MiR-29c-3p and MiR-223-3p Regulate the Proliferation and Drug Resistance of OSCC by Targeting ANGPTL4

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Research

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Abstract

Background

Oral cancer is one of the most common malignant tumors of the head and neck. MicroRNA are reported to be involved in the regulation of posttranscriptional gene expression. The aim of this research is to determine the expression and function of ANGPTL4 in OSCC, and to explore whether miR-29c-3p and miR-223-3p targeting ANGPTL4 regulate the proliferation and cisplatin resistance of OSCC.

Methods

A bioinformatics database was used to identify the expression of ANGPTL4 and miRNAs in tumors, and qPCR and WB were used to determine the protein and mRNA expression of ANGPTL4 and miRNA in tissues and cells. The proliferation of the cells was determined by the plate colony formation and CCK-8 assays, the drug resistance of the cells was determined by IC50 measurements, and a dual luciferase reporter assay was used to determine the regulation of miRNA on ANGPTL4. Independent sample t test was used for the parameters that followed a normal distribution, otherwise, non-parametric test was used.

Results

ANGPTL4 expression was higher in the OSCC tissues than in the normal 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 WT group decreased after miR-29c-3p and miR-223-3p were overexpression, while that of the MUT group was almost unchanged. The overexpression of miR-29c-3p and miR-223-3p decreased the protein levels of ANGPTL4. In addition, the proliferation of OSCC cells decreased, and cell resistance to cisplatin was decreased.

Conclusion

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

Introduction

The incidence of lip and oral cavity cancer in the world is greater than 350,000 every year, and the mortality rate is higher than 170,000^[1]. Oral cancer, as one of the most common malignant tumors in the head and neck, accounting for 1-2% of all malignant tumors worldwide^[2]. Because OSCC is prone to metastasis and has a poor prognosis, the overall 5-year survival rate is only 55%^[3], and the treatment of OSCC has been a long-standing concern.

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^[4]. Other studies have shown that ANGPTL4 is

considered a potential marker and therapeutic target for preventing the progression and metastasis of tongue squamous cell carcinoma (TSCC) [5]. 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[6]. At the same time, some studies have suggested that ANGPTL4 plays an anticancer role in liver cancer and other cancers[7]. ANGPTL4 plays dual functions as a tumor suppressor and promotor, 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 (HNSCC) and other cancers[8]. 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 the biological behavior of OSCC. Although previous studies have shown that ANGPTL4 may regulate the migration and invasion of tumors, the results of this study showed that miRNAs may have an influence on the proliferation and drug resistance of OSCC by targeting ANGPTL4. We know that the proliferation and drug resistance of OSCC play an important role in the occurrence, development and treatment of OSCC, so we pay attention to the aspects of proliferation and drug resistance. It is expected to contribute to the diagnosis and treatment of OSCC as well as the prognosis of the prognosis.

Materials And Methods

1. Tissue collection

This study was approved by the Ethics Committee of Sun Yat-sen Memorial Hospital, Sun Yat-sen University. 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°C for use. All patients signed informed consent.

2. Cell culture and treatment

Experiments were performed with HOK, SCC9 UM1, HSC6, HSC3, FADU, 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°C humidified incubator containing 5% CO₂, among which HOK, HSC6, HSC3,

FADU, CAL27 and OSCC3 cells were cultured in DMEM, F12/DMEM was used to culture SCC9 and UM1 cells.

3. RNA Extraction and Quantitative Real-time Polymerase Chain Reaction (qRT-PCR)

Total mRNA extraction and reverse transcription: 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^[9]. In qRT-PCR, 1 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. The primers were used to detect the relative expression of mRNA in a Light Cycler 480 II Real-time PCR system (Roche), and statistical software was used for statistical analysis. The reaction conditions were as follows: 94°C for 2 min, 94°C for 20 s, 58°C for 20 s, 72°C 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 ΔC_t or $2^{-\Delta\Delta C_t}$ method. As shown in Table 1, all PCR primers were purchased from IGEBO (Guangzhou, China).

