# OTX1 Exerts an Oncogenic Role and is Negatively Regulated by miR129-5p in Laryngeal Squamous Cell Carcinoma

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# Abstract

Background Orthodenticle homeobox 1 (OTX1) is a transcription factor that plays important roles in various human cancers. However, the function of OTX1 in laryngeal squamous cell carcinoma (LSCC) is largely unknown. We aim to explore the roles of OTX1 in LSCC and possible molecular mechanism.

Methods The expression levels of OTX1 were assessed in LSCC cell lines and tissue samples. We further examined the effect of OTX1 on LSCC progression. The upstream regulator of OTX1 was identified using computer algorithm and confirmed experimentally.

Results OTX1 was highly expressed in 70.7% (70/99) of LSCC patient tissues. The OTX1 expression in LSCC was significantly correlated with lymph node metastasis. High OTX1 expression in LSCC patients was correlated with poor prognosis. Knockdown of OTX1 inhibits proliferation, colony formation, migration and invasion in LSCC cells. Knockdown of OTX1 inhibits tumor growth in a xenograft mouse model. Mechanistically, OTX1 might act as a direct target of miR-129-5p. OTX1 enhances tumorigenicity and tumor growth both *in vitro* and *in vivo*.

Conclusions Our findings support that OTX1 is an oncogene in LSCC tumorigenesis and progression. Furthermore, OTX1as a direct target of miR-129-5p in LSCC cells.Taken together, OTX1 is a promising diagnostic and therapeutic marker for LSCC.


# Backgroud

Laryngeal squamous cell carcinoma (LSCC) is one of the most common malignant cancers in head and neck. In China, the estimated incidence of LSCC was 26,400 new cases and 14,500 related deaths in 2015 [1]. In the United States, the incidence and mortality rates were 13,360 and 3,660 per year in 2017 [2]. Human papilloma virus infection, smoking, drinking and fried foods are considered as the high risk factors for the occurrence and development of laryngeal cancer [3–5]. At present, surgical resection, chemotherapy and radiotherapy are considered as the standard treatments for patients with LSCC [6, 7]. Despite advances in diagnosis and treatment, the prognosis of LSCC remains poor. Therefore, further understanding of the molecular mechanisms of carcinogenesis and development of LSCC is important for finding new diagnostic markers and therapeutic targets for LSCC patients.

Orthodenticle homeobox 1 (OTX1) is clustered on human chromosome 2p13, which includes five exons, encodes a protein containing 354 amino acids and is widely distributed in human tissues [8, 9]. OTX1 is a transcription factor that has a high-affinity binding to TAATCC/T elements on target genes, which plays an essential role in the process of embryo development, such as brain corticogenesis, neuron differentiation, sense organ development, as well as mammary grand development [10–16]. With the continuous progression of study, the role of OTX1 in the carcinogenesis and development of human cancer has drawn more and more attention. It has been reported that overexpression and tumor-promotion effect of OTX1 was observed in hepatocellular carcinoma, colorectal cancer and breast cancer

[17–19]. Meanwhile, the activation of OTX1 expression occurred in aggressive non-Hodgkin lymphoma [20]. These findings suggest OTX1 might be a potential oncogene and anti-cancer therapeutic target.

Based on the above findings, we sought to investigate possible roles of OTX1 in the oncogenesis and progression of LSCC as well as related molecular mechanisms.

## Methods

# LSCC tissue specimens and patients' follow up

In the study, a total of 99 LSCC tissues were obtained from patients who underwent surgical resection at the Sun Yat-sen University Cancer Center from October 2007 to December 2009. All patients with laryngeal squamous cell carcinoma confirmed by pathology. The clinico-pathological features were collected and stored in our database. Patients were followed up every 3 months for survival status. Overall survival (OS) was recorded from the date of surgery to the date of death or last follow-up (31st May 2015), as previously reported [21]. This retrospective study on tumor material was approved by the Research Ethics Committee of Guangdong General Hospital & Guangdong Academy of Medical Sciences and Sun Yat-sen University Cancer Center (No.GDREC2018029H).

# Cell Culture

Two human LSCC cell lines, Hep-2 and TU212 were purchased from the Cell Resource Center of Shanghai Institutes for Biological Sciences (Chinese Academy of Science, Shanghai, China). Cells were cultured in RMPI-1640 medium (Gibco, Waltham, MA,USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Waltham, MA,USA), 100 U/mL penicillin, and 100 μg/mL streptomycin (Hyclone, Logan, UT, USA). Cells were cultured at 37 °C in 5%CO2/95% air in a humidified atmosphere.

# Plasmid Construction And Cell Transfection

Two lentivector-mediated short-hairpin OTX1 (sh-OTX1-1 and sh-OTX1-2) and non-targeting plasmids (sh-control) were designed and synthesized by GenePharma (Shanghai, China). with a scramble, empty lentiviral vector as the control. Lipofectamine 2000 system was used for transfection. The lentivirial vectors carried puromycin resistance. The transfected cells were screened with 0.5 mg/ml puromycin. Stably transfected cell lines was tested by quantitative real-time PCR (qRT-PCR) and western blot analysis. The transfection procedure was performed according to the manufacturer's instruction. The following shRNA sequences were used: sh-OTX1-1:GCAACACCTCGTGTATGCA;sh-OTX1-2: GCCGACTGCTTGGATTACA

# Immunohistochemistry (ihc) Analysis

LSCC tissues were paraffin-embedded and sliced. Slides were then subjected to de-paraffinization/ rehydration, followed by the antigen retrieval in a microwave. Slides were blocked by 5% milk for 1 hour at 25 °C, and later incubated with the corresponding anti-human OTX1 antibodies (1:400, MAD5602, Millipore, USA) at 4 °C overnight. After PBS washes, slides were incubated with secondary antibodies at 25 °C for 1 hour. Signals for each slide were developed in pre-made 0.05% diaminobenzidine (DAB) containing 0.01% hydrogen peroxidise ($H_2O_2$). The results of IHC were evaluated by two independent investigators who were blinded to the clinical and prognostic data based on the Shimizu criteria [22]. Based on the score criteria, the expression of OTX1 in LSCC tissues was graded as negative (-), weakly positive (+), moderate positive (++), and strongly positive (+++). The expression levels of OTX1 protein in LSCC tissues were dichotomized as the low (-/+) and high (++/+++) expression groups, respectively.

