HIF-1α regulated tongue squamous cell carcinoma cell growth via regulating VEGF expression in a xenograft model

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Objective: We aimed to study the mechanism of hypoxia-inducible factor 1 alpha (HIF-1α) regulating the cell proliferation of tongue squamous cell carcinoma (TSCC) via vascular endothelial growth factor (VEGF).

Methods: We used RNA interference (RNAi) technique, transfected chemically synthesized siRNA against HIF-1α into CAL-27 cells, and detected the expression of HIF-1α and VEGF by real time-PCR and Western blotting in order to find out if HIF-1α regulated the expression of VEGF. A xenograft experiment was carried out to observe the role of HIF-1α on the tumor growth of tongue squamous cell carcinoma.

Results: HIF-1α and VEGF mRNA expression was significantly downregulated 36 and 48 h after transfection ($P<0.05$); the protein expression of HIF-1α and VEGF was also significantly suppressed by siRNA against HIF-1α. Furthermore, intratumoraly injection of HIF-1α targeting siRNA suppressed tumor growth in nude mice.

Conclusions: HIF-1α regulated VEGF expression, and they may contribute to TSCC cell tumor growth.

Keywords: Tongue squamous cell carcinoma (TSCC); hypoxia-inducible factor-1 alpha (HIF-1α); vascular endothelial growth factor (VEGF); RNA interference (RNAi)

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Introduction

Tongue squamous cell carcinoma (TSCC) is the most common type of the oral cancers (1). And its incidence is prone to increase recently. In the past several decades, many kinds of therapies such as operation, chemo- and radio-therapy were used for treating TSCC patients, but the prognosis was not satisfactory, the five-year survival rate is no more than 50% (2). Therefore, it is important to find out new special bio-markers, which are closely related to bionomic characteristics, prognosis, and its mechanisms that regulate tumorigenesis and tumor growth in tongue squamous cell carcinoma cells.

Hypoxia-inducible factor-1 (HIF-1) is a heterodimer consisting of hypoxia-inducible factor-1 alpha (HIF-1α) and HIF-1β (3). HIF-1α is an important factor contributed to the tumor aggressiveness, metastasis and mortality by controlling oncogenic and tumor suppressor gene pathways (4-6). And it regulates the expression of many genes involved in adaptive responses to oxygen deprivation and stimulates the development of new blood vessels by releasing various angiogenic-stimulating cytokines and growth factors (7-9). Vascular endothelial growth factor (VEGF) is a significant mediator in the vascularization, proliferation, invasion and metastasis of tumor, and its receptors play an important role in tumor angiogenesis (10,11). Sometimes, VEGF may be secreted from cells under hypoxia conditions and bind to its receptors on endothelial cells, then activate endothelial cell division and angiogenesis (12).

In recent years, the roles that HIF-1α and VEGF play in tumorigenesis have been given special attention. HIF-1α and VEGF expression was up-regulated in a variety of tumors.

In addition, HIF-1α can stimulate the expression of VEGF. Some questions about the roles of these proteins in TSCC have not yet been explored: how does their expression affect the growth of TSCC? What is their relationship in TSCC? And what is the specific mechanism responsible for those effects? In this study, we first transfected HIF-1α specific siRNA in TSCC cell lines *in vitro* and investigated the expression of HIF-1α and VEGF. And then, we evaluated the role of HIF-1α in tumor formation in nude mice inoculated subcutaneously with TSCC cells.

Materials and methods

Cell culture

The human tongue cancer cell lines (CAL-27), obtained from the Ninth People's Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, China were thawed and maintained in DMEM a high glucose culture media (Gibco USA) with 10% fetal bovine serum (FBS) (Gibco USA). Cells were cultured at 37 ℃ with 5% CO_2 in a constant temperature cell incubator, and moved into new media approximately every 3 days.

Cell transfection

Negative control siRNA (synthesised by the Guangzhou Ruibo Company) fluorescently labeled with cy3 was transfected into CAL-27 cells. The day before transfection, cells were seeded onto 24-well plates with a density of 1×10^5 per well in DMEM containing high glucose and no antibiotics to achieve normal growth. On the day of transfection, RNAiMAX liposome suspension (Invitrogen, USA) was used to dilute siRNA and mixed with the CAL-27 cells in OPTI-MEM I culture media without serum (Invitrogen, USA). Eight hours after transfection the medium was replaced. And 24 hours later fluorescence microscopy was used to monitor the transfection rate of tumor cells. Negative control siRNA and HIF-1α-specific-siRNA (Sense: 5'-CTGATGACCAGCAACTTGA-3') were transfected into CAL-27 cells as mentioned above.

