MiR-429 Inhibits Oral Squamous Cell Carcinoma Growth by Targeting ZEB1

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Background:
Oral squamous cell carcinoma (OSCC) is the sixth most common human malignancy worldwide. To develop new therapeutics requires elucidation of the underlying mechanism of OSCC pathogenesis. The role of miR-429 in OSCC remains unknown.

Material/Methods:
The level of miR-429 and ZEB1 in OSCC tissues and cell lines was measured by qRT-PCR. MiR-429 was down-regulated by miRNAs antisense oligonucleotides (ASO) transfection and up-regulated by miRNAs mimics. Cell proliferation was analyzed by MTT assay. Cell apoptosis was revealed by FACS analysis. Targeted genes were predicted by a bioinformatics algorithm and confirmed by a dual luciferase reporter assay.

Results:
MiR-429 was down-regulated in OSCC tissues, and miR-429 overexpression inhibited OSCC cell lines growth and vice versa. Further, we found that miR-429 could inhibit zinc finger E-boxbinding homebox 1 (ZEB1) expression, and that miR-429 and ZEB1 expression in OSCC tissues were negatively correlated.

Conclusions:
Our data demonstrate the tumor suppressor role of miR-429 in OSCC, and may provide a potential therapeutic target that warrants further investigation.

MeSH Keywords:
Cell Growth Processes • MicroRNAs • Mouth Neoplasms

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Background

Oral squamous cell carcinoma (OSCC) is a significant global disease and is the sixth most common human malignancy worldwide. Importantly, the incidence rate is increasing, especially in younger people [1,2]. Although the incidence of OSCC accounts for only 3% of all cases of cancer worldwide, this disease is considered a critical public health threat due to the relatively low survival compared to the major cancers [3]. In China, over 11,900 cases of oral cancers are diagnosed each year and approximately 5,000 patients die of the disease [4]. A number of factors are associated with the increase of risks of oral cancer. The risk factors include age, tobacco and alcohol consumption, human papilloma virus infection, and race [5–7]. OSCC carcinogenesis occurs by a multistep process in which gene mutation or modifications were accumulated [8]. Although many molecules involved in the OSCC pathogenesis have been identified, the 5-year survival rate of OSCC remains very low [9]. The development of new therapies requires elucidation of the underlying mechanism of OSCC pathogenesis.

MicroRNAs (miRNAs) are a class of endogenous, non-coding, single-stranded, small regulatory RNA molecules, which are approximately 22 nucleotides in length [10]. MiRNAs inhibit translation and cleave mRNA by base-pairing to the 3’ untranslated region of the target genes [11–13]. Derepression or dysfunction of miRNAs contributes to cancer development [14–19]. In OSCC, for example, down-regulation of miR-375 promotes proliferation via MYC protein [20], and decreased expression of miR-125b and miR-100 in oral cancer cells contributes to malignancy [21].

A previous study suggested that miR-429 may be involved in the pathogenesis of OSCC [22]. MiR-429 belongs to the miR-200 family, which plays a very important role in epithelial-mesenchymal transition (EMT) [23]. Another study revealed that miR-429 up-regulation induces apoptosis and suppresses invasion by targeting Bcl-2 and SP-1 in esophageal carcinoma [24].

In this study, we focused on the role of miR-429 in OSCC. We found that miR-429 could inhibit OSCC cell lines growth, and revealed the gene which miR-429 targeted. We hope that the results of this study will be useful in further investigation of OSCC pathogenesis and provide a potential therapeutic target.

Material and Methods

Patients and sample

Surgical specimens from 66 PDAC OSCC patients and matched tumor-adjacent normal oral tissues were obtained postoperatively from 2006 onward from the Department of Oral and Maxillofacial Surgery, West China College of Stomatology, Sichuan University (Chengdu, China). All patients gave signed, informed consent for their tissues to be used for scientific research. Ethics approval for the study was obtained from West China Hospital, Sichuan University (Chengdu, China). All diagnoses were based on pathological and/or cytological evidence. The histological features of the specimens were evaluated by senior pathologists according to the World Health Organization classification criteria. Tissues were obtained prior to chemotherapy and radiotherapy and were immediately frozen and stored at −80°C prior to qRT-PCR assay. Matched tumor-adjacent normal tissues were used as control.

Cell culture and reagent

HEK293, SCC-25, and CAL27 were obtained from the Cell Bank of the Chinese Academy of Science (Shanghai, China) and cultured in DMEM medium (Hyclone, South Logan, UT, USA) supplemented with 10% fetal bovine serum (HyClone), 2 mM L-glutamine, and 100 μg/mL penicillin/streptomycin (Bio Light, Shanghai, China) as described in previous studies [25,26].

