Original Article

MiR-29c-3p and MiR-223-3p regulate the proliferation and drug resistance of oral squamous cell carcinoma by targeting ANGPTL4

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Background: The aim of this research is to determine the expression and function of angiopoietin-like protein 4 (ANGPTL4) in oral squamous cell carcinoma (OSCC), and to explore whether miR-29C-3p and miR-223-3p targeting ANGPTL4 regulate the proliferation and cisplatin resistance of OSCC.

Methods: Bioinformatics database was used to identify the expression of ANGPTL4 and microRNAs (miRNAs) in tumors, and qPCR and western blot were used to determine the protein and mRNA expression of ANGPTL4 and miRNA in tissues and cells. The proliferation and drug resistance of the cells were determined by the plate colony formation, Cell Counting Kit-8 (CCK-8) assays and IC50, and a dual luciferase reporter assay was used to determine the regulation of miRNA on ANGPTL4.

Results: ANGPTL4 expression was higher in the OSCC tissues. After knocking down ANGPTL4, the proliferation of OSCC cells decreased, and their sensitivity to cisplatin increased. A dual luciferase reporting assay showed that the fluorescence intensity of the wild-type (WT) group decreased after miR-29c-3p and miR-223-3p were overexpression, while that of the mutant (MUT) group was almost unchanged. The overexpression of miRNA decreased the protein levels of ANGPTL4 and thus affect the proliferation and drug resistance of OSCC cells.

Conclusions: miR-29c-3p and miR-223-3p can regulate cell proliferation and cisplatin resistance in OSCC by targeting ANGPTL4.

Keywords: Oral squamous cell carcinoma (OSCC); microRNA (miRNA); angiopoietin-like protein 4 (ANGPTL4); proliferation; cisplatin resistance

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Introduction

Oral cancer, as one of the most common malignant tumors in the head and neck, accounting for 1–2% of all malignant tumors worldwide (1). Because oral squamous cell carcinoma (OSCC) is prone to metastasis and has a poor prognosis, the overall 5-year survival rate is only 55% (2). Cisplatin is the first-line drug for postoperative chemotherapy in OSCC (3), but tumor resistance to cisplatin chemotherapy is increasingly serious (4). How to effectively improve the sensitivity of postoperative chemotherapy is a hot and difficult problem in OSCC research.

Recent studies have shown that angiopoietin-like protein 4 (ANGPTL4) may be involved in the occurrence of OSCC and is related to the metastasis of OSCC (5)

and may be a potential marker and therapeutic target for preventing the progression and metastasis of tongue squamous cell carcinoma (TSCC) (6).

Previous studies in our group have shown that downregulation of ANGPTL4 can inhibit its migration and proliferation in TSCC, and high levels of ANGPTL4 are associated with T stage, lymphatic metastasis, angiogenesis, and poor overall survival (7). The *ANGPTL4* gene is located on chromosome 19p131 and contains 7 exons and 6 introns. Currently, it is believed that the main function of the ANGPTL4 family is participation in the regulation of substance and energy metabolism, but many recent studies have confirmed that ANGPTL4 plays an important role in the regulation of tumor development. Some studies have suggested that ANGPTL4 plays an anticancer role in liver cancer and other cancers (8). ANGPTL4 plays dual functions as a tumor suppressor and oncogene, and the regulatory mechanisms remain unclear. Therefore, the role of ANGPTL4 in OSCC is worthy of further study.

MicroRNAs(miRNAs) are noncoding single-stranded RNA molecules with a length of approximately 22 nucleotides, that are involved in the regulation of posttranscriptional gene expression. Many studies have proven that abnormal expression of miRNA can lead to the occurrence and development of head and neck squamous carcinoma (HNSC) and other cancers (9). MiRNA can regulate target genes, induce mRNA degradation or inhibit protein translation. In this study, we explored the expression and influence of ANGPTL4 in OSCC, as predicted through the miRNA targeting of ANGPTL4, and sought to determine whether miRNA targeting to ANGPTL4 had an impact on proliferation and drug resistance of OSCC. It is expected to contribute to the diagnosis and treatment of OSCC as well as the prediction of the prognosis. We present the following article in accordance with the MDAR reporting checklist (available at https://fomm.amegroups.com/article/view/10.21037/fomm-21-114/rc).

Methods

Tissue collection

The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Sun Yat-sen Memorial Hospital Institutional Review Board (No. SYSEC-KY-KS-2021-028). A total of 36 pairs of tumor tissues (T) and paracancerous tissues (P) from patients with OSCC were obtained from the Department of Oral and Maxillofacial Surgery, Sun Yat-sen Memorial Hospital. All patients had primary OSCC and had not received preoperative radiotherapy or chemotherapy. All samples were stored at –80 ℃ for use. All patients signed informed consent. The Cancer Genome Atlas (TCGA) database was used to analysis ANGPTL4 expression in head and neck squamous cell carcinoma and normal tissues.

Cell culture and treatment

Experiments were performed with HOK, SCC9, UM1, HSC6, HSC3, CAL27 and OSCC3 cell lines purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). All cell lines were routinely cultured in DMEM (Thermo Fisher, USA) and DMEM-F12 (Thermo Fisher, USA) medium supplemented with 10% FBS (Thermo Fisher, USA) and 1% Penicillin-Streptomycin Solution (Thermo Fisher, USA) in a 37 ℃ humidified incubator containing 5% CO_2, among which HOK, HSC6, HSC3, CAL27 and OSCC3 cells were cultured in DMEM, F12/DMEM was used to culture SCC9 and UM1 cells.

RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted using TRIzol reagent (TaKaRa, Japan), according to the manufacturer's instructions and reverse transcribed into cDNA using an ABI 9700 Real-time PCR instrument (ABI, USA). MicroRNA reverse transcription primers were designed according to the stem loop method (10). One μL of synthesized cDNA was mixed with TB Green® Premix Ex Taq™ II (#RR820B, TaKaRa, Japan) in a reaction volume of 20 μL, according to the manufacturer's instructions. Relative expression of mRNA in a Light Cycler 480 II Real-time PCR system (Roche, Basel, Switzerland), and statistical software was used for statistical analysis. The reaction conditions were as follows: 94 ℃ for 2 min, 94 ℃ for 20 s, 58 ℃ for 20 s, 72 ℃ for 20 s for 40 cycles, and all the reactions were repeated in triplicate. After normalization with reference to expression of GAPDH and U6, the relative expression levels of ANGPTL4 and microRNA were calculated by the $2^{-\Delta\Delta Ct}$ method. As shown in Table S1, all PCR primers were purchased from IGEBIO (Guangzhou, China).

