Effect of HIF-1α on biological activation of human tongue squamous cell carcinoma SCC-15 cells in vitro

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Abstract. Hypoxia-inducible factor-1α (HIF-1α) is a key regulator for tumor cells and tissues to adapt to hypoxic condition. Suppressing the expression of HIF-1α is important to evaluate its effect on cancer cells. This study was carried out to analyze the effect of HIF-1α on the biological activation of human tongue squamous cell carcinoma (TSCC) SCC-15 cells. In this experiment, deferoxamine mesylate (DFO) was used to induce hypoxic condition. HIF-1α gene was suppressed by lentiviral vector. The effect of the level of HIF-1α expression was tested on the proliferation, cell cycle, cell apoptosis and cell invasion of SCC-15 cells. We demonstrated that SCC-15 cells showed a more aggressive phenotype after treated with DFO. Additionally, DFO was able to induce the expression of HIF-1α protein. Lentiviral vector can effectively inhibit HIF-1α expression on mRNA and protein level. Under normoxic or hypoxic conditions, downregulation of HIF-1α for SCC-15 cells induced cell apoptosis and inhibited growth and invasion. These results showed that suppressing the expression of HIF-1α inhibited the aggressive potential of SCC-15 cells under normoxic and hypoxic condition. Thus, finding an effective and safe pathway to inhibit the expression of HIF-1α can help us to improve the survival rate of human TSCC patients.

Introduction

Cells with indefinite proliferation, spreading to adjacent tissues, regional lymph nodes and distant organs are characteristics of cancer. Among the oral and maxillofacial cancers, squamous cell carcinoma is the most common one. Every year >410,000 new oral squamous cell carcinoma patients are diagnosed, accounting for 1-5% of all cancers (1). In oral malignant tumors tongue squamous cell carcinoma (TSCC) is the most common cause of cancer-related deaths. Although chemotherapy, radiotherapy, and surgical therapy for TSCC have developed rapidly in the past years, the 5-year survival rate is still poor (2,3). Most cancers including TSCC are considered as a gene-related disease and associated with the activation of oncopgenes and inactivation of tumor-suppressor genes. Hence, finding a safe and effective therapy to change the abnormal expression of genes and to improve the rate of survival with TSCC is imperative. RNA interference (RNAi) has emerged as a powerful method for gene suppression in molecular medicine. RNAi is the process of silencing genes by the sequence specific double-stranded RNA (dsRNA). Hence it is post-transcriptional gene silencing in animals and plants. Fire and Mello were awarded the Nobel Prize for Medicine in 2006 for discovering RNAi in 1998 (4). Studies have shown that RNAi is a promising anticancer therapeutic tool (5,6).

The center of the solid tumor is often in a hypoxic microenvironment because of its rapid growth (7). The hypoxic conditions can lead to a more malignant tumor. It can enhance abnormal angiogenesis, invasion, metastasis of tumors, and result in poor prognosis (8,9). To adapt to the hypoxic microenvironment, many normal and abnormal factors are regulated, including hypoxia-inducible factor-1 (HIF-1) which plays an important role in the process. HIF-1, a transcription factor was found in 1992 (10). It is composed of two subunits, a strictly regulated α subunit and a constitutive β subunit. HIF-1β is also called aryl hydrocarbon receptor nuclear translocator (ARNT) (11). HIF-1β levels of mRNA and protein are maintained constant regardless of oxygen tension (12), whereas, HIF-1α is an oxygen-labile subunit. In normoxia, HIF-1α can be degraded by rapid ubiquitination [its protein has a short half-life (1/2~5 min) under normoxia (13)]. However, under hypoxic conditions, the decay of HIF-1α is suppressed, and then it can translocate into the nucleus and dimerizes with HIF-1β and forms the active complex HIF-1 (14). The activated complex associate with hypoxia response element (HRE) to induce expression of its target genes (15).
The target genes, including erythropoiesis, glycolysis and angiogenesis (16), are essential for tumors to adapt to and survive in hypoxic conditions. Previous studies have found overexpression of HIF-1α in various human cancers may play an important role for cancer progression (17,18), which implied that HIF-1α is an essential transcriptional regulator of tumor microenvironment. Therefore, gene silencing HIF-1α by RNAi may be an effective method to control the malignancy of tumors and improve the survival of patients.

