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GROα expression and its prognostic implications in laryngeal squamous cell carcinoma

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The growth-regulated oncogene α (GROα) , which is also designated as CXC chemokine ligand 1 (CXCL1), was first identified as an autocrine growth factor in human malignant melanoma. It is involved in tumor development and invasion, and is highly expressed in various human cancers. However, little is known about the association between GROα expression and the clinical attributes of laryngeal squamous cell carcinoma (LSCC).

One-step quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and immunohistochemical staining of tissue microarrays were employed to evaluate the relationship between GROα expression and LSCC clinicopathological attributes.

GROα mRNA and protein expression levels were significantly greater in LSCC than in non-cancerous tumor-adjacent tissues. GROα protein expression in LSCC was also significantly associated with TNM stage, lymph node metastasis, and histopathological grade. Kaplan-Meier and Cox multi-factor analyses suggested that increased GROα expression and positive lymph node metastasis were significantly associated with the poor survival of LSCC patients.

These data indicate that GROα may be a novel prognostic marker of LSCC.

Key words: GROα, LSCC, qPCR, immunohistochemistry, prognosis

Laryngeal squamous cell carcinoma (LSCC) is the most common malignant neoplasm of the head and neck. Its incidence rate is nearly 48,000 cases in the United States and over 500,000 cases worldwide each year [1,2]. The most critical carcinogenic agents for LSCC are cigarette smoke and alcohol while other exogenic and endogenic factors are mostly promoters or carcinogens in multiplied carcinogenesis processes [3]. The larynx is an important organ required for pronunciation, breathing and swallowing and patients with LSCC have problems with these functions such as dysphonia, dysphagia and dyspnea. The most effective treatment of LSCC, laryngectomy, is highly invasive and is usually only performed for cases in which fundamental changes are accompanied by physiological and psychological function [4]. Despite recent advances in multidisciplinary treatment strategies for LSCC, including targeted surgical extirpation or larynx-preservation protocols implementing chemotherapy or radiotherapy, a large proportion of patients with localized or advanced disease will eventually relapse and die. Indeed, the overall survival of LSCC has not improved much for years [5,6]. Therefore, identifying novel molecular indicators of its malignant behavior is of great interest and could be helpful for early prevention, diagnosis and treatment of LSCC.

The growth-regulated oncogene α (GROα), a 73-amino-acid protein which is also designated CXC chemokine ligand 1 (CXCL1), was first identified as an autocrine growth factor in human malignant melanoma [7]. GROα plays an important role in chemoattraction, wound healing, and angiogenesis through signaling via the seven-transmembrane G-protein-coupled receptor CXCR2 [8,9]. In recent years, GROα has been demonstrated to be involved in tumor development and invasion as a growth and anti-apoptotic factor [10-12]. It is also reported to act as a potent mediator of tumor-associated angiogenesis in bladder cancer and Kaposi's sarcoma [13,14]. In addition, elevated expression of GROα has been detected

in various human cancers [10, 15-18]. In comparison, down-regulation of GROα inhibits tumor growth in colorectal liver metastasis [19]. However, although GROα exhibits oncogenic characteristics, its expression and function in LSCC remain still to be fully determined. Moreover, whether GROα could be used as a biomarker for LSCC also deserves further investigation.

In the present study, the expression of GROα mRNA in LSCC tissue was detected via one-step quantitative reverse-transcription polymerase chain reaction (qPCR) while the expression of GROα protein in LSCC tissue microarray (TMA) was evaluated by immunohistochemistry (IHC). The correlation between GROα expression and clinicopathological attributes of LSCC was then investigated. The results showed that GROα expression was significantly elevated in LSCC in comparison with non-cancerous tumor-adjacent tissue. Its expression was also positively associated with several LSCC traits, as well as reduced patient survival. Together, our results suggest that GROα is a potential biomarker for the diagnosis and prognosis of LSCC.

