European Review for Medical and Pharmacological Sciences 2020; 24: 4803-4809

MiR-92a inhibits proliferation and promotes apoptosis of OSCC cells through Wnt/β-catenin signaling pathway

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Abstract. – OBJECTIVE: The aim of this study was to investigate the role of micro ribonucleic acid (miR)-92a in the pathogenesis of oral squamous cell carcinoma (OSCC).

MATERIALS AND METHODS: The relative expression level of miR-92a in OSCC cell lines and normal oral epithelial keratinocyte cell lines was detected via quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR). Subsequently, the effects of miR-92a silencing on proliferation and apoptosis of OSCC cells were determined via cell counting kit-8 (CCK-8) assay, flow cytometry, and colony formation assay, respectively. Whether miR-92a could bind to the 3'-untranslated region (3'-UTR) of target mRNA was detected using Dual-Luciferase reporter gene assay. Furthermore, changes in Wnt/β-catenin pathway-associated proteins were explored via Western blotting.

RESULTS: The expression of miR-92a in OSCC cell lines was significantly higher than that of normal oral epithelial keratinocyte cell lines ($p<0.05$). Silencing of miR-92a significantly inhibited proliferation and promoted apoptosis of OSCC cells ($p<0.05$). Luciferase reporter gene assay confirmed that miR-92a could bind to the 3'-UTR of Kruppel-like factor 4 (KLF4) gene. After miR-92a silencing, the expressions of Wnt/β-catenin pathway-associated proteins were remarkably down-regulated, including β-catenin, c-Myc, and Wnt3a ($p<0.05$).

CONCLUSIONS: Silencing of miR-92a inactivates the Wnt/β-catenin signaling pathway by targeting KLF4, thereby inhibiting proliferation and promoting apoptosis of OSCC cells. Our findings suggest that miR-92a may be a potential therapeutic target for OSCC patients.

Key Words:
Oral squamous cell carcinoma (OSCC), MiR-92a, KLF4 gene, Wnt/β-catenin signaling pathway, Proliferation and apoptosis.

Introduction

Oral carcinoma originates from oral epithelial cells, which is one of the most common malignant tumors in the known head-neck cancers[1]. Pathologically, more than 90% of patients with oral carcinoma are diagnosed with oral squamous cell carcinoma (OSCC)[2]. According to the pathological type, disease stage, patient's physical condition, and other related factors, adjuvant radiotherapy or systemic chemotherapy are often preferred for the treatment of OSCC patients[3]. Despite constant improvement in treatment means, the 5-year survival rate of OSCC is still far from satisfactory, which is even 50% lower than that of mid- or late-stage disease[4]. Current studies have found that OSCC is prone to local onset with a high risk of recurrence. The poor prognosis of OSCC can be attributed to the combination of factors, including late diagnosis, radiation tolerance, and recurrence[5,6]. Therefore, it is urgent to understand the mechanism of OSCC development, so as to determine the potential targets for molecular therapy of OSCC. Micro ribonucleic acids (miRNAs) are abnormally expressed in human cancers. Meanwhile, they play key roles in the occurrence, development, and metastasis of human malignancies, including OSCC[7]. MiRNAs are a class of small endogenous, non-coding, and single-stranded RNAs with 18-24 nucleotides in length. They can negatively regulate gene expression at the post-transcriptional level through base pairing with the 3'-untranslated region (3'-UTR)[8]. The dysregulation of miRNAs plays a key role in a variety of physiological and biological processes, as well as many diseases, including cancer[9]. It is reported that some dysregulated miRNAs are associated with the development of OSCC. Soga et

