Suppression of miR-886-3p mediated by arecoline (ARE) contributes to the progression of oral squamous cell carcinoma

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ABSTRACT

Previous studies have reported that as the main extract of areca nut, arecoline (ARE) causes DNA damage and in turn contributes to the carcinogenesis of oral epithelial cells. It has been reported that ARE can inhibit the expression of miR-886-3p. In the current study, we aimed to explore the expression and biological functions of miR-886-3p in oral squamous cell carcinoma (OSCC). Herein, we demonstrated that in OSCC cells treated with ARE, the expression level of miR-886-3p was negatively correlated with the concentration of ARE. Compared with adjacent tissue, the expression level of miR-886-3p in OSCC tissue was remarkably downregulated. Transfection of miR-886-3p mimics markedly decreased viability, migration and invasion of OSCC cells. These experimental data implied that miR-886-3p suppression mediated by ARE took part in the proliferation and metastasis of OSCC. This study can help elucidate the mechanism by which areca nut chewing contributes to the malignant transformation of oral epithelial cells.

Significance of this study

What is already known about this subject?

- Arecoline (ARE) can cause DNA damage, which in turn participates in the tumorigenesis of oral squamous cell carcinoma (OSCC).
- miR-886-3p was reported to inhibit the proliferation and migration of cancer cells in several types of tumors.
- ARE could inhibit the expression of miR-886-3p in oral mucosal fibroblasts.

What are the new findings?

- ARE treatment reduced the expression of miR-886-3p in OSCC cell lines in a time-dependent and dose-dependent manner.
- miR-886-3p expression was significantly downregulated in OSCC tissues and cell lines.
- miR-886-3p suppression mediated by ARE took part in the proliferation, migration and invasion of OSCC cells.

How might these results change the focus of research or clinical practice?

- This study helps elucidate the mechanism by which areca nut chewing contributes to the malignant transformation of oral epithelial cells, providing clues for the prevention and treatment of OSCC.

INTRODUCTION

Oral carcinoma is a general term for multiple types of malignant tumors in the oral cavity, of which approximately 90% is oral squamous cell carcinoma (OSCC).[1] It is reported that about 275 000 new cases of OSCC are diagnosed every year worldwide.[2] Despite significant improvements have been achieved in OSCC treatment (such as radiotherapy, chemotherapy, and surgical resection), the 5-year overall survival rate of patients with OSCC is less than 70% and their life quality remains poor.[3] Therefore, it is extremely necessary to clarify the underlying mechanism of OSCC tumorigenesis and progression, so as to find the potential therapeutic strategies of OSCC.

Areca nut chewing is a common habit among people from East and Southeast Asia, UK, USA and South Africa.[4] The international agency for research on cancer has declared areca nut as a psychoactive addictive substance and it is among the group 1 of human carcinogens.[5 6] What is worse, areca nut has been considered to be a major risk factor for oral precancerous lesions.[7] Arecoline (ARE), as the major alkaloid in areca nut, is highly cytotoxic, genotoxic and mutagenic and it leads to histological changes and other biological consequences.[8 9] However, the exact mechanism of ARE in OSCC progression is not fully understood.

MicroRNAs (miRNAs, miRs), as a class of short and endogenous single-stranded RNA molecules with a length of ~22 nucleotides, can bind with 3' UTR of the target mRNAs so as to regulate gene expressions at post-transcriptional level.[10] MiRNAs have various biological functions in cancer progression, including regulating cell differentiation, proliferation, apoptosis, migration and so on.[11] In addition, many studies have proved that miRNAs are related to tumorigenesis of OSCC. For example, miR-155

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is reported to regulate the proliferation, cell cycle progression and apoptosis of OSCC cells by targeting p27Kip1.[12] What is more, miR-133-3p suppresses the proliferation and invasion of OSCC cells through inhibiting COL1A1.[13] Besides, in the familial non-medullary thyroid carcinoma, miR-886-3p inhibits cancer cell proliferation and migration as a tumor suppressor.[14] A recent study reveals that ARE induces the downregulation of miR-886-3p in oral mucosal fibroblasts.[15] However, there was little evidence about the association between miR-886-3p and OSCC tumorigenesis and progression.

