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Original Article

miR-509 inhibits cancer stemness properties in oral carcinomas via directly targeting PLK1

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KEYWORDS

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Abstract *Background/purpose:* Oral cancer is one of the common cancers worldwide. Emerging evidence has indicated that microRNAs (non-coding RNA molecules of approximately 22 nucleotides in length) are implicated in the regulation of cancer stemness. However, the functional role of microRNA-509 (miR-509) in the characteristics of oral cancer stem cells (CSCs) has not been unraveled.

Materials and methods: The expression level of miR-509 in ALDH1⁺ and sphere oral CSCs was examined by qRT-PCR. The aldehyde dehydrogenase 1 (ALDH1) activity and CD44 expression were assessed using flow cytometry. Self-renewal, transwell migration, and colony formation assays were conducted to measure the CSC phenotypes. Besides, a luciferase reporter assay was used to confirm the direct interaction between miR-509 and its target polo-like kinase 1 (plk1).

Results: We showed the expression of miR-509 was downregulated in the CSCs derived from oral cancer cells (SAS), and upregulation of miR-509 diminished the several CSCs features, including ALDH1 activity, self-renewal capacity, CD44 expression, migration, and colony-forming abilities. Moreover, the result from the luciferase reporter assay validated the direct

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binding of miR-509 to plk1.

Conclusion: Our results suggest that the miR-509/plk1 axis may mediate the cancer stemness in oral cancer, and targeting this axis may attenuate the progression of oral cancer.

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Introduction

Oral cancer includes cancers of the 'oral cavity and oropharynx', excluding the salivary glands and other pharyngeal sites. It ranks as the sixth leading cancer in the world and the high incidence areas comprise countries in South Asia, such as Sri Lanka, India, Pakistan, Taiwan.¹ Numerous studies have suggested that cancer stem cells (CSCs) are implicated in cancer relapse and distant metastasis due to their self-renewal capacity, pluripotency, and aggressiveness.² To study the effects of CSCs on cancer progression, several approaches have been used to identify CSCs in oral cancer, such as examination of aldehyde dehydrogenase 1 (ALDH1) activity,³ the expression of surface marker CD44⁴ or cells with sphere-forming ability.⁵

Various factors have been known to be associated with cancer stemness, such as p53 expression⁶ or Wnt/ β -catenin signaling.⁷ Accumulating evidence has suggested that microRNAs may also serve pivotal roles in the maintenance of oral cancer stemness.⁸ MicroRNAs belong to non-coding RNAs, which refer to RNAs that do not encode proteins. MicroRNAs (~19–22 nucleotides in length) are a type of short non-coding RNAs, and there are long non-coding RNAs (lncRNAs; more than 200 nucleotides in length) that can affect oral carcinogenesis as well.⁹ Additionally, microRNAs (miRs) have been shown to exert their regulatory effects via binding to the targeted mRNAs with the 3'-untranslated region (3'-UTR).

Previous studies have shown that miR-509 was aberrantly expressed in several types of cancers,^{10,11} and it also has been reported to function as a tumor suppressor in tongue squamous cell carcinoma through suppressing epidermal growth factor receptor (EGFR).¹⁰ Nevertheless, whether miR-509 affects the cancer stemness in oral cancer has not been investigated. As such, we aimed to assess the functional role of miR-509 in oral CSCs and explore the potential downstream target of miR-509 in the present study.

Materials and methods

OSCC cell culture, OSCC tissues and oral cancer stem cells

The OSCC cell line SAS was cultivated as previously described.⁵ OSCC tissues were collected after obtaining written informed consent. All procedures were conducted in accordance with the Declaration of Helsinki, and approved by The Institutional Review Board in Chung Shan Medical University Hospital. OSCC tissues were snap-frozen

in liquid nitrogen and stored at -80°C for miR-509 and plk1 expression by qRT-PCR analysis. Isolation of ALDH1+ oral cancer stem cells were conducted by staining cells with ALDEFUOR assay kit (StemCell Technologies, Inc., Vancouver, BC, Canada) and sorted with FACSAria II cell sorter (BD Biosciences, San Jose, CA, USA).⁵

