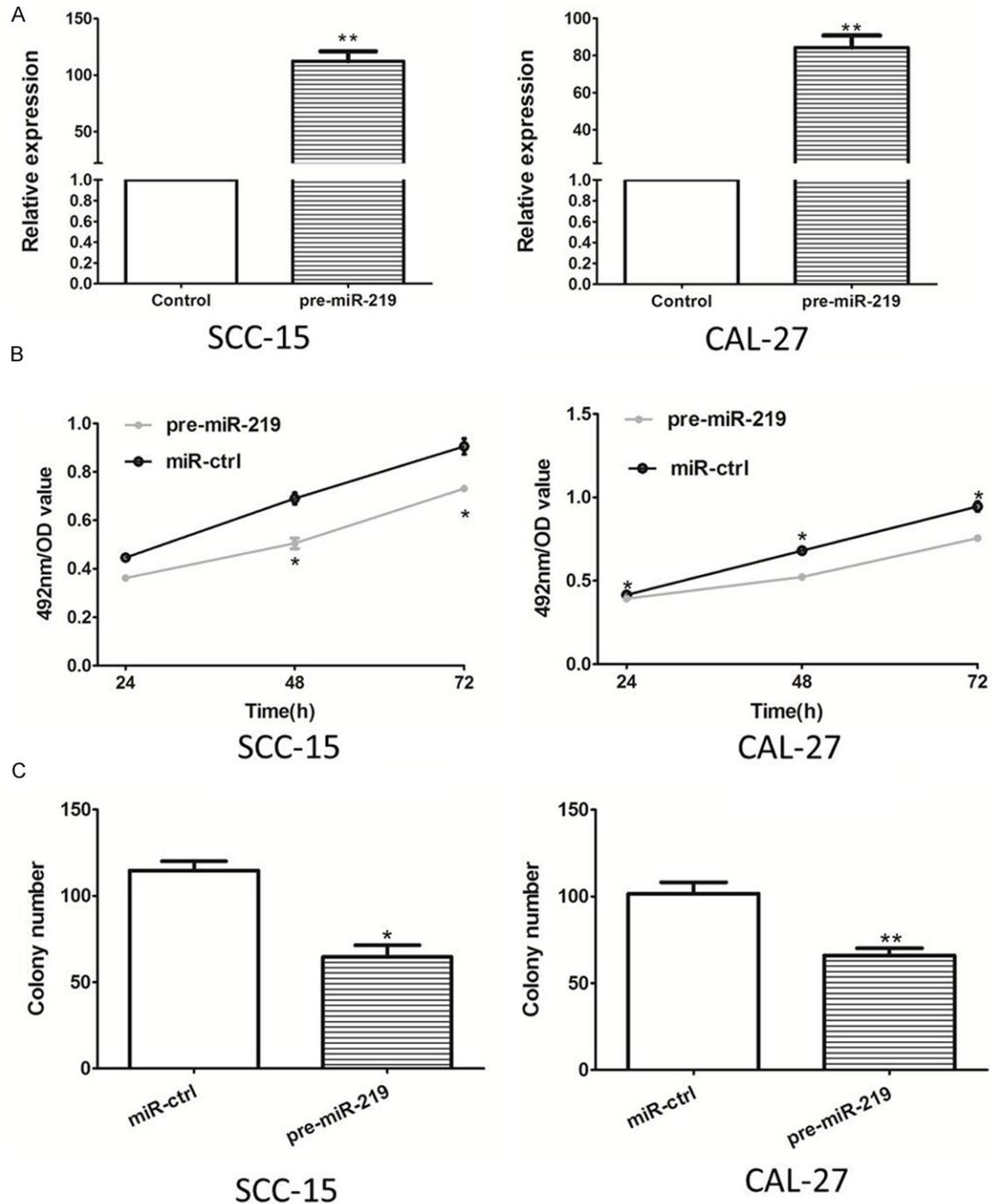


Figure 2. miR-219 inhibited TSCC cell proliferation and colony formation. A. Expression of miR-219 in SCC-15 or CAL-27 cells transfected with miR-219. B. MTT assay of SCC-15 or CAL-27 cells transfected with miR-219 or miR-control. C. Colony formation of SCC-15 or CAL-27 cells transfected with miR-219 or miR-control. Data were presented as means \pm SEM. * $P < 0.05$, ** $P < 0.001$ compared with the control group.

