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Running title: HIF-1α/HOXA9 and HOTTIP/CTCF/HOXA9 axis in HNSCC

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Abstract

Head and neck squamous cell carcinoma (HNSCC) is the sixth most frequently diagnosed cancer worldwide. However, the clinical outcomes remain unsatisfactory. The aim of this study is to unravel the functional role and regulatory mechanism of HOXA9 in HNSCC. A cohort of 25 HNSCC tumor tissues and normal tissue counterparts were collected. qRT-PCR and western blotting were performed to determine the levels of HOXA9, and EMT-related markers. CCK-8 and colony formation assay were conducted to monitor cell viability and cytotoxicity. Transwell and wound healing assays were used to determine cell migration and invasion. Annexin-V-FITC/PI staining was performed to detect cell apoptosis. Bioinformatic analysis, EMSA and ChIP assays were performed to investigate the direct binding between HIF-1α or CTCF and HOXA9. GST pull-down and RNA pull-down assays were used to validate the interaction between CTCF and HOTTIP. HOXA9 was up-regulated in HNSCC tissues and cells. Knockdown of HOXA9 inhibited cell proliferation, migration, invasion and chemoresistance, but promoted apoptosis in CAL-27 and KB cells. Knockdown of HOXA9 also regulated EMT-related marker via targeting YAP1/β-catenin. Silencing of HOTTIP or CTCF exerted similar tumor-suppressive effects in HNSCC. Mechanistically, HIF-1α or CTCF transcriptionally regulated HOXA9, and HOTTIP/CTCF cooperatively regulated HOXA9 in KB cells. HIF-1α or HOTTIP/CTCF transcriptionally modulates HOXA9 expression to regulate HNSCC progression and drug resistance.

Keywords: Head and neck squamous cell carcinoma (HNSCC), HOXA9, HOTTIP, CTCF
Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most frequently diagnosed cancer worldwide. The therapeutic outcomes of HNSCC remain poor due to late diagnosis, and the 5-year survival rate for patients with HNSCC is only ~50%\(^1\). HNSCC, which accounts for ~90% of head and neck malignancies, can originate from different subsites, including the lip, oral cavity, oropharynx, hypopharynx and larynx\(^2\). It is well-established that tobacco and alcohol abuse, as well as human papilloma virus (HPV) or Epstein-Barr virus (EBV) infections are independent risk factors for HNSCC\(^3,4\). Despite advances in surgery, chemo- and radiotherapies over last 4 decades, the survival rate of HNSCC have not improved\(^5\). The poor survival rate could be attributed to local recurrence, distant metastases and chemoresistance. Therefore, a better understanding of the mechanism involved in tumor progression and identification of reliable biomarkers are essential for improving HNSCC survival.

Honmeobox (HOX) genes encode homeoproteins which are a family of homeodomain containing transcription factors. In mammalian cells, the HOX family consists of 39 transcription factors which play important roles in embryonic development\(^6\). In addition, emerging evidence indicates that HOX genes function as either transcriptional activators or repressors in cancers\(^7\). Homeobox A9 (HOXA9) is dysregulated in hematopoietic malignancies and solid tumors, such as acute myeloid leukemia (AML), breast cancer, ovarian cancer and cervical cancer\(^8\)\(^-\)\(^11\). More important, a recent clinical study has demonstrated that HOXA9 promoter hypermethylation is associated with HNSCC progression and metastasis\(^12\). However, the biological function and regulatory mechanism of HOXA9 during HNSCC progression remain poorly understood.

Long noncoding RNAs (lncRNAs) are a class of non-coding RNAs which consist of at least 200 nucleotides in length. LncRNAs has been demonstrated to be novel regulators of tumor progression\(^13\). HOXA transcript at the distal tip (HOTTIP), a lncRNA located at the 5’ tip of the
HOXA locus, enhances gene transcription and H3 lysine 4 trimethylation via direct interaction with WDR5/MLL complex \(^{14}\). HOTTIP was found to promote HOXA9 expression by binding to WDR5 in pancreatic cancer cells \(^{15}\). Recent study has reported that HOTTIP physically associates with a well-known insulator factor CCCTC-binding factor (CTCF) to modulate HOXA gene expression in human foreskin fibroblasts \(^{16}\). Moreover, a clinical study has revealed that HOTTIP is overexpressed in tongue squamous cell carcinoma (TSCC), and increased HOTTIP expression is positively correlated with distant metastasis and clinical stage \(^{17}\), indicating the critical role of HOTTIP in HNSCC.

In the present study, we have demonstrated that HOXA9 was significantly up-regulated in HNSCC tissues and cells. Silencing of HOXA9 inhibited cell proliferation, migration, invasion and chemoresistance, but promoted apoptosis in CAL-27 and KB cells. Knockdown of HOXA9 regulated EMT-related markers via targeting YAP1/β-catenin. Similarly, HOTTIP or CTCF exerted oncogenic effects in HNSCC. Mechanistically, we found that HIF-1α transcriptionally suppressed HOXA9 expression, and HOTTIP and CTCF cooperatively regulated HOXA9 in KB cells.
Results

**HOXA9 is up-regulated in HNSCC tissues and cells.**

To determine the expression level of HOXA9, we first analyzed mRNA level of HOXA9 using data from the Cancer Genome Atlas (TCGA) database. As shown in Fig. 1A, HOXA9 was significantly elevated in HNSCC tissues. qRT-PCR was further performed with HNSCC tissues and normal counterparts. HOXA9 mRNA was markedly higher in HNSCC tissues compared with that in paired adjacent normal tissues (Fig. 1B). A similar expression pattern was also observed at the protein level by western blotting (Fig. 1C). In parallel, we examined the level of HOXA9 in normal human oral keratinocytes (NHOK) cells and two HNSCC cell lines. Consistently, the mRNA and protein levels of HOXA9 were significantly up-regulated in CAL-27 and KB cells compared to NHOK cells (Fig. 1D and 1E). Taken together, HOXA9 was significantly up-regulated in HNSCC tissues and cells.