Quantitative real-time PCR (qRT-PCR)

The qRT-PCR analysis for miR-429 was performed by Shengong Company (Shanghai) using standard protocols on an Applied Biosystem’s 7500 HT sequence detection system. This assay includes a reverse-transcription step performed using a High-Capacity Complementary DNA Archive Kit (Applied Biosystems), in which a stem-loop reverse-transcription primer specifically hybridizes with an miRNA and then is reverse-transcribed with MultiScribe reverse transcriptase. miR-429 expression was assessed using a miVana™ qRT-PCR miRNA Detection Kit (Ambion, USA). The following primers were designed and synthesized by Shengong Company (Shanghai, China): MiR-429 sense strand UAAUACUGUCUGGUAACCCGU; miR-429 antisense strand CAAGAUCGGAUCUACGGGUUUU; NC sense strand UUCUCGGACUGUGUCAGUTT; and NC antisense strand ACCUGACAGGUUCGAGAATT. MiRNA-U6 was used as an internal control.

MTT assay

For MTT assay, 5×10^3 cells per well were seeded in triplicate in a 96-well plate with complete growth medium. The viability of the cells was measured over a period of 5 days using the MTT assay (Promega, Fitchburg, WI, USA) as described previously [17,27,28]. The data were read by Microtiter plate reader 570-nm filters (Promega, Fitchburg, WI, USA).

MicroRNA target prediction [29–32]

MiRNA targets were predicted using the following algorithms: TargetScan (https://www.targetscan.org) [33], miRand
MiRNA, plasmid and transfection

MiR-429 mimics, negative control miRNAs, and miR-429 antitense oligonucleotides (miR-429 ASO) were obtained from GenePharma (GenePharma, China). Plasmids were constructed by Genema (Genema, Shanghai). The mutant reporter plasmids were constructed using the QuikChange Mutagenesis Kit (Stratagene, La Jolla, CA). Cells were transfected with synthetic miR-429 (mimics) (Genema, Shanghai), pre-miR-429, negative control (NC), or pcDNA3.1-ZEB1 at a concentration of 50 nM using Lipofectamine 2000 (Invitrogen, Canada) transfection reagent according to the manufacturer’s instructions. Cells were used for further experiments 48 h later.

Luciferase reporter assay

The 3’UTR fragments of ZEB1 containing putative binding sites for miR-429 were cloned into pMIR-Report construct (Ambion, Austin, TX). The primers were constructed by Biomart (Shanghai, China) according to previous publications [38,39] and the details are provided in previous papers [39,40]. Mutant 3’UTR of ZEB1, which carried a mutated sequence in the complementary site for the seed region of miR-429, was generated using the fusion PCR method. Luciferase reporter assay was performed in HEK293 cells as described previously [41].

Western blot and antibodies

Tumor tissues were collected, lysed, and blotted as described previously [16]. Membranes were blocked with blocking solution (5% skim milk in TBST) and incubated with primary antibody, followed by the incubation with appropriate HRP-conjugated secondary antibody. The ZEB1 antibody (anti-ZEB1) was purchased from Santa Cruz Biotechnology, Inc [42]. The densitometry of Western blot results was measured using ImageJ software.

Statistical analysis

Data are presented as the mean ±sd from at least three independent experiments. The difference between the groups was analyzed using two-tailed Student’s t test when only two groups were compared. The difference between the groups was analyzed using ANOVA when three or more groups were compared. The Wilcoxon matched-pairs signed rank test was used to determine if there was a statistically significant difference in the expression of miR-429 between matched pairs. Correlation analysis was performed by two-tailed Pearson’s correlation coefficient analysis. Statistical analyses were performed using SPSS software (version 17.0). P<0.05 was considered significantly different.

Results

Expression of miR-429 in OSCC tissues

Initially, we collected 66 pairs of OSCC and its matched tumor-adjacent normal oral tissues. Then these tissues were analyzed by qRT-PCR for the miR-429 level. We found that in 52 pairs of OSCC and its matched tumor-adjacent normal oral tissues, miR-429 level in OSCC tissue was lower than in its matched tumor-adjacent normal oral tissues (Figure 1A) and the mean level of miR-429 was lower in OSCC tissues than in matched tumor-adjacent normal oral tissues (Figure 1B). These data indicate that miR-429 may play a role in the pathogenesis of OSCC.

MiR-429 overexpression inhibited OSCC cell lines growth

To further investigate the role of miR-429 in OSCC, we firstly measured the miR-429 level in two OSCC cell lines – SCC-25 and CAL27. We found that the miR-429 levels in SCC-25 and CAL27 were lower in than in normal oral tissues and HEK293 cell line (Figure 2A). Then, we up-regulated the miR-429 level in SCC-25 and CAL27 by miR-429 mimics transfection. The effectiveness of transfection was verified by qRT-PCR (Figure 2B). After miR-429 mimics transfection, cellular proliferation was significantly reduced.
assayed by MTT assay, and we found that up-regulation of miR-429 inhibited SCC-25 and CAL27 proliferation (Figure 2C).

