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# Overexpression of long non-coding RNA GASL1 induces apoptosis and $G_0/G_1$ cell cycle arrest in human oral cancer cells

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**Oral cancer is one of the commonly reported malignancies of the human oral cavity and pharynx. It accounts for a significant level of cancer-based mortality across the globe. Long non-coding RNAs (lncRNAs) are emerging as important study targets in cancer therapy. The present study aimed to characterize the role of lncRNA GASL1 in regulating the growth, migration, and invasion of human oral cancer cells. The qRT-PCR showed significant ($P<0.05$) upregulation of GASL1 in oral cancer cells. Overexpression of GASL1 led to the loss of viability of HN6 oral cancer cells by inducing apoptosis which was associated with upregulation of Bax and downregulation of Bcl-2. The apoptotic cell percentage increased from 2. 81% in control to 25.89% upon GASL1 overexpression. Cell cycle analysis showed that overexpression of GASL1 increased the $G_1$ cells from 35.19% in control to 84.52% upon GASL1 overexpression indicative of $G_0/G_1$ cell cycle arrest. Cell cycle arrest was also accompanied by inhibition of cyclin D1 and CDK4 protein expression. Wound healing and transwell assays showed that overexpression of GASL1 significantly ($P<0.05$) inhibited the migration and invasion of HN6 oral cancer cells. The invasion of the HN6 oral cancer cells was found to be decreased by more than 70%. Finally, the results of *in vivo* study revealed that GASL1 overexpression inhibits the xenografted tumor growth *in vivo*. Thus, the results are thus suggestive of the tumor-suppressive molecular role of GASL1 in oral cancer cells.**

**Keywords**: oral cancer, long non-coding RNA, GASL1, apoptosis, metastasis, prognosis

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**Abbreviations**: CDK4, Cyclin-dependent kinase-4; cDNA, Complementary DNA; DAPI, 4′,6-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle's medium; EdU, 5-ethynyl-2′-deoxyuridine; FBS, Fetal bovine serum; lncRNAs, Long non-coding RNAs; ncRNAs, Non-coding RNAs; OSCC, Oral squamous cell carcinoma; PVDF, Polyvinylidene difluoride membranes; qRT-PCR, Quantitative real time polymerase chain reaction

## INTRODUCTION

Oral cancer is ranked among the most prevalent cancers of the human oral cavity and pharynx (Chaturvedi *et al*., 2022). This malignancy is responsible for a significant level of mortality and morbidity throughout the world (Dhanuthai *et al*., 2018). Among the different histological sub-types, oral squamous cell carcinoma (OSCC) is the most dominant type of oral cancer which is often detected at advanced stages and exhibits poor prognosis (Meng *et al*., 2021; Panarese *et al*., 2019). In addition, this neoplastic disorder is linked with lymph node metastasis (Panarese *et al*., 2019). OSCC is highly lethal owing to the invasion of cancer to distant body parts (Peng *et al*., 2020). The currently applied chemotherapy is often reported to exhibit less soothing clinical results. Moreover, it is linked with a number of side effects negatively affecting overall human health. Pertinent to this backdrop, there is vast scope for the development of efficient and reliable prognostic and therapeutic targets against oral cancer.

The non-coding RNAs (ncRNAs) have attained considerable research attention over the past years. Several studies have put forward that ncRNAs exhibit frequent involvement in tumorigenesis of different types of human cancers including oral cancer (Yi *et al*., 2019; Irimie *et al*., 2017). Long non-coding RNAs (lncRNAs), a subclass of single-stranded ncRNAs with an average size of more than 200 nucleotides, are known to regulate the key hallmarks of human cancer (Vafadar *et al*., 2019). There is growing support that lncRNAs play diverse roles in the human body by regulating the expression of several target genes at transcriptional, post-transcriptional/translational or epigenetic levels (Statello *et al*., 2021). The molecular dysregulations of lncRNAs have been shown to be associated with tumor development and progression (Sun *et al*., 2018). LncRNAs regulate the crucial aspects of cancer cells including proliferation, apoptosis, migration, and invasion (de Oliveira *et al*., 2019). They have been shown to be involved in the remodeling of the micro-environment and metastasis of human tumors (Lin *et al*., 2018). Furthermore, the lncRNAs are involved in maintaining the stemness of different types of human cancers (Ma *et al*., 2019). Considering the pivotal role of this group of regulatory RNAs, there is mounting evidence that lncRNA-based cancer therapeutics might soon change from a mere concept to reality, soon.

