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MiR-25-3p attenuates the proliferation of tongue squamous cell carcinoma cell line Tca8113

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ABSTRACT

Objective: To investigate the effects of miR-25-3p on the occurrence, development and proliferation of tongue squamous cell carcinoma cells. **Methods:** To establish tongue squamous cell carcinoma cell line Tca8113 that stably and highly express miR-25-3p using recombinant retroviral vector-mediated gene transfer method. The proliferation of transfected Tca8113 was detected by thiazolyl blue tetrazolium bromide (MTT) and cell colony formation assays. cyclinD1, $p21^{cip1}$ and $p27^{kip1}$mRNA expressions in the transfected Tca-8113 were detected by quantitative PCR. cyclinD1, $p21^{cip1}$, $p27^{kip1}$, AKT, p-AKT, FOXO1 and p-FOXO1 expressions in the transfected Tca8113 were detected by western blot analysis. In addition, miR-25-3p expression in the tongue squamous cell carcinoma cell line and tissue specimen was also detected by quantitative PCR. **Results:** Quantitative PCR showed that miR-25-3p expression in the tongue squamous cell carcinoma cell lines and tissue specimen was significantly lower than that in the adjacent tissue. MTT and cell colony formation assays showed that after miR-25-3p overexpression, the proliferation of transfected Tca8113 was obviously attenuated. Western blot analysis and quantitative PCR showed that after miR-25-3p overexpression, $p21^{cip1}$ and $p27^{kip1}$ expressions were upregulated, while cyclinD1, AKT, FOXO1 expressions were downregulated, and AKT and FOXO1 phosphorylation was inactivated in the transfected Tca8113 cells. **Conclusions:** MiR-25-3p inhibited the proliferation of tongue squamous cell carcinoma cells and regulated cell cycle-related protein expression, playing an important role in the occurrence and development of squamous cell carcinoma of the tongue.

1. Introduction

Squamous cell carcinoma of the tongue is the most common malignant tumor in the oromaxillo-facial region. Conventional adjuvant radiation therapy and chemotherapy do not increase the 1-year survival rate and healing rate of the patients. With increasing attention to biological targeted therapy, targeted therapy against tumors has been preferred, but the mechanism underlying molecular treatment remains poorly understood. MicroRNA (miRNA), a tissue-specific biomarker of cancer, functions as an oncogene or a tumor suppressor and is involved in a diverse range of physiological and pathological processes including organism development, cancer and immunity[1-8].

miRNA expression abnormalities can be easily detected in the early stage and therefore can be used for screening of early-stage cancer[9]. According to a previous study[10], different expression ratios of miR-221 to miR-375 can be used to distinguish between normal tissues and tumors and showed a 92% sensitivity and 93% specificity for disease prediction, indicating that difference in miRNA expression pattern is likely to be an alternative diagnostic method for cancer. There is evidence that miR-25 is expressed in tissues of colon cancer, gastric cancer, prostatic cancer,

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and ovarian cancer, participates in cellular proliferation, apoptosis and migration, and is closely related to the prognosis[11-14]. However, to the best of our knowledge, there are few reports about miR-25-3p in squamous cell carcinoma of the tongue, and the underlying molecular mechanism is unclear. Our previous findings have demonstrated that miR-25-3p expression in either tongue squamous cell carcinoma cell line or tissue specimen is significantly lower than that in the normal tongue epithelial tissue. This suggests that miR-25-3p is possibly related to the occurrence and development of tongue squamous cell carcinoma and affects tumor growth, invasion and metastasis. This study was designed to investigate whether upregulated miR-25-3p expression inhibit the proliferation of tongue squamous cell carcinoma cells and whether regulation of AKT/FOXO signaling pathway influences cell proliferation.

2. Materials and methods

2.1. Cells and plasmids

Tongue squamous cell carcinoma cell lines SCC-9, SCC-2, TSCCa and Tca8113, normal tongue epithelial cells, and FT293 cells were purchased from Chinese Academy of Sciences, China. pMSCV-puro-miR-25-3p plasmids were constructed and preserved by Guangzhou Laura biotech Ltd., Guangzhou, China.