In this study, we hypothesized that ARE could participate in the development and progression of OSCC by reducing the expression level of miR-886-3p. To validate this scientific hypothesis, we explored the effect of ARE with different concentrations and treatment time on the expression level of miR-886-3p in OSCC cells. Besides, we explored the biological functions of miR-886-3p in OSCC cells. Our work helps elucidate the mechanism by which areca nut chewing contributes to the malignant transformation of oral epithelial cells.

MATERIALS AND METHODS

Tissue samples

A total of 54 paired OSCC and adjacent normal oral epithelial tissue samples were collected during surgery, from patients during the period of 2012–2017 in Affiliated Hospital of Chengde Medical University. No patients received targeted therapeutic drugs, radiotherapy or chemotherapy before surgery. All the participants in this study agreed to be enrolled and they signed the informed consent. Tumor tissues and paired normal tissues were collected and preserved at −80°C. The collection and use of human tissue samples were performed under the instruction of the principles of the Declaration of Helsinki.

Cell culture

OSCC cell lines (including CAL-27, TCA-8113, SCC-4, SCC-9 and SCC-15) and normal oral epithelial cell line (HIOEC) were purchased from Cell Bank, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). OSCC cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Carlsbad, California, USA) supplemented 10% (v/v) fetal bovine serum (FBS) (Gibco, Carlsbad, California, USA). HIOEC cells were maintained in defined keratinocyte serum-free medium (Gibco, Carlsbad, California, USA). These cells were placed in an incubator in 5% CO_2 at 37 °C. The cells were then treated with a series of concentrations of ARE (Selleck, Shanghai, China) (0, 100, 200 µg/mL) for different treatment time (0, 3, 6 and 9 hours), respectively.

Quantitative real-time polymerase chain reaction (qRT-PCR)

RNAiso Plus reagent (Takara, Dalian, China) was used to extract the total RNA from cancer tissues/cells. To measure miR-886-3p expression, the total RNA was reverse-transcribed using Moloney murine leukemia virus (MMLV) reverse transcriptase (Takara, Dalian, China). The obtained cDNA was then used as the template for qRT-PCR, and qRT-PCR was performed with SYBR Green Master Mix (Takara, Dalian, China). U6 was used as the endogenous control for miR-886-3p. The cycle threshold (Ct) value was employed to calculate the relative expression of miR-886-3p (formula=$2^{-\triangle\triangle Ct}$). The detailed information of the primer sequences was shown in table 1.

Table 1 qRT-PCR primer sequence (5′–3′)

Name	Primer sequences
miR-886-3p	Forward: 5'-CACGCGGGTGCTTACTGAC-3'
	Reverse: 5'-GTGCAGGGTCCGAGGT-3'
U6	Forward: 5'-CTCGCTTCGGCAGCACA-3'
	Reverse: 5'-ACGCTTCACGAATTTGCGT-3'

Cell transfection

The miRNA precursor (pre-miR-886-3p, Applied Biosystems, USA) was transfected into cells by Lipofectamine RNAiMAX (Invitrogen, Carlsbad, California, USA) according to the manufacturer's protocol. The negative control (miR-NC) was the random sequence of pre-miR (pre-miR-Negative Control, Applied Biosystems, USA).

Cell counting kit-8 (CCK-8) assay

The OSCC cells in logarithmic growth phase were harvested and inoculated in a 96-well plate (1×10^3 cells / well). One hundred microliters of complete medium containing 10 µL of CCK-8 reagent (Dojindo, Kumamoto, Japan) was then added to replace the original medium at different time points (12 hours, 24 hours, 48 hours, 72 hours, 96 hours, respectively, after cell inoculation). Next, the cells were cultured at 37°C for 1 hour. Next, the absorbance of the cells in each well was determined by the Vmax Microplate Spectrophotometer (Molecular Devices, Sunnyvale, USA) with a wavelength of 450 nm. Ultimately, the cell proliferation curves were plotted based on the absorbance values.

Colony formation assay

After transfection of mimics NC or miR-886-3p mimics, CAL-27 and SCC-4 cells were inoculated into 6-well plates (1000 cells/well) and cultured for 2 weeks. Cells with the ability to survive and proliferate would form a colony. The medium was then discarded and the cells were fixed with 10% formaldehyde. These cells were then stained with crystal violet solution (0.1%, w/w) for 15 min. Next, the colonies were gently rinsed with running water. Finally, the number of colonies containing at least 10 cells was counted with naked eyes.

