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FFAR4 promotes cell proliferation and migration and servers as a potential biomarker for clinicopathological characteristics and prognosis in laryngocarcinoma

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Abstract. – OBJECTIVE: The poor prognosis of advanced laryngocarcinoma was associated with the epithelial-mesenchymal transformation (EMT), which was related to the dysregulated expression of free fatty acids receptor 4 (FFAR4). By detecting the expression of FFAR4 in laryngocarcinoma and its relation with the clinicopathological characteristics and prognosis of laryngocarcinoma, as well as conducting *in vitro* experiments, our aim is to explore the role of FFAR4 in laryngocarcinoma biological and clinical process.

PATIENTS AND METHODS: The protein expression level of FFAR4 in 54 cases of laryngocarcinoma and 30 cases of laryngocarcinoma adjacent tissues was detected by immunohistochemistry. Combined with clinical follow-up data, the Kaplan-Meier survival curve and log-rank test were conducted to compare the relation between the expression of FFAR4, the clinicopathological characteristics, and the 5-year survival rate in laryngocarcinoma. Multivariable Cox regression analysis revealed the independent predictors for the prognosis of laryngocarcinoma. CCK-8 and migration assay were used to test cell proliferation and migration abilities.

RESULTS: FFAR4 was upregulated in laryngocarcinoma tissues and influenced cell proliferation and migration abilities. The FFAR4 expression was related to the age and lymph node metastasis in laryngocarcinoma patients and indicated a reduced 5-year survival rate and increased lymph node metastasis.

CONCLUSIONS: The upregulation FFAR4 expression was associated with the lymph node metastasis and the prognosis. FFAR4 can significantly promote laryngocarcinoma cell proliferation and migration *in vitro*.

Key Words:
Laryngocarcinoma, FFAR4, Metastasis, Prognosis, Proliferation.

Introduction

Laryngeal squamous cell carcinoma (Laryngocarcinoma) is one of the most common malignant head and neck tumors. The incidence rate accounts 1-5% in the total malignant tumors, and 3.3-8.1% in the malignant tumors of the head and neck[1]. Advanced laryngocarcinoma is often accompanied by cervical lymph node metastasis, which leads to the poor prognosis[2]. According to the epidemiological data from 2004 to 2006, the 5-year survival rate of laryngocarcinoma patients in Asia is 72.5%, of which, more than 90% died of tumor metastasis[3]. Although the 5-year survival rate of laryngocarcinoma has been improved by the surgical adjuvant radiotherapy and chemotherapy, the 5-year survival rate and the life quality of advanced laryngocarcinoma patients remains poor[4]. Free fatty acids receptor 4 (FFAR4), also known as GPR120, is a newly discovered G-protein coupled receptors (GPCRs). As an important member of the GPCRs family, FFAR4 can be bound by long-chain polyunsaturated fatty acids (FAs), and then, activates the downstream molecular signaling pathway to regulate the cell metabolism, endocrine, and immune function[5,6]. In recent years, the abnormal expression of FFAR4 has been found in many malignant tumors, which may be related to the promotion of tumor cell invasion and metastasis by inducing tumor EMT. FFAR4 has been reported to be involved in the development of digestive system tumors, lung cancer, breast cancer, melanoma, and prostate cancer[7]. However, the expression of FFAR4 in laryngocarcinoma and its role in the progression of laryngocarcinoma is

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rarely reported at home and abroad. In this study, we determined for the first time the expression of FFAR4 in human laryngocarcinoma and adjacent tissues by immunohistochemistry. Also, we analyzed its relation with clinicopathological characteristics and prognosis.

Patients and Methods

Patient Specimens and Ethics Statement

54 cases of laryngocarcinoma tissues pathological specimens and 30 cases of laryngeal mucosa (tissue more than 1.5 cm from the safe edge of the operation as adjacent tissues) were collected by surgical resection from the Department of Otolaryngology-Head and Neck Surgery at the First Affiliated Hospital of Bengbu Medical College during January 2012 to October 2013. 50 cases were male, 4 females, aged from 30 to 70 years old (mean 62.8, median 64). 33 patients received partial laryngectomy and 21 received total laryngectomy according to tumor range. All the patients did not receive any radiotherapy and/or chemotherapy before the operation. The TNM staging of the tumor was defined according to the Standard (2002) of the International Union Against Cancer (UICC). All human tissues were collected after obtaining written informed consent from the patients. This research was approved by the Ethics Committee of the First Affiliated Hospital of Bengbu Medical College.