Down-regulation of miR-429 promoted OSCC cell lines growth

We then down-regulated the miR-429 level in SCC-25, CAL27, and HEK293 cell lines by transfecting with miR-429 ASO. The level of miR-429 in the three cell lines was assayed by qRT-PCR 48 h after transfection and we found miR-429 ASO transfection down-regulated the miR-429 level in the three cell lines (Figure 3A). Then the cellular proliferation was assayed by MTT assay. We found that miR-429 ASO transfection mildly promoted cells growth in SCC-25 and CAL27 and greatly promoted cells growth in HEK293 cell lines (Figure 3B).

ZEB1 was targeted by miR-429

Epithelial-mesenchymal transition (EMT) is a critical step in tumor cell invasion and metastasis, and correlates positively with poor patient prognosis [43,44]. E-cadherin transcriptional repressors, ZEB1, are the EMT-inducing transcriptional factors. ZEB1 repress E-cadherin expression and promote cancer cell migration and invasion [45–48]. Previous studies have shown that EMT is also a critical step of the pathogenesis of OSCC [49], and clinically, the level of ZEB1 expression was higher in recurrent OSCC tumor samples but lower in primary lesions [50]. The bioinformatics algorithm predicted that ZEB1 was a potential target gene of miR-429. The five binding sites and mutated sites in ZEB1 are shown in Figure 4A. To confirm that ZEB1 was a potential target gene of miR-429 and the miRNA-target interaction, five binding sites in the 3'UTR of ZEB1 and its mutated version were cloned into luciferase reporter plasmids. MiR-429 mimics and the reporter plasmids were co-transfected into HEK293 cells. We found that in wild type 3' UTR, miR-429 mimics reduced the luciferase activity; however, in the mutated version, the mutated binding site partly restored the luciferase activity and the five mutated binding sites almost restored the luciferase activity (Figure 4B). Next, we transfected CAL27 cell line with miR-429 mimics; 48 h later, the data from Western blot analysis showed that the ZEB1 protein level was inhibited (Figure 4C). Further, the ZEB1 mRNA expression in the 66
OSCC tissues were examined by qRT-PCR, and we found that ZEB1 mRNA level and miR-429 level were negative correlated (Figure 4D). Therefore, our results show that miR-429 plays a role in OSCC via ZEB1.

**Discussion**

Our results show that miR-429 expression is lower in OSCC tissues than in normal control oral tissues. MiR-429 overexpression inhibited OSCC cell lines growth and vice versa. 3’UTR of ZEB1 was targeted by miR-429. Therefore, we conclude that the low miR-429 level in OSCC promoted tumor cells growth.

Our data highlights the role of miR-429 in the growth of OSCC cells. Our study may be the first to reveal the role of miR-429 in OSCC. Previously, miR-429 was shown to have two different roles in cancer. In most cancers, miR-429 level is down-regulated [51,52], which agrees with our data. In endometrial carcinoma and bladder cancer, miR-429 level was up-regulated [53,54]. Higher expression levels of miR-429 are correlated with a poor prognosis in patients with serious ovarian carcinoma [55]. In hepatocellular carcinoma, miR-429 plays the role of an oncogene, is overexpressed in liver cancer tissues, and can be considered as a potential prognostic factor for HCC. Furthermore, overexpression of this miR-429 promoted proliferation and inhibited apoptosis in liver cancer cells [56]. The different roles of miR-429 are possibly due to the fact that miRNAs can down-regulate numerous targets, including oncogenes and tumor suppressor genes. A previous study proved that miR-429 could not only inhibit the proliferation of osteosarcoma cell lines, but also induce more cell apoptosis. MiR-429 plays its role in osteosarcoma by binding the 3’UTR of ZEB1 mRNA, and overexpression of ZEB1 could reverse the proliferation inhibitory effect of miR-429 [40]. This study also revealed the miR-429-ZEB1 pathway in osteosarcoma.

Our data show that ZEB1 mRNA level and miR-429 level were negative correlated, and that miR-429 targeted ZEB1. ZEB1 is a very important protein in epithelial-mesenchymal transition (EMT), which is a critical step in the pathogenesis in various cancers. Whether ZEB1 plays a direct role or are targeted by other miRNAs in OSCC needs further study.
Conclusions

In conclusion, our data proves that low miR-429 level and in OSCC tissues promotes tumor cell growth. MiR-429 exerts its role by targeting ZEB1. We anticipate the results of our study will provide useful information and be a basis for further studies.

Conflict of interest

The authors have declared that no competing interests exist.

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