

HIF-1 α restricts proliferation and apoptosis of Tca8113 cells through up regulation of Hippo signaling pathway under hypoxic conditions

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Abstract. – **OBJECTIVE:** The hypoxia-inducible factor-1 α (HIF-1 α) is a key factor for tumor cells adaptation to hypoxia. Studies have shown that under hypoxic conditions, HIF-1 α expression was significantly increased in human tongue squamous cell carcinoma cells (Tca8113). This research aims to determine the exact mechanism of HIF-1 α on the proliferation and apoptosis of Tca8113 cells.

MATERIALS AND METHODS: Tca8113 cells were cultured under normoxia and hypoxia. Real Time-PCR and Western blot were used to measure the expression levels of HIF-1 α and TAZ. Under hypoxic condition, HIF-1 α siRNA was transfected into Tca8113 cells. CCK8 was used to measure the proliferation of Tca8113 cells. Flow cytometry was used to detect apoptosis of Tca8113 cells.

RESULTS: Under hypoxic condition, the expression levels of HIF-1 α and TAZ at both mRNA and protein levels were significantly increased ($p < 0.05$). The downregulation of HIF-1 α by siRNA significantly inhibited Tca8113 cells proliferation, increased their apoptosis, and reduced the expression level of TAZ.

CONCLUSIONS: Under hypoxic conditions, HIF-1 α inhibits the proliferation and apoptosis of Tca8113 cells via the elevation of the Hippo signaling pathway.

Key Words:

Human tongue squamous cell carcinoma, Hypoxia, HIF-1 α , Hippo signaling pathway.

Introduction

Squamous cell carcinoma is a type of common oral cancer, the incidence rate of which is gradually increased. Of note, the amount of younger

patients with squamous cell carcinoma gradually increases and poses a serious threat to human health. Important factors involved in the failure of treatment of tongue tumor include invasion, metastasis, and resistance to chemotherapy, among which, hypoxia plays an essential role. Therefore, hypoxic condition ought to be a key point for the treatment of tongue cancer. Most solid tumors exist in the anoxic environment because of insufficient blood supply¹. To survive in hypoxic conditions, the tumor cells gradually adapt to hypoxic stress through regulation of the expression of a large number of genes, associated with tumor cell proliferation, differentiation, apoptosis, invasion, metastasis, radiotherapy resistance, and chemotherapy resistance². Thus, hypoxic environment contributes to the change of the biological characteristics of tumor cells, enhances tumor angiogenesis and chemotherapy resistance. Effect and mechanism of hypoxia on the biological characteristics of tumor cells have become the focus of the research. HIF-1 α is a nuclear transcription factor used by solid tumor cells to adapt hypoxia. It exists in mammals and humans under anoxic conditions. As a key regulator of hypoxia adaptive response³, HIF-1 α can regulate the transcription of many genes when it is activated. It is a gene regulatory protein, which involved in tumor angiogenesis, cell proliferation, invasion, and metastasis⁴. Zhang et al⁵ found that HIF-1 α expression was increased under the anoxic environment in tongue squamous cell carcinoma cells Tca8113. Song et al⁶ also confirmed this phenomenon. But HIF-1 α expression levels for the biological behavior Tca8113 cells were rarely reported. Hippo signaling pathway partici-

pates in various bioprocesses. The central part of the Hippo pathway is cascade from Hippo to oncoprotein YAP/TAZ kinase⁷. It has been found^{8,9} that TAZ levels were closely related to tumor occurrence, development, and prognosis. TAZ has been shown highly expressed in squamous cell cancer, and closely related to the prognosis of tongue cancer patients¹⁰. Accumulative evidence^{11,12} also revealed that HIF-1 α regulated TAZ expression. Yan et al¹³ showed that TAZ expression levels were affected by hypoxia. Thus, the present work aims to investigate the mechanism of TAZ on proliferation and apoptosis of Tca8113 cells under hypoxia.

