HIF-1α and HIF-2α Play Distinct and Functionally Overlapping Roles in Oral
Squamous Cell Carcinoma

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Running title: Distinct and Overlapping Roles of HIF-1α and HIF-2α in OSCC

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Statement of Translational Relevance

In this study, we have shown that HIF-1α and HIF-2α correlated with different clinical-pathological parameters, stabilized at different oxygen levels, contributed differently to cell proliferation, and regulated different target genes in OSCC. Despite these differences, both HIF-1α and HIF-2α showed promoting roles in the tumor growth, and simultaneously knocking down HIF-1α and HIF-2α would acquire a better inhibitory effect than knocking down HIF-1α or HIF-2α individually. As such, we suggest that therapeutic outcome in terms of inhibiting tumor growth and angiogenesis may benefit from nonselectively targeting HIF-1α and HIF-2α in OSCC. Fortunately, a small-molecule inhibitor, NSC-134754, has been tested to block both HIF-1α and HIF-2α in VHL-defective renal carcinoma cells. Whether NSC-134754 can be applied to oral squamous cell carcinomas therapy needs further exploration.
Abstract

Purpose: To investigate the functional difference between HIF-1α and HIF-2α in oral squamous cell carcinomas.

Experimental Design: We evaluated the correlations between HIF-1α and HIF-2α expression and clinical-pathological characteristics of 97 patients with OSCC by immunohistochemical staining. OSCC cell lines transfected with lentivirus encoding shRNA against HIF-1α/2α were used to investigate the HIF-1α/2α dependent target genes. Xenograft tumors in nude mice were established using cells affected by lentivirus, and tumor growth, angiogenesis, proliferation and apoptosis were measured.

Results: HIF-1α expression was significantly associated with T stage (P=0.004), lymph node involvement (P=0.006), histological differentiation (P=0.013), and MVD (P=0.014) while that of HIF-2α was associated with T stage (P=0.011) and MVD (P=0.005). Patients with positive HIF-1α nuclear staining had a significantly worse overall survival (P<0.001) and disease-free survival (P<0.001) than those with negative HIF-1α staining. When OSCC cells were cultured at 5% O₂, only HIF-2α contributed to the expression of VEGF. At 1% O₂, VEGF was regulated by both HIF-1α and HIF-2α, but GLUT-1, CA-IX, and uPAR were regulated by HIF-1α rather than HIF-2α. Knocking down HIF-1α or HIF-2α individually inhibited the xenograft
tumor angiogenesis and growth, and simultaneous knockdown of them revealed a better inhibitory effect than knocking down either unit alone.

Conclusions: HIF-1α and HIF-2α correlated with different clinical-pathological parameters, stabilized at different oxygen levels, and regulated different genes in OSCC. However, both HIF-1α and HIF-2α showed promoting roles in the tumor angiogenesis and growth, and therapeutic outcome may benefit from combined targeting HIF-1α and HIF-2α.

Key words: Hypoxia, HIF-1α, HIF-2α, Oral Squamous Cell Carcinoma
Introduction

Robust tumor growth requires the presence of a local vascular network that supplies both oxygen and nutrients to tumor cells. However, a highly proliferating mass of tumor cells develops faster than the vasculature, and tumor cells rapidly meet up with an avascular environment deficient in oxygen(1). The oxygen pressure within solid tumors is heterogeneous, ranging from approximately 5% O₂ in well-vascularized regions to anoxia near necrotic regions, but is on average in the hypoxic range (about 1% O₂)(2). This in turn means that most tumor cells have adapted to and can grow in a hypoxic condition. Such hypoxic zones have been postulated to increase patient treatment resistance and favor tumor progression(1, 3). Two of the most important transcription factors mediating the cellular response to hypoxia are hypoxia inducible factor (HIF)-1α and HIF-2α(4).

Although HIF-1α and HIF-2α have striking similarities in structure, function and regulation, many lines of evidence suggest that there is little redundancy between the two HIF-α units. In vitro, HIF-2α, as opposed to HIF-1α protein, is strongly induced at physiological oxygen concentrations (5% O₂)(2). Besides the differences in activation pattern, HIF-1α and HIF-2α exhibit distinct roles in hypoxic activation of...
target genes (5-11). For example, HIF-1α was suggested to primarily regulate the expression of vascular endothelial growth factor (VEGF) in MCF-7 cells (breast cancer)(9), and the expression of carbonic anhydrase 9 (CA-IX), glucose transporter 1 (GLUT-1), and urokinase-type plasminogen activator receptor (uPAR) in MDA 468 cells (breast cancer)(10). HIF-2α, however, appears to regulate VEGF, GLUT-1, uPAR, and plasminogen activator inhibitor-1 (PAI-1) in RCC4 cells (renal carcinoma)(9), erythropoietin in Hep3B cells(8), and PAI-1 in adenocarcinoma A549 cells(12). Thus, the roles of HIF-1α and HIF-2α in the response to hypoxia vary among cell types and biological functions of them may be different.