4. 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°C 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 1:1000 in 5% bovine serum albumin with 0.1% Tween-20 Tris buffer saline) for 16 h (incubation overnight) in a 4°C 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 1:1000 in 5% bovine serum albumin with 0.1% Tween-20 Tris buffer saline) for 1 h in room temperature. Chemiluminescence, photography and gel image analysis were carried out according to the standard protocol.

5. Small interfering RNA (siRNA) and miRNA-mimic transfection

MiR-29c-3p mimics, miR-223-3p mimics and a negative control (miR-NC), small interfering RNAs (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 2. We performed siRNA transfection with Lipofectamine 3000 (Invitrogen, USA) according to the manufacturer's instructions.

6. 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 cells 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).

7. Growth curve

The cell growth curve was generated according to the instructions of the manufacturer of the CCK-8 kit (#K1018, APExBio, USA). 24 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 OD value was read 450nm with an TECAN Spark10M and recorded.

8. 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 was added to 96-well plates according to the concentration gradient and cultured in a constant temperature incubator at 37°C, with 5% CO₂ 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 inhibition rate was as follows: cell proliferation inhibition rate = (control group OD value - experimental group OD value) / control group OD value × 100%.

9. Predicted miRNAs targeting ANGPTL and dual luciferase reporter assay

The interaction between miR-29c-3p or miR-223-3p and ANGPTL4 mRNA was predicted by analysis using TargetScan (<http://www.targetscan.org>) online datasets. We further analysed the expression of these MiRNAs in OSCC with the OncomiR database (<http://www.oncomir.org/>). Wild-type and mutant ANGPTL4 sequences were inserted into the psiCHECK-2 vector. Luciferase reporter genes were co-transfected with miRNA-mimics or miRNA-NC into cells using LIP2000 transfection reagents (Invitrogen, USA). The luciferase activity was measured using a dual-luciferase reporter assay system (#E1910, Promega, China).

10. 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 $\pm$ standard deviation ($M \pm SD$). 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 were 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

1. Expression of ANGPTL4 in OSCC

ANGPTL4 plays different functions in different tissues. Currently, ANGPTL4 is a poor prognostic marker of renal cancer, colorectal cancer and lung cancer. 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 of 7 OSCC cell lines was higher than that in HOK cells (Figure 1c). Therefore, the results of this study show that ANGPTL4 is highly expressed in OSCC.

2. 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 WB and qPCR (Figure 2a). The results of plate colony formation experiments and CCK-8 assays showed that the proliferation efficiency of OSCC decreased after knocking down ANGPTL4 (Figure 2b, c). The cells were treated with CIS, a common chemotherapy drug used to treat OSCC, and it was found that the sensitivity of HSC6 and OSCC3 cells to cisplatin was increased after ANGPTL4 was knocked down (Figure 2d).

3. Screening miRNAs that potentially regulate ANGPTL4

MiRNAs are involved in the regulation of posttranscriptional gene expression. The dysregulated expression of miRNAs usually leads to the abnormal expression of target genes. To explore the mechanism of ANGPTL4 upregulation in tumors, we analysed miRNAs that may target ANGPTL4. We used TargetScan (<http://www.targetscan.org>) and StarBase (<http://starbase.sysu.edu.cn/>) 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 OSCC with the OncomiR database (<http://www.oncomir.org/>). It was found that the expression of miR-29c-p 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 expression of miR-29c-3p was positively correlated with the survival rate of HNSC patients (Figure 3d). In general, miR-29c-3p and miR-223-3p may play regulatory roles by targeting ANGPTL4 in OSCC.