Materials and Methods

Instruments and Reagents

Human tongue squamous cell carcinoma cells (Tca8113) were obtained from ATCC (Manassas, VA, USA). Roswell Park Memorial Institute-1640 (RPMI-1640) medium and fetal calf serum (FCS) were purchased from Gibco (Waltham, MA, USA). CCK8 kit was collected from Solarbio (Beijing, China). Gallios flow cytometer was acquired from Beckman (Brea, CA, USA). Apoptosis detection kit was from Biyuntian Biotechnology (Beijing, China). RNA extraction reagent TRIzol and LipofectamineTM 2000 were provided from Invitrogen (Carlsbad, CA, USA). Real-Time quantitative reverse transcription polymerase chain reaction (RT-qPCR) kit was purchased from TaKaRa (Otsu, Shiga, Japan). Gel imaging system (ViiA7) was from ABI (Waltham, MA, USA). Total protein extraction kit was offered from Shanghai Best-Bio (Shanghai, China). Coomassie brilliant blue protein assay kit was purchased from Shanghai Jimei Biotechnology (Shanghai, China). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), phosphate-buffered solution tween-20 (PBST) solution, vertical electrophoresis set, and GIS-2020D gel image system were purchased from Sigma-Aldrich (St. Louis, Mo, USA). HIF-1 α , TAZ, and β -actin antibodies were received from Abcam (Cambridge, MA, USA). HIF-1 α small interfering Ribonucleic acid (siRNA) and negative control siRNA were purchased from Ambion (Manassas, VA, USA).

Tca8113 Cell Culture and Transfection

Tca8113 cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum. The

culture conditions of control group were 37°C, 20% O₂, and 5% CO₂. The culture conditions of hypoxia group were 37°C, 5% O₂, and 5% CO₂. Cells were passaged once every 2 to 3 days, and cells at logarithmic growth were used in the experiment. HIF-1 α siRNA and negative control siRNA were transfected using LipofectamineTM 2000 according to the kit instructions. Subsequent experiments were performed 24-72 h after transfection.

Cell Proliferation Assay Using CCK8

si-HIF-1 α , si-Control, and control cells were seeded in 96-well plates and cultured under 37°C, 5% O₂, and 5% CO₂. Cell proliferation assay was performed at 24 h, 48 h, 72 h after transfection. Cells were washed with PBS for 3 times. 100 μ l CCK8 mixture (CCK8 reagents: medium = 1:10) was added to each well and incubated at 37°C for 2 h in the dark. Absorbance at 450 nm wavelength was measured by a microplate reader. Each group has 5 wells.

Flow Cytometry Assay of Apoptosis

Cells were trypsinized, counted, and cultured in 6-well plates at 37°C, 5% O₂, and 5% CO₂. 4 h after transfection, cells were trypsinized, washed with PBS for 2 times, span at 1000 g for 5 min, and counted. 100,000 cells were re-suspended in 195 μ l Annexin V-fluorescein isothiocyanate (FITC). 5 μ l Annexin V-FITC were added and mixed gently. Then, 10 μ l propidium iodide (PI) staining solution was added and mixed gently. Cells mixture was incubated in the dark at room temperature for 20 min and then placed on ice for detection of cell apoptosis.

Measurement of mRNA Levels of HIF-1 α and TAZ

RNA was extracted using TRIzol and quantified using Nanodrop. The integrity of RNA was measured using 1% agarose gel electrophoresis. 1 μ g RNA was used to do the reverse transcription to obtain cDNA according to manufacturer's instruction (TaKaRa, Otsu, Shiga, Japan). The Real-Time PCR reaction was performed using ABI7500 quantitative PCR instrument (Manassas, VA, US) with the following profile: an initial 10 min incubation at 95°C, 40 cycles of (15 s at 95°C, 45 s at 50°C, and 40 s at 72°C), and a final extension of 3 min at 72°C. Reaction mixtures contained the following ingredients: 2 $\times$ SYBR Green Mixture 5 μ l, cDNA 0.5 μ l, primer (10 μ M) 0.5

