RETRACTED ARTICLE: MiR-29a inhibits the progression of oral squamous cell carcinoma by targeting Wnt/β-catenin signalling pathway

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ABSTRACT

Aim: The study aimed to investigate the role of miR-29a in the progression of oral squamous cell carcinoma (OSCC), as well as its molecular mechanism.

Methods: Tissue samples were collected from 103 OSCC patients. Quantitative real-time polymerase chain reaction (qRT-PCR) was used to detect the relative expression of miR-29a in OSCC tissues and cell line. Cell proliferation, motility and apoptosis were detected using MTT, transwell and flow cytometry methods, respectively. Western blot was used to measure the protein expression.

Results: The expression of miR-29a was decreased in OSCC tissue and cells (p < .05 for both), and its down-regulation was negatively associated with the lymph node metastasis (p = .017) and TNM stage (p = .027). Enforcing miR-29a expression in OSCC cells using mimic transfection could significantly inhibit the proliferation, migration and invasion, and promote cells apoptosis. Furthermore, miR-29a over-expression could suppress Wnt/β-catenin pathway activity. Meanwhile, LiCl, the activator of Wnt/β-catenin pathway, could reverse the anti-tumour action induced by miR-29a over-expression.

Conclusions: MiR-29a may inhibit the malignant progression of OSCC by suppressing Wnt/β-catenin signalling pathway.

ARTICLE HISTORY

Received 30 November 2018
Accepted 16 January 2019

KEYWORDS

MiR-29a; Wnt/β-catenin signalling; oral squamous cell carcinoma; OSCC

Introduction

Oral squamous cell carcinoma (OSCC) is a malignancy arising from the squamous epithelium of oral cavity [1]. OSCC is the most frequently diagnosed head and neck cancer, posing a serious threat to human healthy worldwide [2]. Despite of the great advances in treatments, including surgery, chemotherapy and radiotherapy, the prognosis of the OSCC patients with advanced stages is still unsatisfactory [3,4]. It has been reported that the five-year survival rate of OSCC patients at early stages is up to 80%, while those with late stages have a five-year survival only 20–40% [5]. Although a variety of risk factors have been confirmed for OSCC, such as smoking, alcohol abuse, poor oral hygiene, etc. [6], its pathogenesis still remains unclear. In order to improve the management of OSCC, it is key to explore the molecular alterations in progression of the cancer.

MicroRNAs (miRNAs) are a group of endogenous RNAs without protein coding ability, and their length is about 17–25 nucleotides [7]. MiRNAs play regulatory roles in gene expression through binding to the 3’ untranslated regions (UTRs) of their target mRNAs [8]. Given their roles in gene expression, miRNAs are involved in various biological processes, such as development, differentiation, inflammatory, as well as tumourigenesis [9]. In human cancers, miRNA as oncogenes or tumour suppressors take part in tumour progression and development that can be used for anti-tumour treatments [10]. MicroRNA-29a (miR-29a) is a common member of miRNA family, and its dysregulation has been observed in several types of cancer, such as lung cancer [11], breast cancer [12], papillary thyroid carcinoma [13], prostate cancer [14], etc. MiR-29a may serve as a tumour suppressor via targeting multiple cellular pathways. In OSCC, it has been reported that miR-29a might play anti-tumour action through inhibiting cell invasion [15]. However, the molecular mechanisms underlying the functional roles of miR-29a in OSCC progression were poorly known.

In this study, we investigated the expression of miR-29a in OSCC tissues and cell line, as well as its association with clinical characteristics of the patients. In addition, cell experiments were carried out to explore the molecular mechanisms of miR-29a in progression of OSCC.

