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LncRNA INHBA-AS1 promotes cell growth, migration, and invasion of oral squamous cell carcinoma by sponging miR-143-3p

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Abstract. – OBJECTIVE: Recent studies have revealed that long noncoding RNAs (lncRNAs) play important roles in the progression of tumorigenesis. Oral squamous cell carcinoma is a disease widely widespread all over the world. The aim of this study was to identify how lncRNA INHBA-AS1 functions in the progression of OSCC.

PATIENTS AND METHODS: LncRNA INHBA-AS1 expression in both OSCC cells and 48 paired tissue samples was detected by Quantitative Real Time-quantitative Polymerase Chain Reaction (RT-qPCR). The function of INHBA-AS1 was identified by the transwell assay, wound healing assay, and proliferation assay *in vitro*. Meanwhile, the role of INHBA-AS1 was investigated through tumor formation assay *in vivo*. Furthermore, the underlying mechanism was explored by the luciferase assays and RNA binding protein immunoprecipitation assay (RIP).

RESULTS: INHBA-AS1 was significantly overexpressed in OSCC tissues when compared with adjacent tissue samples. Moreover, the proliferation, invasion, and migration of OSCC cells were significantly inhibited after the knockdown of INHBA-AS1 *in vitro*. Meanwhile, the knockdown of INHBA-AS1 remarkably inhibited tumor growth and metastasis in vivo. Besides, miR-143-3p was down-regulated after the knockdown of INHBA-AS1 *in vitro*. The expression of miR-143-3p negatively correlated with the expression of INHBA-AS1 in OSCC tissues. In addition, miR-143-3p was directly targeted by INHBA-AS1.

CONCLUSIONS: The knockdown of INHBA-AS1 repressed cell migration, invasion, proliferation in OSCC by sponging miR-143-3p, which might offer a new therapeutic intervention for OSCC patients.

Key Words:
Long noncoding RNA, INHBA-AS1, Oral squamous cell carcinoma (OSCC), MiR-143-3p.

Introduction

Oral cancer is the most prevalent cancer in the world. Meanwhile, it ranks third among all malignancies in the developing countries[1]. As the main subtype of oral cancer, oral squamous cell carcinoma (OSCC) contributes to almost 3% newly diagnosed cancer cases. Statistics[2] have shown OSCC has the highest morbidity among head and neck cancers. A rapid development has been achieved in the diagnosis and treatment of OSCC. However, the survival rate of patients remains less than 50%, which has not been remarkably improved in decades. To improve the therapeutic efficacy and to understand the molecular mechanism of OSCC, it is essential and urgent to find out important molecular markers in the development of OSCC.

Long non-coding RNAs (lncRNAs) are a cluster of non-coding RNA transcripts with over 200 nucleotides (nt) in length, with no protein-coding function. Evidence has shown that lncRNAs act as vital regulators in many biological behaviors in malignancies, including carcinogenesis, cell apoptosis, cell proliferation, and cell metastasis. In fact, through binding to SRSF6, lncRNA LINC01133 promotes colorectal cancer metastasis by inducing epithelial-mesenchymal transition (EMT)[3]. LncRNA MALAT1 accelerates cell migration and invasion in hepatocellular carcinoma via targeting miR-204[4]. LncRNA GHET1 expression level is correlated with TNM staging and prognosis of pancreatic cancer patients, which promotes cell proliferation as well[5]. Besides, lncRNA CRNDE-h has been reported to serve as a novel serum biomarker for colorectal cancer[6]. However, the exact role of lncRNA INHBA-AS1 in the development of OSCC remains unclear.

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Our study revealed that the expression level of INHBA-AS1 was remarkably up-regulated in OSCC tissues. Subsequent function experiments indicated that the knockdown of INHBA-AS1 significantly suppressed OSCC growth, invasion, and migration *in vitro* and *in vivo*. Furthermore, INHBA-AS1 played its role in OSCC by sponging miR-143-3p.