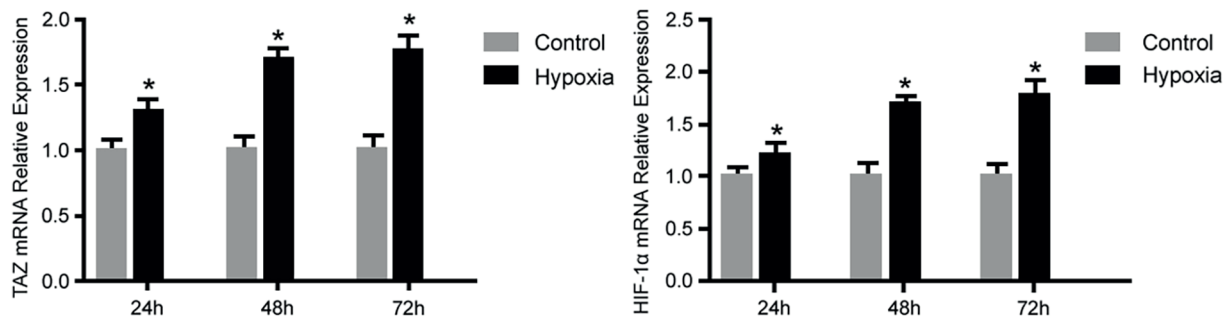


Figure 1. Effect of hypoxia on the expression of HIF-1α and TAZ mRNA (* $p < 0.05$, compared to control group).

μl, ddH₂O 4 μl. β-actin was used as a reference gene.

Western Blot Assay of HIF-1α and TAZ Protein

Cells were homogenized to extract total protein. Coomassie brilliant blue protein assay kit was used for protein quantification. Proteins were resolved on SDS-PAGE gel, transferred into polyvinylidene difluoride (PVDF) membranes, blocked with 5% nonfat milk for 1 h, washed with PBST for 3 times, and incubated with primary antibody overnight at 4°C. The membrane was then washed with PBST for 30 min, followed with incubation with secondary antibody for 60 min. After the membrane was washed three times with PBST, chemiluminescence detection reagent was used to develop and fix. GIS-2020D gel image system was used to analyze the band density of HIF-1α, TAZ, and β-actin.

Statistical Analysis

SPSS 20.0 software (IBM, Armonk, NY, USA) was used for statistical analysis. Data were expressed as mean ± standard deviation. Differences between multiple groups were compared using analysis of variance with the Tukey's post-hoc test. Differences between the two groups were compared using the *t*-test. $p < 0.05$ was considered statistically significant.

Results

Effect of Hypoxia on HIF-1α, TAZ mRNA Expression in Tca8113 Cells

Real-Time quantitative PCR results (Figure 1) showed that hypoxia significantly increased the mRNA levels of HIF-1α and TAZ ($p < 0.05$). Moreover, the levels of HIF-1α and TAZ mRNA at 48h and 72h of hypoxia were significantly higher than that of 24h hypoxia ($p < 0.05$), but no significant difference was found between the levels of HIF-1α and TAZ mRNA at 48h and 72h ($p > 0.05$).

Effect of Hypoxia on HIF-1α, TAZ Protein Expression in Tca8113 Cells

Western blot results (Figure 2 and Table I) showed that hypoxia significantly up-regulated the protein levels of HIF-1α and TAZ ($p < 0.05$).

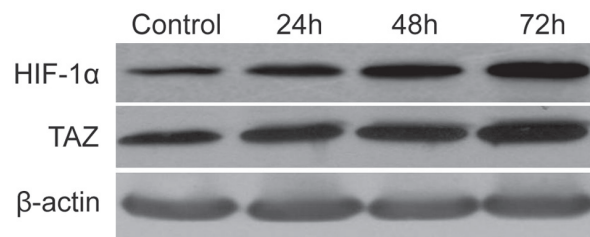


Figure 2. Effect of hypoxia on the expression of HIF-1α and TAZ protein.

Table I. Effect of hypoxia on the expression of HIF-1α and TAZ protein in Tca8113 cells.

Protein	Control	24h	48h	72h	<i>p</i>
HIF-1α	0.48±0.06	0.61±0.05*	0.84±0.04*	0.99±0.08*	<0.001
TAZ	0.59±0.07	0.68±0.08	0.88±0.07*	1.01±0.05*	<0.001

* $p < 0.05$, compared to control group.