In clinical specimens, elevated HIF-1α expression correlates with poor patient outcome in colorectal, pancreatic, breast, cervical, endometrial, ovarian, bladder, gastric, and head and neck carcinomas, while HIF-2α expression correlates with poor patient outcome in hepatocellular, colorectal carcinoma, melanoma, ovarian, and non-small cell lung cancers(13). In xenograft tumor model, HIF-2α was found to enhance the growth of renal cell carcinoma(14-16) while HIF-1α had an inhibitory effect (16). In colon cancer, on the contrary, HIF-1α promoted the growth of colon cancer cells but HIF-2α appeared to restrain growth (11). These findings suggest that the two HIF-α units play important and unique roles in different types of tumors.

There’s a growing body of evidence suggesting that HIF-1α is involved in the progression of oral carcinomas(17-20). In vitro model has shown that over-expression of HIF-1α was associated with increased oral carcinoma cell invasiveness (18). In
clinical studies, elevated expression of HIF-1α was found to be correlated with lymph node involvement, TNM classification, poor survival, and resistance to chemo- and radio-therapy in patients with oral squamous cell cancer (OSCC)(19, 21, 22). Furthermore, the use of anti-sense oligonucleotides and chemical inhibitor targeted to HIF-1 exhibited pro-apoptosis and anti-tumor activity(17, 20).

However, contradicting results related to HIF-1α’s roles in oral carcinomas have been reported as well, e.g. HIF-1α over-expression was found to indicate a favorable prognosis of patients with T1 and T2 OSCC(23). Moreover, no study, to the best of our knowledge, has investigated the functional role of HIF-2α in oral carcinomas. To better understand the differences between these two HIF-α units, we therefore investigated the correlation between HIF-1α/HIF-2α and clinical-pathological characteristics in patients with OSCC respectively. We knocked down HIF-1α/HIF-2α by lentivirus encoding shRNA to further address the question as to whether certain genes are preferentially regulated by HIF-1α or by HIF-2α in human OSCC cell lines. Finally, we examined the contribution of HIF-1α/ HIF-2α knockdown to the xenograft tumor growth as well as angiogenesis, proliferation, and apoptosis.
Materials and Methods

Patients and specimens

The cohort was assembled from patients who were histologically diagnosed with OSCC and underwent radical surgery at West China Hospital of Stomatology, Sichuan University between 2003 and 2004. Exclusion criteria included recurrence at presentation, preoperative radiotherapy, chemotherapy, or hormone therapy, with residual tumor at surgical margin, and incomplete medical records. We retrospectively reviewed the medical records of patients with OSCC according to the inclusion and exclusion criteria. Finally, 97 patients (52 male and 45 female, median age 58 years, range 40–79) were recruited in this study. The formalin-fixed, paraffin-embedded specimens from these patients were used for immunohistochemical analysis. Follow-up time was calculated from the date of surgery to the date of death, loss of follow, or the 60th month, whichever came first (median follow-up period of 47 months). A group of 36 tumors was early stage (T1/T2), and 61 were late stage
(T3/T4). Among 97 cases, 18 with T1 stage received local resection only, 31 with clinically negative nodal involvement underwent radical resection plus selective neck dissection (level I–III), 48 with clinically positive nodal involvement underwent radical resection plus modified radical neck dissection (level I–V), 19 received postoperative radiotherapy, and 20 received postoperative chemotherapy because of their advanced stage. During the whole follow-up period, lymph node metastases were detected histologically in 64 patients. This study was approved by the Institutional Ethics Committee of Sichuan University. The principal clinical and pathological characteristics of the patient cohort are summarized in Table 1.

**Immunohistochemical staining**

Sections (4 μm) were deparaffinized in xylene, rehydrated, and endogenous peroxidase blocked with 3% H2O2. Antigen retrieval was accomplished by 0.01 M citrate buffer solution (pH=6.0) in a microwave oven 700W for 15 min. After incubated with 5% normal goat serum for 20 min, the slides were exposed for 1 hour at 37°C and overnight at 4°C to the mouse anti-HIF-1α (1:200, NOVUS, Littleton, CO, USA), rabbit anti-HIF-2α(1:100, Abcam, Cambridge, MA, USA), rabbit anti-VEGF (1:200, Santa Cruz, Santa Cruz, CA, USA), rabbit anti-CA-IX (1:100, Santa Cruz), rabbit anti-GLUT-1(1:200, Santa Cruz), mouse anti-CD34(1:100, Zymed, San Francisco, CA, USA), and mouse anti-uPAR(1:100, Santa Cruz). Sections were then incubated with biotinylated goat anti-rabbit IgG/goat anti-mouse IgG (Zhongshan Goldenbridge Biotechnology, Beijing, China) for 1 h, and
streptavidin-peroxidase for 30 min. The 0.02% diaminobenzidine tetrahydrochloride (DAB) was used as a chromogen, and the slides were counterstained with haematoxylin. The percentage of positive cells was estimated using an image analysis system (Leica, Germany). Microvessel density (MVD) was assessed by the microscope as described previously(19). The median values for VEGF, CA-IX, GLUT-1, uPAR, and MDV were regarded as the cut-off values for low and high expression. Nuclear expressions of HIF-1α and HIF-2α were graded as negative and positive.

