MiR-127-3p targets KIF3B to inhibit the development of oral squamous cell carcinoma

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Abstract. – OBJECTIVE: Recently, increased microRNAs have been shown to play an important role in the pathogenesis and progression of human cancers, including oral squamous cell carcinoma (OSCC). In this study, we focused on the function of microRNA-127-3p (miR-127-3p) associated with OSCC carcinogenesis.

PATIENTS AND METHODS: MiR-127-3p and KIF3B expressions were observed via quantitative Real-time polymerase chain reaction (qRT-PCR) or Western blot in OSCC. The functions of miR-127-3p and KIF3B were investigated through MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) and transwell assays. And luciferase reporter assay was performed to confirm the relationship between miR-127-3p and KIF3B.

RESULTS: First, down-regulation of miR-127-3p was identified in OSCC, which was associated with malignant clinicopathological features and poor prognosis in OSCC patients. Functionally, overexpression of miR-127-3p led to inhibition of cell proliferation and metastasis in OSCC. Further, KIF3B was confirmed to be a direct target of miR-127-3p. Moreover, upregulation of KIF3B was also observed in OSCC, which promoted tumorigenesis of OSCC. In particular, the upregulation of KIF3B partially attenuated the inhibitory effect of miR-127-3p on the development of OSCC.

CONCLUSIONS: MiR-127-3p targeted KIF3B to inhibit the development of OSCC through suppressing cell proliferation, migration and invasion.

Key Words: miR-127-3p, Proliferation, Metastasis, Prognosis, KIF3B, Oral squamous cell carcinoma.

Introduction

Oral squamous cell carcinoma (OSCC) is the malignant tumor derived from the oral epithelium lining, including lip cancer, tongue cancer, oral cancer, oropharyngeal cancer, etc. In recent years, the incidence of OSCC has increased, which often occurs in men. In the worldwide, the incidence of OSCC ranks sixth among human malignancies. More seriously, the overall 5-year survival rate of OSCC is lower, below 50% due to the high rate of lymph nodes metastasis. In addition, the recurrence rate of OSCC is above 40%, which seriously threatens human life. Therefore, early diagnosis, timely prevention and improvement of survival rate of OSCC patients are very important. Recently, abnormal expressions of microRNAs (miRNAs) have been proposed to modulate the expression of corresponding genes to further regulate their downstream biological activities. In addition, miRNAs aberrations have been shown to be closely associated with the pathogenesis and clinical significance of head and neck cancer. It has been reported that miRNAs as small molecules have a potential role in OSCC. To date, various miRNAs have been found to be involved in the pathogenesis and development of OSCC. For example, miR-497 and miR-654 were upregulated in OSCC, which promoted proliferation, metastasis. Besides that, downregulation of miR-186 and miR-377 was also identified in...
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OSCC. In particular, miR-127 has been reported to be abnormally expressed in different human diseases and cancers. Previous studies have shown that miR-127 was upregulated in inflammatory injury and lung cancer. In contrast, downregulation of miR-127 was also found in gastric cancer and esophageal squamous cell carcinoma. Furthermore, it has been suggested that miR-127-3p functions as a tumor suppressor in epithelial ovarian cancer via inhibiting BAG5 expression. However, the specific role of miR-127-3p in OSCC cells remains unclear.

KIF3B is a member of kinesin superfamily proteins (KIFs) that regulate various cellular processes, such as meiosis, mitosis, and transport macromolecules. Especially, exception of mitosis may contribute to the carcinogenic effects of human cancers. Moreover, KIF3A and KIF3B are heterodimers with several cargo proteins, such as fodrin and the tumor suppressor gene APC in the KIF3 subfamily. Furthermore, it has been reported that KIF3B has a novel role in the seminoma cell cycle. All of these studies suggest that KIF3B may be involved in the development of human cancers. Therefore, we conducted this study to investigate the role of miR-127-3p and KIF3B in OSCC. At the same time, we analyzed the relationship between miR-127-3p and clinicopathological features or prognosis in OSCC patients. More importantly, we also demonstrated that miR-127-3p inhibited the development of OSCC by targeting KIF3B.

