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Original Article

Gastric adenocarcinoma predictive long intergenic noncoding RNA (GAPLINC) promotes oral cancer stemness by acting as a molecular sponge of miR331-3p

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Abstract *Background/purpose:* Accumulating evidence has suggested that treatment failure of cancer therapy can be attributed to cancer stem cells (CSCs). Among numerous regulators of cancer stemness, non-coding RNAs (ncRNAs) have gained significant attention recently. In this study, we examined the role of gastric adenocarcinoma predictive long intergenic noncoding RNA (GAPLINC) in oral CSCs (OCSCs).
Materials and methods: RNA Sequencing and quantitative real-time polymerase chain reaction (qRT-PCR) were used to determine the expression of GAPLINC. Flow cytometry and sphere-forming assay were exploited to isolate OCSCs. Measurement of aldehyde dehydrogenase 1

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(ALDH1) activity, CD44 expressing cells, and various phenotypic assays, such as self-renewal, migration, invasion, and colony-forming abilities, were conducted in CSCs of two types of oral cancer cells (SAS and GNM) following the knockdown of GAPLINC. A luciferase reporter was also carried out to validate the direct interaction between GAPLINC and microRNA (miR)-331-3p.
Results: Our results showed that GAPLINC was overexpressed in OCSCs from patient-derived and oral cancer cell lines. We demonstrated that silencing of GAPLINC in OCSCs downregulated various CSC hallmarks, such as ALDH1 activity, percentage of CD44-expressing cells, self-renewal capacity, and colony-forming ability. Moreover, our results revealed that the effect of GAPLINC on cancer stemness was mediated by direct repression of miR-331-3p.
Conclusion: These data have potential clinical implications in that we unraveled the aberrant upregulation of GAPLINC and demonstrated that suppression of GAPLINC may reduce cancer stemness via sequestering miR-331-3p.

Introduction

Oral cancer is one of the most common malignancies in the head and neck region, and its mortality rate has increased over the past decade.[1] Moreover, patients with oral cancer often suffer from various functional impairments in speaking, eating, and swallowing and these adverse effects may eventually lead to a deterioration of quality of life.[2] It has been recognized that drug resistance, metastasis, and tumor relapse are attributed to the existence of a small fraction of cancer stem cells (CSCs) with properties of self-renewal, pluripotency, and aggressiveness. Multiple biomarkers of oral CSCs (OCSCs) have been proposed, with aldehyde dehydrogenase 1 (ALDH1), CD44, and CD133 being the most extensively validated and related to prognostic significance.[3–5] Also, cancer cells that aggregated and formed sphere-like bodies have been shown to possess OCSCs properties,[3] so sphere cells can be used to identify OCSCs. These approaches have enabled the study of investing the effect of anti-cancer treatment targeting OCSCs.

Emerging evidence has suggested the significance of non-coding RNAs (ncRNAs) in the regulation of cancer stemness of oral cancer, including both long ncRNAs (lncRNAs; >200 nucleotides) and microRNAs (miRNAs; ~22 nucleotides).[6–10] To date, various modes of lncRNA action have been discovered. Aside from their roles in transcription regulation and nuclear organization, lncRNAs mainly exhibit their ability to establish interactions with proteins and nucleic acids and act as post-transcriptional, translational, and post-translational regulators. It has been known that various lncRNAs bearing multiple miRNA-complementary sites can regulate abundant gene expression by functioning as competitive endogenous RNAs (ceRNAs) or 'sponges' of miRNAs to reduce miRNA availability to target genes.[11] In fact, several studies have demonstrated that lncRNAs modulate the cancer stemness of oral cancer via sequestering miRNAs.[12,13]

GAPLINC (gastric adenocarcinoma predictive long intergenic noncoding RNA) is a 924-bp-long lncRNA located on chromosome 18p11.31 and was first reported in 2014.[14] It was highly expressed in gastric cancer tissues and strongly correlated with poor survival.[14] Hu et al. showed that GAPLINC can act as a molecular decoy for miR-211-3p, which targets CD44 for degradation.[14] Later, lots of studies have revealed that the expression of GAPLINC can be used to predict poor prognosis of various types of cancers, such as non-small cell lung cancer, perihilar cholangiocarcinoma, bladder, and renal cancers.[15–18] However, its role in oral tumorigenesis has not been investigated. On the other hand, the hyaluronan-CD44 interaction has been demonstrated to promote cancer stemness of oral cancer.[19,20] Given that GAPLINC can regulate CD44, we postulate that dysregulation of GAPLINC may affect cancer stemness.