Spheres formation assay

OSCC cells were dissociated and cultured as moral spheres in modified DMEM/F-12 supplemented with N2 (R&D), 10 ng/mL epidermal growth factor (EGF, Invitrogen), 10 ng/mL basic fibroblast growth factor (bFGF, Invitrogen), and penicillin/streptomycin at 10^3 live cells/low-attachment six-well plate (Corning Inc., Corning, NY), and the medium were changed every other day until the moral spheres formation observed in about 2 weeks. For the self-renewal ability evaluation, single cells will be obtained from accurtase treated spheroids and the cell density of passage will be 1000 cells/ml in the serum-free medium.⁵

Quantitative real-time PCR (qRT-PCR)

Total RNA is prepared from cells using Trizol reagent according to the manufacturer's protocol (Invitrogen Life Technologies, Carlsbad, CA). qRT-PCR of mRNAs are reverse-transcribed using the Superscript III first-strand synthesis system for RT-PCR (Invitrogen Life Technologies, Carlsbad, CA). qRT-PCR reactions on resulting cDNAs will be performed on an ABI StepOne™ Real-Time PCR Systems (Applied Biosystems).⁵

Western blot analysis

Western blot analysis followed previously described protocols. The primary antibodies were those against Plk1 and GAPDH markers.⁵

Reporter construction and assay

The 3'UTR sequence of Plk1 was cloned into the pMIR-REPORT vector (Life Technologies, Grand Island, NY) to generate the wild type reporter (WT). A mutant reporter (MUT) construct was generated from the WT by replacing the targeted sequence. Firefly luciferase activity after normalizing to transfection efficiency represented reporter activity. Transfectin lipid transfection reagent was purchased from BioRad Lab (Hercules, CA).¹²

Migration and colony-formation assay

All procedures followed the previously described protocol.⁵

Statistical analysis

Statistical Package of Social Sciences software (version 13.0) (SPSS, Inc., Chicago, IL) was used for statistical analysis. Data from at least triplicate analysis was shown as

mean \pm SD. Student's *t* test was used to determine statistical significance of the differences between experimental groups; *p* values less than 0.05 will be considered statistically significant.

Results

To detect the aberrant expression of non-coding RNA transcripts among parental, ALDH1⁻, ALDH1⁺, and sphere

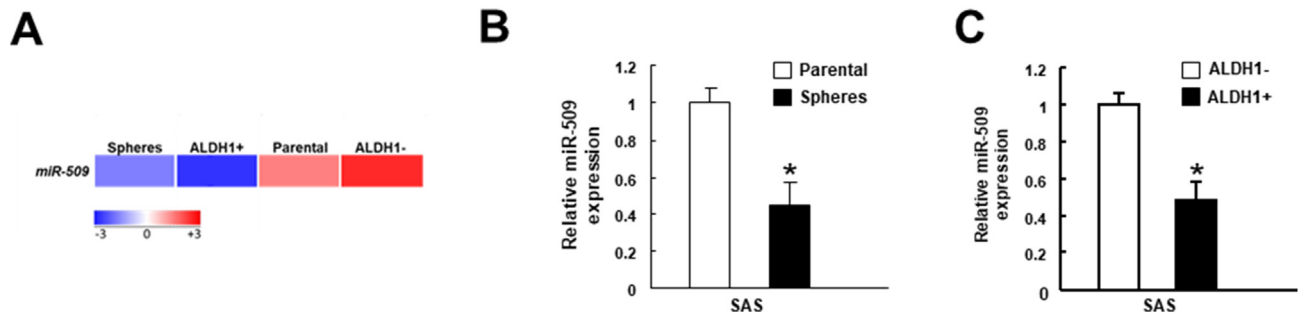


Figure 1 MiR-509 is downregulated in oral cancer stem cells (CSCs) (A) A heatmap showing miR-509 was downregulated in ALDH1⁺ and sphere oral CSCs; The relative expression of miR-509 in parental and sphere oral CSCs (B) as well as ALDH1⁻ non-CSC cells and ALDH1⁺ CSCs derived from SAS cells. **p* < 0.05 compared to parental or ALDH1⁻ non-CSC cells.