**Patients and Methods**

**Clinical Tissues**

Forty-five surgical OSCC specimens and neighboring tissue samples were obtained from the Affiliated Stomatological Hospital of Nanchang University after receiving written informed consent. All participants received no other treatment prior to the operation. The tissues were then frozen in liquid nitrogen and stored in a -80°C refrigerator for further experiment. The experiment was approved by the Institutional Ethics Committee of the Affiliated Stomatological Hospital of Nanchang University.

**Cell Culture and Transfection**

The Tca-8113, SCC-4, SCC-9, SCC-25 cell lines and normal human oral keratinocytes Normal Human Oral Keratinocyte (NHOK) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). All of these cell lines were inoculated into Dulbecco’s modified eagle medium (DMEM) (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) and cultured at 37°C with 5% CO2. The miR-127-3p mimic and inhibitor, KIF3B vector and siRNA were purchased from RiboBio (Guangzhou, China) and then transferred to OSCC cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) based on the manufactures’ protocols.

**Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)**

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was applied for extracting total RNA to quantitate miR-127-3p expression level in OSCC cells according to the manufacturer’s protocol. QRT-PCR was carried out by using TaKaRa SYBR Green PCR Kit (TaKaRa, Otsu, Shiga, Japan) on ABI 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). Next, U6 and GAPDH were used as control for miR-127-3p and KIF3B. And their expressions were calculated using the 2−∆∆ct method. The following primers were used: miR-127-3p, (F) GGAAGATCTGTA-GTCCTGTCTGTTGGTCAG, (R) CCCAAC-GCTTCTTGAGAAGACTGCTTCCC; KIF3B (F) GATGTTAAGCTGGGGCAGGT, (R) TTTGCCTCCACTAGAGCAG; U6, (F) CTC-GCTCGGCAAGCCA, (R) AACGCTTCAC-GAATTTCGCT; Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (F) GAGACTGGA-GGTCGAGTGC, (R) GAGATGGTGATGG-GATTC.

**MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide) assay**

The transfected cells were then cultured in 96-well plates (1×104 cells/well) at 24, 48, 72 and 96 h, and then cultured with 20 µL MTT (Sigma-Aldrich, St. Louis, MO, USA). MTT assay was performed to assess cell viability. The absorbance at 490 nm (OD=490 nm) was recorded with a spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

**Transwell Assays**

The migratory and invasive abilities of OSCC cells in 24-well plates were evaluated using a transwell chambers (8 μm pore size, Millipore, Billerica, MA, USA). Serum-free OSCC cells
were placed in the upper chamber on the uncoated membrane, and lower chamber was filled with 10% fetal bovine serum (FBS) to induce OSCC cells to migrate or invade through the membrane. Besides that, some cells were placed in the upper chamber, and an invasion assay was performed using the coated membrane. These cells were then incubated for cell migration and invasion for 48 h. A microscope was used to count migrated and invading cells.

Western Blot Analysis
Protein samples were obtained using radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China). The protein was then separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and incubated in polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA) with 5% skim milk at room temperature. Next, we incubated the membranes overnight at 4°C with rabbit monoclonal anti-KIF3B (1:1000; Abcam, Cambridge, MA, USA) and rabbit monoclonal anti-GAPDH antibody (1:1000; Epitomics, Burlingame, CA, USA) and subsequently incubated with goat polyclonal anti-rabbit IgG secondary antibody (1:1000; Abcam, Cambridge, MA, USA). Protein expression levels were then measured by electrochemiluminescence (ECL, Pierce, Rockford, IL, USA).