# Quantitative Real-time Pcr

The total RNA was extracted with Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions from LSCC tissue samples and cells. The cDNA was synthesized from total RNA using the cDNA Takara Kit (NHK, Japan). The transcriptional levels were detected in amplification by RT-PCR using the Power SYBR Green PCR Master Mix (NHK, Japan). The relative OTX1 mRNA expression was calculated using the $2^{-\Delta\Delta CT}$ method and normalized to the internal control β-actin. The primers used in the study were as follow: OTX1 forward, 5'-GCGTCGTCGCTGAGTACAC-3' and reverse, 5'-ACATGGGATAAGAGGCTG CTG-3'; β-actin forward, 5'-TCACCAACTGGGACGACAT-3' and reverse, 5'-GCACAGCCTGGATAGCAAC-3'.

# Western Blot

Cells were lysed in ice-cold RIPA lysis buffer with 0.1% protease inhibitor. The concentration of total protein was measured using BCA assay kit (Boster, Wuhan, China). Equal amounts of protein was loaded onto 10% sodium dodecyl sulfate ploy acrylamide (SDS-PAGE) gel and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). After blocking with 5% skimmed milk, the membranes were incubated with anti-OTX1 (1:1000 dilutions, MAD5602, Millipore, MA, USA), β-actin (1:3000 dilutions, CST4967, Cell Signaling Technology, Boston, USA) at 4 °C overnight. Subsequently, the membranes were washed with TBST buffer three times and incubated with the corresponding secondary antibodies for 1 hour at room temperature. Finally, the immunoreactive bands on the membrane were visualized with enhanced chemiluminescene (ECL) reagents (Millipore, Plano, TX, USA).

# Cell Proliferation

Cell proliferation was evaluated using the MTS (Qiagen, Hilden, German) assay. Briefly, Logarithmically growing cells were seed at 96-well plates at a density of 3,000 cells/well in triplicate. After incubation for 24, 48, 72, 96, or 120 h, 20 μL of working solution containing MTS and serum-free RPMI-1640 medium

was added into each well, followed by another incubation in humidified air with 5% CO2 at 37 °C for 2 h. The absorbance was measured at 490 nm using a Synergy™ Multi-Mode Microplate Reader (Biotek, Vermont, USA).

# Colony Formation Assay

As for colony formation assay, 500 cells were seeded in a 6-well plate and cultured with complete medium for 10 days. Cell colonies were fixed with methanol and stained with 0.2% crystal violet for 30 min at room temperature. The number of colonies was counted using Quantity One software (Bio-Rad, Hercules, CA, USA).

# Migration, And Invasion Assay

Cell migration was evaluated using Transwell migration assay (8-μm pore; BD Biosciences). Briefly, cells were starved in serum-free medium for 2 h. Next, cells were resuspended in serum-free RPMI-1640 medium, and 200 μL of the cell suspension ($3 \times 10^6$ cells) was added to the upper chamber. A volume of 600 μL of RPMI-1640 medium containing 10% FBS was added to the bottom chamber. After 24 h, cells that attached to the lower surface of the membrane were fixed with methanol and stained with 0.2% crystal violet for 30 min. Finally, migratory cells were counted from random five fields. Cell invasion assay was performed following the same procedures as described above, except that diluted Matrigel (Corning, New York, USA) was precoated on the upper well of the transwell chambers and incubated 48 h.

# Xenografted Tumors In Nude Mice

All animal experiments were performed in accordance with the guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health. This study was approved by the Ethics Committee of Guangdong Provincial People's Hospital (No.GDREC2018029A). Four-to-five-week old BALB/c nude mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. and were raised under the specific pathogen-free environment at 20–22 °C and 40–60% humidity. After 1 week of adaptive feeding, the nude mice were randomly divided into six groups (n = 5 for each group) in Hep-2 and TU212 cells: Control, sh-OTX1-1, sh-OTX1-2. To stably knock down OTX1, the shRNA-OTX1-1 and shRNA-OTX1-2 were constructed, packaged into lentiviruses, and infected Hep-2 and TU212 cells. After this, Hep-2 and TU212 cells infected with shRNA-OTX1-1 and shRNA-OTX1-2 or their corresponding controls were suspended in normal saline ($1 \times 10^6$ cells in 0.1 mL) and then subcutaneously injected into the right armpit of each mouse to establish a xenograft tumor mouse model. The tumor volume was calculated every 4 days. Twenty-eight days later, the mice were sacrificed under anesthesia. All animals were anesthetized by intravenous injection of barbiturateat a final concentration of 100 mg/kg. The tumors were isolated and fixed in 4% paraformaldehyde solutions and embedded in paraffin before being

cut into 4 μm thick sections. The prepared sections were stained using hematoxylin and eosin (H&E) and ki-67 following the routine staining procedure and examined using a microscope.

# Luciferase Assay

MiR-129-5p or miR-129-5p mut mimics were purchased form RiboBio Co., LTD (Guangzhou, China). DNA fragment form the 3'-untranslate region (UTR) of OTX1 containing the predicted complementary sites of miR-129-5p were constructed into a pGL3-basic plasmid (Addgene, Cambridge, USA). $1 \times 10^4$ LSCC cells were seeded in 48-well plates in triplicate and settled for 24 h. Then, the pGL3-OTX1 -3'UTR reporter plasmids (100 ng) plus 5 ng of pRL-TK renilla plasmid (Promega, Madison, USA), and increasing amounts (10 and 50 nM) of negative control (NC), miR-129-5p or miR- 129-5p mut mimic were co-transfected into LSCC cells using Lipofectamine LTX reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's recommendation. Luciferase and renilla signals were measured 24 h after transfection using the Dual Luciferase Reporter Assay Kit (Promega, Madison, USA) according to a protocol provided by the manufacturer.

# Statistical analysis

All experimental assays were conducted at least three times. Data were represented as means ± standard deviation (SD). The two-tailed Student t-test was used to determine the statistical significance of difference between groups. The chi-square or Fisher's exact tests was used to analyse the categorical data. Survival analysis was used to the Kaplan-Meier method and compared using log-rank test. Statistical analysis was performed using the Statistical Program for Social Sciences 19.0 software (SPSS, CA, USA) and presented with GraphPad Prism 6.0 (GraphPad Software, CA, USA). $p < 0.05$ was considered as statistical significance.