4. 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 OSC3 cells was verified by qPCR (Figure 4a). The protein expression level of ANGPTL4 was decreased after the overexpression of miR-29c-3p was induced in OSCC and HSC6 cells. Similarly, the level of ANGPTL4 decreased after miR-29c-3p was overexpressed (Figure 4b). However, mRNA expression level of ANGPTL4 in HSC6 cell was not affected by overexpression of miR-29C-3p and miR-223-3p, and it was decreased in OSCC3 cell after overexpression of miR-29C-3p (Figure 4b).

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.

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

To determine whether ANGPTL4 regulate the function of 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, cisplatin resistance was reduced (Figure 5c).

Discussion

The ANGPTL4 gene is located on chromosome 19p131 and contains 7 exons and 6 introns. The full length of ANGPTL4 cDNA is 1943 bp, and the open reading frame is 1218 bp. The molecular weight is approximately 60 kD^[10], Full-length ANGPTL4 contains an N-terminal domain and a COOH terminal fibrinoid domain. 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. One view is that ANGPTL4 can

stimulate the antiapoptotic effect of tumor cells and promote tumor growth^[11]. This has been verified in colorectal cancer^[12] and liver cancer^[13], and ANGPTL4 promotes tumor proliferation and affects chemotherapy sensitivity. In addition, through its involvement in DNA damage repair, ANGPTL4 has a significant effect on the radiotherapy sensitivity of cervical squamous cell carcinoma^[14, 15]. Moreover, ANGPTL4 is highly expressed in gastric cancer, oesophageal cancer and other tumors^[16]. However, the role of ANGPTL4 is currently debated, as it plays a tumor suppressive role in lung cancer and hepatoblastoma^[17]. Therefore, the role of ANGPTL4 has not been determined. 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, findings consistent with previous studies on oral tumors^[18].

Many studies have shown that ANGPTL4 is a powerful hypoxia-related gene^[19], that promotes angiogenesis and increases vascular permeability^[20, 21], and can promote the invasion and metastasis of breast cancer^[22], lung cancer, liver cancer^[23], and gastrointestinal tumors^[24]. However, in this study, no effect of abnormal ANGPTL4 expression on the migration and invasion of OSCC cells was observed.

Studies have shown that miR-29c-3p is expressed at low in colorectal cancer and affects cell proliferation and migration by targeting SPARC^[25]. In addition, in laryngeal squamous cell carcinoma, its expression is closely related to tumor TNM stage and lymph node metastasis^[26]. In head and neck squamous cell carcinoma, the expression of miR-29c-3p is correlated with survival and recurrence rates^[27]. 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^[28], ossified fibroma^[29], and primary biliary cholangitis^[30], 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. RT-qPCR, WB and dual luciferase reporting 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, numerous studies have shown that microRNAs can induce mRNA affect mRNA translation^[31, 32]. 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^[33, 34]. Our result showed that the protein levels of ANGPTL4 were decreased after the overexpression of microRNA (Fig. 4B). The results of those studies may also explain the phenomenon of decreased mRNA level of ANGPTL4 when miR-29C-3p was overexpressed in OSCC3 cells (Fig. 4B). In conclusion, miR-29c-3p and miR-223-3p may affect cell proliferation and cisplatin sensitivity in OSCC by targeting ANGPTL4.

Conclusion

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).

Abbreviations

MiR: micro RNA; OSCC: oral squamous cell carcinoma; ANGPTL4: angiopoietin-like 4; qPCR: quantitative real-time PCR; RT-qPCR: real-time quantitative real-time PCR; WB: western-blot

CCK-8: cell counting kit-8; IC50: half maximal inhibitory concentration; TSCC: tongue squamous cell carcinoma; HNSCC: head and neck squamous carcinoma; PBS: phosphate balanced solution; siRNA: Small interfering RNA; NC: negative control; SPARC: secreted protein acidic and rich in cysteine.

Declarations

Ethics approval and consent to participate

The Ethics Committee of Sun Yat-sen Memorial Hospital has approved this project (SYSEC-KY-KS-2021-028).

