

Received: 2015.07.10  
Accepted: 2015.09.02  
Published: 2016.02.17

# Downregulation of Microrna-126 Contributes to Tumorigenesis of Squamous Tongue Cell Carcinoma via Targeting KRAS

Authors' Contribution:  
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Statistical Analysis C  
Data Interpretation D  
Manuscript Preparation E  
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**Source of support:** Departmental sources

**Background:** miR-126 has been reported to be differentially expressed in various malignancies, whereas its role in the pathogenesis of tongue squamous cell carcinoma (TSCC) remains largely unknown.





**Material/Methods:** In this study, we collected 21 pairs of TSCC cancerous and adjacent non-cancerous tissue samples, with which we performed real-time PCR to determine and compare the expression of 6 candidate miRNAs that are reportedly associated with tumorigenesis of TSCC, including miR-100, miR-451, miR-221, let-7a, miR-21, and miR-126. We further performed luciferase assay to validate KRAS as a target of miR-126, and conducted transfection to study the effect of miR-126 on proliferation and apoptosis of the cells.

**Results:** We identified that miR-126 was significantly downregulated in the cancerous tissue samples compared with the non-cancerous control tissue samples. By using computational analysis, we identified that KRAS is a virtual target of miR-126, and such association was verified by using luciferase assay. In addition, we found that mRNA and protein expression level of KRAS was significantly higher in the tumor tissue than the control tissue samples.

**Conclusions:** The following *in vitro* experiment showed that both mRNA and protein KRAS expression were significantly decreased in SCC-15 cells in which miR-126 was overexpressed, in comparison with similar cells transfected with a negative control, while downregulation of miR-126 by transfecting the cells with miR-126 inhibitors significantly upregulated the mRNA and protein expression of KRAS. **Conclusions:** miR-126 might be a promising diagnostic and therapeutic target in the prevention and management of TSCC patients.

**MeSH Keywords:** **Apoptosis • Carcinoma, Adenosquamous • MicroRNAs**

**Full-text PDF:** <http://www.medscimonit.com/abstract/index/idArt/895306>

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## Background

Neck and head squamous cell carcinoma, including tumors of oropharynx, oral cavity, hypopharynx and larynx, is the sixth leading solid cancer worldwide [1]. The most frequent type of oral cancer is tongue squamous cell carcinoma (TSCC), which is well-recognized for its high rate of proliferation and metastasis of lymph nodes [2]. Previous studies showed that approximately 50% of patients were already at stage III and IV on onset, although TSCC obviously presents in the oral cavity [3,4]. A better understanding of the molecular pathogenesis of the disease may help with development of new diagnostic methods, and discovery of new therapeutic targets.

MicroRNAs (miRNAs), a class of small noncoding RNAs that are expressed endogenously, suppress gene expression via binding the "seed sequence" within the 3'-untranslated regions (3'-UTRs) of their target messenger RNAs (mRNAs) [5]. miRNAs are believed to play important roles in the control of biological processes such as cell growth, metastasis, apoptosis, and transformation due to their extensive regulation of gene expression [6–8]. In TSCC, miR-184 functions as an oncogene as evidenced by the overexpression of miR-184 in tumor tissue [9]. miR-138 has been reported to be crucial in regulating migration and invasion of cells [10], and miR-21 signals activation was thought to be associated with poor prognosis in patients with TSCC [11].

Recent studies have shown that the expression of miR-126 was significantly reduced in a variety of malignancies, ranged from adult T cell leukemia, breast cancer, colorectal cancer (CRC) and malignant mesothelioma [12–14] compared with nonmalignant tissue. However, miR-126 was significantly up-regulated in some other tumor types such as adrenocortical adenomas [15] and breast cancer [16]. Nevertheless, the clinical importance and prognostic potential of miR-126 in TSCC remained to be further studied.

Li et al. performed miRNA microarray analysis to compare the expression profiles between the TSCC tumor tissue and adjacent non-cancerous control, and reported a list of differentially expressed miRNAs [17]. In this study, we collected 21 pairs of TSCC cancerous and adjacent non-cancerous tissue samples, with which we performed real-time PCR to determine and compare the expression of 6 candidate miRNAs that have been reportedly associated with tumorigenesis of TSCC including miR-100, miR-451, miR-221, let-7a, miR-21 and miR-126, and identified that miR-126 was significantly downregulated in the cancerous tissue samples compared with the non-cancerous control tissue samples. In addition, we validated KRAS as a target of miR-126, and verified the regulatory association between miR-126 and KRAS and their roles in the development of TSCC.