## Results

# The expression of OTX1 is correlated with the clinicopathologic features and prognosis of LSCC patients

To investigate the clinical significance of OTX1 expression in LSCC patients, we firstly examined the expression level of OTX1 in human LSCC tissues using IHC (Fig. 1). The result indicated that high OTX1 expression was detected in 70.7% (70/99) of LSCC patient tissues. Next, we analyzed the correlation of OTX1 expression with clinicopathologic features according to the IHC results. The expression level of OTX1 in LSCC was significantly correlated with lymph node metastasis and smoking history (all $p < 0.05$), while there was no significant correlation between the OTX1 expression and age, sex, drinking history, T classification, histological grade or primary location in LSCC (all $p > 0.05$) (Table 1). Lastly, we further found that the 1-, 3-, 5-year overall survival (OS) rates were 90.0%, 70.0%, 55.7% in LSCC patients with

high OTX1 expression compared to 96.6%, 89.7%, 86.2% in LSCC patients with low OTX1 expression ($p = 0.021$, Fig. 2).

Table 1
Correlation between OTX1 expression and Clinicopathologic Variables of LSCC patients

| | | OTX1 | | |
|---|---|---|---|---|
| **Variables** | **Cases (n = 99)** | **Low expression (n = 29)** | **High expression (n = 70)** | P |
| Age(years) | | | | |
| ≤ 60 | 47 | 15(66.9%) | 32(33.1%) | |
| > 60 | 52 | 14(61.4%) | 38(38.6%) | 0.586 |
| Sex | | | | |
| Male | 95 | 27(63.5%) | 68(36.5%) | |
| Female | 2 | 2(87.5%) | 2(12.5%) | 0.353 |
| Smoking history | | | | |
| Yes | 86 | 29(64.0%) | 57(36.0%) | |
| No | 13 | 0(64.5%) | 13(35.5%) | 0.013 |
| Drinking history | | | | |
| Yes | 31 | 12(62.5%) | 19(37.5%) | |
| No | 68 | 17(64.7%) | 51(35.3%) | 0.165 |
| T classification | | | | |
| T1-T2 | 58 | 21(69.0%) | 37(31.0%) | |
| T3-T4 | 41 | 8(55.7%) | 33(44.3%) | 0.072 |
| Lymph node metastasis | | | | |
| Negative | 75 | 26(65.5%) | 49(34.5%) | |
| Positive | 24 | 3(57.7%) | 21(42.3%) | 0.038 |
| Histological grade | | | | |
| Well | 39 | 14(66.1%) | 25(33.9%) | |
| Moderately | 44 | 11(63.2%) | 33(36.8%) | |
| Poorly | 16 | 4(61.4%) | 12(38.6%) | 0.508 |
| Primary location | | | | |

OTX1, Orthodenticle homeobox 1; LSCC, Laryngeal squamous cell carcinoma.

| | | OTX1 | | |
|---|---|---|---|---|
| Supraglottic | 22 | 4(65.4%) | 18(34.6%) | |
| Glottic | 70 | 21(55.3%) | 49(44.7%) | |
| Subglottic | 7 | 4(58.3%) | 3(41.7%) | 0.139 |
| OTX1, Orthodenticle homeobox 1; LSCC, Laryngeal squamous cell carcinoma. | | | | |

**OTX1 promotes the proliferation and colony formation of LSCC cells**

To identify whether OTX1 plays a role in LSCC progression, we knocked down the expression of OTX1 by shRNAs and performed both cell proliferation and colony formation assays in LSCC cell lines Hep-2 and TU212. The inhibitory efficiency of shRNAs targeting OTX1 was evaluated by qRT-PCR and western blot analysis. Compared to the scrambled control shRNA, shRNA-OTX1#1 and shRNA-OTX1#2 effectively reduced OTX1 expression in Hep-2 and TU212 cells (Fig. 3A and B). Next, we determined the effect of OTX1 expression on cell proliferation using MTS assay. The results showed that OTX1 knockdown significantly inhibited the proliferation of Hep-2 and TU212 cells cells (Fig. 3C and D). Colony formation assays also indicated that OTX1 knockdown prominently suppressed the ability of colony formation of Hep-2 and TU212 cells (Fig. 3E and F).

# Otx1 Promotes The Migration And Invasion Of Lscc Cells

To evaluate whether OTX1 has an effect on metastasis of LSCC cells, we analyzed the abilities of migration and invasion of Hep-2 and TU212 cells after downregulating OTX1 expression. The ability of migration of Hep-2 and TU212 was distinctly descended following knockdown OTX1 compared with matched control group through transwell migration assay (Fig. 4A and B). Compared to matched control group, the ability of invasion of Hep-2 and TU212 was distinctly curbed after downregulating OTX1 expression using transwell invasion assay (Fig. 4C and D).

**OTX1 promotes tumor growth** in vivo

To further demonstrate the physiological relevance of OTX1 in LSCC *in vivo*, we investigated the effect of OTX1 on tumor growth using a xenotransplantation model. As expected, the OTX1 knockdown groups clearly reduced tumor growth and decrease tumor weight compared with the matched control groups (Fig. 5A-F). Moreover, OTX1 knockdown cells-induced tumor samples more reduced proliferation indices (Ki-67 positive) compared to those formed by matched control in Hep-2 (Fig. 5G) and TU212 (Fig. 5H). In summary, these data suggested that knockdown of OTX1 inhibited tumor growth *in vivo*.

# Otx1 Is Negatively Regulated By Mir-129-5p

To further explore the mechanisms that regulate OTX1 expression in LSCC, we firstly predicted potential miRNAs that could bind to the 3'UTR of OTX1 by Targetsan and miRDB. There were 14 and 63 miRNAs identified by Targetsan and miRDB, respectively. Among them, miR-129-5p simultaneously appeared the above two algorithms (Fig. 6A). Therefore, we chose miR-129-5p for further investigation. The predicted binding site of miR-129-5p to the sequence OTX1 was illustrated in Fig. 6B. In both mRNA (Fig. 6C) and protein (Fig. 6D) levels, OTX1 were decreased after the overexpression of miR-129-5p by transfecting miR-129-5p into Hep2 and TU212 cells. Subsequently, we tested the direct binding affinity between miR-129-5p and the 3'-UTR of OTX1 by luciferase reporter assay. The results indicated that the miR-129-5p mimics attenuated luciferase activity of OTX1- 3' UTR whereas the mutant miR-129-5p mimics did not suppress OTX1-3' UTR luciferase activity (Fig. 6E and F). These suggested that OTX1 might act as a direct target of miR-129-5p.

