

## Clinical significance of serum expression of GRO $\beta$ in esophageal squamous cell carcinoma

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ESCC patients than in healthy controls (median: 645 ng/L vs 269 ng/L,  $P < 0.05$ ). Serum GRO $\beta$  levels were correlated positively with tumor size, lymph node metastasis, and tumor-node-metastasis (TNM) staging, but not with gender or the histological grade of tumors in ESCC patients. The sensitivity and specificity of the assay for serum GRO $\beta$  were 73.61% and 56.63%, respectively.

**CONCLUSION:** GRO $\beta$  may function as an oncogene product and contribute to tumorigenesis and metastasis of ESCC.

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**Key words:** GRO $\beta$ ; Esophageal squamous cell carcinoma; Metastasis; Cytokine; Tumor markers

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### Abstract

**AIM:** To determine the association between serum levels of growth-related gene product  $\beta$  (GRO $\beta$ ) and clinical parameters in esophageal squamous cell carcinoma (ESCC).

**METHODS:** Using enzyme-linked immunosorbent assay, serum GRO $\beta$  levels were measured in ESCC patients ( $n = 72$ ) and healthy volunteers ( $n = 83$ ). The association between serum levels of GRO $\beta$  and clinical parameters of ESCC was analyzed statistically.

**RESULTS:** The serum GRO $\beta$  levels were much higher in

### INTRODUCTION

Esophageal squamous cell carcinoma (ESCC) is one of the most aggressive malignancies in China, South Africa, and some other developing countries<sup>[1,2]</sup>. In China, patients with ESCC have a poor prognosis, with an average 5-year survival rate of less than 30%<sup>[3-5]</sup>. It is thus imperative to find new markers, especially serum protein markers, to facilitate the early detection and diagnosis of ESCC.

CXC chemokine receptors are integral membrane proteins that specifically bind and are activated by cytokines

of the CXC chemokine family, a group of small cytokines<sup>[6]</sup> involved in the response to injury<sup>[7]</sup> and inflammatory reactions<sup>[8]</sup> as well tumorigenesis, cancer progression, and metastasis<sup>[9,10]</sup>. CXCL16 expression is increased in multiple tumor tissues and cell lines, and upregulation is associated with tumor progression and metastasis<sup>[11]</sup>. The plasma levels of CXCL4 and CXCL6 are much higher in patients with osteosarcoma than in healthy controls, and elevated plasma CXCL4 and CXCL6 levels are associated with poor prognosis<sup>[10]</sup>. Growth-related gene product (GRO) is a member of the CXC chemokine family, which is composed of GRO $\alpha$ , GRO $\beta$  and GRO $\gamma$ <sup>[12,13]</sup>. GRO $\alpha$  is highly expressed in a variety of tumors and plays an important role in tumor proliferation<sup>[14]</sup>, angiogenesis<sup>[15]</sup>, and metastasis<sup>[16]</sup>. Compared with GRO $\alpha$ , the roles of GRO $\beta$  in tumors are poorly understood.

Recently, authors from South Africa reported that GRO $\beta$  is highly expressed in ESCC tissues and cell lines<sup>[17]</sup>. Although this suggests that GRO $\beta$  may be secreted from tumor tissues into the extracellular milieu, no report has yet investigated serum GRO $\beta$  expression levels in human ESCC. In this study, we measured the serum levels of GRO $\beta$  in ESCC patients and healthy controls by enzyme-linked immunosorbent assay (ELISA), and analyzed the association between clinical parameters of ESCC and serum GRO $\beta$  levels.

## MATERIALS AND METHODS

### Clinical specimen collection and preparation

Serum samples were collected from 72 ESCC patients [52 males and 20 females; mean age, 61  $\pm$  7.7 years (SD); range 41-76 years] and 83 healthy controls [45 males and 38 females; mean age, 57  $\pm$  8.4 years (SD); range 41-70 years] after the informed consent was obtained. Pathological diagnosis was independently conducted by two senior pathologists. The healthy individuals were negative for hepatitis B virus, hepatitis C virus, and human immunodeficiency virus. Abdominal ultrasound examination, routine blood tests, and biochemistry tests were performed for the healthy controls, and the results were within normal ranges. Sample preparation was referred to the reference<sup>[18]</sup>. The clinical characteristics of the serum samples are shown in Table 1.