Western blot assay

For protein extraction, the cells were washed twice with cool PBS, harvested by scraping and then lysed in lysis buffer (#01408, Beyotime, China) containing 1% protease Inhibitor Cocktail (#CW2200S, CWBIO, China). Following centrifugation, the supernatant was collected, and the protein concentration was determined using the BCA Protein Assay Kit (#CW0014S, CWBIO, China). The loading buffer (#CW0027S, CWBIO, China) was diluted and mixed, and the protein was denatured at 95 ℃ for 5 min. SDS-PAGE gels were prepared according to the instructions of a Beyotime polyacrylamide gel kit. After electrophoresis (90 V, 1.3 h), the proteins were transferred to a PVDF membrane (#P0021S, Beyotime, China) (250 mA, 70 min). The PVDF membrane was incubated with TRIS-buffered saline containing 5% skim milk with 0.1% Tween-20 for 1 h at room temperature for protein blocking. The primary antibody [ANGPTL4 (#18374-1-AP, Proteintech, USA) and GAPDH (60004-1-Ig, Proteintech, USA)] was incubated (antibody dilution 1:1,000 in 5% bovine serum albumin with 0.1% Tween-20 Tris buffer saline) for 16 h (incubation overnight) in a 4 ℃ shaker. The membrane was washed with TRIS-buffered solution containing 0.1% Tween-20, and the secondary antibody [goat anti-mouse IgG-HRP (#sc-2005, Santa Cruz Biotechnology, USA) and goat anti-rabbit IgG-HRP (#sc-2004, Santa Cruz Biotechnology, USA)] was incubated (antibody dilution 1:1,000 in 5% bovine serum albumin with 0.1% Tween-20 Tris buffer saline) for 1h in room temperature. Chemiluminescence, photography and gel image analysis were carried out according to the standard protocol.

Small interfering RNA (siRNA) and miRNA-mimic transfection

MiR-29c-3p mimics, miR-223-3p mimics and a negative control (miR-NC), siRNAs targeting ANGPTL4 (siANGPTL4-1, and siANGPTL4-2) and a matched control (si-NC) were obtained from Gemma Pharma Biotechnology (Suzhou, China), shown in Table S2. We performed transfection with Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions.

Plate cloning and colony formation assay

Twenty-four hours after siRNA and miR-mimics transfection, the cells were evenly spread into 6-well plates at 1×10^3–2×10^3 cells/well, and 3 replicate wells were prepared in each group. When visible clones appeared in the culture dish, the cell were rinsed twice with PBS, 4% paraformaldehyde was added to fix the cells for 15 min. The fixing solution was removed, 0.4% crystal violet dye solution was added and incubated for 15 min, the dye was slowly washed away with running water and the cells were air-dried. The clones were counted using an ABI automatic cell counter (Invitrogen, USA).

Growth curve

The cell growth curve was generated according to the instructions of the manufacturer of the Cell Counting Kit-8 (CCK-8) kit (#K1018, APExBio, USA). Twenty-four hours after siRNA and miRNA-mimic transfection, the cells were evenly spread into 96-well plates at 1×10^3 cells/well, 3 replicates were set in each group, the old medium was removed at a fixed time point every day, and complete medium containing 10% CCK-8 reagent was added for 2 hours incubation. The optical density (OD) value was read 450 nm with an TECAN Spark10M and recorded.

Cell sensitivity to cisplatin

Twenty-four hours after siRNA and miRNA-mimic transfection, the cells were counted. The cells were spread into 96 wells at a rate of 1×10^4 cells/well. The next day, cisplatin (#1134357, Sigma, USA) was added to 96-well plates according to the concentration gradient (0, 5, 10, 15, 20, 30 μM) and cultured in a constant temperature incubator at 37 ℃, with 5% CO_2 and saturated humidity for 48 hours. A CCK-8 assay was performed to determine the relative absorbance of the cells, and a conversion formula was used to calculate the IC50 of cisplatin in the cells. The formula for calculating the cell relative survival rate was as follows: cell relative survival rate = (OD value – OD value of blank hole)/(OD value of 0 μM – OD value of blank hole) × 100% (11).

Predicted miRNAs targeting ANGPTL4 and dual luciferase reporter assay

The interaction between miR-29c-3p or miR-223-3p and ANGPTL4 mRNA was predicted by analysis using StarBase (https://starbase.sysu.edu.cn/) online datasets. We further analysed the expression of these miRNAs in OSCC with

the OncomiR database (http://www.oncomir.org/). Wild-type (WT) and mutant (MUT) ANGPTL4 sequences were inserted into the psiCHECK-2 vector. Luciferase reporter genes were co-transfected with miRNA-mimics or miRNA-NC into cells using Lipofectamine 2000 transfection reagents (Invitrogen, USA). The luciferase activity was measured using a dual-luciferase reporter assay system (#E1910, Promega, China).

Statistical analysis

Data analyses were performed using SPSS 22.0 software (IBM, Chicago, Illinois). Kolmogorov test was used to analyze the normal distribution and homogeneity of variance of experimental data in this study, and it was expressed as the mean ± standard deviation (M ± SD). Independent sample *t* test was used for the parameters that followed a normal distribution, otherwise, non-parametric test was used. Repetitive Measure Analysis of Variance was used for comparison of different time and concentration, and Bonferroni method was used for pair comparison between groups for post-test. $P<0.05$ was considered as statistically significant.

Results

Expression of ANGPTL4 in OSCC

ANGPTL4 plays different functions in different tissues. The expression of ANGPTL4 in HNSC was slightly higher in tumor tissues than in normal tissues, according to the TCGA database (*Figure 1A*). A total of 36 pairs of cancer and paracancerous tissues obtained from OSCC patients were collected. The qRT-PCR results showed that the expression of ANGPTL4 was higher in 36 pairs of tumor tissues from OSCC specimens than in the corresponding paracancerous tissues (*Figure 1B*). We also found that the expression of ANGPTL4 in 6 OSCC cell lines (SCC9, UM1, HSC6, HSC3, CAL27, OSCC3) was higher than that in HOK cells (*Figure 1C*). Therefore, the results of this study show that ANGPTL4 is highly expressed in OSCC.

