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Study of miR-26a inhibiting epithelial-mesenchymal transition and invasion of Tu686 cell line through SMAD1

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Abstract. – OBJECTIVE: This study aims to investigate the effect of miR-26a on the epithelial-mesenchymal transition (EMT) and invasion of Tu686 cell line through SMAD1.

MATERIALS AND METHODS: Tu686 Squamous cell carcinoma of head and neck (SCCHN) cell strains were divided into the miR-26a group, miR-NC group, co-transfection group and blank control group. Among them, the miR-26a group only transfected miR-26a mimics, the miR-NC group only transfected miR-26a negative control, the co-transfection group transfected miR-26a mimics and pcDNA3.1-SMAD1 plasmid. The qRT-PCR method was used for the detection of the expressions of miR-26a and SMAD1 in each group of cells, transwell assay for the detection of the invasion ability of each group of cells and Western blot for detecting the expression level of SMAD1 and the expressions of EMT-related proteins E-cadherin and N-cadherin.

RESULTS: The relative expression of miR-26a in the miR-26a group was significantly higher than that in the miR-NC group and blank control group, and the relative expression in the co-transfection group was significantly higher than that in the miR-NC and blank control groups. The relative expression of SMAD1 in the miR-26a group was significantly lower than that in the miR-NC and blank control groups, and the relative expression in the co-transfection group was lower than that in the miR-NC and blank control groups, and higher than that in the miR-26a group (all $p<0.05$). There was no significant difference between the miR-NC group and the blank control group ($p>0.05$).

CONCLUSIONS: miR-26a may reduce the expression level of SMAD1, affect the expression of EMT-related proteins, inhibit the EMT function of Tu686 cells of squamous cell carcinoma of head and neck, and inhibit the invasion of them.

Key Words:
MiR-26a, SMAD1, SCCHN, Epithelial-mesenchymal transition, Invasion.

Introduction

Squamous cell carcinoma of head and neck (SCCHN) refers to squamous cell carcinoma originating from the neck, ears, nose and throat (ENT), oral and maxillofacial regions. The incidence of it has gradually increased in recent years, as well as the number of female patients[1]. Each year, approximately 300,000 people worldwide suffer from SCCHN, and at least 50% of them die as a result[2]. The main treatment methods of SCCHN are surgical resection, radiotherapy and chemotherapy. In recent years, with the process of medical technology, treatment methods have been improving, but its five-year survival rate has not been effectively improved[3]. One of the main reasons different from other tumors is that SCCHN metastasis occurs in the early tumor, and no more than 50% of patients with cervical lymph node metastasis can survive for five years. If distant metastasis occurs, the five-year survival rate will do not exceed 20%. Therefore, metastasis is a very important factor affecting the prognosis of SCCHN[4]. Since SCCHN is an epithelial-derived tumor, if the tightly-connected epithelial cells such as mesenchymal cells transform, wander and migrate, they can cross the basement membrane and migrate to adjacent tissues or even distant organs. This type of epithelial-mesenchymal transition (EMT) is a necessary condition for the invasion and metastasis of tumor cells[5].

MicroRNA (miRNA) is an endogenous single-stranded small-molecule RNA that cannot encode a protein and exerts its function of degrading or inhibiting the translation of target mRNA by binding to the 3' untranslated region of target mRNA[6]. The mutation or abnormal expression of miRNA plays an important role in malignant tumors[7]. Wu et al[8] found that the expression of miR-

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26a down-regulated in laryngeal squamous cell carcinoma tissues and cell strains, while the overexpression of it inhibited cell proliferation, migration and invasion. A variety of studies have shown that miRNA-26a promotes apoptosis and inhibits cell proliferation and migration in tumors[9]. SMADs are cytoplasmic transmitters, mainly involved in the signal transduction of transforming growth factor-β (TGF-β) family. Up to now, eight SMAD proteins have been found. Among them, SMAD1 belongs to the receptor-regulatory SMAD, mainly mediating the bone morphogenetic protein (BMP) pathway[10]. BMPs also belong to the TGF-β superfamily that can activate downstream proteins after activation, phosphorylate SMAD1, form complexes with SMAD4 to enter the nucleus, and bind to DNA sequences, thereby regulating the transcription of the BMP target gene and promoting the occurrence of EMT[11,12]. Icli et al[13] studied endothelial cells and found that miR-26a inhibited the SMAD1 signaling pathway-mediated EMT angiogenesis by targeted binding to the 3'untranslated region of SMAD1.

