LncRNA HOXA-AS3 promotes the progression of oral squamous cell carcinoma via inhibiting miR-218-5p

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Research

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LncRNA HOXA-AS3 promotes the progression of oral squamous cell carcinoma *via* inhibiting miR-218-5p

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Running title: LncRNA HOXA-AS3 promotes the progression of OSCC.

Abstract

Objective: The aim of the present study was to investigate the roles and molecular mechanism of long non-coding RNA (lncRNA) HOXA-AS3 in the progression of oral squamous cell carcinoma (OSCC). **Methods:** The expression of HOXA-AS3 and miR-218-5p was detected in OSCC tissues and cells using quantitative real-time polymerase chain reaction (qRT-PCR). Cell Counting Kit-8 (CCK-8) and colony formation assays were used to examine the effects of HOXA-AS3 and miR-218-5p on the proliferation of OSCC cells. Luciferase reporter gene assay was used to confirm the directly binding condition between lncRNA HOXA-AS3 and miR-218-5p in OSCC cells. Subsequently, a tumor xenograft model was used to determine the function of HOXA-AS3 in OSCC growth *in vivo*. **Results:** The relative expression of lncRNA HOXA-AS3 was observably upregulated in OSCC tissues and cell lines compared with the para-cancerous tissues and normal human oral keratinocyte (NHOK), respectively. Knockdown of HOXA-AS3 significantly inhibited the cell proliferation and colony formation of OSCC *in vitro and in vivo*. Bioinformatics and luciferase reporter assays showed that HOXA-AS3 directly bound to miR-218-5p. Moreover, the expression of miR-218-5p was negatively regulated by HOXA-AS3, and there was an inverse correlation between them. Silencing miR-218-5p reversed the inhibitory effect of lncRNA HOXA-AS3 knockdown on the proliferative potential of OSCC cells. **Conclusion:** In summary, our study illustrated lncRNA HOXA-AS3 promoted cancer cell proliferation in OSCC possibly by inhibiting miR-218-5p for the first time, which provides a new target or a potential diagnostic biomarker of the treatment for OSCC.

Keywords: Oral squamous cell carcinoma, LncRNA, HOXA-AS3, MiR-218-5p, Proliferation

Introduction

Oral squamous cell carcinoma (OSCC) is one of the most common head and neck squamous cell carcinomas and a public health threat. There are about 500,000 new cases in the world every year, and the incidence is increasing year by year [1-3]. Due

to the reasons of quick progression, invasive growth, easy lymph node and distance metastasis, and high recurrence rate, the prognosis of OSCC is relatively poor, with an overall 5-year survival rate of less than 50% [4, 5]. In recent years, despite synthetic serial treatments including of surgery, radiotherapy and chemotherapy have been widely applied in the patients of OSCC, the cure rate is one half only [6]. Therefore, an improved understanding of the molecular mechanisms underlying OSCC tumorigenesis help to provide novel insights into the pathogenesis of OSCC and thus improve the diagnostic and therapeutic methods.

Long non-coding RNAs (lncRNAs) are a family of RNAs with more than 200 nucleotides in length which do not code for proteins [7, 8]. Recently, a growing number of studies has demonstrated that aberrant expression of lncRNAs has important roles in many different types of human cancer including OSCC, osteosarcoma, hepatocellular carcinoma, nasopharyngeal carcinoma and gastric cancer [9-13]. LncRNAs are associated with the biological characteristics of malignant tumors, such as oncogenesis, development and metastasis [14, 15]. Increasing evidence has demonstrated the important roles of lncRNAs in regulating the biological performances of oral squamous cell carcinoma (OSCC). For example, the expression of lncRNA HOTAIR influenced the proliferation, apoptosis and cell cycle in OSCC cells [16]. LncRNA AC132217.4 was significantly upregulated and promoted the cell migration and epithelial-mesenchymal transition *via* the KLF8-AC132217.4-IGF2 signaling pathway in OSCC [17]. The high expression of lncRNA NEAT1 was correlated with advanced TNM stage and poor survival of patients and promoted the proliferation and invasion of OSCC cells *in vitro and in vivo* through the NEAT1/miR-365/RGS20 axis [18]. LncRNA LACAT1 was markedly increased in OSCC tissues and cells and promoted the malignant progression of OSCC by regulating miR-4301 [19].

Previous studies have demonstrated that lncRNA HOXA-AS3 is an important gene involving in tumorigenesis and tumor progression [20, 21]. However, the expression and role of HOXA-AS3 in OSCC have not been reported. In the present study, we found that lncRNA HOXA-AS3 was upregulated in OSCC samples and cell

lines, and the expression level was associated with with patient survival. Furthermore, we designed experiments to investigate the function and mechanism of HOXA-AS3 in OSCC cells. The data suggested that lncRNA HOXA-AS3 promoted the progression of OSCC by sponging miR-218-5p.

Materials and Methods

Patient tissue samples

Human OSCC tissues and para-cancerous specimens (more than 2 cm away from tumor tissues) were obtained from Tianjin Stomatological Hospital, and the category of all OSCC tissues was confirmed by pathological analysis. The patients referred to the 8th edition of UICC/AJCC oral squamous cell carcinoma tumor node metastasis (TNM) staging criteria. Moreover, none of the patients received the radiotherapy or chemotherapy before the operation. Clinical characteristics and demographics of the patients in this study were summarized in Table 1. All patients signed informed consent prior to using the tissues for this study according to the principles of the Declaration of Helsinki. Te tumor tissues were immediately frozen in liquid nitrogen and then stored at -80 °C for further research. This study was approved by the Ethics Committee of Tianjin Stomatological Hospital (Tianjin, People's Republic of China [PRC]).

Cell culture and transfection

Four OSCC cell lines (TSCCA, CAL-27, SCC-9, and Tca8113) and the normal human oral keratinocyte (NHOK) were purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, PRC). All cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Gibco, Waltham, MA, USA), 100 IU/mL of penicillin and 100 μg/mL of streptomycin at 37 °C incubator with 5% CO_2.

shRNA containing the HOXA-AS3 interference sequence (sh-HOXA-AS3) and negative control (sh-NC) were purchased from GeneChem (Shanghai, PRC), and miR-218-5p mimics, anti-miR-218-5p and negative control (miR-NC) were purchased from RiboBio (Guangzhou, PRC). Transfection was performed using Lipofectamine®

2000 Reagent (Invitrogen, Waltham, MA, USA) according to the manufacturer's protocol in OSCC cells. The culture medium was replaced 6 h after transfection, and transfection efficiency was examined with the expression vector of red fluorescent protein (RFP) at 48 h after the transfection.

RNA extraction and quantitative real time-polymerase chain reaction (qRT-PCR)

Total RNA was extracted from OSCC tissues or cells using the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. qRT-PCR was performed using the All-in-One™ miRNA qRT-PCR detection kit (GeneCopoeia, Rockville, MD, USA) for miR-218-5p and U6 as the internal control. The relative expression level of mRNA was detected using SYBR Green qRT-PCR assay (Bio-Rad Laboratories Inc, Hercules, CA, USA), and GAPDH was used as the internal control. All qRT-PCR procedure was performed on the ABI 7500 thermocycler (Thermo Fisher Scientific, Waltham, MA, USA). The sequences of the primers were: HOXA-AS3, F: 5'-GCTGAATTAACGGTGGCTCC-3', R:5'-ATGGCGAGCGAAGGGAAG-3'; GAPDH, F: 5'-GGAATCCACTGGCGTCTTCA-3', R: 5'-GGTTCACGCCCATCACAAAC-3'. The specific primers for miR-218-5p and U6 were purchased from RiboBio (Guangzhou, PRC). The relative expression levels of detective genes were calculated using the $2^{-\Delta\Delta Ct}$ method.