**Cloning, lentivirus preparation, and titration**

Lenti-X shRNA expression system (Clontech, Mountain View, CA, USA) was used for the construction of the lentiviral expression construct according to the manufacturer's instructions. Short pairs of sense and antisense DNA oligo encoding a sense-loop-antisense sequence to HIF-1α and HIF-2α genes were synthesized for the validated corresponding siRNAs(10), and sequences are listed in Table S1. The complementary DNA oligos were annealed and cloned to the BamH I/Eco RI-digested pLVX-shRNA1 vectors (Clontech). The recombinant vectors were purified and cotransfected with Lenti-X HT packaging Mix (Clontech) into HEK 293T packaging cells using lipofectamine 2000 (Invitrogen). The virus-containing cell culture supernatants were collected 48h after transfection, passed through a 0.45-μm filter, and stored at −80°C. The virus titration was determined using puromycin selection following the manufacturer’s protocol.
Cell culture and virus infection

Two malignant oral squamous cell carcinoma (OSCC) cells line—Tca8113(24) and BcaCD885(25) were obtained from the State Key Laboratory of Oral disease, Sichuan University. Cells were cultured in RPMI 1640 Medium (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal calf serum (Hyclone, Logan, UT, USA), 2mM L-glutamine, 25mM 2-(4-(2-hydroxyethyl)-1-piperazinyl) ethanesulfonic acid (HEPES), and 100 units/ml penicillin and streptomycin in a humidified 5% CO2 atmosphere. The cells were cultured under 1% O2, 5% O2, and 20% O2 conditions respectively, balanced with N2 in a 3-Gas incubator (Binder, Tuttlington, Germany). After 24h, cells were infected with recombinant lentivirus vectors at a multiplicity of infection (MOI) of 5.

Immunofluorescence

OSCC cells were seeded onto coverslips at a density of 10^4/ml and cultured in 6-well culture plate for 24h. Cells grown on coverslips were washed in cold PBS and fixed in 2% paraformaldehyde-PBS for 20 min, permeabilized in 0.5% Triton X-100 in PBS for 10 min at 4°C, and blocked in 1% BSA for 30 min at room temperature. Cells were incubated overnight with 1:100 dilution of mouse anti- HIF-1α (NOVUS) or 1:100 dilution of rabbit anti- HIF-2α (Abcam), and then incubated with FITC conjugated goat anti-mouse/rabbit IgG (1:500, Zhongshan Goldenbridge Biotechnology) at 37°C for 1h. Cells were counterstained with DAPI (1 μg/μl), and examined using a fluorescence microscope (Olympus BX51).
Quantitative real-time RT-PCR

Total RNA was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and treated with RNase-free DNase I (Takara, Kyoto, Japan) to avoid genomic DNA contamination. Samples with a 260:280 nm absorbance ratio of ≥1.9 were reverse-transcribed using a RevertAid First-Strand cDNA Synthesis Kit (Fermentas, Lithuania) with random hexamer primer. PCR amplification of the cDNA template was performed using Thunderbird SYBR qPCR mix (TOYOBO, Osaka, Japan) on ABI PRISM 7300 sequence detection system (Applied Biosystems, Foster City, CA, USA). PCR conditions were 95 °C for 1min followed by 40 cycles of amplification consisting of 95 °C for 15s, 55 °C for 15s, and 72 °C for 1min. Reactions were run in triplicate, and results were averaged. Each value was normalized to GAPDH as the housekeeping gene to control for variations in the amount of input cDNA. Table S1 shows the sequences of PCR primers used in this study. The relative expression level of the genes was calculated using the 2ΔΔCt method comparing to cells transfected with shRNA-neg.