Tu686 cell line is a typical SCCHN cell line. In this paper, we investigated the effect of Tu686 cell line miR-26a on SMAD1-mediated EMT and cell invasion in order to provide a theoretical basis and research points for the follow-up study of miR-26a combined with SMAD1 in SCCHN, and new molecular biological markers and therapeutic targets for SCCHN metastasis.

Materials and Methods

Reagents and Materials

Tu686 cells of squamous cell carcinoma of head and neck (GuangZhou Jennio Biotechnology Co., Ltd., Guangzhou, China); fetal bovine serum (FBS) and Dulbecco's Modified Eagle Medium (DMEM) F12 medium (Gibco, Grand Island, NY, USA); TRIzol reagent, RIPA lysate and SDS-PAGE buffer solution (Yisen Biotechnology Co., Ltd., Beijing, China); rabbit anti-human SMAD1 polyclonal antibody, rabbit anti-human E-cadherin/N-cadherin polyclonal antibody, rabbit anti-human GAPDH polyclonal antibody and goat anti-rabbit secondary antibody labeled with horseradish peroxidase (Beyotime Biotechnology Co., Ltd., Shanghai, China); transwell chamber and Matrigel (BD Biosciences, Franklin Lakes, NJ, USA); miR-26a mimics and pcDNA3.1-SMAD1 plasmid (Sangon Biotech Co., Ltd., Shanghai, China); Lipofectamine® 3000 transfection reagent (Invitrogen™, Carlsband, CA, USA); TransScript Green miRNA Two-Step qRT-PCR SuperMix and TransScript® Green Two-Step qRT-PCR SuperMix (TransGen Biotechnology, Beijing, China); qPCR primer sequences (Thermo Fisher, Waltham, MA, USA).

Cell Transfection

The Tu686 SCCHN cell strain was cultured with the DMEM F12 medium containing 10% fetal bovine serum at 37°C in a 5% CO_2 incubator and observed. When the confluence reached 50%, transfection was performed in strict accordance with the Lipofectamine® 3000 transfection reagent instruction. The miR-26a group only transfected miR-26a mimics, the miR-NC group only transfected miR-26a negative control mimic, the co-transfection group transfected miR-26a mimics and pcDNA3.1-SMAD1 plasmid, and the blank control group was not transfected. The equal amount of medium was added, placed and incubated in a 5% CO_2 37°C incubator; the detection analysis was performed after 48 h.

qRT-PCR Test

The total RNA was extracted and lysed by the TRIzol method. After purification, 2 μl of total RNA was diluted with 98 μl of diethyl pyrocarbonate (DEPC), that was used as a blank control. An ultraviolet spectrophotometer was used to measure absorbance values at 260 nm and 280 nm, and the concentration and purity of RNA were detected. The reverse transcription reaction system was prepared according to the reverse TransScript® miRNA RT Enzyme Mix. The above reagents were gently mixed, and the PCR tube was placed on the PCR apparatus. The reverse transcription reaction was performed under the following conditions: incubated at 37 °C for 1 h and heated at 85 °C for 5 s to inactivate RT Enzyme Mix. The detection of miR-26a was performed according to the TransScript Green miRNA Two-Step qRT-PCR SuperMix to prepare the reaction system. The detection of SMAD1 was performed according to the TransScript® Green Two-Step qRT-PCR SuperMix operating. The amplification reaction was performed under the following conditions: incubated at 37°C for 1 h, at 94°C for 5 s, and at 60°C for 30 s for a total of 40 cycles. U6 was used as an internal reference. Primer sequences are shown in Table I. $2^{-\Delta Ct}$ method was used to statistically process the data.

Transwell Invasion Test

Cells were cultured in a 37°C and 5% CO_2 incubator for 24 h, then changing to a serum-free

Table I. Primer sequences.

Genes	Upstream primer	Downstream primer
GAPDH	5'-CCCATCACCATCTTCCAGGAG-3'	5'-GTTGTCATGGATGACCTTGGC-3'
miR-26a	5'-GGATCCGCAGAAACTCCA GAGA-3'	5'-TTGGAGGAAAGACGATTT CCGT-3'
SMAD1	5'-CCGCCTGCTTACCTGCCTC CTGAA-3'	5'-GAACGCTTCGCCCACACG GTTGT-3'

medium, continuously cultured for 12 h and were removed and digested with trypsin. They were re-suspended with a serum-free medium to prepare 1×10^5/ml cell suspension, added to the transwell upper chamber (100 μl/well); 600 μl of DMEM F12 medium containing 10% fetal bovine serum was added to the transwell lower chamber, continuously incubated at 37°C and 5% CO_2 for 48 h. The chamber was removed, and the residual cells on the chamber surface were wiped off with a cotton swab, with the chamber bottom up; after air-dried for 10 min, the chamber was fixed with 100% ethanol for 10 min, washed with PBS once and stained with crystal violet for 10 min; washed slowly with PBS 3 times, count was observed under a microscope at 400 times. Five visual fields were randomly selected from each well and the average value was obtained.