Realtime-PCR

Negative control siRNA and HIF-1α-specific-siRNA, both synthesized by the Guangzhou Ruibo Company, were transfected into CAL-27 cells respectively. The cells were divided into three groups. The control group was transfected with negative control siRNA and its mRNA was extracted 48 h after transfection. The other two groups were transfected with HIF-1α-specific-siRNA, which have been verified against the HIF-1α gene (13). The total RNA was extracted 36 and 48 h after transfection. Reverse transcription reaction was carried out with M-MLV reverse transcriptase and dNTP which bought from PROMEGA. Real-time PCR was done using SYBR Master Mixture kit (TAKARA JPN) for the extracted cellular RNA (each group repeated three times and quantitative analysis for the mean).

The primer sequences were as follows:

hACTIN-F: CATGTACGTTGCTATCCAGGC;
hACTIN-R: CTCCTTAATGTCACGCACGAT;
HIF-1A-F: GCACAGGCCACATTCACGTATAT;
HIF-1A-R: GGTTCACAAATCAGCACCAAGC;
VEGF-F: GAGATGAGCTTCCTACAGCACAAC;
VEGF-R: GTACAAACAAATGCTTTCTCCGC.

Western blotting

Cells and tissue samples were harvested and lysed in RIPA buffer with protease inhibitors (Applygen Technologies, Inc., Beijing, China). Concentration was examined by the BCA Protein Assay. Proteins were separated on a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride membrane. The membranes were blocked in 5% non-fat dry milk for 1h and incubated in antibodies against HIF-1α, VEGF, and GAPDH (Dako, Carpinteria, California, USA) separately at 4 ℃ overnight. Following incubation with peroxidase-linked secondary antibodies, proteins were visualized by enhanced chemiluminescence reagent. Image J was used for quantification of Western blotting results.

Tumor xenografts

Ten female BALB/c-nu mice of 8 weeks old were prepared for tumor implantation. The BALB/c-nu mice were purchased from Shanghai Super-B&K laboratory animal Corp. Ltd. All animals were maintained in a sterile environment on a daily 12-h light/12-h dark cycle. CAL-27 cells (2×10^6/mouse) were injected subcutaneously into the flanks of the nude mice. When the tumor became palpable, the mice were divided into two groups randomly. The experimental group was intratumorally injected with 4 μg chemically synthesized siRNA of HIF-1α (synthesised by the Shanghai Jima Company) and RNAiMAX every day. The control group is treated with the same amount of