Cell Culture

293T and hep-2 cell lines were purchased from American Type Culture Collection (Manassas, VA, USA). All cells were maintained under standard culture conditions (37°C, 5% CO_2) in a culture medium recommended by the American Type Culture Collection.

Immunohistochemical Assay

SP staining was used for the immunohistochemical analysis and phosphate-buffered saline (PBS) was used as the negative control. The specific steps were as follows. Paraffin embedding sections were prepared at 4 μm thickness and heated at 60°C for 20 min, then deparaffinized with dimethylbenzene, hydrated with gradient ethanol (100%, 95%, 75%), and washed with PBS. The sections were heated in the pressure cooker with citrate buffer (pH 6.0) for 5 min and treated by 3% H_2O_2 for 10 min to block the endogenous peroxidase activity, and washed by PBS for 3 times (3 min/time). The rabbit anti-human polyclonal antibody to FFAR4 was diluted 100 times and added into the sections, cultured at 4°C for overnight. Next, the sections were washed 3 times (3 min/time) and stained with diaminobenzidine (DAB) under microscope control. Finally, the sections were counter-stained with hematoxylin, dehydrated in graded ethanol, cleared in dimethylbenzene, fixed with neutral balata, and examined and taken pictures under a microscope. In addition, in negative control group, the main antibody was replaced by PBS under the same conditions. The FFAR4 was mainly located in the cytoplasm, with diffuse brown or yellow around the nucleus of laryngocarcinoma, but with less staining in the interstitial cells of the tumor. Score was based on dyeing intensity and area (percentage of positive cells), as described in Table I. Multiply the results of the two scores, total scores ≤3 means low expression, >3 means high expression[8]. All immunohistochemical sections were scored independently by two experienced pathologists. Results were corrected by a joint assessment.

Cell Counting Kit-8 (CCK-8) Assay

The CCK-8 assay was used to assess cell proliferation ability. Cell Counting Kit-8 (CCK8) kit (Dojindo Laboratories, Kumamoto, Japan) was used according to the manufacturer's protocol. After culturing for 12, 24, 48, and 72 h each group, different groups of Hep-2 cells were plated at a 96-well plate with a concentration gradient. After using the colorant, cells were cultured for another 2 h in the incubator and then the absorbance was measured at a 450 nm wavelength. Three independent tests were conducted for each experiment.

Table I. Scoring principle.

	Percentage of positive cells				Dyeing intensity			
Classification	≤ 10%	10%-30%	≥ 30%	NC	Light yellow	Yellow	Brown-yellow	No positive
Scores	1	2	3	0	1	2	3	0

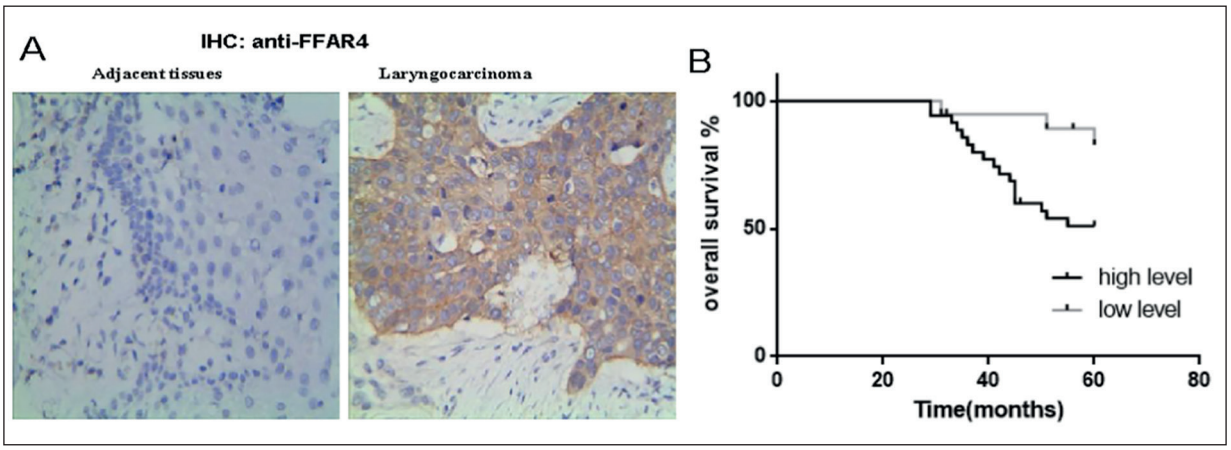

Figure 1. ***A,*** FFAR4 proteins were mainly located in the cytoplasm among laryngocarcinoma and adjacent tissues (magnification: 400×). ***B,*** 5-year survival rate was 51.0% in the high expression group, while 78.4% in the low expression group.