Western Blot Test

The total protein was extracted by RIPA lysis method, and its concentration was measured by the BCA method. The calculated protein was added to 1/4 volume of 5x SDS upper sample buffer solution, separated by SDS-PAGE electrophoresis when the constant pressure was 80 V at 8% spacer gel, until entering 5% separation gel, it changed to 120 V; transferring membrane with 80V under ice bath to difluoroethylene membrane for 100 min, stained in the Ponceau S staining solution. When the protein band appeared, the membrane was immersed in PBST for 5 min and blocked overnight with 5% skim milk at 4°C. Each antibody was diluted with PBS containing 1% skim milk, the dilution ratio of rabbit anti-human E-cadherin/N-cadherin/SMAD1 polyclonal antibody was 1:1000, and rabbit anti-human GAPDH polyclonal antibody was used as an internal reference at a dilution ratio of 1:3000, after shaking for 2 h at room temperature, immersed in TBST and rinsed. Goat anti-rabbit secondary antibody labeled with horseradish peroxidase was added at a dilution ratio of 1:5000, after shaking gently for 1 h at room temperature, rinsed with TBST 5 times, each time for 5 minutes. The membrane was developed in a dark room, the liquid on it was blotted dry with filter paper and the ECL luminescent agent was dripped, exposed after 5 min. The protein band was scanned, and the gray value was analyzed in the Quantity One software, where the relative expression level of the protein = the gray value of target protein band/the gray value of GAPDH protein band.

Data Processing

SPSS 20.0 (SPSS IBM, Armonk, NY, USA) software was used to count the data. The measurement data were expressed as mean ± standard deviation (Mean ± SD). Independent sample *t*-test was used for the comparison between the two groups. ANOVA analysis of variance was used for the overall test of comparison of means between multiple groups. If there was a difference, then LSD-t was used for pairwise comparison. The significance level is $\alpha=0.05$.

Results

qRT-PCR Test for the Detection of MiR-26a Expression in Each Group

The relative expressions of miR-26a in the miR-26a group, miR-NC group, co-transfection group and blank control group were (3.72±0.56), (0.94±0.21), (3.81±0.62), and (0.88±0.19), respectively. The relative expression in the miR-26a group was significantly higher than that in the miR-NC and blank control groups, and that in the co-transfection group was markedly higher than in the miR-NC and blank control groups (all $p<0.05$). There was no significant

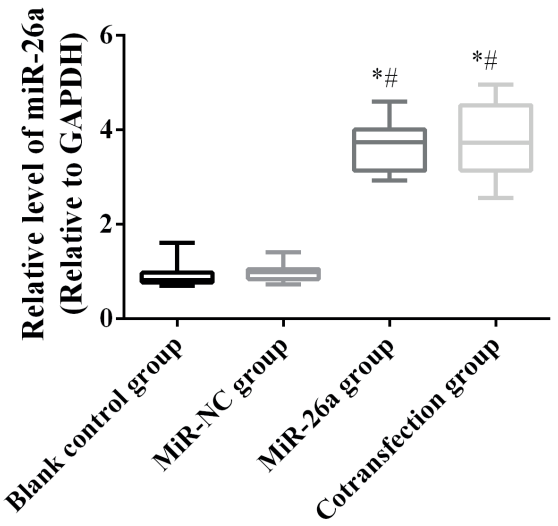

Figure 1. qRT-PCR test for the detection of miR-26a expression in each group. The relative expression of miR-26a in the miR-26a group was significantly higher than that in the miR-NC and blank control groups, and that in the co-transfection group was markedly higher than that in the miR-NC and blank control groups (all $p<0.05$). There was no significant difference between the miR-NC group and the blank control group, and between the co-transfection group and the miR-26a group ($p>0.05$). Note: *: compared with the blank control group, $p<0.05$. #: compared with the miR-NC group, $p<0.05$.

