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EXPRESSION OF HYPOXIA-INDUCIBLE FACTOR-1 Α AND ENOLASE-1 IN LARYNGEAL CARCINOMA AND CORRELATION ANALYSIS

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ABSTRACT

Objective: *To investigate the expression of hypoxia-inducible factor-1 α and enolase-1 in laryngeal carcinoma and correlation analysis.*

Methods: *A total of 45 male patients with LSCC were selected from the Department of Otorhinolaryngology in our hospital from December 2015 to December 2016. The LSCC tissue and adjacent tissues (more than 0.5 cm from the cancerous tissue) were taken. The immunohistochemical method was used to detect the positive expression rate of HIF-1α and ENO1 in LSCC tissue and adjacent tissues, and further analyze the correlation between the two in LSCC tissue.*

Results: *The positive expression rate of HIF-1α in LSCC tissues was 75.55%, which was significantly higher than the positive expression rate of HIF-1α in adjacent tissues of 22.22% (P<0.05). The positive expression rate of ENO1 in LSCC tissues—80.00%—was significantly higher than that of ENO1 in adjacent tissues: 22.22% (P<0.05). The expression level of HIF-1α was correlated with lymph node metastasis and differentiation in LSCC patients (P<0.05). This was unrelated to the patient's age, daily smoking habits, and clinical stage (P>0.05). ENO1 was correlated with lymph node metastasis and differentiation in LSCC patients (P<0.05). This was also unrelated to the patient's age, smoking habits, and clinical stage (P>0.05). After correlation analysis, HIF-1α and ENO1 were found to be positively correlated in LSCC tissues (P<0.05, r=0.591).*

Conclusions: *The expressions of HIF-1α and ENO1 in LSCC tissues are both up-regulated, and their expression levels are correlated with the degree of lymph node metastasis and differentiation in LSCC patients. In addition, HIF-1α and ENO1 are positively correlated in LSCC tissues, and the two are expected to become potential biomarkers of LSCC, providing important references for clinical evaluation and disease prevention.*

Keywords: *LSCC, HIF-1α, ENO1, lymph node metastasis.*

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Introduction

Laryngeal squamous cell carcinoma (LSCC) originates from the epithelial tissue of the head and neck and is extremely malignant(1). Investigations have shown that in 2015, the number of new cases of LSCC in my country exceeded 26,000; the number of deaths exceeded 14,000; and the incidence rate is increasing(2). Surgery is the main treatment method for LSCC; however, this operation can seriously damage the patient's laryngeal function and has a poor prognosis, threatening the patient's health and quality of life(3). Therefore, identifying the biological characteristics of LSCC and improving the survival rate of patients has become a significant topic in clinical research. Hypoxia-inducible factor 1(HIF-1) was discovered in 1992 and is mainly composed of α and β subunits. It is recognized that α is an oxygen-regulating subunit that can determine the activity of HIF-1(4). Reports have confirmed that HIF-1α exists

in many tumor cells, and the expression continues to be at a high level. If hypoxia can induce the activation of HIF-1α, make it bind to HIF-1β, then activate the related structural gene regulatory sequences, it will play an important role in the biological functions related to energy metabolism(5).

One report found that there is an overexpression of HIF-1α in LSCC tissues, but the specific mechanism of action has not been elucidated(6). Endase1 (ENO1) can participate in cell metabolism and play a key role. Clinical reports have confirmed that ENO1 could participate in tumor onset and progression as a tumor-related protein(7). Another report shows that ENO1 may be present as an oncogene in the onset, progression, and metastasis of tumors. Therefore, this study aimed to explore the expression and correlation of HIF-1α and ENO1 in laryngeal squamous cell carcinoma.

Materials and methods

Materials

A total of 45 male patients with LSCC were selected from the Department of Otorhinolaryngology in our hospital from December 2015 to December 2016. Among them, 21 cases were ≤60 years old and 24 cases were >60 years old. There were 24 cases with daily smoking habits of up to 20 cigarettes and 21 cases of more than 20 cigarettes daily. Twenty-eight cases were well-differentiated, and 17 cases were poorly differentiated. There were 29 cases with lymph node metastasis and 16 cases without. Seventeen cases were in stage I+II, while 28 cases were in stage III+IV.

The entry criteria were as follows:

• Diagnosed as LSCC by pathological examination;

• Diagnosed as LSCC after double-blind reading by two pathologists;

• Have complete clinicopathological and follow-up data;

• Male;

• Before the study, the patients and their families knew the contents of the experiment and signed the informed consent form;

• Approved by the ethics committee of our hospital.