Patients and Methods

OSCC Tissue Specimens

Paired OSCC tissues and adjacent non-tumor tissues were sequentially gathered from 48 OSCC patients who received surgery in the Stomatological Hospital, Southern Medical University, Guangzhou, China. This study was approved by the Ethics Committee of Stomatological Hospital, Southern Medical University. Informed consents were obtained from all subjects before the study.

OSCC Cell Lines

Human OSCC cell lines (Tca8113, TSC[illegible], CAL-27, SCC-9) and normal cervical epi[illegible] cell line (NHOK) were offered by the C[illegible]se Academy of Science (Shanghai, China). All [illegible] were cultured in Roswell Park Memorial [illegible]stitute-1640 (RPMI-1640; HyC[illegible] South L[illegible]gan, UT, USA) consisting o[illegible]l bovin[illegible] serum (FBS; Life Techno[illegible]es, G[illegible]ersburg, MD, USA) and penicillin[illegible]les, [illegible]re maintained in an incu[illegible]tor [illegible] CO_2 at [illegible].

Cell Transfecti[illegible]

The lentivir[illegible]ing short-h[illegible]n RNA (shRNA) direc[illegible]d aga[illegible]HBA-AS1 was provided by [illegible]ePharma ([illegible]hai, China). Subsequent[illegible]we amplified [illegible] encoding INHBA[illegible] and i[illegible]erted it into pcDNA3.1 (Invitro[illegible]arls[illegible], CA, USA). Synthesized pcDNA3.[illegible]nsfecte[illegible]to Tca8113 OSCC cells [illegible]ordin[illegible]e i[illegible]ctions of Lipofectamine [illegible](Invit[illegible]rlsbad, CA, USA). The INH[illegible]AS1 exp[illegible]ion level in transfected cells w[illegible]d using Real Time-quantitative Polyase [illegible]Reaction (RT-qPCR).

[illegible] Extraction and RT-qPCR

[illegible] RNA in tissues and cells was extracted by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Subsequent, the extracted total RNA was reverse-transcribed into cDNAs through reverse Transcription Kit (TaKaRa Biotechnology Co., Ltd., Dalian, China). The thermocycling conditions were as follows: 30 s at 95°C, 5s for 40 cycles at 95°C, and 35 s at 60°C. Primers [illegible] for RT-qPCR were as follows: INHBA-AS[illegible] 5′-CCTACTACACACAGGGGCTC-3[illegible] revers[illegible] 5-TTCCAGAAGCTCCTCATGGC[illegible] Glyceraldehyde 3-phosphate dehydroge[illegible]APDH), forward 5′-CCAAAATC[illegible]GATG[illegible]AAT-GCTGG-3′ and reverse 5′-[illegible]GGCA[illegible] GTGGTCATTCA-3′. Th[illegible]tive e[illegible]ression genes was calculated [illegible] $2^{-\Delta\Delta C}$[illegible]thod.

Cell Proliferati[illegible] Assa[illegible]

Cell prolife[illegible] of transf[illegible] 96-well plates was [illegible]d every 24 [illegible]trict accordance wi[illegible]el[illegible]ing kit-8 (CCK-8) assay (Dojind[illegible] Molecula[illegible]nologies, Inc., Kumamot[illegible]pan). Absorba[illegible]450 nm was measu[illegible]by a spectrophotom[illegible]r (Thermo Scientific, R[illegible]ford, IL, USA).

W[illegible]d Heali[illegible]ssay

[illegible]irst seeded into 6-well plates and cu[illegible]in RPIM-1640 medium overnight. After scratched with a plastic tip, the cells were [illegible]in serum-free RPMI-1640. The wound [illegible]was viewed at specific time points. Each assay was independently repeated for three times.

Transwell Assay

24 h after transfection, 2 ×10^5 cells in 100 µL serum-free RPMI-1640 were transformed to the upper chamber of an 8-µm culture insert (Corning, Corning, NY, USA) coated with 50 µg Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). Meanwhile, 20% of FBS-RPIM-1640 was added to the lower chamber of culture inserts. 24 h later, these inserts were fixed with methanol for 30 min and stained with hematoxylin for 20 min. The number of migrated and invaded cells was counted under an inverted microscope (×20). Three fields were randomly selected for each sample.