Results

miR-219 was downregulated in TSCC tissues and cell lines

Expression of miR-219 in 26 paired TSCC tissue samples was examined by qRT-PCR. We

found that miR-219 was significantly downregulated in TSCC tissues compared with the adjacent non-tumor normal tissues ($P < 0.001$, **Figure 1A**). In addition, miR-219 was dramatically decreased in two TSCC cell lines (SCC-15 and CAL-27) compared with normal oral muco-

The inhibitory roles of miR-219 in TSCC

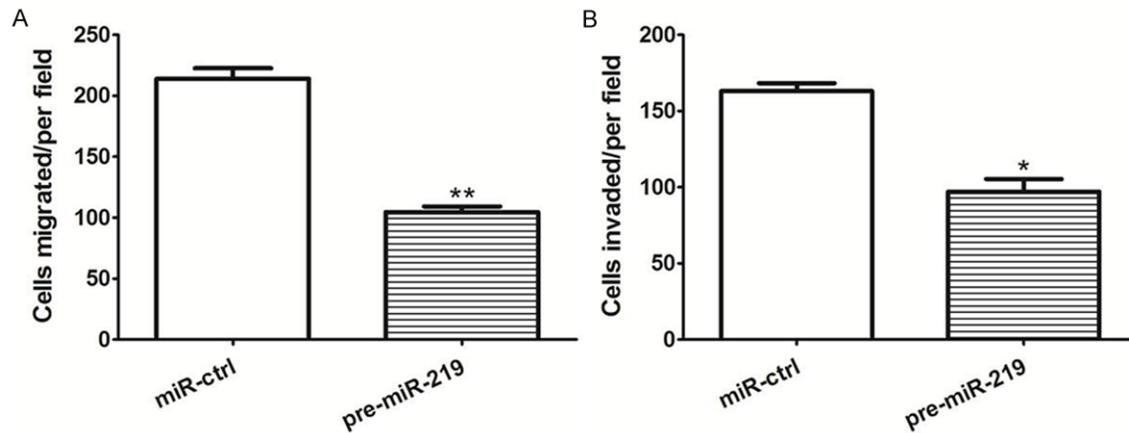


Figure 3. miR-219 suppressed TSCC cell migration and invasion. A. Migration assay of SCC-15 cells transfected with miR-219 or miR-control. B. Invasion assay of SCC-15 cells transfected with miR-219 or miR-control. Data were presented as means \pm SEM from three independent experiments. * $P < 0.05$, ** $P < 0.001$ compared with the control group.

sa cells ($P = 0.001$, $P < 0.001$, respectively, **Figure 1B**).

miR-219 inhibited TSCC cell proliferation and colony formation

We further evaluated the effects of miR-219 on growth of TSCC cells. The expression of miR-219 was identified by qRT-PCR in SCC-15 and CAL-27 cells ($P < 0.001$, **Figure 2A**). MTT assay showed that overexpression of miR-219 significantly suppressed cell proliferation of SCC-15 and CAL-27 cells compared with the miR-ctrl group ($P < 0.05$, **Figure 2B**). Similarly, miR-219 overexpression remarkably suppressed colony formation of SCC-15 and CAL-27 cells ($P < 0.05$, **Figure 2C**).

miR-219 suppressed TSCC cell migration and invasion

We further investigate the role of miR-219 for the motility of TSCC cells, as shown in **Figure 3A**, overexpression of miR-219 significantly suppressed tumor cell migration in SCC-15 cells compared with the miR-ctrl group ($P < 0.001$). Similarly, transwell assays with Matrigel demonstrated that miR-219 dramatically decreased the invasive capacity of SCC-15 cells ($P = 0.003$, **Figure 3B**).