**Knockdown of HOXA9 inhibits cell proliferation, migration, invasion and chemoresistance, but promotes apoptosis in CAL-27 and KB cells.**

We further explored the potential effects of HOXA9 on cell growth, metastatic properties, chemoresistance and apoptosis in HNSCC cells. Loss-of-function experiments were performed in CAL-27 and KB cells. As shown in Fig. 2A, sh-HOXA9 successfully decreased the expression of HOXA9 in both CAL-27 and KB cells. The CCK-8 and colony formation assays indicated that knockdown of HOXA9 markedly attenuated cell proliferation and impaired clonogenic ability in CAL-27 and KB cells (Fig. 2B and 2C). Wound-healing migration assay and transwell assays revealed that silencing of HOXA9 significantly decreased migration and invasive capability compared with that of control cells (Fig. 2D and 2E). In addition, we next examined the effect of HOXA9 on cell apoptosis and chemoresistance. Annexin-V-FITC/PI staining and cytotoxicity assay showed that knockdown of HOXA9 dramatically increased the proportion of apoptotic cells (Fig. 2F) and increased sensitivity to cisplatin and 5-FU in both CAL-17 and KB cells (Fig. 2G),
respectively. Moreover, in vivo xenograft study was conducted to validate the function of HOXA9 on cell growth. In accordance with in vitro findings, tumor growth was remarkably slower in sh-HOXA9 group than the sh-NC group (Fig. 2H). Consistently, tumor weight was significantly lower in sh-HOXA9 group than that in sh-NC group at 4 weeks after inoculation (Fig. 2H). Taken together, these data suggest that knockdown of HOXA9 inhibits cell proliferation, migration, invasion and chemoresistance, but promotes apoptosis in CAL-27 and KB cells.

**Knockdown of HOXA9 regulates EMT-related marker via targeting YAP1/β-catenin.**

Epithelial-mesenchymal transition (EMT) is a well-characterized process contributes to the migration and invasion of cancers. In order to further investigate the biological roles of HOXA9 on EMT, several known EMT or mesenchymal-epithelial transition (MET) biomarkers were detected by western blotting, including cell surface proteins E-cadherin and N-cadherin, cytoskeleton protein β-catenin, as well as transcription factors Twist and Slug-1. Given the regulatory role of YAP1 on β-catenin level in laryngeal cancer cells, we also examined the effect of YAP1 during EMT in HNSCC cells. The results showed that silencing of HOXA9 led to a significant reduction of YAP1, further inducing down-regulation of β-catenin (Fig. 3). Down-regulation of Twist, N-cadherin and Slug-1 and up-regulation of E-cadherin were found in HOXA9-knockdown CAL-27 and KB cells (Fig. 3). These data indicate that knockdown of HOXA9 regulates EMT-related marker via targeting YAP1/β-catenin.

**HIF-1α transcriptionally regulates HOXA9.**

Previous studies have illustrated that HOXA9 regulates HIF-1α on the transcriptional level. Conversely, bioinformatics analysis predicted hypoxia response elements (HREs) in HOXA9 promoter region using JASPAR (http://jaspar.genereg.net/). HIF-1α was identified as a putative transcription factor bound to HOXA9 promoter using UCSC genome browser database (https://genome-cancer.ucsc.edu), and the binding site was determined by JASPAR database. To
further validate the results of bioinformatics analysis, we investigated the effect of sh-HIF-1α on HOXA9 expression. As shown in Fig. 4A, HOXA9 expression was significantly decreased by sh-HIF-1α. EMSA was performed to detect the direct binding between purified HIF-1α protein and the predicted binding motif (shown in Fig. 4B). The results of EMSA showed that DNA-protein complex was formed when native probe was incubated with purified HIF-1α protein, whereas the mutated probe diminished the binding activity. Antibody supershift assay illustrated that HIF-1α was the transcription factor bound to this motif (Fig. 4C). ChIP assay further confirmed that lack of HIF-1α resulted in a significant reduction in the binding enrichment of HIF-1α at the binding site of HOXA9 (Fig. 4D). In short, these data suggest that HIF-1α regulates HOXA9 on the transcriptional level.