Cell proliferation analysis

Cell Counting Kit-8 reagent (Dojindo, Kumamoto, Japan) was used to measure the proliferation of OSCC cells. The OSCC cells were seeded into 96-well plates after transfection for 48 h. After culturing for 12 h, 24 h, 48 h and 72 h respectively, and then CCK-8 reagent was added to each well with 10 μl and further incubated for 4 h. The optical density (OD) value of each well was detected using an enzyme labeling instrument at 450 nm.

Colony formation assay

The OSCC cells were inoculated into 6-well plates with 200 cells in each well after transfection for 48 h. Subsequently, the cells were cultured in the complete medium for 14 d. At the first time, the medium was replaced after 5 d, and then replaced every

3 d. When the cell colonies formed, the medium was sucked dry. Then, the cells were washed twice with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde at 4 °C for 1 h. Next, the cells were stained with 0.1% crystal violet staining solution for 20 min. Finally, the number of cell colonies containing >50 cells in each well was calculated and photographed.

Xenograft tumor model in vivo

BALB/c nude male mice, six-week-old and weighing 22-28 g, were purchased from Beijing Huafukang Bioscience Co. Inc. (Beijing, PRC) and maintained in an environmental room with a constant temperature of 20 °C, humidity of 60% and a programmed 12 h light / 12 h dark cycle for circadian control. All mice were allowed free access to drinking water and a sterilized standard diet. A total of 2×10^6 SCC-9 cells transfected with sh-HOXA-AS3 or sh-NC were subcutaneously injected into the posterior flank of nude mice. The length (L) and width (W) of the tumors were measured every 5 days with calipers, and the tumor volume in nude mice was calculated according to the following formula: tumor volume (mm3) = $(L\times W^2)/2$. The mice were sacrificed by cervical dislocation after 25 days. All experimental animals received care in compliance with the Principles of Laboratory Animal Care. Reasonable efforts were made to minimize animal suffering. The animal experiments were approved by The Ethics Committee of Tianjin Stomatological Hospital (Tianjin, PRC).

Luciferase reporter assay

Online Software Starbase v2.0 (http://starbase.sysu.edu.cn) was used to predict the target miRNAs of lncRNA HOXA-AS3. The wild-type HOXA-AS3 3'-UTR containing the miR-218-5p seed sequence fragment (HOXA-AS3 Wt) and mutant-type (HOXA-AS3 Mut) luciferase vectors were conducted. The OSCC cells were seeded into 48-well plates and were co-transfected with the luciferase vectors and miR-218-5p mimics or negative control using Lipofectamine® 2000 Reagent for 48 h. This assay was normalized with 0.05 μg of the RFP expression vector pDsRed2-N1 (Clontech, USA). Subsequently, cells were lysed with RIPA lysis buffer, and the luciferase activity and RFP intensity were detected with the F-4500

Fluorescence Spectrophotometer (Hitachi, Japan) according to the manufacturer's instructions.

RNA immunoprecipitation (RIP) assay

RIP assay was used to detect the sponge function of HOXA-AS3 on miR-218-5p by using Magna RIPTM RNA Immunoprecipitation Kit (Millipore, Bedford, MA,USA). Briefly, the OSCC cells were transfected with miR-218-5p mimics, Vector-HOXA-AS3 or corresponding controls for 48 h, and then were lysed using the lysis buffer. Next, cell lysates were incubated with anti-Ago2 (Abcam, UK) or anti-IgG (Abcam, UK) and protein A/G magnetic beads. Finally, co-precipitated RNAs were detected by qRT-PCR.

Statistical analysis

Statistical analyse was performed using SPSS version 20.0 (IBM, Armonk, NY, USA) Data are presented as the mean ± standard deviation. Differences among multiple groups were analyzed by ANOVA (one-way) followed by Tukey *t*-test, and differences between the two groups were analyzed using the student's *t*-test. Correlation analysis between HOXA-AS3 and miR-218-5p expression was assessed using Pearson's correlation coefficient. The prognosis survival time of patients was evaluated using Kaplan-Meier analysis, and Log-rank test was used to examine the difference between different curves. $P<0.05$ was considered to indicate a statistically significant difference.

Results

LncRNA HOXA-AS3 was upregulated in OSCC tissues and cell lines, and significantly correlated with patient survival

In this study, we examined the expression of lncRNA HOXA-AS3 in 38 paired human OSCC tissues and the corresponding para-carcinoma tissues, as well as OSCC cell lines using qRT-PCR assay. As shown in Figure 1A, the expression level of lncRNA HOXA-AS3 was significantly increased in human OSCC tissues compared with that in the corresponding para-carcinoma tissues. Meanwhile, the expression levels of lncRNA HOXA-AS3 in four OSCC cell lines were obviously higher than that of

NHOK cells (Fig. 1B). The associations between lncRNA HOXA-AS3 expression and age, gender, clinical stage and distant metastasis of OSCC patients were shown in Table 1, indicating that patients with higher HOXA-AS3 expression had a higher clinical stage compared to those with lower HOXA-AS3 expression level. Moreover, as shown in Figure 1C, the lncRNA HOXA-AS3 expression in the 38 OSCC tissues was significantly associated with the overall survival of OSCC patients. The patients with low HOXA-AS3 expression had better overall survival compared to those with high expression.

Downregulation of lncRNA HOXA-AS3 inhibited the growth of OSCC cells in vitro and in vivo

Among the four selected OSCC cell lines, SCC-9 and CAL-27 cells expressed the relatively high level of lncRNA HOXA-AS3 (Fig. 1B), which were selected for transfection and subsequent experiments. To investigate the effect of lncRNA HOXA-AS3 on the proliferation of OSCC cells, short hairpin RNA targeting HOXA-AS3 mRNA (sh-HOXA-AS3) or sh-NC was transfected into SCC-9 and CAL-27 cells. As shown in Figure 2A, sh-HOXA-AS3 significantly decreased the HOXA-AS3 expression compared with sh-NC group both in the SCC-9 and CAL-27 cells. CCK-8 data revealed that downregulation of HOXA-AS3 observably inhibited the proliferation of SCC-9 and CAL-27 cells compared with sh-NC group (Fig. 2B and 2C). Furthermore, we found that downregulation of HOXA-AS3 decreased the colony formation ability of SCC-9 and CAL-27 cells by the colony formation assay (Fig. 2D). The potential involvement of HOXA-AS3 in tumorigenesis of OSCC *in vivo* was examined by injecting transfected SCC-9 cells into nude mice. As shown in Figure 2E, the tumors were smaller in the sh-HOXA-AS3 group compared with those in sh-NC group. The mean volume of xenograft tumors was markedly smaller in the sh-HOXA-AS3 group compared with sh-NC group (Fig. 2F). Thus, it was concluded that downregulation of lncRNA HOXA-AS3 suppressed the cell growth of OSCC *in vitro and in vivo*.

LncRNA HOXA-AS3 could directly bind miR-218-5p in OSCC

To explore the possible mechanism of lncRNA HOXA-AS3 in regulating the

biological behaviors of OSCC cells, we predicted the binding sites of lncRNA HOXA-AS3 using Online Software Starbase v2.0. As shown in Figure 3A, lncRNA HOXA-AS3 could bind to miR-218-5p. Subsequently, we validated the targeted effect of miR-218-5p on HOXA-AS3 via the luciferase reporter assay. The results revealed that luciferase activity significantly decreased in OSCC cells co-transfected with miR-218-5p mimics and wt HOXA-AS3 3'UTR; however, there has no marked change of luciferase activity in the mutated HOXA-AS3 3'UTR group, demonstrating the binding between miR-218-5p and HOXA-AS3 (Fig. 3B and 3C). Furthermore, RIP assay revealed that HOXA-AS3 was substantially enriched by miR-218-5p overexpression with anti-Ago2 in OSCC cells. The data suggested that there was an endogenous interaction between HOXA-AS3 and miR-218-5p, and that HOXA-AS3 might work as a miR-218-5p sponge (Fig. 3D and 3E). In short, these results suggested that lncRNA HOXA-AS3 could directly bind miR-218-5p in OSCC.