PRKCI was a target of miR-219 in TSCC cells

To investigate the downstream target of miR-219, TargetScan 6.2 was used ([\[getscan.org/\]\(http://getscan.org/\)\). PRKCI was predicted to be a target of miR-219, wild type \(WT\) or the mutated \(MT\) PRKCI 3'-UTR sequences were amplified and cloned into the luciferase reporter vector \(**Figure 4A**\). Luciferase activity assay showed that miR-219 significantly inhibited the WT but not the MT luciferase activity of PRKCI in HEK-293 cells \(\$P < 0.001\$, **Figure 4B**\). In addition, qRT-PCR showed miR-219 overexpression had no effect on PRKCI mRNA level \(\$P = 0.087\$, **Figure 4C**\), however, the protein levels of PRKCI was significantly reduced in cells transfected with miR-219 compared with miR-ctrl group by western blot \(**Figure 4D**\). These data suggest that miR-219 post-transcriptionally regulated protein expression level of PRKCI by directly targeting its 3'UTR.](http://www.tar-</p></div><div data-bbox=)

Restoration of PRKCI attenuated the effects of miR-219

We further investigated that whether miR-219 suppressed TSCC progression by targeting PRKCI. SCC-15 cells were transfected with PRKCI vector, and qRT-PCR and western blot were used to detect the expression of PRKCI (**Figure 5A** and **5B**). Then, SCC-15 cells were co-transfected with miR-219 and PRKCI or control vector. MTT assay, colony formation, migration and invasion showed that restoration of PRKCI could significantly attenuated the tumor suppressive effects of miR-219 ($P < 0.05$, **Figure 5C-F**).