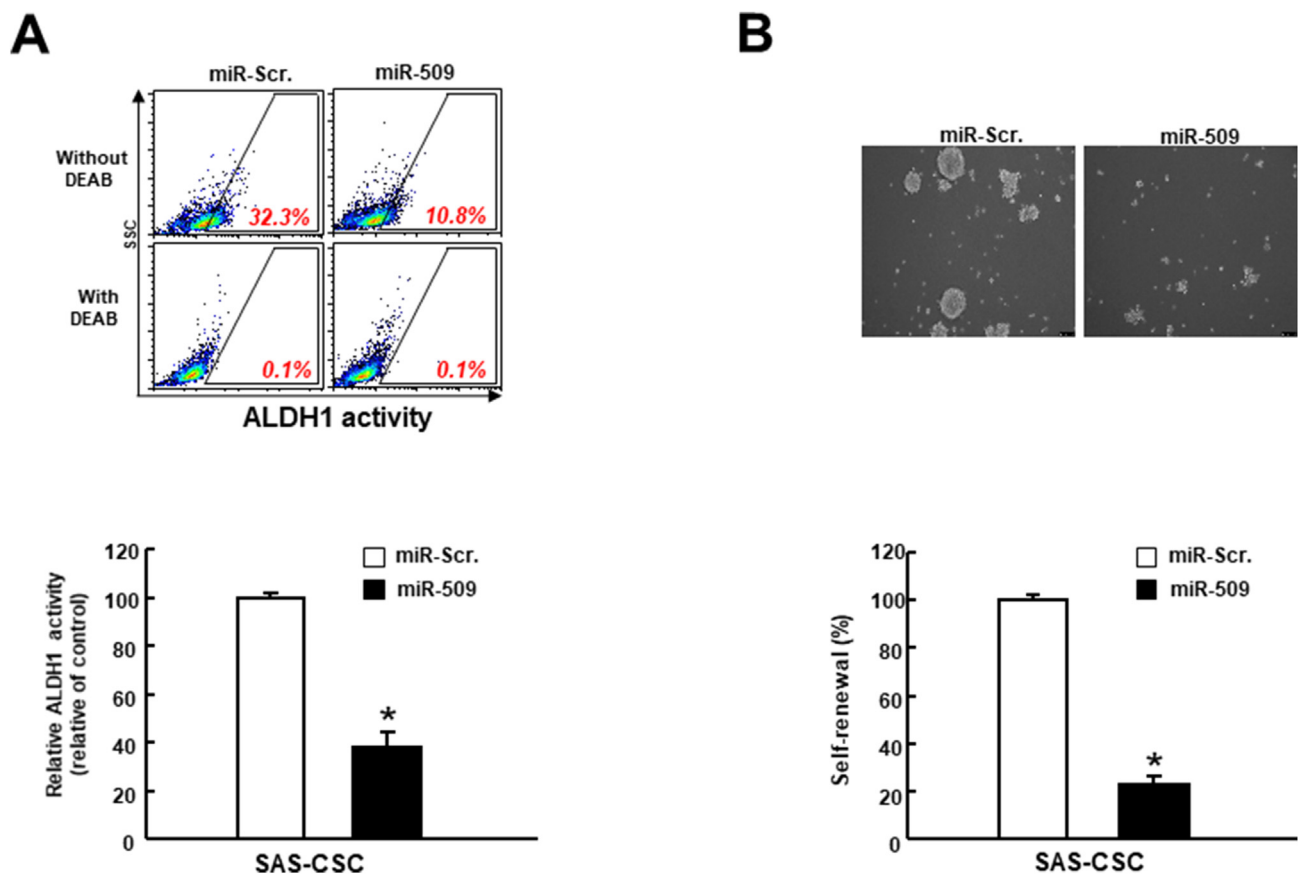


Figure 2 Ectopic expression of miR-509 diminishes ALDH1 activity and self-renewal capacity of oral CSCs. Overexpression of miR-509 in two types of oral CSCs (SAS-CSCs) reduced the ALDH1 activity using flow-cytometry (A) and self-renewal ability using secondary sphere formation assay (B). **p* < 0.05 compared to miR-Scrambled (miR-Scr.).

cells, high-throughput RNA-sequencing was employed and miR-509 was found as one of the differentially expressed genes (Fig. 1A). To validate this finding, qRT-PCR was conducted and we showed that miR-509 was downregulated in sphere cells of SAS (Fig. 1B). Similarly, the expression of miR-509 was reduced in ALDH1⁺ cells compared to ALDH1⁻ cells (Fig. 1C). These results suggested that miR-509 may be suppressed in the oral CSCs.