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al[10] analyzed miRNA expression profiles of OSCC and normal oral mucosal tissues. Their findings have indicated the unique miRNA profile of OSCC tissues compared with normal tissues. Manikandan et al[11] have identified 46 differentially expressed miRNAs in OSCC. Similarly, Siow et al[12] have identified 15 up-regulated and 4 down-regulated miRNAs in OSCC compared with normal tissues. Li et al[13] studied the effects of miR-92a-3p on the proliferation, migration, and invasion of esophageal squamous cell carcinoma (ESCC). They have found that the expression of miR-92a-3p was significantly higher in ESCC tissues than that of adjacent normal tissues. In addition, the expression of miR-92a-3p in ESCC cell lines is remarkably higher than normal esophageal cells. MiR-92a-3p facilitates the proliferation, migration, and invasion of ESCC cells by regulating PTEN. However, the biological function of miR-92a in OSCC has not been fully elucidated. Therefore, the aim of this study was to explore the effects of miR-92a on proliferation and apoptosis of OSCC.

Materials and Methods

Materials

Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), and apoptosis assay kit were purchased from Sigma-Aldrich (St. Louis, MO, USA). MiRNA extraction kit, miRNA reverse transcription (RT) kit, and miRNA fluorescence quantitative assay kit were purchased from Tiangen (Beijing, China). Lipofectamine 2000 transfection reagent from Invitrogen (Carlsbad, CA, USA), cell counting kit-8 (CCK-8) reagent from Dojindo Molecular Technologies, (Kumamoto, Japan), pmirGLO plasmids, and Dual-Luciferase reporter assay system from Promega (Madison, WI, USA). Quantitative RT-Polymerase Chain Reaction (qRT-PCR) instrument from Thermo Fisher Scientific (Waltham, MA, USA), and flow cytometer from BD Biosciences (Franklin Lakes, NJ, USA).

Cell Culture and MiRNA Transfection

Human OSCC cell lines (HSC3, SSC-25, and Tca-8113) and normal oral epithelial keratinocyte cell line (HOK) were purchased from the Shanghai Cell Bank, Chinese Academy of Sciences (Shanghai, China). All cells were cultured in DMEM containing 10% FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin (Roche, Basel, Switzerland) in an incubator with 5% CO_2 at 37°C. MiR-92a mimics or inhibitor, and negative control miR-NC or anti-miR-NC, si-Kruppel-like factor 4 (KLF4) and negative control si-NC were designed and chemically synthesized by GenePharma (Shanghai, China). Cell transfection was performed according to the instructions of Lipofectamine 2000.

RNA Extraction and QRT-PCR

48 h after transfection, total RNA was extracted from cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Subsequently, extracted RNA was synthesized into first-strand complementary deoxyribonucleic acid (cDNA) *via* RT using random primers and RT kit. With 1 μL of cDNA as the template, qRT-PCR was performed using the miRNA fluorescence quantitative assay kit as follows: pre-denaturation at 95°C for 10 min, and 95°C for 2 s, 60°C for 30 s, and 70°C for 8 s, for a total of 40 cycles. The relative expression of miR-92a was calculated by the $2^{-\Delta\Delta Ct}$ method, $\Delta Ct = Ct_{miR\text{-}92a} - Ct_{U6}$. Primers used in this study were as follows: miR-92a forward: 5'-TGTACAATCACGAACCAGTGA-3', reverse: 5'-AGCATAGCTCGGAAAGAACCTC-3'. KLF4 forward: 5'-CGAACCCACACAGGTGAGAA-3', reverse: 5'-TACGGTAGTGCCTGGTCAGTTC-3'. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) forward: 5'-GGTGGTCTCCTCTGACTTCAACA-3', reverse: 5'-GTGGTCGTTGAGGGCAATG-3'. U6 forward: 5'-GCGCGTCGTGAAGCGTTC-3', reverse: 5'-GTGCAGGGTCCGAGGT-3'.

Cell Proliferation Assay

Transfected cells were inoculated into 96-well plates at a density of 2000 cells/well. After culture for 24, 48, 72, and 96 h, respectively, 10 μL of CCK-8 solution was added into each well, followed by incubation for 3 h in the dark. The absorbance at 450 nm was measured using a micro-plate reader.