Materials and methods

Patients and tissue specimens

A total of 103 OSCC patients were recruited from The First Affiliated Hospital of Jinzhou Medical University during
October 2016 and February 2018. Diagnosis of OSCC was made by pathological examinations. None of the patients had received any pre-operative treatments, such as chemotherapy, radiotherapy or drug treatments. The cancer tissues and adjacent normal tissues were collected from the patients, and stored in liquid nitrogen immediately. Then, the tissue samples were stored at −80°C for further analysis. The clinical information of the patients was collected from the original medical records. The present study was approved by the Ethic Committee of the hospital. The written informed contents were collected from all the patients.

**Cell lines and cell culture**

Human OSCC cell line SCC-25 (ATCC® CRL-1628™) and normal oral mucosal epithelial cells HOK (human oral keratinocytes, HOKs) (ATCC® PCS-200–014™) were purchased from American type culture collection (ATCC, Manassas, VA). SCC-25 cells were cultured in a 1:1 mixture of DMEM/F-12 medium (Invitrogen, Carlsbad, CA) supplemented with 10% foetal bovine serum (FBS) and 0.4 μg/ml hydrocortisone. The HOK cells severity as normal control was cultured in oral keratinocyte medium (OKM) containing 500 ml basal medium, 5 ml oral keratinocyte growth supplement. Then, the cultures were incubated at 37°C in a humidified atmosphere with 5% CO₂.

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

The relative expression of mir-29a in OSCC tissues and cell lines were estimated using qRT-PCR method. Total RNA was isolated from OSCC tissue and cell lines using TRizol reagent (Invitrogen, Carlsbad, CA), and the experiment procedures were carried out according to the manufacturer’s instructions. The quality of the obtained RNA samples was estimated using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE). The RNA samples with OD260/280 ratio of 1.8–2.0 were used for the subsequence analyses. The first-strand cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Then qRT-PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) in the 7900 Real-time PCR System (Applied Biosystems, Foster City, CA). RNU6B (U6) was employed as the internal control, and the specific primer sequences were as follows: U6 forward: 5'-CTCGCTTCGGCAGCACATATAC-3' reverse: 5'-TCTGAAA-3'; mir-29a forward: 5'-UUCUGAAGUGUGCAUUUGCC-3'; reverse: 5'-UCAGUGAGGUCCGUAGATT-3'. The relative expression of mir-29a was calculated using 2^ΔΔct method. Each test was repeated three times.

**Cell transfection**

The mir-29a mimic and negative control (NC) were synthesized by GenePharma (Shanghai, China). The plasmids were transfected to the cells at log phase using LipofectamineTM 2000™. The transfection effects were estimated at 48 h after transfection using qRT-PCR method.

**Cell proliferation assay**

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to evaluate cell proliferation. Forty eight hours after transfection, the cells were seeded to 96-well plate at the concentration of 5 × 10⁴ cells/well. The cells were cultured at 37°C with 5% CO₂. After cultured for 0 h, 24 h, 48 h and 72 h, 20 μl MTT (5 mg/ml; Sigma, St. Louis, MO) was added, and cultured for an additional 4 h. Then, the medium was removed, and 150 μl dimethyl sulfoxide (Sigma, St. Louis, MO) was added to dissolve the purple formazan. Fifteen minutes later, the absorbance at 490 nm was read with a Microplate Reader (TECAN, Salzburg, Austria).

**Transwell assay**

Transwell chambers coated without or with Matrigel (Corning Glass Works, Corning, NY) on the upper chamber were used for cell migration and invasion analyses, respectively. The upper chamber of transwell (Corning Glass Works, Corning, NY) contained 200 μl serum-free medium, while the medium with 10% FBS was added at the bottom chamber. The transfected cells were seeded to the upper chamber, with the density of 4 × 10⁴ cells, and incubated for 48 h. Then, the numbers of cells migrated or invaded towards the bottom chamber were determined by crystal violet staining and quantified using an inverted microscope (Leica, Malvern, PA) in 10 random fields.