# Discussion

OTX1 is a homeobox gene, which belongs to the OTX family (OTX1, OTX2, OTX3 and CRX). OTX1 played a fundamentally important role in the development of early human mammary gland, and fetal retina [11, 14]. Recently, it has been reported that OTX1 is highly expressed in aggressive non-Hodgkin lymphoma, and medulloblastomas [20, 23]. High expression of OTX1 is also observed in solid tumors, such as gastric cancer, sinonasal carcinoma and olfactory neuroblastoma [24, 25]. However, studies on the expression and role of OTX1 in human LSCC have not been reported.

In the current study, we firstly confirmed that OTX1 was overexpressed in LSCC tissues, and that high OTX1 expression in LSCC was associated with lymph node metastasis and poor prognosis. Next, we explored the potential role of OTX1 in tumor progression and metastasis. The knockdown of OTX1 expression by shRNA in human LSCC cells inhibited cell proliferation, migration and invasion *in vitro* and repressed tumor growth *in vivo*. we provide the first evidence that OTX1 plays a key role in development and metastasis of laryngeal squamous cell carcinoma. These results suggested that OTX1 might be a potential oncogene in laryngeal carcinogenesis.

Previous studies showed that OTX1 was determined as the target gene for p53 in breast cancer [19]. The expression of OTX1was regulated by nitric oxide in the rat myenteric plexus after intestinal ischemia-reperfusion injury [26]. To clarify the mechanisms how OTX1 is regulated in LSCC, we first predicted that OTX1 might interact with miR-129-5p via bioinformatic tools. We then discovered that the overexpression of miR-129-5p inhibited the expression of OTX1 at both mRNA and proteins levels in LSCC cells. Further, the luciferase reporter assay showed that miR-129-5p could directly bind to the 3'UTR of OTX1. Aberrant expression of miR-129-5p was observed in different types of cancers [27–29]. Li *et al* demonstrated that down-regulation of miR-129-5p inhibited cell growth and induced apoptosis in LSCC by targeting adenomatous polyposis coli [30]. Our results suggested that miR-129-5p might target and suppress OTX1 in LSCC. Hence, the overexpression of miR-129-5p can be a potential therapeutic strategy for LSCC patients with high OTX1 expression.

# Conclusions

In summary, our study demonstrated that OTX1 is highly expressed in LSCC and related with lymph node metastasis and poor prognosis. OTX1 promotes the progression and metastasis of LSCC *in vitro* and *in vivo*. MiR-129-5p is an upstream regulator of OTX1 expression, which might potentially shed light on new therapeutic method to alleviate LSCC progression.

# Abbreviations

OTX1
orthodenticle homeobox 1; LSCC:laryngeal squamous cell carcinoma; OS:overall survival; FBS:fetal bovine serum; qRT-PCR:quantitative real-time PCR; IHC:Immunohistochemistry; DAB:diaminobenzidine; H2O2:hydrogen peroxidise; SDS-PAGE:sodium dodecyl sulfate ployacrylamide; PVDF:polyvinylidene fluoride; ECL:enhanced chemiluminescene; H&E:hematoxylin and eosin; NC:negative control; UTR:untranslate region; SD:standard deviation; SPSS:Statistical Program for Social Sciences.

# Declarations


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None.


### Authors' contributions

Tu XP, Zhang SY and Chen SH designed and conceived the study. Tu XP, and Li H performed the experiments and analyzed the data. Tu XP, Li H, Chen LS, Luo XN and Lu ZM collected and analyzed the clinical data. Tu XP wrote the manuscript. Tu XP obtained funding. All authors read and approved the final manuscript.


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### Availability of data and materials

Not applicable.

### Ethics approval and consent to participate

This retrospective study on tumor material was approved by the Research Ethics Committee of Guangdong General Hospital & Guangdong Academy of Medical Sciences and Sun Yat-sen University Cancer Center (No.GDREC2018029H).

All animal experiments were performed in accordance with the guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health. This study was approved by the Ethics Committee of Guangdong Provincial People's Hospital (No.GDREC2018029A).

### Consent for publication

Not applicable

### Competing interests

The authors declare that they have no competing interests.