Here, we assessed the expression of GAPLINC in the patient-derived OCSCs, ALDH1-positive, and sphere-forming oral cancer cells. Next, we examined the effect of GAPLINC on the alteration of cancer stemness, including the expression of CSC markers and CSC phenotypes. Most importantly, we revealed a putative miRNA that was under the regulation of GAPLINC to modulate oral cancer stemness. Lastly, we validated the upregulation of GAPLINC in oral cancer tissues and its oncogenic role.

Materials and methods

Cell culture

Oral squamous cell carcinoma (OSCC) cell lines (SAS and GNM) were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Invitrogen Life Technologies, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS), 100 μg/ml streptomycin, 100 U/ml penicillin, and 1% L-glutamine at 37 °C with 5% CO_2: 95% air in Corning tissue culture-treated plastic (Corning, Inc., Corning, NY, USA).

Quantitative real-time polymerase chain reaction analysis

Total RNA was prepared from cells using Trizol reagent according to the manufacturer's protocol (Invitrogen Life Technologies). Quantitative real-time polymerase chain

reaction (qRT-PCR) of mRNAs was reverse-transcribed using the Superscript III first-strand synthesis system (Invitrogen Life Technologies). QRT-PCR reactions on resulting cDNAs were performed on an ABI StepOne™ Real-Time PCR Systems (Applied Biosystems, Foster City, CA, USA). The tissue section for qPCR was provided by Chung Shan Medical University Hospital. For the qRT-PCR analysis of OSCC specimen, written informed consent were received from all patients before collection, and the study was approved by the Institutional Review Board of Chung Shan Medical University Hospital. The patients' medical records were reviewed, and we ensure that identities are protected. The primer sequences are provided below: GAPLINC, CTCAGCAGTGGCTCAGGAAA (forward) and TGATAGCCCCTGGATGTGGA (reverse); GAPDH, CTGGTGGCTGGCTCAGAAAA (forward) and GGAGATTCAG TGTGGTGGGG (reverse).

Lentiviral-mediated RNAi for silencing GAPLINC

The pLV-RNAi vector is purchased from Biosettia Inc. (Biosettia, San Diego, CA, USA). The method of cloning the double-stranded short-hairpin (sh) RNA sequence is described in the manufacturer's protocol. Oligonucleotide sequence of lentiviral vectors expressing shRNA that targets GAPLINC were synthesized and cloned into pLVRNAi to generate a lentiviral expression vector.

Tumor spheres assay, migration, invasion, and colony-forming assays

These assays and procedures were followed the previously described protocol.[6,7]

Flow cytometry analysis

The cancer stemness marker expression of ALDH1 activity was e measured with a FACSCalibur (Becton Dickinson, Franklin Lakes, NJ, USA) followed by CellQuest software.

Luciferase reporter assay

The 3′UTR region of GAPLINC was inserted into the pMIR-REPORT vector (Invitrogen Life Technologies) to construct the wild-type (wt) reporter vector. The mutant version (mut) was created by altering the sequence of the miR-331-3p binding site within the wild-type construct. For the following transfection experiments, cells were transfected with the respective pMIR reporter vector along with miR-331-3p mimics or a scrambled miR control (miR-Scr) using Lipofectamine 2000 (Invitrogen Life Technologies). The firefly luciferase signal was measured and normalized based on transfection efficiency to assess the reporter activity.[21]

Statistical analysis

Statistical Package of Social Sciences software (version 13.0, SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Data from at least triplicate analysis were shown as mean ± SD. Student's *t* test was used to determine the statistical significance of the differences between experimental groups; *P*-values less than 0.05 will be considered statistically significant.

Results

First, we found that the expression of GAPLINC was overexpressed in the ALDH1-positive oral cancer cells derived from two patients compared to ALDH1-negative cells using RNA Sequencing (Fig. 1A). In accordance with this finding, the expression level of GAPLINC was elevated in both ALDH1-positive (Fig. 1B) and sphere-forming (Fig. 1C) oral cancer cells, suggesting that GAPLINC was increased in OCSCs.