MiR-218-5p was downregulated in human OSCC tissues and cell lines, and inversely correlated with HOXA-AS3 expression

QRT-PCR was performed to detect expression level of miR-218-5p in OSCC cell lines, and 38 pairs of OSCC and para-cancerous tissues. As shown in Figure 4A, the expression level of miR-218-5p was markedly lower in OSCC cells than that of NHOK cells. Meanwhile, the expression of miR-218-5p was remarkably lower in OSCC tissues compared with that of para-cancerous tissues (Fig. 4B). And then, Pearson's correlation analysis showed there was a correlation between HOXA-AS3 and miR-218-5p expression in OSCC tissues. As shown in Figure 4C, the expression of miR-218-5p was inversely correlated with HOXA-AS3 expression level in OSCC tissues. Furthermore, the expression of miR-218-5p was markedly increased after HOXA-AS3 knockdown (Fig. 4D).

Overexpression of miR-218-5p inhibited the proliferation of OSCC cells

To investigate the role of miR-218-5p in the proliferation of OSCC cells, miR-218-5p mimics or miR-NC was transfected into SCC-9 and CAL-27 cells. As shown in Figure 5A, the expression level of miR-218-5p was significantly increased in miR-218-5p mimics group compared to that of miR-NC group both in SCC-9 and CAL-27 cells.

Then, the proliferation of OSCC cells was measured using CCK-8 and colony formation assay, respectively. The data revealed that the proliferation ability of SCC-9 and CAL-27 cells in the miR-218-5p mimics group was obviously reduced when compared to miR-NC group (Fig. 5B-5D). Together, overexpression of miR-218-5p inhibited the OSCC cells proliferation, which was consistent with the result of downregulation of HOXA-AS3.

Anti-miR-218-5p reversed the effect of lncRNA HOXA-AS3 knockdown on the proliferation of OSCC cells

To further study the interaction between HOXA-AS3 and miR-218-5p in OSCC cells, anti-miR-218-5p was transfected into SCC-9 and CAL-27 cells with HOXA-AS3 silencing. The expression level of HOXA-AS3 in each group was measured by qRT-PCR. As shown in Figure 6A and 6B, the expression of HOXA-AS3 in the co-transfected with si-HOXA-AS3 and anti-miR-218-5p group was observably higher than that of the co-transfected with si-HOXA-AS3 and miR-NC group. Moreover, CCK-8 assay showed that HOXA-AS3 knockdown could significantly suppress the proliferative activity of SCC-9 and CAL-27 cells, but was further reversed by miR-218-5p knockdown (Fig. 6C and 6D). As shown in Figure 6E and 6F, colony formation assay yielded the identical results.

Discussion

OSCC is a kind of malignant tumors that seriously threaten human health, and it has the characteristics of easy invasion and lymph node metastasis. Although great progress has been made in exploring new treatments, the prognosis of OSCC is still unsatisfactory due to its high malignancy [22]. Risk factors for OSCC include betel nut, tobacco, low-quality edible pigment, human papillomavirus infection, etc [23]. At present, the pathogenesis of OSCC is still not very clear, which may involve a multi-gene epigenetic and metabolic process, such as the loss of the function of cancer suppressor gene and activation of function of oncogene. [24, 25]. Thus, the research on the molecular biological mechanism of OSCC, especially the detection and diagnosis of specific early tumor markers, which is important for early diagnosis and

improvement of prognosis of OSCC patients.

HOXA-AS3 is a novel lncRNA located in chromosome 7p15.2, and belongs to the clusters of HOX genes, a group of highly homologous transcription factors that regulate embryological development [26]. HOXA-AS3 interacts with Enhancer Of Zeste 2 (EZH2) and acts as an epigenetic switch that determines the lineage specification of mesenchymal stem cells [27]. At present, there has been only two published researches expounding the roles of lncRNA HOXA-AS3 in glioma and lung adenocarcinoma, respectively [20, 21]. Wu et al.[20] reported that the expression of HOXA-AS3 was significantly increased in glioma tissues and cell lines, and knockdown of HOXA-AS3 inhibited the cell growth in vitro and vivo, promoted cell apoptosis, and impaired cell migration in glioma cells. Similarly, HOXA-AS3 was markedly upregulated in tissues and cells of lung adenocarcinoma, and promoted cancer cell progression [21]. However, there is no report on the function and molecular mechanism of HOXA-AS3 in OSCC. In this study, the expression levels of lncRNA HOXA-AS3 in OSCC tissues and cell lines were measured. We discovered that lncRNA HOXA-AS3 was more highly expressed in OSCC tissues and cells than that of in para-carcinoma tissues and NHOK cells, respectively. Moreover, the high expression of HOXA-AS3 was obviously correlated with pathological stage and overall survival of OSCC patients. To further investigate the function of lncRNA HOXA-AS3 on the biological performances of OSCC cells, HOXA-AS3 knockdown model was constructed in the SCC-9 and CAL-27 cells. The data revealed that knockdown of HOXA-AS3 suppressed the cell proliferation and growth *in vitro and in vivo.* This means that the high expression of HOXA-AS3 might be closely related to the progression of OSCC.

Currently, lncRNAs have been demonstrated to function as competing endogenous RNAs (ceRNA) by sponging miRNA and inhibiting intracellular miRNA function [28, 29]. Therefore, establishing the interrelationship of lncRNA and its regulation miRNA may help further understanding of the molecular mechanism underlying tumor progression and provide potential therapeutic targets for the clinical treatment of tumors. Bioinformatics analysis predicted that there was a binding site of

miR-218-5p in HOXA-AS3 3'UTR, and luciferase reporter assay confirmed the direct interaction in the SCC-9 and CAL-27 cells. Meanwhile, the expression of miR-218-5p was markedly decreased in OSCC tissues compared with para-carcinoma tissues, and was negatively correlated with HOXA-AS3 expression. RIP assay showed that HOXA-AS3 might work as a miR-218-5p sponge in OSCC. These results suggested that lncRNA HOXA-AS3 might influence the function of OSCC cells *via* sponging and regulating the miR-218-5p.

Previous studies have demonstrated that miR-218-5p as a tumor suppressor miRNA in various types of cancers, such as gastric cancer [30], colorectal cancer[31], prostate cancer [32], and pancreatic cancer [33]. Additionally, miR-218-5p was also illustrated to function as an anti-metastasis miRNA in non-small cell lung cancer [34], cervical cancer [35], and hepatocellular carcinoma [36]. Our study showed that the expression of miR-218-5p was significantly downregulated in OSCC cell lines, and overexpression of miR-218-5p remarkably inhibited the proliferation and colony formation of OSCC cells. Furthermore, to investigate whether HOXA-AS3 promoted the development of OSCC through regulating miR-218-5p, anti-miR-218-5p was transfected into SCC-9 and CAL-27 cells with knockdown of HOXA-AS3. The results revealed that knockdown of miR-218-5p could restore OSCC cell proliferation and colony formation activities after HOXA-AS3 silencing, suggesting that HOXA-AS3 might promote malignant progression of OSCC by inhibiting miR-218-5p.

Conclusion

In a word, our study showed that lncRNA HOXA-AS3 is significantly upregulated in OSCC and this high expression positively correlated with the pathological stage and poor prognosis of patients, which promotes the development of OSCC through sponging and inhibiting miR-218-5p.

Declarations

Ethics approval and consent to participate

All the OSCC tissue samples were collected with written informed consent in

accordance with the Declaration of Helsinki and with the approval of the Ethics Committee of Tianjin Stomatological Hospital (No. TJSH2017010, Date: 02/10/2017, Tianjin, PRC). The animal experiment was performed in accordance with the Basel Declaration and approved by the Ethics Committee of Tianjin Stomatological Hospital (No. TJSH2018062, Date: 07/12/2018, Tianjin, PRC).