difference between the miR-NC group and the blank control group, and between the co-transfection group and the miR-26a group ($p>0.05$) (Figure 1).

qRT-PCR Test for the Detection of SMAD1 Expression in Each Group

The relative expressions of SMAD1 in the miR-26a group, miR-NC group, co-transfection group and blank control group were (0.08±0.01), (1.38±0.08), (0.67±0.07), and (1.22±0.06), respectively. The expression in the miR-26a group was significantly lower than that in the miR-NC and blank control groups, and that in the co-transfection group was lower than in the miR-NC and blank control groups, and higher than that in the miR-26a group (all $p<0.05$). There was no significant difference between the miR-NC group and the blank control group ($p>0.05$) (Figure 2).

Detection of Invasion Ability in Each Group

The number of invasive cells in the miR-26a group, miR-NC group, co-transfection group and blank control group were (36.24±3.73), (98.22±6.73), (73.52±5.24) and (101.34±5.43), respectively. The number in the miR-26a group was markedly lower than in the miR-NC group, blank control group and co-transfection group, and that in the co-transfection group was lower than in the miR-NC group and blank control group (all $p<0.05$). There was no significant difference between the miR-NC group and blank control group ($p>0.05$) (Figure 3).

Detection of E-cadherin/N-cadherin/ SMAD1 Proteins in Each Group

The relative protein content of E-cadherin in the miR-26a group, miR-NC group, co-transfection group and blank control group were (6.24±1.73), (1.43±0.28), (3.52±1.24) and (1.34±0.23), respectively. The relative protein content in the miR-26a group was significantly higher than that in the miR-NC group, co-transfection group and blank control group, and that in the co-transfection group was remarkably higher than in miR-NC group and blank control group (all $p<0.05$). There was no significant difference between the miR-NC group and the blank control group ($p>0.05$). The relative pro-

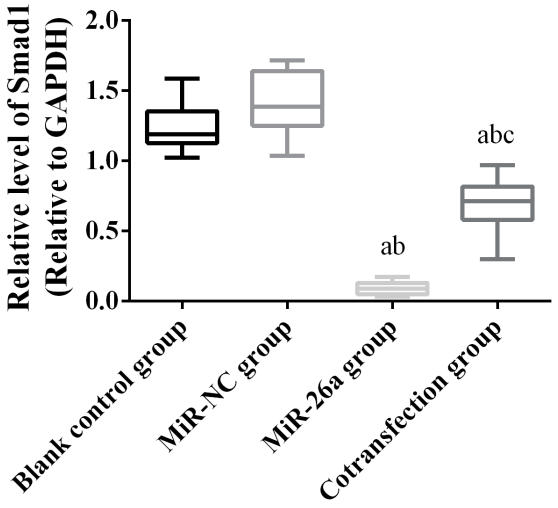

Figure 2. qRT-PCR test for the detection of SMAD1expression in each group. The relative expression of SMAD1 in the miR-26a group was significantly lower than that in the miR-NC and blank control groups; that in the co-transfection group was lower than that in the miR-NC and blank control groups, and higher than that in the miR-26a group (all $p<0.05$). There was no significant difference between the miR-NC group and the blank control group ($p>0.05$). Note: a: compared with the blank control group, $p<0.05$. b: compared with the miR-NC group, $p<0.05$. c: compared with the miR-26a group, $p<0.05$.

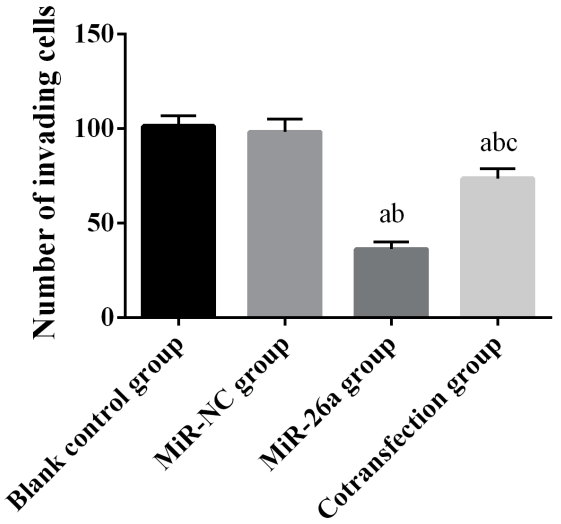

Figure 3. Detection of invasion ability in each group. The number of invasive cells in the miR-26a group was significantly lower than that in the miR-NC group, blank control group and co-transfection group, and that in the co-transfection group was lower than that in the miR-NC and blank control groups (all $p<0.05$). There was no significant difference between the miR-NC group and the blank control group ($p>0.05$). Note: a: compared with the blank control group, $p<0.05$. b: compared with the miR-NC group, $p<0.05$. c: compared with the miR-26a group, $p<0.05$.