ANGPTL4 promotes the proliferation and cisplatin resistance of OSCC cell

Since ANGPTL4 is highly expressed in OSCC, we explored the function of ANGPTL4 in OSCC. OSCC3 and HSC6 cell lines with high expression of ANGPTL4 were selected, and the knockdown efficiency of ANGPTL4 was verified by western blot and qPCR (*Figure 2A*). The results of plate colony formation experiments showed that the proliferation efficiency of OSCC decreased after knocking down ANGPTL4 (*Figure 2B*). CCK-8 assay also showed that HSC6 and OSCC3 began to proliferate more on the second day after ANGPTL4 knockdown than the control group. OSCC3 cells in all groups were almost full of plates on the fourth day, therefore, the proliferation of OSCC3 cells reached a same level on day four (*Figure 2C*). The cells were treated with different concentrations of cisplatin, and it was found that the sensitivity of HSC6 and OSCC3 cells to cisplatin was increased after ANGPTL4 was knocked down (*Figure 2D*).

Screening miRNAs that potentially regulate ANGPTL4

To explore the mechanism of ANGPTL4 upregulation in tumors, we analysed miRNAs that may target ANGPTL4. We used StarBase online databases to forecast the microRNA target ANGPTL4, we found that miR-29c-3p and miR-223-3p can target ANGPTL4 (*Figure 3A*). We further analysed the expression of these miRNAs in HNSC with the OncomiR database. It was found that the expression of miR-29c-3p and miR-223-3p differed in a variety of tumors, and the expression of miR-29c-3p was significantly lower in HNSC (*Figure 3B*). MiR-29c-3p and miR-223-3p were correlated with clinical and pathological T and N stages (*Figure 3C*). Moreover, the OncomiR database also showed that the expression of miR-29c-3p was positively correlated with the survival rate of HNSC patients (*Figure 3D*). In general, miR-29c-p and miR-223-3p may play regulatory roles by targeting ANGPTL4 in HNSC.

ANGPTL4 is a target of miR-29C-3p and miR-223-3p

To determine whether miR-29C-3p and miR-223-3p target ANGPTL4, we transfected mimics to overexpress miRNA, the overexpression of miR-29c-3p and miR-223-3p in HSC6 and OSCC3 cells was verified by qPCR (*Figure 4A*). The results of the western blot experiment showed that the protein expression level of ANGPTL4 decreased after increased the expression of miR-29c-3p in OSCC3 and HSC6 cells. Similarly, the level of ANGPTL4 decreased after miR-223-3p was overexpressed (*Figure 4B*). It is known that miRNA can trigger the cleavage of mRNA through