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Authors' Contributions

YZ and RY designed the study and performed the experiments; YZ collected experimental data and drafted the manuscript; RY performed the statistical analysis and helped to revise the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets during and analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

References

1. Krishna Rao SV, Mejia G, Roberts-Thomson K, Logan R. Epidemiology of oral cancer in Asia in the past decade--an update (2000-2012). Asian Pac J Cancer Prev. 2013;14(10):5567-77.

2. Huang WC, Chan SH, Jang TH, Chang JW, Ko YC, Yen TC, Chiang SL, Chiang WF, Shieh TY, Liao CT, et al. miRNA-491-5p and GIT1 serve as modulators and biomarkers for oral squamous cell carcinoma invasion and metastasis. Cancer Res. 2014;74(3):751-64.

3. Chi AC, Day TA, Neville BW. Oral cavity and oropharyngeal squamous cell

carcinoma-an update. CA Cancer J Clin. 2015;65(5):401-21.
4. Afzali P, Ward BB. Management of the neck in oral squamous cell carcinoma: background, classification, and current philosophy. Oral Maxillofac Surg Clin North Am. 2019;31(1):69-84.
5. Kessler P, Grabenbauer G, Leher A, Bloch-Birkholz A, Vairaktaris E, Neukam FW. Neoadjuvant and adjuvant therapy in patients with oral squamous cell carcinoma Long-term survival in a prospective, non-randomized study. Br J Oral Maxillofac Surg. 2008;46(1):1-5.
6. Sacco AG, Cohen EE. Current Treatment Options for Recurrent or Metastatic Head and Neck Squamous Cell Carcinoma. J Clin Oncol. 2015;33(29):3305-13.
7. Mercer TR, Dinger ME, Mattick JS. Long non-coding RNAs: insights into functions. Nat Rev Genet. 2009;10(3):155-9.
8. Yang L, Froberg JE, Lee JT. Long noncoding RNAs: fresh perspectives into the RNA world. Trends Biochem Sci. 2014;39(1):35-43.
9. Su W, Tang J, Wang Y, Sun S, Shen Y, Yang H. Long non-coding RNA highly up-regulated in liver cancer promotes epithelial-to-mesenchymal transition process in oral squamous cell carcinoma. J Cell Mol Med. 2019;23(4):2645-55.
10. Fei D, Zhang X, Liu J, Tan L, Xing J, Zhao D, Zhang Y. Long Noncoding RNA FER1L4 Suppresses Tumorigenesis by Regulating the Expression of PTEN Targeting miR-18a-5p in Osteosarcoma. Cell Physiol Biochem. 2018;51(3):1364-75.
11. Zhang Y, Xu J, Zhang S, An J, Zhang J, Huang J, Jin Y. HOXA-AS2 Promotes Proliferation and Induces Epithelial-Mesenchymal Transition via the miR-520c-3p/GPC3 Axis in Hepatocellular Carcinoma. Cell Physiol Biochem. 2018;50(6):2124-38.
12. Lian Y, Xiong F, Yang L, Bo H, Gong Z, Wang Y, Wei F, Tang Y, Li X, Liao Q, et al. Long noncoding RNA AFAP1-AS1 acts as a competing endogenous RNA of miR-423-5p to facilitate nasopharyngeal carcinoma metastasis through regulating the Rho/Rac pathway. J Exp Clin Cancer Res. 2018;37(1):253.
13. Zhuo W, Liu Y, Li S, Guo D, Sun Q, Jin J, Rao X, Li M, Sun M, Jiang M, et al. Long Noncoding RNA GMAN, Up-regulated in Gastric Cancer Tissues, Is Associated With Metastasis in Patients and Promotes Translation of Ephrin A1 by Competitively Binding GMAN-AS. Gastroenterology. 2019;156(3):676-91.e11.
14. Rinn JL, Kertesz M, Wang JK, Squazzo SL, Xu X, Brugmann SA, Goodnough LH, Helms JA, Farnham PJ, Segal E, et al. Functional demarcation of active and silent chromatin domains in human HOX loci by noncoding RNAs. Cell. 2007;129(7):1311-23.
15. Liu H, Luo J, Luan S, He C, Li Z. Long non-coding RNAs involved in cancer metabolic reprogramming. Cell Mol Life Sci. 2019;76(3):495-504.
16. Liu H, Li Z, Wang C, Feng L, Huang H, Liu C, Li F. Expression of long non-coding RNA-HOTAIR in oral squamous cell carcinoma Tca8113 cells and its associated biological behavior. Am J Transl Res. 2016;8(11):4726-34.
17. Li X, Ma C, Zhang L, Li N, Zhang X, He J, He R, Shao M, Wang J, Kang L, et al. LncRNAAC132217.4, a KLF8-regulated long non-coding RNA, facilitates oral squamous cell carcinoma metastasis by upregulating IGF2 expression. Cancer Lett.

2017; 407:45-56.
18. Huang G, He X, Wei XL. lncRNA NEAT1 promotes cell proliferation and invasion by regulating miR-365/RGS20 in oral squamous cell carcinoma. Oncol Rep. 2018;39(4):1948-56.
19. Wang J, Huo F, Wang XR, Xu YY. LncRNA LACAT1 promotes proliferation of oral squamous cell carcinoma cells by inhibiting microRNA-4301. Eur Rev Med Pharmacol Sci. 2019;23(6):2427-35.
20. Wu F, Zhang C, Cai J, Yang F, Liang T, Yan X, Wang H, Wang W, Chen J, Jiang T. Upregulation of long noncoding RNA HOXA-AS3 promotes tumor progression and predicts poor prognosis in glioma. Oncotarget. 2017;8(32):53110-23.
21. Zhang H, Liu Y, Yan L, Zhang M, Yu X, Du W, Wang S, Li Q, Chen H, Zhang Y, et al. Increased levels of the long noncoding RNA, HOXA-AS3, promote proliferation of A549 cells. Cell Death Dis. 2018;9(6):707-20.
22. Bergamini C, Locati L, Bossi P, Granata R, Alfieri S, Resteghini C, Imbimbo M, Fallai C, Orlandi E, Tana S, et al. Does a multidisciplinary team approach in a tertiary referral centre impact on the initial management of head and neck cancer? Oral Oncol. 2016;54:54-7.
23. Rosenblatt KA, Daling JR, Chen C, Sherman KJ, Schwartz SM. Marijuana use and risk of oral squamous cell carcinoma. Cancer Res. 2004;64(11):4049-54.
24. Wang Y, Fan H, Zheng L. Biological information analysis of differentially expressed genes in oral squamous cell carcinoma tissues in GEO database. J BUON. 2018;23(6):1662-70.
25. Huang TH, Li KY, Choi WS. Lymph node ratio as prognostic variable in oral squamous cell carcinomas: Systematic review and meta-analysis. Oral Oncol. 2019; 89:133-43.
26. Duboule D. The rise and fall of Hox gene clusters. Development. 2007;134(14):2549-60.
27. Zhu XX, Yan YW, Chen D, Ai CZ, Lu X, Xu SS, Jiang S, Zhong GS, Chen DB, Jiang YZ. Long non-coding RNA HoxA-AS3 interacts with EZH2 to regulate lineage commitment of mesenchymal stem cells. Oncotarget. 2016;7(39):63561-70.
28. Kartha RV, Subramanian S. Competing endogenous RNAs (ceRNAs): new entrants to the intricacies of gene regulation. Front Genet. 2014;5:8.
29. Wang W, Zhuang Q, Ji K, Wen B, Lin P, Zhao Y, Li W, Yan C. Identification of miRNA, lncRNA and mRNA-associated ceRNA networks and potential biomarker for MELAS with mitochondrial DNA A3243G mutation. Sci Rep. 2017;7:41639.
30. Deng M, Zeng C, Lu X, He X, Zhang R, Qiu Q, Zheng G, Jia X, Liu H, He Z. miR-218 suppresses gastric cancer cell cycle progression through the CDK6/Cyclin D1/E2F1 axis in a feedback loop. Cancer Lett. 2017;403:175-85.
31. Liu M, Yin K, Guo X, Feng H, Yuan M, Liu Y, Zhang J, Guo B, Wang C, Zhou G, et al. Diphthamide Biosynthesis 1 is a Novel Oncogene in Colorectal Cancer Cells and is Regulated by MiR-218-5p. Cell Physiol Biochem. 2017;44(2):505-14.
32. Zhang G, He X, Ren C, Lin J, Wang Q. Long noncoding RNA PCA3 regulates prostate cancer through sponging miR-218-5p and modulating high mobility group box 1. J Cell Physiol. 2019;234(8):13097-109.

