The prognostic implications of growth-related gene product β in laryngeal squamous cell carcinoma

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Abstract. Growth-related gene product β (GROβ) is an angiogenic chemokine that belongs to the CXC chemokine family, and a number of studies have suggested that GROβ is associated with tumor development and progression. However, a number of studies have investigated the association between GROβ expression and the clinical attributes of laryngeal squamous cell carcinoma (LSCC). In the present study, one-step quantitative polymerase chain reaction and immunohistochemistry analysis were used to detect GROβ expression and evaluate the association between its expression and the clinicopathological characteristics of LSCC. The results demonstrated that the GROβ mRNA and protein expression levels were significantly increased in LSCC compared with the corresponding non-cancerous tissues. GROβ protein expression in LSCC was associated with tumor-node-metastasis stage, lymph node metastasis and histopathological grade. The Kaplan-Meier method and Cox multi-factor analysis indicated that high GROβ expression, lymph node metastasis and histopathological grade were significantly associated with poor survival of patients with LSCC. These data indicated that GROβ may be a novel prognostic biomarker of LSCC.

Introduction

Laryngeal cancer, which is a common type of head and neck malignancy, is the 11th most common neoplasm in males (1). Laryngeal squamous cell carcinomas (LSCC) account for >95% of laryngeal cancers, and LSCC accounts for ~25% of all types of head and neck cancers (2). In the last decade, the incidence rate of LSCC has increased, with invasion and metastasis being the primary factors that affect the prognosis of patients, with a 5-year survival rate of ~60% (3,4). At present, laryngectomy is the major effective treatment strategy used for LSCC (5). In addition, larynx-preservation protocols using chemotherapy or radiotherapy are also developing (6). However, the aforementioned treatments often do not achieve a satisfactory clinical outcome and the overall survival of LSCC has not improved for years (7). There is a lack of sensitive and specific biomarkers to identify LSCC characteristics and to predict LSCC outcomes.

Growth-related gene product β (GROβ) is an angiogenic chemokine belonging to the CXC chemokine family, and growing evidence has indicated that chemokines are associated with tumor development and progression (8,9). GROβ was initially identified in melanoma cell lines, and high expression of GROβ was observed in human melanomas (10). Several previous studies have reported the involvement of GROβ in tumorigenesis (11,12). GROβ also attracted tumor cells and contributed to esophageal cancer cell transformation and growth (13). Blocking GROβ expression resulted in reduced proliferation and colonization capacity of esophageal cancer cells (14). Upregulation of GROβ-chemokine receptor 2 (CXCR2) signaling significantly increased the proliferation of cancer cells by modulating epithelial growth factor-1 (EGR-1) via extracellular signal-regulated kinase 1/2 (ERK1/2) (15). Previous studies have detected differentiated expression of GROβ, and high GROβ expression was associated with several malignant features of colorectal cancer, hepatocellular carcinoma and esophageal cancer (16-18). However, although GROβ exhibits oncogenic functions, the association between GROβ expression and LSCC characteristics remains to be fully determined. Additional studies are required to determine whether GROβ may serve as a biomarker for LSCC.

In the present study, the expression of GROβ in LSCC tissue was detected via one-step quantitative reverse-transcription polymerase chain reaction (RT-qPCR) and immunohistochemistry (IHC). The associations between GROβ expression and clinicopathological attributes of LSCC, in particular the prognostic status, were investigated.

Materials and methods

Patient specimens. A total of 20 samples of fresh LSCC tissues and corresponding non-cancerous tissues were collected

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from the Department of Pathology, the Affiliated Hospital of Nantong University (Nantong, China) between January 2013 and December 2014. All of the 20 samples were obtained from males (range, 48-70 years; average age, 60.30±5.42 years). Simultaneously, a total of 126 paraffin-embedded LSCC tissue samples (124 males and 2 females; range, 42-87 years; average age, 64.10±8.59 years) and 28 matched adjacent paracancerous tissue (<1 cm) samples obtained from males (range, 52-76 years; average age, 62.93±5.80 years), were collected from the archives of the Department of Pathology, the Affiliated Hospital of Nantong University, between January 2002 and December 2012. Diagnosis of LSCC was confirmed according to the latest World Health Organization criteria and tumor-node-metastasis (TNM) stage classification (19,20). The original clinical data were obtained from hospital medical records, and include details pertaining to patient sex and age, tobacco use, alcohol consumption, TNM stage, lymph node metastasis status and histopathological grade. None of the patients received radiotherapy or chemotherapy prior to surgery. Written informed consent was acquired from each patient enrolled in the present study. Ethical approval to perform the present study was granted by the Human Research Ethics Committee of the Affiliated Hospital of Nantong University.

