Reduced expression of secretogranin VGF in laryngeal squamous cell carcinoma

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Abstract. Laryngeal cancer accounts for one-third of all head and neck tumors, with squamous cell carcinoma (SCC) being the most predominant type, followed by neuroendocrine tumors. Chromogranins, are commonly used as biomarkers for neuroendocrine tumors, including laryngeal cancer. It has been reported that secretogranin VGF, a member of the chromogranin family, can be also used as a significant biomarker for neuroendocrine tumors. However, the expression and role of VGF in laryngeal carcinomas have not been previously investigated. Therefore, the present study aimed to determine the expression levels of VGF in laryngeal SCC (LSCC). The present study collected tumor tissues, as well as serum samples, from a cohort of 15 patients with LSCC. The results of reverse transcription-quantitative PCR, western blot analysis and immunofluorescence assays showed that the selective VGF precursor was downregulated in patients with LSCC. Notably, in tumor tissue, the immunoreactivity for VGF was found in vimentin-positive cells, probably corresponding to T lymphocytes. The current preliminary study suggested that the reduced expression levels of VGF observed in tumor tissue and at the systemic level could sustain LSCC phenotype. Overall, VGF could be a potential biomarker for detecting neoplastic lesions with a higher risk of tumor invasiveness, even in non-neuroendocrine tumors.

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Abbreviations: HNC, head and neck cancer; SCC, squamous cell carcinoma; LSCC, laryngeal squamous cell carcinoma; CGA, chromogranin A; NGF, nerve growth factor; proVGF, VGF precursor; FFPE, formalin-fixed paraffin-embedded; qPCR, quantitative real-time PCR; WB, western blotting; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CD3, cluster of differentiation 3

Key words: VGF, secretogranins, chromogranins, larynx cancer, squamous cell carcinomas, laryngeal squamous cell carcinoma, head and neck cancer, squamous cell carcinoma

Introduction

Laryngeal cancer, characterized by an increasing annual incidence, is the second most common head and neck type of cancer, accounting for ~20% of all head and neck cancer cases (1,2). According to the 2018 Global Cancer Statistics report, the incidence of laryngeal cancer was 2/100,000 individuals, with a mortality rate of 1/100,000 (3).

Laryngeal cancers are predominantly (95%) squamous cell carcinomas (SCC), followed by neuroendocrine neoplasms of the larynx, which are the most common non-squamous tumors of the larynx, despite their rarity (4,5).

Comprehensive treatment approaches, such as surgery, radiotherapy, chemotherapy and concurrent chemotherapy and radiotherapy have provided a higher 5-year survival rate (50-80%) in patients with laryngeal cancer (6). However, despite the current therapeutic advances, the survival rate of patients with laryngeal cancer remains poor due to the advanced stage of diagnosis, the high tumor recurrence rate, and distant metastases (7).

Early diagnosis serves a crucial role in the early detection of relapses and in reducing mortality via enhancing the effectiveness of the currently available therapeutic approaches. Therefore, identifying effective diagnostic and prognostic biomarkers for laryngeal cancer is critical to guide disease management and improve treatment outcomes.

The members of the chromogranin family, and more particular chromogranin A (CGA) and its proteolytically derived peptides, are widely used to identify particular types of tumors with a neuroendocrine-like phenotype (8) and allow the assessment of the malignancy grade and metastatic potential of tumors. Bartolomucci *et al* (9) demonstrated that CGA

is expressed in several types of endocrine and neuroendocrine tumors, such as prostate cancer (10), gastrointestinal neuroendocrine tumors (11) and neuroendocrine carcinomas of the head and neck, including laryngeal cancer (11-13).

In addition to CGA, other secretogranins have been also identified as potential endocrine tumor markers (14), including the VGF (non-acronymic) polypeptide, identified from the 'V' clone of the PC12 cDNA library (15).

In humans, VGF encodes a precursor protein (pro-VGF), which produces several peptides involved in food intake, energy balance and metabolism, water and electrolyte homeostasis, reproduction, pain, learning and memory (16). In addition, it has been reported that pro-VGF can promote neuronal growth and prevent apoptosis (17,18), while it serves a significant role in the pathogenesis of several types of neuroendocrine tumors (19-21).

In addition to neuroendocrine tumors, previous studies suggest that VGF could exhibit anticancer effects in non-endocrine tumors, such as breast (22), testicular (23) and ovarian cancer (24), thus indicating that VGF could be a potential biomarker in the above types of cancer. Therefore, dysregulation of VGF expression and processing could be dependent on tumor type.

As studies regarding the expression and role of VGF in laryngeal cancer are lacking, the present study aimed to investigate the expression profile of VGF in LSCC tumor tissues. In addition, since a previous study indicated that CGA levels in the blood of patients with endocrine tumors could act as a potential biomarker (25), the measurement of VGF in blood could provide an additional tool for the diagnosis and monitoring of laryngeal tumors.

Materials and methods

Patients. A total of 15 patients with LSCC were included in the present study. The protocol conformed to the Declaration of Helsinki and its later amendments and was approved by the internal Institutional Review Board (Ethical Committee of Sapienza University and Policlinico Umberto I, Rome, Italy; approval number: 6129).

The sites of the tumors and staging and grading were established according to the American Joint Committee on Cancer (26). Archival formalin-fixed paraffin-embedded (FFPE) tumor tissue from a laryngeal neuroendocrine carcinoma was used as positive control in the immunofluorescence experiments. Serum from five age-matched healthy subjects was used to quantitate VGF by western blotting for comparison with that of five of the patients with LSCC included in the present study. A clinical synopsis of the patients with LSCC included in the present study is in Table I.