Consistently, the present study was designed to investigate the role of lncRNA GASL1 in regulating the growth and metastasis of human oral cancer cells.

## MATERIALS AND METHODS

### Cell lines

The oral cancer cell lines (SCC9, HN4, HN6 and Cal127) as well as the normal human epithelial ovarian cells (NCE) were bought from the ATCC, USA. The cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Gaithersburg, USA) supplemented

with 10% FBS and streptomycin-penicillin (100 U/ml). The cells were maintained using humidified incubated $CO_2$ incubator at 37°C with 5% $CO_2$.

### Transfection

LncRNA GASL1 was amplified using cDNA prepared from the HN6 cell line and cloned into the pcDNA3.1 over-expression vector. Cell transfection was carried out in serum-free medium using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. 6 h post-transfection, fresh complete medium was used to incubate cells. About 48 h later, the cells were collected for detection. The pcNAD3.1 vector alone transfected cells were used to serve as the negative control cells.

### qRT-PCR expression analysis

Trizol reagent and Revert Aid First Strand cDNA synthesis kit (both from Thermo Fisher Scientific) was used for total RNA isolation and reverse transcription of RNA, respectively as per the manufacturer guidelines. SYBR PCR master mix (Takara, Japan) was used to perform the qRT-PCR on QuantStudio 3.0 PCR system (Applied Biosystems). The cycling conditions were 95°C for 5 min, followed by 40 cycles of 95°C for 15 s, and 60°C for 60 s. GAPDH genes were used as an internal control in the expression study. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression levels of GASL1. The qRT-PCR primers used were: GASL1, 5'-CTGAGGCCAAAGTTTCCAAC-3' (forward) and 5'-CAGCCTGACTTTCCCTCTTCT-3' (reverse); GADPH, 5'-GTCTCCTCTGACTTCAACAGCG-3' (forward) and 5'-ACCACCCTGTTGCTGTAGCCAA-3' (reverse).

### CCK-8 assay

Cell viability was determined with the help of a Cell Counting Kit-8 (Beyotime). In brief, $1\times10^5$ HN6 cells transfected with pcDNA-GASL1 or control vector (pcDNA3.1) were added per well of a 96-well plate and cultured for 24 h at 37°C. At this, the CCK-8 solution was added to each well and 37°C incubation was extended for 1 h. Next, the absorbance at 570 nm ($OD_{570}$) was determined for each well with the help of a microplate reader to analyze the cell viability.

### EdU assay

The proliferation of HN6 cancer cells over-expressing GASL1 was studied with reference to negative control cells with the help of an EdU Apollo *in vitro* flow cytometry kit (RiboBio, Guangzhou, China) in accordance with the manufacturer's recommendations. Approximately, $1.5\times10^6$ transfected cells /well were cultured in 12 well plates for 24 h at 37°C. Afterwards, the cells were added with EdU solution at a concentration of 50 μmol/L and incubated for 2 h. The cells were then PBS washed, fixed with 4% paraformaldehyde, and stained using Apollo staining solution (San Francisco, CA) for 25 min. The cells were also counterstained with DAPI. The cells were visualized under a fluorescence microscope (Olympus, Japan) for nuclear fluorescence.