### ELISA for GRO $\beta$

A human GRO $\beta$  ELISA development kit (PeproTech, Rocky Hill, NJ, USA) was used to measure GRO $\beta$  in human serum. Briefly, 96-well ELISA microplates were coated overnight with 100  $\mu$ L GRO $\beta$  antibody (PeproTech, Rocky Hill, NJ, USA) at a final concentration of 0.25 mg/L in PBS. After washing with PBS/0.05% (w/v) Tween-20 (PBST, pH 7.4), the wells were blocked with blocking buffer at room temperature for 1 h. Then, 100  $\mu$ L diluted serum samples (at 1:5 dilution) were added and incubated at room temperature for 2 h. Similarly, 100  $\mu$ L PBST lacking antibody was used as a negative control. Following three washes with PBST, 100  $\mu$ L antibody diluted to a concen-

Table 1 Clinical parameters of esophageal squamous cell carcinoma patients and healthy controls *n* (%)

	ESCC patients ( <i>n</i> = 72)	Healthy controls ( <i>n</i> = 83)
Age (yr)		
< 50	5 (7)	16 (19)
$\geq$ 50	67 (93)	67 (81)
Gender		
Male	52 (72)	45 (54)
Female	20 (28)	38 (46)
Histological grade		
Well	16 (22)	-
Well/moderate	2 (3)	-
Moderate	33 (46)	-
Moderate/poor	3 (4)	-
Poor	15 (21)	-
Unknown	3 (4)	-
Tumor size (cm)		
< 5	32 (44)	-
$\geq$ 5	37 (51)	-
Unknown	3 (5)	-
TNM stage		
I	14 (19)	-
II	21 (29)	-
III	31 (43)	-
Unknown	6 (8)	-
Lymph node metastasis		
Yes	34 (47)	-
No	31 (43)	-
Unknown	7 (10)	-

ESCC: Esophageal squamous cell carcinoma.

tration of 0.25 mg/L was added. After incubation at room temperature for 2 h, 100  $\mu$ L avidin-horseradish peroxidase-conjugated secondary antibody (at 1:2000 dilution) was added, and plates were incubated at room temperature for 30 min. The excess conjugate was removed by washing the plates three times with PBST. The amount of bound conjugate was determined by adding ABTS liquid substrate solution to each well, and plates were incubated at room temperature for color development. The absorbance was measured at 405 nm using a Model 680 microplate reader (Bio-Rad Lab. Inc., Hercules, CA, USA). All analyses were performed in triplicate. The coefficient of variation was lower than 15% between analyses.

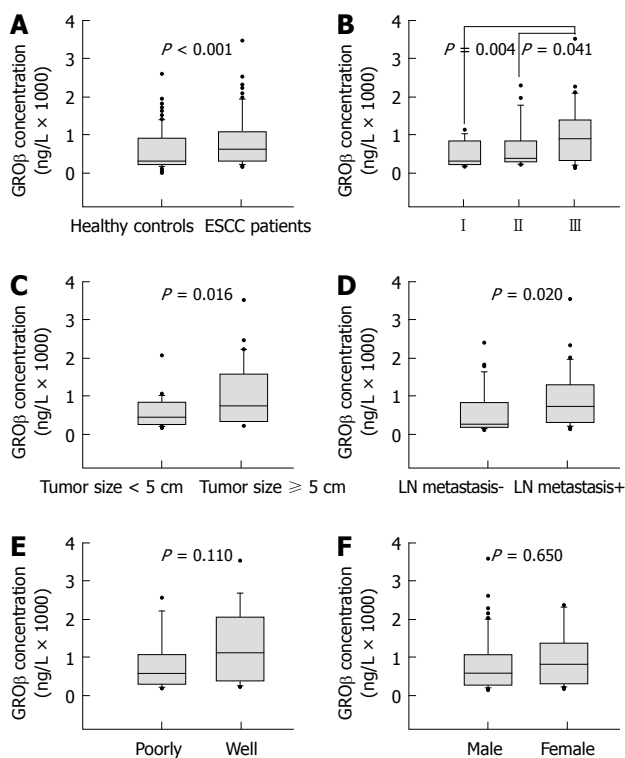
### Statistical analysis

Statistical significance was determined with the nonparametric Mann-Whitney *U*-test (differences between two groups) and the Kruskal-Wallis nonparametric test (differences among more than two groups) with SPSS 16.0 software (Chicago, IL, USA). Values of *P* < 0.05 were considered statistically significant.

## RESULTS

### Serum GRO $\beta$ levels in ESCC patients and healthy controls

A total of 155 participants were enrolled in this study. The serum levels of GRO $\beta$  in ESCC patients (*n* = 72) and healthy controls (*n* = 83) were assessed by ELISA.



**Figure 1** Correlations between serum growth-related gene product  $\beta$  level and clinicopathological parameters in esophageal squamous cell carcinoma.