Patients and methods

Patient samples and clinical data. A total of 135 paraffin-embedded LSCC tissue samples and 27 control samples were collected from the archives of the Department of Pathology, at the Affiliated Hospital of Nantong University, between 2002 and 2012. Diagnosis of LSCC was confirmed according to the latest WHO criteria [20] and TNM (Tumour, Node, Metastasis) stage classification (UICC 2009)[21]. The original clinical data were obtained from hospital medical records, and include details pertaining to patient gender and age, tobacco use, alcohol consumption, TNM stage, lymph node metastasis status, histopathological grade and overall survival. None of the patients received preoperative radiotherapy or chemotherapy before surgery. Written informed consent was obtained from each patient for publication of this study. Ethical approval to perform this research was approved by the Human Research Ethics Committee of the Affiliated Hospital of Nantong University.

One-step quantitative polymerase chain reaction. Ten samples of fresh LSCC tissues and matched tumor-adjacent tissues were collected from the Department of Pathology, the Affiliated Hospital of Nantong University. Total RNA was extracted from samples using Trizol reagent (Invitrogen, Carlsbad, CA). Expression levels of GROα and GAPDH were determined by real-time PCR with IQ5 (Bio-Rad, Hercules, CA) using the SensiMix One-Step Kit-based SYBR Green method (Quantace, London, UK). The primers for GROα were as follows: forward primer 5'-GATTGTGCCTAATGTGTT-3'and reverse primer 5'-ATCCAGATTGAACTAACTTG-3'; for GAPDH, forward primer 5'-TAT TAC CTG GAC GAG ATT CCCC-3' and reverse primer 5'-TAT TAC CTG GAC GAG ATT CCCC-3'. Amplification conditions consisted of 30 min at 42°C for reverse transcription and 2 min at 94°C for Taq activation, followed by 35 cycles of 95°C for 20 s, 56°C for 20 s, and elongation at 72°C for 30 s. Each measurement was performed in triplicate. Tissue microarray (TMA) construction and immunohistochemistry (IHC) TMAs were produced by Xinchao Biotech Co. Ltd. (Shanghai, China). Core tissue biopsies (2 mm in diameter) were taken from individual paraffin-embedded sections and arranged in the new paraffin blocks. The TMAs were cut into 4 μm sections and placed on TMA-specific adhesive-coated glass slides. IHC analysis was performed as described previously [22,23]. Briefly, TMA sections were incubated with a primary monoclonal mouse anti-GROα antibody (1:200, Abcam, England) diluted in phosphate-buffered saline (PBS). Following washing with PBS, sections were incubated with horseradish peroxidase-conjugated goat anti-mouse antibody (Dako Cytomation, Carpinteria, CA) for 15 min and then washed. The color was developed by a 15-min incubation with diaminobenzidine solution (Kem-En-Tec Diagnostics, Taastrup, Denmark), and sections were weakly counterstained with hematoxylin. Negative control reactions used PBS instead of the primary antibody. The results of IHC were evaluated by a double-blind method whereby the staining results were determined under an optical microscope by two pathologists independently. In the case of disagreement, the slides were reviewed by a third pathologist until a consensus score was established. Expression levels of GROα protein were assessed by observing the incidence and staining intensity of immunohistochemically positive cells, as described previously [22,23]. The incidence of positive cells was scored as follows: negative as 0; 1–10% positive cells as 1 point; 10–50% positive cells as 2 points; and >50% positive cells as 3 points. Staining intensity was scored as: no color as 0; yellow for weak positive as 1 point; light brown for medium positive as 2 points; and brown for strong positive as 3 points. The two components were produced to obtain an overall expression score, as follows: 0 as (-); 1–3 as (+); 4–6 as (++); and7- 9 as (+++).The degree of GROa staining was quantified using a two-level grading system, and staining scores were defined as follows: 0-3, low expression, and 4-9, high expression.