**Knockdown of lncRNA HOTTIP inhibits cell proliferation, migration and invasion via targeting HOXA9 in CAL-27 and KB cells.**

Emerging evidence illustrated the critical roles of lncRNAs in a variety of cellular processes during tumor progression, including cell proliferation, migration and invasion. Recent study has demonstrated that lncRNA HOTTIP promotes HOXA9 in pancreatic cancer stem cells (PCSCs) by binding to WDR5 15. To unravel the regulatory mechanism of HOXA9 in HNSCC cells, functional experiments were conducted to evaluate the biological role of HOTTIP in CAL-27 and KB cells. HOTTIP was significantly elevated in CAL-27 and KB cells compared with normal NHOK cells (Fig. 5A). As shown in Fig. 5B, sh-HOTTIP-mediated knockdown successfully decreased HOTTIP levels in both CAL-27 and KB cells. Both CCK-8 and colony formation assays showed that knockdown of HOTTIP inhibited cell growth in these two HNSCC cell lines (Fig. 5C and 5D). Cell migration capability was monitored by wound healing assay, and Fig. 5E showed significant slower wound closure in sh-HOTTIP knockdown cells at 24 h after creation of the linear wounds. In addition, transwell assay revealed that knockdown of HOTTIP also inhibited the invasive and migrated capabilities in CAL-27 and KB cells (Fig. 5F). It is apparent that HOTTIP exerted similar
effects on cell proliferation, migration and invasion as HOXA9 in HNSCC cells. To validate whether HOTTIP played oncogenic roles through HOXA9, the expression of HOXA9 and its downstream molecules were examined. As presented in Fig. 5G, knockdown of HOTTIP caused markedly reduction of HOXA9. Consistent with previous results, down-regulation of HOXA9 reduced YAP1 and EMT biomarker β-catenin in CAL-27 and KB cells (Fig. 5G). Taken together, these results suggest that HOTTIP is highly expressed in HNSCC cells, and knockdown of HOTTIP inhibits cell proliferation, migration and invasion via targeting HOXA9 in CAL-27 and KB cells.

Knockdown of CTCF inhibits cell proliferation, migration, invasion and chemoresistance in CAL-27 and KB cells.

A previous study has showed that HOTTIP physically associates with CCCTC-binding factor (CTCF) to coordinate HOXA gene expression\(^{16}\). Hence, it is of interest to investigate the biological role of CTCF in HNSCC cells. As shown in Fig. 6A, sh-CTCF dramatically decreased CTCF protein levels in CAL-27 and KB cells. Proliferation curves determined by CCK-8 assays showed that cells transfected with sh-CTCF grew slower than control cells (Fig. 6B). Wound healing coupled with transwell assays demonstrated that knockdown of CTCF remarkably inhibited cell migration and invasion in both CAL-27 and KB cells (Fig. 6C and 6D). In addition, enhanced sensitivity to cisplatin or 5-FU treatment was found in CTCF-knockdown CAL-27 and KB cells (Fig. 6E). Furthermore, the in vivo findings illustrated that tumor growth was impeded by sh-CTCF in which the tumor volumes and weights were significantly reduced in sh-CTCF group (Fig. 6F). Collectively, knockdown of CTCF inhibits cell proliferation, migration, invasion and chemoresistance in CAL-27 and KB cells.

CTCF transcriptionally induces HOXA9 expression.

To further test whether CTCF was involved in the transcriptional regulation of HOXA9, a series of experiments were conducted. We found that knockdown of CTCF significantly decreased HOXA9
protein level in HNSCC cells (Fig. 7A). The predicted CTCF binding sites in HOXA9 promoter region were shown in Fig. 7B as determined by JASPAR. Subsequent EMSA assay showed that CTCF protein and native probe formed a DNA-protein complex, and antibody supershift assay further confirmed that CTCF directly bound to native probe (Fig. 7C). Consistently, ChIP assay indicated that the binding enrichment of CTCF at HOXA9 locus was significantly decreased by sh-CTCF (Fig. 7D). These findings suggested that CTCF transcriptionally induces HOXA9 expression.

**HOTTIP and CTCF cooperatively regulate HOXA9 in HNSCC cells.**

In order to validate whether HOTTIP physically interacted with CTCF to modulate HOXA9 expression, recombinant GST tagged CTCF (GST-CTCF) was expressed and purified (Fig. 8A). Purified GST-CTCF was then used as bait protein to retrieve full-length HOTTIP or control histone RNA. As shown in Fig. 8B, HOTTIP RNA specifically bound to GST-CTCF, but not to GST. Consistent with GST pull-down assay, immunoprecipitation assay showed that endogenous CTCF successfully retrieved HOTTIP, but not negative control U1 spliceosomal RNA (Fig. 8C upper panel). The results of western blotting confirmed the success of IP (Fig. 8C lower panel). RNA pull-down assay was further performed to verify the direct interaction between HOTTIP and CTCF. Biotinylated HOTTIP specifically retrieved CTCF, whereas GFP or antisense HOTTIP failed to capture CTCF (Fig. 8D). These results suggest that HOTTIP physically interacts with CTCF to cooperatively regulate HOXA9 in HNSCC cells.
Discussion

HOX genes were dysregulated in most solid tumors, and they acted as either transcriptional activators or repressors to promote tumorigenesis \(^7\). Of the 39 human HOX family members, HOXA9 was reported to be dysregulated in leukemia, breast and ovarian cancers \(^9, 10, 21\). Interestingly, our findings showed that HOXA9 was highly expressed in HNSCC compared to their normal tissue counterparts which was consistent with TCGA data. These results indicate that HOXA9 might play critical roles in HNSCC. *In vitro* and *in vivo* experiments showed that HOXA9 promoted cell proliferation, migration, invasion and chemoresistance in HNSCC cells. In addition, subsequent knockdown experiments revealed that knockdown of HOXA9 decreased YAP1 expression, thereby leading to reduction of \(\beta\)-catenin. Previous study has demonstrated that YAP1 regulates Hep-2 laryngeal cancer cell growth via mediating EMT and Wnt/\(\beta\)-catenin pathway \(^18\). These findings suggest that YAP1 plays an important role in regulating EMT in HNSCC cells via modulating \(\beta\)-catenin expression. Future investigations are needed to connect HOXA9 and YAP1 in HNSCC.

