EXPRESSION LEVEL OF GLYCOGEN SYNTHESIZING KINASE-3B IN PATIENTS WITH LARYNGEAL CANCER AND ITS CORRELATION WITH CLINICOPATHOLOGICAL FEATURES AND PROGNOSIS

Qiang Liu[1], Yuye Shi[2], Gang Ren[3], Bin Li[1], Bingliang Ma[3,*]
[1]Department of Otolaryngology, Affiliated Hospital of Shandong Medical College, No. 80, Jintan Road, Lanshan District, Linyi City, Shandong Province - [2]Operating Room, Huzhou First People's Hospital, No.158, Square Back Road, Huzhou City - [3]Department of Otorhinolaryngology, Head and Neck Surgery, Huzhou First People's Hospital, No.158, Square Back Road, Huzhou City

ABSTRACT

Purpose*: To investigate the correlation between the expression level of glycogen synthesis kinase-3 (GSK-3β) and clinicopathological features and prognosis in patients with laryngeal cancer.*

Methods: *Thirty-four cases of laryngeal cancer tissues archived in our hospital from February 2012 to November 2014 were collected, along with another 34 cases of normal laryngeal mucosal tissues from patients without malignant tumors. The expression of GSK-3β in laryngeal cancer tissues and normal laryngeal mucosal tissues was detected with immunohistochemistry, and the correlation of GSK-3β with the clinicopathological features and prognosis of patients with laryngeal cancer was further explored.*

Results: *The positive expression rate of GSK-3β in laryngeal cancer tissues was 67.65% (23/34), while in normal laryngeal mucosal tissues the rate was 23.53% (8/34). The difference between the positive expression rates of the two groups was significant (P<0.05). GSK-3β expression levels were correlated with histological differentiation, TNM stage, and lymph node metastasis in patients with laryngeal cancer (P<0.05) and was independent of age, gender, and smoking history of the patient. The five-year survival rate of patients with GSK-3β - positive expression was 34.79% (8/23), which was 63.64% (7/11) in patients with GSK-3β - negative expression. The difference in the five-year survival rate between the two groups was significant (P<0.05). Histological differentiation, TNM stage, lymph node metastasis, and GSK-3β expression levels were all independent risk factors for the prognosis of patients with laryngeal cancer.*

Conclusion: *The increased expression of GSK-3β in laryngeal cancer can promote the development of this disease. GSK-3β expression level is related to histological differentiation, TNM stage, and lymph node metastasis. Moreover, the higher the expression level observed, the worse the prognosis of patients. These findings indicate that GSK-3β may be used as a potential histological biomarker for the evaluation of laryngeal cancer.*

Keywords: *Laryngeal cancer, glycogen synthesis kinase 3β, lymph node metastasis, TNM stage.*

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Introduction

Laryngeal cancer is a malignant tumor of the head and neck. Previous research in China has shown that laryngeal cancer occupies the third place in the malignant tumors of the head and neck, including laryngeal squamous cell carcinoma, which accounts for about 96% of all laryngeal cancers[(1)]. Currently, most treatments for laryngeal cancer involve surgery and chemoradiotherapy, which can improve the condition of patients to a certain extent. However, the prognosis of patients is poor, and the five-year survival rate is extremely low due to local lymph node metastasis or distant lymph node metastasis[(2)]. In recent years, the incidence of postoperative cancer has been gradually increasing, seriously threatening the life and health of patients[(3)]. Many variables affect the prognosis of laryngeal cancer patients, such as tumor location and TNM stage, but the precise factors affecting the prognosis

of this disease have not been clarified[4]. As a result, there is a significant need for effective approaches for monitoring the pathogenesis and progression of laryngeal cancer as well as for evaluating the prognosis of patients. It has also been reported that GSK-3β has dual effects, playing an important role in the onset and progression of cancer. However, it still remains controversial sounds after years of exploration[5].

Clinical studies have shown that GSK-3β is a tumor promoter: abnormal expression of GSK-3β promotes excessive proliferation of tumor cells and negatively regulates proto-oncogene proteins and cell cycle regulators[6]. In contrast, other reports have indicated that GSK-3β may inhibit tumor progression and transformation[7]. Therefore, in this study, we examined the expression of GSK-3β in laryngeal cancer tissues and examined correlations of GSK-3β with the clinicopathological features and prognosis of patients with laryngeal cancer.

Materials and methods

General materials

Thirty-four cases of laryngeal cancer tissues archived in our hospital from February 2012 to November 2014 were collected, in addition to another 34 cases of normal laryngeal mucosal tissues from patients without malignant tumors.