2.2. Main reagents

Fetal bovine serum, RPMI-1640 medium, 6-well plate were purchased from Gibco (Carlsbad, CA, USA); matrigel from BD Becton Dickinson (San Jose, CA, USA); puromycin from Invitrogen (Paisley, UK); cyclinD1, $p21^{cip1}$, $p27^{kip1}$, AKT, phosphorylated AKT (p-AKT), FOXO1, phosphorylated FOXO1(p-FOXO1) primary antibodies and goat anti-rabbit secondary antibody from Abcam (Hangzhou, China); thiazolyl blue tetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) from Sigma Chemical Co., (St Louis,. MO., USA); real time fluorescent quantitative PCR kit from FulenGen Co.,Ltd., (Guangzhou, China); other routine reagents and medical consumables from Guangzhou Laura biotech Ltd., (Guangzhou, China).

2.3. Establishment of stable cell lines Tca8113/miR-25-3p

Retroviral expression plasmids, pMSCV-miR-25-3p and pMSCV-Vector, together with PIK packaging plasmid were co-transfected into FT293 packaging cell lines by calcium phosphate transfection method. The culture medium was refreshed 5 h after cell transfection. Viruses were filtered through a 0.45-μm-pore-size filter and preserved at -80 ℃ before use.

When Tca8113 cells were appropriately 70 confluent after seeding, viruses were added. To increase the efficiency of viral infection, polybrene was also added at a final concentration of 8 μg/mL. After 48 h of infection, culture medium was supplemented with 0.5 μg/mL puromycin (Sigma) to select transfected cells. Passage 7 cells were used for detection of miR-25-3p expression by RT-PCR.

2.4. Detection of cell proliferation

Determination of cell growth curves by MTT assay: Cells in the logarithmic phase were digested and seeded into 96-well plates at a density of 1×10^3 per well in 200 μL culture medium. Equal amounts of culture medium were used as the control. 20 μL of sterile MTT solution (5 mg/mL) was added to each well every other 24 h. After incubation at 37 ℃ for 4 h, the culture medium was discarded, 150 μL of DMSO was added to each well, and the 96-well plates were oscillated for 10 min with a trace oscillator to allow the crystals to fully dissolve. Absorbance values at 490 nm were recorded from three parallel wells per day for 4 d using an ELISA plate reader.

Cell colony formation assays: Cells in the logarithmic phase were digested and seeded into 6-well plates at a density of 1×10^3 per well. The culture was stopped when the colonies were clearly visible by the naked eyes. Following methanol fixation, cells were stained with hematoxylin. The number of colonies each containing more than 50 cells was counted in three parallel wells.

2.5. Detection of miR-25-3p expression in tongue squamous cell carcinoma cell lines and tissue specimen and cyclinD1, $p21^{cip1}$ and $p27^{kip1}$ gene expression in transfected Tca8113 cells by quantitative PCR

Cells in the logarithmic phase were lysed with Trizol and total RNA was extracted with chloroform. The concentration of RNA was measured by UV spectrophotometry. Then RNA sample was preserved at -20 ℃ for later use. β-Actin, a housekeeping gene, was used as an internal control gene to detect the expression of cyclinD1, $p21^{cip1}$, $p27^{kip1}$ genes in different cell lines. Primer sequences are shown in Table 1.

Table 1
Primer sequences.

Gene	Forward primer	Reverse primer
cyclinD1	5′-AAC TAC CTG GAC CGC TTC CT-3′	5′-CCA CTT GAG CTT GTT CAC CA-3′
$p21^{cip1}$	5′-CGA TGC CAA CCT CCT CAA CGA-3′	5′-TCG CAG ACC TCC AGC ATC CA-3′
$p27^{kip1}$	5′-TGC AAC CGA CGA TTC TTC TAC TCA A-3′	5′-CAA GCA GTG ATG TAT CTG ATA AAC AAG GA-3′
β-Actin	5′-TGG CAC CCA GCA CAA TGA A-3′	5′-CTA AGT CAT AGT CCG CCT AGA AGC A-3′

2.6. Detection of cell cycle and the expression of AKT/FOXO signaling pathway-related proteins in transfected Tca8113 cells by western blot analysis

Cells in the logarithmic phase were lysed and protein concentration was measured with BCA Protein Assay Kit. 20 μg of total protein extracts were separated by 9% SDS-PAGE. PCR products were confirmed by electrophoresis at 70 V for about 4 h. Following membrane transfer, blocking and incubation with antibody, bands were visualized by a streptavidin-horseradish peroxidase ECL detection assay and exposure to X-ray film for detection of cyclinD1, $p21^{cip1}$, $p27^{kip1}$, AKT, pAKT, p-FOXO1 and FOXO1 expression.