## Material and Methods

### Tissue specimens

Paired primary TSCC samples from anterior regions of the tongue and nearby tissues that were histological normal were collected from 21 patients (age: 58.24±8.35, male vs. female: 16 vs. 5), who received surgical treatment in Oral Cavity College of Shandong University. None of patients had been treated prior to operation. Tumor tissues as well as nearby normal tissues at minimum 1.5 cm distant to the tumor edges were collected, followed by being frozen in liquid nitrogen and placed at –80°C for use. The study protocol was approved by the Institutional Ethics Committee of Shandong University, and all patients who were collected for samples signed informed consent for approval of the application of their tissues for the study after operation.

### RNA isolation and quantitative reverse-transcription PCR (qRT-PCR)

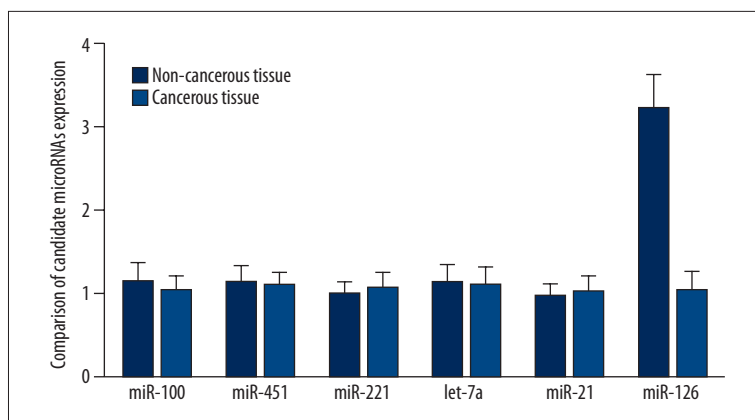
Total RNA was isolated from tissues or cells using TRIzol reagent (Invitrogen, Carlsbad, CA) as per the instructions of the manufacturer. Primer sets for amplification of KRAS, miRNAs and U6 were designed and supplied by Sangon Biotech (Shanghai, China). Quantitative PCR was performed in an ABI 7500 real-time PCR system, at 95°C for 10 min, and then 95°C for 15 sec at a total of 40 cycles, followed by 60°C for 60 sec. U6 was used an internal control, and the expression of miRNA and mRNA were normalized to the expression of U6. Gene expression changes were quantified using the delta-delta CT method.

### Cell cultures and transfection

SCC-15 were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA), and the cells were incubated at 37°C in Dulbecco's modified Eagle's medium (DMEM) containing 100 U/ml penicillin, 100 mg/ml streptomycin and 10% fetal calf serum (Invitrogen, Carlsbad, CA). Before transfection, SCC-15 cells were incubated in 6-well plates to make sure the cells grown to 80% confluence. miR-126 mimics/inhibitors, and the small interfering RNA (siRNA) that acted on siRNA control and human KRAS transcripts were obtained from Integrated Biotech Solutions Company (Ibsbio, Shanghai, China). Lipofectamine 2000 (Invitrogen, Carlsbad, CA) was used to transfect cells as per the instruction of the manufacturer.

### Cell proliferation assays

The Cell Counting Kit-8 (CCK-8, Dojindo, Kumamoto, Japan) was used to determine the impacts of upregulation or downregulation of miR-126 and downregulation of KRAS on proliferation of SCC-15 cells. In brief, the cells were plated into 96-well plates (10<sup>3</sup> cells/well). miRNA analogues or suppressor were



**Figure 1.** Real-time PCR was performed to screen for the differentially expressed miRNAs, and miR-126 was found to be significantly downregulated in cancerous tissue of TSCC compared with non-cancerous controls.

used to transfect cells. After transfection, CCK-8 was pipetted to all wells and cultured at 37°C for 3 h. The microplate spectrophotometer (Bio-Tek Instruments Inc, Winooski, VT) was used to determine the absorbance at 450 nm.