### Annexin V/PI staining assay

To analyze the effect of GASL1 over-expression on HN6 cancer cell apoptosis, pcDNA-GASL1 transfected cells ($1.5\times10^6$/well) as well as the negative control cells were initially cultured in DMEM for 24 h in a 12-well plate at 37°C. Next, the cells were harvested and washed three times with PBS. Annexin V-FITC/PI double staining was performed using an Annexin V-FITC Apoptosis Detection Kit (BD Biosciences, San Jose, CA) as per the manufacturer's instructions. The cells were examined using a BD FACSCantoII flow cytometer (BD Biosciences) and analyzed by FlowJo 6.0 software to determine the relative percentage of apoptotic cells.

### Cell cycle analysis

Approximately, $1.5\times10^6$ HN6 cells/well transfected with NC and pcDNA-GASL1 were harvested 48 h after culture. Subsequently, the cells were PBS washed and fixed in 500 μl of 75% cold ethanol at 4°C overnight. The cells were then washed again with PBS, and stained by 100 μl propidium iodide (prepared in 3.8 nM sodium citrate, pH 7.0) containing RNase A (10 mg/ml) for 35 min in the dark at 37°C. The percentage of cells in different phases of the cell cycle was determined by flow cytometry (Beckman Coulter, Inc., Brea, CA, USA), and analyzed using ModiFit software.

### Wound healing assay

For studying the migration of GASL1 over-expressing HN6 cancer cells with respect to the negative control cells, the wound-healing assay was performed. For the wound-healing assay, $5\times10^5$ transfected HN6 cells were placed into each well of a six-well plate. The cells were allowed to grow for 24 h. At this, the growth medium was removed, and the cell surface was scratched with a pipette tip to carve a uniform linear scratch which was photographed under a light microscope (Olympus). The plate was incubated at 37°C for 24 h and then the wound was again analyzed and photographed under a microscope.

### Transwell assay

The transwell assay was performed to investigate the invasion of the transfected HN6 cancer cells. Here, the transwell chamber (8 μM, Corning Incorporated, Corning, NY) plated with Matrigel was used for invasion assay. 2.5 $x10^4$ cells suspended in 200 μl of serum-free medium were placed in the upper chamber while the lower chamber was added with serum-free medium alone. After 24 h of incubation at 37°C, the cells invading the lower chamber were fixed with 70% ethanol, stained with 0.2% crystal violet solution, and analyzed under a light microscope.

### Western blotting

The RIPA lysis and extraction buffer (Thermo Fisher Scientific) was used to extract total proteins from the transfected HN6 cancer cells. Bradford's assay was performed to examine the concentration of proteins isolated. Exactly, 35 μg of protein samples were isolated using the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Resolved proteins were transferred onto the activated polyvinylidene difluoride membranes (PVDF; Millipore, Billerica, MA, USA). 5% skimmed milk powder was used to block the membranes for 1 h. Next, the PVDF membranes were incubated overnight 4°C with the primary antibodies like anti-Bax (Abcam, cat no. ab216494, 1:1000), anti-Bcl-2 (Abcam, cat no. A95253, 1:800), anti-Cyclin D1 (Abcam, cat no. ab226977, 1:600) anti-CDK4 (Bio-Rad, cat no. AHP2454, 1:1000) and β-actin (Santa Cruz, cat no. sc-47778, 1:1000). This was followed by the incubation of the membranes with peroxidase-conjugated anti-rabbit secondary antibody (Cell signaling technology,