One-step RT-qPCR. A total of 20 fresh LSCC tissue samples and corresponding non-cancerous tissue samples were used to perform one-step qPCR. Total RNA was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Expression levels of GROβ and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were determined by RT-qPCR using the iQ5 detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and the SensiMix One-Step SYBR-Green kit (Quantace Ltd., London, UK). The primers used were as follows: GROβ forward, 5'-CAC CAA CCA CCA GGC TAC-3' and reverse, 5'-CTT CAG GGT CAA GGC AAA-3'; and GAPDH forward, 5'-TAT TAC CTG GAC GAG ATT CCCC-3' and reverse, 5'-TAT TAC CTG GAC GAG ATT CCCC-3'. Amplification conditions were as described in previous studies (21-23). GAPDH was used as the reference gene to normalize the Cq values of cancer and control tissue samples. The calculation formula of all sample is $2^{-\Delta\Delta Cq}$ (24). Each experiment was repeated 3 times.

Tissue microarray (TMA) construction and IHC. A total of 126 LSCC tissues were prepared and TMAs were produced by Xinchao Biotech Co. Ltd. (Shanghai, China) to proceed IHC analysis. Core tissue biopsies (diameter, 2 mm) were taken from individual paraffin-embedded sections and arranged in the new paraffin blocks. The tissue microarray was cut into 4 μm sections and placed on microscope slides. IHC was performed as described previously (25,26). Briefly, TMA sections were incubated with a primary polyclonal anti-GROβ antibody (1:200; cat. no. ab10366; Abcam, Cambridge, UK) diluted in PBS at 4˚C for 8 h. Following washing with PBS at 37˚C for 30 min, sections were incubated with horseradish peroxidase-conjugated secondary antibody (1:2,000; cat. no. P0160; Dako; Agilent Technologies, Inc., Santa Clara, CA, USA). 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 a

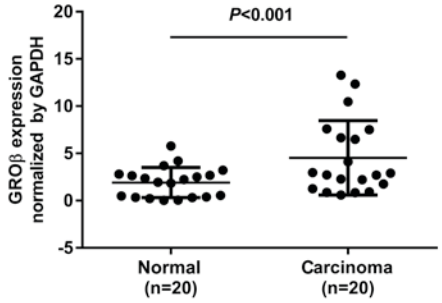

Figure 1. GROβ mRNA levels in LSCC tissues were significantly increased compared with corresponding non-cancerous tissues, as determined by reverse transcription-quantitative polymerase chain reaction, with data normalized to GAPDH mRNA levels (P=0.009). GROβ, growth-related gene product β; LSCC, laryngeal squamous cell carcinoma; GADPH, glyceraldehyde 3-phosphate dehydrogenase.