Western-blot

Total proteins were isolated from the cultured monolayer cells with a total protein extraction kit (Keygen, Nanjing, China), and protein concentrations were detected by a BCA protein assay kit (Pierce, Rockford, IL, USA) as described previously(26). Thirty microgram proteins from each sample were separated on 8% SDS-PAGE and transferred electrophoretically to PVDF membranes (Millipore, Boston, MA, USA).
Membranes were blocked with 2% BSA in TBS containing 0.1% Tween20 at 37°C for 2h and then incubated for 2h respectively with 1:500 dilution of mouse anti-HIF-1α, 1:200 dilution of rabbit anti- HIF-2α, 1:400 dilution of mouse anti-uPAR, 1:500 dilution of rabbit anti-GLUT1, and 1:1000 dilution of mouse anti-GAPDH. Horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG were used as secondary antibody (1:5000 diluted in TBST with 2% BSA, incubated for 1h). Bands were scanned using a densitometer (GS-700; Bio-Rad Laboratories, Hercules, CA, USA), and quantification was performed using Quantity One 4.4.0 software.

**ELISA assay**

VEGF protein contents in supernatant were measured using the Human VEGF Quantikine ELISA Kit (R&D systems, Minneapolis, MN, USA) following the manufacture’s instructions. The intensity of color was measured at 540nm in a microtest plate spectrophotometer. The concentration of protein in culture media was determined in triplicate wells and was normalized to standard curves generated for each set of samples assayed.

**Cell proliferation**

The cell proliferation was quantified by the colorimetric MTT assay. In brief, cells were incubated with 0.5 mg/ml MTT for 4 h at 37°C. Then supernatant was removed and 150 mg of dimethyl sulfoxide (DMSO) was added. Optical densities at 490 nm were measured using culture medium as a blank.

**Xenografts in Nude Mice**
The nude mice (female, 6 weeks of age) were obtained from Laboratory Animal Center of Sichuan University (Chengdu, Sichuan, China). Thirty-two mice were randomized and divided into 4 groups (shRNA-1, shRNA-2, shRNA-1+shRNA-2, and shRNA-neg), 8 mice each. Lentivirus transfected cells were then injected subcutaneously (5×10^6 cells/200 μl PBS/mouse) on the back of nude mice. The tumor size was monitored by measuring diameters using vernier caliper weekly, and was calculated as πls^2/6, where l=long side and s=short side as described previously(2). Tumors were harvested at the 7th week and fixed by 4% paraformaldehyde and then embedded by paraffin for immunohistochemistry analyses.

**Proliferation and apoptosis in OSCC xenograft tumors**

The proliferation was measured by immunohistochemical staining of the proliferation marker ki-67 (1:200, Santa Cruz). The cell apoptosis was detected by TUNEL method using In Situ Cell Death Detection Kit, POD (Roche Applied Science, Basel, Switzerland) according to the manufacturer’s protocol. Cells were observed in a bright field microscope, and the number of apoptotic cells was counted in 10 different microscopic fields.

**Statistics**

The association between the levels of the HIF-1α/HIF-2α and patients’ sex, age, T stage, lymph node involvement, MVD, histological differentiation, and recurrence was compared using Pearson’s χ^2 test. Overall survival and disease-free survival curves were estimated using the Kaplan-Meier method and differences between
groups were compared using the log-rank test. A Cox proportional hazards model was applied to identify prognostic variables that predict overall survival and disease-free survival. The power calculation was done with PASS (V. 08.0.3, NCSS, Kaysville, Utah, USA), assuming that 5-year survival rate is 50% (27), hazard ratio vary from 2.17 to 4.53 (21, 28-30), proportion in control group 1 is 47.5%(HIF-1α negative) or 39.2%(HIF-2α negative), and two side alpha is 0.05. The log-rank test with an overall sample size of 97 subjects achieves 81.72%-99.94% power for HIF-1α and 76.69%-99.74% power for HIF-2α. The comparisons of means among groups were analyzed by one-way ANOVA, and the Dunn’s Multiple Comparison Test was further used to determine the specific differences between groups. All statistical analyses were performed using the SPSS package (version 13.0, Chicago, IL, USA). A value of $P < 0.05$ was considered statistically significant.

**Results**

**HIF-1α and HIF-2α expression and clinical outcomes**

The representative immunohistochemical images were shown in figure 1. We found that the nuclear staining of HIF-1α was detected in 51 of 97 (52.5%) OSCC specimens, and that of HIF-2α was detected in 59 of 97 (60.8%). The nuclear staining of HIF-1α was significantly associated with T stage ($P=0.004$), lymph node involvement ($P=0.006$), histological differentiation ($P=0.013$), and MVD ($P=0.014$)
while that of HIF-2α was associated with T stage (P=0.011) and MVD (P=0.005).
(Table 1, Figure S1)