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# Figures

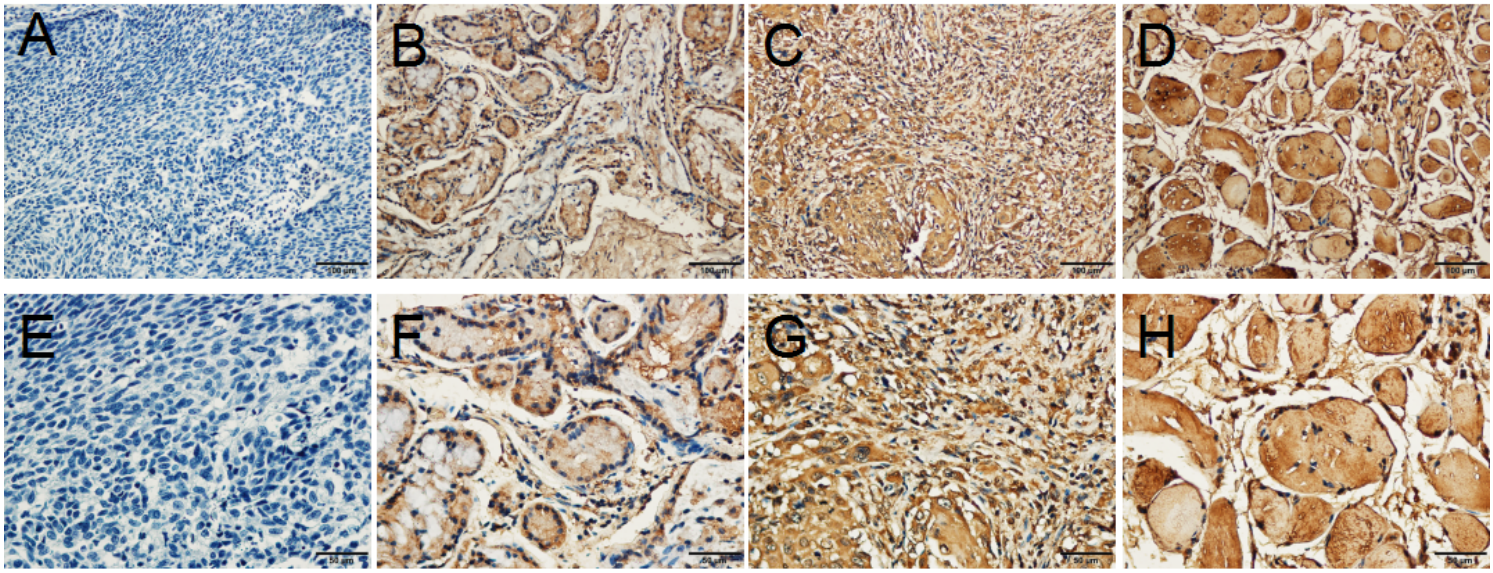
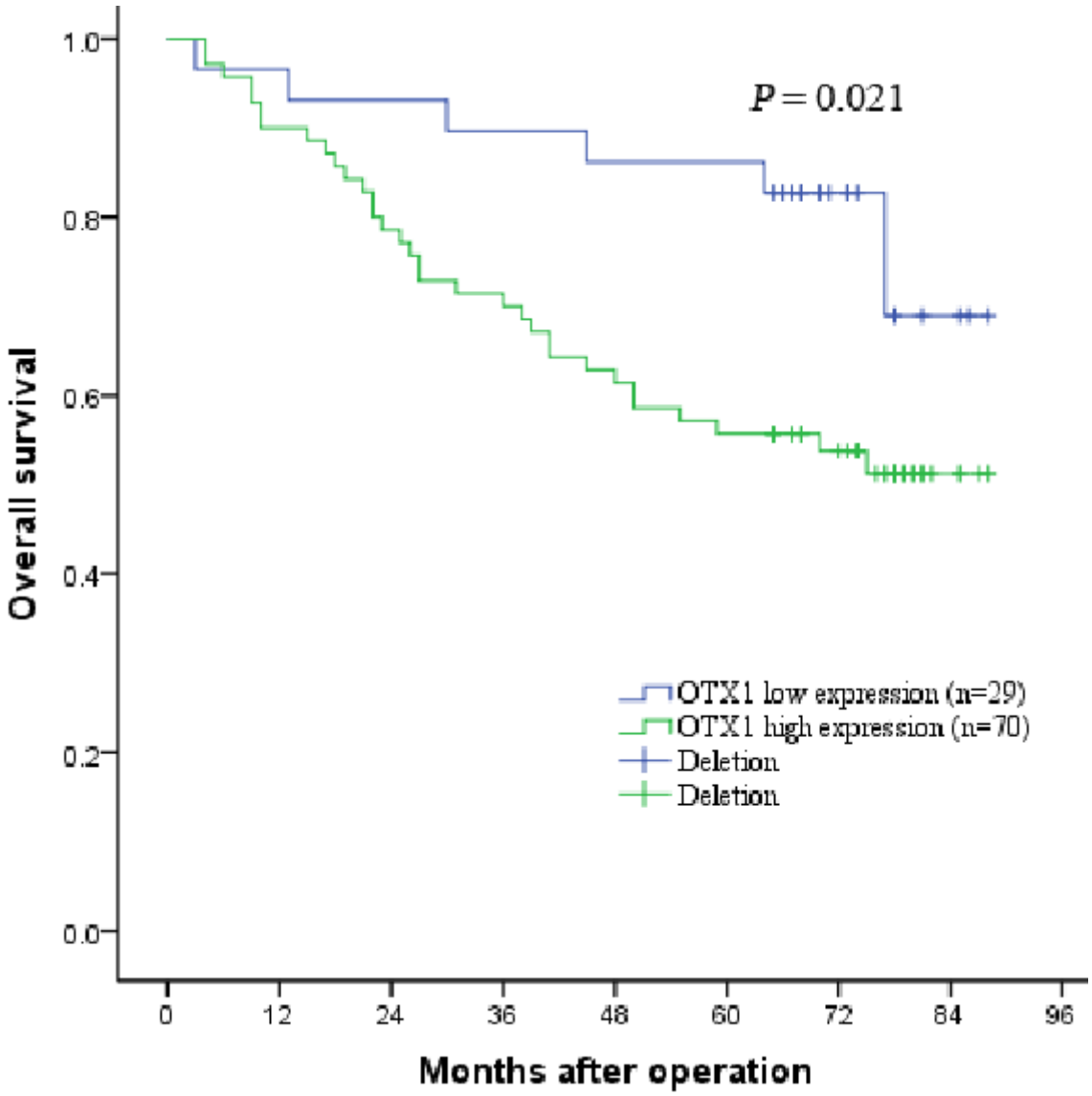