### Apoptosis analysis

The apoptosis status was evaluated using flow cytometry. The SCC-15 cells were transfected as described previously in this methods section. 48 h after transfection, the cells were harvested and re-suspended in phosphate-buffered saline (PBS) and then blocked in ethanol under room temperature overnight. After wash with PBS, the cells were re-suspended in staining solution containing 1 mg/ml RNase A, 50 mg/ml propidium iodide, and annexin-V-FLUOS Staining kit (Roche, Mannheim, Germany). The apoptosis of stained cells were then analyzed using The FACSCanto II (BD Biosciences, San Jose, CA), and CellQuest software (BD Biosciences, San Jose, CA).

### Luciferase reporter assay

PCR was used to amplify the human KRAS 3'-UTR containing estimated targeting sites of miR-126. After amplification, they were cloned into a pcDNA3.1(+) with modification containing a firefly luciferase reporter gene where at downstream of the luciferase reporter. SCC-15 cells were incubated in a 48-well plate. 24 h after co-transfection with 40 ng of the firefly luciferase reporter plasmid that contained the 3'-UTR of the target gene, 400 ng of pcDNA3.0 or miR-126, and 4 ng of pRL-TK which was a plasmid that expressed Renilla luciferase (Promega, Madison, WI).

### Western blot analysis

Cells were lysed with RIPA lysis buffer (250 mM NaCl, 1% NP40, 50 mM Tris/HCl, pH 8.0, 0.1% sodium dodecylsulfate 0.5%, (w/v) sodium deoxycholate). 12% SDS polyacrylamide gel electrophoresis was performed, and the separated protein extracts were transferred to polyvinylidene difluoride membrane (Millipore,

Billerica, MA). The membranes were incubated with primary antibodies (anti-KRAS antibody 1: 2000, and anti-β-actin antibody 1:10000, purchased from Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C for one night. After washing thoroughly, the membranes were incubated with horseradish peroxidase-conjugated second antibodies at 1:10,000 dilution (Zhongshan Goldenbridge, Beijing, China) for 1 h at room temperature and determined using ECL kit (Applygen, Beijing, China).

### Statistical analysis

The comparison between the groups was performed using Student's t test and one-way ANOVA. SPSS 16.0 (SPSS, Chicago, IL) was used for all statistical analyses. Experiments were performed at least three times. Data were indicated as mean ± standard deviation (SD). The statistical significance was set at a two-tailed value of  $P < 0.05$ .

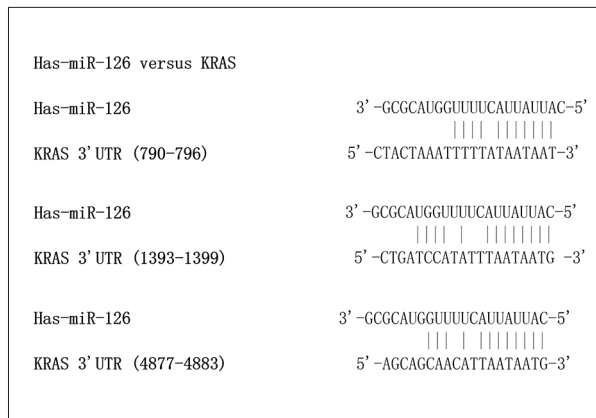
## Results

### miR-126 Expression was downregulated in TSCC

In this study, we collected 21 pairs of TSCC cancerous and adjacent non-cancerous tissue samples, with which we performed real-time PCR to determine and compare the expression of 6 candidate miRNAs that have been reportedly associated with tumorigenesis of TSCC including miR-100, miR-451, miR-221, let-7a, miR-21 and miR-126, and identified that miR-126 was significantly downregulated in the cancerous tissue samples compared with the non-cancerous control tissue samples, as shown in Figure 1.

### miR-126 targets KRAS in STCC cells

By using computational analysis, we identified that KRAS is a virtual target of miR-126 with multiple hits in the 3'UTR of the gene, as shown in Figure 2. To further confirm the interactive between KRAS 3'UTR and miR-126, we subcloned the full