Dual Luciferase Assay
The 3′-Untranslated Region (3′-UTR) of wild or mutant type KIF3B was inserted into a pGL3 control vector (Promega, Madison, WI, USA) for luciferase reporter experiments. The 3′-UTR of wild or mutant type KIF3B and miR-127-3p mimic were then transfected into OSCC cells. Subsequently, dual luciferase assay system (Promega, Madison, WI, USA) was used to analyze luciferase activity.

Statistical Analysis
Data were analyzed by Statistical Product and Service Solutions (SPSS) 19.0 (SPSS, Inc., IBM, Armonk, NY, USA) and Graphpad Prism 6 (Graphpad Software, Inc., La Jolla, CA, USA). The difference was calculated according to the \( \chi^2 \)-test. The relationship between miR-127-3p level and survival rate was performed by Kaplan-Meier method and the log-rank test. \( p<0.05 \) was defined as significant difference.

Results
The Alteration of miR-127-3p and KIF3B Expressions was Identified in OSCC
First, the expression of miR-127-3p and KIF3B was evaluated by qRT-PCR to observe their changes in OSCC. Furthermore, we found that miR-127-3p expression was significantly decreased in OSCC tissues compared to that of normal tissues (Figure 1A; \( p<0.01 \)). In addition, miR-127-3p expression was observed in Tca-8113, SCC-4, SCC-9, SCC-25 and NHOK cell lines. Similarly to the trend of OSCC tissues, miR-127-3p was also downregulated in these four cell lines compared with NHOK cells (Figure 1B; \( p<0.05 \) or 0.01). At the same time, the opposite result of KIF3B expression was examined in OSCC. The qRT-PCR experiment showed that KIF3B was upregulated in OSCC tissues (Figure 1C; \( p<0.01 \)) and cell lines compared to the control group (Figure 1D; \( p<0.05 \) or 0.01).

Downregulation of miR-127-3p was Associated with Poor Clinicopathological Features and Prognosis of OSCC Patients
After confirming the downregulation of miR-127-3p in OSCC, we analyzed the association between abnormal miR-127-3p expression and clinic-pathological characteristics of OSCC patients in this study. The association between low miR-127-3p expression and TNM stage (\( p=0.048 \)) was identified as shown in Table I. In addition, Kaplan-Meier analysis showed that OSCC patients with low miR-127-3p expression had a shorter overall survival (\( p=0.0345 \), Figure 2) compared with high miR-127-3p expression. Based on these results, we suggested that miR-127-3p may be involved in tumorigenesis and prognosis of OSCC.

The Inhibition of Cell Proliferation was Caused by Overexpression of miR-127-3p in OSCC
We then transfected the miR-127-3p mimics or inhibitor into SCC-9 cells to explore its specific role in OSCC. We found that, when miR-127-3p mimics significantly promote its expression (Figure 3A; \( p<0.01 \)), miR-127-3p expression was apparently inhibited by miR-127-3p inhibitor (Figure 3B; \( p<0.01 \)). Thereafter, we performed MTT assay to measure the cell viability regulated by miR-127-3p in transfected SCC-9 cells. The MTT assay showed that miR-127-3p overexpression repressed the proliferation of SCC-9 cells (Figure
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Figure 1. The alteration of miR-127-3p and KIF3B expressions was identified in OSCC. (A) The expressions of miR-127-3p in OSCC tissues were detected via qRT-PCR. (B) The miR-127-3p expression was observed in Tca-8113, SCC-4, SCC-9, SCC-25 and NHOK cell lines. (C) The expressions of KIF3B in OSCC tissues were detected via qRT-PCR. (D) The KIF3B expression was examined in Tca-8113, SCC-4, SCC-9, SCC-25 and NHOK cell lines. *p<0.05, **p<0.01.

Table I. Relationship between miR-127-3p expression and their clinic-pathological characteristics of OSCC patients.