Statistical analysis. The mRNA expression of GROα in fresh LSCC tissues compared with that of matched tumor-adjacent tissues was analyzed by the Wilcoxon signed rank nonparametric test. The influence of GROα expression on clinicopathological attributes of LSCC was analyzed by the chi-square test. The Kaplan-Meier method was employed to evaluate the association between GROα expression and LSCC prognosis. Univariate and multivariate analyses were executed by Cox proportional hazards regression models to determine factors that were independently associated with overall survival. For all analyses, a p value less than 0.05 was regarded as statistically significant. All statistical analyses were performed using STATA Version 12.0 (Stata Corporation, College Station, TX) and SPSS 18.0 statistical software (SPSS Inc., Chicago, IL).

Results

Clinicopathological attributes of LSCC patients. The main clinicopathological attributes of LSCC are shown in Table 1. A total of 135 LSCC patients, of median age 60 years (range 42–79 years) were enrolled in this study. Seventy-four patients had a history of cigarette use while the remaining 33 did not smoke. Fifty-two patients drank alcohol while the other 54 had never drunk. Regarding tumor TNM stage, 13 patients were stage I, 57 were stage II, and the 33 other patients were stages III and IV. In terms of histopathological grade, 11 patients were low grade, 59 were moderate grade and 61 were high grade. There were 20 patients with positive lymph node metastasis while 114 were negative.

Analysis of GROα mRNA expression in LSCC by qPCR. To assess the expression profile of GROα in LSCC, total RNA was extracted from LSCC tissues and non-cancerous tumor-adjacent tissues then GROα mRNA expression was evaluated by one-step qPCR analysis. When normalized to GAPDH, the mean expression of GROα mRNA in LSCC and corresponding tumor-adjacent tissues was 88.03 ± 28.186 and 25.64 ± 7.194, respectively (F=4.599, p=0.046). Therefore, GROα expression was on average 3.43-fold greater in LSCC samples than in non-cancerous tissues (Figure 1).

Detection of GROα expression in LSCC by IHC. IHC analysis was performed to investigate the expression of GROα in LSCC. A significant difference was detected between GROα expression in LSCC tissues and normal tumor-adjacent tissues; indeed, elevated GROα expression was detected in 81 of 135 (60%) LSCC tissues, while only six cases of 27 normal tumor-adjacent tissues (22.2%) exhibited GROα expression (p<0.05). Analysis of the GROα expression pattern revealed that positive staining was mainly localized in the cytoplasm of LSCC cells while strong staining was not observed in the non-cancerous tumor-adjacent areas. Typical IHC staining patterns of GROα in LSCC are shown in Figure 2.

Association between GROα expression and clinical attributes

The associations between expression of GROα protein and the clinical attributes of LSCC patients are shown in Table 1. Elevated GROα expression was significantly associated with TNM stage (p=0.035), lymph node metastasis (p=0.018) and histopathological grade (p=0.001). In contrast, no correlation was detected between GROα expression and other

Table 1. Association of GROα expression with clinical attributes of LSCC

Groups	No.	GROα		χ^2	p value
		High expression (%)	Low expression (%)		
Total	135	81(60.0)	54(40.0)		
Age(years)					
≤60 y	46	25(55.3)	21(45.7)	0.9288	0.335
>60 y	89	56(62.9)	33(37.1)		
Tobacco consumption					
Yes	74	53(71.6)	21(28.4)	0.1283	0.720
No	33	24(75.8)	8(24.2)		
Unknown	29	4(13.8)	25(86.2)		
Alcohol consumption					
Yes	52	38(73.1)	14(26.9)	0.0097	0.921
No	54	39(72.2)	15(27.8)		
Unknown	29	4(13.8)	25(86.2)		
TNM stage					
Stage I	13	9(69.2)	4(30.8)	4.4303	0.035*
Stage II	57	39(68.4)	18(31.6)		
Stage III, IV	33	29(87.9)	4(12.1)		
Unknown	32	4(12.5)	28(87.5)		
Lymph node metastasis					
Yes	20	17(85.0)	3(15.0)	5.6104	0.018*
No	114	65(57.0)	49(43.0)		
Unknown	1	0(0.0)	1(100.0)		
Histopathological grade					
High	61	28(45.9)	33(54.1)	15.5373	0.001*
Moderate	59	42(71.2)	17(28.8)		
Low	11	11(100.0)	0(0.0)		
Unknown	4	0(0.0)	4(100.0)		