Flow Cytometry

Cell apoptosis was evaluated at 48 h after transfection. Briefly, transfected cells were washed with ice-cold phosphate-buffered saline (PBS), centrifuged, and re-suspended in 100 μL of binding buffer. Then, the cells were stained with 5 μL of Annexin V-fluorescein isothiocyanate (FITC) and 5 μL of propidium iodide (PI) at room temperature in the dark for 30 min. Finally, the number of apoptotic cells was counted using a flow cytometer.

Colony Formation Assay

Transfected cells were seeded into 6-well plates and cultured for 14 d. When there were colonies visible to naked eyes, the cells were washed with PBS, fixed with paraformaldehyde, and stained with crystal violet. Finally, formed colonies were observed under a microscope, and the number of colonies was counted.

Western Blotting

Total protein was isolated from cultured cells using cell lysis buffer. Protein concentration was measured using the bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). An equal number of proteins were separated *via* 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). After sealing with 5% skimmed milk, the membranes were incubated with primary antibodies at 4°C overnight. On the next day, the membranes were incubated again with corresponding secondary antibodies at room temperature for 1 h. Immunoreactive bands were finally exposed by the enhanced chemiluminescence (ECL) developing system (Pierce, Rockville, MD, USA).

Luciferase Activity Assay

Target genes for miR-92a were predicted online using TargetScan (http://www.targetscan.org/). The miR-92a-targeted KLF4 3'UTR was amplified *via* PCR and cloned into luciferase reporter plasmids (pmiR-KLF4-wt) and corresponding mutant-type reporter plasmids (pmiR-KLF4-mut). Next, pmiR-KLF4-wt or pmiR-KLF4-mut and miR-92a mimic or its negative control miR-NC were co-transfected into cells using Lipofectamine 2000. At 48 h after transfection, luciferase activity was determined using the Dual-Luciferase reporter assay system.

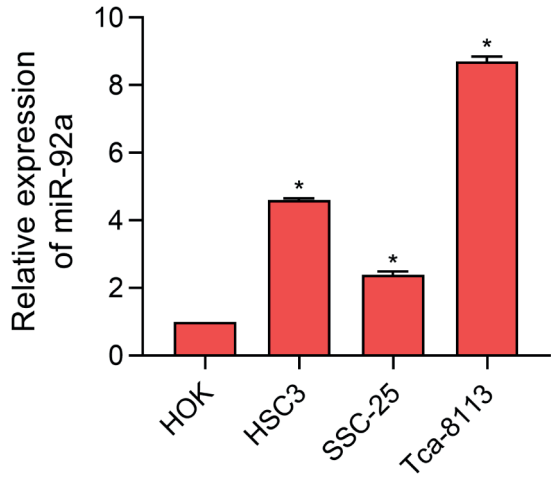

Figure 1. Expression level of miR-92a in OSCC cell lines detected *via* qRT-PCR. *$p<0.05$.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 19.0 software (IBM, Armonk, NY, USA) was utilized for all statistical analysis. The *t*-test was used for analyzing measurement data. Differences between two groups were analyzed by using the Student's *t*-test. One-way ANOVA was applied to compare the differences among different groups, followed by post-hoc test (Least Significant Difference). $p<0.05$ was considered statistically significant.

Results

Expression Level of MiR-92a in OSCC Cell Lines

The expression level of miR-92a in OSCC cell lines was detected *via* qRT-PCR. As shown in Figure 1, the expression of miR-92a increased significantly in three OSCC cell lines (HSC3, SSC-25, and Tca-8113) compared with HOK cells ($p<0.05$). Among the three OSCC cell lines, Tca-8113 cells expressed a relatively higher level of miR-92a. Therefore, they were selected for subsequent experiments.