To test the functional role of GAPLINC in OCSCs, shRNA-mediated knockdown of GAPLINC in two types of oral CSCs (SAS and GNM; OCSCs) was conducted. The knockdown

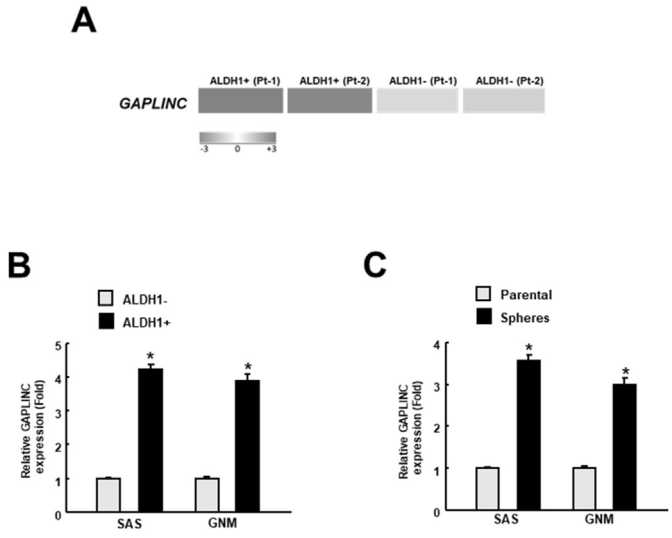

Figure 1 LncRNA GAPLINC is upregulated in oral cancer stem cells isolated from patient-derived tumor tissues and oral cancer cell lines. (A) Heatmap showing differentially expressed GAPLINC between aldehyde dehydrogenase 1-positive ($ALDH1^+$) and ALDH1-negative ($ALDH1^-$) tumor samples. QRT-PCR analysis revealed the relative expression of GAPLINC in (B) ALDH1-positive and (C) sphere-forming cells from two oral cancer cell lines (SAS and GNM). *$P < 0.05$ compared to ALDH1-negative and parental cells, respectively. Patient (Pt); Luciferase short hairpin RNA (sh-Luc., as a control vector).

efficiency was confirmed by measuring the expression levels following transfection of the sh-Luc. and sh-RNA GAPLINC vectors into cells (Fig. 2A). We showed that silencing of GAPLINC reduced ALDH1 activity compared to the sh-Luc group (Fig. 2B). In addition, the proportion of CD44-positive cells (Fig. 2C) and self-renewal capacity (Fig. 2D) were all diminished after knocking down GAPLINC. Besides, cells silenced for GAPLINC showed a marked decrease in transwell migration (Fig. 3A) and colony-forming (Fig. 3B) abilities. These results all indicated that the expression of GAPLINC contributes to cancer stemness of oral cancer cells.

One of the previous studies has revealed that GAPLINC can negatively modulate miR-331-3p and affect the progression of glioblastoma cells.[22] Here, we performed a luciferase reporter assay to verify their paired binding, and the sequences of wild-type (wt)-GAPLINC and mutated (mut)-GAPLINC were inserted into the luciferase reporter vector followed by cotransfection with miR-331-3p mimic (Fig. 4A). The result showed that only the miR-331-3p mimic effectively inhibited the luciferase activity (Fig. 4B). Our findings verified that GAPLINC can directly interact with miR-331-3p.

Subsequently, we investigated whether miR-331-3p was implicated in the oncogenic effect of GAPLINC in oral cancer cells. Our results demonstrated that miR-331-3p inhibitor reversed the reduced self-renewal capacity (Fig. 5A) and invasion ability (Fig. 5B) in GAPLINC-silenced cells, suggesting that GAPLINC contributes to cancer stemness of oral cancer via suppression of miR-331-3p. Lastly, results from RNA Sequencing showed that GAPLINC was aberrantly upregulated in oral cancer tissues (Fig. 6A) and qRT-PCR confirmed the overexpression of GAPLINC in oral cancer specimens. Taken together, these results were all in support of the critical role of GAPLINC in the maintenance of cancer stemness of oral cancer cells via inhibiting miR-331-3p.