Previously it was found that HIF-1α might be a significant prognostic predictor for TSCC patients (19). Another study showed that HIF-1α can regulate angiogenesis and survival of oral squamous cell carcinoma (20). Also, we that HIF-1α was expressed in oral squamous cell carcinoma, and found that the levels of HIF-1α in human TSCC seemed to be correlated with human prognosis (21). These findings implied that HIF-1α is an important factor in development and treatment of TSCC. In the present study, according to the principles of RNAi, we constructed lentiviral vector targeting HIF-1α and infected TSCC cell line SCC-15 cells to investigate the effect of HIF-1α on the biological behavior of SCC-15 cells.

Materials and methods

Cell lines and reagents. Human TSCC SCC-15 cell line was provided by Dr Huang Xin, Beijing Stomatological Hospital, Capital Medical University, Beijing, China. SCC-15 cells were cultured in Dulbecco's modified Eagle's medium/ F12 (DMEM/F12=1:1). Medium was supplement with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin. The cells were incubated at 37˚C in a humidified atmosphere with 5% CO2. Eight hours later, the medium was removed and replaced with fresh, complete medium. Seventy-two hours later, the medium was replaced with fresh, complete medium containing 2 µg/ml of puromycin to select for stably transduced cells. The medium was replaced with fresh medium containing puromycin every 3 days until puromycin-resistant colonies were identified. The cells transduced with lentiviral vectors targeting HIF-1α were named as RNAi cells. The LV-shCon affected cells were named as Con cells, and the untransduced cells were named as Mock.

Construction of lentiviral vector mediated RNAi. We use siRNA design software (www.ambion.com) to choose the RNAi target gene sequence. The target gene sequence of HIF-1α (NM_001530) is GATGAAAGAATTACCGAAT. The control target sequence is TTCTCCGAACGTGTCACGT. In this experiment, we generated the double-stranded oligonucleotides targeting the endogenous HIF-1α gene cloned into GV248 vector (hu6-MCS-UBiquitin-EFGP-RES-puromycin), and named it lentiviral/shRNA-HIF-1α (LV-shHIF-1α) (Table I). The sequence was not related to HIF-1α target genes which was designed and used as negative control and termed letiviral/shRNA-control (LV-shCon) (Table I). The two vectors were confirmed by DNA sequencing.

Lentiviral vector production. According to the manufacturer's instructions, first, the packaging 293T cells were trypsinized, collected, and resuspended at a density of 1.2x10⁶ cells/20 ml in growth medium containing 10% serum. The cells were seeded in a 15-cm dish. Next, DNA-Lipofectamine 2000 complexes containing 20 µg pGC-LV vector, 15 µg pHelper 1.0 vector, 10 µg pHelper 2.0 vectors and 100 µl Lipofectamine 2000 were prepared and mixed with Opti-MEM medium ≤5 ml, and incubated at room temperature for 20 min. After the 293T cells reaching 80% confluence, the medium was replaced with serum-free medium. Two hours later, the DNA-Lipofectamine 2000 complexes were added to the serum-free medium and incubated at 37˚C in a humidified atmosphere with 5% CO2. Eight hours later, the medium was removed and replaced with the fresh medium containing 10% serum. Forty-eight hours later, the medium containing lentivirus was centrifuged at 4000 g for 10 min at 4˚C to pellet cell debris, and to concentrate the lentivirus at 4000 g for 15 min and stored at -80˚C.

Transduction of target cells and selection. SCC-15 cells (2x10⁵) were collected and seeded in each well of a 6-well plate with 1 ml of complete media and transduced by lentiviral vectors at a MOI (Manual Optical Inspector) 10. Transduction was carried out with 5 µg/ml of Polybrene. Twelve hours later the medium was removed and replaced with fresh, complete medium. Seventy-two hours later, the medium was replaced with fresh, complete medium containing 2 µg/ml of puromycin to select for stably transduced cells. The medium was replaced with fresh medium containing puromycin every 3 days until puromycin-resistant colonies were identified. The cells transduced with lentiviral vectors targeting HIF-1α were named as RNAi cells. The LV-shCon affected cells were named as Con cells, and the untransduced cells were named as Mock.