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

Not applicable.

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Authors' contributions

Zhiquan Huang: Conceptualization, Methodology, Funding acquisition, Writing - Review & Editing.

Lijuan Bian: Methodology, Project administration, Writing - Original Draft, Validation.

Xi Rui: Data Curation, Data Analysis, Writing - Original Draft.

Zixian Huang: Data Analysis, Data Interpretation, Validation.

Yan Wang: Data Curation, Data Interpretation.

All authors read and approved the final manuscript.

References

- [1]. Bray, F., et al., Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*, 2018. 68(6): p. 394-424.
- [2]. Ferlay, J., et al., Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer*, 2015. 136(5): p. E359-86.
- [3]. Torre, L.A., et al., Global cancer statistics, 2012. *CA Cancer J Clin*, 2015. 65(2): p. 87-108.
- [4]. Tanaka, J., et al., ANGPTL4 regulates the metastatic potential of oral squamous cell carcinoma. *J Oral Pathol Med*, 2015. 44(2): p. 126-33.
- [5]. Wang, Z., et al., Expression of angiopoietin-like 4 and tenascin C but not cathepsin C mRNA predicts prognosis of oral tongue squamous cell carcinoma. *Biomarkers*, 2010. 15(1): p. 39-46.
- [6]. Huang, Z., et al., The downregulation of ANGPTL4 inhibits the migration and proliferation of tongue squamous cell carcinoma. *Arch Oral Biol*, 2016. 71: p. 144-149.
- [7]. Xu, A., et al., Angiopoietin-like protein 4 decreases blood glucose and improves glucose tolerance but induces hyperlipidemia and hepatic steatosis in mice. *Proc Natl Acad Sci U S A*, 2005. 102(17): p. 6086-91.
- [8]. Lan, H., et al., MicroRNAs as potential biomarkers in cancer: opportunities and challenges. *Biomed Res Int*, 2015. 2015: p. 125094.
- [9]. Chen, C., et al., Real-time quantification of microRNAs by stem-loop RT-PCR. *Nucleic Acids Res*, 2005. 33(20): p. e179.
- [10]. Kim, I., et al., Hepatic expression, synthesis and secretion of a novel fibrinogen/angiopoietin-related protein that prevents endothelial-cell apoptosis. *Biochem J*, 2000. 346 Pt 3: p. 603-10.