To assess the functional role of miR-509 in cancer stemness features, we transfected oral CSCs with mimics and examined the ALDH1 activity, self-renewal ability, and CD44 expression. As shown in Fig. 2A, ALDH1 activity was decreased in SAS CSCs transfected with miR-509 mimics, and N,N-diethylaminobenzaldehyde (DEAB) was used as a selective inhibitor of ALDH1 (negative control). Overexpression of miR-509 also inhibited the self-renewal ability in these two types of oral CSCs using secondary sphere assay (Fig. 2B). Besides, CD44 expression was markedly reduced in miR-509-overexpressing oral CSCs (Fig. 3).

Additionally, ectopic expression of miR-509 in SAS oral CSCs suppressed their migration capacity (Fig. 4A), indicating the anti-metastasis potential of miR-509. The result from the clonogenic assay demonstrated that elevation of miR-509 in SAS oral CSCs mitigated the ability of a single

cell to grow into a colony (Fig. 4B). These findings showed that an increase of miR-509 in oral CSCs may attenuate the cancer stemness properties.

To unravel the target gene of miR-509, we used a bioinformatics tool to predict that polo-like kinase 1 (plk1) may serve as one of the putative targets of miR-509. In order to confirm the direct relationship between miR-509 and plk1, we employed the luciferase reporter assay to validate the binding site. Fig. 5A illustrated the complementarity between the 3' UTRs of plk1 and miR-509 to pinpoint the target sequence of miR-509. Reporter plasmids containing either full-length (Wt-plk1) or mutated (mut-plk1) forms of the miR-509-binding region were constructed and co-transfected with miR-509 mimics into oral CSCs. We demonstrated that the luciferase activity of Wt-plk1 vector was reduced when co-transfected with miR-509 mimics, whereas no significant change was observed in the mut-plk1 vector group (Fig. 5B). Our results showed that miR-509 binds to the 3'UTR of plk1 through imperfect base pairing. Furthermore, we showed that the expression of plk1 in SAS CSCs were both abolished following miR-509 overexpression. Clinically, miR-509 is negatively correlated with plk1 expression in Taiwanese OSCC specimens (Fig. 6).

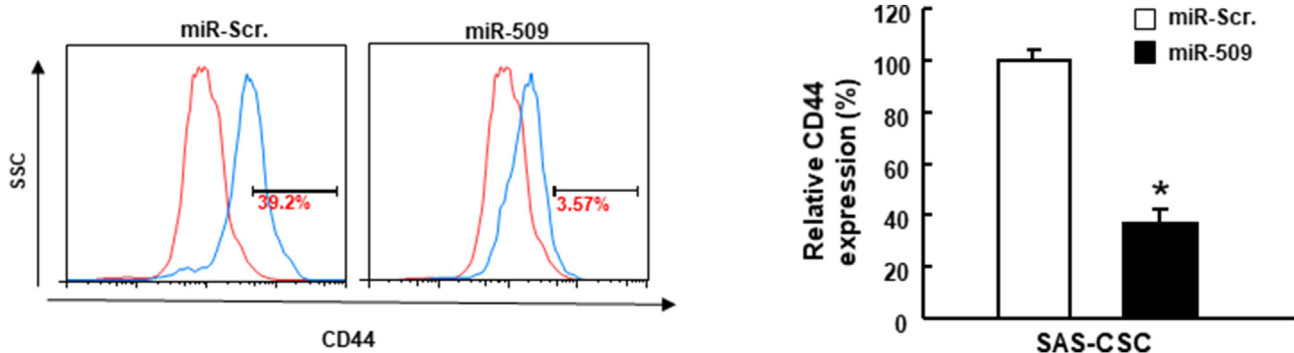


Figure 3 Overexpression of miR-509 mitigates CD44 expression in two oral CSCs. Upregulation of miR-509 in two types of oral CSCs (SAS-CSCs) suppressed the percentage of cells expressing CSC marker, CD44, using flow-cytometry. * $p < 0.05$ compared to miR-Scr.