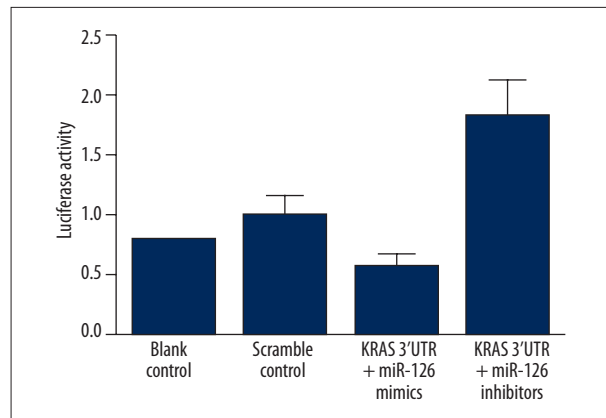


**Figure 2.** Computational analysis showed that there were three virtual seed sequences of miR-126 in the 3'UTR of KRAS.

length of KRAS 3'UTR into a vector and cotransfected it with miR-126 mimics or inhibitors, and we found that the luciferase activity of the cells cotransfected with KRAS 3'UTR and miR-126 mimics were significantly lower than that of the cells transfected with KRAS 3'UTR and scramble controls, while the luciferase activity of the cells transfected with KRAS 3'UTR and miR-126 inhibitors were substantially higher than that of the cells transfected with scramble controls and KRAS 3'UTR (Figure 3).

#### Determination and comparison of mRNA and protein expression of KRAS in the cancerous and non-cancerous tissue samples

To further confirm the relationship between miR-126 and KRAS, we evaluated the mRNA and protein expression of KRAS in the cancerous and non-cancerous tissue samples by using real-time PCR and western blot. As shown in Figure 4, we found that mRNA expression level of KRAS was significantly higher in the tumor tissue than the control tissue samples. Consistently, the protein expression level of KRAS was also significantly higher in the cancerous groups than the non-cancerous group. The

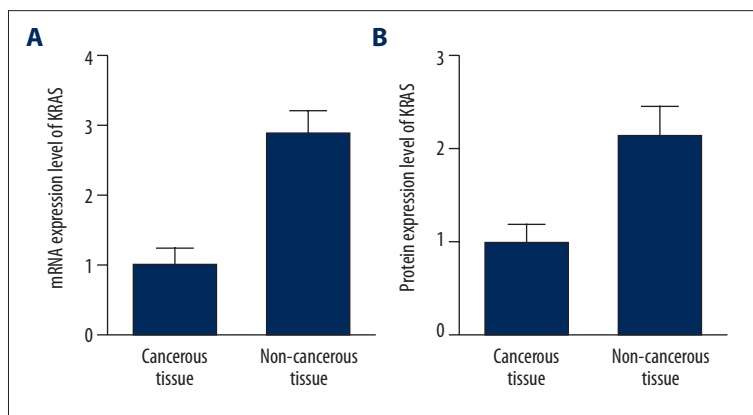


**Figure 3.** The results of luciferase reporter assay showed that the luciferase activity of the cells cotransfected with KRAS 3'UTR and miR-126 mimics were significantly lower than that of the cells transfected with KRAS 3'UTR and scramble controls, while the luciferase activity of the cells transfected with KRAS 3'UTR and miR-126 inhibitors were substantially higher than that of the cells transfected with scramble controls and KRAS 3'UTR.

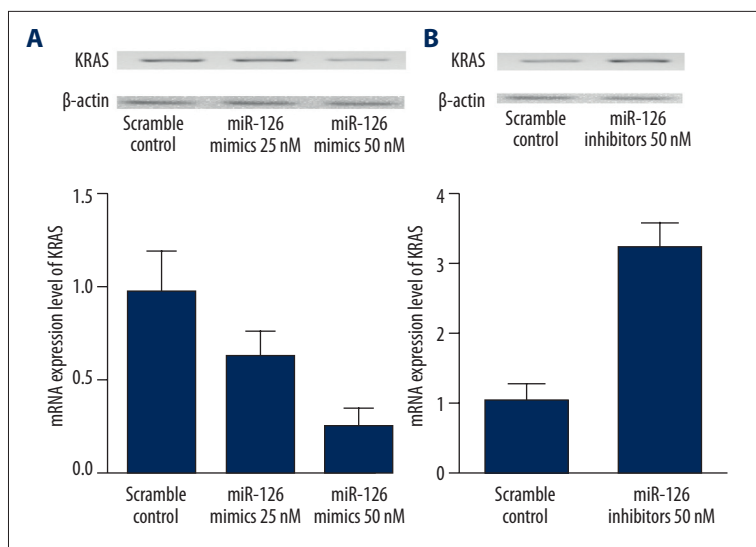
results further confirmed the regulatory relationship between the miR-126 and KRAS in the STCC cells.