- [11]. Zhu, P., et al., Angiopoietin-like 4 protein elevates the prosurvival intracellular O₂(-):H₂O₂ ratio and confers anoikis resistance to tumors. *Cancer Cell*, 2011. 19(3): p. 401-15.
- [12]. Kim, S.H., et al., ANGPTL4 induction by prostaglandin E₂ under hypoxic conditions promotes colorectal cancer progression. *Cancer Res*, 2011. 71(22): p. 7010-20.
- [13]. Zhang, Z., et al., Acquisition of anoikis resistance reveals a synoikis-like survival style in BEL7402 hepatoma cells. *Cancer Lett*, 2008. 267(1): p. 106-15.
- [14]. Xu, A., et al., Angiopoietin-like protein 4 decreases blood glucose and improves glucose tolerance but induces hyperlipidemia and hepatic steatosis in mice. *Proc Natl Acad Sci U S A*, 2005. 102(17): p. 6086-91.
- [15]. Clement, L.C., et al., Podocyte-secreted angiopoietin-like-4 mediates proteinuria in glucocorticoid-sensitive nephrotic syndrome. *Nat Med*, 2011. 17(1): p. 117-22.
- [16]. Yi, J., et al., Clinical significance of angiopoietin-like protein 4 expression in tissue and serum of esophageal squamous cell carcinoma patients. *Med Oncol*, 2013. 30(3): p. 680.
- [17]. Li, K.Q., et al., Anti-tumor effect of recombinant retroviral vector-mediated human ANGPTL4 gene transfection. *Chin Med J (Engl)*, 2004. 117(9): p. 1364-9.
- [18]. Hu, J., et al., Angiopoietin-like 4: a novel molecular hallmark in oral Kaposi's sarcoma. *Oral Oncol*, 2011. 47(5): p. 371-5.
- [19]. Le Jan, S., et al., Angiopoietin-like 4 is a proangiogenic factor produced during ischemia and in conventional renal cell carcinoma. *Am J Pathol*, 2003. 162(5): p. 1521-8.
- [20]. Katanasaka, Y., et al., Epidermal growth factor receptor variant type III markedly accelerates angiogenesis and tumor growth via inducing c-myc mediated angiopoietin-like 4 expression in malignant glioma. *Mol Cancer*, 2013. 12: p. 31.
- [21]. Huang, R.L., et al., ANGPTL4 modulates vascular junction integrity by integrin signaling and disruption of intercellular VE-cadherin and claudin-5 clusters. *Blood*, 2011. 118(14): p. 3990-4002.
- [22]. Izraely, S., et al., The metastatic microenvironment: brain-residing melanoma metastasis and dormant micrometastasis. *Int J Cancer*, 2012. 131(5): p. 1071-82.
- [23]. Li, H., et al., Hypoxia-inducible factor 1 alpha-activated angiopoietin-like protein 4 contributes to tumor metastasis via vascular cell adhesion molecule-1/integrin beta1 signaling in human hepatocellular carcinoma. *Hepatology*, 2011. 54(3): p. 910-9.
- [24]. Nakayama, T., et al., Expression of angiopoietin-like 4 (ANGPTL4) in human colorectal cancer: ANGPTL4 promotes venous invasion and distant metastasis. *Oncol Rep*, 2011. 25(4): p. 929-35.

- [25]. Zhang, S., et al., hsa-miR-29c-3p regulates biological function of colorectal cancer by targeting SPARC. *Oncotarget*, 2017. 8(61): p. 104508-104524.
- [26]. Fang, R., et al., Downregulation of miR-29c-3p is associated with a poor prognosis in patients with laryngeal squamous cell carcinoma. *Diagn Pathol*, 2019. 14(1): p. 109.
- [27]. Hudcova, K., et al., Expression profiles of miR-29c, miR-200b and miR-375 in tumour and tumour-adjacent tissues of head and neck cancers. *Tumour Biol*, 2016. 37(9): p. 12627-12633.
- [28]. Qin, S., et al., Transcription Factor and miRNA Interplays Can Manifest the Survival of ccRCC Patients. *Cancers (Basel)*, 2019. 11(11).
- [29]. Pereira, T., et al., MicroRNA profiling reveals dysregulated microRNAs and their target gene regulatory networks in cemento-ossifying fibroma. *J Oral Pathol Med*, 2018. 47(1): p. 78-85.
- [30]. Wang, X., et al., MicroRNA-223 and microRNA-21 in peripheral blood B cells associated with progression of primary biliary cholangitis patients. *PLoS One*, 2017. 12(9): p. e0184292.
- [31]. Bartel, D.P., MicroRNAs: target recognition and regulatory functions. *Cell*, 2009. 136(2): p. 215-33.
- [32]. Baek, D., et al., The impact of microRNAs on protein output. *Nature*, 2008. 455(7209): p. 64-71.
- [33]. Guo, H., et al., Mammalian microRNAs predominantly act to decrease target mRNA levels. *Nature*, 2010. 466(7308): p. 835-40.
- [34]. Eichhorn, S.W., et al., mRNA destabilization is the dominant effect of mammalian microRNAs by the time substantial repression ensues. *Mol Cell*, 2014. 56(1): p. 104-15.