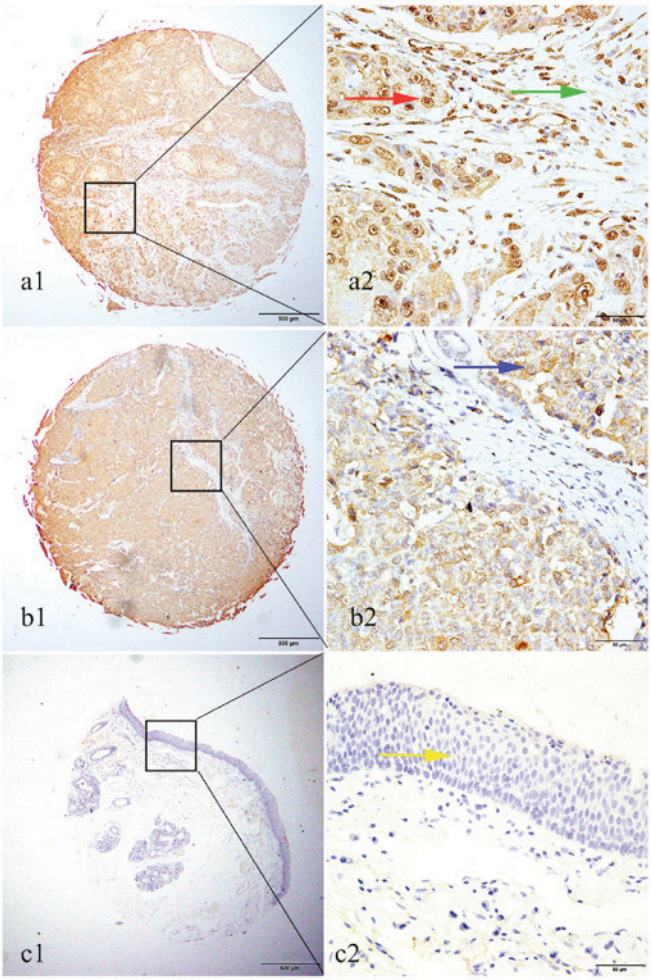

Figure 2. Representative pattern of GROβ protein expression in LSCC samples and corresponding non-cancerous tissue samples. (A1) Strong nuclear staining of GROβ in an LSCC tissue sample (original magnification, x40). (A2) Red arrow indicates positive staining in the nucleus of cancer cells. Green arrow indicates positive staining in the stroma of cancer cells (magnification, x400). (B1) Strong cytoplasmic staining of GROβ in an LSCC tissue sample (original magnification, x40). (B2) Blue arrow indicates positive staining in the cytoplasm of cancer cells (magnification, x400). (C1) Negative staining of GROβ in a corresponding non-cancerous tissue sample (original magnification, x40). (C2) Yellow arrow indicates negative staining in the epithelial cells (magnification, x400). GROβ, growth-related gene product β; LSCC, laryngeal squamous cell carcinoma.

Table I. Association of GROβ expression with clinical attributes of laryngeal squamous cell carcinoma.

Groups	n	GROβ expression, n		χ^2	P-value
		High expression	Low expression		
Total	126	72	54		
Age					
≤60 years	41	23	18	0.027	0.869
>60 years	85	49	36		
Tobacco consumption					
Yes	68	41	27	0.261	0.609
No	31	17	14		
Unknown	27	14	13		
Alcohol consumption					
Yes	48	31	17	1.381	0.240
No	51	27	24		
Unknown	27	14	13		
TNM stage					
Stage I, II	65	31	33	4.383	0.036
Stage III, IV	49	34	16		
Unknown	12	7	5		
Lymph node metastasis					
Yes	18	15	3	5.667	0.017
No	105	56	49		
Unknown	3	1	2		
Histopathological grade					
High	11	9	0	9.861	0.007
Moderate	65	37	28		
Low	47	26	21		
Unknown	3	0	5		

GROβ, growth-related gene product β; TNM, tumor-node-metastasis.

light optical microscope at magnifications x40 and x400, by two independent pathologists.

Expression levels of GROβ protein were evaluated by observing the staining density and intensity of positive cells as described previously (27,28). Staining density of positive cells was scored as follows: 0, negative; 1, 1-10% positive cells; 2, 10-50% positive cells, and 3, >50% positive cells. Similarly, staining intensity was scored as: 0, no color; 1, yellow for weak positive; 2, light brown for medium positive and 3, brown for strong positive. The two components were produced to obtain an overall expression score, as follows: 0, (-); 1-3, (+); 4-6, (++); and 7-9, (+++). The degree of GROβ staining was quantified using a two-level grading system, and staining scores were defined as follows: ≤3, low expression and 4-9, high expression.

Statistical analysis. The GROβ mRNA expression in fresh LSCC tissues compared with corresponding non-cancerous tissues was analyzed with the Wilcoxon signed-rank nonparametric test. The association of GROβ expression on clinicopathological items of LSCC was calculated by the χ^2 test. Univariate and multivariate analyses were performed using Cox proportional hazards regression models to explore the prognostic factors. The Kaplan-Meier method was utilized to evaluate the association between GROβ expression and LSCC outcomes. For all tests, $P<0.05$ was considered to indicate a statistically significant difference. All the statistical analyses were conducted using uSTATA (version 12.0; StataCorp LLC, College Station, TX, USA) and SPSS 18.0 statistical software (SPSS, Inc., Chicago, IL, USA).

Results

Detection of GROβ mRNA expression in LSCC by RT-qPCR. When normalized to GAPDH, the means of *GROβ* mRNA in LSCC and corresponding non-cancerous tissues were 4.53±0.882 and 1.91±0.358, respectively (t=2.746; P=0.009; Fig. 1). GROβ expression averaged 2.41-fold higher in the LSCC samples compared with the non-cancerous tissues (Fig. 1).

Detection of GROβ protein expression in LSCC by IHC. High GROβ expression was detected in 72 of 126 (57.1%) LSCC tissues, while only 2 cases of 28 non-cancerous tissues (7.1%) exhibited high GROβ expression. There was a significant difference in high expression rate

Table II. Univariate and multivariate analysis of prognostic factors in laryngeal squamous cell carcinoma for overall survival time.