#### Overexpression of miR-126 decreased expression of KRAS in STCC cells

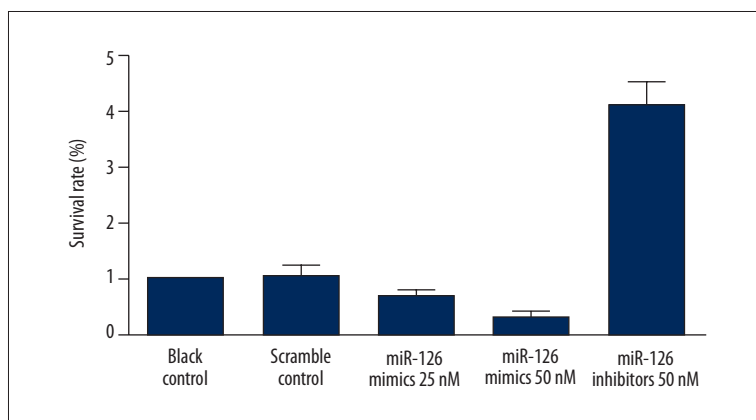
To further establish the regulatory relationship between the miR-126 and KRAS, we examined the effects of upregulation and downregulation of miR-126 on the endogenous expression of KRAS mRNA and proteins in a STCC cell line. Both mRNA and protein KRAS expression were significantly decreased in SCC-15 cells in which miR-126 was overexpressed, in comparison with similar cells transfected with a negative control (Figure 5A). On the other hand, downregulation of miR-126 by transfecting the cells with miR-126 inhibitors significantly up-regulated the mRNA and protein expression of KRAS (Figure



**Figure 4.** mRNA (A) and protein (B) expression levels of KRAS were significantly higher in the tumor tissue than the control tissue samples.



**Figure 5.** Both mRNA and protein KRAS expression were significantly decreased in SCC-15 cells in which miR-126 was overexpressed, in comparison with a negative control (A). On the other hand, downregulation of miR-126 by transfecting the cells with miR-126 inhibitors significantly upregulated the mRNA and protein expression of KRAS (B).



**Figure 6.** Overexpression of miR-126 inhibited the viability of SCC-15, while downregulation of miR-126 significantly promoted the proliferation of SCC-15.

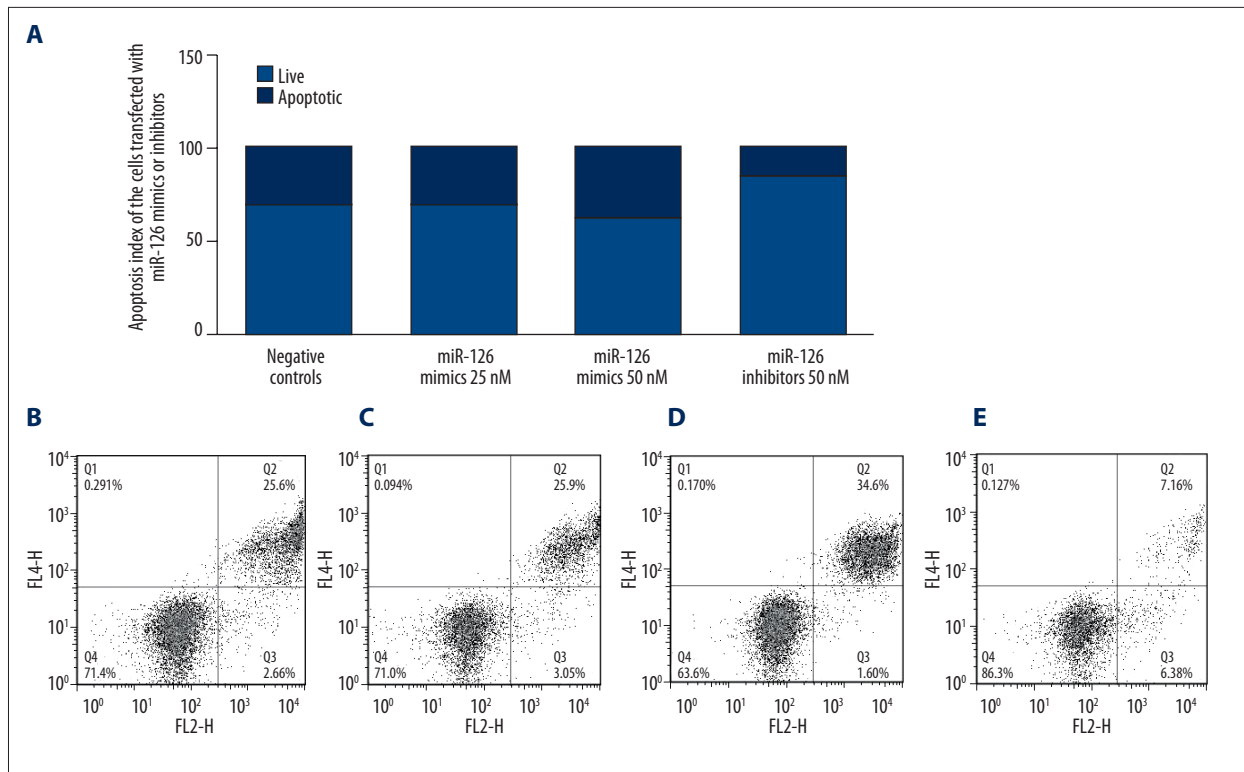
5B). These findings further demonstrated that KRAS is a direct target of miR-126 in TSCC cell lines.