* $p < 0.05$

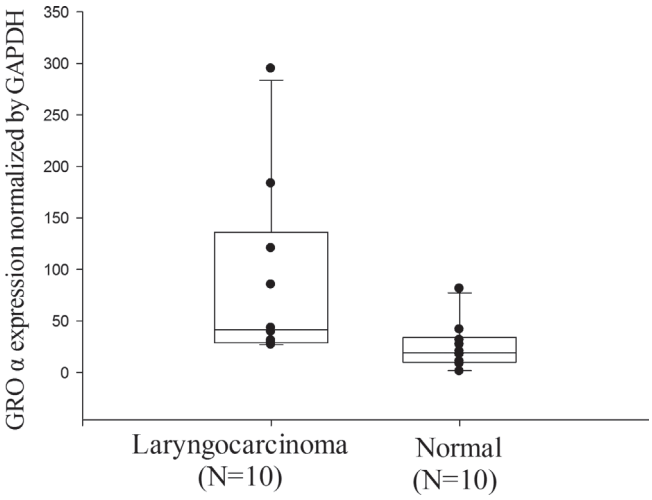

Figure 1. One-step quantitative real-time polymerase chain reaction (qPCR) evaluation of GROα mRNA expression levels in LSCC (cancer) and matched tumor-adjacent tissues (non-cancerous). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA levels were used for normalization (* p=0.001).

clinical items, such as age, use of either tobacco or alcohol consumption.

Survival analysis. Univariate analysis showed that the life span of LSCC patients was correlated with elevated GROα expression (p=0.001), TNM stage (p=0.003), and lymph node metastasis (p=0.001). Multivariate analysis further revealed that both increased GROα protein level (p=0.048) and lymph node metastasis (p=0.008) are two independent prognostic factors for overall survival (Table 2). Furthermore, Kaplan-Meier survival curves indicated that LSCC patients with low GROα expression and negative lymph node metastasis had a significantly longer mean survival time (Figure 3).

Discussion

Chemokines are a huge family of chemotactic signaling molecules that are attracting increasing attention for their role in tumorigenic mechanisms within malignant cells and the tumor microenvironment. Chemokines secreted by tumors not only attract infiltrating cells into tumor sites but may also contribute to tumor cell growth [24]. Accumulating evidence has indicated that chemokines play substantial roles in the proliferation, survival, and migration of tumor cells, suggesting their involvement in tumor development and invasion [10]. The chemokine GROα was first identified as an autostimulatory melanoma mitogen from the human malignant melanoma cell line Hs0294 [25]. Multiple studies have since implicated a relationship between the expression of GROα and various human cancers [10,15-18]. Recently, it has been reported that GROα plays a critical role in human cancers through several potential signaling pathways, including NF-κB [26], phosphatidylinositol-3-kinase [19] and MEKK1/p38 [27].