Real-time RT-PCR analysis for HIF-1α. RNAi, Con and Mock cells were treated with or without DFO for 24 h. Total RNA was extracted by using TRIzol reagent (Invitrogen, USA). Additionally, cDNA was reverse transcribed by Transcriptor First Strand cDNA Synthesis kit (Roche, USA). HIF-1α mRNA expression was evaluated quantitatively by real-time RT-PCR using LightCycler® 96 Instrument system (Roche). The thermocycler conditions were preincubation at 95˚C for 60 sec, 45 cycles of 95˚C for 10 sec, 60˚C for 10 sec and 72˚C for 20 sec, melting of 95˚C for 10 sec, 65˚C for 60 sec and 97˚C for 1 sec. Reactions were run in triplicate and repeated three times. As an internal control of each sample, the β-actin gene was used for standardization. The relative mRNA expression level of the gene was calculated using the 2¹∆∆Ct method. The primers were synthesized (Invitrogen) and the sequences were as follows: HIF-1α: forward, 5'-GTCGCTTCGGCCAGTGTG-3; reverse, 5'-GGA AAGGCAAGTCCAGAGGTG-3. β-actin: forward, 5'-TGG CACCCAGCACAATGAA-3; reverse, 5'-CTAAGTCATAAGTC CGCTTAGAAGCA-3.

Western blot analysis. After the three groups of cells were treated with or without DFO for 24 h. Cells were washed with ice-cold PBS twice, treated with buffer [50 mmol/l Tris-HCl (pH 7.5), 5 mmol/l EDTA, 150 mmol/l NaCl,0.5% Triton X-100, 10 mmol/l sodium fluoride, 20 mmol/l 1 h-mercaptoethanol, 250 mmol/l sodium orthovanadate, 1 mmol/l phenylmethylsulfonyl fluoride], and incubated at 4˚C for 30 min. The lysates were centrifuged at 10,000 g for 10 min, followed by collecting the supernatants and stored at -80˚C. Protein concentrations were examined by bicinchoninic acid assay methods (BCA protein assay kit, Thermo, USA). Equivalent amount of protein were loaded into 8% SDS-PAGE gels and electroblotted onto PVDF membrane (Sigma, USA). The PVDF membrane was washed three times with TBST solution (0.1% Tween-20 in TBS, pH 7.5) and blocked it for 1 h with
the addition of 10 µl of CCk-8 to the culture medium in each well or without DFO and then cultured for 24, 48 and 72 h before 96-multiwell plates (Corning, USA). Cells were treated with or without DFO for 24 h. After treatment the cells were trypsinized and washed twice with PBS.

**Expression of HIF-1α in SCC-15 cells and regulation by DFO.**

The level of HIF-1α mRNA in SCC-15 cells under normoxic or hypoxic condition was analyzed by real-time RT-PCR. After treated with DFO, no significant change in HIF-1α mRNA transcript was observed (Fig. 1A). However, the level of HIF-1α protein tested by western blotting demonstrated that DFO induced a significant increase of HIF-1α protein in SCC-15 cells and regulation by DFO.

**Statistical analysis.** Statistical analysis was calculated using SPSS 20.0 software. Data are presented as the means ± SD. Student's t-test was used for statistical analysis, with significant differences determined as p<0.05.

| Name          | Sequence                                                                                   |
|---------------|-------------------------------------------------------------------------------------------|
| LV-shHIF-1α-a | CCGGTTGATGAAAGAATTACCAGAATCTCGAGATTTCGTAATTTCGTTACATCACATTTCGTTACATCACATTTCGTTACATCA |
| LV-shHIF-1α-b | AATTCAAAAAGTGAAGAATTACCAGAATCTCGAGATTTCGTAATTTCGTTACATCACATTTCGTTACATCACATTTCGTTACATCA |
inhibited compared to the Con and Mock cells under normoxic (Fig. 4B) and hypoxic conditions (Fig. 4C).

**Cell cycle.** Cell cycle for cells in G1 phase or S/G2 phase was tested by FACS with DNA staining by propidium iodide (Fig. 5A). DFO can induce a significant increase of SCC-15 cells in G1 phase and a decrease of cells in the S/G2 phase compared to normoxic cells (Fig. 5B). SCC-15 cells treated with lentiviral vector targeting HIF-1α showed a significant decrease of G1 phase cells and an increase of S/G2 phase cells compared to Mock and Con cells after treated with DFO for 24 h (Fig. 5C).