The inhibitory roles of miR-219 in TSCC

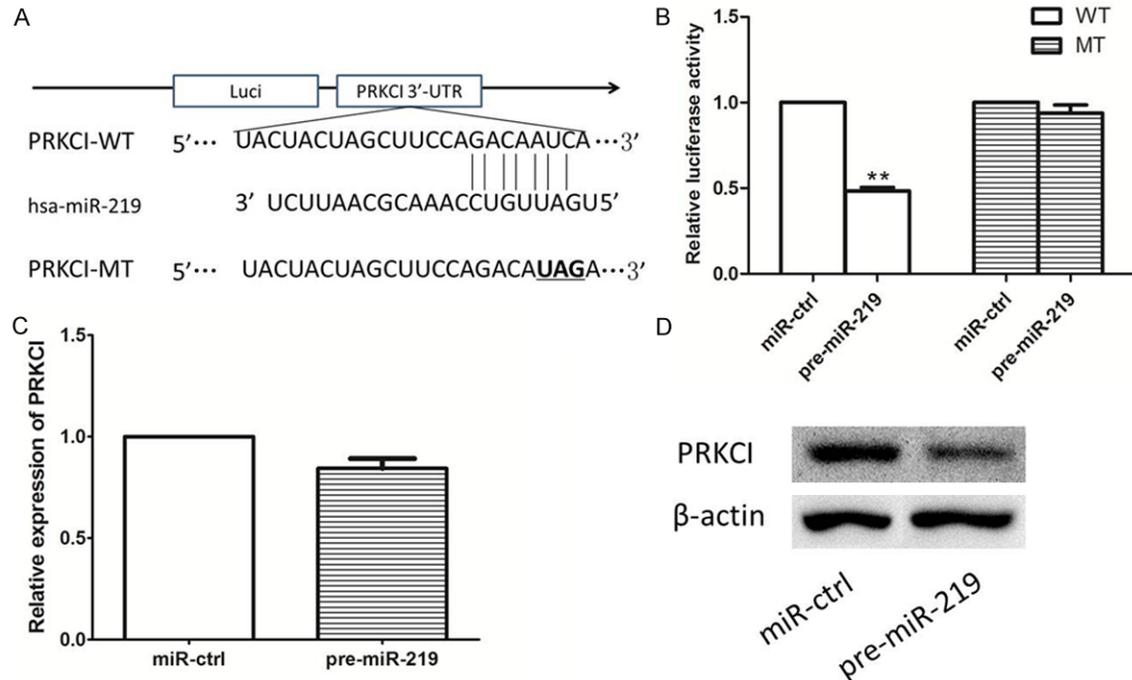


Figure 4. PRKCI was a target of miR-219 in TSCC cells. A. The predicted miR-219 binding site within PRKCI 3'-UTR and its mutated type by site mutagenesis were as shown. B. HEK293 cells were co-transfected with miR-219 or miR-ctrl with WT or MT PRKCI 3'-UTR. Luciferase activity was detected. C. Expression of PRKCI mRNA was detected by qRT-PCR in SCC-15 cells transfected with miR-219 or miR-control. D. PRKCI protein level was detected by western blotting in SCC-15 cells transfected with miR-219 or miR-control. Data were presented as means \pm SEM from three independent experiments. ** $P < 0.001$ compared with the control group.

miR-219 was inversely correlated with PRKCI expression in TSCC tissues

Expression of PRKCI in 26 paired TSCC tissues was examined by qRT-PCR. We found that the expression of PRKCI was significantly increased in TSCC tissue samples compared with the adjacent adjacent non-tumor normal tissues ($P < 0.001$, **Figure 6A**). Furthermore, PRKCI mRNA level was inversely correlated with miR-219 level in TSCC tissues ($r = -0.984$, $P < 0.001$, **Figure 6B**).

Discussion

In this study, our findings demonstrated that the tumor suppressive role of miR-219 in TSCC development and progression *in vitro*. We found that miR-219 was downregulated in TSCC tissue samples and cell lines. In addition, PRKCI was identified as a direct target of miR-219 in TSCC cells. Restoration of PRKCI dramatically attenuated the tumor-suppressive effects of miR-219 in TSCC cells. Finally, we also found that PRKCI mRNA level was inversely correlated with miR-219 in TSCC tissues.

miR-219 was decreased in various types of cancer and exerted tumor-suppressive effects. The downregulation of miR-219 may be induced by methylation. In gastric cancer, MGC-803 and HGC-27 cells were treated with 5-aza-2'-deoxycytidine and trichostatin A, the expression of miR-219 was increased and exerted antiproliferative, proapoptotic, and anti-metastatic roles and reduced levels of p-ERK1/2 in gastric cancer cells [12]. In addition, Huang et al. [11] demonstrated that miR-219 was significantly decreased in HCC tissues and cell lines, miR-219-5p could inhibit cell proliferation *in vitro* and arrest cell cycle at the G1 to S transition through negative regulation of GPC3 expression in hepatic carcinogenesis. Rao et al. [10] found that miR-219 was downregulated in glioblastoma and the overexpression of miR-219 in glioma cell lines inhibited the proliferation, anchorage independent growth and migration by targeting EGFR 3'-UTR. While they indicated that miR-219 inhibited MAPK and PI3K pathways in glioma cell lines in concordance with its ability to target EGFR. These data were consistent with the study of hepatocellular carcinoma