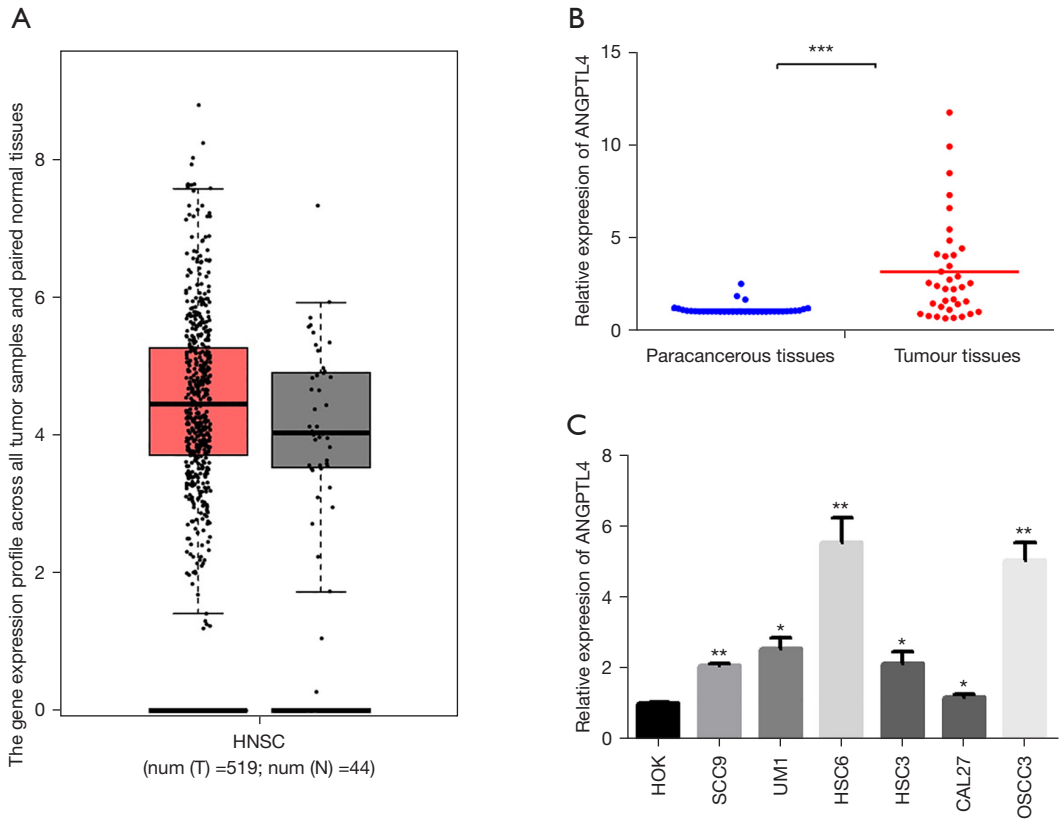


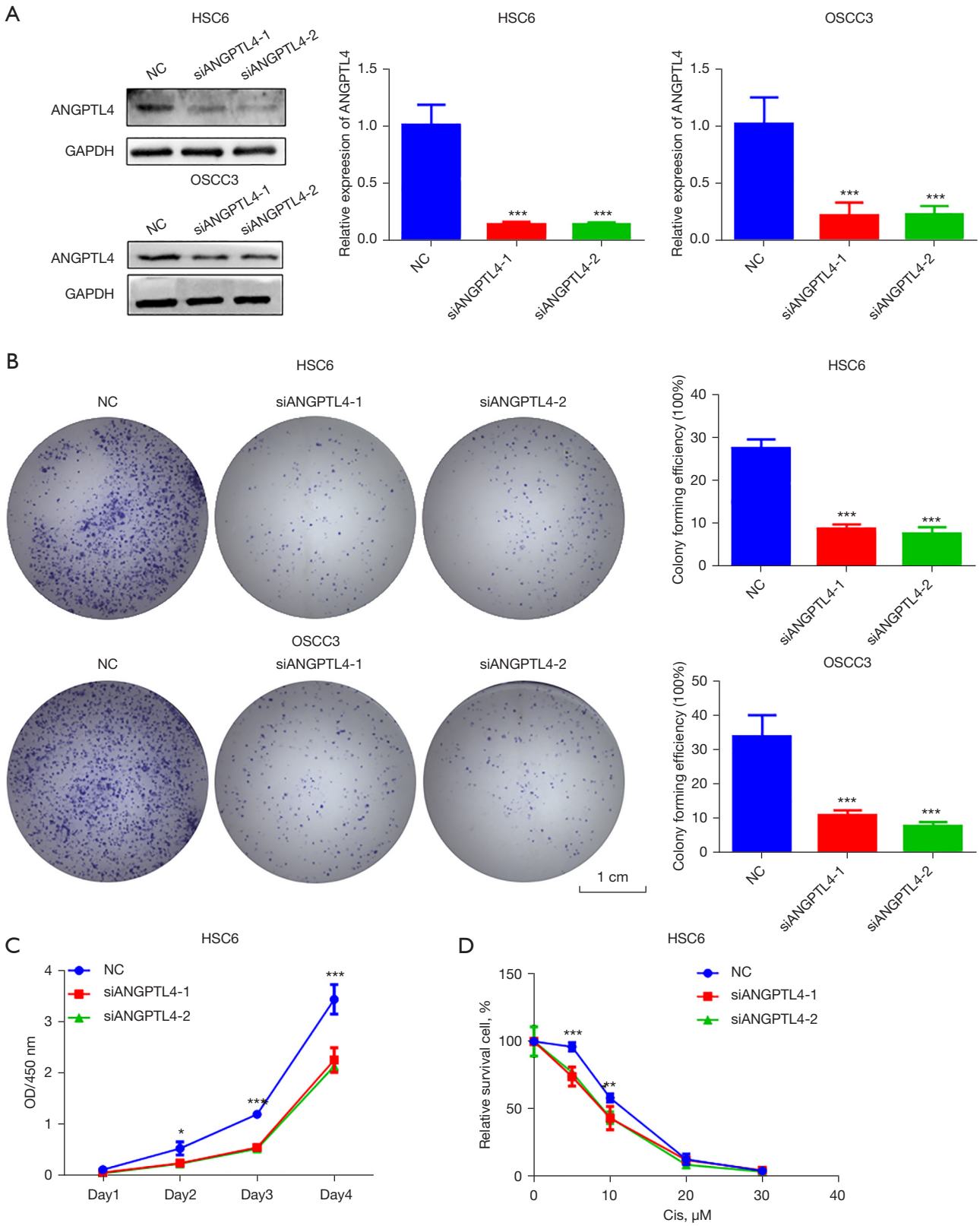
Figure 1 Expression of ANGPTL4 in OSCC. (A) TCGA database analysis showed that ANGPTL4 expression in head and neck squamous cell carcinoma was slightly higher than that in normal tissues. (B) The qRT-PCR results of 36 OSCC clinical specimens showed that the mRNA expression of ANGPTL4 in cancer tissues was higher than that in adjacent tissues. (C) The mRNA expression level of ANGPTL4 in oral cancer cell lines (SCC9, UM1, HSC6, HSC3, CAL27 and OSCC3 cells) was higher than that in normal cells (HOK cells). *, P<0.05; **, P<0.01; ***, P<0.001. ANGPTL4, angiopoietin-like protein 4; OSCC, oral squamous cell carcinoma; HNSC, head and neck squamous cell carcinoma.

RNAi mechanism and reduce the level of target mRNA, on the other hand, miRNA can also inhibit mRNA translation without significantly affecting mRNA stability (12,13). In this study, mRNA expression level of ANGPTL4 in OSCC cell was not affected by overexpression of miR-29C-3p and miR-223-3p, however, it was decreased in OSCC3 cell after overexpression of miR-29C-3p (*Figure 4B*). How miR-29C-3p and miR-223-3p affects ANGPTL4 expression needs to be further verified in future experiments.

In addition, we cloned the 3'UTR segments with the miR-29c-3p and miR-223-3p targeted in ANGPTL4 mRNA and expressed them in the psiCHECK-2 vector for use in dual-luciferase reporter assays, and the mutant miRNA-targeting region was also cloned (*Figure 4C*). In the dual luciferase reporting assay, two kinds of psiCHECK-2 and miRNA-mimics or miR-NC were co-transfected. As shown in *Figure 4D*, compared to the effect in the miR-29c-3p-NC group, overexpression of miR-29c-3p significantly repressed the relative luciferase activity in the co-transfected psiCHECK-2-WT group. However, there was no significant difference in the co-transfected psiCHECK-2-MUT group. Similarly, overexpression of miR-223-3p significantly repressed the relative luciferase activity in the co-transfected psiCHECK-2-WT group cells. All these experiments demonstrate that miR-29c-3p and miR-223-3p can target ANGPTL4 and regulate the protein expression level of ANGPTL4.