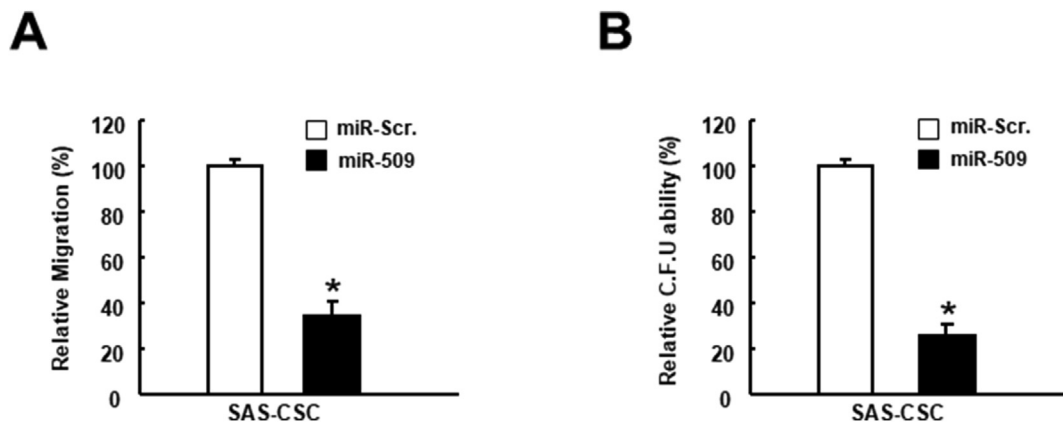


Figure 4 Upregulation of miR-509 lessens CSCs phenotypes. Ectopic expression of miR-509 in two types of oral CSCs (SAS-CSCs) attenuated the transwell migration (A) and colony-forming (B) abilities. * $p < 0.05$ compared to miR-Scr.

Discussion

Currently, research regarding the significance of miR-509 in cancer progression is still limited. The majority of studies suggested that miR-509 may be downregulated and play a tumor-suppressive role in several types of cancer, such as renal cancer,¹³ papillary thyroid carcinoma,¹⁴ gliomas,¹¹ and non-small cell lung cancer.¹⁵ It has been demonstrated that miR-509 inhibited the proliferative and migratory abilities of renal cancer cells, thyroid cancers, or gliomas via down-regulating the mitogen-activated protein kinase 8 (MAP3K8),¹³ paired box 6 (PAX6),¹⁴ or X-linked inhibitor of apoptosis,¹¹ respectively. Aside from repressing cell viability, migration, and invasion capacities, overexpression of miR-509 also attenuated the colony formation through forkhead box M1 (FOXM1)¹⁵ and increased the chemosensitivity through Axl¹⁶ in lung cancer or osteosarcoma. As for tongue squamous cell carcinoma, miR-509 has been shown to mitigate the proliferation and invasion abilities via EGFR.¹⁰ In the present study, we showed that miR-509 exerted an inhibitory effect on oral CSCs through plk1.

Plk1 is a serine/threonine-protein kinase that has been implicated in various stages of mitosis, meiosis, and

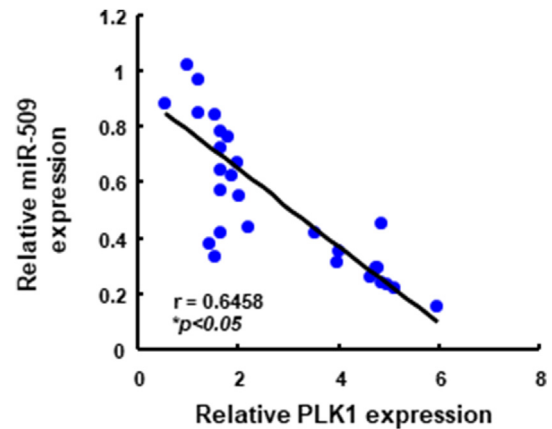
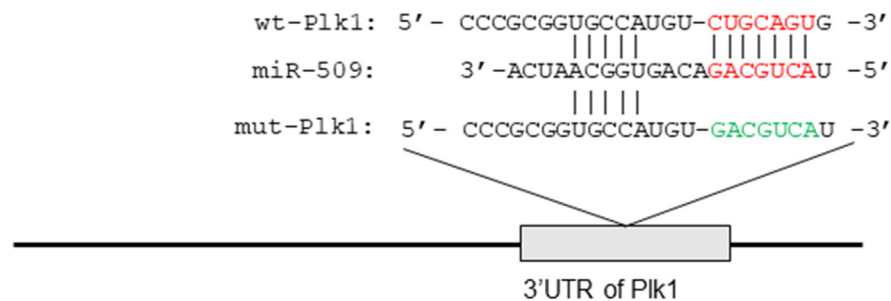