	Univariate analysis		Multivariate analysis	
	P-value	95% CI	P-value	95% CI
GROβ expression				
High vs. low	0.004	1.411-6.249	0.043	1.025-5.164
Age				
≤60 vs. >60 years	0.197		0.125	
Tobacco consumption				
Yes vs. no	0.210		0.398	
Alcohol consumption				
Yes vs. no	0.595		0.226	
TNM stage				
Stage I, II vs. Stage III, IV	0.002	1.409-5.046	0.226	0.774-3.633
Lymph node metastasis				
Yes vs. no	0.001	2.122-8.706	0.007	1.418-9.339
Histopathological grade				
High vs. moderate vs. low	0.017	1.112-2.971	0.038	1.031-3.073

GROβ, growth-related gene product β; TNM, tumor-node-metastasis; CI, confidence interval.

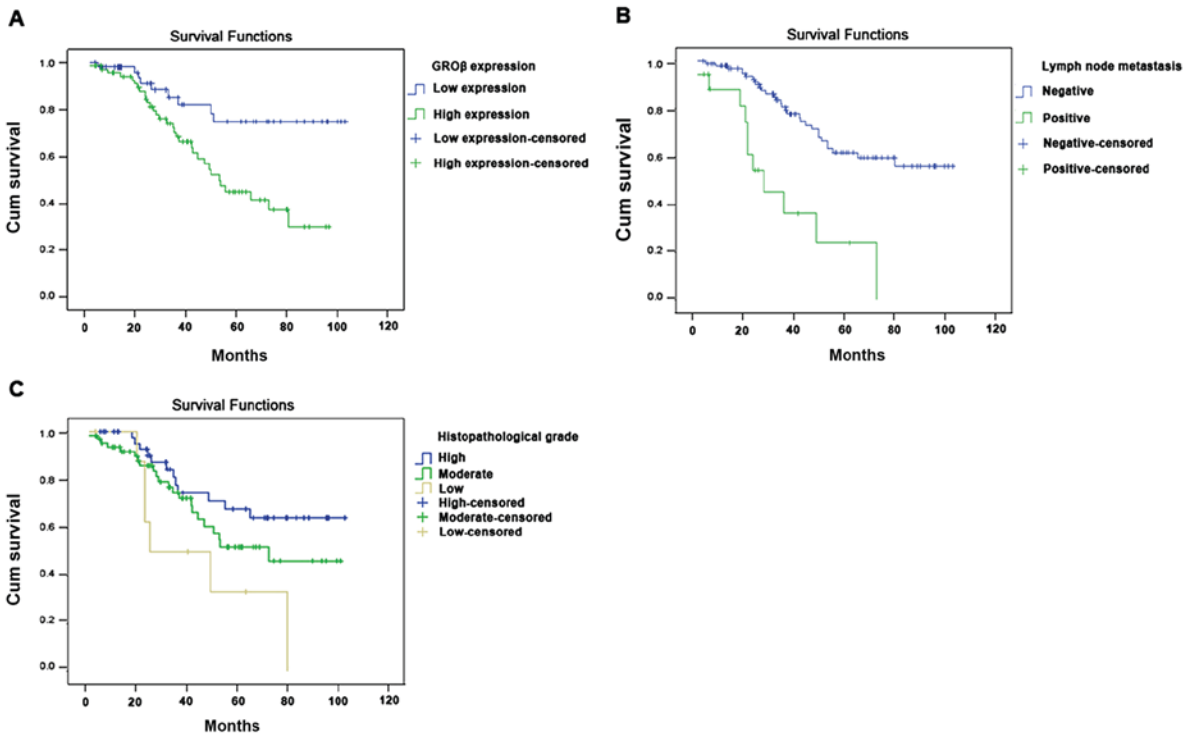

Figure 3. Survival analysis of patients with laryngeal squamous cell carcinoma using the Kaplan-Meier method. (A) Overall survival rate in patients with high GROβ expression (green line) was significantly lower compared with patients with low GROβ expression (blue line). (B) Overall survival rate in patients with positive lymph node metastasis (green line) was significantly lower compared with patients with negative lymph node metastasis (blue line). (C) Overall survival rate in patients with low histopathological grade (yellow line) was significantly lower compared with patients with moderate and high histopathological grade (green and blue line, respectively). GROβ, growth-related gene product β; Cum, cumulative.

of GROβ between LSCC tissues and non-cancerous tissues (P<0.001). Positive staining of GROβ was mainly localized in the nucleus of cancer cells (Fig. 2). Although positive cytoplasmic and stromal staining of GROβ was observed in certain cases, the case number was too small to perform statistics (Fig. 2).