Cell Migration Assay

Cell migration assay was used according to the manufacturer's protocol. SiNC and siFFAR4 group cells were transfected and digested with trypsin, resuspend, and counted. Transwell chambers with an aperture of 8.0 m were placed. Counted cells were inoculated on 24 well plates in the upper chamber and the graded medium was added to the lower chamber. After culturing for 36 h, the chamber was removed and the unmigrated ones were swiped with a cotton swab. Cells were fixed with formaldehyde for 30 min, washed with pure water, stained with crystal violet for 20 min. After drying, pictures were taken under the microscope and the cells were counted. Three independent tests were conducted for each experiment.

Statistical Analysis

The FFAR4 expression levels among groups and the relations between clinicopathological characteristics and lymph node metastasis were analyzed by the χ^2-test. The survival curve was drawn by Kaplan-Meier, the difference in survival rate between high and low expression groups of FFAR4 was compared by the Log-rank test. Multivariable Cox regression analysis revealed the independent predictors for the prognosis of laryngocarcinoma. All the data were statistically analyzed by Statistical Product and Service Solutions (SPSS) 15.0 software package (SPSS Inc., (Chicago, IL, USA), $p<0.05$ was statistically significant.

Results

FFAR4 is Upregulated in Laryngocarcinoma

We first detected the FFAR4 expression in laryngocarcinoma and adjacent tissues by immunohistochemical analysis. As shown in Figure 1A, FFAR4 proteins were mainly located in the cytoplasm among laryngocarcinoma and adjacent tissues. The expression of FFAR4 in laryngocarcinoma (65.8%, 35/54) was significantly higher than that in adjacent normal tissues (40.3%, 11/30) and the difference was statistically significant ($p<0.05$, Table II). These results suggest that FFAR4 is upregulated in laryngocarcinoma, indicating its potential as an oncogene.

Table II. Expression of FFAR4 in laryngocarcinoma and adjacent tissues.

Tissues	Cases	FFAR4		χ^2	*p*
		High expression	Low expression		
Laryngocarcinoma	54	35	19	6.17	0.01
Adjacent tissues	30	11	19		

Relationship Between FFAR4 Expression and Clinicopathological Characteristics in Laryngocarcinoma

We analyzed the relation between FFAR4 expression and clinicopathological characteristics of laryngeal carcinoma using χ^2-test. As showed in Table III, the age and N-grade were related to the high expression of FFAR4 ($p<0.05$). While there was no significant correlation between FFAR4 expression and sex, T grade, and pathological grade of patients ($p>0.05$). Cox univariate analysis was used to study the influence of clinicopathological parameters and the FFAR4 expression on the prognosis of the patients. Significant prognostic factors were screened in a 95% confidence interval. Results showed that the FFAR4 expression was related to the prognosis of laryngocarcinoma ($p<0.05$), while age and pathological grade were not associated with the prognosis of laryngocarcinoma. Further multivariate Cox risk proportional model analysis showed that FFAR4 expression was an independent predictor of laryngocarcinoma prognosis. And the risk of lymph node metastasis in laryngocarcinoma with a stage above N0 was 1.39 times higher than that in stage N0 (RR = 1.39, 95% CI = 0.12-0.66). The other parameters, such as clinical tumor classification, T grade, and pathological grade, could not predict the prognosis of the disease alone (Table IV).

Relationship Between FFAR4 Expression and 5-Year Survival Rate in Laryngocarcinoma

The above results inspired us to further analyze the clinical survival role of FFAR4. According to the expression level of FFAR4 in laryngocarcinoma, the patients were divided into two groups, FFAR4 high expression group (n=35) and low expression group (n=20). As shown in Figure 1B, the 5-year survival rate was 51.0% in high expression group, while 78.4% in low expression group. The 5-year overall survival rate of FFAR4 high expression group was lower than that in low expression group, and the difference was statistically significant ($\chi^2=3.94$, $p<0.05$). These results suggest that the upregulation of FFAR4 expression predicts poor survival rate in laryngocarcinoma.

FFAR4 Significantly Promotes Cell Proliferation and Migration In Vitro

To determine the biological function of FFAR4 in laryngocarcinoma, we down-regulated FFAR4 in hep-2 cell line by siRNA. As shown in Figure 2A-2B, the downregulation was detected by Western blot (WB) protein level. We conducted the CCK-8 assay to assess cell proliferation, which shows that the downregulation of FFAR4 can decrease the cell number significantly (Figure 2C). Given that FFAR4 has been reported to get

Table III. Relationship between FFAR4 expression and clinicopathological characteristics of laryngocarcinoma.