33. Liu S, Zhang W, Liu K, Liu Y. LncRNA SNHG16 promotes tumor growth of pancreatic cancer by targeting miR-218-5p. Biomed Pharmacother. 2019;114:108862.
34. Zhang L, Hu J, Li J, Yang Q, Hao M, Bu L. Long noncoding RNA LINC-PINT inhibits non-small cell lung cancer progression through sponging miR-218-5p/PDCD4. Artif Cells Nanomed Biotechnol. 2019;47(1):1595-602.
35. Xu Y, He Q, Lu Y, Tao F, Zhao L, Ou R. MicroRNA-218-5p inhibits cell growth and metastasis in cervical cancer via /NF-κB signaling pathway. Cancer Cell Int. 2018;18:198.
36. Ji D, Wang Y, Sun B, Yang J, Luo X. Long non-coding RNA MNX1-AS1 promotes hepatocellular carcinoma proliferation and invasion through targeting miR-218-5p/COMMD8 axis. Biochem Biophys Res Commun. 2019;513(3):669-74.

Legends for figures

Fig. 1 HOXA-AS3 expression is upregulated in OSCC tissues and cell lines. (**A**) The expression of HOXA-AS3 in OSCC tissues and para-cancerous tissues was measured using qRT-PCR. (**B**) Expression levels of HOXA-AS3 in NHOK and OSCC cell lines (TSCCA, CAL-27, SCC-9, and Tca8113) were detected via qRT-PCR. (**C**) The Kaplan-Meier survival curve indicated that the prognosis of patients in HOXA-AS3 high-expression group was significantly worse than that of patients in low-expression group. *P<0.05, **P<0.01.

Fig. 2 HOXA-AS3 knockdown inhibited OSCC cell proliferation and colony formation in vitro and in vivo. (**A**) Transfection efficacy of sh-HOXA-AS3 in SCC-9 and CAL-27 cells. (**B, C**) Cell Counting Kit-8 assay showed that HOXA-AS3 knockdown inhibited cell proliferation in SCC-9 and CAL-27 cells. (**D**) Colony formation assay showed that HOXA-AS3 knockdown significantly reduced the number of colonies. (**E**) A representative image of the xenograft tumors is shown (n = 6 per group). (**F**) The growth curve of xenograft tumors derived by sh-HOXA-AS3-treated SCC-9 cells was determined in vivo. *P<0.05, **P<0.01.

Fig. 3 HOXA-AS3 directly interacted with miR-218-5p. (**A**) Predicted binding of human miR-218-5p with the wild-type 3'UTR region of HOXA-AS3 mRNA and a mutated 3'UTR of HOXA-AS3. (**B, C**) Luciferase reporter gene assay verified that HOXA-AS3 could directly bind to miR-218-5p in SCC-9 and CAL-27 cells. (**D, E**) SCC-9 and CAL-27 cells were transfected with miR-218-5p mimics or control,

followed by the measurement of HOXA-AS3 mRNA enrichment with anti-Ago2 by qRT-PCR, and anti-IgG used as control. *P<0.05, **P<0.01.

Fig. 4 MiR-218-5p was downregulated in OSCC tissues and cells, and inversely correlated with HOXA-AS3 expression. (**A**) The expression of miR-218-5p in OSCC cell lines and NHOK was detected by qRT-PCR. (**B**) The expression of miR-218-5p in OSCC tissues and para-cancerous tissues was detected by qRT-PCR. (**C**) HOXA-AS3 and miR-218-5p expression level was negatively correlated in OSCC tissues (r=-0.759, P<0.01, n=38). (**D**) qRT-PCR was used to measure the expression level of miR-218-5p after HOXA-AS3 knockdown in OSCC cell lines. *P<0.05, **P<0.01.

Fig. 5 Overexpression of miR-218-5p inhibited the proliferation of OSCC cells. (**A**) Transfection efficacy of miR-218-5p mimics in SCC-9 and CAL-27 cells. (**B, C**) Cell Counting Kit-8 assay showed that overexpression of miR-218-5p inhibited cell proliferation in OSCC cells. (**D**) Colony formation assay showed that overexpression of miR-218-5p significantly reduced the number of colonies. *P<0.05, **P<0.01.

Fig. 6 LncRNA HOXA-AS3 promoted OSCC development through regulating miR-218-5p. (**A, B**) The expression level of HOXA-AS3 in cells co-transfected with sh-HOXA-AS3 and anti-miR-218-5p was detected by qRT-PCR. (**C-F**) Inhibited proliferation of SCC-9 and CAL-27 cells by HOXA-AS3 knockdown was reversed by anti-miR-218-5p. *P<0.05, **P<0.01.