3. Results

3.1. Aberrant expression of miR-25-3p in the tongue squamous cell carcinoma cell line and tissue specimen

Normal tongue epithelial cells served as controls. Quantitative PCR showed that miR-25-3p expression in the tongue squamous cell carcinoma cell line and tissue specimen was significantly lower than that in the normal tongue epithelial cells (Figure 1).

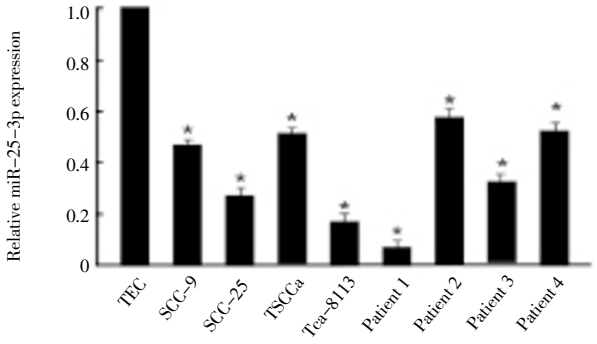

Figure 1. Detection of relative expression of miR-25-3p in tongue squamous cell carcinoma tissue and cell lines by quantitative PCR.

3.2. Successful establishment of tongue squamous cell carcinoma cell lines expressing high level of exogenous miR-25-3p

Tca8113 cells were transfected with vector and miR-25-3p separately using recombinant retroviral vector-mediated gene transfer method and then selected with 0.5 μg/mL puromycin. Total RNA was extracted from passage 7 cells. Quantitative PCR showed that miR-25-3p expression in the Tca8113 cells transfected with miR-25-3p was significantly upregulated compared to the Tca8113 cells transfected with vector. This suggests that the tongue squamous cell carcinoma cell line Tca8113/miR-25-3p that stably expresses high level of miR-25-3p was successfully established (Figure 2).

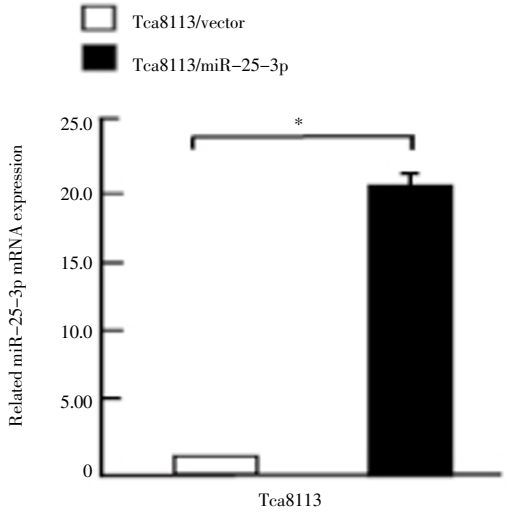

Figure 2. MiR-25-3p mRNA expression in Tca8113 cells transfected with vector and miR-25-3p.

3.3. Effects of miR-25-3p overexpression on Tca8113 cell proliferation

Detection of cell proliferation by MTT assay: Through the growth curve, the logarithmic phase of the Tca8113 cells transfected either with vector or miR-25-3p was both 7 days after seeding, but the doubling time of Tca8113 transfected with miR-25-3p was relatively prolonged, and the growth rate was significantly downregulated, and therefore, the proliferation of Tca8113 cells transfected with miR-25-3p was inhibited (Figure 3A).

Detection of cell colony formation by cell colony formation assays: Cell colony formation assay results showed that the number of colonies of Tca8113 transfected with miR-25-3p was significantly small than that of colonies of Tca8113 transfected with vector ($P<0.05$) (Figure 3B). This findings suggest that miR-25-3p overexpression decreased cell colony formation ability.

3.4. Effects of miR-25-3p overexpression on regulation of the mechanism behind Tca8113 cell proliferation

The expressions of molecules related to Tca8113 cell proliferation regulation and AKT signaling pathway after miR-25-3p overexpression were detected by western blot analysis and quantitative PCR. After miR-25-3p overexpression, cyclinD1 expression in the Tca8113 cells was significantly downregulated, while $p21^{cip1}$ and $p27^{kip1}$ expressions were significantly increased and phosphorylation of AKT/FOXO proteins was obviously inhibited (Figure 4).