**Apoptosis analysis**

Cell apoptosis analysis was performed using flow cytometry with Annexin V/PI double staining method. Forty eight hours after transfection, the cells were harvested and adjusted to a density of 1 × 10⁶/ml. Then, the cells were double-stained using AnnexinV-FITC/PI Apoptosis Detection Kit (Beyotime, Shanghai, China) according to the manufacturer’s instructions. The rate of apoptotic cells was assessed by flow cytometry (BD Biosciences, San Jose, CA).

**Western blot analysis**

The proteins were isolated from the cell samples using pre-cold RIPA buffer (Beyotime, Shanghai, China) with protease inhibitor, and quantified using BCA protein assay kit (Thermo Fisher Scientific, San Jose, CA). The equal protein was separated through 10% SDS-PAGE gel, and transferred into the polyvinylidene fluoride membrane (PVDF) (Millipore, Billerica, MA) at 48 V for 3.5 h. Then the membranes were blocked by 5% bovine serum albumin (BSA) for 2 h at room temperature, and washed using 1× Tris-buffered saline with Tween 20 (TBST). After then, the membranes were probed by the primary antibodies namely, mouse anti-human β-actin (1: 1000), C-myc (1:1000), Bcl-2 (1:1000), MMP-9 (1:1000) and CyclinDI (1:1000) (Cell Signaling Technologies, Beverly, MA). The membranes were washed by 1× TBST. Next, the membranes were incubated by the secondary goat anti-rabbit antibody (1: 4000; Cell Signaling Technologies, Beverly, MA), and
visualized under UV transilluminator (Uvitec Ltd., Avebury House, Cambridge, UK). GAPDH served as an internal control to calculate the relative expression of the targeted proteins.

**Statistical analyses**

The continuous variables were shown as mean±SD, and their comparisons between two groups were performed using Student’s t test. The association of miR-29a with clinical characteristics of OSCC patients was estimated using Chi-square test. All the analyses were performed using SPSS 18.0 software (SPSS Inc., Chicago, IL), and figures were plotted using GraphPad Prism version 5.0 (GraphPad, San Diego, CA). p Values less than .05 were considered as statistically significant.

**Results**

**The demographic and clinical characteristics of the study population**

In our study, tissue samples were collected from 103 OSCC patients, including 60 men and 43 women. The average age of the patients was 55.78±10.57 years. Metastasis was observed in 43 patients, accounting for 47.75%. According to the TNM stage, 68 patients were at stages I and II, while the rest 35 patients were grouped to stages III and IV. The baseline characteristics of the patients are summarized in Table 1.

The expression of miR-29a in OSCC tissues and cell line

The relative expression levels of miR-29a were detected in OSCC tissues and cell line using qRT-PCR method. The results demonstrated that miR-29a expression exhibited decreased trend in OSCC tissues and cell line, compared to the non-cancerous tissues and cell (**p < .01, ***p < .001, Figure 1).**

Association of miR-29a with clinical characteristics of OSCC patients

According to the mean expression levels of miR-29a in OSCC tissues, the included patients were divided into high expression group (n = 55) and low expression group (n = 48). Chi-square was performed to estimate the association of miR-29a expression with clinical parameters of OSCC patients. Analysis results suggested that miR-29a was negatively correlated with lymph node metastasis (p = .017) and TNM stage (p = .007). Meanwhile, the expression of miR-29a did not show significant association with age, gender, drinking, smoking or tumour size (p > .05 for all) (Table 1).

Effects of miR-29a expression on biological behaviours of OSCC cells

In order to investigate the effects of miR-29a expression on biological behaviours of OSCC cells, SCC-25 cells were transfected by miR-29a mimic vector. QRT-PCR detection indicated that the expression of miR-29a was significantly increased after transfection (**p < .001, Figure 2).

MTT method was used to investigate the influences of miR-29a expression on cell proliferation in OSCC. The enforced expression of miR-29a could significantly inhibit cell proliferation in vitro (*p < .05, **p < .01, Figure 3(A)). Transwell assay suggested that the up-regulation of miR-29a could suppress cell migration and invasion (**p < .01, Figure 3(B,C)). Additionally, the apoptotic rate of the transfected cells was obviously higher than the cells transfected by NC (***p < .01, Figure 3(D)). All the evidence might reveal that miR-29a might hold the capacity to suppress the malignant behaviours of OSCC cells in vitro.