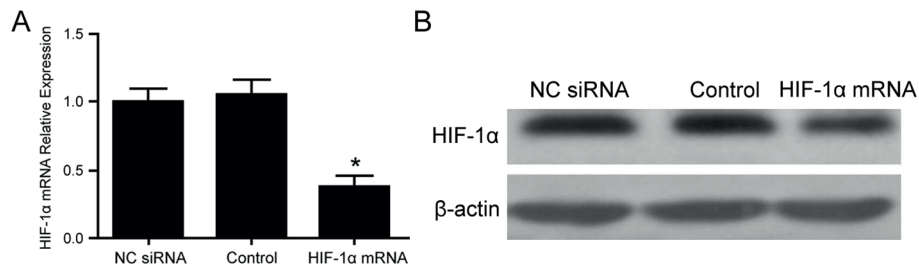


Figure 3. Expression changes of HIF-1 α mRNA and protein after transfection.

The levels of HIF-1 α and TAZ protein were also gradually increased along with the extension of hypoxia, suggesting that the effect of hypoxia on HIF-1 α , TAZ protein expression was similar to that at mRNA levels.

Manipulation of HIF-1 α Expression in Tca8113 Cells

To further verify the effect of HIF-1 α , its expression was downregulated by specific siRNA. The data on Real-Time PCR and Western blotting indicated that under hypoxic condition, both mRNA and protein levels of HIF-1 α were significantly lower than that of control group and si-control group ($p < 0.05$) (Figure 3).

Effect of Silence of HIF-1 α on the Proliferation and Apoptosis of Tca8113 Cells Under Hypoxic Conditions

CCK8 kit was then used to detect the effect of a decrease of HIF-1 α on the proliferation of Tca8113 cells under hypoxic conditions. Results presented that the proliferation of cells was significantly inhibited by the transfection of HIF-1 α siRNA, compared to that of si-control group and control

Table II. Effect of silence of HIF-1 α on the proliferation of Tca8113 cells.

Group	24h	48h	72h
Control	0.68 $\pm$ 0.03	0.79 $\pm$ 0.05	0.91 $\pm$ 0.04
si-control	0.67 $\pm$ 0.04	0.80 $\pm$ 0.03	0.90 $\pm$ 0.05
si-HIF-1 α	0.51 $\pm$ 0.03 ^{ab}	0.58 $\pm$ 0.04 ^{ab}	0.69 $\pm$ 0.04 ^{ab}

^a, $p < 0.05$, compared to control ^b, $p < 0.05$, compared to si-control

group ($p < 0.05$) (Table II). There was no significant difference in the number of cells between si-control group and control group ($p > 0.05$). Flow cytometry was also performed to determine the effect of HIF-1 α on the apoptosis of Tca8113 cells under hypoxic conditions. Our data revealed that at 48 h after the transfection, the apoptosis rate of si-HIF-1 α group was $25.78 \pm 2.01\%$, which was significantly higher than that of si-control group ($9.27 \pm 1.11\%$) and control group ($9.86 \pm 1.32\%$) ($p < 0.05$). No significant difference of apoptosis rate was found in si-control group and control group ($p > 0.05$) (Figure 4).

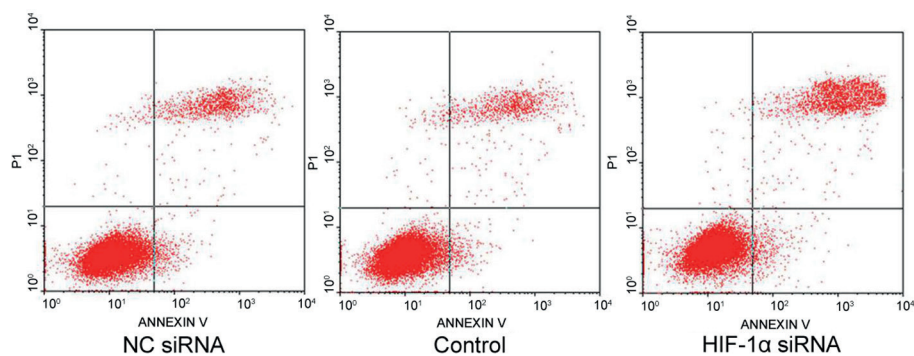


Figure 4. Effect of silence of HIF-1 α on the apoptosis of Tca8113 cells.