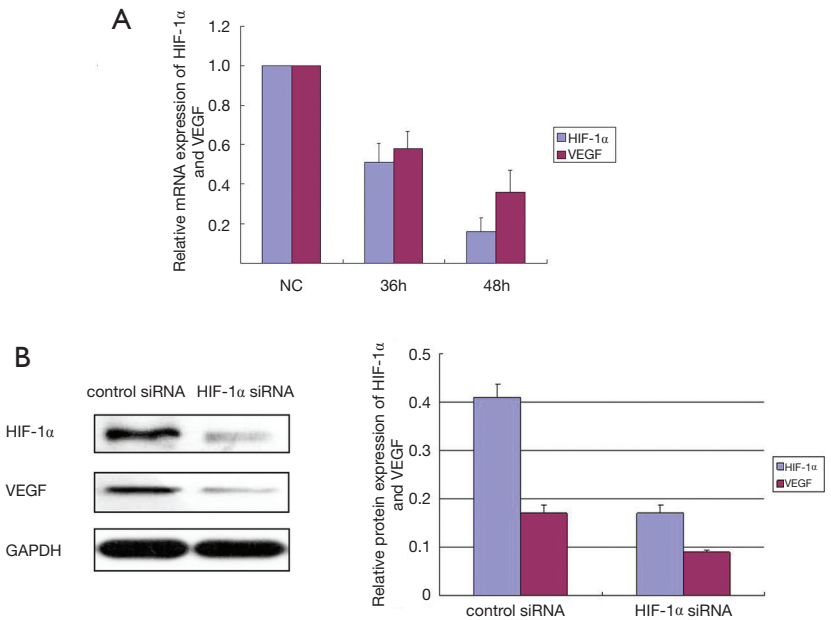

Figure 1 HIF-1α-siRNA suppressed HIF-1α and VEGF expression. (A) mRNA expression of HIF-1α and VEGF was suppressed by HIF-1α-siRNA in Cal-27 cells; (B) protein expression of HIF-1α and VEGF was suppressed by HIF-1α-siRNA in Cal-27 cells at 48 h. The right panel is quantification of Western blotting results. Data were expressed as mean ± SD (n=5). HIF-1α, hypoxia-inducible factor-1 alpha; VEGF, vascular endothelial growth factor.

negative control siRNA and RNAiMAX. Tumor volume was calculated every other day for 2 weeks according to this formula (14), TV (mm^3) = length × $width^2$ ×0.5. Tumor xenografts were harvested and weighted two weeks after the first siRNA injection. All the experiment protocol has been approved by the animal care and use committee in the Fifth Affiliated Hospital of Sun Yat-sen University.

Immunohistochemistry

Cryosections were cut at a thickness of 4 μm and stained with H&E. Immunohistochemical staining CD34 (Dako, Carpinteria, California, USA) was performed. Microvessel densities (MVD) determination was based on the criteria of the Weidner method: Under a low-power objective lens, the entire section was examined to search for regions of high capillary density, referred to as hot spots. A 200× objective lens was then used and the clusters of vascular endothelial cells with brown staining was counted. The final microvessel density was the average of three different microscopic fields (15).

Statistical analysis

All statistical analyses were performed by the SPSS WIN program package 13.0 (SPSS Inc., Chicago, IL, USA). We used the one-way anova test to analyze the microvessel densities, PCR and tumor xenografts results between experimental and control groups. Differences were considered statistically significant for a P value <0.05.

Results

High transfection efficiency of chemically synthesized siRNA by liposome

Chemically synthesized siRNA fluorescently labeled with cy3 was transmitted into the CAL-27 cells effectively by liposome. The cells with fluorescence were counted under a fluorescent inverted microscope. The transfection rate was analyzed by comparing the number of fluorescent cells with total cells. And we found that the transfection rate was over 90%.

HIF-1α siRNA significantly down-regulated the HIF-1α and VEGF expression in CAL-27 cells

PCR amplification curve was observed with fluorescence quantitative PCR. Our data showed that the HIF-1α and VEGF mRNA expression were significantly suppressed in the experimental groups 36 and 48 h after transfection (*Figure 1A*).