Serum growth-related gene product  $\beta$  (GRO $\beta$ ) levels were measured in esophageal squamous cell carcinoma (ESCC) patients ( $n = 72$ ) and healthy volunteers ( $n = 83$ ) using enzyme-linked immunosorbent assay. Serum levels of GRO $\beta$  were significantly increased in ESCC patients (A). Serum levels of GRO $\beta$  were correlated positively with tumor-node-metastasis stage (B), tumor size (C), and lymph node (LN) metastasis (D), but not with histological grade (E) or gender (F) in ESCC patients. The boxes represent the distribution of the 25th and 75th percentile data (the line in the box is the median, and the dots are the outlying points).

Serum concentrations of GRO $\beta$  ranged from 146 ng/L to 3721 ng/L (median = 645 ng/L) in ESCC patients, and from 0 ng/L to 2781 ng/L (median = 269 ng/L) in healthy controls. GRO $\beta$  levels were significantly higher in ESCC patients than in healthy controls (Mann-Whitney  $U$ -test,  $P < 0.001$ , Figure 1A). The sensitivity and specificity of serum GRO $\beta$  were 73.61% and 56.63%, respectively.

#### Association between serum concentrations of GRO $\beta$ and clinical parameters in ESCC

We assessed the potential correlations between serum levels of GRO $\beta$  and clinical parameters, including tumor-node-metastasis (TNM) stage, tumor size, lymph node metastasis, histological grade, and gender. The median levels of GRO $\beta$  in the ESCC patients at different stages were: stage I, 284 ng/L (range, 146-1141 ng/L); stage II, 360 ng/L (range, 211-2453 ng/L); and stage III, 966 ng/L (range, 165-3721 ng/L). The serum GRO $\beta$  levels were significantly different (Kruskal-Wallis nonparametric test,  $P = 0.006$ ) among the three TNM stages, and significantly higher in patients at stage III than in those at stage II (Mann-Whitney  $U$ -test,  $P = 0.041$ , Figure 1B) and stage I (Mann-Whitney  $U$ -test,  $P = 0.004$ , Figure 1B). However, there was no signif-

icant difference between stage I and stage II (Mann-Whitney  $U$ -test,  $P = 0.135$ ). Furthermore, patients with tumor diameters  $\geq 5$  cm had higher serum levels of GRO $\beta$  (median = 786 ng/L; range, 191-3721 ng/L) than those with smaller tumors (median = 456 ng/L; range, 146-2008 ng/L; Mann-Whitney  $U$ -test,  $P = 0.016$ ; Figure 1C). Serum levels of GRO $\beta$  in patients with lymph node metastasis (median = 819 ng/L; range, 165-3721 ng/L) were significantly higher than those without lymph node metastasis (median = 330 ng/L; range, 146-2453 ng/L; Mann-Whitney  $U$ -test,  $P = 0.020$ ; Figure 1D). However, there was no significant difference between serum levels of GRO $\beta$  in patients with poorly differentiated tumors (median = 607 ng/L; range, 191-2705 ng/L) and well-differentiated tumors (median = 1178 ng/L; range, 239-3721 ng/L; Mann-Whitney  $U$ -test,  $P = 0.110$ , Figure 1E). Serum levels of GRO $\beta$  were not gender dependent (Mann-Whitney  $U$ -test,  $P = 0.650$ , Figure 1F).

## DISCUSSION

Identification of targets for early detection of ESCC is important to improve the prognosis of the patients with this pernicious disease. Currently, carcinoembryonic antigen, cytokeratin 19 fragments, and squamous cell carcinoma-associated antigen are routinely used as serum markers for detection of ESCC. Due to the low sensitivity and specificity of detection of these markers<sup>[19]</sup>, additional serum markers must be established for early detection and diagnosis of ESCC.

GRO $\beta$ , also known as the chemokine CXCL2, is a member of the CXC chemokine family and shares the same receptor with interleukin (IL)-8, interleukinIL-8RB/CXCR2. GRO $\beta$  is widely expressed in the central nervous system<sup>[20]</sup> and possesses important functions, such as attracting neutrophils to sites of inflammation, mobilizing stem cells<sup>[21-25]</sup>, and modulating neurotransmitter release<sup>[26]</sup>. The roles of GRO $\beta$  in tumor formation and development have been investigated<sup>[27]</sup>, and a recent report described significant elevation of GRO $\beta$  mRNA in colon cancer tissues<sup>[28]</sup>. Upregulation of GRO $\beta$  promotes colony formation by melanocytes in soft agar and tumor formation in nude mice<sup>[13,29]</sup>. Conversely, decreased expression of GRO $\beta$  inhibits the proliferation and colonization capacity of esophageal cancer cells<sup>[30]</sup>. Through binding to its receptor CXCR2, GRO $\beta$  forms an autocrine loop that activates the Ras-Erk1/2 signaling pathway, which is important for cell proliferation<sup>[17,25,26,31]</sup>. This pathway in turn activates the transcription and expression of the early growth response 1 gene (*egr1*), a transcription factor that regulates the expression of downstream factors related to cell growth and cell cycle regulation (p65, p27), thereby promoting tumor growth<sup>[32-34]</sup>.