Hypoxia has been recognized as a common character of solid tumors due to the increase of tumor volume and poorly formed vasculature. It is well-established that HIF-1\(\alpha\) plays a crucial role in adaptive responses of the cancer cells in the harsh microenvironment. For instance, HIF-1\(\alpha\) transcriptionally regulates downstream genes which involved in cell proliferation, migration, invasion, glucose metabolism and angiogenesis \(^22\). In the current study, bioinformatic analysis coupled with EMSA and ChIP assays showed that HIF-1\(\alpha\) positively regulated HOXA9 expression via directly binding to its promoter region. On the contrary, previous study has illustrated that HOXA9 inhibits glycolysis in cutaneous squamous cell carcinoma by negatively regulating HIF-1\(\alpha\) and its downstream effectors \(^20\). Our findings identified a feedback regulation between HOXA9 and HIF-1\(\alpha\).
A number of lncRNAs are dysregulated in HNSCC and correlated with tumor progression, metastasis, clinical stage and poor prognosis \(^{23}\). Bioinformatic analysis has illustrated the prognostic value of HOTTIP in HNSCC. HOTTIP is highly expressed in HNSCC tissues and cells, and correlated with clinical stage and histological grade of HNSCC patients \(^{24}\), indicating that HOTTIP might act as a key candidate biomarker in HNSCC. Consistent with this study, our results showed that HOTTIP was up-regulated in CAL-27 and KB cells, and it played an oncogenic role in HNSCC cells. In addition, EMSA, ChIP, GST pull-down and RNA pull-down assays demonstrated that HOTTIP and CTCF worked in concert to modulate HOXA9 expression in HNSCC cells. These findings were in accordance with a previous study which illustrated the functional cooperation between HOTTIP and CTCF in human foreskin fibroblasts \(^{16}\).

In conclusion, HIF-1\(\alpha\) or HOTTIP/CTCF transcriptionally modulates HOXA9 expression to regulate HNSCC progression and drug resistance.
Methods

Human tissues and cells

A cohort of 25 HNSCC tumor tissue and normal tissue counterparts were collected post-operatively from patients with HNSCC in The First Affiliated Hospital of Zhengzhou University. Consents were obtained from all patients.

Human oral squamous cell carcinoma cell line CAL-27 cells, head and neck squamous cell carcinoma cell line KB cells and normal human oral keratinocytes (NHOK) cells were purchased from Cell Bank of Type Culture Collection, Chinese Academy of Science (Shanghai, China). Cells were grown in DMEM containing 10% FBS (Gibco, Thermo Fisher Scientific, Waltham, MA, USA), 10 µg/ml streptomycin and 100 units/ml penicillin. Cultures were maintained at 37 °C in humidified atmosphere with 5% CO₂ in air.

Cell transduction and transfection

Non-specific sh-Negative control (sh-NC), sh-HOXA9, sh-HOTTIP or sh-HIF-1α were designed and synthesized by HanBio (Shanghai, China). HEK293 cells were used to produce adenovirus using Lipofiter™ transfection reagent (HanBio) according to the manufacturer’s instructions. Adenoviruses were purified and titered. CAL-27 or KB cells were infected with adenovirus. After 48 h, the infected cells were subjected to subsequent analysis.

qRT-PCR

Total RNA was isolated from tissues or cells using Trizol reagent (Invitrogen, Thermo Fisher Scientific) following the manufacturer’s protocols. cDNA was reverse transcribed using SuperScript III reverse transcriptase (Invitrogen) and qPCR performed using PowerUp SYBR Green Master Mix (Applied Biosystems, Thermo Fisher Scientific) and the following primers targeting the indicated human gene: HOTTIP forward primer 5’-CACACTCACATTGCACACT-
3’, reverse primer 5’-TCCAGAAGTGCTTACGCCATA-3’; HOX A9 forward primer 5’-
TGATTATTTTGTAGGTCCGT-3’, reverse primer 5’-
TAAAATTATTTTCTCACCCTTT-3’; U6 (internal control for lncRNA) forward primer 5’-
CTGCTTCCGGCAGCACA-3’, reverse primer 5’-AACGCTTACGAAATTTCGT-3’. GAPDH
(internal control for mRNAs) forward primer 5’-TGTGGGGAATGATGATTTGG-3’, reverse
primer 5’-ACACCATGTATTCGCGGTCAAT-3’. The relative expression of a target gene to that
of the internal control was calculated using 2^{-ΔΔCt} method.