The exclusion criteria were as follows:

• Those who have previously undergone radiotherapy and chemotherapy;

• Those with autoimmune diseases, malignant tumors, or infections;

• Those with mental disorders or poor compliance;

• Lost visitors. The LSCC and adjacent (more than 0.5 cm from the cancerous tissues) tissues of 45 patients were taken in 4% paraformaldehyde; 24 hours later, they were embedded in paraffin and sliced, and the thickness was set to 5 μm.

Reagents and instruments

Reagents

HIF-1α and ENO1 polyclonal antibodies were purchased from Wuhan Elite Biotechnology Co., Ltd; HRP-labeled goat anti-rabbit mouse was provided by Jiangxi Jianglan Pure Biological Reagent Co., Ltd; xylene was purchased from Beijing Kairuiji Biotechnology Co., Ltd.; hematoxylin was obtained from Chengdu Refines Biotechnology Co., Ltd; DAB color reagent kit was purchased from Shanghai Bangyi Biotechnology Co., Ltd; and EDTA (PH9.0) antigen retrieval solution was provided by Nanjing Senbega Biotechnology Co., Ltd.

Instruments

The embedding machine was purchased from Dongguan Pubiao Experimental Equipment Technology Co., Ltd; the slicer was provided by Beijing Jiayuan Xingye Technology Co., Ltd; the 37°C constant temperature incubator was purchased from Shanghai Aladdin Biochemical Technology Co., Ltd; and the microscope was obtained from Prime Medical Technology (Beijing) Co., Ltd.

Methods

Immunohistochemistry was used to detect the expression levels of HIF-1α and ENO1 in LSCC tissues and adjacent tissues. The sections were deparaffinized in xylene I, II, and II solutions for 15 minutes each. After dewaxing, the sections were placed in 85% and 75% alcohol for hydration treatment for 5 minutes each and then rinse with distilled water for 5 minutes. The slices were then placed in EDTA antigen retrieval solution and heated for antigen retrieval. They were first treated with medium heat for 8 minutes until boiling, then medium-low heat for 7 minutes before being rinsed with PBS solution three times, for 5 minutes each. The slices were placed in 3% hydrogen peroxide water and incubated in the dark for 30 minutes, then rinsed with PBS solution three times, for 5 minutes each. Normal goat serum was placed on the section, which was then placed in a constant temperature incubator at 37°C

and incubated for 30 minutes. The remaining serum was then removed and the surrounding serum blotted with filter paper. The primary antibody was put into a dropper (1:100) and placed in a 4°C environment overnight. On the second day, after taking out the sections and returning them to room temperature for 30 minutes, the secondary antibody (HRP labeled) was added via a dropper and incubated in a 37°C constant temperature incubator for 30 minutes. Next, they were rinsed with PBS solution three times, for 5 minutes each. After rinsing, the slices were dried and DAB chromogenic solution added via a dropper, before being placed under a microscope for observation. The color development time was determined according to the staining of the slices.

After the color development was complete, hematoxylin counterstain was used in the slides for observation under a microscope, followed by 1% hydrochloric acid alcohol solution for differentiation, 1% ammonia water inverse blue, xylene transparent, and neutral gum.

Interpretation standard

HIF-1α and ENO1 were expressed in the cytoplasm and nucleus, respectively, and the appearance of yellow or brown particles indicated positive expression. According to the staining intensity score and the percentage score of positive cells in the microscope field, the staining intensity was as follows: colorless = 0 points; yellow = 1 point; brown-yellow = 2 points; and tan = 3 points. The percentage of positive cells was as follows: no positive cells = 0 points; <25% = 1 point; 26%-50% = 2 points; 51%-75% = 3 points; and >75% = 4 points. The product of the staining intensity score and the percentage score of positive cells was as follows: 0-1 point = negative (-); 2-3 points = weakly positive (+); 4-6 points = moderately positive (++); and >6 points = strong positive. The final score <4 is divided into negative expression (-); >4 points = positive expression (+).

Statistical method

The positive expression rates of HIF-1α and ENO1 in LSCC tissues and adjacent tissues, and their relationship with clinicopathological parameters of LSCC patients, are expressed in (n(%)). The χ^2 test was used for comparison between the two groups. The correlation between HIF-1α and ENO1 in LSCC was analyzed by Spearman. All data in this study were processed by SPSS23.0. P<0.05 was regarded as a significant difference.

Results

The expression level of HIF-1α in LSCC tissues and adjacent tissues

The positive expression rate of HIF-1α in LSCC tissues was 75.55%, which was significantly higher than the positive expression rate of HIF-1α in adjacent tissues of 22.22% (P<0.05). The results are shown in Table 1 and Figure 1.