Discussion

Growing evidence suggests that deregulated lncRNAs mediate oncogenic or tumor-suppressing properties. Nevertheless, a large number of lncRNAs remain functionally uncharacterized. In this study, we showed that lncRNA GAPLINC was overexpressed in oral cancer tissues and associated with cancer stemness. Our data revealed that GAPLINC was elevated in ALDH1-positive and sphere-forming OCSCs. Moreover, downregulation of GAPLINC reduced various features of cancer stemness such as lower ALDH1 activity, percentage of CD44-expressing cells, self-renewal capacity, transwell migration, and colony-forming ability in OCSCs. Apart from the present study, there is a lack of investigation demonstrating the functional role of GAPLINC in the maintenance of cancer stemness. Nonetheless, recent studies have shown that knockdown of GAPLINC repressed the *in vivo* tumorigenicity of non-small cell lung cancer and renal cancer by acting as a sponge of miR-661 and miR-135b-5p, respectively.[15,18] Besides, ALDH1 has been known to promote the hallmark of cancer

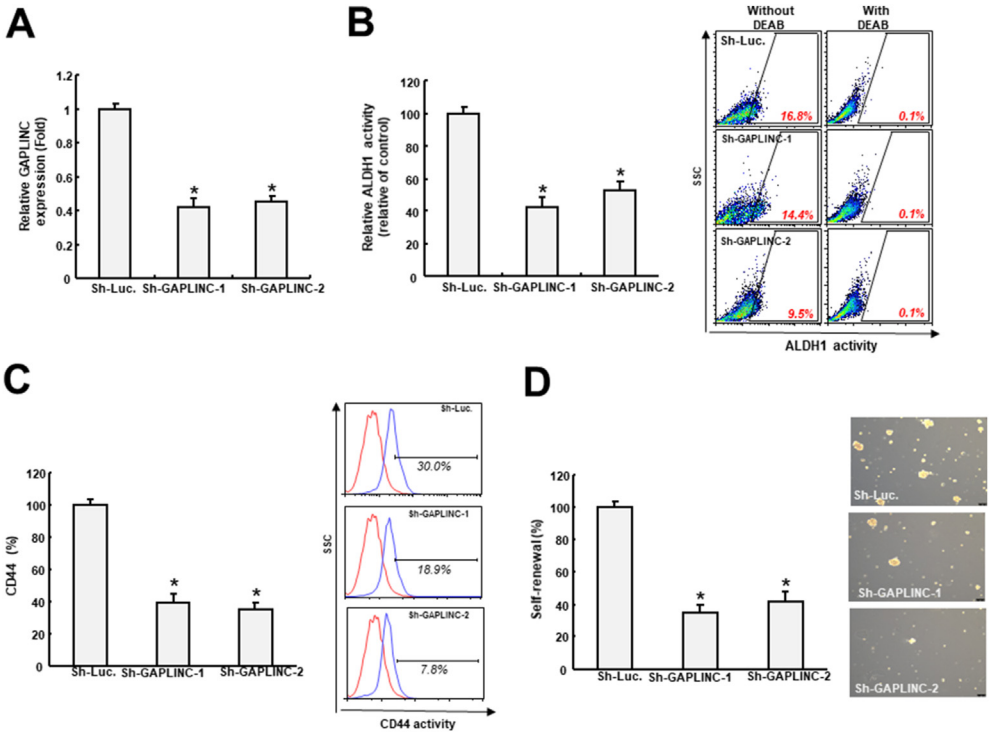

Figure 2 Silencing of GAPLINC downregulates the features of cancer stemness in oral cancer stem cells. (A) Silencing efficiency of shRNA-mediated knockdown of GAPLINC in OCSCs. (B) ALDH1 activity, (C) percentage of CD44-expressing cells, and (D) self-renewal ability in cells transfected with sh-Luc. or shRNA-GAPLINC. *$P < 0.05$ compared to sh-Luc. Side scatter, SSC; N,N-diethylaminobenzaldehyde (DEAB, as a selective inhibitor of ALDH1).