tein content of N-cadherin in the miR-26a group, miR-NC group, co-transfection group and blank control group were (0.24±0.13), (1.22±0.24), (0.72±0.14), and (1.34±0.21), respectively. The relative protein content in the miR-26a group was markedly lower than in the miR-NC group, co-transfection group and blank control group, and that in the co-transfection group was significantly lower than that in the miR-NC group and blank control group (all $p<0.05$). There was no significant difference between the miR-NC group and the blank control group ($p>0.05$). The relative protein content of SMAD1 in the miR-26a group, miR-NC group, co-transfection group and blank control group were (0.24±0.12), (0.89±0.13), (0.52±0.11), and (0.92±0.15), respectively. The relative protein content in the miR-26a group was remarkably lower than that in the miR-NC group, co-transfection group and blank control group, and that in the co-transfection group was significantly lower than that in the miR-NC group and blank control group (all $p<0.05$). There was no significant difference between the miR-NC group and the blank control group ($p>0.05$) (Figure 4).

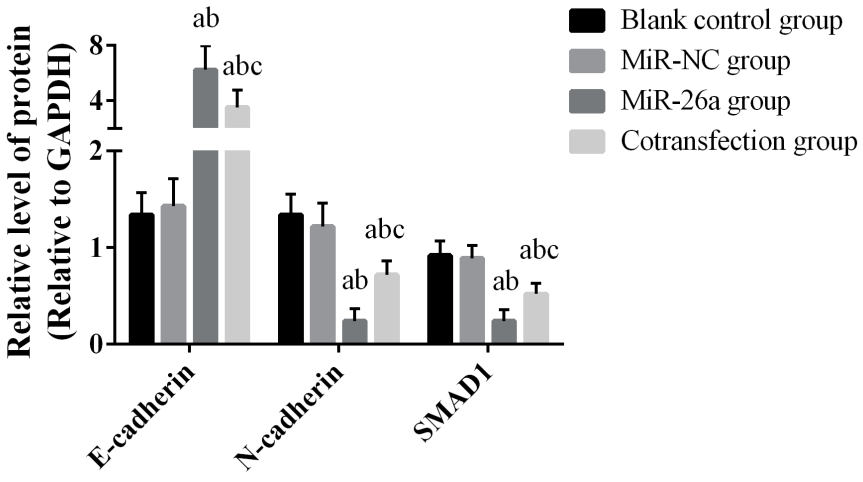

Figure 4. Detection of E-cadherin/N-cadherin/SMAD1 proteins in each group**.** The relative protein content of E-cadherin in the miR-26a group was significantly higher than that in the miR-NC group, co-transfection group and blank control group; that in co-transfection group was markedly higher than that in the miR-NC and blank control groups (all $p<0.05$). There was no significant difference between the miR-NC group and the blank control group ($p>0.05$). The relative protein content of N-cadherin in the miR-26a group was significantly lower than that in the miR-NC group, co-transfection group and blank control group, and that in the co-transfection group was markedly lower than that in the miR-NC and blank control groups (all $p<0.05$). There was no significant difference between the miR-NC group and the blank control group ($p>0.05$). The relative protein content of SMAD1 in the miR-26a group was remarkably lower than that in the miR-NC group, co-transfection group and blank control group; that in the co-transfection group was significantly lower than that in the miR-NC and blank control groups (all $p<0.05$). There was no significant difference between the miR-NC group and the blank control group ($p>0.05$). Note: a: compared with the blank control group, $p<0.05$. b: compared with the miR-NC group, $p<0.05$. c: compared with the miR-26a group, $p<0.05$.

Discussion

SCCHN is one of the most common cancers in the world. The number of newly-increased patients is approximately 500,000 every year. More than 50% of them are diagnosed as SCCHN at the advanced stage, and more than 20% of them have already metastasized. Medical science level continuously improves, as well as the treatment method of SCCHN, but its prognosis is still very poor with a 5-year survival rate of less than 50%[14,15]. Studies[16,17] have shown that miRNA-26 can inhibit the proliferation, migration and EMT of various tumor cells. SMAD1 is an important signal transduction medium in EMT. The activation of BMPs leads to the phosphorylation of downstream protein SMAD1. Phosphorylated SMAD1 binds to Smad4 to form a complex and transfer to the nucleus, up-regulating the expression of nuclear transcription factors, thereby inhibiting the transcriptional activity of epithelial cell marker proteins such as E. Cadherin, and promoting the formation of EMT. It has been reported that miRNA-26 can inhibit SMAD1, to inhibit EMT[18]. It is also reported that the expression of miR-26a in SCCHN is down-regulated by SMAD1.