Inclusion criteria:

• The patient met the TNM staging criteria for laryngeal cancer issued by the American Joint Committee on Cancer (AJCC) in 2002;

• Laryngeal cancer was diagnosed for the first time;

• The patient received surgical treatment;

• Preoperative chemoradiotherapy or adjuvant therapy was not performed;

• (5) Complete clinicopathological data and follow-up data were available;

• The patient was informed about the study, and informed consent was obtained prior to specimen collection;

• Participation in the study was approved by the Ethics Committee of our hospital.

Exclusion criteria:

• Patients with endocrine diseases and primary tumors;

• Patients with abnormal heart, liver and kidney function;

• Patients who are lactating women and pregnant women.

Main reagents and instruments

Reagents

Rabbit anti-human GSK-3β rabbit monoclonal antibody was purchased from Wuhan Biofavor Biotechnology Co., LTD. Immune chromogenic reagent was purchased from FUJI FILM Wako Pure Chemical (Guang Zhou) Co., LTD. DAB dyeing liquid was purchased from Shanghai Kemin Biotechnology Co., LTD. PBS powder was purchased from Beijing Taize Jiaye Technology Development Co., LTD. Hematoxiglin liquid was purchased from Beijing CHREAGEN Biotechnology Co., LTD. An immunohistochemical SP kit was purchased from Xi'an Ruixi Biotechnology Co., LTD. Citrate antigen repair solution was purchased from Nanjing Senbeijia Biotechnology Co., LTD.

Instrument

A tissue crushing instrument was purchased from Monad (Suzhou) Biotechnology Co., LTD. A chemiluminescence imager was purchased from Suzhou Abitope Biotechnology Co., LTD. A tissue slicer was purchased from Hunan Yuanxiang Biotechnology Co., LTD. An oven was purchased from Guangzhou Koster Scientific Instrument Co., LTD. A benchtop was purchased from Dongguan Pubiao Equipment Technology Co., LTD.

Methods

• The expression of GSK-3β in laryngeal cancer tissues and normal laryngeal mucosal tissues was detected with immunohistochemistry. The specific steps were as follows: the laryngeal cancer tissues and normal laryngeal mucosal tissues were fixed with 10% formaldehyde and embedded in paraffin. Paraffin-embedded sections with a thickness of about 5μm were prepared. The slices were baked in the oven for 60min at 60°C.

The slices were removed from the oven and were dewaxed in xylene and a gradient of alcohol to water. The slices were then placed in sodium citrate solution for high pressure heat treatment, cooled to room temperature, and then 410% H_2O_2 was used to block endogenous peroxidase. The rabbit anti-human GSK-3β rabbit monoclonal antibody (1:100) working fluid was added drops at 4°C overnight. Biotinylated secondary antibody was added and incubated at room temperature.

After 20min, PBS solution was used to wash the slices 3 times, 5min each wash. The sections were removed and observed under a microscope following

DAB color development, hematlignin re-staining, and neutral gum sealing.

• All patients with laryngeal cancer included in the study were followed up for five years, including routine physical examination and dynamic laryngoscope of chest film.

Criteria

GSK-3β was found to be mainly expressed in the cytoplasm, and the positive expression of GSK-3β produced brownish yellow granules. The immunohistochemical results were mostly evaluated with a positive cell percentage score and a cell staining degree score. The score of cell staining degree was determined with the following scale: 0 points for no staining, 1 point for light yellow, 2 points for brownish, and 3 points for tawny.

Percentage of positive cells was determined with the following scale: 0 points for <5%, 1 point for 5%~25%, 2 points for 26%~50%, 3 points for 51%~80%, and 4 points for >80%. The percentage score of positive cells was multiplied by the score of staining degree. If the score <4 was negative expression, >4 was positive expression.

Statistical methods

SAS8.2 software was used to process the data in this study. The expression rate of GSK-3β in laryngeal cancer tissues and normal laryngeal mucosa tissues was indicated, and comparison between groups was performed by χ^2. Spearman's correlation coefficient was used to test the correlation between the GSK-3β expression level and the clinicopathological characteristics of patients with laryngeal cancer.

The survival of laryngeal cancer patients was analyzed with the Kaplan-Meier method, and the log-rank test was compared between groups. Additionally, the COX model was used to analyze the independent risk factors affecting the survival of liver cancer patients. P<0.05 was considered statistically significant.