**Western blot analysis**

Protein lysates from tissues or cells were prepared in IP lysis buffer supplemented with protease
inhibitor cocktail (Pierce, Thermo Fisher Scientific). Protein estimation was performed using BCA
protein assay kit (Pierce). Equal amount of protein lysates were resolved by SDS-PAGE and
transferred onto PVDF membrane for western blotting. Membranes were blocked with 5% non-fat
milk, followed by the incubation with primary antibody at 4°C overnight: anti-HOXA9 (ab140631,
Abcam, Cambridge, UK), anti-β-catenin (71-2700, Invitrogen), anti-Twist (ab50581, Abcam), anti-
E-cadherin (3195, Cell signaling technologies, Beverly, MA, USA), anti-N-cadherin (33-3900,
Invitrogen), anti-Slug-1 (9585, Cell signaling technologies), anti-YAP1 (sc-15407, Santa Cruz
Biotechnology, Santa Cruz, CA, USA), anti-HIF-1α (sc-10790, Santa Cruz), anti-CTCF (ab70303,
Abcam) and anti-GAPDH (sc-25778, Santa Cruz). Membranes were then incubated with a HRP-
conjugated secondary antibody (1:5000, Bioworld Technology, Louis Park, MN, USA) at room
temperature for 1 h. Western blots were visualized using Immobilon Western Chemiluminescent
HRP substrate (Merck Millipore, Burlington, MA, USA) followed by film exposure. The relative
expression of a target protein was normalized with internal control.
Cell Counting Kit-8 (CCK-8) assay

Cell viability in cell proliferation or cytotoxicity assays was determined by CCK-8 assay. CCK-8 assay was performed using Cell Counting Kit-8 (Beyotime, Haimen, China) according to the manufacturer’s instructions. In brief, cells (2×10^4 cells/ml) were seeded in 96-well plates 24 h prior to the treatment. For cytotoxicity assay, cells were then treated with different doses of cisplatin or 5-FU (Sigma-Aldrich, St Louis, MO, USA) for 48 h. 10 µl of CCK-8 solution was added into each well and incubated for 1 h at 37°C. Absorbance was measured at a wavelength of 490 nm by the use of microplate reader (Bio-Rad Laboratories, Hercules, CA, USA).

Annexin V-FITC/PI staining

Cell apoptosis was assessed using Dead Cell Apoptosis Kit with Annexin V-FITC and PI (Thermo Fisher Scientific). In brief, cells were harvested and resuspended in binding buffer at 24 h post-transfection. Dual staining of Annexin V-FITC and PI was performed according to the manufacturer’s instructions. 100 µl of cell suspension (1×10^5 cells) was added into a test tube and stained with 5 µl Annexin-V-FITC reagent and 1 µl PI solution for 15 min at room temperature. 400 µl binding buffer was then added into each tube. The stained cells were analyzed by flow cytometry with BD FACSCalibur (BD Biosciences, San Diego, CA, USA).

Colony-forming assay

CAL-27 or KB cells were seeded on 60 mm culture plates (2×10^2 cells per plate) and transfected with shRNA or corresponding control. After 10 days, the colonies were fixed with 10% formaldehyde for 5 min and stained with 1% crystal violet for 30 seconds. Viable containing at least 50 cells were counted.
**Wound-healing migration assay**

CAL-27 and KB cells were seeded in 6-well plates for 100% confluence in 24 h. The cell monolayer was lightly scratched using a sterile 1000 µl micropipette tip. The cell debris was carefully removed. Wound closure was observed and evaluated in five random fields using and inverted microscope (Carl Zeiss, Germany) at 24 h. Plates were photographed at 0 and 24 h after scratching at an identical location, respectively. The width (W) of the scratch measured. The wound closure was calculated as \((W_{0h} - W_{24h}) / W_{0h} \times 100\%\). All experiments were performed in triplicate.

**Transwell invasion and migration assay**

For transwell invasion assay, Transwell chambers (Corning, Lowell, MA, USA) were coated with Matrigel (BD Biosciences). At 24 h post-transfection, cells were deprived of FBS for 6 h. CAL-27 and KB cells were then seeded in the upper chambers. The lower chambers were filled with DMEM supplemented with 10% FBS. After 48 h incubation, cells remaining in the upper chamber were removed with cotton swabs. Cells which migrated to the lower chambers were fixed with 4% paraformaldehyde (PFA) and stained with 0.2% crystal violet. The number of invading cells was counted under a light microscope. Transwell migration assay were performed with the similar approach without coating of Matrigel. All experiments were performed in triplicate.

**In vivo xenograft assay**

Female BALB/c nude mice (6~8-week-old, n=20) were purchased from Shanghai SLAC Laboratory Animal Co. Ltd. All mice were housed in a temperature-controlled environment and maintained on a 12 h light/darkness cycle. All experiments were approved by the Animal Care and Use Committee of The First Affiliated Hospital of Zhengzhou University. Stable transfected CAL-27 or KB cells were implanted subcutaneously under the mice dorsa. The length (L) and width (W) of the tumors were measured weekly. The tumor volume (V) was calculated using the formula: \(V = L \times W^2 / 2\). The tumors were harvested and weighted after 4 weeks.
Chromatin immunoprecipitation (ChIP) assay

ChIP assay was conducted using Pierce Agarose ChIP kit (Pierce) according to the manufacturer’s instructions. In brief, KB cells were transfected with sh-NC or sh-HIF-1α/sh-CTCF. Cells were crosslinked with 1% formaldehyde and lysed to prepare nuclei. Chromatin was sheared using microneclease digestion. Sheared DNA was then incubated with anti-HIF-1α/anti-CTCF antibody. Normal IgG was served as a negative control. DNA was purified and analyzed by qRT-PCR.