Therefore, this work investigated the effect of miR-26a on the SMAD1-mediated EMT and invasion of SCCHN to provide new understanding ideas and molecular markers for the mechanism of SCCHN metastasis.

The qRT-PCR test for the detection of miR-26a expression in each group found that the relative expression of miR-26a in the miR-26a group was significantly higher than that in the miR-NC group and blank control group, and that in the co-transfection group was markedly higher than that in the miR-NC group and blank control group. There was no significant difference between the co-transfection group and the miR-26a group. These results indicate that miR-26a is successfully transfected into the Tu686 cell strain of squamous cell carcinoma of head and neck in the miR-26a group and co-transfection group, and SMAD1 will not affect the miR-26a expression when co-transfected. The qRT-PCR test for the detection of SMAD1 expression in each group found that the relative expression of SMAD1 in the miR-26a group was significantly lower than that in the miR-NC group and blank control group, indicating that the miR-26a group inhibits the SMAD1 expression after transfection with miR-26a. The relative expression of SMAD1 in the co-transfection group was lower than that in the miR-NC group and blank control group, and higher than that in the miR-26a group, showing that the co-transfection group reverses the inhibitory effect of miR-26a on SMAD1 after transfection with SMAD1 and miR-26a, but it is still lower than that in the miR-NC group and blank control group. The detection of the invasion ability in each group found that the number of invasive cells in the miR-26a group was remarkably lower than that in the miR-NC group, blank control group and co-transfection group, and that in the co-transfection group was lower than that in the miR-NC group and blank control group. It indicates that the overexpression of miR-26a can significantly inhibit the invasion ability of squamous cell carcinoma of head and neck cells, and the overexpression of miR-26a and SMAD1 can also inhibit the invasion of them, but its ability reduces compared with that of the overexpression of miR-26a alone, indicating that SMAD1 has an opposite effect of miR-26a in invasion. The relative protein content of SMAD1 in the miR-26a group was markedly lower than that in the miR-NC group, co-transfection group and blank control group, and that in the co-transfection group was significantly lower than that in the miR-NC group and blank control group, consistent with qRT-PCR test for the detection of the SMAD1 expression in each group. The detection of E-cadherin/N-cadherin proteins in each group found that the relative protein content of E-cadherin in the miR-26a group was remarkably higher than that in the miR-NC group, co-transfection group and blank control group, and that in the co-transfection group was significantly higher than that in the miR-NC group and blank control group. The relative protein content of N-cadherin in the miR-26a group was markedly lower than that in the miR-NC group, co-transfection group and blank control group; the relative protein content in the co-transfection group was significantly lower than that in the miR-NC group and blank control group. E-cadherin is a typical epithelial cell marker, and N-cadherin is a mesenchymal cell marker. In EMT, the E-cadherin expression reduces but the N-cadherin expression increases[19]. Therefore, the test for the detection of E-cadherin/N-cadherin proteins suggests that the overexpression of miR-26a alone can inhibit the EMT of Tu686 cells. After the overexpression of miR-26a and SMAD1, the effect of miR-26a inhibiting the EMT of Tu686 cells reduces. It was found that the cell invasion and EMT were inhibited after the overexpression of miR-26a in Tu686 cells, and that the SMAD1 expression down-regulated from detection of RNA level and protein level. A reversal experiment of the overexpression of miR-26a and SMAD1 was performed and revealed that the effective rever-

sal of miR-26a could inhibit the invasion and EMT of Tu686 cells. Study results of Fukumoto et al[20] are consistent with ours. MiR-26a is lowly expressed in SCCHN cancer tissues and can inhibit the invasion and metastasis of SCCHN.

Conclusions

We demonstrated that miR-26a may reduce the expression level of SMAD1, affect the expression of EMT-related proteins, inhibit the EMT function of Tu686 cells of squamous cell carcinoma of head and neck, and inhibit the invasion of them.

Conflict of Interest

The Authors declare that they have no conflict of interest.

Acknowledgements

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