Xenograft Model

For tumor formation assay, the transfected Tca8113 cells were subcutaneously injected into NOD/SCID mice (4-5 weeks old). The tumor diameters were detected every 5 days after inoculation. The tumor volume was calculated as the formula: volume = length × width^2 × 1/2. The mice were sacrificed, and the tumors were extracted after 4 weeks. For tumor metastasis assay, the transfected Tca8113 cells were injected into the

tail vein of NOD/SCID mice (4-5 weeks old). The mice were sacrificed, and lung tissues were extracted after 4 weeks. The number of metastatic nodules in lung tissues was counted. The animal experiments were approved by the Animal Ethics Committee of Southern Medical University.

Luciferase Assays

The 3′-UTR of INHBA-AS1 was first cloned into the pGL3 vector (Promega, Madison, WI, USA). Site-directed mutagenesis of the miR-143-3p binding site in INHBA-AS1 3′-UTR was performed using Quick-change site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). The cells were transfected with INHBA-AS1 WT-3′-UTR or XIST MUT-3′-UTR and miR-ctrl or miR-143-3p for 48 h. Finally, the dual luciferase reporter assay system (Promega, Madison, WI, USA) was utilized for the luciferase assays.

RNA Immunoprecipitation (RIP) Assay

To confirm the endogenous relationship between INHBA-AS1 and miR-143-3p, the RIP assay was carried out using EZMagna RIP RNA-binding protein immunoprecipitation (Millipore, Billerica, MA, USA). The transfected Tca8113 cells were collected and lysed in RIP lysis buffer containing protease inhibitor and RNase inhibitor. Then, the cells were incubated with the RIP buffer containing magnetic beads coated with Ago2 antibodies (Millipore, Billerica, MA, USA). IgG used as control (input group). After incubation for 2 h at 4°C, the coprecipitated RNAs were isolated and measured by RT-qPCR.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 18.0 (SPSS Inc., PASW Statistics for Windows, Chicago, IL, USA) was used for all statistical analysis. The Chi-square test and the Student *t*-test were selected when appropriate. The experimental data were presented by mean ± SD (standard deviation). $p<0.05$ was considered statistically significant.

Results

High Expression Level of INHBA-AS1 in OSCC Tissues and Cells

First, RT-qPCR was conducted to detect the INHBA-AS1 expression in 48 patients' tissues and OSCC cells. The results demonstrated that INHBA-AS1 was significantly up-regulated in OSCC tissues when compared with adjacent tissues (Figure 1A). Meanwhile, INHBA-AS1 expression in OSCC cells was significantly higher than that of NHOK cells (Figure 1B).

INHBA-AS1 Knockdown Inhibited OSCC Cell Proliferation In Vitro

According to INHBA-AS1 expression in OSCC cells, Tca8113 OSCC cell line was chosen for the knockdown of INHBA-AS1. The