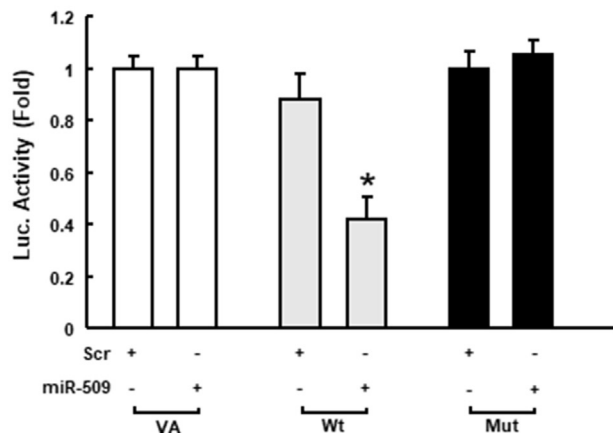
Figure 6 The negative correlation between miR-509 expression and plk1 in Taiwanese OSCC specimens by qRT-PCR and linear regression analysis.

cytokinesis.^{17,18} It has been shown that silencing of plk1 resulted in inhibition of proliferation in cancer cells.¹⁹ In oral cancer, plk1 has been shown to be overexpressed and

A



B



C

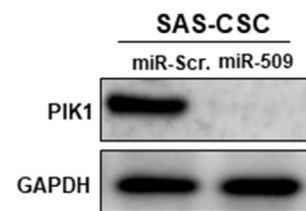


Figure 5 MiR-509 directly interacts with plk1 (A) Schematic of miR-509, the putative binding sequence along with the mutant sequence at the 3'-untranslated region (3'UTR) of plk1 (B) Luciferase activity decreased when cells were co-transfected with Wt-plk1 and miR-509 mimic (C) The expression of plk1 in SAS-CSC transfected with miR-Scr. or miR-509 mimics. * $p < 0.05$ compared to miR-Scr.

considered as one of the most potent genes required for oral cancer cell proliferation.²⁰ Likewise, plk1 is crucial to cell proliferation in CSCs. It has been demonstrated that suppression of plk1 inhibited Bmi1, an important regulator of CSC phenotype, by upregulating the miRNA-200c/141 cluster in breast cancer cells.²¹ Besides, plk1 activity has been known to be imperative for maintaining forkhead box protein C2 (FOXC2) protein stability, which could regulate the G2/M transition of breast CSCs.²² Another study showed that plk1 activity modulated the mitotic entry and cell polarity of glioblastoma CSCs and targeting plk1 reduced glioblastoma cell growth.²³ In line with our finding, it has been shown that plk1 was a direct target of miR-509 in smooth muscle cells.²⁴ Taken together, these results suggested that downregulation of plk1 by miR-509 may affect the stemness features, proliferation, and migration abilities of CSCs.

In conclusion, this study demonstrated that the expression of miR-509 was repressed in oral CSCs, and upregulation of miR-509 mitigated several CSCs characteristics. In addition, we showed that plk1 was a direct target of miR-509. Our findings suggested that approaches to increase the expression of miR-509 may be beneficial to oral cancer treatment.

Declaration of competing interest

All authors have no conflicts of interest relevant to this article.