Figures

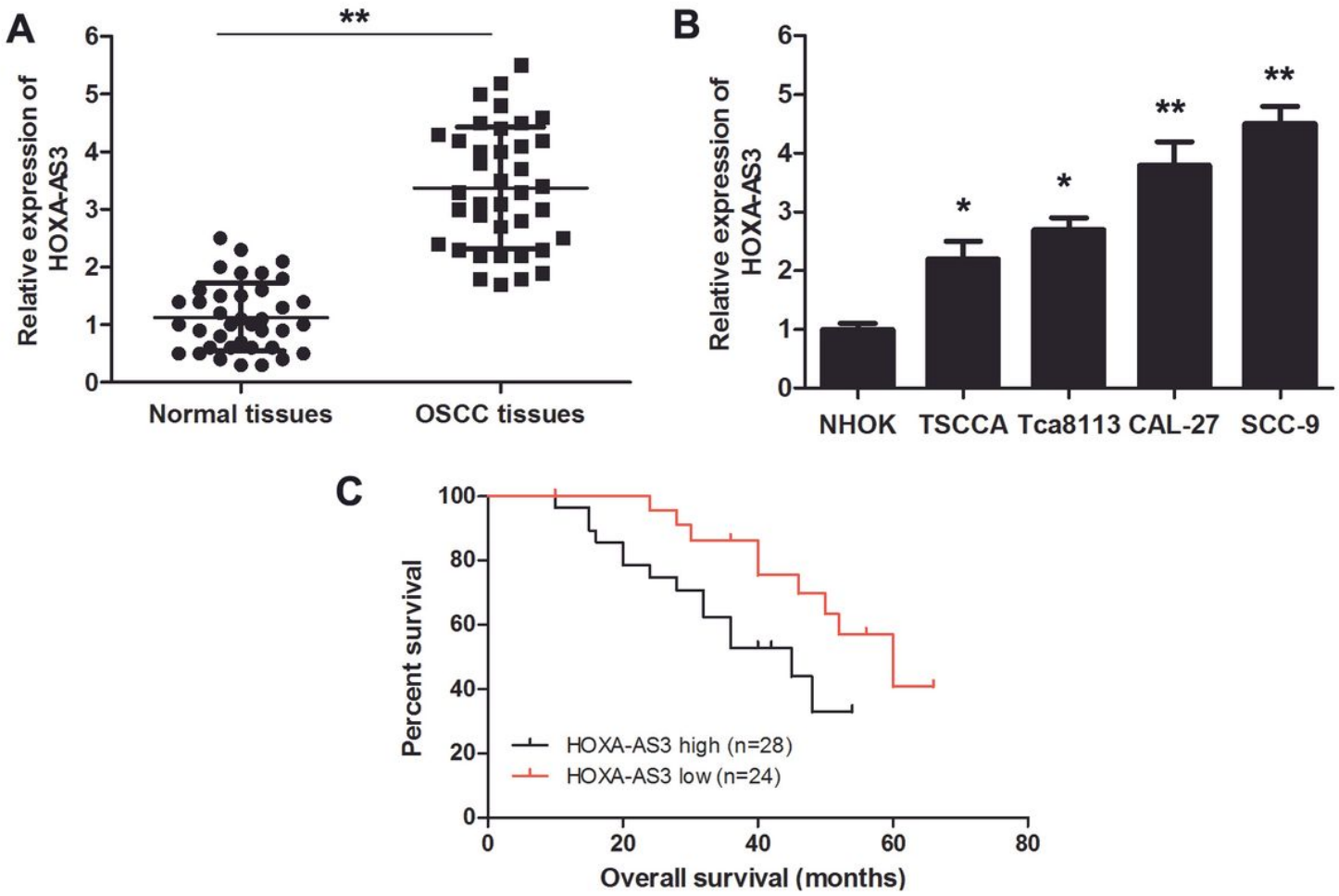

Figure 2

HOXA-AS3 expression is upregulated in OSCC tissues and cell lines. (A) The expression of HOXA-AS3 in OSCC tissues and para-cancerous tissues was measured using qRT-PCR. (B) Expression levels of HOXA-AS3 in NHOK and OSCC cell lines (TSCCA, CAL-27, SCC-9, and Tca8113) were detected via qRT-PCR. (C) The Kaplan-Meier survival curve indicated that the prognosis of patients in HOXA-AS3 high-expression group was significantly worse than that of patients in low-expression group. *P<0.05, **P<0.01.

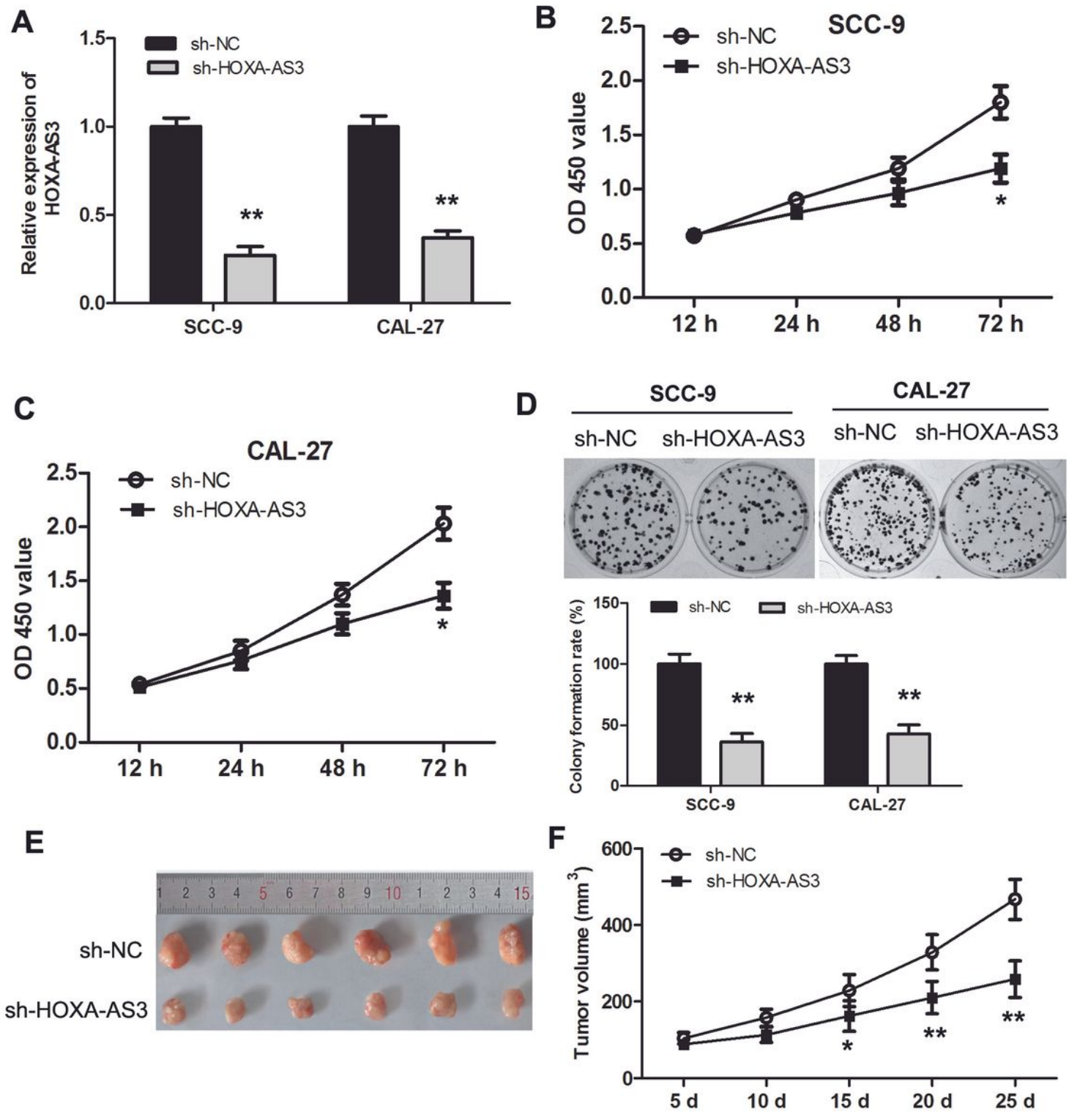

Figure 3

HOXA-AS3 knockdown inhibited OSCC cell proliferation and colony formation in vitro and in vivo. (A) Transfection efficacy of sh-HOXA-AS3 in SCC-9 and CAL-27 cells. (B, C) Cell Counting Kit-8 assay showed that HOXA-AS3 knockdown inhibited cell proliferation in SCC-9 and CAL-27 cells. (D) Colony formation assay showed that HOXA-AS3 knockdown significantly reduced the number of colonies. (E) A