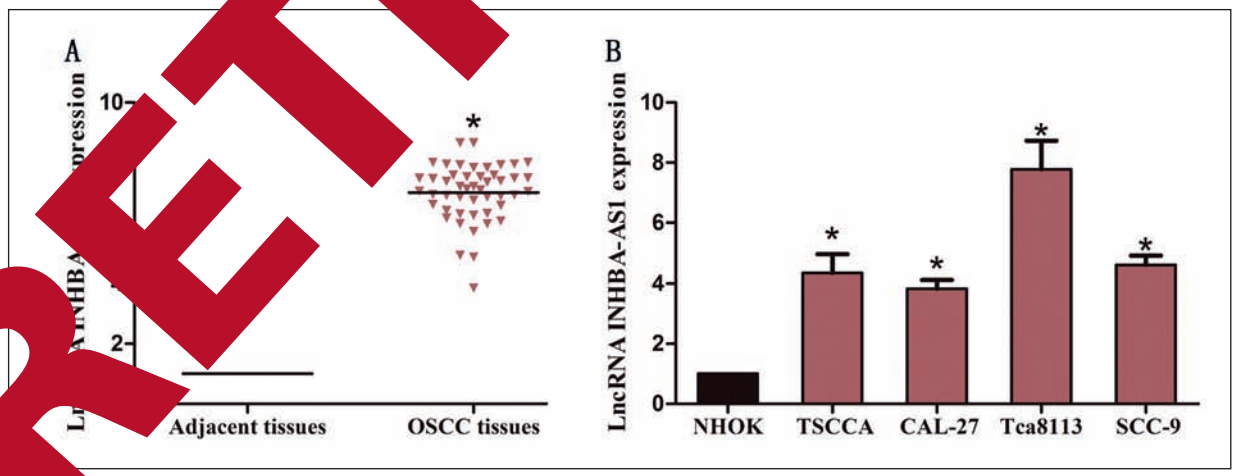

Figure 1. Expression level of INHBA-AS1 increased significantly in both OSCC tissues and cell lines. **A,** INHBA-AS1 expression was significantly up-regulated in OSCC tissues compared with adjacent tissues. **B,** Expression levels of INHBA-AS1 relative to GAPDH in human OSCC cell lines and normal human oral keratinocyte (NHOK) were determined by RT-qPCR. The data were presented as mean ± standard error of the mean. *$p<0.05$.

INHBA-AS1 shRNA (sh-INHBA-AS1) and the empty vector were synthesized and transduced into Tca8113 cells. Later, the INHBA-AS1 expression was determined by RT-qPCR (Figure 2A). The results of CCK8 assay showed that the proliferation of OSCC cells was significantly repressed after INHBA-AS1 was knocked down (Figure 2B).