**miR-29a could suppress the activity of Wnt/β-catenin signalling pathway in OSCC**

In order to investigate the molecular mechanisms of miR-29a in OSCC progression, Western blot assay was performed to investigate the expression of Wnt/β-catenin signalling pathway-related proteins in the SCC-25 cells transfected by miR-29a mimic. Compared to NC, miR-29a mimic transfection significantly inhibit the expression of β-catenin, C-myc, Bcl-2, MMP-9 and CyclinD1 (*p < .05, Figure 4).

**MiR-29a could suppress aggressive behaviours of OSCC cell through inhibiting Wnt/β-catenin signalling pathway**

To explore the molecular mechanisms underlying the anti-tumour action of miR-29a in OSCC, LiCl, an activator of Wnt/β-catenin signalling pathway was used in current study. Forty eight hours after transfection, the cells transfected by miR-29a mimic were treated by 20 mM LiCl for 24 h. The expression of the proteins in Wnt/β-catenin signalling pathway was detected using Western blot analysis. The results confirmed that the expression of β-catenin, C-myc, Bcl-2, MMP-9 and

**Table 1. The association of miR-29a mRNA expression with clinical parameters of OSCC patients.**

| Characteristics | N (n = 103) | miR-29a low expression (n = 48) | miR-29a high expression (n = 55) | p Values |
|----------------|------------|---------------------------------|---------------------------------|----------|
| Age (years)   |            |                                 |                                 |          |
| ≥60           | 52         | 26                              | 26                              | .485     |
| <60           | 51         | 22                              | 29                              |          |
| Gender        |            |                                 |                                 |          |
| Male          | 60         | 24                              | 36                              | .113     |
| Female        | 43         | 24                              | 19                              |          |
| Drinking      |            |                                 |                                 |          |
| Yes           | 58         | 30                              | 28                              | .237     |
| No            | 45         | 18                              | 27                              |          |
| Smoking       |            |                                 |                                 | .988     |
| Yes           | 60         | 28                              | 32                              |          |
| No            | 43         | 20                              | 23                              |          |
| Tumour size (cm) |        |                                 |                                 | .535     |
| ≤3            | 57         | 25                              | 32                              |          |
| >3            | 46         | 23                              | 23                              |          |
| Lymph node metastasis | |                                 |                                 | .017     |
| Yes           | 43         | 26                              | 17                              |          |
| No            | 60         | 22                              | 38                              |          |
| TNM stage     |            |                                 |                                 | .007     |
| I–II          | 68         | 22                              | 46                              |          |
| III–IV        | 35         | 26                              | 19                              |          |
CyclinD1 proteins was significantly increased after LiCl treatment \((p < .05, **p < .01, ***p < .001, \text{Figure 5})\). The Wnt/β-catenin signalling pathway was activated in the transfected cells after LiCl treatment.

In addition, we also investigated the biological behaviours of the transfected cells after LiCl treatment. We found that the cell proliferation rate was obviously enhanced, and the numbers of migrated and invasive cells were increased. Furthermore, the rate of apoptotic cells was remarkably decreased \((p < .05, **p < .01, ***p < .001, \text{Figure 6})\). The activation of Wnt/β-catenin signalling pathway might reverse the anti-tumour effects caused by the up-regulation of miR-29a in OSCC.

**Discussion**

MiRNAs could regulate gene expression at post-transcriptional level, and their dysregulation may contribute to human diseases, like cancer \([16,17]\). Growing evidence has demonstrated that the expression patterns of miRNAs show close association with cancer development and progression, which could be employed as biomarkers for early detection and prognosis evaluation, as well as therapeutic targets \([18,19]\).