Group	n	Negative expression rate (%)	Positive expression rate (%)
LSCC tissues	45	11 (24.44)	34 (75.55)
Adjacent tissues	45	35 (77.78)	10 (22.22)
χ^2			25.613
P			<0.001

Table 1: The expression level of HIF-1α in LSCC tissues and adjacent tissues (n(%)).

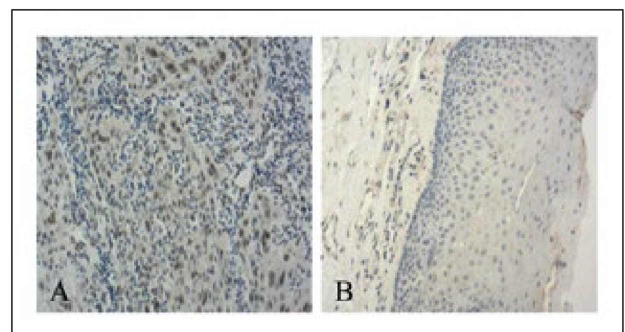

Figure 1: The expression level of HIF-1α in LSCC tissue and adjacent tissues.

Note: Figure A: Positive expression of HIF-1α in LSCC tissue. Figure B: Negative expression of HIF-1α in adjacent tissues.

The expression level of ENO1 in LSCC tissues and adjacent tissues

The positive expression rate of ENO1 in LSCC tissues was 80.00%, which was significantly higher than the positive expression rate of ENO1 in adjacent tissues of 22.22% (P<0.05). The results are shown in Table 2 and Figure 2.

Group	n	Negative expression rate (%)	Positive expression rate (%)
LSCC tissues	45	9 (20.00)	36 (80.00))
Adjacent tissues	45	35 (77.78)	10 (22.22)
χ^2			30.059
P			<0.001

Table 2: The expression level of ENO1 in LSCC tissues and adjacent tissues (n(%)).

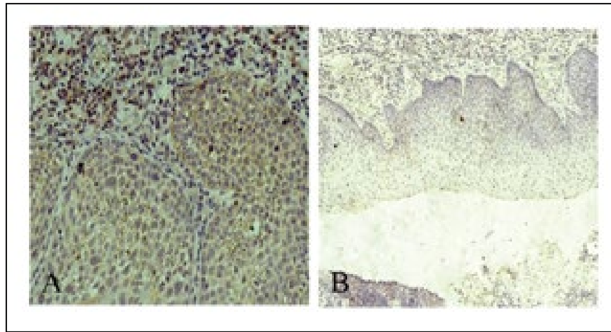

Figure 2: The expression level of ENO1 in LSCC tissues and adjacent tissues.
Note: Figure A: Positive expression of HIF-1α in LSCC tissue. Figure B: Negative expression of HIF-1α in adjacent tissues.

Correlation between HIF-1α expression level and clinicopathological characteristics of LSCC patients

The expression level of HIF-1α is correlated with lymph node metastasis and differentiation in LSCC patients (P<0.05); it is unrelated to patient age, daily smoking habits, and clinical stage (P>0.05). The results are shown in Table 3.

Clinicopathological parameters	n	HIF-1α		*P*
		Negative expression (n=11)	Positive expression (n=34)	
Age (years)				0.926
≤60	21	5 (45.45)	16 (40.06)	
>60	24	6 (54.54)	18 (52.94)	
Cigarettes daily				0.926
≤20	24	6 (54.54)	18 (52.94)	
>20	21	5 (45.45)	16 (40.06)	
Differentiation				0.001
Well-differentiated	28	2 (18.18)	26 (76.47)	
Poorly differentiated	17	9 (81.82)	8 (23.53)	
Lymph node metastasis				0.003
No	16	8 (72.73)	8 (23.53)	
Yes	29	3 (27.27)	26 (76.47)	
Clinical stage				0.911
I+II	17	4 (36.36)	13 (38.24)	
III+IV	28	7 (63.64)	21 (61.76)	

Table 3: Correlation between HIF-1α expression level and clinicopathological characteristics of LSCC patients (n(%)).