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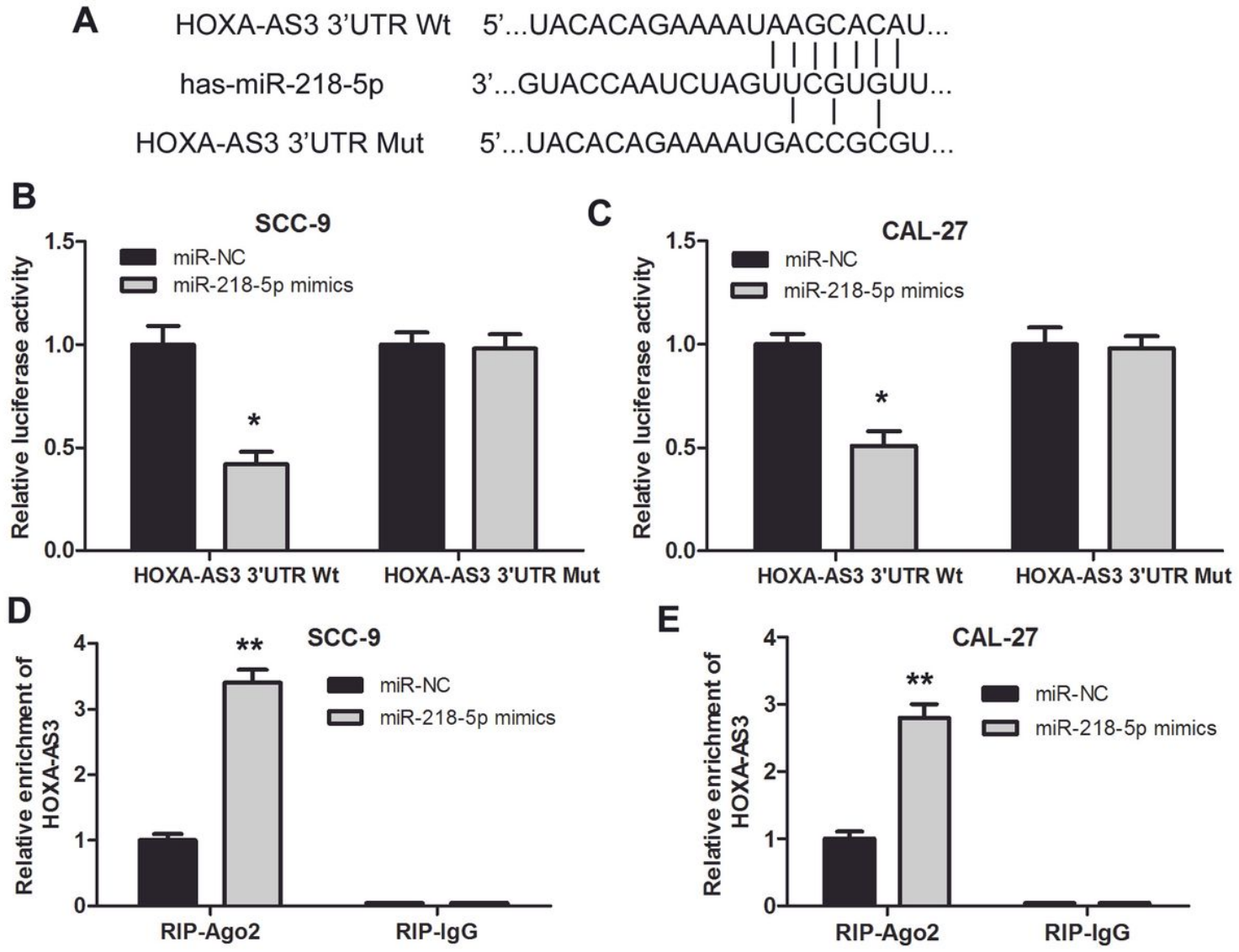

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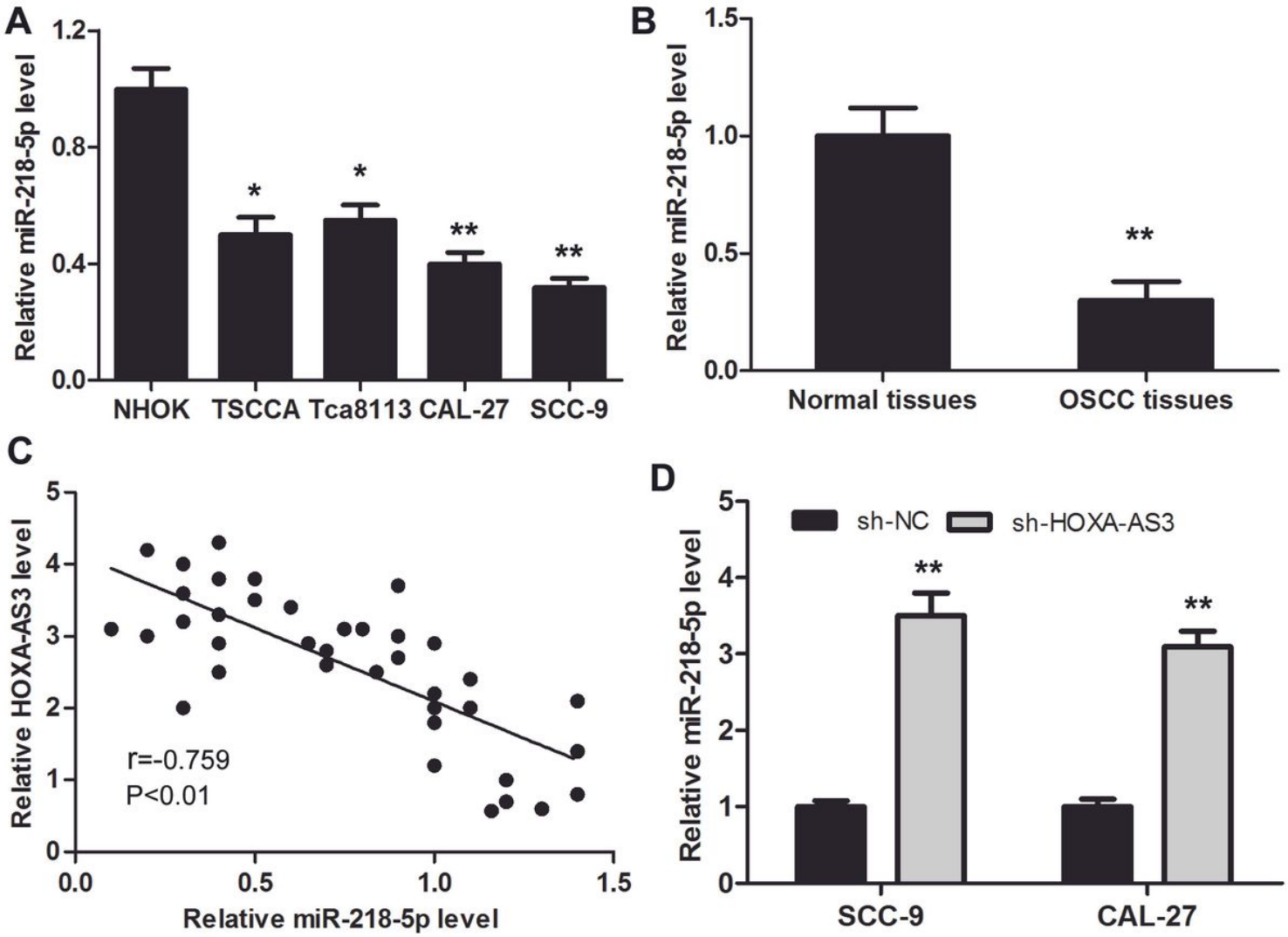

Figure 8

MiR-218-5p was downregulated in OSCC tissues and cells, and inversely correlated with HOXA-AS3 expression. (A) The expression of miR-218-5p in OSCC cell lines and NHOK was detected by qRT-PCR. (B) The expression of miR-218-5p in OSCC tissues and para-cancerous tissues was detected by qRT-PCR. (C) HOXA-AS3 and miR-218-5p expression level was negatively correlated in OSCC tissues (r=-0.759, P<0.01, n=38). (D) qRT-PCR was used to measure the expression level of miR-218-5p after HOXA-AS3 knockdown in OSCC cell lines. *P<0.05, **P<0.01.