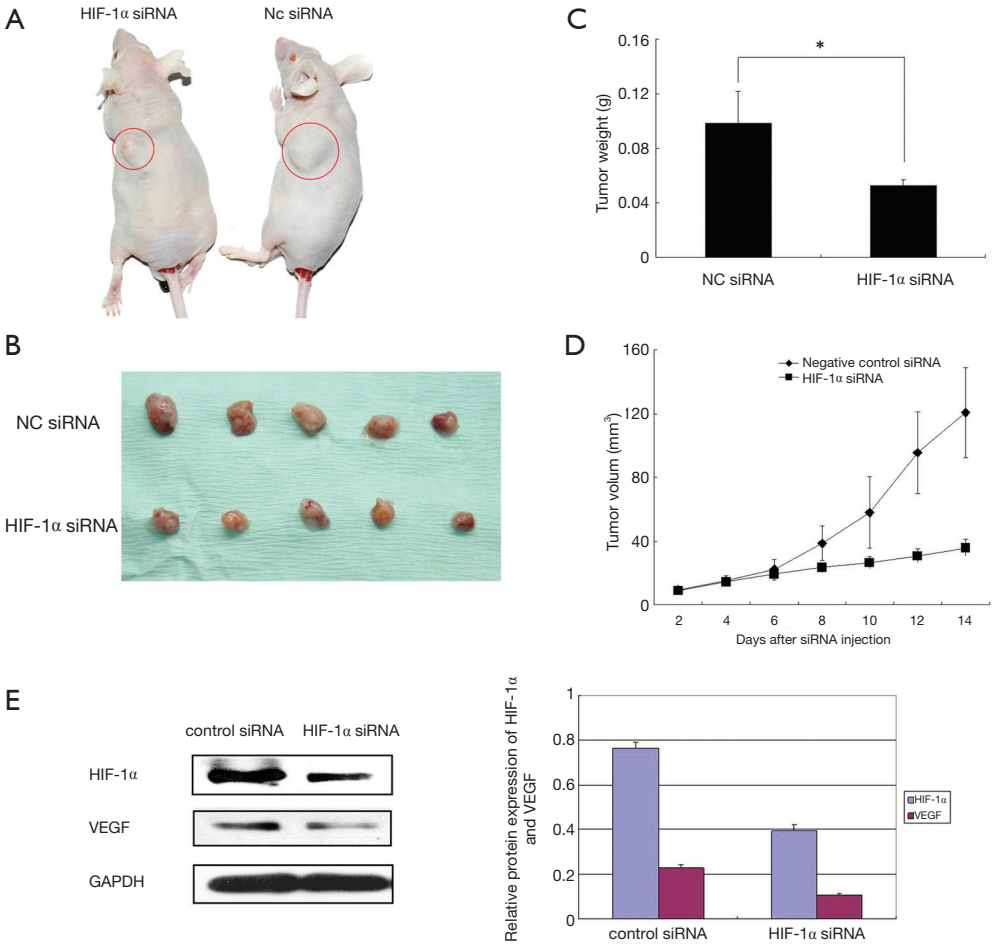

Figure 2 HIF-1α siRNA Suppressed growth of TSCC xenografts. (A,B) Xenografted tumor injected with HIF-1α siRNA grew slower than with Negative control siRNA in nude mice. The xenografted tumor weight (C) and volumn (D) were lower in HIF-1α siRNA group than Negative control siRNA group. (E) Protein expression of HIF-1α and VEGF was suppressed by HIF-1α-siRNA in xenografted tissue samples. HIF-1α and VEGF expression were significantly suppressed by HIF-1α siRNA. The right panel is quantification of Western blotting results. Data were expressed as mean ± SD (n=5). HIF-1α, hypoxia-inducible factor-1 alpha; TSCC, tongue squamous cell carcinoma; VEGF, vascular endothelial growth factor.

Previous studies have showed that the same siRNA sequence effectively suppressed protein expression of HIF-1α *in vivo* or *in vitro* (16,17). Moreover, knocking down HIF-1α expression by siRNA induced apoptosis in tongue squamous cell carcinoma cells (18). Our data (*Figure 1B*) showed that HIF-1α siRNA significantly suppressed the protein expression of HIF-1α and VEGF in CAL-27 cells at 48 h comparing with control siRNA (P<0.05).

HIF-1α siRNA suppressed growth of TSCC xenografts

All the xenografted tumors were conformed as squamous cell carcinoma. Tremendously reduced tumor size and tumor weight were observed in mice treated with HIF-1α siRNA comparing with control siRNA (P<0.05) (*Figure 2A-D*). The tumor and surrounding tissue boundaries is clearer, and no obvious infiltration was found in the experimental group. However, the tumor in control group grew more rapidly and obviously infiltrated muscle. HIF-1α siRNA significantly suppressed the protein expression of HIF-1α and VEGF in xenografted tissue samples comparing with control siRNA (P<0.05) (*Figure 2E*). Immunochemical staining CD34 showed that the microvessel densities in tumor could be suppressed by target siRNA of HIF-1α (*Table 1*) (*Figure 3*).

Discussion

Vascular networks are important for angiogenesis and it participates in the development, maintain of normal tissues and many pathological and physiological processes (19,20). Based upon the tumor angiogenesis theory suggested by Folkman (21), the development of solid tumors can be divided into two steps. The first is a pre-angiogenic phase in which the tumor grows slowly. But when blood vessels grow into tumors, it then enters the second step. At that time, the tumor grows rapidly and metastasis easily. Because the new blood vessels in the tumor provide adequate nutrients and remove much metabolic products, while also accelerating the formation of solid tumors (19,22). Weidner's (23) study show that tumor MVD could serve as a reliable factor in patients with breast carcinoma for clinical outcome. MVD detection is usually used to determine angiogenesis. And it reflects the correlation of the angiogenesis and the invading behavior of tumours. So the prognostic value of MVD was soon verified in many different types of tumors, including oral tumors (11,24).