Results

Expression of GSK-3β in laryngeal cancer tissues and in normal laryngeal mucosal tissues

The positive expression rate of GSK-3β in laryngeal cancer tissues was 67.65% (23/34), while the expression rate in normal laryngeal mucosal tissues was 23.53% (8/34); the difference between the two groups was significant (P<0.05). (See Figure 1 A-D).

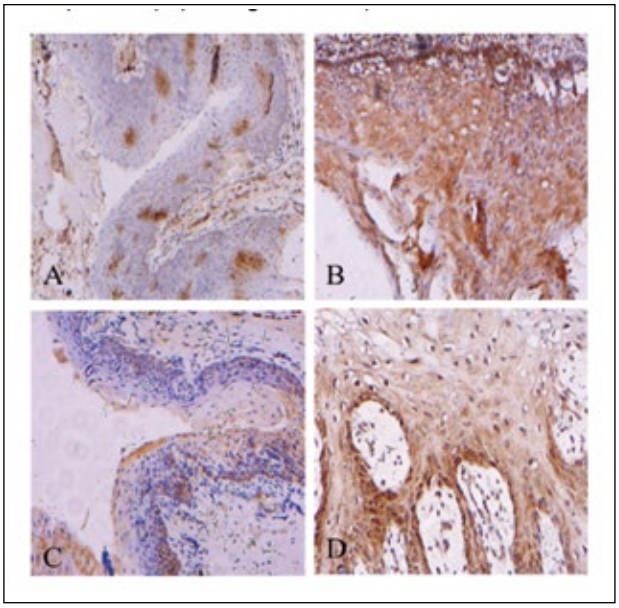

Figure 1: Expression of GSK-3β in laryngeal cancer and normal laryngeal mucosal tissues (×100).
Note: Figure A: GSK-3β was negatively expressed in laryngeal cancer tissues; Figure B: GSK-3β was positively expressed in laryngeal cancer tissues. Figure C: GSK-3β was negatively expressed in normal mucosal tissues. Figure D: GSK-3β was positively expressed in normal mucosal tissues.

Correlation between the GSK-3β expression level and clinicopathological features of patients with laryngeal cancer

GSK-3β expression level was correlated with histological differentiation, TNM stage, and lymph node metastasis in patients with laryngeal cancer (P<0.05) and was independent of age, gender, and smoking history of the patient. (See Table 1).

Clinicopathological characteristics	NNT	GSK-3β expression		*P*
		Negative expression (n=11)	Positive expression (n=23)	
Age				0.568
<65	17	5	12	
≥65	17	6	11	
Gender				1.011
Male	28	9	19	
Female	6	2	4	
Smoking History				0.404
Yes	9	2	7	
No	25	9	16	
Histological differentiation				0.022
Medium/low differentiation	9	4	5	
High differentiation	25	7	18	
TNM stage				0.019
I~II	15	8	7	
III~IV	19	3	16	
Lymph node metastasis				0.024
No	15	10	5	
Yes	19	1	18	

Table 1: Correlation between the GSK-3β expression level and clinicopathological characteristics of patients with laryngeal cancer.

Correlation between the GSK-3β expression level and the prognosis of patients with laryngeal cancer

The five-year survival rate of patients with GSK-3β - positive expression was 34.79% (8/23), while the survival rate of patients with GSK-3β - negative expression was 63.64% (7/11). The difference in the five-year survival rate between the two groups was significant (P<0.05). (See Figure 2).

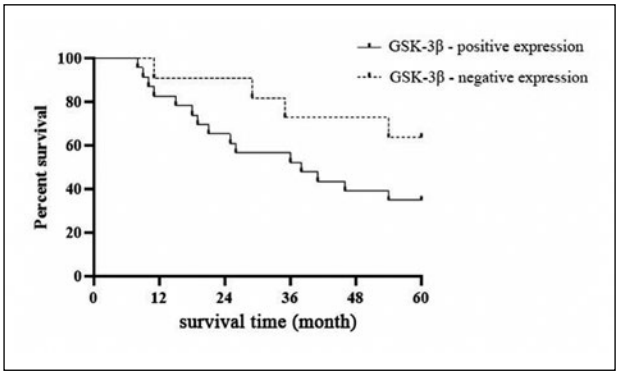

Figure 2: Correlation between the GSK-3β expression level and the prognosis of patients with laryngeal cancer.

Analysis of COX proportional hazard regression model in patients with laryngeal cancer

Histological differentiation, TNM stage, lymph node metastasis, and GSK-3β expression levels were all independent risk factors for the prognosis of patients with laryngeal cancer. (See Table 2).