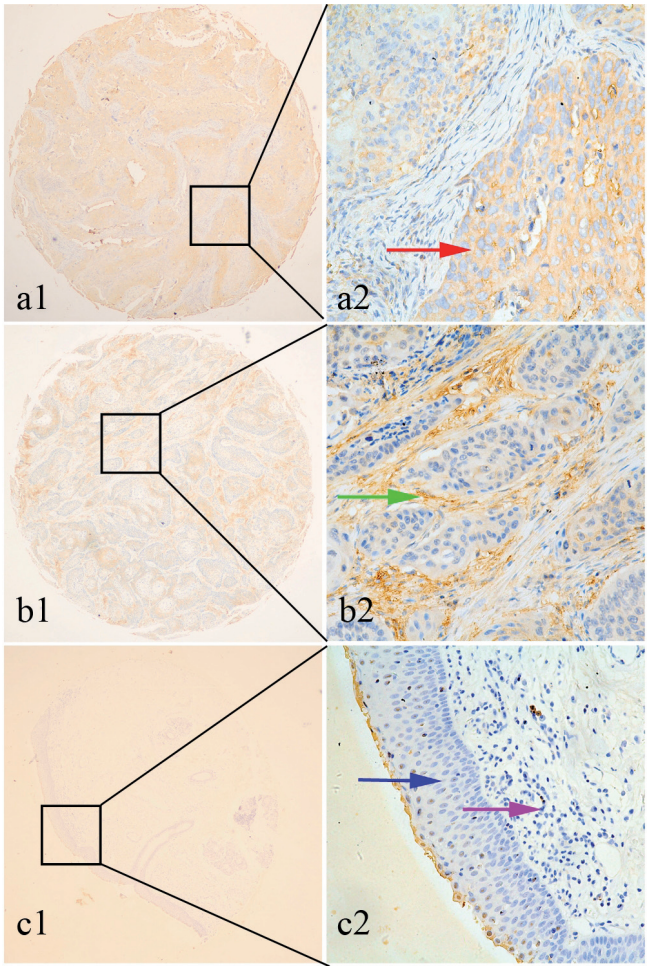

Figure 2. Representative hematoxylin-eosin (H&E) staining and patterning of GROα protein expression in tissue microarray (TMA) sections prepared from LSCC and adjacent non-cancerous tissue. a1 GROα-positive staining in LSCC tissue sample. a2 GROα-positive staining in LSCC cytoplasm (red arrow). b1 GROα-positive staining in LSCC tissue sample b2 GROα-positive staining in LSCC stromal cells (green arrow). c1 GROα-negative staining in tumor-adjacent non-cancerous tissue sample. c2 GROα-negative staining in LSCC (blue arrow) and in tumor-adjacent non-cancerous tissue sample. GROα-negative staining in LSCC stromal cells (purple arrow). Original magnification = ×40 in a1, b1, and c1; ×400 in a2, b2, and c2.

CXCR2, the receptor of GROα, interacts with this chemokine with high affinity. In our previous research we demonstrated that elevated expression of CXCR2 is significantly associated with the poor prognosis of LSCC [22]. Thus, although the exact function of GROα in LSCC remains to be elucidated, it appears reasonable to speculate that the GROα/CXCR2 axis is involved in its pathophysiology. In the present study, the clinicopathological significance of GROα in LSCC was evaluated with a particular focus on its prognostic value.

The results of qPCR showed that the mRNA level of GROα in LSCC was greater than that in non-cancerous tumor-adjacent tissues. The data are consistent with that reported in