Electrophoretic mobility shift assay (EMSA)

Recombinant HIF-1α and CTCF protein were purchased from Abcam. Native or mutated probe was biotinylated using Biotin 3’-end DNA labeling kit (Pierce) and annealed at room temperature for 1 h. EMSA assay was performed using the Lightshift Chemiluminescent DNA EMSA kit (Pierce) according to the manufacturer’s instructions. Briefly, binding reactions were performed with or without anti-HIF-1α/anti-CTCF antibody. DNA-protein complexes were electrophoresed and transferred onto a positive charged nylon membrane (Pierce), followed by UV light crosslinking. The signal was visualized by Chemiluminescent Substrate, followed by film exposure.

GST pull-down assay

GST pull-down assay was performed as previously described. Briefly, GST-tagged CTCF was expressed in E. coli, purified and bound to glutathione beads (GE Healthcare, Buckinghamshire, UK) as the bait protein. HOTTIP and histone H2B1 mRNA were in vitro transcribed using T7 polymerase (Promega, Madison, WI, USA) according to the manufacturer’s instructions. The mRNAs were then denatured, refolded and incubated with the CTCF-bound beads at room temperature for 1 h. The bound RNAs were extracted and determined by qRT-PCR.
RNA pull-down assay

The RNAs were *in vitro* transcribed using T7 polymerase (Promega) and biotin-labeled using Pierce RNA 3’ End Desthiobiotinylation Kit (Pierce). RNA pull-down assay was performed using Pierce Magnetic RNA-Protein Pull-Down Kit (Pierce) according the manufacturer’s instructions. In brief, biotinylated RNA was incubated with streptavidin beads and mixed with cell lysates. The eluted RNA-binding proteins were detected by western blotting.

Statistical analysis

All experiments were performed at least three times. Data are presented as the means ±S.D. Statistical analysis was performed using Student’s *t* test (two-tailed) between two groups or one-way analysis of variance (ANOVA) followed by Tukey post hoc test for multiple comparison. Statistical analysis was performed using the SPSS22.0 (SPSS Inc., Chicago, IL, USA). Differences were considered significant if *P* < 0.05. **P** < 0.01.
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Conflict of interest

The authors declare no conflicts of interest.
References

1. Siegel, RL, Miller, KD, and Jemal, A (2019). Cancer statistics, 2019. CA: a cancer journal for clinicians 69: 7-34.

2. Leemans, CR, Braakhuis, BJ, and Brakenhoff, RH (2011). The molecular biology of head and neck cancer. Nature reviews Cancer 11: 9-22.

3. Sturgis, EM, and Cinciripini, PM (2007). Trends in head and neck cancer incidence in relation to smoking prevalence: an emerging epidemic of human papillomavirus-associated cancers? Cancer 110: 1429-1435.

4. Jung, AC, Briolat, J, Millon, R, de Reynies, A, Rickman, D, Thomas, E, et al. (2010). Biological and clinical relevance of transcriptionally active human papillomavirus (HPV) infection in oropharynx squamous cell carcinoma. International journal of cancer 126: 1882-1894.

5. Gupta, S, Kong, W, Peng, Y, Miao, Q, and Mackillop, WJ (2009). Temporal trends in the incidence and survival of cancers of the upper aerodigestive tract in Ontario and the United States. International journal of cancer 125: 2159-2165.

6. Gehring, WJ, and Hiromi, Y (1986). Homeotic genes and the homeobox. Annual review of genetics 20: 147-173.

7. Bhatlekar, S, Fields, JZ, and Boman, BM (2014). HOX genes and their role in the development of human cancers. Journal of molecular medicine 92: 811-823.

8. Dorsam, ST, Ferrell, CM, Dorsam, GP, Derynck, MK, Vijapurkar, U, Khodabakhsh, D, et al. (2004). The transcriptome of the leukemogenic homeoprotein HOXA9 in human hematopoietic cells. Blood 103: 1676-1684.

9. Ko, SY, Barengo, N, Ladanyi, A, Lee, JS, Marini, F, Lengyel, E, et al. (2012). HOXA9 promotes ovarian cancer growth by stimulating cancer-associated fibroblasts. The Journal of clinical investigation 122: 3603-3617.
10. Sun, M, Song, CX, Huang, H, Frankenberger, CA, Sankarasharma, D, Gomes, S, et al. (2013). HMGA2/TET1/HOXA9 signaling pathway regulates breast cancer growth and metastasis. *Proceedings of the National Academy of Sciences of the United States of America* **110**: 9920-9925.

11. Alvarado-Ruiz, L, Martinez-Silva, MG, Torres-Reyes, LA, Pina-Sanchez, P, Ortiz-Lazarenno, P, Bravo-Cuellar, A, et al. (2016). HOXA9 is Underexpressed in Cervical Cancer Cells and its Restoration Decreases Proliferation, Migration and Expression of Epithelial-to-Mesenchymal Transition Genes. *Asian Pacific journal of cancer prevention : APJCP* **17**: 1037-1047.