The Kaplan-Meier survival analysis revealed a significantly worse overall survival
(P<0.001) and disease-free survival (P<0.001) for patients who had nuclear staining
of HIF-1α compared with those who had negative HIF-1α staining (Figure 1, B and
C). HIF-2α had no significant association with either overall survival (P=0.398,) or
disease-free survival (P=0.279) (Figure 1, E and F). HIF-1α and HIF-2α expression
levels as well as patients’ gender, age, T stage, lymph node involvement, histological
differentiation, neck dissection, postoperative radiotherapy, and postoperative
chemotherapy were included in the univariate and multivariate analysis performed by
Cox proportional hazard regression model. Multivariate analysis were performed
using a forward variable selection technique (entry P<0.05, removal, P>0.1). In the
univariate analysis, HIF-1α, T stage, lymph node involvement, histological
differentiation, and neck dissection were correlated with the overall survival, while
HIF-1α, T stage, lymph node involvement, histological differentiation, and
postoperative radiotherapy were correlated with the disease-free survival (Table S2).
In the multivariate analysis, nuclear HIF-1α staining (P=0.002, HR=2.641, 95%CI
1.433-4.868) and neck dissection (P=0.055, HR=2.767, 95%CI 0.98-7.81) remained
in the final model for overall survival, and nuclear HIF-1α staining (P=0.013,
HR=1.976, 95%CI 1.154-3.385) and differentiation (P=0.023, HR=1.554, 95%CI
1.064-2.27) were correlated with disease-free survival. (Table S2) These results
suggest that HIF-1α but not HIF-2α expression may predict a worse survival of patients.

The OSCC specimens were simultaneously immunostained for several hypoxia related proteins VEGF, CA-IX, GLUT-1, and uPAR that are potentially relevant to different aspects of tumor biology. (Figure S1) HIF-2α protein was significantly associated with VEGF ($P=0.005$), while HIF-1α was found to be significantly associated with VEGF ($P=0.012$), GLUT-1 ($P=0.018$), and CA-IX ($P=0.004$). (Table S3)

**HIF-1α and HIF-2α induction pattern in OSCC cells**

We exposed OSCC cells to normoxic condition (20% O$_2$), physiological conditioned oxygen level (5% O$_2$), and hypoxic condition (1% O$_2$). After 24h exposure, HIF-α expression was detected by immunofluorescence. At 20% O$_2$, neither HIF-1α nor HIF-2α was detected in either cell line. Nuclear staining of HIF-1α was detected in both cell lines that cultured at 1% O$_2$, but was undetectable in those cultured at 5% O$_2$. HIF-2α, however, was detected in cells that cultured at both 5% O$_2$ and 1% O$_2$ (Figure 2, A). The protein levels of HIF-α were further analyzed by western blot. HIF-1α protein was hardly detectable under 20% O$_2$ and 5% O$_2$ conditions, and was remarkably increased at 1% O$_2$ in both cell lines. HIF-2α protein was undetectable at 20% O$_2$, and was remarkably increased at 5% O$_2$ and 1% O$_2$ in both cell lines. (Figure 2, B) The mRNA levels of HIF-α were analyzed by quantitative RT-PCR after 24h exposure. The HIF-1α mRNA levels were significantly increased.
at 1% O₂, but not at 5% O₂, while HIF-2α mRNA levels were significantly up-regulated at 5% O₂ and 1% O₂ in both cell lines. (Figure 2, C). These results suggest that HIF-2α protein express at a physiological oxygen level, while HIF-1α protein should be stabilized at a much lower oxygen level (1% O₂).

**HIF-1α primarily contributed to cell proliferation under hypoxia.**

We exposed cells to different oxygen levels, and infected respectively with lentivirus encoding shRNA targeting HIF-1α (shRNA1), lentivirus encoding shRNA targeting HIF-2α (shRNA2), shRNA1 plus shRNA2 (shRNA1+siRNA2), and lentivirus encoding a negative control shRNA (shRNA-neg). Cell proliferation was measured by MTT assay. In both cell lines, no significant difference was observed among four groups at 20% O₂ or 5% O₂. (Figure S2, A, B, C, and D) However, at 1% O₂, the cells treated by shRNA1 and shRNA1+shRNA2 had significant lower proliferation rate than those treated by shRNA2 and shRNA-neg (Figure S2, E and F). These results suggest that under hypoxic condition (1% O₂), HIF-1α but not HIF-2α primarily contributes to the cell proliferation.

**Different genes regulated by HIF-1α and HIF-2α**

We analyzed the mRNA and protein expression of VEGF, GLUT-1, CA-IX, and uPAR in Tca8113 and BcaCD885 cells after respectively treated with shRNA-neg, shRNA1, shRNA2, and shRNA1+shRNA2.

The mRNA levels of genes were measured by quantitative RT-PCR, and the protein levels were measured by ELISA (VEGF) or western-blot (GLUT-1, CA-IX,
and uPAR). In both cell lines that cultured at 5% O₂, VEGF mRNA (Figure 3, A and C) and protein levels (Figure 4, A) were significantly decreased after shRNA2 and shRNA1+shRNA2 treatments, but were not significantly affected by shRNA1 in comparison with shRNA-neg. GLUT-1, CA IX, and uPAR mRNA (Figure 3, B and D-H) and protein levels (Figure 4, B) were not significantly affected by either shRNA1 or shRNA2.