To explore, the molecular mechanisms of miRNAs in cancer progression may provide a new insight into cancer etiology. In the current study, we investigated the function of miR-29a in OSCC. We found that the down-regulation of miR-29a might contribute to malignant progression of OSCC through activating Wnt/β-catenin signalling pathway.

OSCC is a common type of head and neck cancer around the world. Despite various available treatments, the clinical outcomes of the OSCC patients are still dismal, due to the unclear mechanisms underlying the malignant tumour progression \([20,21]\). MiRNAs play diverse roles in multiple biological processes, which may provide new insight into the etiology of OSCC. In the current study, we found that the expression of miR-29a was significantly decreased in OSCC tissues and cell lines. Furthermore, the down-regulation of miR-29a was closely correlated with lymph node metastasis and advanced tumour stage. MiR-29a might play anti-tumour action in OSCC, and its expression loss predicted malignant tumour progression. The conclusions were consistent with the published articles. Lu et al. reported that the expression of miR-29a was lower in OSCC tissues than that in adjacent normal tissues \([15]\). The down-regulation of miR-29a might be a risk hallmark in pathogenesis of OSCC \([22]\).

Cell experiments were carried to investigate the function of miR-29a in etiology of OSCC. We found that the
restoration of miR-29a in OSCC cell line via mimic transfection could inhibit cell proliferation, migration and invasion, and promote cell apoptosis. MiR-29a might play tumour suppressive roles in OSCC through inhibiting malignant behaviours of the cancer cells. In addition, the activity of Wnt/β-catenin signalling pathway was significantly reduced after the transfection of miR-29a mimic in OSCC cell in vitro. The activation of Wnt/β-catenin pathway reversed the
anti-tumour action caused by enforced expression of miR-29a in OSCC. All the data revealed that miR-29a acted as a tumour suppressor in OSCC through inhibiting Wnt/β-catenin signalling pathway. It has been reported that miR-29a could regulate chemotherapy resistance through Wnt/β-catenin signalling pathway in pancreatic cancer [23]. Tan et al. suggested that miR-29a could enhance the demethylation of WIF-1, thus suppressing Wnt signalling pathway in non-small cell lung cancer [24]. However, the molecular mechanisms underlying the regulation of miR-29a on Wnt/β-catenin signalling pathway in OSCC had not been explored in current study. Further studies are required.

Wnt/β-catenin signalling pathway is a signal transduction pathway, which could pass signals into cell through cell surface receptors [24]. Dysregulation of wnt/β-catenin pathway may lead to human disease, like cancer. Accumulating evidence has demonstrated that miRNA dysregulation and the Wnt/β-catenin signalling pathway jointly contribute to carcinogenesis, including OSCC [25]. For examples, the study carried out by Qiao et al. indicated that miR-27a-3p could modulate Wnt/β-catenin signalling pathway to enhance epithelial-mesenchymal transition in OSCC [26]. Yu et al. reported that miR-9 could suppress cell proliferation of OSCC via inhibiting Wnt/β-catenin pathway [27]. In this study, we found that miR-29a could suppress malignant progression of OSCC through inhibiting Wnt/β-catenin signalling pathway. The interaction between miRNAs and Wnt/β-catenin signalling pathway takes part in etiology of OSCC, which may be employed as a therapeutic target for the malignant disease.

In conclusion, miR-29a is downregulated in OSCC tissues and cell line, and its decreased expression is significantly correlated with advanced tumour stage and metastasis. MiR-29a may play anti-tumour action in progression of OSCC through inhibiting Wnt/β-catenin signalling pathway. However, several limitations in the current study should be stated. First, the sample size was relatively small. Second, miR-29a might take part in pathogenesis of OSCC through multiple signalling pathways. The relationship between miR-29a and other pathways had not been explored in our study. Further investigations with a larger sample size will be performed to identify and improve our results.

Disclosure statement
No potential conflict of interest was reported by the authors.

Funding
The study was supported by the Natural Science Foundation of Liaoning [No. 2014022003].

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