12. Zhou, C, Li, J, Li, Q, Liu, H, Ye, D, Wu, Z, et al. (2019). The clinical significance of HOXA9 promoter hypermethylation in head and neck squamous cell carcinoma. *Journal of clinical laboratory analysis: e22873*.

13. Cheetham, SW, Gruhl, F, Mattick, JS, and Dinger, ME (2013). Long noncoding RNAs and the genetics of cancer. *British journal of cancer* **108**: 2419-2425.

14. Wang, KC, Yang, YW, Liu, B, Sanyal, A, Corces-Zimmerman, R, Chen, Y, et al. (2011). A long noncoding RNA maintains active chromatin to coordinate homeotic gene expression. *Nature* **472**: 120-124.

15. Fu, Z, Chen, C, Zhou, Q, Wang, Y, Zhao, Y, Zhao, X, et al. (2017). LncRNA HOTTIP modulates cancer stem cell properties in human pancreatic cancer by regulating HOXA9. *Cancer letters* **410**: 68-81.

16. Wang, F, Tang, Z, Shao, H, Guo, J, Tan, T, Dong, Y, et al. (2018). Long noncoding RNA HOTTIP cooperates with CCCTC-binding factor to coordinate HOXA gene expression. *Biochemical and biophysical research communications* **500**: 852-859.

17. Zhang, H, Zhao, L, Wang, YX, Xi, M, Liu, SL, and Luo, LL (2015). Long non-coding RNA HOTTIP is correlated with progression and prognosis in tongue squamous cell carcinoma.
18. Tang, X, Sun, Y, Wan, G, Sun, J, Sun, J, and Pan, C (2019). Knockdown of YAP inhibits growth in Hep-2 laryngeal cancer cells via epithelial-mesenchymal transition and the Wnt/beta-catenin pathway. *BMC cancer* 19: 654.

19. Kocabas, F, Xie, L, Xie, J, Yu, Z, DeBerardinis, RJ, Kimura, W, *et al.* (2015). Hypoxic metabolism in human hematopoietic stem cells. *Cell & bioscience* 5: 39.

20. Zhou, L, Wang, Y, Zhou, M, Zhang, Y, Wang, P, Li, X, *et al.* (2018). HOXA9 inhibits HIF-1alpha-mediated glycolysis through interacting with CRIP2 to repress cutaneous squamous cell carcinoma development. *Nature communications* 9: 1480.

21. Collins, CT, and Hess, JL (2016). Role of HOXA9 in leukemia: dysregulation, cofactors and essential targets. *Oncogene* 35: 1090-1098.

22. Masoud, GN, and Li, W (2015). HIF-1alpha pathway: role, regulation and intervention for cancer therapy. *Acta pharmaceutica Sinica B* 5: 378-389.

23. Guglas, K, Bogaczynska, M, Kolenda, T, Rys, M, Teresiak, A, Blizniak, R, *et al.* (2017). lncRNA in HNSCC: challenges and potential. *Contemporary oncology* 21: 259-266.

24. Yin, X, Yang, W, Xie, J, Wei, Z, Tang, C, Song, C, *et al.* (2019). HOTTIP Functions as a Key Candidate Biomarker in Head and Neck Squamous Cell Carcinoma by Integrated Bioinformatic Analysis. *BioMed research international* 2019: 5450617.
Figure Legends

Figure 1. HOXA9 expression in HNSCC tissues and cell lines. (A) Analysis of HOXA9 expression using TCGA data. (B) HOXA9 mRNA level in HNSCC tissues and normal counterparts were determined by qRT-PCR. GAPDH acted as an internal control. (C) The protein level of HOXA9 in HNSCC tissues and paired adjacent normal tissues were determined by western blotting. GAPDH served as a loading control. (D) HOXA9 mRNA levels in different cell lines were determined by qRT-PCR. GAPDH served as an internal control. (E) The protein level of HOXA9 in different cell lines were determined by western blotting. GAPDH served as a loading control. Data were representative images or were expressed as the mean ± SD. * $P < 0.05$, ** $P < 0.01$.

Figure 2. HOXA9 knockdown inhibits HNSCC cell growth, migration, invasion and chemoresistance, but inhibits apoptosis. (A) The protein level of HOXA9 was determined by western blotting. GAPDH served as a loading control. (B) Cell proliferation was monitored by CCK-8 assay. (C) Clonogenic ability was determined by colony formation assay. (D) The migration capacities were detected by wound healing assay. (E) The migration and invasive capacities were detected by transwell assays. (F) Cell apoptosis was detected by FACS analysis. Early and late apoptotic cells were defined as PI-/Annexin V+ and PI-/Annexin-V+, respectively. (G) CAL-27 or KB cells transfected with sh-NC or sh-HOXA9 were treated with different doses of cisplatin or 5-FU for 48 h. Cell cytotoxicity was monitored by CCK-8 assay. (H) 4 weeks after inoculation of cells transfected with sh-NC or sh-HOXA9, tumors were harvested from nude mice. Representative photographs of tumors at 4 weeks after inoculation. Tumor volumes were measured every week after inoculation. Tumor weights were measured at 4 weeks after inoculation. Each bar is a mean±SD of $n = 3$ experiments. * indicates $P<0.05$, ** indicates $P<0.01$.