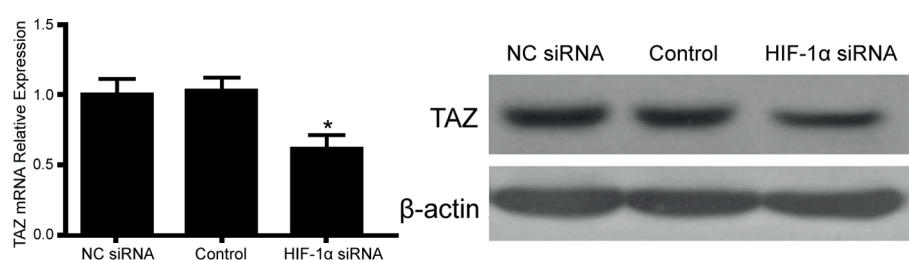


Figure 5. Effect of silence of HIF-1 α on the expression level of TAZ under hypoxic conditions.

Effect of Silence of HIF-1 α on the Expression Level of TAZ Under Hypoxic Conditions

The expression levels of TAZ in Tca8113 cells were measured at both mRNA and protein levels at 48 h after transfection of HIF-1 α siRNA. We found that the reduction of HIF-1 α resulted in the down-regulation of TAZ at both mRNA and protein levels compared with that of si-control group and control group ($p < 0.05$) (Figure 5).

Discussion

Hypoxia can lead to tumor progression and resistance to chemotherapy^{14,15}. A multitude of findings^{16,17} showed that as a key regulator of hypoxia adaptive response, the expression level of HIF-1 α was closely related to tumor occurrence, development, and treatment. Under hypoxic conditions, suppression of HIF-1 α expression can lead to changes of tumor cell proliferation, invasion, metastasis, and apoptosis^{18,19}, and thus, HIF-1 α became a potential target in the treatment of cancer gradually becoming a hot topic²⁰. Our results showed that, under hypoxic conditions, the expression levels of mRNA, protein of HIF-1 α and TAZ were significantly increased in Tca8113 cells, which were consistent with the previous study⁵. Additionally, under hypoxic conditions, the block of HIF-1 α increased the apoptosis of Tca8113 cells, suggesting that under hypoxic conditions, HIF-1 α can regulate the proliferation of Tca8113 cells. Zhou et al²¹ found that reducing the expression of HIF-1 α inhibited the proliferation of tongue squamous cell carcinoma SCC-15 cells. Liang et al²² also found that HIF-1 α expression is closely related with the proliferation of tongue squamous cell carcinoma CAL-27 cells. Combined with our findings, these studies provide a theoretical basis for targeting HIF-1 α in the treatment of carcinoma; they

were confirmed in animal models²³. This study also found that, under hypoxic conditions, the suppression of HIF-1 α resulted in the downregulation of TAZ at both mRNA and protein levels, suggesting that the expression of TAZ was regulated by HIF-1 α . Since TAZ is the main effector of Hippo signaling pathway, the results of this study indicated that HIF-1 α regulated proliferation and apoptosis under hypoxic conditions via regulation of Hippo signaling pathway in Tca8113 cells, which has been hitherto confirmed in breast cancer cells¹². The effect of HIF-1 α on the proliferation of Tca8113 cells also lays insights for the potential targets of the treatment of oral tongue squamous cell carcinoma besides recent finding of Semaphorin-7A²⁴.

Conclusions

We demonstrate that, under hypoxic conditions, HIF-1 α modulates the proliferation and apoptosis of Tca8113 cells via upregulating Hippo signaling pathway, suggesting that HIF-1 α can be used as a potential target in the treatment of tongue cancer.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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