<table>
<thead>
<tr>
<th>Characteristics</th>
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<th>miR-127-3p</th>
<th>p-value</th>
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<tbody>
<tr>
<td></td>
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<td>Low</td>
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<td>Age (years)</td>
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<td>7</td>
<td>13</td>
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<td>9</td>
<td>8</td>
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<tr>
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<td>≥ 4</td>
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Statistical analyses were performed by the χ² test. *p<0.05 was considered significant.
The upregulation of miR-127-3p led to suppression of cell migration and invasion in OSCC

Transwell assays were then performed to measure migration and invasion abilities in transfected cells. Furthermore, we found that miR-127-3p overexpression significantly suppressed migration and invasion of SCC-9 cells (Figure 4A, 4B; p<0.01). On the contrary, downregulation of miR-127-3p promoted migration and invasion in SCC-9 cells (Figure 4A, 4B; p<0.01). Collectively, miR-127-3p may impede the progression of OSCC by inhibiting migration, invasion and proliferation of OSCC cells.

Figure 2. MiR-127-3p could predict the prognosis of OSCC patients. OSCC patients with high miR-127-3p expression showed longer OS.

Figure 3. The inhibition of cell proliferation was caused by overexpression of miR-127-3p in OSCC. (A, B) The miR-127-3p expression was examined in cells contained miR-127-3p mimics or inhibitor via qRT-PCR. (C, D) The cell proliferation was measured in cells with miR-139-5p mimics or inhibitor via MTT assay. **p<0.01.
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KIF3B was a Downstream Target of miR-127-3p in OSCC

Further, downstream targets of miR-127-3p were investigated after verifying the inhibitory effect of miR-127-3p in OSCC cells. We selected KIF3B as a candidate target gene based on TargetScan prediction (http://www.targetscan.org/vert_71/). It shows the binding sites of KIF3B to miR-127-3p (Figure 5A). Then, the results of the luciferase assay indicated that the miR-127-3p mimics inhibited the luciferase activity of wild-type KIF3B (Figure 5B, p<0.01), which was not identified to affect the luciferase activity of the mutant KIF3B. More importantly, we also found a negative correlation between KIF3B and miR-127-3p expression in OSCC tissues (R²=0.4391, p=0.0052; Figure 5C). To further confirm, we also observed the expression of KIF3B in cells with miR-127-3p mimics or inhibitors. Consistent with above observation, upregulation of miR-127-3p significantly reduced KIF3B expression, whereas downregulation of miR-127-3p had opposite results (Figure 5D, 5E; p<0.01). Therefore, KIF3B is considered to be a direct target of miR-127-3p, which has a negative correlation with miR-127-3p in OSCC.

KIF3B Silencing Showed the Inhibitory Effect on the Development of OSCC

Next, KIF3B siRNA was transfected into SCC-9 cells to investigate its function in OSCC. And we performed qRT-PCR to confirm transfection efficiency (Figure 6A, p<0.01). Moreover, we found that KIF3B silencing inhibited cell proliferation in SCC-9 cells (Figure 6B, p<0.01). Similarly, KIF3B silencing also suppressed migration and invasion of OSCC cells (Figure 6C, 6D; p<0.01). Taken together, KIF3B silencing has an inhibitory effect on the development of OSCC by inhibiting cell proliferation and metastasis.

Overexpression of KIF3B Partially Weakened the Inhibitory Action of miR-127-3p in OSCC

Finally, the miR-127-3p mimics and the KIF3B vector were co-transfected into SCC-9 cells to further analyze their interactions. The results showed that there was almost no change in KIF3B expression in co-transfected cells compared to the control group, whereas KIF3B expression was significantly decreased in cells with only miR-127-3p mimics (Figure 7A, p<0.01). Besides that, upregulation of KIF3B was found to impair

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**Figure 4.** The suppression of cell migration and invasion was also due to the upregulation of miR-127-3p in OSCC. (A-B) The cell migration and invasion was measured in cells with miR-127-3p mimics or inhibitor via transwell analysis. **p<0.01.
the inhibitory function of miR-127-3p in SCC-9 cells (Figure 7B, 7C, 7D; \(p<0.01\)). Based on these findings, it was found that overexpression of KIF3B partially impaired the inhibitory effect of miR-127-3p in OSCC.