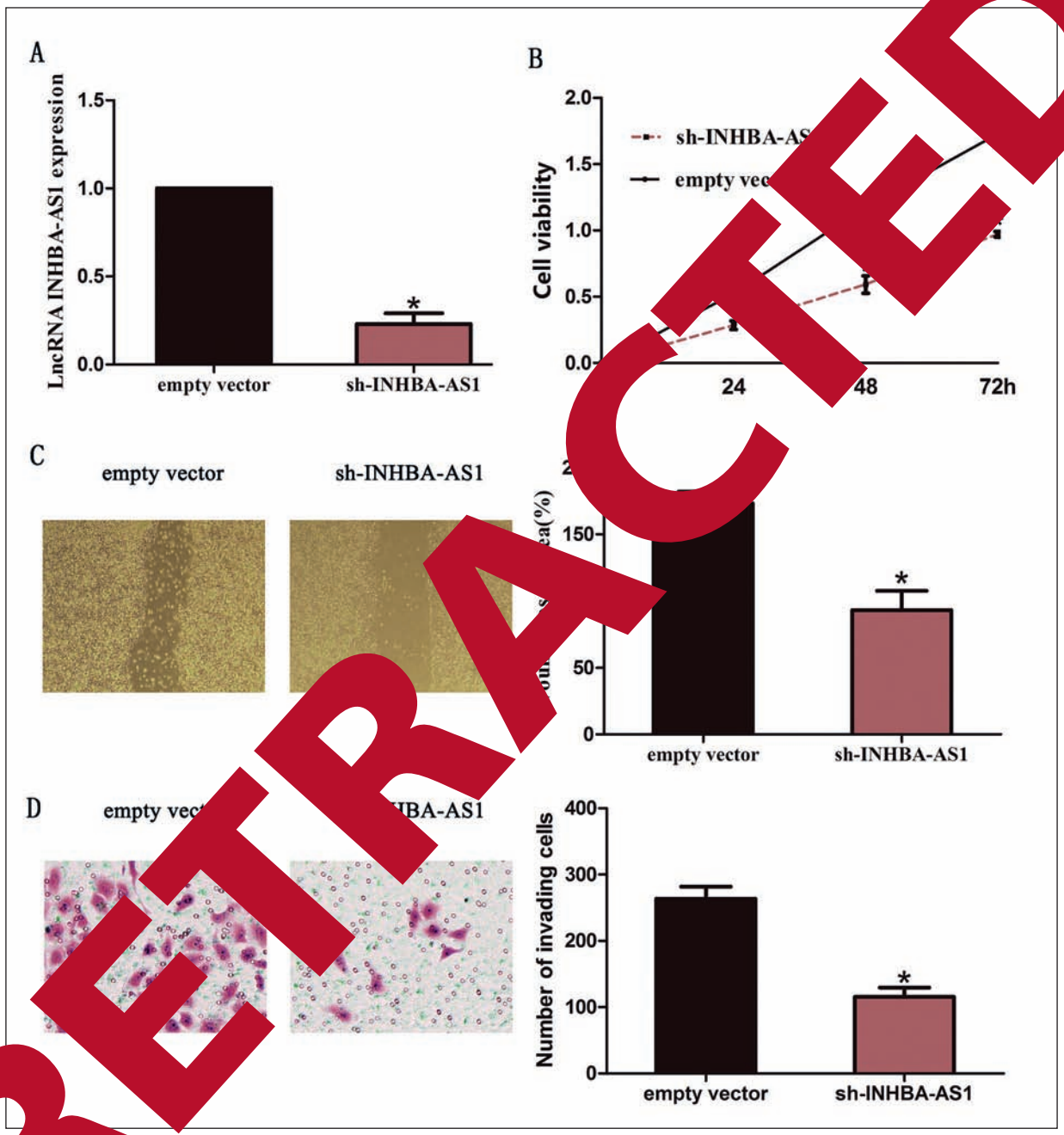

Figure 2. Knockdown of INHBA-AS1 inhibited Tca8113 OSCC cell proliferation, migration and invasion. **A,** INHBA-AS1 expression in OSCC cells transfected with empty vector or INHBA-AS1 lentivirus (sh-INHBA-AS1) was detected by RT-qPCR. GAPDH was used as an internal control. **B,** CCK-8 assay showed that the knockdown of INHBA-AS1 significantly repressed the proliferation of Tca8113 OSCC cells. **C,** Wound healing assay showed that the migrated length of the cells in INHBA-AS1 lentivirus group significantly decreased when compared with the empty control group. **D,** The transwell assay showed that the knockdown of INHBA-AS1 significantly repressed the invasion of Tca8113 OSCC cells (magnification ×20). The results represented the average of three independent experiments (mean ± standard error of the mean). *p<0.05, as compared with the control cells.

INHBA-AS1 Knockdown Inhibited OSCC Cell Migration and Invasion In Vitro

The subsequent wound healing assay indicated that the knockdown of INHBA-AS1 significantly inhibited OSCC cell migration (Figure 2C). Moreover, the invasion of OSCC cells was remarkably inhibited after INHBA-AS1 knockdown *in vitro* (Figure 2D).

INHBA-AS1 Knockdown Inhibited Tumor Formation and Metastasis In Vivo

The ability of INHBA-AS1 in tumor formation and metastasis was detected *in vivo*. The tumor size in shRNA group was significantly smaller than that of the empty vector group (Figure 3A). The weight of the dissected tumors in shRNA group was significantly smaller than that of the empty vector group, as well (Figure 3B). The number of metastatic nodules in lung tissues of the shRNA group was significantly reduced when compared with the empty vector group (Figure 3C). The expression level of INHBA-AS1 in dissected tumor tissues was then detected by RT-qPCR. The results showed that INHBA-AS1 was lowly expressed in the shRNA group compared with the empty vector group (Figure 3D). The above results suggested that INHBA-AS1 could induce tumor formation and metastasis *in vivo*.