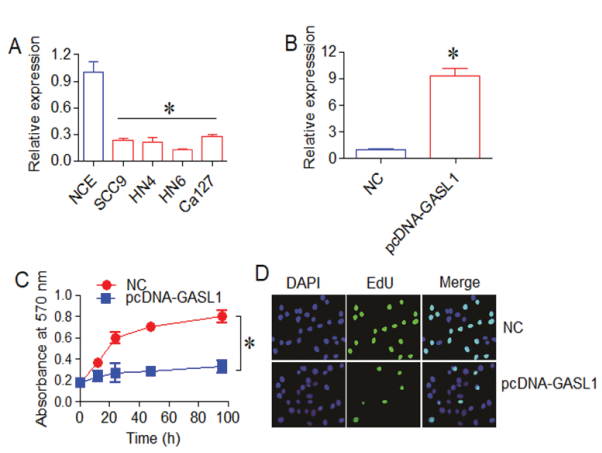


**Figure 1. GASL1 restrained the growth of oral cancer cells.** (**A**) Relative expression of GASL1 in oral cancer cell lines (SCC9, HN4, HN6 and Cal127) with respect to NCE normal oral epithelial cells (**B**) relative expression of GASL1 in pcDNA-GASL1 transfected HN6 cancer cells with respect to pcDNA3.1 vector transfected HN6 cells (**C**) CCK-8 assay of viability analysis of transfected HN6 cells (**D**) EdU incorporation assay of transfected HN6 cells. Three replicates were used for performing the experiments (*$P$<0.05)

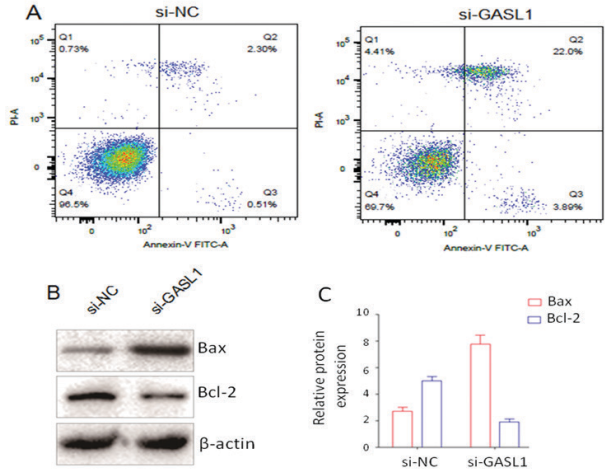


**Figure 2. GASL1 induced apoptosis in oral cancer cells.** (**A**) Flow cytometric analysis for percentage cell apoptosis analysis of transfected HN6 cancer cells double stained with Annexin V-FITC/PI (**B**) western blot analysis of Bax and Bcl-2 proteins from transfected HN6 cells. (**C**) Densitometry analysis of western blot. Three replicates were used for performing the experiments.

cat no. 7074, 1:3000) for 1 h at room temperature. Finally, the protein bands were detected through chemiluminescence and analyzed with Image J software. β-actin served as the endogenous control.

### *In vivo* study

Around 7-8 weeks old female SCID/NOD mice were maintained in well ventilated rooms under standard conditions on a 12-h light–dark cycle and given access to sterilized food and water. The study was approved by the animal research ethics committee of Sichuan University, Chengdu, China (SU/223/2021-23). HN6 cells at the density of $4\times10^6$ to induce tumor development. As the tumors reached 0.15–0.20 $cm^3$, the mice were randomly divided into 2 groups NC group and pcDNA-GASL1 group ($n$=10 for each group). Thereafter the mice were administered with intra-tumor injections (five in total, each after 3 days) carrying NC (empty vector) or pcDNA-GASL1 constructs. At the end of 3 weeks, the mice were sacrificed, and tumor weight (g) and volume ($cm^3$) were determined. The volume of tumors was calculated following this formula: $0.5\times length\times width^2$ ($mm^3$).

### Statistical analysis

The experiments were performed in triplicates and final values were given as the mean ± standard deviation. Student's $t$-test was performed using GraphPad Prism v6.0 (GraphPad Software, Inc.) to analyze the statistical difference between the two groups. The $p$-values less than 0.05 were considered to be representative of a statistically significant difference.

## RESULTS

### GASL1 is downregulated in oral cancer cells

In order to examine the expression levels of lncRNA GASL1 in different oral cancer cell lines (SCC9, HN4, HN6 and Cal127) with reference to normal oral epithelial cells (NCE), qRT-PCR was performed. The results showed that GASL1 expression is significantly down-regulated ($P$<0.05) in all the oral cancer cells (Fig. 1A). HN6 cancer cells were shown to express the least transcript levels of GASL1 and were thus chosen for further experimentation.