Effect of MiR-92a Silencing on Proliferation of Tca-8113 Cells

To explore the functional gain and loss of miR-92a in OSCC cells, Tca-8113 cells were transfected with miR-92a inhibitor. QRT-PCR results indicated that at 48 h after transfection, the expression of miR-92a significantly declined in cells ($p<0.05$, Figure 2A). CCK-8 results showed that the proliferation of cells transfected with miR-92a inhibitor was significantly inhibited when compared with those transfected with anti-miR-NC ($p<0.05$, Figure 2B).

Effect of MiR-92a Silencing on Colony Formation Ability of Tca-8113 Cells

Tca-8113 cells transfected with miR-92a inhibitor and those transfected with anti-miR-NC were first inoculated into 6-well plates, respectively.

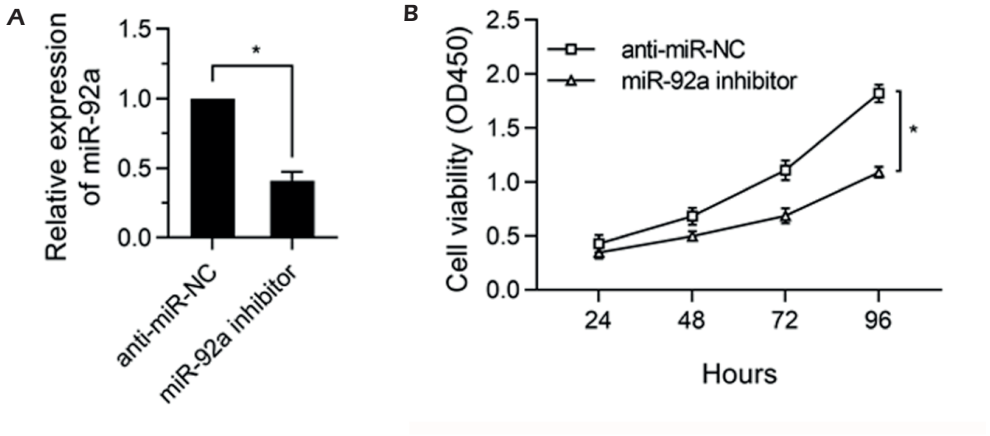

Figure 2. A, Silencing effect of miR-92a detected *via* qRT-PCR. **B,** Cell proliferation after miR-92a silencing detected *via* CCK-8 assay (*p<0.05).

After 2 weeks, the number of formed colonies of cells transfected with miR-92a inhibitor was evidently smaller than that of cells transfected with anti-miR-NC (p<0.05, Figure 3).

Effect of MiR-92a Silencing on Apoptosis of Tca-8113 Cells

To study the effect of miR-92a on apoptosis of OSCC cells, the apoptosis rate was determined through flow cytometry at 48 h after transfection of miR-92a inhibitor in Tca-8113 cells. The results demonstrated that the apoptosis rate of cells transfected with miR-92a inhibitor was significantly higher than that of cells transfected with anti-miR-NC (p<0.05, Figure 4). All these findings indicated that miR-92a silencing induced apoptosis of Tca-8113 cells.

MiR-92a Bound to KLF4 3′-UTR

The target gene for miR-92a was predicted using TargetScan. KLF4 was finally identified as the potential target gene for miR-92a in OSCC (Figure 5A). To further confirm such a conjecture, pmiR-KLF4-wt or pmiR-KLF4-mut and miR-92a mimics or miR-NC were co-transfected into Tca-8113 cells. Dual-Luciferase reporter gene assay manifested that transfection of miR-92a mimics remarkably decreased the luciferase activity of pmiR-KLF4-wt in Tca-8113 cells (p<0.05, Figure 5B). However, transfection of miR-92a inhibitor