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References

- Warnakulasuriya S. Living with oral cancer: epidemiology with particular reference to prevalence and life-style changes that influence survival. *Oral Oncol* 2010;46:407–10.
- Bighetti-Trevisan RL, Sousa LO, Castilho RM, Almeida LO. Cancer stem cells: powerful targets to improve current anti-cancer therapeutics. *Stem Cell Int* 2019;2019:9618065.
- Chen YC, Chen YW, Hsu HS, et al. Aldehyde dehydrogenase 1 is a putative marker for cancer stem cells in head and neck squamous cancer. *Biochem Biophys Res Commun* 2009;385:307–13.
- Prince ME, Sivanandan R, Kaczorowski A, et al. Identification of a subpopulation of cells with cancer stem cell properties in head and neck squamous cell carcinoma. *Proc Natl Acad Sci U S A* 2007;104:973–8.
- Chiou SH, Yu CC, Huang CY, et al. Positive correlations of oct-4 and nanog in oral cancer stem-like cells and high-grade oral squamous cell carcinoma. *Clin Cancer Res* 2008;14:4085–95.
- Ghatak D, Das Ghosh D, Roychoudhury S. Cancer stemness: p53 at the wheel. *Front Oncol* 2020;10:604124.
- Lee SH, Koo BS, Kim JM, et al. Wnt/ β -catenin signalling maintains self-renewal and tumourigenicity of head and neck squamous cell carcinoma stem-like cells by activating Oct4. *J Pathol* 2014;234:99–107.
- Hsieh PL, Liao YW, Pichler M, Yu CC. MicroRNAs as theranostics targets in oral carcinoma stem cells. *Cancers (Basel)* 2020;12:340.
- Momen-Heravi F, Bala S. Emerging role of non-coding RNA in oral cancer. *Cell Signal* 2018;42:134–43.
- Hou C, Dong Y, Zhang F, Du B. MicroRNA-509 acts as a tumor suppressor in tongue squamous cell carcinoma by targeting epidermal growth factor receptor. *Mol Med Rep* 2017;16:7245–52.
- Du P, Luan X, Liao Y, et al. MicroRNA-509-3p inhibits cell proliferation and invasion via downregulation of X-linked inhibitor of apoptosis in glioma. *Oncol Lett* 2018;15:1307–12.
- Yang HW, Yu CC, Hsieh PL, et al. Arecoline enhances miR-21 to promote buccal mucosal fibroblasts activation. *J Formos Med Assoc* 2021;120:1108–13.
- Su Z, Chen D, Zhang E, et al. MicroRNA-509-3p inhibits cancer cell proliferation and migration by targeting the mitogen-activated protein kinase kinase kinase 8 oncogene in renal cell carcinoma. *Mol Med Rep* 2015;12:1535–43.
- Zhang S, Wang Q, Li D, et al. MicroRNA-509 targets PAX6 to inhibit cell proliferation and invasion in papillary thyroid carcinoma. *Mol Med Rep* 2019;19:1403–9.
- Ma N, Zhang W, Qiao C, et al. The tumor suppressive role of miRNA-509-5p by targeting FOXM1 in non-small cell lung cancer. *Cell Physiol Biochem* 2016;38:1435–46.
- Patil SL, Palat A, Pan Y, et al. MicroRNA-509-3p inhibits cellular migration, invasion, and proliferation, and sensitizes osteosarcoma to cisplatin. *Sci Rep* 2019;9:19089.
- Petronczki M, Lénárt P, Peters JM. Polo on the rise-from mitotic entry to cytokinesis with Plk1. *Dev Cell* 2008;14:646–59.
- Barr FA, Silljé HH, Nigg EA. Polo-like kinases and the orchestration of cell division. *Nat Rev Mol Cell Biol* 2004;5:429–40.
- Matthess Y, Kappel S, Spänkuch B, Zimmer B, Kaufmann M, Strebhardt K. Conditional inhibition of cancer cell proliferation by tetracycline-responsive, H1 promoter-driven silencing of PLK1. *Oncogene* 2005;24:2973–80.
- Goan YG, Liu PF, Chang HW, et al. Kinome-wide screening with small interfering RNA identified polo-like kinase 1 as a key regulator of proliferation in oral cancer cells. *Cancers (Basel)* 2019;11:1117.
- Dimri M, Cho JH, Kang M, Dimri GP. PLK1 inhibition down-regulates polycomb group protein BMI1 via modulation of the miR-200c/141 cluster. *J Biol Chem* 2015;290:3033–44.
- Pietilä M, Vijay GV, Soundararajan R, et al. FOXC2 regulates the G2/M transition of stem cell-rich breast cancer cells and sensitizes them to PLK1 inhibition. *Sci Rep* 2016;6:23070.
- Lerner RG, Grossauer S, Kadkhodaei B, et al. Targeting a Plk1-controlled polarity checkpoint in therapy-resistant glioblastoma-propagating cells. *Cancer Res* 2015;75:5355–66.
- Liao G, Wang R, Rezey AC, Gerlach BD, Tang DD. MicroRNA miR-509 regulates ERK1/2, the Vimentin network, and focal adhesions by targeting Plk1. *Sci Rep* 2018;8:12635.