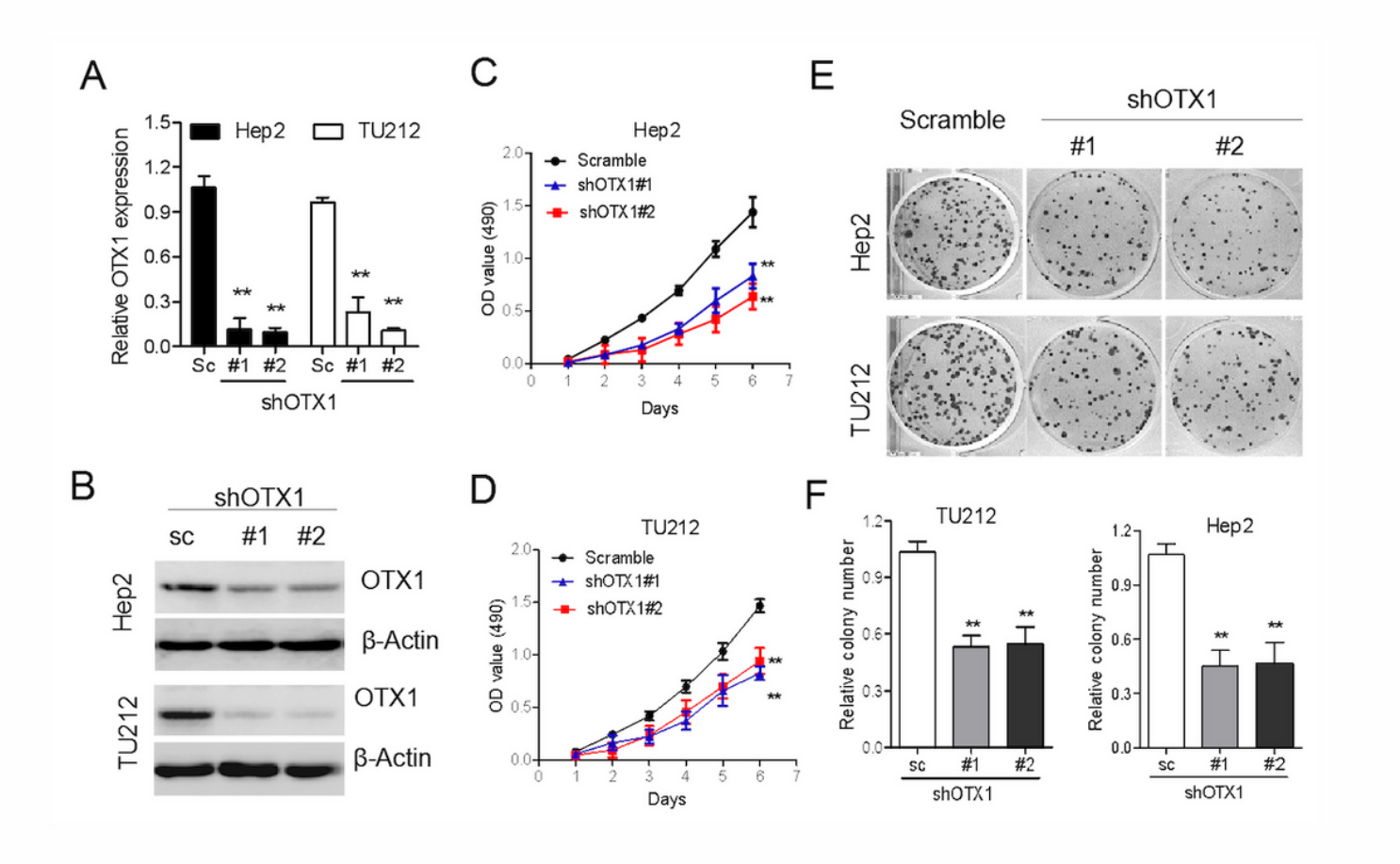

**Figure 1**

OTX1 expression in LSCC tissues. Immunohistochemical staining analysis was to performed for detection of OTX1 in human LSCC tissues. Representative images of negative (A,E), weakly positive (B,F), moderate positive (C,G), and strongly positive (D,F). Original magnification, ×100 (A-D); ×400 (E-H).



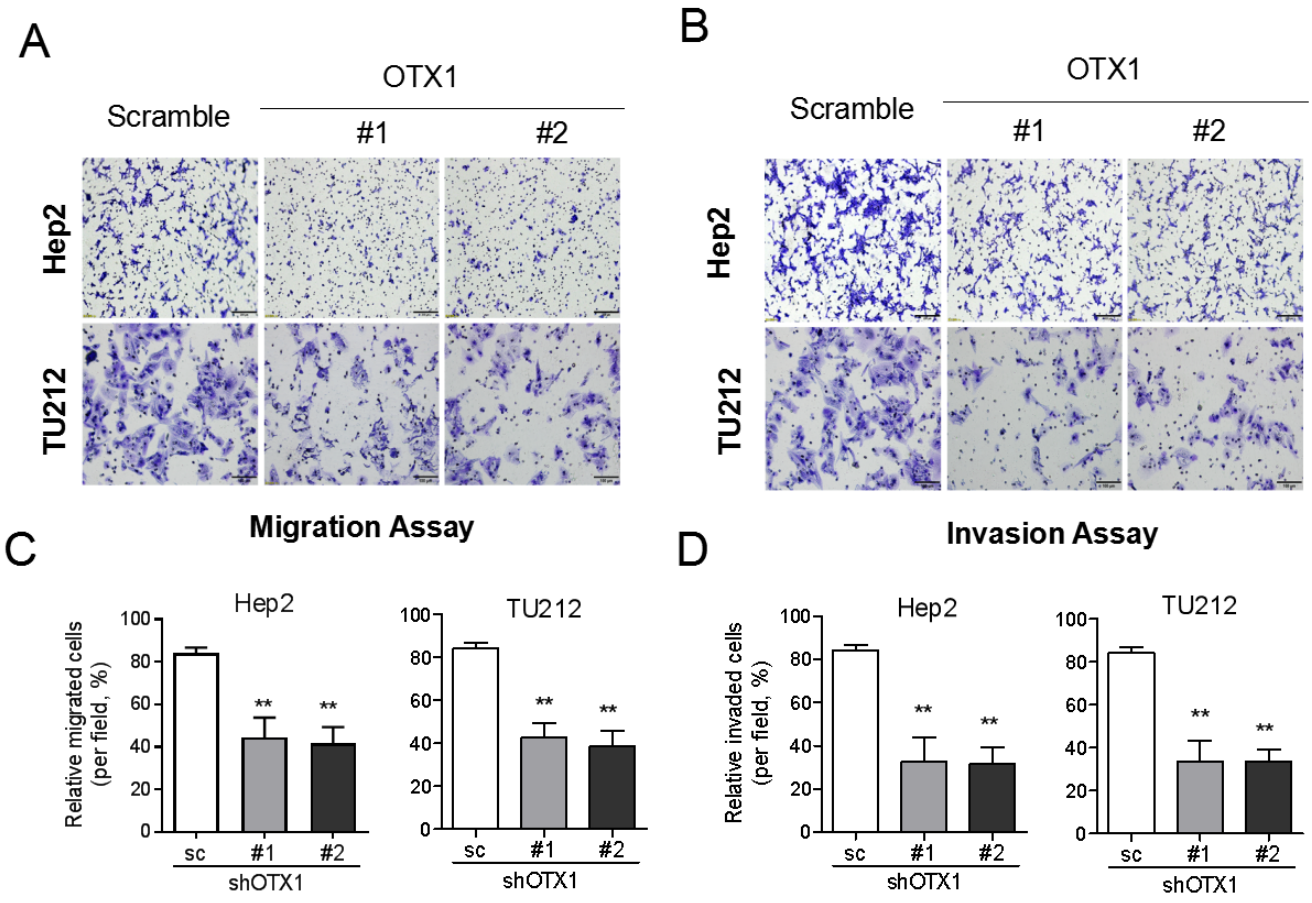

## Figure 2

Association between OTX1 expression and prognosis in human LSCC. Kaplan-Meier survival analysis showed LSCC patients with OTX1 high expression had worse prognosis than those with OTX1 low expression.