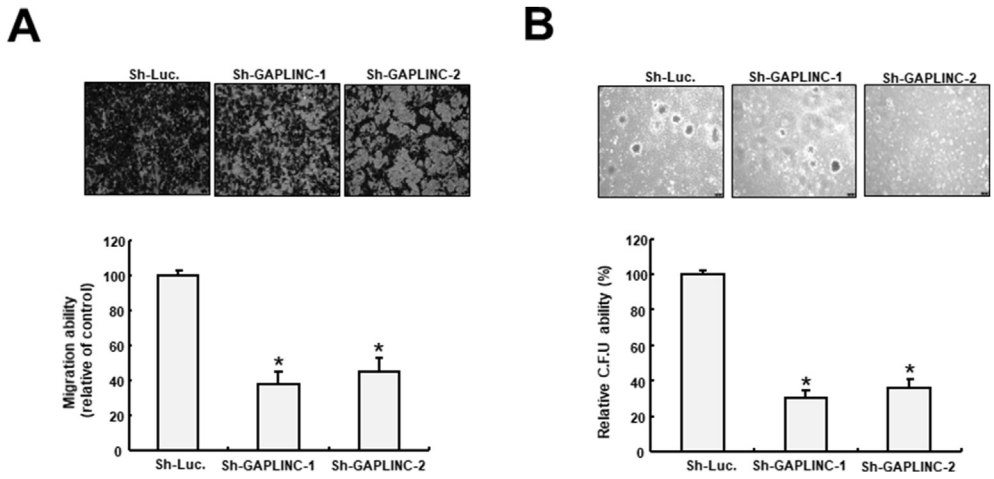

Figure 3 Downregulation of GAPLINC suppresses the phenotypes of oral cancer stem cells. (A) Transwell migration and (B) colony-forming unit (C.F.U.) assays were conducted in OCSCs transfected with sh-Luc. or shRNA-GAPLINC. *$P < 0.05$ compared to sh-Luc.

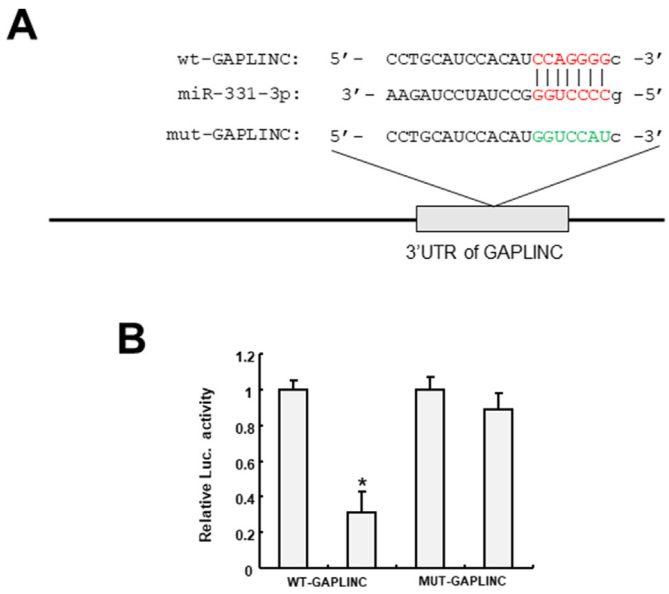

Figure 4 MiR-331-3p is a direct target of GAPLINC. (A) An illustration of the putative miR-331-3p binding sites in wild-type (wt) and mutant (mut) GAPLINC at the 3'-untranslated region (3'UTR). (B) Luciferase (Luc.) activity was decreased in cells co-transfected with WT-GAPLINC and miR-331-3p mimic. *$P < 0.05$ compared to with miR-Scr (scramble).

stemness through taking part in retinoic acid-mediated signaling and reducing reactive oxygen species.[23,24] Hyaluronan-CD44 signaling has been proven to affect the expression of various stemness markers, including octamer-binding transcription factor 4 (Oct4), sex determining region Y 2 (Sox2), and Nanog.[25] Our results were in line with these findings and showed that the silencing of GAPLINC downregulated the expression levels of ALDH1 and CD44, which may partly explain the reduced CSC behaviors (e.g. self-renewal and colony formation).