INHBA-AS1 Promoted OSCC Tumorigenesis Via Sponging MiR-143-3p in OSCC

The starBase v2.0 (http://starbase.sysu.edu.cn/mirLncRNA.php) was used to predict miRNAs that contained complementary sites with INHBA-AS1. miR-143-3p contained a binding area with INHBA-AS1 was selected (Figure 4A). Previous studies have demonstrated that miR-143-3p suppresses the tumorigenesis of several tumors. In the present study, RT-qPCR results

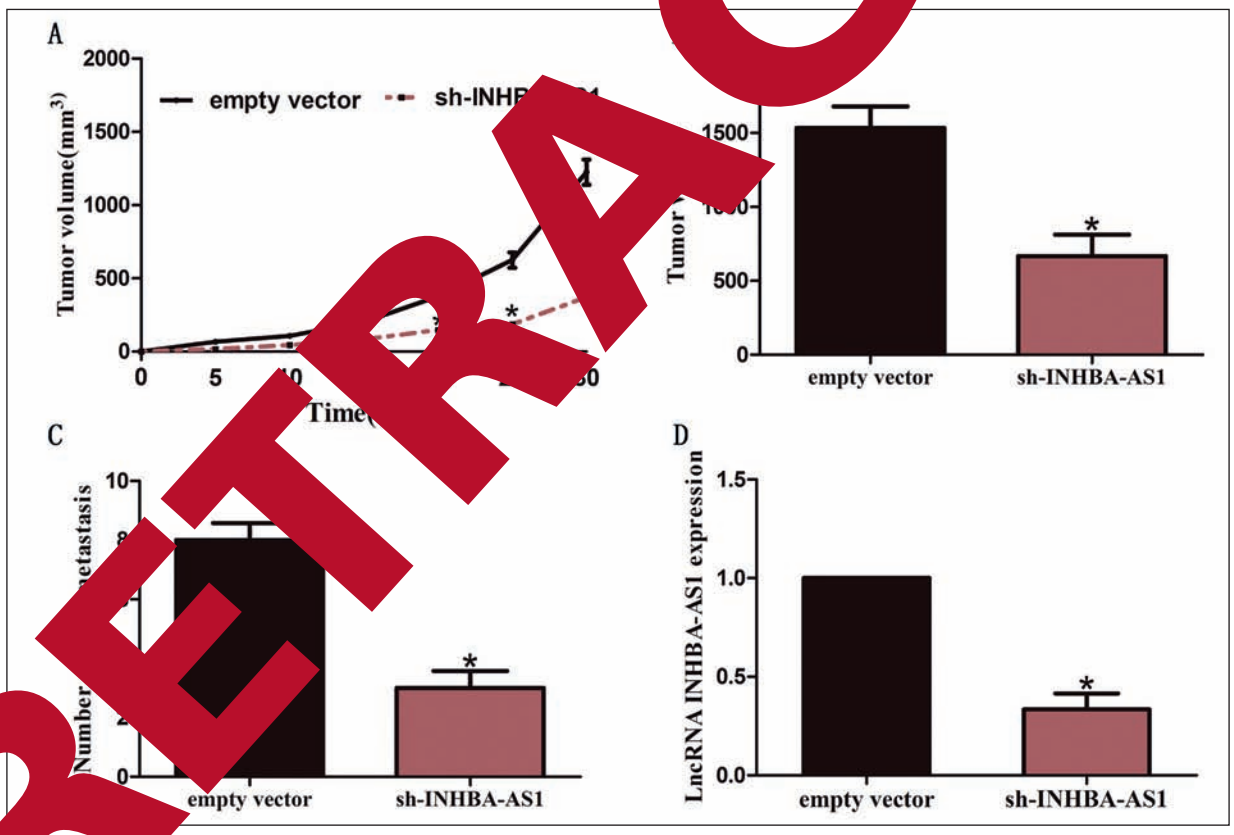

Figure 3. Knockdown of INHBA-AS1 inhibited tumor formation and metastasis of OSCC *in vivo*. **A,** The tumor size in the sh-INHBA-AS1 group was significantly smaller compared with the empty vector group. **B,** The weight of the dissected tumors in the sh-INHBA-AS1 group was remarkably smaller than the empty vector group. **C,** The number of metastatic nodules in lung tissues of sh-INHBA-AS1 group was significantly reduced when compared with the empty vector group. **D,** INHBA-AS1 was lowly expressed in the dissected tumors of the sh-INHBA-AS1 group compared with the empty vector group. The results represented the average of three independent experiments (mean ± standard error of the mean). *$p<0.05$, as compared with the control cells.