## Figure 3

OTX1 promotes LSCC proliferation and colony formation in vitro. A. The expression of OTX1 was detected in Hep2 and TU212 cells using qRT-PCR after the transduction of lentiviruses encoding OTX1 short hairpin RNA (shRNA)-1, shRNA-2 or scrambled shRNA. B. The expression of OTX1 was detected in Hep2 and TU212 cells using Western blot after the transduction of lentiviruses encoding OTX1 short hairpin RNA (shRNA)-1, shRNA-2 or scrambled shRNA. C. MTT assays were performed to assess the proliferation of Hep2 in response to transfection with OTX1 shRNA-1 or OTX1 shRNA-2 compared with the sh-NC group. D. MTT assays were performed to assess the proliferation of TU212 in response to transfection with OTX1 shRNA-1 or OTX1 shRNA-2 compared with the sh-NC group. E. A colony formation assay was performed to determine the colony formation capacity of Hep2 and TU212 in response to transfection with OTX1 shRNA-1 or OTX1 shRNA-2 compared with the sh-NC group. F. The quantitative graph of colony formation assay was performed to determine the colony formation capacity of Hep2 and TU212 in response to transfection with OTX1 shRNA-1 or OTX1shRNA-2 compared with the sh-NC group. *P < 0.05; **P < 0.01.




## Figure 4

OTX1 promotes LSCC migration ans invasion in vitro. A. Transwell migration assay of Hep2 and TU212 cells transfected with OTX1 shRNA-1 or OTX1 shRNA-2 compared with the sh-NC group. B. Transwell invasion assay of Hep2 and TU212 cells transfected with OTX1 shRNA-1 or OTX1 shRNA-2 compared with the sh-NC group. C. The quantitative graph of Transwell migration assay. D. The quantitative graph of Transwell invasion assay. *P < 0.05; **P < 0.01.

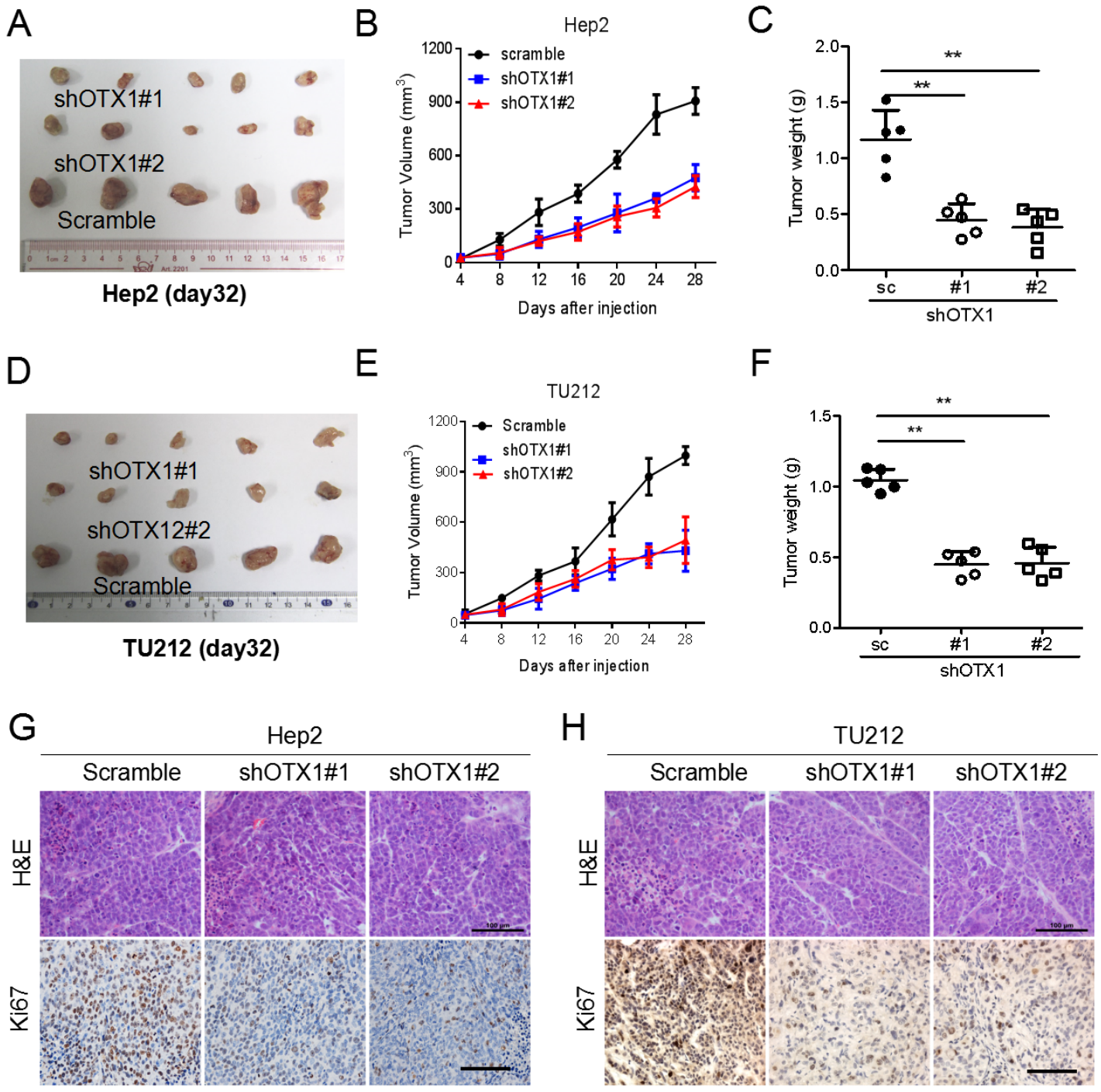
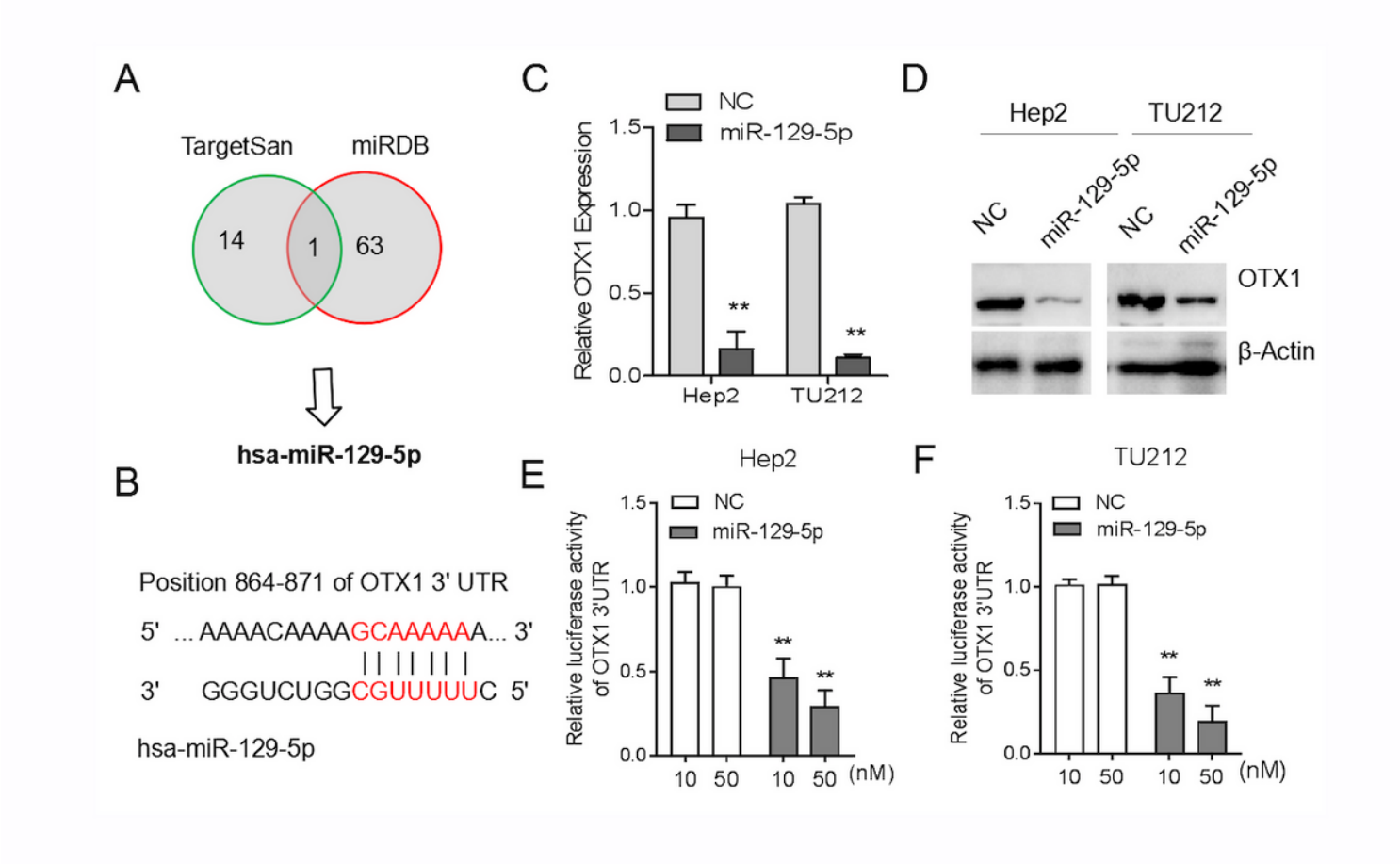