Clinicopathological Parameters	B	SE	Wald	Sig	Exp(B)	95%CI
Age	0.139	0.994	2.053	0.891	1.891	1.139~4.069
Gender	0.125	1.616	2.562	0.557	3.198	3.362~4.565
Smoking History	0.941	1.036	3.379	0.569	1.265	0.064~2.951
Histological differentiation	0.966	0.252	15.758	<0.001	2.609	1.632~4.174
TNM Stage	0.604	0.261	5.696	0.028	1.821	1.122~2.957
Lymph node metastasis	1.344	0.407	11.373	0.002	3.805	1.758~8.249
GSK-3β expression	0.738	0.366	4.309	0.039	2.081	1.043±4.157

Table 2: Analysis of COX proportional hazard regression model in patients with laryngeal cancer.

Discussion

Laryngeal cancer is second only to nasopharyngeal cancer in the incidence of head and neck malignancies. Laryngeal cancer is more common in people aged between 50 and 70, and is found more commonly in male patients than female patients. Currently, on average there are 150,000 new cases of laryngeal cancer every year. While early treatment of laryngeal cancer is relatively effective, late treatment is not obvious and the prognosis is unsatisfactory, which is associated with complications and outcomes that seriously affect the health and the quality of life of patients[8]. Human squamous cell progression involves multiple factors and genes, but the specific mechanism of action is not yet clear[9]. Therefore, it is critical to explore novel and effective diagnostic indicators for treating patients with laryngeal cancer.

GSK-3β is widely distributed in organisms and was originally identified in skeletal muscle. However, subsequent studies have found that GSK-3β is abundantly expressed in all tissues, especially in brain tissue[10]. GSK-3β was first discovered to phosphorylate and inhibit glycogen synthase activity, thus improving glucose catabolism[11]. Clinical studies have shown that this protein can also phosphorylate a series of first substrates such as metabolic-related proteins and transcription factors, and can exist in the body as regulatory kinases, affecting cell proliferation, apoptosis, and migration[12]. Clinical studies have shown that GSK-3β can cause tumors when its function is abnormal. However, research results are inconsistent, with conflicting views[13]. Many reports have confirmed that GSK-3β can prevent the onset of skin cancer.

Using a mouse multistage skin cancer model, studies have found that the expression level of GSK-3β in advanced squamous cell carcinoma is significantly higher than the expression level in normal tissues[14]. In addition, nonmelanoma skin cancer studies have shown that GSK-3β expression levels in cancer cells is significantly lower compared to the expression levels in normal glial cells. The expression level of GSK-3β in the mouse epidermal skin cancer transformation model JB6 P + cell study was observed to be significantly lower compared to the control group. However, while the expression of JB6 P was higher in the non-malignant transformation of JB6 P-, the expression level of JB7 was lowest in the successfully transformed skin cancer cells, which may be caused by the deactivation of GSK-3 due to the phosphorylation of epidermal growth factor and tumor derived polypeptide antigen[15].

In this study, we first measured GSK-3β expression in laryngeal cancer tissues and normal laryngeal mucosal tissues using a immunohistochemical method. The results showed that the positive expression rate of GSK-3β in laryngeal cancer tissues was 67.65% (23/34), which was significantly higher than the positive expression rate of GSK-3β in normal laryngeal mucosal

tissues (23.53% (8/34)) (P<0.05), suggesting that GSK-3β may participate in the initiation and progression of laryngeal cancer. Next, we further analyzed correlations between GSK-3β expression levels and the clinicopathological characteristics of patients with laryngeal cancer and found that the GSK-3β expression level was correlated with tissue differentiation, TNM stage, and lymph node metastasis in patients with laryngeal cancer (P<0.05).

In addition, the five-year survival rate of patients with GSK-3β - positive expression was 34.79% (8/23), which was significantly lower than the five-year survival rate of patients with GSK-3β - negative expression (63.64%) (7/11). These findings indicate that GSK-3β expression level is associated with the prognosis of patients with laryngeal cancer. In addition, we established using the COX proportional hazard regression model in this study and found that tissue differentiation, TNM stage, lymph node metastasis, and GSK-3β expression level were independent risk factors affecting the prognosis of patients with laryngeal cancer.

In summary, the increased GSK-3β expression level observed in laryngeal cancer tissue can promote the progression of this disease. GSK-3β expression level was associated with tissue differentiation, TNM stage, and lymph node metastasis, and higher expression levels were associated with a worse prognosis of patients. The results of this study suggest that GSK-3β levels could be used as a potential tissue biomarker for the evaluation of laryngeal cancer.

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Corresponding Author:
Bingliang Ma
Email: kg90gn@163.com
(China)