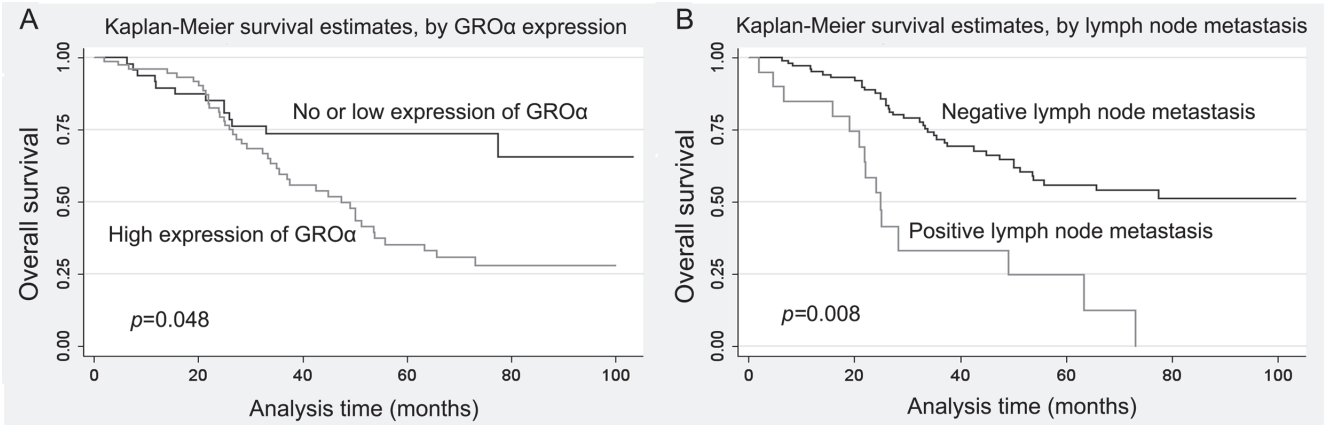

Figure 3. Survival analysis of LSCC patients by the Kaplan-Meier method. A Overall survival rate of patients with elevated (gray line) or low to no GROα expression (black line). B Overall survival rate of patients with positive (gray line) and negative (black line) lymph node metastasis.

a previous study, in which the mRNA expression of GROα was found to be significantly increased in colon adenocarcinoma tissue when compared with that in adjacent normal tissue [27]. We further investigated the expression of GROα protein levels in TMAs prepared from LSCC specimens. Consistent with the results of qPCR, IHC analysis also showed greater GROα expression in LSCC tissues than in normal tumor-adjacent tissues. The IHC staining pattern revealed that GROα protein was localized in the cytoplasm of cancer cells. It has been previously reported that positive GROα staining is diffuse and not associated with any specific cell types [28]. The reason for the different localization of GROα might be because of the use of dissimilar antibodies as well as differences in the samples collected.

Table 2. Univariate and multivariate analysis of prognostic factors in LSCC for overall survival

	Univariate analysis	Multivariate analysis	
	p >|z|	p >|z|	95% CI
GROα expression			
High vs Low	0.001*	0.048*	1.009-6.267
Age (years)			
≤ 60y vs > 60y	0.295		
Tobacco consumption			
Yes vs No	0.100		
Alcohol consumption			
Yes vs No	0.782		
TNM stage			
Stage I, II vs Stage III, IV	0.003*	0.098	0.934-2.248
Lymph node metastasis			
Yes vs No	0.001*	0.008*	1.278-4.999
Histopathological grade			
High vs Moderate vs Low	0.089		

* $p < 0.05$

GROα overexpression is also associated with the poor prognosis of various cancers, including breast cancer [29] and colorectal cancer [30]. In our study, increased GROα expression in LSCC was correlated with TNM stage, lymph node metastasis and histopathological grade. Univariate analysis showed that the overall survival of LSCC patients was associated with TNM stage, lymph node metastasis, and GROα expression. Multivariate analysis further revealed that the strong expression of GROα and positive lymph node metastasis correspond with the poor prognosis of LSCC patients. The Kaplan-Meier curve analysis of LSCC patients with elevated GROα expression and positive lymph node metastasis demonstrated unfavorable overall survival. All the above results suggest that GROα expression may be associated with the development and progression of LSCC; hence elevated GROα expression may serve as an independent prognostic marker of this disease. Further studies that enroll a larger scale of LSCC clinical samples will be necessary to confirm our results.

In conclusion, this study is the first to evaluate GROα mRNA expression with qPCR and protein expression with TMA in LSCC. Our results showed that elevation of GROα expression correlates with an aggressive malignant phenotype of LSCC. Therefore, GROα may be a valuable and promising prognostic marker of LSCC.

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