**Cell apoptosis.** Cell apoptosis was evaluated by FACS with Annexin V-keyFlour647 Apoptosis Detection kit (Fig. 6A). There was no significant change in the percentage of apoptotic cells after SCC-15 cells were cultured with DFO for 24 h when compared with non-treated incubated cells (Fig. 6B). It was revealed that the apoptotic cells for RNAi cells were
Figure 4. The effect of HIF-1α on cell viability of SCC-15 cells. (A) The viability for Mock cells treated with DFO compared to normoxic cells [Cell viability = (ODHOX-ODblank)/(ODNOX-ODblank)]. (B) The difference of viability between Mock, Con and RNAi cells in normoxia [Cell viability = (ODexperiment-ODblank)/(ODMock-NOX-ODblank)]. (C) The difference of viability between Mock, Con and RNAi cells under hypoxic condition [Cell viability = (ODexperiment-ODblank)/(ODMock-HOX-ODblank)] (bars, ± SD, *p<0.01, compared to normoxic Mock group, **p<0.01, compared to Mock and Con cells). NOX, normoxia; HOX, hypoxia.

Figure 5. The effect of HIF-1α on cell cycle of SCC-15 cells. (A) Cell cycle of the Mock, Con and RNAi cells were tested by FACS. (B) DFO can significantly increase the number of G1 phase cells and decreased the number of S/G2 phase cells. (C) SCC-15 cells treated with lentiviral vector targeting HIF-1α showed a significant increase on G1 phase cells, and a significant decrease on S/G2 phase cells (bars, ± SD, *p<0.05, compared to normoxic Mock cells, **p<0.05, compared to Mock and Con cells). NOX, normoxia; HOX, hypoxia.
significantly increase compared to Mock and Con cells when incubated with or without DFO for 24 h (Fig. 6B).

**Cell invasion.** Studies have shown that HIF-1α can regulate the invasion of tumor cells (22). To investigate the effect of HIF-1α on cancer cell invasion of SCC-15 cells in vitro, we used the transwell assay (Fig. 7A). After the three groups of cells were treated with or without DFO and incubated in the transwell chamber for 24 h, 0.1% crystal violet staining was used to detect the number of cells to pass through the Matrigel membrane which represent the invasive abilities. As shown, after treated with DFO, the number of SCC-15 cells passing through the Matrigel membrane significantly increased (Fig. 7B). When HIF-1α was suppressed, the number of cells was significantly decreased compared to the Mock and Con cells under normoxic or hypoxic conditions (Fig. 7C).

**Discussion**

Oxygen is one of essential factors for cells and tissues to maintain their function. Hypoxia-inducible factor-1 (HIF-1), a transcription factor, is a key regulator for cells and tissues to adapt to and survive from hypoxia (11,23). HIF-1 contains HIF-1α and HIF-1β, and HIF-1α is highly regulated by oxygen (13). DFO has been proved to induce expression of HIF-1α (24). In the present study, we used DFO to simulate the hypoxic condition, and analyzed the effect of HIF-1α on proliferation, cell cycle, apoptosis and invasion of SCC-15 cells which were correlated to the growth, survival and metastasis of tumors. When the SCC-15 cells were cultured with DFO (100 µmol/l), the expression of HIF-1α was significantly increased, which was similar to our previous study in SCC-6 cells (25). After treated with DFO, the proliferation of SCC-15 cells was...
inhibited, which was like the other cell types (26). We observed
a significant increase of cells in G1 phase and a decrease of
cells in the S/G2 phase in DFO-treated SCC-15 cells, which
indicated the G1 arrest. However, there was no significant
change in the percent of apoptotic cells. These findings were
similar to the other cell types in hypoxia (27), which meant a
hypoxia-induced growth arrest in SCC-15 cells. The invasion
ability of SCC-15 cells was significantly increased after treated
with DFO. Therefore, HIF-1α may be an important regulator
for the different biological characteristics of SCC-15 cells.

It has been reported that HIF-1α can be regulated by DFO
(24). In the present study, we found the level of HIF-1α protein
increased after treated with DFO. Then HIF-1α associates
with HIF-1β to form HIF-1, and HIF-1 activates hundreds
of target genes to regulate angiogenesis, blood vessel tone,
vascular remodeling; cell proliferation and viability; erythro-
poiesis and iron metabolism; glucose transport and glycolysis
(11). Therefore, HIF-1α is very important for cells to adapt to
hypoxia. Studies found that HIF-1α is overexpressed in most of
human tumor cells (18,28). In head and neck carcinomas, study
showed that the expression of HIF-1α protein is associated
with the microvessel vascular density and vascular endothelial
growth factor (VEGF) expression (28). In our previous study,
we also found HIF-1α was overexpressed in oral squamous
cell carcinoma (21). In the present study, we found that HIF-1α
enhanced malignant phenotype of SCC-15 cells.