MiR-29c-3p and miR-223-3p regulate the proliferation and cisplatin resistance of OSCC cell

To determine whether ANGPTL4 regulate the function of



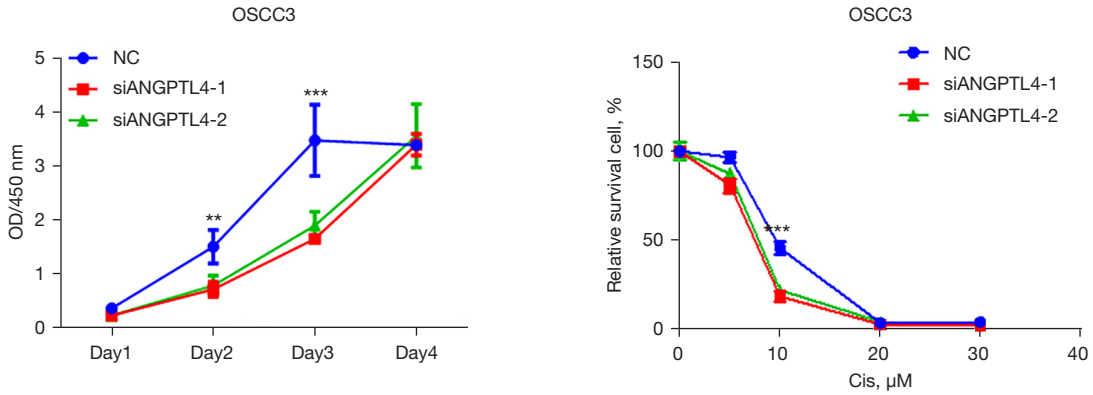


Figure 2 Effects of ANGPTL4 on the biological function of OSCC cells. (A) Western blot and qPCR experiments showed that the protein and mRNA levels of ANGPTL4 of HSC6 and OSCC3 cells were decreased after transfection with siANGPTL4. (B) After fixing solution, 0.4% crystal violet dye solution was added and incubated for 15 min, the dye was slowly washed away with running water and the cells were air-dried. Plate colony formation assay showed that the colony formation efficiency of HSC6 and OSCC3 cells decreased after ANGPTL4 knockdown of (colony formation efficiency = number of clones/number of inoculated cells ×100%). (C) OSCC cell proliferation was inhibited upon downregulation of ANGPTL4, according to the CCK-8 assay. (D) Cisplatin chemoresistance upon downregulation of ANGPTL4. Cell relative survival rate = (OD value – OD value of blank hole)/(OD value of 0 μM – OD value of blank hole) ×100%. *, P<0.05; **, P<0.01; ***, P<0.001. NC, normal contrast; ANGPTL4, angiopoietin-like protein 4; OSCC, oral squamous cell carcinoma; CCK-8, Cell Counting Kit-8; OD, optical density.

OSCC through the involvement of miR-29c-3p and miR-223-3p, we explored the function of microRNA in OSCC. The results of the plate colony formation assay showed that after overexpression of miR-29c-3p or miR-223-3p in HSC6 and OSCC3 cells, the colony formation rate decreased (*Figure 5A*). According to the CCK-8 assay, the growth rate of the HSC6 and OSC3 cells was reduced after miR-29c-3p and miR-223-3p were overexpressed (*Figure 5B*). In addition, it was found that the sensitivity of HSC6 and OSCC3 cells to cisplatin was reduced after miR-29c-3p and miR-223-3p was overexpressed (*Figure 5C*).

Discussion

One view is that ANGPTL4 can stimulate the antiapoptotic effect of tumor cells, promote tumor growth and affects chemotherapy sensitivity (14-16). However, the role of ANGPTL4 is currently debated (17). In this study, high expression of ANGPTL4 was found in OSCC. *In vitro* experiments confirmed that high expression of ANGPTL4 promoted cell proliferation and cisplatin resistance in OSCC.

Many studies have shown that ANGPTL4 is a powerful hypoxia-related gene, that promotes angiogenesis and increases vascular permeability, and can promote the invasion and metastasis of many cancers (18,19). However, in the preliminary experiment of this study, no effect of abnormal ANGPTL4 expression on the migration and invasion of OSCC cells was observed.

MiRNAs regulate protein synthesis by base-pairing to target mRNAs. Studies have shown that miR-29c-3p is expressed at low in colorectal cancer and affects cell proliferation and migration by targeting SPARC (20). In addition, in laryngeal squamous cell carcinoma, its expression is closely related to tumor TNM (tumor node metastasis) stage and lymph node metastasis (21). In head and neck squamous cell carcinoma, the expression of miR-29c-3p is correlated with survival and recurrence rates (22). In this study, consistent with previous results, miR-29c-3p targeted ANGPTL4, and its high expression in OSCC cells led to the downregulation of ANGPTL4, thus reducing cell proliferation and cisplatin resistance. The expression of miR-223-3p is abnormal in a variety of diseases. For example, the expression of miR-223-3p downregulated in clear cell renal cell carcinoma (23) and ossified fibroma (24), suggesting that miR-223-3p may be involved in the occurrence and development of a variety of diseases.

In this study, the role of ANGPTL4 in OSCC was verified, and it is believed that ANGPTL4 can promote the proliferation and cisplatin resistance of OSCC cells.

A

miRNA: miR-29c-3p
Target region: 19–38

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miRNA:   3'  auUGGCUAAAGUUUACCACGAu  5'
                |  ||     ||   ||||||||
Target:  5'  agAGCGCCCTC - - ATGGTGCTg  3'
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miRNA: miR-223-3p
Target region: 14–37

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miRNA:   3'  accccauaaACUGU--UUGACUGu  5'
                          |  |||  : ||||||
Target:  5'  cggactcagTCACA TTGACTGACg  3'
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B

Cancer Abbreviation	Tumor Log2 Mean Expression	Normal Log2 Mean Expression	T-Test P-value
BRCA	10.50	10.74	6.75e-03
CHOL	10.38	11.37	3.29e-03
ESCA	10.53	11.78	3.38e-03
HNSC	10.39	12.45	2.57e-12
KICH	10.71	11.50	2.30e-04
KIRC	10.28	10.62	9.75e-04
KIRP	10.49	11.73	5.21e-07
LIHC	11.02	12.04	4.55e-08
LUSC	10.73	12.29	5.96e-13
STAD	11.35	12.10	3.68e-03
THCA	12.32	12.94	1.45e-12

C

miRNA Name	Clinical Parameter	ANOVA P-value
MiR-29c-3p	Clinical N Status	6.30e-01
MiR-29c-3p	Clinical T Status	4.98e-02
MiR-29c-3p	Histologic Grade	3.09e-04
MiR-29c-3p	Pathologic N Status	1.42e-02
MiR-29c-3p	Pathologic Stage	2.86e-02
MiR-29c-3p	Pathologic T Status	3.13e-04
MiR-223-3p	Clinical M Status	6.09e-02
MiR-223-3p	Clinical N Status	4.01e-02
MiR-223-3p	Clinical Status	4.98e-02
MiR-223-3p	Clinical T Status	7.05e-02
MiR-223-3p	Histologic Grade	8.41e-02
MiR-223-3p	Pathologic N Status	4.25e-02
MiR-223-3p	Pathologic Stage	8.74e-02
MiR-223-3p	Pathologic T Status	1.15e-01