Transwell assay

Cells were harvested and resuspended with serum-free medium. Then, the cells (10^5 cells/well) were inoculated into the upper chamber of the Transwell system (pore size: 8 µm, BD Biosciences, Franklin Lakes, New Jersey, USA). The lower chamber was added with 500 µL of DMEM containing 10% (v/v) FBS. Twenty-four hours after incubation, the cells on the upper surface of the membrane were gently wiped off using a cotton swab, whereas the migrated cells which passed through the membrane were fixed by methanol and stained with crystal violet solution. With an inverted microscope, the numbers of migrated cells were

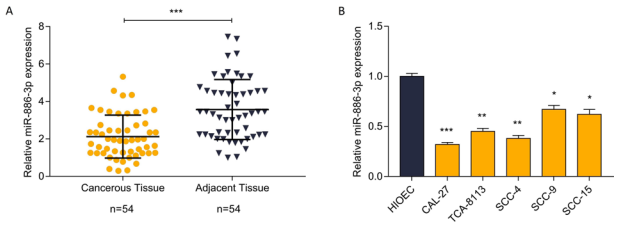

Figure 1 The relative miR-886-3p expression was detected by qRT-PCR in (A) OSCC tissues and adjacent normal tissues; (B) the HIOEC and OSCC cell lines (CAL-27, TCA-8113, SCC-4, SCC-9 and SCC-15). *P<0.05; **p<0.01; ***p<0.001. HIOEC, normal oral epithelial cell line; OSCC, oral squamous cell carcinoma.

counted in five randomly selected fields of view (including the center and periphery of the membrane). As for the invasion assay, the experiment was conducted with Transwell chamber whose membranes were coated with a layer of Matrigel (Sigma-Aldrich, St Louis, Missouri, USA), and the other steps were the same as those of the migration assay.

Statistical analysis

SPSS software (V.21.0, SPSS, Chicago, Illinois, USA) was applied to carry out all statistical analyses. The data in this study (represented by mean±SD) followed a normal distribution. Student's *t*-test or one-way analysis of variance was adopted to compare the differences between/among the groups. It was considered to be statistically significant if the p value was less than 0.05.

RESULTS

miR-886-3p was downregulated in OSCC

To verify whether miR-886-3p was abnormally expressed in OSCC tissues and cell lines, the expression of miR-886-3p was detected in 54 pairs of OSCC tissues and corresponding adjacent normal tissues. As shown (figure 1A), compared with the adjacent normal tissues, the expression of miR-886-3p in cancerous tissue was dramatically downregulated (p<0.001). Besides, the relative expression level of miR-886-3p in OSCC cell lines (including CAL-27, TCA-8113, SCC-4, SCC-9 and SCC-15) was significantly lower than that of the oral epithelial line (HIOEC) (p<0.05) (figure 1B).

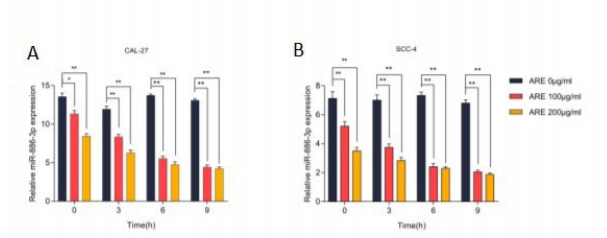

Figure 2 The relative miR-886-3p expression was downregulated in CAL-27 and SCC-4 cells with ARE treatment. qRT-PCR was applied to detect the relative expression of miR-886-3p at different time points (0, 3, 6, 9 hours) with different doses of ARE (0, 100, 200 μg/mL) treatment in CAL-27 cells (A) and SCC-4 cells (B). *P<0.05; **p<0.01. ARE, arecoline.