#### Overexpression of miR-126 suppressed proliferation and induced apoptosis, and downregulation of miR-126 promoted proliferation and inhibited apoptosis in TSCC cell lines

To understand the biological function of miR-126 in TSCC, miR-126 was upregulated and downregulated by transfecting miR-126 mimics or inhibitors in TSCC cell lines. In the first of these experiments, we transfected TSCC cells with miR-126 mimics or inhibitors and performed proliferation assays to evaluate the effects of miR-126 expression on cell proliferation and viability. Overexpression of miR-126 inhibited the viability of SCC-15, while downregulation of miR-126 significantly promoted the proliferation of SCC-15 (Figure 6). Meanwhile, to explore the molecular mechanism underlying, we evaluated the apoptosis status of the differently treated SCC-15 cells, and found that overexpression of miR-126 induced the apoptosis of SCC-15, while downregulation of miR-126 significantly promoted the apoptosis of SCC-15 (Figure 7).

## Discussion

Although miRNAs are not directly associated with coding of the protein, it is believed that they regulate the expression of over one-third of the genes that codes the protein in the human genome [18–20]. By binding to the certain sequences in the target mRNA molecules on the basis of ‘imperfect complementarity’, each miRNA has the potential to modulate numerous genes at post-transcriptional levels. But the accurate relationships between miRNA and its target genes remain to be further determined from the perspective of the micro-environment and cell types. In this study, we collected 21 pairs of TSCC cancerous and adjacent non-cancerous tissue samples, with which we performed real-time PCR to determine and compare the expression of 6 candidate miRNAs that have been reportedly associated with tumorigenesis of TSCC including miR-100, miR-451, miR-221, let-7a, miR-21 and miR-126, and identified that miR-126 was significantly downregulated in the cancerous tissue samples compared with the non-cancerous control tissue samples.



**Figure 7.** Summary of the effects of miR-126 mimics and inhibitors on the apoptosis status in SCC-15 cells: **(A)** Schematic summary of the effects of miR-126 mimics and inhibitors on the apoptosis status; **(B)** Scramble controls; **(C)** transfection of 25 nM miR-126 mimics had minimal effect on apoptosis of the cells; **(D)** transfection of 50 nM miR-126 mimics substantially promoted apoptosis of the cells; **(E)** transfection of 50 nM miR-126 inhibitors substantially suppressed apoptosis of the cells.