In both cell lines that cultured at 1% O₂, VEGF mRNA (Figure 3, A and C) and protein levels (Figure 4, A) were significantly decreased in the shRNA1 and shRNA2 groups, and were further decreased in the shRNA1+shRNA2 groups compared with shRNA-neg group. GLUT-1, CA IX, and uPAR mRNA (Figure 3, B and D-H) and protein levels (Figure 4, B) were significantly decreased in the shRNA1 and shRNA1+shRNA2 groups but were not decreased in shRNA2 group compared with shRNA-neg group. These results indicate that at 1% O₂, VEGF was regulated by both HIF-1α and HIF-2α, while GLUT-1, CA IX, and uPAR were regulated by HIF-1α but not HIF-2α.

**Different roles of HIF-1α and HIF-2α in the xenograft growth**

We established xenograft using cells treated respectively with shRNA-neg, shRNA1, shRNA2, and shRNA1+shRNA2, and documented the tumor volume weekly.

As shown in figure 5, no difference of tumor volume was observed between shRNA1 and shRNA-neg groups in the first 3 weeks, but the growth of tumor
significantly slowed down since the 4th week in the shRNA1 group compared with shRNA-neg group. In contrast, the volume of shRNA2 treated tumor was significantly smaller than that of shRNA-neg treated tumor since the 2nd week. Although it was still significantly slower than shRNA-neg treated tumor, there was a tendency to speed up after the 5th week. In addition, the shRNA1+shRNA2 treated tumor kept a much slower growth than other three groups during the whole period (figure 5, A and B).

We harvested the tumors at the 7th week, and then evaluated the tumors’ proliferation, apoptosis, and MVD. The ki-67 immunohistochemical staining in the shRNA1, shRNA2, and shRNA1+shRNA2 treated tumors were remarkably lower than that in shRNA-neg treated tumors (Figure 5, C, D, and E), indicating that interference for either or both HIF-α units may restrain the proliferation of tumor cells. The TUNEL assay showed a stronger apoptosis in shRNA1, shRNA2, and shRNA1+shRNA2 treated tumors than shRNA-neg treated tumors (Figure 5, F, G, and H). The MVD in shRNA1 and shRNA2 treated tumor was significantly lower than that in shRNA-neg treated tumor (Figure 5, I, J, and K). Moreover, shRNA1+shRNA2 treated tumor had a much lower ki-67 staining, more apoptosis cells, and lower MVD than both shRNA1 and shRNA2 treated tumors alone (Figure 5, E, H, and K).

Discussion

A number of previous studies have provided evidence that HIF-1α and HIF-2α play
different roles in different types of tumors(2, 9, 11, 16). Microarray analyses have identified a set of unique and common target genes regulated by HIF-1α and HIF-2α in a cell type-specific manner (2, 5-7). In the present study, we analyzed four approved HIF-α target genes (VEGF, GLUT-1, CA-IX, and uPAR) that are potentially correlated with different aspects of tumor biology. VEGF, a key angiogenic stimulator, was a well known target gene of HIF-1α (31). Recently, regulatory roles of HIF-2α on VEGF have been reported in a few types of cells (2, 9, 16). Here, we show that both HIF-1α and HIF-2α regulated the expression of VEGF in OSCC cells, and that both HIF-1α and HIF-2α were correlated with VEGF and MVD in OSCC specimens. These results suggest essential roles of HIF-1α and HIF-2α in the angiogenesis of OSCC. GLUT-1 regulates the glucose uptake, and plays an important role in cell adaptation to hypoxic environment. CA-IX, an enzyme that catalyzes the reversible conversion of CO2 to carbonic acid, allows cancer cells to maintain an alkaline intracellular pH and an acidic extracellular pH, which are critical for cell proliferation and invasion, respectively(1, 32). We found that HIF-1α, but not HIF-2α, regulated the expression of GLUT-1 and CA-IX at 1% O2, indicating that HIF-1α rather than HIF-2α mediates the OSCC cellular adaptation to hypoxia. This may explain why knockdown of HIF-2α had no effect on cell proliferation at either 5% O2 or 1% O2, while knockdown of HIF-1α significantly inhibited the cell proliferation at 1% O2. uPAR, a serine proteinase receptor, activates plasminogen and matrix metalloproteinase, and finally facilitates matrix remodeling and cell proliferation.
migration(24). We observed that the hypoxic induction of uPAR was regulated by HIF-1α, but not HIF-2α, in OSCC cells, indicating a pro-metastatic role of HIF-1α in OSCC. We also observed a correlation between HIF-1α and lymph node involvement in patients with OSCC, which further supported the notion that HIF-1α may participate in the metastasis process of OSCC. Moreover, consistent with the results reported by Holmquist-Mengelbier et al.(2), we found that HIF-2α protein was stabilized at 5% O₂ and 1% O₂, while HIF-1α protein was stabilized at 1% O₂. Since HIF-1α and HIF-2α stabilized at different oxygen levels and regulated different target genes, we therefore argue that the specific roles of HIF-1α and HIF-2α in the tumor biology depend not only on which genes are transcribed by HIF-1α or HIF-2α, but also on the conditions under which HIF-1α and HIF-2α are stabilized.