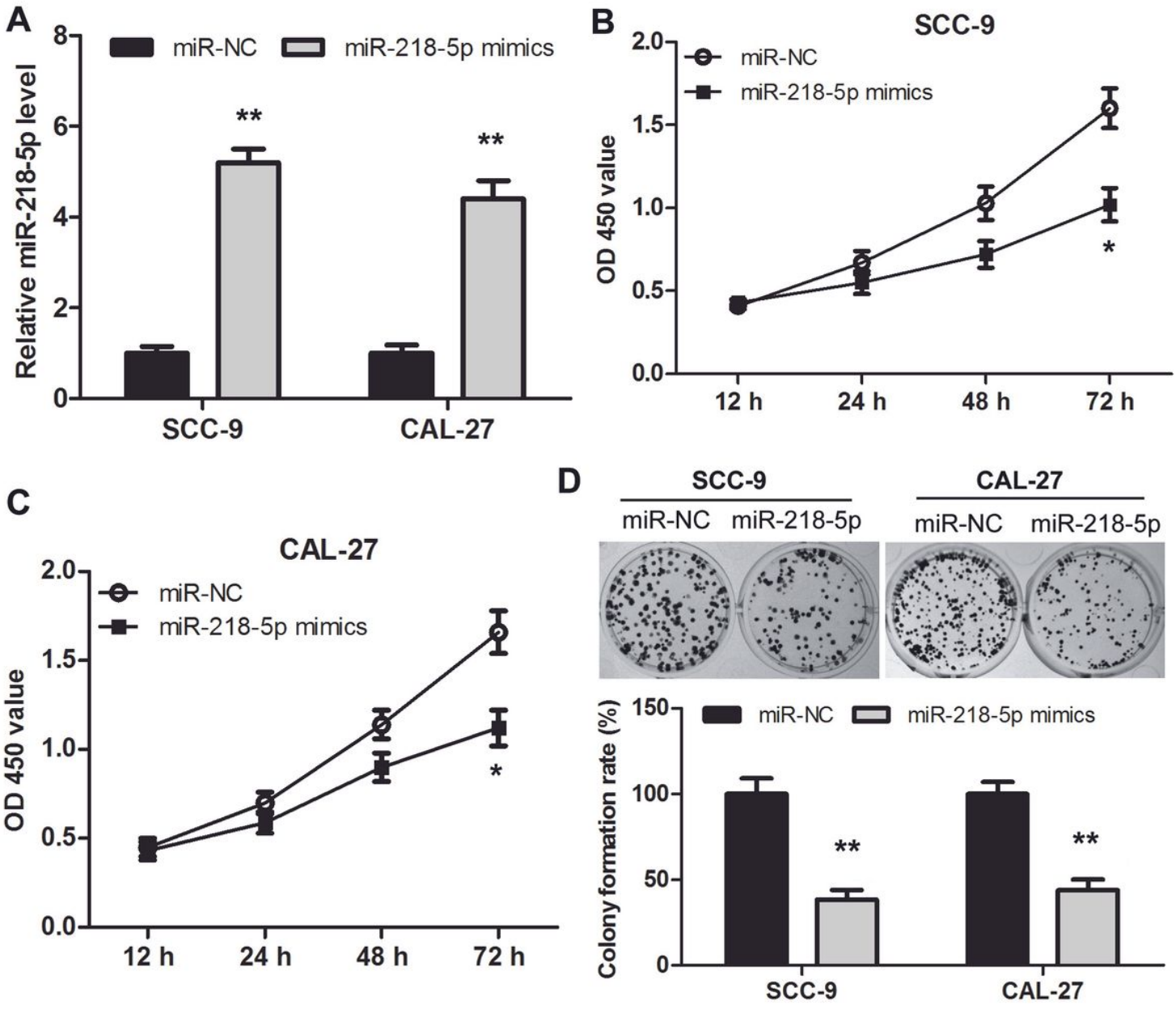

Figure 10

Overexpression of miR-218-5p inhibited the proliferation of OSCC cells. (A) Transfection efficacy of miR-218-5p mimics in SCC-9 and CAL-27 cells. (B, C) Cell Counting Kit-8 assay showed that overexpression of miR-218-5p inhibited cell proliferation in OSCC cells. (D) Colony formation assay showed that overexpression of miR-218-5p significantly reduced the number of colonies. *P<0.05, **P<0.01.

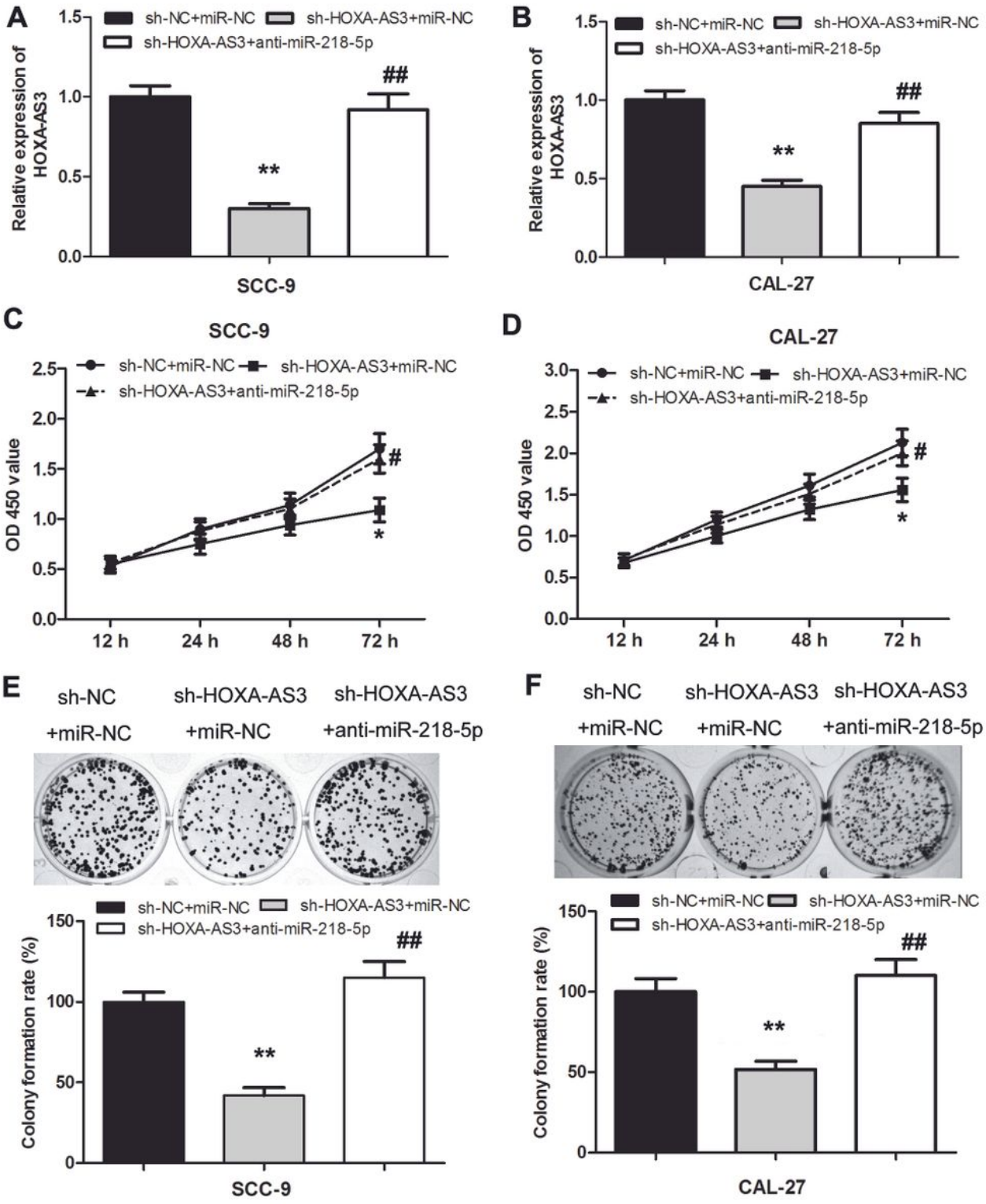

Figure 11

LncRNA HOXA-AS3 promoted OSCC development through regulating miR-218-5p. (A, B) The expression level of HOXA-AS3 in cells co-transfected with sh-HOXA-AS3 and anti-miR-218-5p was detected by qRT-PCR. (C-F) Inhibited proliferation of SCC-9 and CAL-27 cells by HOXA-AS3 knockdown was reversed by anti-miR-218-5p. *P<0.05, **P<0.01.