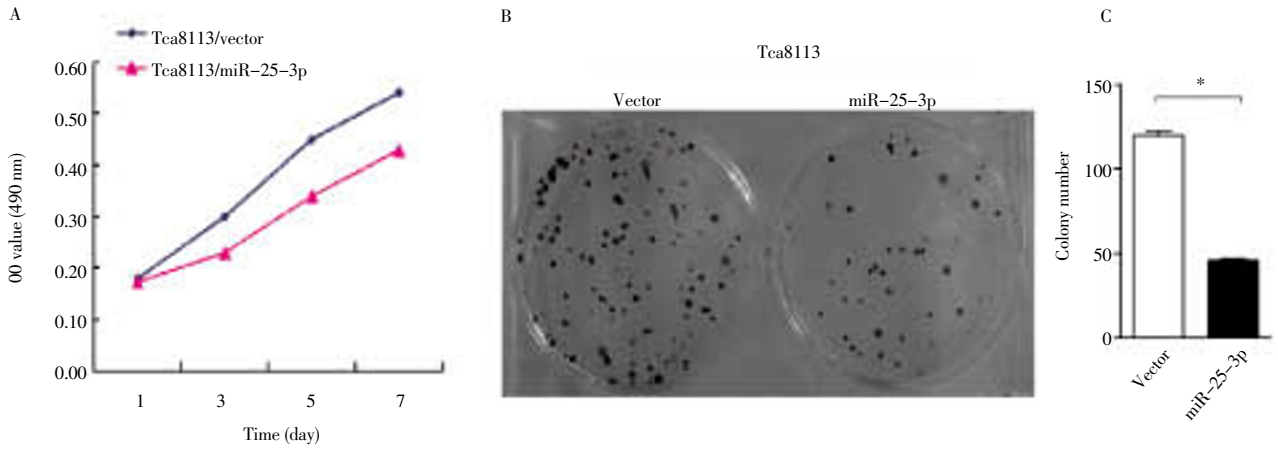

Figure 3. Detection of Tca8113 cell proliferation after miR-25-3p overexpression by MTT assay (A) and colony formation assay (B).

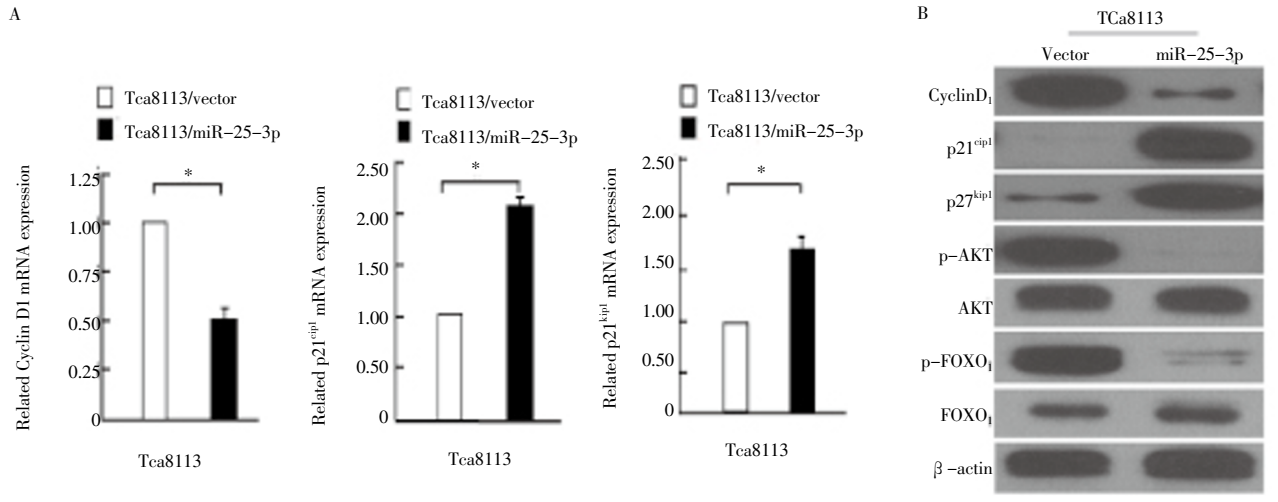

Figure 4. Detection of cell cycle related mRNA expression in the Tca8113 cells after miR-25-3p overexpression by quantitative PCR (A) and western blot analysis (B).