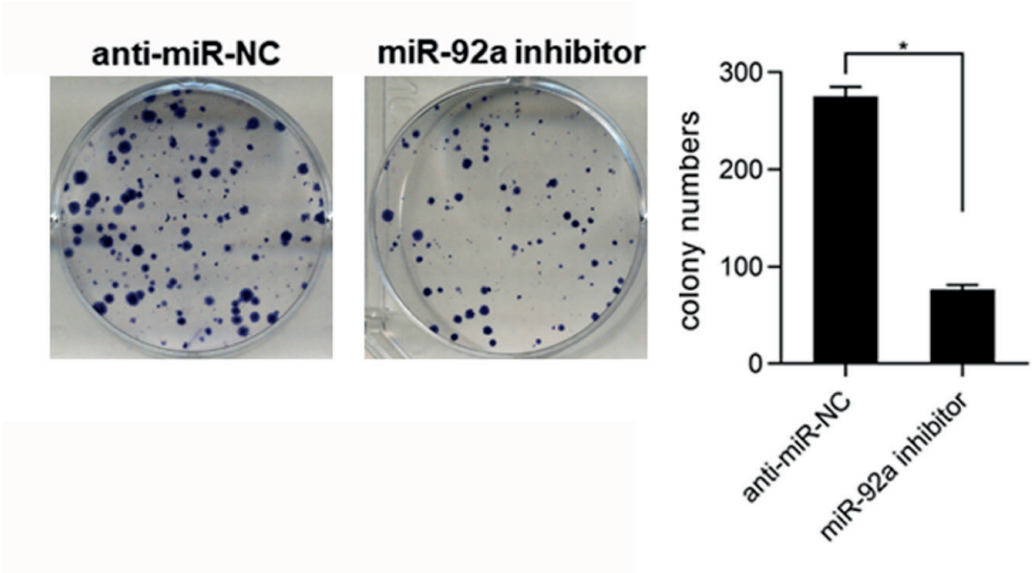

Figure 3. Colony formation after miR-92a silencing determined using colony formation assay (magnification x100) (*p<0.05).

remarkably increased the luciferase activity of pmiR-KLF4-wt ($p<0.05$, Figure 5C). However, there were no significant changes in the luciferase activity of pmiR-KLF4-mut in cells.

Effects of MiR-92a on Wnt/β-Catenin Signaling Pathway-Associated Proteins

KLF4 is one of the negative regulators of Wnt/β-catenin signaling pathway. Therefore, the relation between miR-92a and Wnt/β-catenin signaling pathway was then explored. After miR-92a silencing, changes in Wnt/β-catenin signaling pathway-associated proteins were detected using Western blotting, including β-catenin, c-Myc, and Wnt3a. The results manifested that the protein expressions of β-catenin, c-Myc, and Wnt3a were significantly down-regulated after miR-92a silencing (Figure 6). These findings indicated that silencing of miR-92a inactivated the Wnt/β-catenin signaling pathway.

Discussion

OSCC is the most common head-neck cancer, which is also one of the major causes of oral carcinoma-related death due to poor prognosis worldwide[14]. Great progress has been made in the treatment of OSCC through surgery, chemotherapy or radiotherapy. However, the 5-year survival rate of OSCC is still lower than 60%. Similar to other tumors, the pathogenesis of OSCC is a complex process involving multiple events and steps[15].

Dysregulation of miRNAs is involved in the occurrence and progression of various types of cancers, including OSCC[9]. Therefore, it is still of great significance to explore the roles of miRNAs in specific diseases or biological processes. Many miRNAs have shown significant potential in the diagnosis and treatment of malignancies. In the present study, we found that the expression of miR-92a in OSCC cell lines was significantly higher than that of normal oral epithelial keratinocyte cell lines. Subsequent *in vitro* functional assays revealed that dysregulation of miR-92a expression affected proliferation and apoptosis of OSCC cells. Therefore, we hypothesized that miR-92a might act as a carcinogen in human OSCC. Meanwhile, its increased expression might be related to the development of OSCC. All our findings demonstrated that miR-92a participated in the regulation of the occurrence and development of OSCC, serving as a potential therapeutic target for OSCC.