Clinicopathological characteristics	Cases	FFAR4		χ^2	p
		High expression	Low expression		
Age					
≤ 60	20	8	12	8.51	0.00
>60	34	27	7		
Sex					
Male	50	32	18	0.00	1.00
Female	4	3	1		
T grade					
T1	10	6	4	0.54	0.9
T2	19	11	8		
T3	12	8	4		
T4	13	9	4		
N grade					
N0	40	22	18	5.35	0.03
N1	12	11	1		
N2	2	2	0		
Pathological grade					
Well-differentiated	24	14	10	1.42	0.49
Moderately differentiated	27	20	7		
Poorly differentiated	3	2	1		

Table IV. Multivariate analysis of FFAR4 expression and clinical prognosis in laryngocarcinoma.

Pathological parameters	COX univariate analysis		COX multivariate analysis	
	Risk value	*p*	Risk value	*p*
Age ≤ 60 > 60	0.05	0.05	3.44	0.06
T grade T1-T2 T3-T4	-1.02	0.03	2.45	0.12
N grade N0 N+	-1.39	< 0.01	-1.40	< 0.01
Pathological grade Well-differentiated Poorly differentiated	0.00	0.99	0.23	0.65
FFAR4 expression High expression Low expression	1.33	0.03	1.56	0.035

involved in the EMT process, we further tested the migration using the transwell experiment. As shown in Figure 2D-2E, the downregulation of FFAR4 has a negative impact on the cell migration ability. These results indicate that FFAR4 significantly promotes cell proliferation and migration in laryngocarcinoma.

Discussion

Previously Balenga et al[9] suggested that FFAR4 and related signaling pathway dysregulation can be used as tumor markers. The abnormal expression of GPCR triggers a series of intracellular signal abnormalities, leading to the proliferation,

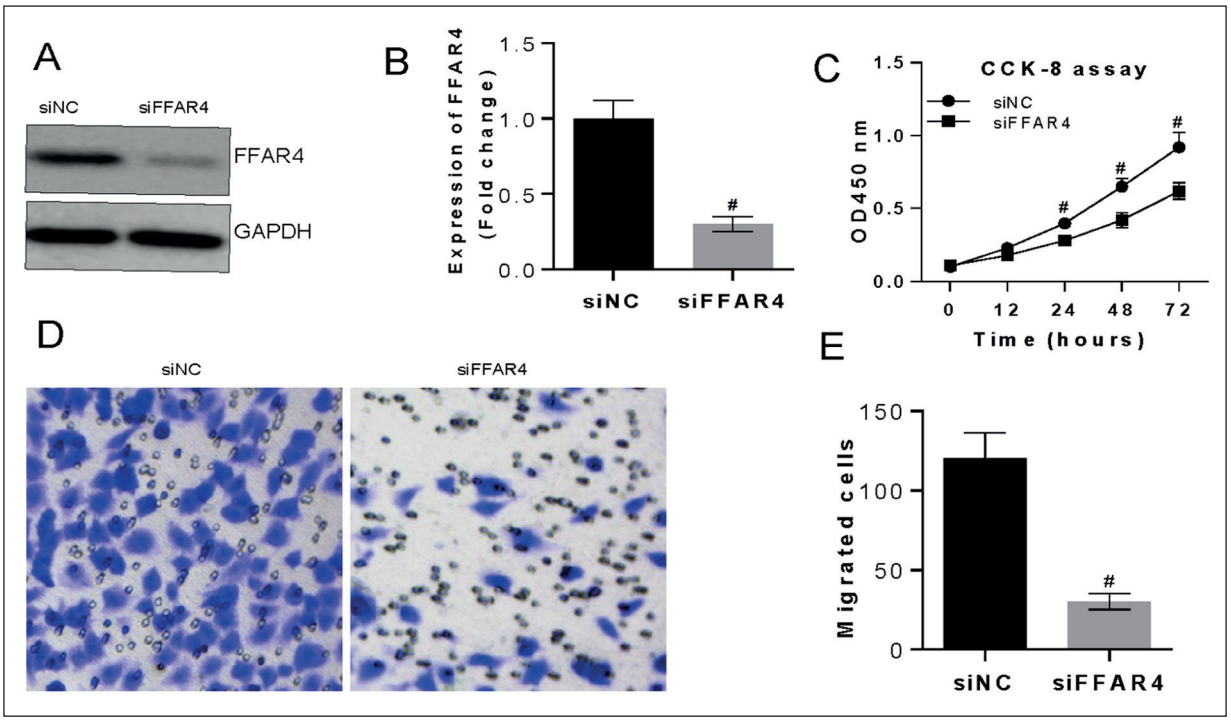

Figure 2. ***A, B,*** FFAR4 was down-regulated in hep-2 cell line by testing WB protein level. ***C,*** Down regulation of FFAR4 can decrease cell number significantly by CCK-8 assay. ***D-E,*** Down regulation of FFAR4 has a negative impact on cell migration ability (magnification: 100×).