4. Discussion

Cell proliferation is a complex process, and the primary factors controlling this process are the growth factors from the cell environment and intracellular signal transduction. Under normal circumstance, cellular signal transduction is strictly controlled. During abnormal regulation of signal transduction pathways, abnormal cellular proliferation will occur and finally lead to malignant transformation[15]. Previous studies have demonstrated that cancer cell proliferation is related to many signaling pathways, including PI3K/AKT, EGFR, MAPK, NF-κB, Wnt/β-Catenin and mTOR[16,17]. It has been clearly confirmed that PI3K/AKT signal plays a critical role in the regulation of cell proliferation. The FoxO family of forkhead transcription factors, FoxO1, FoxO3a, and FoxO4, are the primary target proteins of AKT kinase, can be transported from the nucleus to the cytoplasm and plays an important role in cellular proliferation, apoptosis, differentiation and anti-oxidation[18]. AKT-phosphorylated FoxO1/3a can be ubiquitinated in the cytoplasm and then degraded by proteasomes, leading to decreases in protein level and transcription activity[19,20]. The activated AKT can phosphorylate its downstream transcription factors, which leads to a relocalization of FOXO protein from the nucleus to the cytoplasm and loss of transcription activity.

FOXO1 can directly upregulate $p21^{cip1}$ and $p27^{kip1}$ expressions or downregulate cyclinD1 expression, playing an inhibitory effect on cellular proliferation[21-23]. Inactivated AKT kinase downregulates cyclinD1 expression and upregulates $p21^{cip1}$ and $p27^{kip1}$ expressions, which inactivate FOXO1 phosphorylation, inhibit cell cycle conversion and attenuate the rapid proliferation of cancer cells.

miRNAs are widely distributed non-coding small RNAs that negatively regulate target mRNAs. They regulate many biological signaling pathways. miRNAs widely exists in the eukaryotes and are evolutionarily conserved and tissue-specific. The wide existence and evolutionary conservation of miRNAs indicate that miRNAs are indispensable for vital movement and play a critical role in the regulation of gene expression, growth and development of organism and

behaviors. There is recent evidence that aberrant expression of miRNAs is correlated with specific tumors and some miRNAs serve as anti-oncogenes[24,25]. Results from this study showed that miR-25-3p expression in the tongue squamous cell carcinoma cell line and tissue specimen was significantly lower than that in the normal tongue epithelial cells. Using recombinant retroviral vector-mediated gene transfer method, this study successfully established tongue squamous cell carcinoma cell models Tca8113/miR-25-3p that stably express miR-25-3p.

Real time fluorescent quantitative PCR results showed that high miR-25-3p expression persisted in the tongue squamous cell carcinoma cell line. The use of tongue squamous cell carcinoma cell models in MTT assay, cell colony formation assay and detection of expressions of cell proliferation-related markers provides more repeatable results. During routine culture, no morphological changes in Tca8113/miR-25-3p cells were observed. After miR-25-3p overexpression, cell proliferation and colony-forming ability of Tca8113 cells were obviously inhibited. Western blot analysis and quantitative PCR results showed that cyclinD1 expression was lower in Tca8113/miR-25-3p cells, while $p21^{cip1}$ and $p27^{kip1}$ mRNA and protein expressions in the transfected Tca8113 cells were obviously increased. However, western blot results showed that AKT, FOXO protein phosphorylation was obviously inhibited. This suggests that upregulated miR-25-3p expression inhibited the proliferation of tongue squamous cell carcinoma cell line Tca8113 cells by inhibition of AKT/FOXO signaling pathway. Taken together, upregulated miR-25-3p expression inhibited the proliferation of tongue squamous cell carcinoma cell line Tca8113 cells by inhibiting AKT/FOXO signaling pathway and regulating cell cycle-related proteins. This finding suggests that miR-25-3p functions as an oncogene and plays an important role in the occurrence and development of tongue squamous cell carcinoma. MiR-25-3p may be a promising biomarker and new target for predicting lymph node metastasis and prognosis in squamous cell carcinoma of the tongue.

Conflict of interest statement

We declare that we have no conflict of interest.

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