**Figure 5**

Knockdown of OTX1 inhibits tumor growth in a xenograft mouse model. (A). Representative images of tumors formed in nude mice that had been subcutaneously injected with Hep2 cells transfected with OTX1 shRNA-1 and shRNA-2 compared with the sh-NC group. (B). The volumes of tumors dissected from the shRNA- OTX1-1 and shRNA- OTX1- 2 group was lower than the sh-NC group. **P <0 .01. (C). The tumor weight were significantly reduced following the injection of shRNA- OTX1-1 or shRNA- OTX1- 2-expressing tumor cells compared with the sh-NC group. **P <0 .01. (D) Representative images of tumors formed in nude mice that had been subcutaneously injected with TU212 cells transfected with OTX1 shRNA-1 and shRNA-2 compared with the sh-NC group. (E) The volumes of tumors dissected from the

shRNA- OTX1-1 and shRNA- OTX1- 2 group was lower than the sh-NC group. **P <0 .01. (F). The tumor weight were significantly reduced following the injection of shRNA- OTX1-1 or shRNA- OTX1- 2-expressing tumor cells compared with the sh-NC group. **P <0 .01. (G). Representative HE and ki67 IHC staining image showing OTX1 shRNA-1 expression in xenograft tumors from nude mice injected subcutaneously with Hep2 cells transfected with OTX1 shRNA-1 and OTX1 shRNA-2 construct compared with the sh-NC group. (H) Representative HE and ki67 IHC staining image showing OTX1 shRNA-1 expression in xenograft tumors from nude mice injected subcutaneously with TU212 cells transfected with OTX1 shRNA-1 and OTX1 shRNA-2 construct compared with the sh-NC group.




## Figure 6

OTX1 acted as a direct target of the miR-129-5p in LSCC cells. A. miR-129-5p could target OTX1 by using the human miRNA targets prediction tool miRanda combined with the data of TCGA. B. Bioinformatics analysis identified the putative binding sites for miR-129-5p in the OTX1 sequence. C. The expression of OTX1 was detected in Hep2 and TU212 cells using qRT-PCR after the transduction of miR-129-5p mimics. D. The expression of OTX1 was detected in Hep2 and TU212 cells using Western blot after the transduction of miR-129-5p mimics. E. Luciferase activity was determined in Hep2 cells cotransfected with miRNAs (control mimics or miR-129-5p mimics) and a reporter vector containing miR-129-5p segments (WT or MUT) that bind to OTX1. **P <0 .01. F. Luciferase activity was determined in TU212 cells cotransfected with miRNAs (control mimics or miR-129-5p mimics) and a reporter vector containing miR-129-5p segments (WT or MUT) that bind to OTX1. **P <0 .01.

# Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- OriginalWBimage.pptx