MiR-92a, located on chromosome 13q32-33, belongs to the miR-17-92a cluster. It is reported that the up-regulation of miR-92a has been found in B-cell lymphoma, colon cancer, gastric cancer, lung cancer, prostate cancer, liver cancer, thyroid cancer, and nasopharyngeal cancer[16]. MiR-92a expression is up-regulated in hepatocellular carcinoma and multiple myeloma tissues. However, its expression in plasma remarkably declines compared with that in healthy subjects[17]. In addition, the level of miR-92a is remarkably higher in acute myeloid leukemia and acute lymphoblastic leuke-

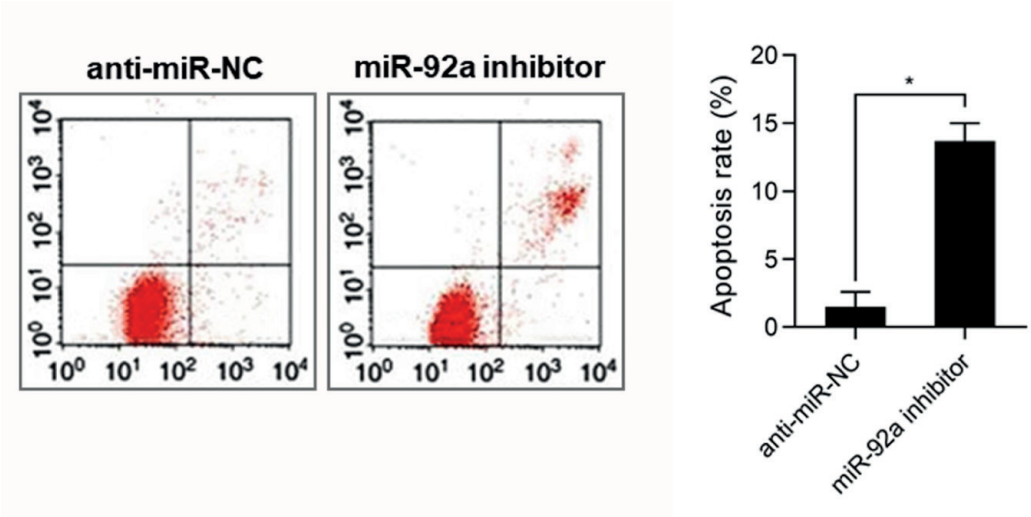

Figure 4. Apoptosis after miR-92a silencing determined using flow cytometry (*$p<0.05$).