### GASL1 inhibits proliferation and colony formation of oral cancer cells

GASL1 was over-expressed in HN6 oral cancer cells, and its upregulation was confirmed by qRT-PCR (Fig. 1B). Whether GASL1 has any bearing on the viability of oral cancer cells, a CCK-8 assay was performed to examine the effect of GASL1 over-expression on HN6 host cell proliferation. It was found that HN6 cancer cells over-expressing GASL1 showed a significant decline in their proliferation with reference to the corresponding negative control cells (Fig. 1C). In addition, over-expression of GASL1 was found to markedly decline the incorporation of EdU into HN6 cancer cells indicative of the loss of cell viability (Fig. 1D).

### GASL1 induces apoptosis in oral cancer cells

To look for the possible mechanism mediating the antiproliferative effects of GASL1 against oral cancer cells, the study of cell apoptosis was performed using Annexin V-FITC/PI staining method. It was shown that GASL1 over-expression in HN6 oral cancer cells significantly enhanced ($P$<0.05) the percentage of both early and late apoptotic host cells with reference to the negative control cancer cells (Fig. 2A). The apoptotic HN6 cell percentage increased from 2. 81% in control to 25.89% upon GASL1 overexpression. The western blotting study also showed that HN6 cancer cells up-regulating GASL1 exhibited significantly higher ($P$<0.05) expression of Bax protein while expression of Bcl-2 protein was markedly repressed with respect to the negative control cells (Fig. 2B).

### GASL1 induces G0/G1 cell cycle arrest in oral cancer cells

To determine whether GASL1 overexpression affects cell cycle progression, cell cycle distribution was

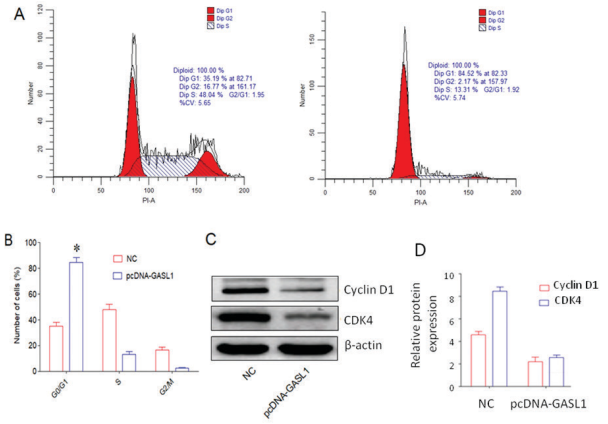


**Figure 3. Cell cycle analysis:**
(**A, B**) Flow cytometry analysis showed that G0/G1 cells increased significantly ($P<0.05$) in HN6 cells upon GASL1 overexpression. (**C**) Western blots analysis for cyclin D1 and CDK4 expression. (**D**) Densitometry analysis of western blot. Three replicates were used for performing the experiments.

investigated by flow cytometry. The results showed that the proportions of G0/G1 cells increased significantly ($P<0.05$) in HN6 cells upon GASL1 overexpression indicative of G0/G1 arrest (Fig. 3A and 3B). To further evaluate the effect of GASL1 overexpression on cell cycle progression, cycle-related protein expression was determined by western blot analysis. It was found that GASL1 inhibited the expression of cyclin D and CDK4, consistent with a G0/G1 cell cycle arrest (Fig. 3C).