The inhibitory roles of miR-219 in TSCC

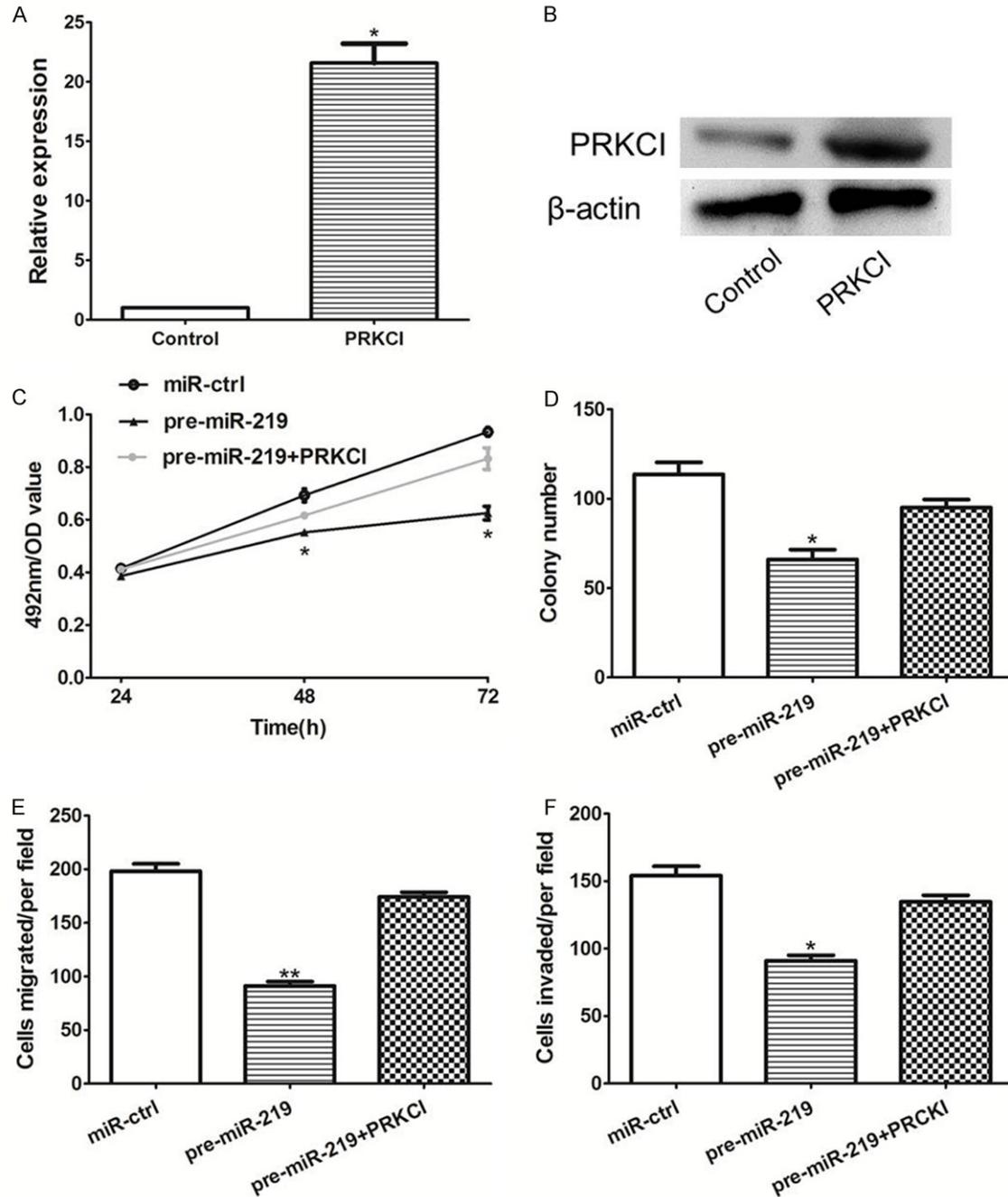


Figure 5. PRKCI overexpression attenuated the tumor suppressive effects of miR-219. A. PRKCI mRNA level was detected by qRT-PCR in SCC-15 cells transfected with PRKCI plasmid. B. PRKCI protein level was detected by western blotting in SCC-15 cells transfected with PRKCI plasmid. C. SCC-15 cells were transfected with miR-219 with/without PRKCI overexpression plasmid, and MTT assay was performed. D. Colony formation assay. E. Migration assay. F. Invasion assay. Data were presented as means \pm SEM from three independent experiments. ** $P < 0.001$ compared with the control group.

cells by Zhou et al. [13]. Furthermore, miR-219 could suppress the proliferation, migration and invasion of medulloblastoma and pancreatic cancer cells by targeting 3'-UTRs of CD164 and

mucin MUC4 respectively. In this study, we also found that forced expression of miR-219 suppressed TSCC cells proliferation, colony formation, migration and invasion. Our data firstly