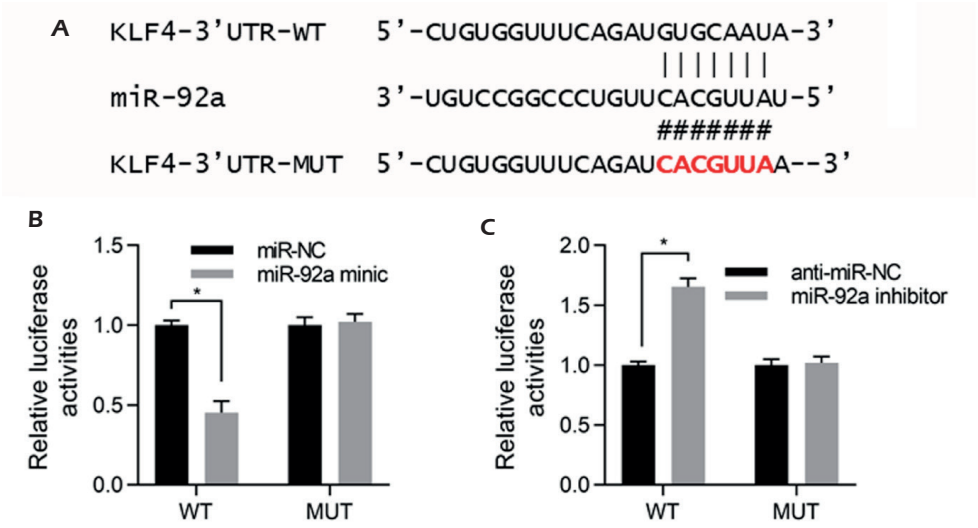

Figure 5. A, Target sites of miR-92a in KLF4 3'-UTR. **B-C,** Luciferase activity. It proves that KLF4 is a direct target for miR-92a in Tca-8113 cells (*p<0.05).

mia cells than that in peripheral blood mononuclear cells in healthy volunteers. Meanwhile, the prognosis of patients with miR-92a overexpression is relatively poor[18]. In the present study, it was found that miR-92a was remarkably up-regulated in OSCC tissues, suggesting that miR-92a acted as an oncogene in OSCC.

KLF4 is a zinc finger-containing transcription factor that plays an important role in cell cycle, apoptosis, and differentiation[19]. KLF4 inhibits cell proliferation *via* inducing cell cycle arrest in colorectal cancer, which depends on targeting CDX gene[20]. Moreover, it regulates the occurrence of esophageal cancer by regulating cell proliferation, apoptosis, and invasion[21]. In this research KLF4 was determined as one of the potential targets for miR-92a in OSCC cells. Our results clearly showed that KLF4 was directly targeted and down-regulated by miR-92a. The target sequences of miR-92a were confirmed *via* Dual-Luciferase reporter gene assay. The results manifested that down-regulation of KLF4 was mediated by direct binding of miR-92a to KLF4 3'-UTR. Such an effect was eliminated by changes in the KLF4 3'-UTR. KLF4 is one of the negative regulators of Wnt/β-catenin signaling pathway according to relevant reports. In this study, the relationship between miR-92a and Wnt/β-catenin signaling pathway was explored. Western blotting results demonstrated that the protein expressions of β-catenin, c-Myc, and Wnt3a all were significantly down-regulated after miR-92a silencing. These findings indicated that silencing of miR-92a inactivated the Wnt/β-catenin signaling pathway.

Conclusions

In summary, miR-92a expression is significantly up-regulated in OSCC cell lines. Silencing of

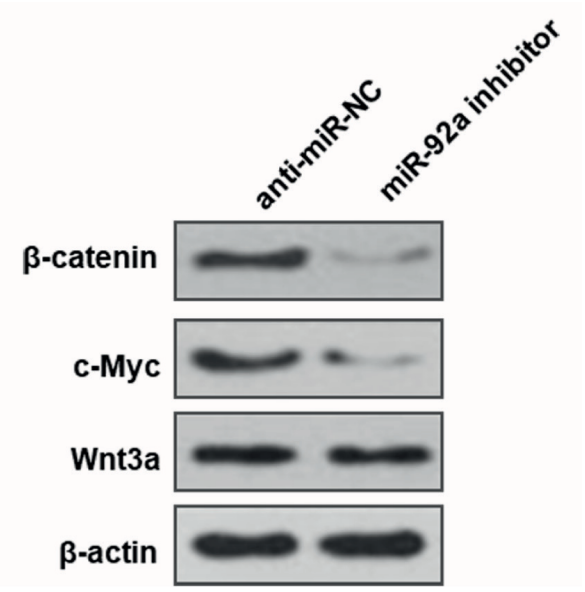

Figure 6. Changes in Wnt/β-catenin signaling pathway-associated proteins β-catenin, c-Myc, and Wnt3a detected using Western blotting.

miR-92a has effects on proliferation and apoptosis of OSCC cells. In addition, the effects of miR-92a on cell proliferation and apoptosis may be related to the Wnt/β-catenin signaling pathway. All our findings suggest that miR-92a can serve as a potential therapeutic target for OSCC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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