metastasis, and angiogenesis of cancer cells. As an important member of the free fatty acid receptor (FFAR) in the GPCR family, FFAR4 was initially concerned about its role in metabolic and inflammatory diseases such as obesity and type 2 diabetes. With the deepening of research, many reports[10] have found that FFAR4 plays an important role in the occurrence and development of multiple tumors. Results showed that the expression of FFAR4 was upregulated in laryngocarcinoma and its high expression was correlated with lymph node metastasis. It has been reported that the increased expression of FFAR4 was closely related to tumor formation, migration, and metastasis. FFAR4 was involved in the occurrence and development of digestive system tumors, lung cancer, breast cancer, melanoma, and prostate cancer[7]. Wu et al[11] reported that the expression of FFAR4 in colon cancer tissues was higher than that in normal colonic mucosa tissues. Also, it was closely related to the poorly differentiated of colon cancer and the progression of clinical stage. It has been shown that FFAR4 signal stimulates angiogenesis and enhances migration and motility in human colorectal cancer cells, which is regulated by VEGF, IL-8, and COX2. In addition, the activation of FFAR4 enhanced the viability of colorectal cancer cells and induced EMT[12]. Takahashi et al[5] have shown that the FFAR4 knockout can inhibit the metastasis of pancreatic cancer cells. This metastasis behavior was regulated by MMP2 and the level of MMP2 was decreased after the FFAR4 knockout. Moreover, FFAR4 also participated in the occurrence and development of esophageal cancer. Cell experiments *in vitro* showed that the cell proliferation, clone formation, migration, and invasion of esophageal cancer cell line decreased after the knockout of FFAR4. In the nude mouse with heterotopic transplantation tumor, the volume of the tumor was significantly reduced after transfection with siFFAR4. FFAR4 could upregulate the expression of PI3K and I-κB signal pathway, induced EMT, promote the angiogenesis of esophageal carcinoma, and induce the expression of tumor-associated inflammatory factor (IL8, COX2, PGE2), thus promoting the development of esophageal cancer. FFAR4 participates in tumor migration by promoting EMT in laryngocarcinoma. In addition, Meng et al[13] found that the upregulation of FFAR4 expression was associated with the EMT and poor prognosis in pancreatic carcinoma. Besides, some researchers have found that linoleic acid (LA) can induce the increased expression of IR and IGF1R in human breast cancer cells MDA-MB-231b through the FFAR4-EGFR and PI3K/Akt dependent signaling pathway, thus promoting the proliferation and migration of breast cancer[14]. However, some scholars have suggested that FFAR4 can play an anticancer role in some tumors. In melanoma, the down-regulation of FFAR4 expression by small RNA interference can enhance the migration of melanoma. These findings suggest that FFAR4 may play a different role in different tumors[15]. Also, the *in vitro* cell experiments showed that ω-3 FAs could activate the expression of FFAR4 in human prostate cancer cells and inhibit the proliferation of prostate cancer cells[16]. FFAR4 expressed in host cells may participate in the inhibition of prostate cancer by regulating ω-3 FAs[17]. Zhu et al[18] reported that the expression of FFAR4 was upregulated in human breast cancer cells, which could promote the proliferation of human breast cancer cells, but it did not participate in the inhibition and anti-apoptosis of human breast cancer cells by ω-3 FAs. This explains to some extent, the difference of FFAR4 in tumor cells. This research revealed the relation between the upregulation of FFAR4 expression in laryngocarcinoma and the reduction of the 5-year survival rate of laryngeal carcinoma. However, there was not sufficient evidence to discuss the relation between the expression of FFAR4 and the clinicopathology and prognosis of laryngocarcinoma due to the small sample size involved in this work. We also found that FFAR4 significantly promotes cell proliferation and migration *in vitro*. The future report will focus on exploring the further mechanism of FFAR4 *in vivo* and its underlining mechanism. Therefore, this study suggests that the upregulation expression of FFAR4 was associated with clinical and biological functions in laryngocarcinoma tissues, which may provide a potential target for the treatment of laryngocarcinoma.

Conclusions

The upregulation expression of FFAR4 was associated with clinical and biological functions in laryngocarcinoma tissues, which may provide a potential target for the treatment of laryngocarcinoma.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Fund
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