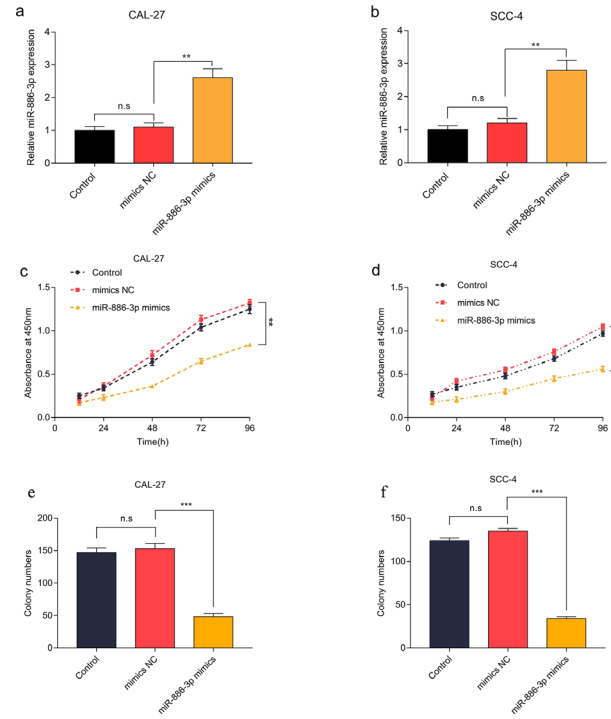

Figure 3 miR-886-3p overexpression inhibited the proliferation of OSCC cell lines. (A,B) qRT-PCR was applied to detect the relative expression of miR-886-3p after CAL-27 and SCC-4 cells were transfected with mimics NC or miR-886-3p mimics. (C,D) CCK-8 assay was used to measure the proliferation of OSCC cell lines (CAL-27 and SCC-4). (E,F) Colony formation assay was performed to detect the colony formation ability of OSCC cell lines (CAL-27 and SCC-4). **P<0.01; ***p<0.001. OSCC, oral squamous cell carcinoma.

ARE treatment downregulated the expression of miR-886-3p

Next, different concentrations of ARE were used to treat CAL-27 and SCC-4 cell lines. Subsequently, qRT-PCR was used to detect the expression level of miR-886-3p in OSCC cells treated with different concentrations of ARE (0, 100 and 200 μg/mL) for different treatment time (0, 3, 6 and 9 hours). The results showed that the relative expression of miR-886-3p in both CAL-27 and SCC-4 cell lines was reduced by ARE treatment in a time-dependent and dose-dependent manner (p<0.05) (figure 2A,B).

Overexpression of miR-886-3p inhibited the proliferation of OSCC cells

The transfection efficiency of miR-NC and miR-mimics was validated by qRT-PCR. In both CAL-27 and SCC-4 cell lines, compared with miR-NC group, miR-886-3p was upregulated in miR-886-3p mimics group (p<0.01) (figure 3A,B). CCK-8 assay was performed to verify the inhibitory effect of miR-886-3p on the proliferation of OSCC cells. As shown, the results from CCK-8 assay indicated that the proliferation of both CAL-27 and SCC-4 cells was significantly suppressed by miR-886-3p overexpression (p<0.01) (figure 3C,D). Moreover, in colony formation assay, the colony numbers in the miR-886-3p overexpression group were remarkably lower than that of miR-NC

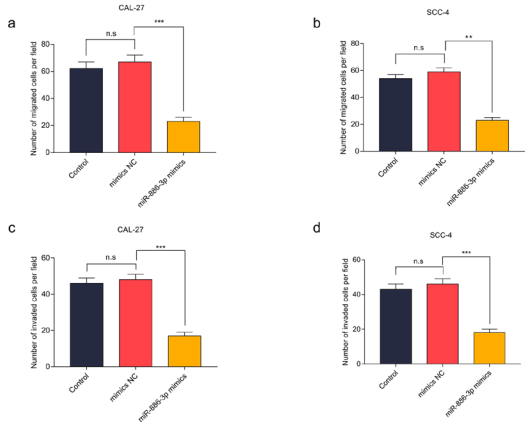

Figure 4 miR-886-3p overexpression suppressed the migration and invasion of OSCC cell lines. Transwell assay was applied to assess the numbers of migrated cells (A,B) and invaded cells (C,D). **P<0.01; ***p<0.001. OSCC, oral squamous cell carcinoma.

group (p<0.001) (figure 3E,F). Collectively, we concluded that the overexpression of miR-886-3p inhibited the proliferation of OSCC cells.