D

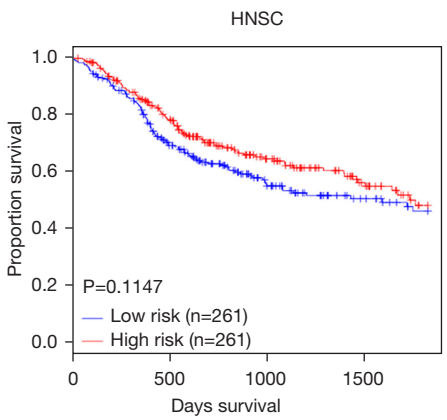


Figure 3 ANGPTL4 is a target of miR-29c-3p and miR-223-3p. (A) The target sites of miR-29c-3p and miR-223-3p on ANGPTL4 mRNA. (B) The OncomiR database showed low expression of miR-29c-3p in a variety of tumours. (C) The OncomiR database showed that the expression of miR-29c-3p and miR-223-3p were correlated with clinical and pathological characteristics of HNSC. (D) The OncomiR database showed patients with high expression of miR-29c-3p in HNSC has a high survival rate. ANGPTL4, angiopoietin-like protein 4; HNSC, head and neck squamous carcinoma; miRNA, microRNA; BRCA, breast invasive carcinoma; CHOL, cholangiocarcinoma; HNSC, head and neck squamous cell carcinoma; KICH, kidney chromophobe; KIRC, kidney renal clear cell carcinoma; KIRP, kidney renal papillary cell carcinoma; LIHC, liver hepatocellular carcinoma; LUSC, lung squamous cell carcinoma; STAD, stomach adenocarcinoma; THCA, thyroid carcinoma.

Our experiments confirmed that miR-29c-3p and miR-223-3p can regulate ANGPTL4, and the extent of cell proliferation and cisplatin resistance was changed after miR-29c-3p and miR-223-3p were overexpressed. Consistent with our results, many studies have shown that microRNAs can induce mRNA affect mRNA translation (25). In addition, miRNA can also reduce the stability of mRNA by promoting the aggregation of protein complexes, resulting in down-regulation of the target gene (26). Our result showed that the protein levels of ANGPTL4 were decreased after the overexpression of microRNA (*Figure 4B*). However, how miR-29C-3p and miR-223-3p affects ANGPTL4 expression needs to be further verified in future experiments.