## GASL1 inhibits the migration and invasion of oral cancer cells

The effect of GASL1 was also studied on the migration and invasion of oral cancer cells *in vitro*. Wound-healing assay showed that migration of HN6 cancer cells significantly decreased ($P<0.05$) by GASL1 overexpression (Fig. 4A). The percentage of cell migrationhas been

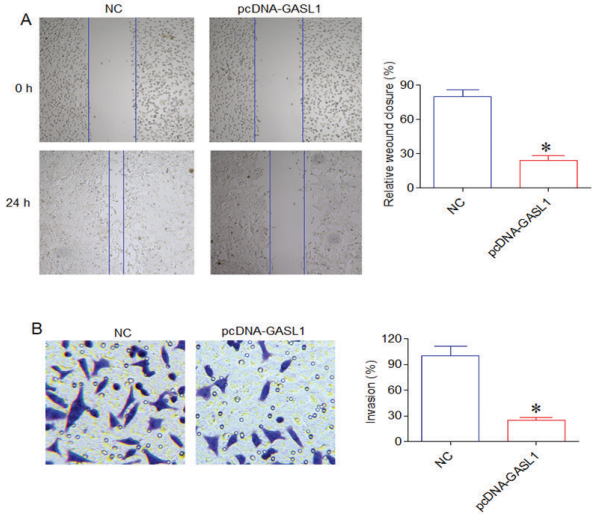


**Figure 4. (A) Wound-healing migration assay of HN6 cells transfected with pcDNA-GASL1 or pcDNA3.1 control vector (B) Transwell chamber invasion assay of HN6 cells transfected with pcDNA-GASL1 or pcDNA3.1 control vector.**
Three replicates were used for performing the experiment (*$P<0.05$). Three replicates were used for performing the experiment (*$P<0.05$).

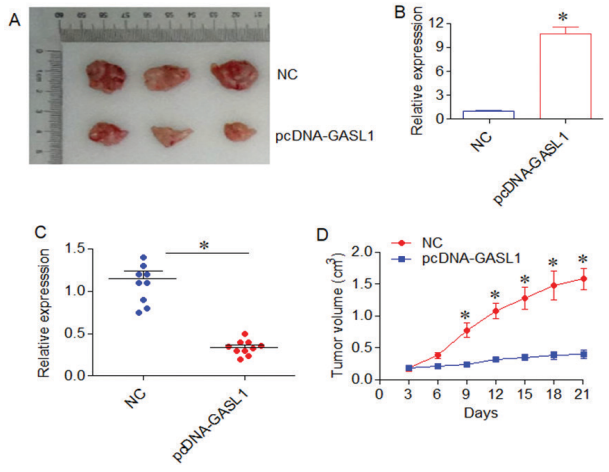


**Figure 5. Overexpression of GASL1 inhibits xenografted tumor growth *in vivo*.**
(**A**) Effects of GASL1 on xenografted tumor growth *in vivo*. (**B, C**) Showed significant upregulation of GASL1 in pcDNA-GASL1 tumors. (**D**) Showed that the volume and tumor weight of pcDNA-GASL1 tumors was significantly lower than the NC tumors.

shown to be decreased by more than 70% in HN6 cells over-expressing GASL1 with respect to negative control cells. Similarly, the invasion of HN6 cancer cells was significantly reduced under GASL1 up-regulation (Fig. 4B). The HN6 oral cancer cells over-expressing GASL1 showed only one-fourth of the rate of invasion of the negative control cells.

## GASL1 inhibits tumor growth *in vivo*

The effects of GASL1 were also investigated on xenografted tumor growth *in vivo* (Fig. 5A). To confirm the overexpression of GASL1 in xenografted tumors, expression analysis was carried out which showed significant upregulation of GASL1 in pcDNA-GASL1 tumors (Fig. 5B). Moreover, tumor volume and tumor weight of pcDNA-GASL1 tumors were significantly lower than the NC tumors (Fig. 5C). These findings indicate that overexpression of GASL1 inhibits xenografted tumor growth *in vivo*.