Additionally, we demonstrated that GAPLINC acted as a ceRNA of miR-331-3p to sustain cancer stemness of OCSCs, which was consistent with a previous study showing that GAPLINC promoted the progression and metastasis of glioblastoma by directly inhibiting miR-331-3p.[22] The tumor suppressive effect of miR-331-3p on cervical cancer has been shown to be associated with suppression of LIM zinc finger domain containing 2 (LIMS2) methylation by targeting the DNA methyltransferase 3 alpha (DNMT3A).[26] Of note, suppression of DNMT3A downregulated the phenotypes of lung CSCs via Wnt/β-catenin signaling and whether the GAPLINC/miR-331-3p/DNMT3A axis contributed to cancer stemness requires further investigation.[27] Besides, a mitochondrial citrate carrier, solute carrier family 25 member 1 (SLC25A1), also has been found to be a direct target of miR-331-3p. It has been demonstrated that overexpression of miR-331-3p or reduction of SLC25A1 led to lower proliferation, migration, and invasion of papillary thyroid

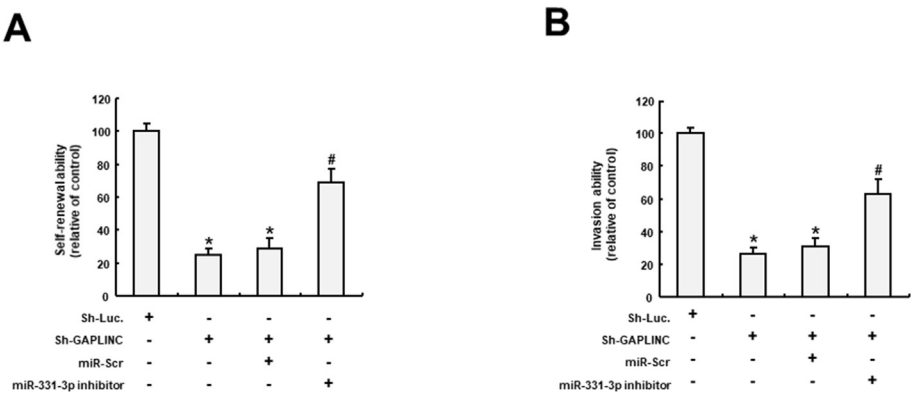

Figure 5 The effects of GAPLINC downregulation on self-renewal and invasion capacities of oral cancer stem cells require a reduction of miR-331-3p. Self-renewal (A) and invasion (B) abilities of cells transfected with sh-GAPLINC with or without miR-33-3p inhibitors were evaluated. *$P < 0.05$ compared with sh-Luc group; $^{\#}P < 0.05$ compared with sh-GAPLINC group.

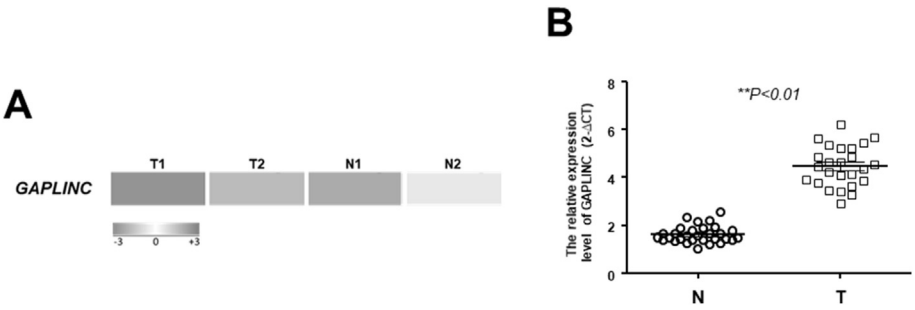

Figure 6 GAPLINC is overexpressed in oral cancer tissues. (A) Upregulation of GAPLINC was observed in oral cancer tissues (T1 and T2) compared to normal specimens (N1 and N2) using RNA-sequencing. The increased expression level of GAPLINC in oral cancer (T) compared to normal (N) tissues (n = 25) was validated using qRT-PCR.

carcinoma cells.[28] Since SLC25A1 was crucial to maintaining the mitochondrial redox balance in CSCs and their self-renewal capability of non-small cell lung cancer and liver cancer,[29,30] it is worthwhile to further elucidate whether the metabolic oncogene SLC25A1 participated in the survival of OCSCs in the future.

Collectively, our results showed that GAPLINC was an oncogenic lncRNA in oral cancer and may promote cancer stemness through direct inhibition of miR-331-3p. Our results also demonstrated that GAPLINC can be a molecular therapeutic target of oral cancer as suppression of GAPLINC holds the potential to impair cancer progression by reducing OCSCs.

Declaration of competing interest

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

Acknowledgments

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