Figure 3. Knockdown of HOXA9 regulates EMT-related marker via targeting YAP1/β-catenin. CAL-27 or KB cells were transfected with sh-NC or sh-HOXA9. Cells were harvested 48
h post-transfection. The protein levels of HOXA9, YAP1, β-catenin, Twist, E-cadherin, N-cadherin and Slug-1 were determined by western blotting. GAPDH served as a loading control. Data were representative images or were expressed as the mean ± SD. * \( P < 0.05 \), ** \( P < 0.01 \).

Figure 4. HIF-1α transcriptionally suppresses HOXA9. (A) KB cells transfected with sh-NC or sh-HIF-1α. The protein levels of HIF-1α and HOXA9 were determined by western blotting. GAPDH served as a loading control. (B) Predicted binding site of HIF-1α in the promoter region of HOXA9 as determined by JASPAR. (C) EMSA of predicted binding motif. DNA-protein complex was indicated by black arrow, and antibody supershift band was indicated by white arrow. (D) The binding enrichment of HIF-1α on the promoter region of HOXA9 was detected by ChIP-PCR. Data were representative images or were expressed as the mean ± SD. * \( P < 0.05 \), ** \( P < 0.01 \).

Figure 5. Knockdown of HOTTIP inhibits cell proliferation, migration and invasion via targeting HOXA9 in CAL-27 and KB cells. (A) HOTTIP mRNA level in different cell lines was determined by qRT-PCR. U6 served as an internal control. (B) CAL-27 or KB cells were transfected with sh-NC or sh-HOTTIP. The mRNA level of HOTTIP in different cell lines was determined by qRT-PCR. GAPDH served as an internal control. (C) Cell proliferation was monitored by CCK-8 assay. (D) Clonogenic ability was determined by colony formation assay. (E) Cell migration was monitored by wound healing assay. (F) Cell invasive and migrated capacities were detected by transwell assays. (G) Protein levels of HOXA9, YAP1 and β-catenin were determined by western blotting. GAPDH served as a loading control. Data were representative images or were expressed as the mean ± SD. * \( P < 0.05 \), ** \( P < 0.01 \).

Figure 6. Knockdown of CTCF inhibits cell proliferation, migration, invasion and chemoresistance in CAL-27 and KB cells. (A) CAL-27 or KB cells were transfected with sh-NC or sh-CTCF. Protein level of CTCF was determined by western blotting. GAPDH served as a
loading control. (B) Cell proliferation was monitored by CCK-8 assay. (C) Cell migration capacity was monitored by wound healing assay. (D) Cell migration and invasive capacities were detected by transwell assays. (E) CAL-27 or KB cells transfected with sh-NC or sh-CTCF were treated with different doses of cisplatin or 5-FU for 48 h. Cell cytotoxicity was monitored by CCK-8 assay. (H) 4 weeks after inoculation of CAL-27 and KB cells transfected with sh-NC or sh-CTCF, tumors were harvested from nude mice. Representative photographs of tumors at 4 weeks after inoculation. Tumor volumes were measured every week after inoculation. Tumor weights were measured at 4 weeks after inoculation. Data were representative images or were expressed as the mean ± SD. * $P < 0.05$, ** $P < 0.01$.

**Figure 7. CTCF transcriptionally induces HOXA9 expression.** (A) KB cells were transfected with sh-NC or sh-CTCF. The protein level of HOXA9 was determined by western blotting. GAPDH served as a loading control. (B) Predicted binding site of CTCF in the promoter region of HOXA9 as determined by JASPAR. (C) EMSA of predicted binding motif. DNA-protein complex was indicated by black arrow, and antibody supershift band was indicated by white arrow. (D) The binding enrichment of CTCF on the promoter region of HOXA9 was detected by ChIP-PCR. Data were representative images or were expressed as the mean ± SD. * $P < 0.05$, ** $P < 0.01$.

**Figure 8. HOTTIP and CTCF cooperatively regulate HOXA9 in HNSCC cells.** (A) Recombinant GST and GST-tagged CTCF were purified and detected by western blotting. (B) Purified GST and GST-tagged CTCF were used as bait protein to retrieve HOTTIP or control histone mRNA. (C) Interaction between endogenous CTCF and HOTTIP was determined by IP. U1 spliceosomal RNA acted as negative control. Successful IP was confirmed by western blotting. (D) Directed interaction between CTCF and HOTTIP was verified by RNA pull-down assay. α-tubulin served as negative control. Data were representative images or were expressed as the mean ± SD. * $P < 0.05$, ** $P < 0.01$. 
A. HOXA9
   Relative protein level of HOXA9
   shNC  shHOXA9
   GAPDH
   HOXA9
   CAL-27  KB

B. CAL-27
   shNC  shHOXA9
   KB
   OD 490nm
   0h 24h 48h 72h

C. CAL-27
   shNC  shHOXA9
   KB
   Colony number
   Migration
   Invasion

D. CAL-27
   shNC  shHOXA9
   KB
   Annexin V
   Annexin V

E. CAL-27
   shNC  shHOXA9
   KB
   The number of migrated and invaded cells

F. CAL-27
   shNC  shHOXA9
   KB
   Relative migration rate (%)
   Apoptosis rate (%)

G. CAL-27
   shNC  shHOXA9
   KB
   Relative cell viability (%)

H. CAL-27
   shNC  shHOXA9
   KB
   Tumor volume (mm³)
   Days

Tumor weight (g)
   Days