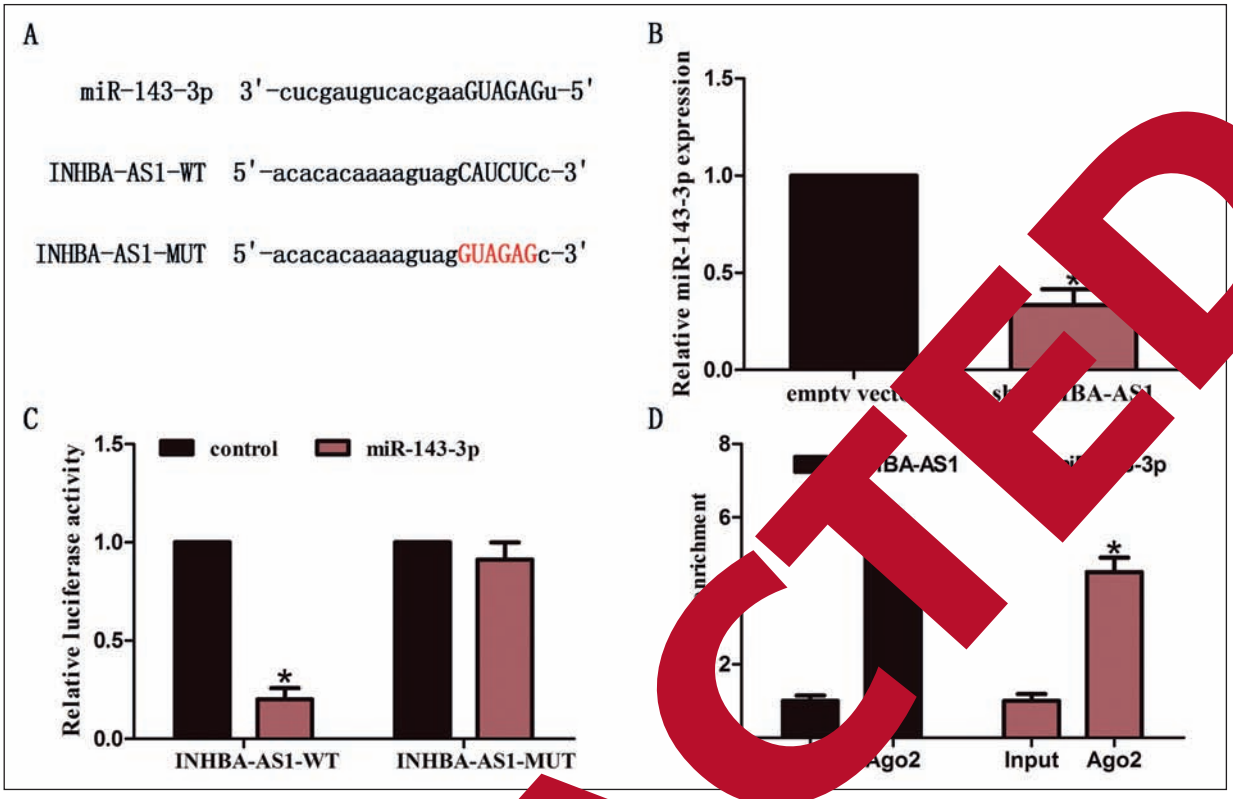

Figure 4. The association between INHBA-AS1 ... OSCC. **A,** The binding sites of miR-143-3p on INHBA-AS1. **B,** MiR-143-3p expression significantly increa... the ... group compared with the empty vector group. **C,** Co-transfection of miR-143-3p and INHBA-AS1-W... ongly ... e luciferase activity, while the co-transfection of miR-control and INHBA-AS1-WT did not change the l... ...ivity. **D,** The RIP assay showed the enrichment of INHBA-AS1 and miR-143-3p Ago2-containing b... The resul... ...nted the average of three independent experiments. The data were presented as mean ± standard e... ...ean. *p-... ...s compared with the control cells.