Tables

Table 1. 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	GTCGTATCCAGTGCAGGGTCCGAGGTATTGCGCACTGGATACGACTAACCGAT
Has-miR-223-3p	TCGGCAGGTGTCAGTTTGTCAAATACCC
Has-miR-223-3p ^c	GTCGTATCCAGTGCAGGGTCCGAGGTATTGCGCACTGGATACGACTGGGGTAT
universal- Reverse	GTCGTATCCAGTGCAGGGTCCG
GAPDH ^a	GAGTCAACGGATTTGGTCGT
GAPDH ^b	GACAAGCTTCCCGTTCTCAG
U6 ^a	CTCGCTTCGGCAGCACA
U6 ^b	AACGCTTCACGAATTTGCGT
ANGPTL4- miR-29c-3p-WT	CAGGGCTTGTGTGGGTGCGAGAGCGCCCTCATGGTGCTGGTGCTGTTGTGTGTA
ANGPTL4- miR-29c-3p-Mut	CAGGGCTTGTGTGGGTGCGAGAGCGCCCTCAACCACGACCACGAGTTGTGTGTA
ANGPTL4- miR-223-3p-WT	CTGGCCTCAATGGCGGACTCAGTCACATTGACTGACGGGGACCAGG
ANGPTL4- miR-223-3p-Mut	CTGGCCTCAATGGCGGACTCAGTCACATTGTGACTGGGGGACCAGG

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

Table 2. Small interfering RNA (siRNA) and miRNA-mimic used in transfection.

Name	Sense5'-3'	Antisense5'-3'
siANGPTL4-1	GCCUGCAGACACAACUCAATT	UUGAGUUGUGUCUGCAGGCTT
siANGPTL4-2	CCAUGUUGAUCCAGCCCAUTT	AUGGGCUGGAUCAACAUGGTT
siRNA-NC	UUCUCCGAACGUGUCACGUTT	ACGUGACACGUUCGGAGAATT
miR-29c-3p-mimic	UAGCACCAUUUGAAAUCGGUUA	ACCGAUUUCAA AUGGUGCUAUU
miR-223-3p-mimic	UGUCAGUUUGUCAAUACCCCA	GGGUUUUGACAAACUGACAUU
mimic-NC	UUCUCCGAACGUGUCACGUTT	ACGUGACACGUUCGGAGAATT

Figures

Figure 1

Expression of ANGPTL4 in OSCC. A. TCGA database analysis showed that ANGPTL4 expression in head and neck squamous cell carcinoma was 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 most oral cancer cell lines (SCC9, UM1, HSC6, HSC3, and OSCC3 cells) was higher than that in normal cells (HOK cells). * $P < 0.05$; * * $P < 0.01$; * * * $P < 0.001$

Figure 2

Effects of ANGPTL4 on the biological function of OSCC cells. A. WB and qPCR experiments showed that the protein and mRNA levels of HSC6 and OSCC3 cells were decreased after transfection with ANGPTL4. B. 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.

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 tumors. C. Expression of miR-29c-3p and miR-223-3p were correlated with clinical and pathological characteristics of HNSC. D. Patients with high expression of miR-29c-3p in HNSC has a high survival rate.

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. WB and qRT-PCR verified the changes in ANGPTL4 protein and mRNA levels after overexpression of miR-29c-3p and miR-223-3p. C. The dual luciferase reporter assay demonstrated that ANGPTL4 targeted by miR-29c-3p and miR-223-3p.

Figure 5

MiR-29c-3p and miR-223-3p regulate the proliferation and cisplatin resistance of OSCC cells. A. The plate colony formation assay showed that the colony formation efficiency of HSC6 and OSCC3 decreased after miR-29c-3p and miR-223-3p were overexpressed. B. OSCC cell proliferation was inhibited upon the overexpression of miR-29c-3p and miR-223-3p, according to the CCK-8 assay. C. Cisplatin

chemoresistance upon overexpression of miR-29c-3p and miR-29c-3p D. Mechanism schematic model of the miR-29c-3p/ANGPTL4 and miR-29c-3p/ANGPTL4 axes in OSCC.