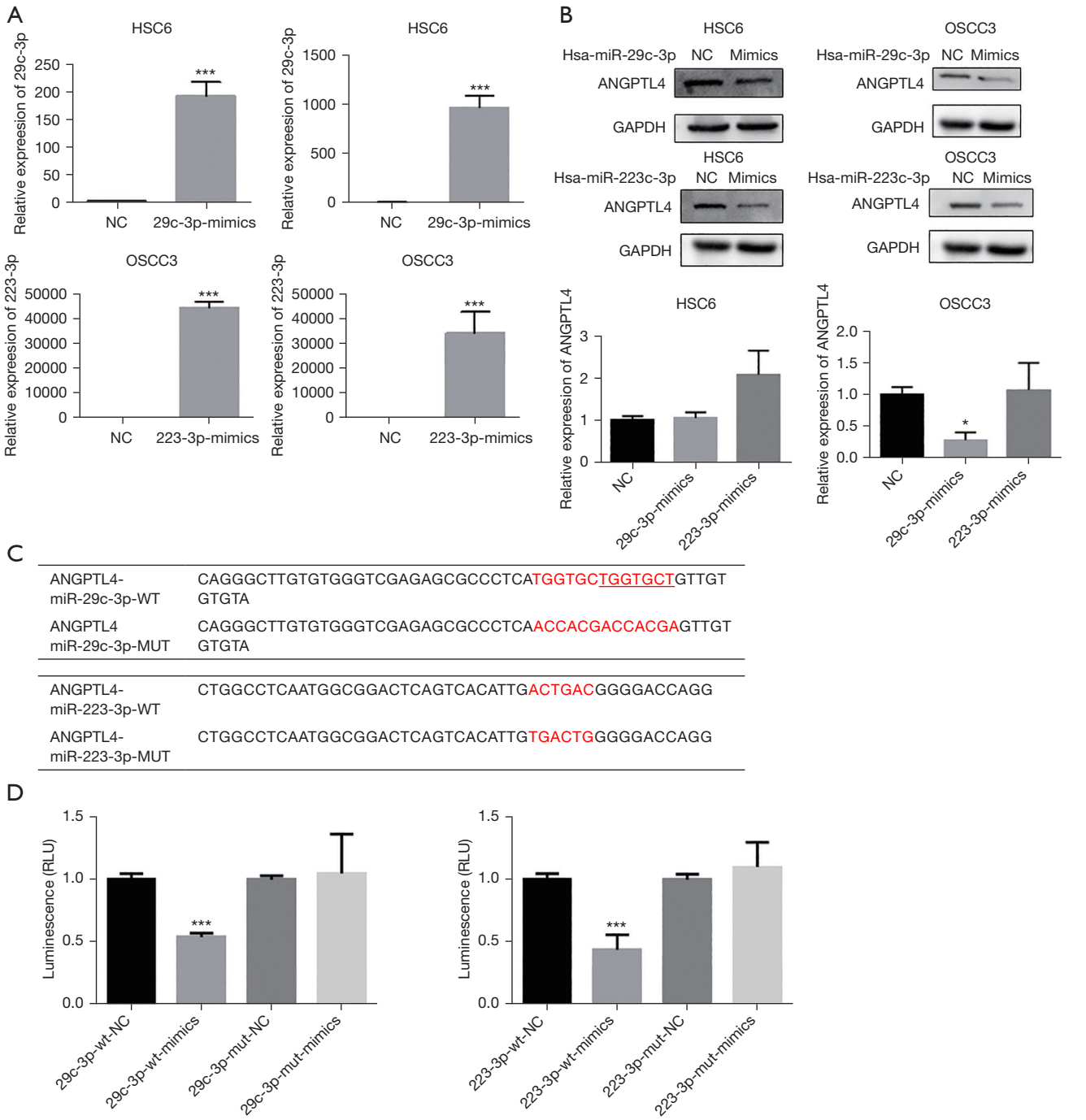
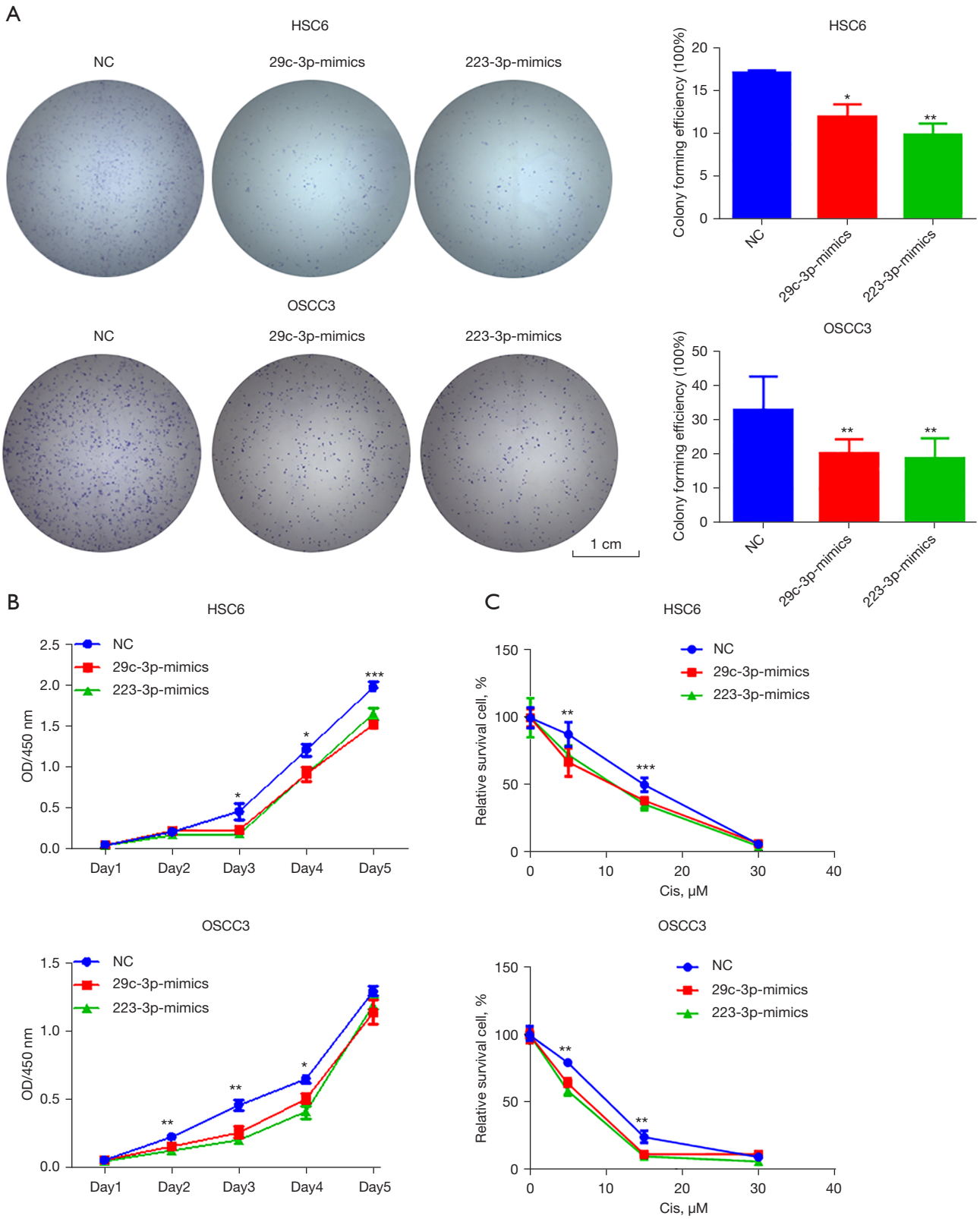


Figure 4 ANGPTL4 mRNA targeted by miR-29c-3p and miR-223-3p. (A) qRT-PCR was used to verify the expression levels of miR-29c-3p and miR-223-3p after ANGPTL4 was overexpressed in OSCC3 and HSC6 cells. (B) Western blot assay verified the protein expression level of ANGPTL4 decreased after increased the expression of miR-29c-3p in OSCC3 and HSC6 cells. RT-qPCR show that mRNA expression level of ANGPTL4 in OSCC cells was not affected by overexpression of two miRNA but it was decreased in OSCC3 cell after overexpression of miR-29C-3p. (C) The 3'UTR segments with the miR-29c-3p and miR-223-3p targeted in ANGPTL4 mRNA was cloned and expressed in the psiCHECK-2 vector, and the plasmid mutated miRNA-targeting region was also constructed. (D) The dual luciferase reporter assay demonstrated that ANGPTL4 targeted by miR-29c-3p and miR-223-3p. *, P <0.05; ***, P<0.001. ANGPTL4, angiopoietin-like protein 4; OSCC, oral squamous cell carcinoma; NC, normal contrast.