Using cDNA microarray analysis, authors from South Africa showed that GRO $\beta$  was highly expressed in ESCC tissues and cultured cells<sup>[17]</sup>. As a chemokine, GRO $\beta$  may be secreted into the extracellular matrix. However, no report has investigated the clinical significance of serum GRO $\beta$  levels in ESCC. To compare the levels of serum GRO $\beta$  in patients with ESCC and healthy controls, we measured

serum GRO $\beta$  concentrations with ELISA. The level of serum GRO $\beta$  was much higher in patients with ESCC than in healthy controls, which supported the hypothesis that GRO $\beta$  may function as an oncogene in ESCC.

We assessed the potential correlations between serum levels of GRO $\beta$  and several clinical parameters of ESCC, including tumor size, TNM stage, lymph node metastasis, histological grade and gender. Serum levels of GRO $\beta$  were correlated significantly with tumor size and the extent of metastasis. Patients with larger tumors ( $\geq 5$  cm in diameter) had higher levels of serum GRO $\beta$  than those with smaller tumors ( $P < 0.05$ ). Likewise, serum GRO $\beta$  levels in patients with lymph node metastasis were significantly higher than those without lymph node metastasis ( $P < 0.05$ ). The elevation of serum GRO $\beta$  in ESCC patients may therefore reflect the enhanced potential of tumor cells to proliferate and metastasize, which is consistent with TNM staging<sup>[35]</sup>. Indeed, GRO $\beta$  serum levels were significantly increased in stage III patients compared with stage II and stage I patients ( $P < 0.05$ ). The histological grade of tumors is also an important indicator associated with the development and prognosis of cancer<sup>[36]</sup>. However, no significant differences were found in the serum levels of GRO $\beta$  in ESCC patients with different histological tumor grades ( $P > 0.05$ ) in our study. Because our study involved a relatively small number of ESCC patients and the sensitivity and specificity of our assay to measure serum GRO $\beta$  were not sufficiently high, further confirmation in a larger sample size is warranted.

In conclusion, we found that serum GRO $\beta$  levels were increased in ESCC patients and correlated positively with tumor size, TNM stage and lymph node metastasis, but not with gender or the histological grade of ESCC. GRO $\beta$  may function as an oncogene product and play a role in tumor formation, progression and metastasis. Examining and monitoring serum GRO $\beta$  levels may be useful in estimating the prognosis of ESCC.

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## COMMENTS

### Background

Esophageal squamous cell carcinoma (ESCC) is one of the most aggressive malignancies in China, South Africa and some other developing countries. In China, prognosis of the patients with ESCC is poor, with a 5-year survival rate of less than 30%. It is important to find new markers, especially serum protein markers to facilitate the early detection and diagnosis of ESCC.

### Research frontiers

Growth-related gene product  $\beta$  (GRO $\beta$ ) was reported to be highly expressed in ESCC tissues and cell lines. As a chemokine, GRO $\beta$  could be secreted from cells to extracellular matrix. But no report has yet investigated serum GRO $\beta$  expression levels in human ESCC. In the present study, serum level of GRO $\beta$  between patients with ESCC and healthy controls were firstly measured, and

the association between clinical parameters of ESCC and serum GRO $\beta$  levels were also analyzed.

### Innovations and breakthroughs

The serum level of GRO $\beta$  was increased in ESCC patients and was positively correlated with tumor size, tumor-node-metastasis staging and lymph node metastasis of the patients. GRO $\beta$  might serve as an oncogene and contribute to tumorigenesis and metastasis of ESCC.

### Applications

Examining and monitoring serum GRO $\beta$  levels are useful in estimating the prognosis of ESCC patients.

### Terminology

GRO $\beta$ , also known as CXCL2 chemokine, is a member of the CXC chemokine family. It is widespread in the central nervous system and involved in many important functions, such as attracting neutrophils to inflammation sites, mobilization of stem cells and neurotransmitter release modulation.

### Peer review

GRO, a member of the CXC chemokine subfamily, plays a major role in inflammation and wound healing. CXC chemokines have been found to be associated with tumorigenesis, angiogenesis and metastasis. The roles of GRO $\beta$  in tumor formation and development were unclear. The papers from tumor cell lines, animal and human studies on this field showed controversial results.

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