showed that miR-143-3p ...nif... ...ulated in the sh-INH...A-A... ...co...d with the empty ve... group ... 4B). The subsequent lucife... assay show... the luciferase activit... ...nificantly ...uced after the co-tra...ectio... INHBA-AS1-WT and miR-143-3... However, ...nificant changes were ob...ed in the lucife... ctivity after the co-tr...ection... INHBA-AS1-MUT and miR-143... igu... C). Meanwhile, the RIP assay showe... HBA... and miR-143-3p were ...ifica... rich... Ago2-containing beads ...ared w... put group (Figure 4D). All th... data reve... that miR-143-3p was a direct t... HBA-AS1.

Discussion

Numerous researches have demonstrated that lncRNAs participate in a variety of biological behaviors in OSCC, including cell growth, metastasis, and invasion. In fact, lncRNAAC132217.4 facilitates cell metastasis in OSCC via targeting IGF2[7]. LncRNA HOTAIR facilitates the invasion and metastasis of OSCC cells by indirectly recruiting EZH2 and depressing E-cadherin[8]. The downregulated lncRNA CCAT2 inhibits tumorigenesis of OSCC via Wnt/β-catenin pathway[9]. In addition, lncRNA FTH1P3 promotes the development of OSCC by modulating the expression of fizzled 5[10].

LncRNA INHBA antisense RNA 1 (INHBA-AS1) was firstly found aberrantly expressed in gastric cancer. INHBA-AS1 is a cluster of transcripts located on 7p14.1[11]. However, its role in tumor development and metastasis remain unexplored. In the present investigation, INHBA-AS1 was found significantly up-regulated in both OSCC tissues and cells. After INHBA-AS1 was knocked down, the abilities of cell growth, migration, and invasion were remarkably suppressed *in vitro*. Furthermore, the knockdown of INHBA remarkably inhibited the tumor forma-

tion and metastasis *in vivo*. These data indicated that INHBA-AS1 functioned as an oncogene and promoted the tumorigenesis of OSCC.

Recently, lncRNAs have been found to interact with microRNAs. Meanwhile, they can participate in the regulation of tumorigenesis by binding to the related area of microRNAs in malignant tumors. The starBase v2.0 was used to predict the possible targeted microRNAs of INHBA-AS1, among which miR-143-3p was reported to be abnormally expressed in many cancers. Authors[12,13] have demonstrated that miR-143-3p directly correlates with the prognosis and progression of various cancers. Of note, miR-143-3p represses cell migration and invasion in colorectal cancer via regulating ASAP3[14]. MiR-143-3p can reverse the process of epithelial-mesenchymal transition of esophageal squamous cell carcinoma through QKI-5[15]. Sun and Zhang et al[16] have shown that miR-143-3p is down-regulated in OSCC tissues, which also suppresses OSCC proliferation and invasion. In our work, the miR-143-3p expression was significantly upregulated after the knockdown of INHBA-AS1. The luciferase assay indicated that miR-143-3p could directly bind to INHBA-AS1. More[illegible] miR-143-3p was significantly enriched [illegible] HBA-AS1 through RIP assay. The above [illegible]lts revealed that INHBA-AS1 might exert its fu[illegible] in OSCC via sponging miR-143-3p.

Conclusions

The fidings of this study [illegible]at lncRNA INHBA-AS1 promo[illegible]OSCC [illegible]eration and metastasis via sp[illegible]g miR-143[illegible] *vitro* and *in vivo*. Our fi[illegible]gest that [illegible]BA-AS1 may be a pote[illegible]al bi[illegible]r and target for OSCC therap[illegible]

Conflict of Interest

The [illegible]e that th[illegible] have no conflict of interests.

Funding Acknowledgements

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