MicroRNA-126 (miR-126) was derived from a typical precursor structure that was present within intron 7 of epidermal growth factor-like domain 7 (EGFL7) [21]. The expression of miR-126 is high in vascular endothelial cells and acts as a crucial regulator in the control of angiogenesis, which responds to angiogenic growth factors by inhibiting negative modulators of signal transduction pathways [22]. Recently, *in vivo* and *in vitro* experiments have verified the influence of miR-126 on the tumorigenesis and progression of human malignant cancers. For instance, non-small cell lung tumor tissues showed a significant downregulation of miR-126, which was possibly associated with increased microvessel density and clinical outcomes [23]; In breast cancer, the reduced expression of miR-126 may be associated with unfavorable metastasis-free survival [24]; Insufficiency of miR-126 is more likely common in metastatic colorectal cancers [25]. On the contrary, miR-126 has been shown to be a crucial negative indicator for prognosis of squamous cell carcinomas, so it may become an oncogene [26]. These debatable results indicated that there may be discrepancy in the effects of miR-126 in different cancer types, which could be attributed to its high tissue-specificity. In this study, we identified that KRAS is a virtual target of miR-126 with multiple hits in the 3'UTR of the gene (790–796 bp, 1393–1399 bp, and 4877–4883 bp) by searching the online

miRNA database ([www.mirdb.org](http://www.mirdb.org)) and using computational analysis, which was further confirmed by luciferase reporter system, showing that the luciferase activity of the cells co-transfected with KRAS 3'UTR and miR-126 mimics were significantly lower than that of the cells transfected with KRAS 3'UTR and scramble controls, while the luciferase activity of the cells transfected with KRAS 3'UTR and miR-126 inhibitors were substantially higher than that of the cells transfected with scramble controls and KRAS 3'UTR. In addition, we found that mRNA expression level of KRAS was significantly higher in the tumor tissue than the control tissue samples. Consistently, the protein expression level of KRAS was also significantly higher in the cancerous groups than the non-cancerous group. All these findings supported our hypothesis that KRAS was a target of miR-126 and confirmed such regulatory relationship between miR-126 and KRAS.

As one of the three members of the Ras oncogene family (consisted of H-ras, K-ras and N-ras), V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (K-ras) encodes small GTPases that play an important role in the control of cellular activities [27]. Ras signal activation induces complicated downstream signaling cascade, including the phosphatidylinositol 3-kinase (PI3K)/Akt pathway and protein kinase (MAPK) pathway activated by

the mitogen, thereby modulating a variety of cellular processes, ranged from cell growth to differentiation, survival, migration, and apoptosis [27]. K-ras is a member of the Ras family and its mutation and excess expression are believed to lead to malignant cell transformation. The stimulation of K-ras in combination with Akt in neural progenitors could trigger generation of the glioma in mice [28]. Almost all high-grade malignancies showed activation of the MAPK pathway and the PI3K/Akt pathway, and maintenance of malignancies requires MAPK signaling [29]. Several miRNAs including miR-18a\*, miR-143, miR-96, miR-217, and miR-181a have been shown to be involved in the control of oncogenesis via targeting the expression of K-ras, suggesting that miRNAs that target K-ras are very crucial in carcinogenesis and act as possible therapeutic options for human cancer [30–34]. In this study, we examined the effects of upregulation and downregulation of miR-126 on the endogenous expression of KRAS mRNA and proteins in a STCC cell line. Both mRNA and protein KRAS expression were significantly decreased in SCC-15 cells in which miR-126 was overexpressed, in comparison with similar cells transfected with a negative control (Figure 5A). On the other hand, downregulation of miR-126 by transfecting the cells with miR-126 inhibitors significantly upregulated the mRNA and protein expression of KRAS (Figure 5B). In addition, we transfected TSCC cells with miR-126 mimics or inhibitors and performed proliferation

assays to evaluate the effects of miR-126 expression on cell proliferation and viability. Overexpression of miR-126 inhibited the viability of SCC-15, while downregulation of miR-126 significantly promoted the proliferation of SCC-15 (Figure 6). Meanwhile, to explore the molecular mechanism underlying, we evaluated the apoptosis status of the differently treated SCC-15 cells, and found that overexpression of miR-126 inhibited the apoptosis of SCC-15, while downregulation of miR-126 significantly promoted the apoptosis of SCC-15 (Figure 7).

## Conclusions

In conclusion, our findings suggest that miR-126 modulates KRAS signaling at multiple levels. The results shown in the present paper, in combination with previous studies on miR-126 and other proto-oncogenes, indicate that miR-126 is very important in tumorigenesis. The administration of miR-126 as a therapeutic agent may be a potent approach for the treatment of this disease, contributing to the central role of the KRAS signaling pathway in TSCC.

## Conflicts of interest

Authors claim no conflict of interest.

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