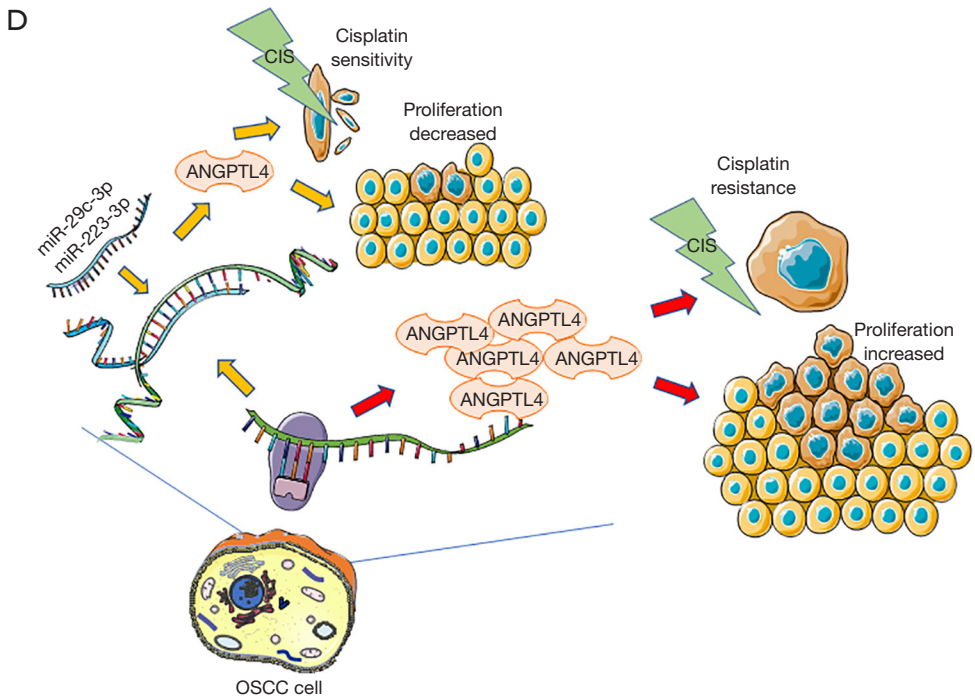


Figure 5 MiR-29c-3p and miR-29c-3p regulate the proliferation and cisplatin resistance of OSCC cells. (A) After fixing solution, 0.4% crystal violet dye solution was added and incubated for 15 min, the dye was slowly washed away with running water and the cells were air-dried. The plate colony formation assay showed that the colony formation efficiency of HSC6 and OSCC3 decreased after miR-29c-3p and miR-29c-3p were overexpressed (colony formation efficiency = number of clones/number of inoculated cells ×100%). (B) OSCC cell proliferation was inhibited upon the overexpression of miR-29c-3p and miR-29c-3p, according to the CCK-8 assay. (C) Cisplatin chemoresistance upon overexpression of miR-29c-3p and miR-29c-3p, according to the CCK-8 assay, cell relative survival rate = (OD value – OD value of blank hole)/(OD value of 0 μM – OD value of blank hole) ×100%. (D) Mechanism schematic model of the miR-29c-3p/ANGPTL4 and miR-29c-3p/ANGPTL4 axes in OSCC. *, P <0.05; **, P<0.01; ***, P<0.001. OSCC, oral squamous cell carcinoma; CCK-8, Cell Counting Kit-8; OD, optical density; NC, normal contrast; CIS, cisplatin; ANGPTL4, angiopoietin-like protein 4.

Conclusions

In general, this study found that ANGPTL4 can play a role in promoting the proliferation of OSCC cells, and the sensitivity of OSCC cells to cisplatin increased after ANGPTL4 knockdown. MiR-29C-3p and miR-223-3p can target ANGPTL4 and affect the protein expression level of ANGPTL4 in OSCC. After miR-29c-3p and miR-223-3p were overexpressed, the level of ANGPTL4 in the OSCC cells decreased, the proliferation ability of the OSCC cells decreased, and cell resistance to cisplatin was decreased. Therefore, miR-29c-3p and miR-223-3p are thought to be diagnostic and therapeutic targets of OSCC (*Figure 5D*).

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://fomm.amegroups.com/article/view/10.21037/fomm-21-114/rc

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Conflicts of Interest: All authors have completed the ICMJE

uniform disclosure form (available at https://fomm.amegroups.com/article/view/10.21037/fomm-21-114/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Sun Yat-sen Memorial Hospital Institutional Review Board (No. SYSEC-KY-KS-2021-028) and informed consent was taken from all the patients.

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Supplementary

Table S1 Information of primers for qRT-PCR and PCR

Primer	Sequence
ANGPTL4[a]	GCTGGACAGTAATTCAGAGGCG
ANGPTL4[b]	AGTGGAGAAGGGTACGGAGAGG
Has-miR-29c-3p	TCGGCAGGTAGCACCATTTGAAATCG
Has-miR-29c-3p[c]	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTAACCGAT
Has-miR-223-3p	TCGGCAGGTGTCAGTTTGTCAAATACCC
Has-miR-223-3p[c]	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTGGGGTAT
universal- Reverse	GTCGTATCCAGTGCAGGGTCCG
GAPDH[a]	GAGTCAACGGATTTGGTCGT
GAPDH[b]	GACAAGCTTCCCGTTCTCAG
U6[a]	CTCGCTTCGGCAGCACA
U6[b]	AACGCTTCACGAATTTGCGT
ANGPTL4-miR-29c-3p-WT	CAGGGCTTGTGTGGGTCGAGAGCGCCCTCATGGTGCTGGTGCTGTTGTGTGTA
ANGPTL4-miR-29c-3p-Mut	CAGGGCTTGTGTGGGTCGAGAGCGCCCTCAACCACGACCACGAGTTGTGTGTA
ANGPTL4-miR-223-3p-WT	CTGGCCTCAATGGCGGACTCAGTCACATTGACTGACGGGGACCAGG
ANGPTL4-miR-223-3p-Mut	CTGGCCTCAATGGCGGACTCAGTCACATTGTGACTGGGGGACCAGG

a: Forward primer; b: Reverse primer; c: Reverse transcription primers.

Table S2 Small interfering RNA (siRNA) and miRNA-mimic used in transfection

Name	Sense (5'-3')	Antisense (5'-3')
siANGPTL4-1	GCCUGCAGACACAACUCAATT	UUGAGUUGUGUCUGCAGGCTT
siANGPTL4-2	CCAUGUUGAUCCAGCCCAUTT	AUGGGCUGGAUCAACAUGGTT
siRNA-NC	UUCUCCGAACGUGUCACGUTT	ACGUGACACGUUCGGAGAATT
miR-29c-3p-mimic	UAGCACCAUUUGAAAUCGGUUA	ACCGAUUUCAAAUGGUGCUAUU
miR-223-3p-mimic	UGUCAGUUUGUCAAAUACCCCA	GGGUAUUUGACAAACUGACAUU
mimic-NC	UUCUCCGAACGUGUCACGUTT	ACGUGACACGUUCGGAGAATT

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