In agreement with previous reports (21, 33, 34), we observed that HIF-1α expression was correlated with T stage, overall survival, and disease-free survival of patients with OSCC. It was suggested that a rapid tumor growth results in a large tumor size and hypoxic area, which thereby induced the stabilization of HIF-1α(21). Once stabilized, HIF-1α transactivates a set of genes that in turn facilitate tumor growth, angiogenesis, and metastasis. In this view, the correlation between HIF-1α and patients’ survival is intelligible. It has been reported that targeting HIF-1α pathway in tumor cells inhibits tumor growth in mouse xenograft(9, 11, 35-37). Consistently, we observed that knockdown of HIF-1α reduced proliferation and MVD, and increased the apoptosis in OSCC xenograft tumors. These findings provide
Further evidence that HIF-1α participates in the progression of OSCC. However, Beasley et al. (4) found in surgically resected head and neck squamous cell carcinoma that HIF-1α was associated with improved disease-free survival. Recent studies have revealed a tumor suppressive role of HIF-1α as well, probably due to HIF-1α-induced apoptosis or transactivation of genes that are themselves targets for negative selection in human cancers (16, 38). In light of these discrepancies among different tumors, there may be tissue-specific differences in response to HIF-1α regulation.

Although HIF-2α has been claimed to have either promoting (2, 16, 39) or suppressing (11, 40) role in different types of tumors, the contribution of HIF-2α to tumor angiogenesis was widely accepted (2, 11, 16, 39, 40). We observed that HIF-2α protein was significantly associated with MVD and VEGF in OSCC specimens, and that knockdown of HIF-2α inhibited the VEGF expression in vitro. In addition, knockdown of HIF-2α inhibited the xenograft tumor growth and reduced the MVD of xenograft tumor. These findings demonstrate an important role of HIF-2α in the angiogenesis and growth of OSCC.

Both HIF-1α and HIF-2α exhibited promoting roles in the xenograft tumor growth individually; however, their patterns are different. Knockdown of HIF-2α inhibited the tumor growth since the early days (2nd week) of xenograft growth, while the inhibitory effects of HIF-1α shRNA occurred during the latter period of time (4th week latter). Holmquist-Mengelbier et al. (2) found that transient knockdown of HIF-1α did not affect neuroblastoma xenograft tumor growth within 15 days. In
agreement with them, we found that knockdown of HIF-1α did not affect the OSCC xenograft tumor growth in the first 3 weeks. The reason for this may be that HIF-2α was stabilized at a wide range of oxygen levels (from physiological oxygen level to hypoxia), but HIF-1α was stabilized at hypoxic condition which arise from large tumor lacking oxygen delivery. More importantly, our results revealed that simultaneously knocking down HIF-1α and HIF-2α acquired a better inhibitory effect on OSCC xenograft than knocking down HIF-1α or HIF-2α individually. Therefore, we suggest that therapeutic outcome in terms of inhibiting tumor growth may benefit from simultaneously targeting HIF-1α and HIF-2α in OSCC. Hitherto, targeting HIF-1α has become an attractive approach for anticancer agents, e.g. PX-478 (a HIF-1α inhibitor) is now undergoing Phase I clinical trials. Though HIF-2α has been demonstrated to exert the effects of an oncogene(2, 16, 39), drugs specifically target HIF-2α have been poorly identified. Recently, a small-molecule inhibitor of HIF-1α, NSC-134754, has been tested to block HIF-2α as well in VHL-defective renal carcinoma cells. Whether NSC-134754 can inhibit both HIF-1α and HIF-2α in OSCC cells needs further exploration.

In conclusion, our results suggest that HIF-1α and HIF-2α may play distinct but functionally overlapping roles in OSCC, and that combined inhibition of HIF-1α and HIF-2α may be a suitable therapeutic strategy for OSCC.
Conflict of interest

None declared.