**Discussion**

OSCC is a common head and neck cancer with a poor prognosis\(^{22}\). Recently, increased miRNAs have been identified in OSCC because of much attention to the regulatory mechanism associated with tumorigenesis of OSCC. For example, miR-1-3p was found to inhibit cell proliferation and migration in OSCC by suppressing DKK1\(^{23}\). Shang et al\(^{24}\) proposed that miR-9 induced OSCC cell arrest and apoptosis by regulating CDK 4/6 pathway. Moreover, miR-375 has been shown to inhibit growth and enhance radiosensitivity in OSCC via targeting IGF-1R\(^{25}\). In this study, the potential function of miR-127-3p associated
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with OSCC progression was investigated. Zhang et al.\textsuperscript{26} reported that miR-127-3p inhibited the proliferation and invasion of osteosarcoma cells through inhibiting SETD8. In contrast, Jiang et al.\textsuperscript{27} demonstrated that miR-127-3p promoted migration and invasion of glioblastoma cells via targeting SEPT7. In the current study, miR-127-3p was downregulated in OSCC, which was similar to the results of previous studies.\textsuperscript{28} We also found that upregulation of miR-127-3p suppressed cell migration, invasion and proliferation in OSCC. The inhibitory effect of miR-127-3p on OSCC progression was consistent with the above studies.\textsuperscript{26} At the same time, it was showed that downregulation of miR-127-3p was associated with poor prognosis in OSCC patients in this study. Similarly, Wang et al.\textsuperscript{29} suggested that low miR-127 expression was closely related

Figure 6. KIF3B silencing showed the inhibitory effect on the development of OSCC. (A) The expression of KIF3B was measured in cells containing KIF3B siRNA. (B) The cell proliferation was measured in cells with si-KIF3B via MTT assay. (C, D) Cell migration and invasion were detected in cells with si-KIF3B by transwell assay. \(*p<0.01\).
to the poor prognosis in breast cancer patients. Furthermore, we observed that KIF3B is a direct target gene of miR-127-3p in OSCC. It has been reported that miR-127 protects proximal tubule cells from ischemia/reperfusion and KIF3B is identified as a miR-127 target\textsuperscript{30}. Moreover, it was also found that the 3’-UTR of KIF3B contains binding sites for miR-372 which directly suppressed its expression\textsuperscript{31}. We also detected a negative association between miR-127-3p and KIF3B in OSCC tissues. In addition, upregulation of KIF3B was observed in OSCC that promoted cell

Figure 7. Overexpression of KIF3B partially weakened the inhibitory action of miR-127-3p in OSCC. (A) The expression of KIF3B was measured in cells containing KIF3B vector and miR-127-3p. (B) The cell proliferation was measured in cells with KIF3B vector and miR-127-3p via MTT assay. (C, D) The cell migration and invasion in cells containing KIF3B vector and miR-127-3p were measured by transwell assay. **p<0.01.
proliferation and metastasis. Consistent with our findings, Huang et al\textsuperscript{2} proposed that KIF3B was upregulated in HCC and suppression of KIF3B inhibited HCC proliferation. All of these findings indicate that upregulation of KIF3B functions as an oncogene in the pathogenesis of OSCC. Furthermore, we also found that overexpression of KIF3B partially attenuated the inhibitory effect of miR-127-3p in OSCC.

Conclusions

We showed that miR-127-3p targeting KIF3B inhibits the development of OSCC by regulating cell proliferation, migration and invasion. Moreover, low miR-127-3p expression was associated with poor prognosis in OSCC patients. We hope that these findings will help diagnose and treat patient with OSCC.

Conflict of Interest

The Authors declare that they have no conflict of interest.

References