Correlation between ENO1 and clinicopathological characteristics of LSCC patients

ENO1 was correlated with lymph node metastasis and differentiation in LSCC patients (P<0.05). It was unrelated to the patient's age, daily smoking habits, and clinical stage (P>0.05). The results are shown in Table 4.

	n	ENO1		*P*
		Negative (n=9)	Positive (n=36)	
Age (years)				0.881
≤60	21	4 (44.44)	17 (47.22)	
>60	24	5 (55.55)	19 (52.78)	
Cigarettes daily				0.179
≤20	24	3 (33.33)	21 (58.33)	
>20	21	6 (66.67)	15 (41.67)	
Differentiation				<0.001
Well-differentiated	28	1 (11.11)	27 (75.00)	
Poorly differentiated	17	8 (88.89)	9 (25.00)	
Lymph node metastasis				0.003
No	16	7 (77.78)	9 (25.00)	
Yes	29	2 (22.22)	27 (75.00)	
Clinical stage				0.655
I+II	22	5 (55.55)	17 (47.22)	
III+IV	23	4 (44.44)	19 (52.78)	

Table 4: Correlation between ENO1 and clinicopathological characteristics of LSCC patients.

The correlation of HIF-1α and ENO1 in LSCC

After correlation analysis, HIF-1α and ENO1 were positively correlated in LSCC (P<0.05, r=0.591).

Discussion

When cancer cells grow vigorously, they need to consume a lot of O2 and energy. If the surrounding tissues lack O2 and energy, the cancer cells will become hypoxic. Clinical reports have shown that HIF-1 is a factor that can regulate the state of cells under hypoxic conditions as well as promote the transcription of erythropoietin, which in turn leads to the production of many red blood cells in the tissues and ultimately more O2[8]. Clinical studies have shown that excessive expression of HIF-1α and phosphodiesterase-4 can accelerate the growth of tumor tissue cells. Other scholars have found that HIF-1α can prolong the survival time of tumor cells[9]. Kim et al.[10] found that HIF-1α can participate in tumor invasion by regulating matrix metalloproteinase-15.

In addition, through the HIF-1α-Myc pathway, HIF-1α can accelerate the transformation of epithelial cells into mesenchymal cells, which in turn leads to decreased cell adhesion, increased activity, and, ultimately, tumor invasion and metastasis[11]. Clinical studies have confirmed that HIF-1α can contribute to the onset and progression

of gastric cancer, and its expression level in many gastric diseases infected by Helicobacter pylori is significantly higher than that of normal gastric mucosal tissue. ENO1 is highly conservative and is overexpressed in many tumor cells, such as head and neck squamous cell carcinoma and breast cancer[12]. Clinical studies have confirmed that there is venous vascular penetration and increased ENQ1 expression in poorly differentiated liver cancer tissues.

Other reports have confirmed that ENO1 has extensive biological functions and can accelerate tumor cell infiltration and migration by binding and activating plasmin. ENO1 can act as a glycolytic enzyme to activate the glycolytic pathway and improve the energy disorder of hypoxic cells. Clinical studies have shown that the connection between ENO1 and cytoskeleton protein is confidential, and it is inseparable from viral transport and replication in viral infectious diseases, such as hepatitis C virus and immunodeficiency virus. In addition, ENO1 has multicellular functions and can participate in many pathophysiological links, especially in the onset and progression of tumors. It is recognized as a diagnostic marker for many types of tumors[13]. Clinical studies have demonstrated that ENO1 overexpression is a common phenomenon in tumors and is closely related to disease progression and poor prognosis. Many previous studies have shown that ENO1 can be regulated at different levels by various factors. For example, the nuclear transcription factor, HIF-1α, and other transcription can activate ENO1, and it can also be obtained from protein-level retinoic acid[14]. Yu[15] and other studies have found that estradiol can increase the half-life of protein under the action of the transcription mechanism, thereby increasing the accumulation of ENO1.

In this study, we first used immunohistochemistry to detect the expression levels of HIF-1α and ENO1 in LSCC tissues and adjacent tissues and determine the relationship between their expression levels and the clinicopathological characteristics of LSCC patients. The results show that the positive expression of HIF-1α and ENO1 in LSCC tissues is higher than that in adjacent tissues.

Furthermore, the expression levels of HIF-1α and ENO1 were correlated with lymph node metastasis and differentiation in LSCC patients ($P<0.05$). This shows that HIF-1α and ENO1 may induce and promote tumor cell proliferation and enhance the malignancy and infiltration of LSCC. Finally, we used Spearman to analyze the relationship between HIF-1α and ENO1 in LSCC tissues. The results show that HIF-1α and ENO1 are positively correlated in LSCC ($P<0.05$, $r=0.591$).

Overall, the expressions of HIF-1α and ENO1 in LSCC tissues are all up-regulated, and their expression levels are correlated with lymph node metastasis and differentiation in LSCC patients. In addition, HIF-1α and ENO1 are positively correlated in LSCC tissues, and the two are expected to become potential biomarkers of LSCC, providing important references for clinical evaluation and disease prevention.

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