## DISCUSSION

There is growing support that long non-coding RNAs (lncRNAs) affect the key biological, cellular, and physiological aspects of the human body (Statello *et al.*, 2021; Li & Chang, 2014). This group of RNAs is seen to lack the protein-coding capability however is crucial for homeostasis at molecular level (Ji *et al.*, 2020; Zhang *et al.*, 2019). The dysregulation of lncRNAs reportedly surfaces the development and progression of different human pathological conditions including cancer (Forrest and Khalil, 2017; Tan *et al.*, 2021). Several studies have implicated that lncRNAs play a key role in regulating the malignant behavior of human cancer cells (Suzuki *et al.*, 2016; Huo *et al.*, 2017; Acha-Sagredo *et al.*, 2020). They have also been shown to be involved in shaping the tumor microenvironment and affect the cellular dynamics related to cancer cell invasion and metastasis (Yuan *et al.*, 2014; Liu *et al.*, 2021; Wu *et al.*, 2015). In the present study, lncRNA GASL1 was studied for its possible involvement in the regulation of growth and metastasis of human oral cancer. As per recent studies, GASL1

exhibits significant down-regulation in human cancers like gastric cancer and esophageal cancer and acts as a tumor-suppressor to regulate cancer cell growth and proliferation (Liu *et al*., 2020; Ren *et al*., 2021). The results of the current study were reflective of GASL1 repression in oral cancer cells. The decline in cell viability by GASL1 overexpression signifies its anti-proliferative regulatory role in oral cancer. GASL1 inhibited oral cancer cell growth by inducing apoptosis and modulating the Bax/Bcl-2 protein ratio. Bax protein was upregulated while Bcl-2 was repressed in GASL1 up-regulating oral cancer cells. This sort of expressional modulation of Bax and Bcl-2 is known to favor the onset of cell apoptosis (Nazeri *et al*., 2020; Wang *et al*., 2016).

Metastasis is the process that promotes the development of secondary tumorigenesis at sites distant from the original tumor development (Fares *et al*., 2020; Chang & Pauklin, 2021). The former is considered the major cause responsible for cancer-based mortality and failure of treatment procedures (Esposito *et al*., 2021; Riggio *et al*., 2021). Moreover, the process of cancer metastasis is poorly understood. It thus becomes interesting to study this important hallmark of human cancer and to identify the various regulators of cancer metastasis. Cancer cell migration and invasion to distant tissues are vital to the process of metastasis (Novikov *et al*., 2021; Winkler *et al*., 2020). The results of the current study indicated that oral cancer cells over-expressing GASL1 showed significantly lower rates of migration and invasion *in vitro*. This is in confirmation with a recent study wherein GASL1 has been shown to be involved in the regulation of esophageal squamous cell carcinoma (Ren *et al*., 2021; Weidle & Birzele, 2022). The finding thus insights into the therapeutic utility of GASL1 against oral cancer. In sum, the study established the role of GASL1 in regulating growth and metastasis of human oral cancer cells however the same needs to be confirmed *in vivo* also.

Our findings imply that lncRNA GASL1 may serve as a new biomarker for the detection and management of oral cancer. Hence, a thorough understanding of the lncRNA GASL1 functioning mechanism will be beneficial for the detection and management of oral cancer. As master controllers of gene expression, lncRNAs are interesting candidates for targeted epigenetic pharmacological therapy. There are now tractable pharmacological methods that can either destroy excessive lncRNAs or competitively decrease their activity. For the first time, medicines that target particular lncRNAs may now be created, allowing for the epigenetic modulation of particular metabolic pathways. If this is the case, lncRNA medicines will significantly improve upon the relatively low specificity of currently available medications that serve as epigenetic regulators.

## CONCLUSION

Taken together, oral cancer cells exhibit significant downregulation of lncRNA GASL1. GASL1 overexpression inhibited the cancer cell proliferation by inducing apoptosis. Interestingly, the oral cancer cell migration and invasion was significantly declined by GASL1 upregulation. The study thus confirmed the tumor-suppressive action of GASL1 in oral cancer however the findings need to be verified further.

### Declarations

**Conflict of interest**. None.

**Acknowledgements.** Declared none.

**Author Contributions**. RZ: Perform experiments, WT: Data collection and analysis, Manuscript writing, LY: Study design, analysis, and interpretation of the results. All authors approved the final manuscript.

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