The hypoxia-inducible transcription factor α subunit was
found to be upregulated in DFO-treated SCC-15 cells. We
demonstrated that the level of HIF-1α mRNA was not signifi-
cantly changed between DFO treated and normal SCC-15
cells. However the level of HIF-1α protein was significantly
increased after treated with DFO up to 24 h. Study showed
that the level of HIF-1α protein is essential for tumor cells to
adapt to and survive from hypoxic microenvironment (29).
HIF-1α is a key regulator of hypoxic regulation, and it was
upregulated mainly on protein level in SCC-15 cells after
treated with DFO, indicating that overexpression of HIF-1α
was an essential factor for SCC-15 cells to show aggressive
phenotype under hypoxic condition. We used RNAi technique
to suppress the expression of HIF-1α, and found that silencing
HIF-1α can significantly decrease the aggressive potential for
SCC-15 cells in hypoxia.

In the present study, we constructed a lentiviral vector to
suppress the expression of HIF-1α, which helps to evaluate the
function of HIF-1α in progression of SCC-15 cells in hypoxia. SCC-15 cells were transduced with lentiviral vector targeting HIF-1α mRNA. The efficacy of interference was assessed by real-time RT-PCR and western blotting. Results showed that the level of HIF-1α mRNA and protein was significantly suppressed compared to Con and Mock cells in both normoxia and hypoxia.

Although our study showed the association between HIF-1α and proliferation, the mechanism is still not fully understood (30). Hypoxia can arrest tumor cells proliferation (31), however, another study showed that the increased hypoxic stress can accelerate the growth of HIF-1α tumors (27). In the present study, we found a significant increase of the G1 phase cells after treated with DFO. It is suggested that DFO can arrest SCC-15 cells in G1 phase. We used RNAi to inhibit the expression of HIF-1α protein, and test the effect of HIF-1α on the cell cycle of SCC-15 cells. We found a significant decrease of G1 phase cells and increase G2/S phase cells compared to the Mock and Con cells under hypoxia. Hence, HIF-1α was the master regulator for SCC-15 cells to adapt to the hypoxic condition.

Furthermore, hypoxia can not only be associated with cell proliferation but also with cell apoptosis in some circumstances. Hypoxia can induce apoptosis, where HIF-1 plays a complex role (27). It was found that HIF-1α was related to apoptosis (32). It has been reported that severe or prolonged hypoxic condition can maintain the stabilization of p53 protein by HIF-1α stimulation, and leads to induction of apoptosis (33). However, another study showed that HIF-1α might inhibit the hypoxia-induced apoptosis (29). These studies suggest that HIF-1α protein plays a key role in mediating apoptosis of tumor cells. We found that there is no significant change between normal SCC-15 cells and hypoxic cells. RNAi technique was used to evaluate the effect of HIF-1α on apoptosis of cultured human SCC-15 cells under normoxia and hypoxia, and we found the number of apoptotic cells was significantly increased in RNAi cells compared to that of Mock or Con cells under normoxic and hypoxic condition. These results suggested that HIF-1α has an important function in apoptosis of SCC-15 cells.

Hypoxia is a key factor for cancer metastasis (34). Cell invasion is involved in tumor metastasis and progression. In a glioma cell line, gene silencing of HIF-1α can downregulate MMP-2/MMP-9 to suppress cell migration and invasion into adjacent normal tissue (35). In the present study, we analyzed the effect of HIF-1α on cell invasion of SCC-15 cells by a transwell assay in vitro. In hypoxia, the number of SCC-15 cells passing through the extracellular matrices (ECM) significantly increases compared with the normoxic cells. Treatment with RNAi lentiviral vector, decreased significantly the amount of cells passing through the Matrigel gel. Thus, HIF-1α may play a major role in regulating the hypoxia-induced invasion of SCC-15 cells to Matrigel in vitro.

In conclusion, our results showed that hypoxic SCC-15 cells expressed high level of HIF-1α protein, and were invasive, whereas downregulation of HIF-1α by RNAi technique can decrease the malignancy of SCC-15 cells. Our study suggests that lentiviral vector target HIF-1α specifically suppressed HIF-1α gene expression, induced cell apoptosis, and inhibited the growth and invasion of SCC-15 cells. Hence gene silencing HIF-1α can consequently reduce the malignant potential of SCC-15 cells. Our present study showed the expression of HIF-1α can regulate proliferation, cell cycle, apoptosis and invasion. Thus, the expression of HIF-1α is an essential factor for human TSCC to regulate its malignancy. The RNAi target HIF-1α can be a potential therapeutic measure and effective tool to improve the survival of TSCC patients.

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