Overexpression of miR-886-3p suppressed the migration and invasion of OSCC cells

Subsequently, we used Transwell assay to examine the migration and invasion of OSCC cells. As shown, overexpression of miR-886-3p suppressed the migration of both CAL-27 and SCC-4 cells (p<0.01) (figure 4A,B). The results of Transwell assay also showed that the invasion of OSCC cells in the miR-886-3p overexpression group was significantly repressed compared with that of miR-NC group (p<0.001) (figure 4C,D).

DISCUSSION

The newly diagnosed OSCC cases continue to rise in developing countries.[16] Due to its low survival rate, more attention should be paid on the potential new therapies for OSCC. MiRNA-based gene therapy is a promising strategy to treat cancers.[17] In the present work, we investigated the expression characteristics, regulatory mechanism and biological functions of miR-886-3p in OSCC.

Accumulating studies suggest that miRNAs play an essential role in tumor progression. For example, it has been reported that miR-628–5 p represses the malignant biological behaviors of ovarian cancer cells through targeting FGFR2.[18] In addition, miR-125a suppresses the proliferation, migration and invasion of cervical cancer cells by targeting STAT3.[19] What is more, miR-124 can directly target SOX9 to suppress the proliferation, migration and invasion of lung adenocarcinoma cells.[20] Besides, some recent studies indicate that miR-886-3p is abnormally expressed in several types of cancers, and functions as a tumor suppressor. For instance, miR-886-3p is downregulated in familial non-medullary thyroid carcinoma, and its defect promotes cancer cell proliferation and migration.[14] Additionally, miR-886-3p is proved to inhibit the malignant phenotypes of small cell lung cancer cells, and its low expression is related to the poor prognosis of patients.[21] Moreover, miR-886-3p expression level is reported to be associated with the sensitivity of cancer cells to chemotherapy in advanced bladder cancer.[22] In this work, qRT-PCR was adopted to investigate the expression pattern of miR-886-3p in OSCC. The data implied that miR-886-3p were dramatically downregulated in OSCC tissues and cell lines. Furthermore, our data showed that the upregulation of miR-886-3p suppressed the proliferation, migration and invasion of OSCC cells. Taken together, for the first time, our findings implied that miR-886-3p acted as a tumor suppressor in OSCC.

The genotoxicity and mutagenicity of ARE have been confirmed by a number of previous studies. ARE was reported to be cytotoxic on a variety of cells including oral fibroblasts and keratinocytes, that is, affecting cell proliferation, disrupting cycle progression and inducing programmed cell death.[23–25] Importantly, ARE is known to induce oral carcinogenesis. Specifically, ARE induces IL-6, STAT3, c-Myc and oncostatin M expressions in OSCC cells.[26] Nonetheless, the mechanism by which ARE induces the tumorigenesis of OSCC is still needed to elucidate. In this work, we measured the changes in the expression of miR-886-3p in OSCC cells after ARE exposure. The results indicated the expression of miR-886-3p in OSCC cells was significantly downregulated after treatment with ARE. Our demonstrations suggested that, the downregulation of miR-886-3p induced by ARE exposure, could probably be an important mechanism of oral mucosa epithelial cells' malignant transformation.

It is worth noting that there are several limitations in this study. First of all, our data are mainly from in vitro experiments, and in vivo models are needed to further verify the inhibitory function of ARE on miR-886-3p expression in oral epithelial cell, and the tumor-suppressive properties of miR-886-3p in OSCC. Second, the mechanism by which miR-886-3p represses the malignant phenotypes of OSCC cells is still obscure, and the downstream targets and pathways of miR-886-3p in OSCC remain to be clarified. Last but not least, the clinical significance of miR-886-3p in OSCC has not been explored, and in the following work, it is necessary to evaluate the potential of miR-886-3p as prognostic biomarker for OSCC.

In conclusion, the current study demonstrates that the downregulation of miR-886-3p mediated by ARE exposure contributes to the proliferation and metastasis of OSCC cells. These data can improve the understanding of the pathogenesis of OSCC. Additionally, our work may also be beneficial to the development of potential therapeutic strategies for OSCC.

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Contributors YW, RL, ZS and FH designed this research. FM and YW carried out the experiments. RL, XW and SH analyzed the data. YZ, YW and RL drew the figures and drafted this manuscript. ZS helped check the manuscript and figures. YZ and FH performed the revision. All authors read and approved the final manuscript.

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Patient consent for publication Not required.

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