VEGF is one of participants in the complex angiogenic response, but it is an important activator of vascular endothelial cells. Moreover, VEGF and its receptors was therapeutic target for treatment of oral squamous cell carcinoma (25). It is reported that in some tumors VEGF and MVD is regulated by HIF-1α (26). In our study we transfected HIF-1α-specific-siRNA in TSCC cell lines *in vitro* and investigated the expression of HIF-1α and VEGF. We can found that when HIF-1α-specific-siRNA is transfected into CAL-27 cells, the mRNA expression of HIF-1α and VEGF decreased at 36 and 48 h ($P<0.05$). VEGF can be regulated by many genes (11), we believed that HIF-1α may be the major one in TSCC cells. In previous study, HIF-1α expression upregulated in injured gastric mucosal and esophageal ulcers, moreover the upregulation of HIF-1α correlated with VEGF gene activation and initiation of angiogenesis (27). And the reason for this is that when the tissue is injured it is under the hypoxic environment, HIF-1α accumulates in endothelial cells inducing VEGF expression. Once the tumor grows, the marginal tissue will necrotized, which makes a hypoxic conditions similar to the injury. Then it will promoted VEGF expression and angiogenesis. Because HIF-1α tries to inhibit apoptosis and cell death by inducing the expression of genes that induce mutations such as VEGF, at the same time, it will result in invasion and metastasis. All this chain of actions easily linked to the malignant alteration through the generation and procession of cancers (28). In order to further study the correlation between HIF-1α and VEGF, we suppressed HIF-1α expression by siRNA in TSCC cells, and found it downregulated VEGF expression. These results implied that HIF-1α may regulate VEGF expression, but the specific mechanism needs further study.

Finally, our results in TSCC xenografts experiment suggested that silencing HIF-1α inhibited the growth

Table 1 The number of MVDs in xenografted tumor in nude mice

Group	The mean value (mean ± SD)
HIF-1α siRNA (n=5)	7.03±1.28
Negative control siRNA (n=5)	16.10±4.07

HIF-1α siRNA *vs*. negative control siRNA, $P<0.05$. MVD, microvessel densities.

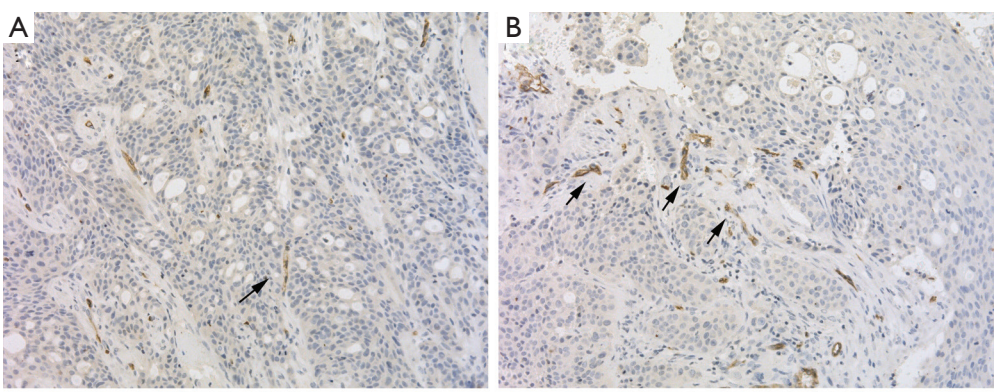

Figure 3 Suppressing HIF-1α significantly inhibited the growth of tumor microvessels (200×). (A) HIF-1α siRNA group; (B) negative control siRNA group; HIF-1α, hypoxia-inducible factor-1 alpha.

and angiogenesis of TSCC cells, which encouraged us to establish a siRNA-based therapy platform. Because the RNA interference (RNAi) technique is one of the most commonly used genetic treatment methods and it may be widely used in the future for targeting angiogenic factors. Therefore HIF-1α may serve as an effective indictor for patient's survival and act as an important target for the tumor therapy.

Conclusions

HIF-1α regulated VEGF expression, and they play important roles in TSCC cell tumor growth. HIF-1α and VEGF may become new therapeutic targets for tongue squamous cell carcinoma.

Acknowledgements

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