Acknowledgments

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Figure legends

**Figure 1** Expression of HIF-1α and HIF-2α in OSCC specimens and their correlations with Overall and Disease-free survival. **(A&D)** Representative image of HIF-1α (A) and HIF-2α (D) expression by immunohistochemical staining. Original magnification:×100, inset:×400, bar: 50μm. **(B&C)** Kaplan-Meier survival curve for Overall survival (B) and Disease-free survival (C) of patients according to HIF-1α nuclear staining. a. HIF-1α negative, b. HIF-1α positive. **(E&F)** Kaplan-Meier survival curve for Overall survival (E) and Disease-free survival (F) according to HIF-2α nuclear staining. a. HIF-2α negative, b. HIF-2α positive.

**Figure 2** HIF-1α and HIF-2α expression in OSCC cell lines cultured at different oxygen levels. **(A)** Immunofluorescence staining of HIF-1α and HIF-2α protein in two
cell lines that cultured at 1% O₂, 5% O₂, and 20% O₂. (B) Protein levels of HIF-1α and HIF-2α were measured by Western blot. The GAPDH levels were used as the internal controls. (C) The mRNA levels of HIF-1α and HIF-2α were analyzed by quantitative real-time RT-PCR, normalized against GAPDH, and indicated % induction or reduction compared with 20% O₂ group. Values are means ±S.D. of triplicate experiments. Error bars indicate S.D. Significantly different with normoxic control values: *P < 0.05.

Figure 3  mRNA levels of VEGF, CA-IX, uPAR, and GLUT-1 in Tca 8113 and BcaCD885 cells cultured at 1% O₂ and 5% O₂. Cells were infected respectively with lentivirus encoding shRNA targeting HIF-1α (shRNA1), shRNA targeting HIF-2α (shRNA2), negative shRNA (shRNA-neg), and shRNA1 plus shRNA2. mRNA levels of VEGF, CA-IX (B&D), uPAR (E&G), and GLUT-1 (F&H) were measured by quantitative real-time RT-PCR, normalized against GAPDH, and indicated % induction or reduction compared with those in cells cultured at 5% O₂ and treated with shRNA-neg. Values are means ±S.D. of triplicate experiments. Error bars indicate S.D. *P < 0.05.

Figure 4  Protein levels of VEGF, CA-IX, uPAR, and GLUT-1 in Tca 8113 and BcaCD885 cells cultured at 1% O₂ and 5% O₂. Cells were treated as described in figure 3. (A) VEGF proteins in the supernatant were analyzed by ELISA analysis. (B)
Protein levels of CA-IX, uPAR, and GLUT-1 were measured by Western blot. The GAPDH levels were used as the internal controls. Values are means ± S.D. of triplicate experiments in each group. Error bars indicate S.D. Statistically significant difference from shRNA-neg control values: *$P < 0.05$.

**Figure 5** Contribution of HIF-1α and HIF-2α to the xenograft growth as well as to the proliferation, apoptosis, and angiogenesis. (A&B) Growth curve of xenograft tumors. (C&D) Representative image of ki-67 expression in xenograft tumor by immunohistochemical staining. magnification:×200, bar: 100μm. (E) The percent of ki-67 positive cells. (F&G) Representative image of TUNEL assay in xenograft tumor. magnification:×200, bar: 100μm. (H) The percent of TUNEL-positive cells (I&J). Representative image of CD34 immunohistochemical staining. magnification:×200, bar: 100μm. (K) Microvessel density (CD34+) in xenograft tumors. Statistical significance was determined by one-way ANOVA. Error bars indicate SEM. statistically significant difference with shRNA-neg control values (*$P < 0.05$).

Figure S1 Representative images of VEGF, uPAR, CA-IX, CD34, GLUT-1 expression in OSCC specimens by immunohistochemical staining. Magnification:×100, Inset:×400, Bar: 50μm.
Figure S2 Growth curve of OSCC cells infected respectively with lentivirus encoding shRNA1, shRNA2, shRNA1+shRNA2, and shRNA-neg. Values are means ±S.D. of triplicate experiments in each group. Error bars indicate S.D. statistically significant difference with shRNA-neg control values (*$P < 0.05$).
Table 1 Clinical-pathological characteristic of 97 patients with OSCC, and association between HIF-1α/HIF-2α expression and these variables

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variables
Figure A: Immunofluorescence images showing HIF-1α and HIF-2α expression in Tca8113 and BcaCD885 cells under different oxygen concentrations (20%, 5%, 1% O2).

Figure B: Western blot analysis of HIF-1α, HIF-2α, and GAPDH in Tca8113 and BcaCD885 cells under different oxygen concentrations. HIF-1α is detected at 97kDa, HIF-2α at 118kDa, and GAPDH at 40kDa.

Figure C: Quantitative analysis of HIF-1α and HIF-2α expression levels using the 2-ΔΔCt method. Significant differences are marked with an asterisk (*) for each oxygen concentration comparison.
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Gui-quan Zhu, Ya-ling Tang, Ling Li, et al.

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