The inhibitory roles of miR-219 in TSCC

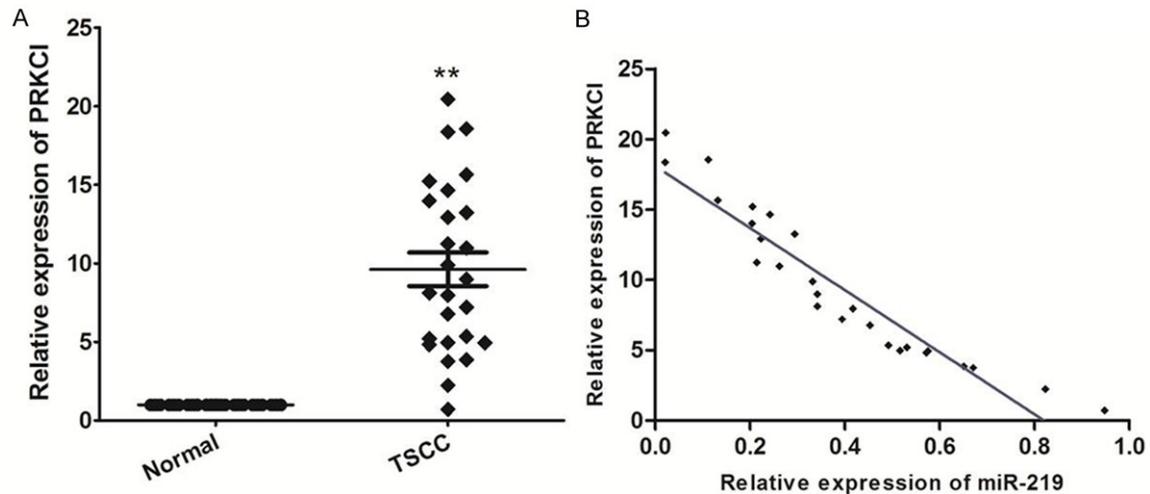


Figure 6. miR-219 was negatively correlated with PRKCI in TSCC tissues A. The expression of PRKCI in 26 pairs of TSCC tissues was measured by qRT-PCR. Data were presented as means \pm SEM from three independent experiments. ** $P < 0.001$ compared with the control group. B. The relationship between miR-219 expression and PRKCI mRNA levels in TSCC samples were analysed using Spearman's correlation analysis.

expanded the tumor suppressive role of miR-219 in TSCC.

PRKCI, encoding the atypical protein kinase C, has been implicated in the transformed growth, invasion, chemoresistance, and survival of tumor cells [14, 15]. Campa et al. [16] speculated that certain allelic variants could modify expression of the PRKCI gene. Two SNPs in the PRKCI gene, rs546950 and rs4955720, correlated with prostate cancer risk. Similarly, Yang et al. [17] found that a positive correlation was found for PRKCI between amplification and tumor size, lymph node metastasis and clinical stage using fluorescence in situ hybridization (FISH), which was consistent with results of immunohistochemistry analysis in esophageal squamous cell carcinomas. Moreover, Regala et al. previously identified PRKCI as an oncogene in lung squamous cell carcinoma (LSCC) [15]. PRKCI expression was also predictive of poor clinical outcome, and PKC α driven LSCC cell invasion and transformed growth *in vitro* and *in vivo* [18-20]. Recently they further indicated that primary LSCC tumors coordinately overexpress PRKCI, SOX2, and HHAT and require PRKCI-SOX2-HHAT signaling to drive tumorigenesis by establishing a cell-autonomous Hh signaling axis [21]. In the present study, we found that PRKCI was a target of miR-219 in TSCC cells, and restoration of PRKCI remarkably attenuated the tumor suppressive effects of miR-219 on TSCC cells. Furthermore, PRKCI was inversely correlated with miR-219 in

TSCC tissues. Taken together, these data suggested that overexpression of PRKCI may be involved in progression of TSCC.

In conclusion, the present study, for the first time, revealed that miR-219 was significantly decreased in TSCC tissues and cell lines. Overexpression of miR-219 inhibited tumor growth and metastasis of TSCC through targeting PRKCI, suggesting that miR-219 might serve as a biomarker for TSCC, and miR-219-based therapies could be a rationale option for treatment of TSCC.

Disclosure of conflict of interest

None.

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The inhibitory roles of miR-219 in TSCC

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