Association between GROβ expression and clinical attributes. The associations between GROβ protein expression and the clinical characteristics of patients with LSCC are listed in Table I. Elevated GROβ expression was significantly associated with TNM stage (P=0.036), lymph node metastasis (P=0.017) and histopathological grade (P=0.007). By contrast, no association was detected between GROβ expression and other clinical characteristics, including age, tobacco or alcohol consumption (Table I).

Survival analysis. Univariate analysis revealed that the overall survival of patients with LSCC was associated with high GROβ expression (P=0.004), TNM stage (P=0.002), lymph node metastasis (P=0.001) and histopathological grade (P=0.017). Multivariate analysis identified that high GROβ expression (P=0.048), lymph node metastasis (P=0.007) and histopathological grade (P=0.038) were independent prognostic factors for overall survival (Table II). Furthermore, Kaplan-Meier survival curves indicated that patients with LSCC with low GROβ expression, negative lymph node metastasis and high histopathological grade had a significantly longer overall survival time (Fig. 3).

Discussion

Chemokines are a superfamily of small, cytokine-like proteins that interact with cell-surface receptors during development of the host immune response (29,30). Previous data have revealed that chemokines are involved in human cancer, in addition to their functions in development and inflammatory responses (31). GRO is a member of the CXC chemokine family, which is composed of GROα, GROβ and GROγ (32). GROα is expressed at high levels in a variety of tumors and is associated with tumor proliferation, angiogenesis and metastasis (33,34). A previous study also confirmed that high GROα expression is associated with an aggressive malignant phenotype of LSCC, and GROα may be a valuable prognostic biomarker for patients with LSCC (35). Several studies have explored the involvement of GROβ in tumor formation and development. For example, Wang *et al* (15) and Dong *et al* (18) reported that GROβ is highly expressed in esophageal squamous cell carcinoma. Doll *et al* (36) also reported a significantly elevated level of GROβ expression in colon carcinoma compared with normal tissue. GROβ may form an autocrine loop by binding its receptor CXCR2 and activating the Ras-ERK1/2 signaling pathway, which is important for cell proliferation (15). This pathway in turn enhances the transcription and expression of EGR-1, a transcription factor that regulates the expression of downstream factors associated with cell growth and cell cycle regulation, thereby promoting tumor progression (37). Based on this information, although the exact function of GROβ in LSCC remains to be investigated, it is reasonable to speculate that the GROβ/CXCR2 axis is involved in LSCC development. In the present study, the clinicopathological significance of GROβ in LSCC was detected with a particular focus on its prognostic characteristics.

The results of RT-qPCR demonstrated that GROβ mRNA levels were increased in LSCC compared with non-cancerous tissues. This data was consistent with that reported in a series of previous studies, in which the expression of GROβ was revealed to be significantly elevated in cancer tissues compared with normal tissues (15,18,36). The expression of GROβ was confirmed by conducting IHC. Consistent with the results of RT-qPCR, the IHC results revealed increased GROβ expression in LSCC tissues compared with non-cancerous tissues. The IHC staining pattern revealed that GROβ protein was mainly localized in the nucleus of LSCC cells. In addition, small LSCC cases exhibited positive cytoplasmic and stromal staining of GROβ. However, Ye *et al* (38) reported that GROβ was principally detected in the cytoplasm in ovarian cancer, and it was presumed that the reason for the differential distribution of GROβ may be due to the differences in cancer type, antibody used and experimental protocol. Additional studies that enroll a larger number of clinical samples of LSCC in particular cancer categories are necessary to validate the findings of the present study.

GROβ overexpression (including in serum, plasma and tissue) has been reported to be associated with several malignant features of human cancers (36,37). In the present study, high GROβ expression in LSCC was associated with three clinical pathological characteristics, namely TNM stage, lymph node metastasis and histopathological grade. In addition, univariate and multivariate analysis revealed the prognostic value of GROβ overexpression, indicating that patients with LSCC with high GROβ expression may have poor prognoses. The Kaplan-Meier curve also implied that high GROβ expression in patients with LSCC indicated unfavorable overall survival. The obtained data were consistent with the results of a previous study, which illustrated that high GROβ expression was associated with poor prognosis and contributed to ovarian cancer tumorigenesis and metastasis (38).

In conclusion, to the best of our knowledge, the present study was the first to examine GROβ mRNA expression with RT-qPCR and protein expression with IHC in LSCC. The results revealed that high GROβ expression may be associated with the development and progression of LSCC. Therefore, GROβ may be a useful biomarker for predicting the prognosis of LSCC, and targeting GROβ may provide a novel strategy for LSCC treatment.

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