Reagents. The following antibodies were used: Anti-VGF mouse monoclonal antibody (cat. no. sc-515482; Santa Cruz Biotechnology, Inc.); dilution for immunofluorescence, 1:100; dilution for western blotting (WB), 1:500; anti-GAPDH mouse monoclonal antibody (cat. no. sc-47724; Santa Cruz Biotechnology, Inc.; dilution for WB, 1:500); anti-Vimentin rabbit monoclonal antibody (cat. no. ab92547; Abcam; dilution for immunofluorescence, 1:500); anti-CD3 monoclonal antibody (cat. no. ab699; Abcam; dilution for immunofluorescence, 1:100). The secondary antibodies conjugated to horseradish peroxidase were purchased from Jackson ImmunoResearch Laboratories (cat. no. 111-035-003; cat. no. 115-035-003) and used at a dilution of 1:5,000. The Alexa Fluor488- and Alexa Fluor594-conjugated secondary antibodies were purchased from Thermo Fisher Scientific Inc. (cat. nos. A-11029 and A-11012) and were used at a dilution of 1:250. TRIzol® was purchased from Thermo Fisher Scientific Inc. Complete protease and phosphatase inhibitor cocktail (cOmplete, EDTA-free Protease and PhosSTOP tablets) were from Roche Diagnostics and the Chemiluminescence ECL kit was from Cytiva. Ponceau S Staining Solution and ProLong with DAPI were from Thermo Fisher Scientific Inc.

RNA extraction, retro-transcription and reverse transcription-quantitative (RT-q) PCR. The total RNA from frozen tumor and adjacent non-tumor tissue samples were extracted using TRIzol® reagent according to the manufacturer's instructions and was then reverse transcribed using a High-Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific). qPCR was performed using an iCycler Detection System (Bio-Rad Laboratories, Inc.). The cDNAs were amplified using iQ SYBR Green Supermix (Bioline; Meridian Bioscience) and specific sense and antisense human primers for the interest gene: VGF (Eurofins Genomics). Each reaction was performed in triplicate under the same thermal cycling conditions as follows: 95˚C for 10 min, followed by 40 cycles at 95˚C for 30 sec, 60˚C and 72˚C for 30 sec, to obtain the cycle time (Ct) mean. A reaction mixture without cDNA was used as control and post-amplification dissociation curves were performed to verify the presence of a single amplification product and the absence of genomic DNA contamination. The Ct mean value of the target gene was normalized to the Ct mean value of the house-keeping gene, 18S rRNA, and the comparative method ($2^{-\Delta\Delta Cq}$) (27) was obtained for each patient using gene expression value of normal tissues as calibrator. Data were reported as fold increase of the target gene mRNA compared to the normal tissues. The human primer sequences used in the present study were: VGF: F: 5'-AGCATAAAGAGCCGGTAGCC-3', R: 5'-GGAAAAGCTCTCCCTCGTCC-3'; 18SrRNA F: 5'-ACCGGGTTGGTTTTGATCTG-3', R: 5'-ATCCTGCCAGTAGCATATGC-3'.

Protein extraction. Protein extraction was carried out as previously described (28). Briefly, frozen tumor and adjacent non-tumor tissue samples were processed in lysis buffer (1% SDS; 1% NP-40, 5% glycerol and 5 mM EDTA) supplemented with complete protease and phosphatase inhibitor cocktail using a homogenizer (7 mm, OMNI International). Samples were boiled for 10 min and centrifuged for 20 min at 12,000 x g at 4˚C. Supernatants were collected and protein concentration was measured by a Qubit fluorometer (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. Protein extracts were stored at -80˚C until use.

WB analysis. WB analysis was carried out as previously described (28). Briefly, protein extracts (30 μg/lane) were electrophoresed through 10% SDS-PAGE and transferred onto nitrocellulose membranes (Cytiva). The membrane was stained with Ponceau Solution for 5 min at room temperature, and then washed. After blocking the proteins with 4% non-fat died milk (PanReac; AppliChem) for 2 h at room temperature, the primary antibodies incubation was performed overnight

Table I. Clinical synopsis of the patients with laryngeal squamous cell carcinoma included in the present study.

Patient number	Sex	Age	Tumor location	pTNM stage	American Joint Committee on Cancer stage	Grade
1	Male	79	Glottis	pT4aN0M0	IVa	G2
2	Male	78	Supraglottis	pT3N3bM0	IVb	G2
3	Male	77	Supraglottis	pT4aN0M0	IVa	G2
4	Male	61	Supraglottis	pT4aN1M0	IVa	G3
5	Female	74	Glottis	pT4aN0M0	IVa	G2
6	Male	63	Glottis	pT4aN2aM0	IVa	G2
7	Male	71	Glottis	pT3N0M0	III	G2
8	Male	56	Supraglottis	pT4aN1M0	IVa	G2
9	Male	58	Glottis	pT3N3bM0	IVb	G2
10	Female	75	Supraglottis	pT3N0M0	III	G2
11	Male	73	Supraglottis	pT3N3bM0	IVb	G2
12	Female	77	Glottis	pT3N0M0	III	G2
13	Male	63	Glottis	pT4aN0M0	IVa	G2
14	Male	78	Supraglottis	pT3N3bM0	IVb	G2
15	Male	78	Glottis	pT3N0M0	III	G2

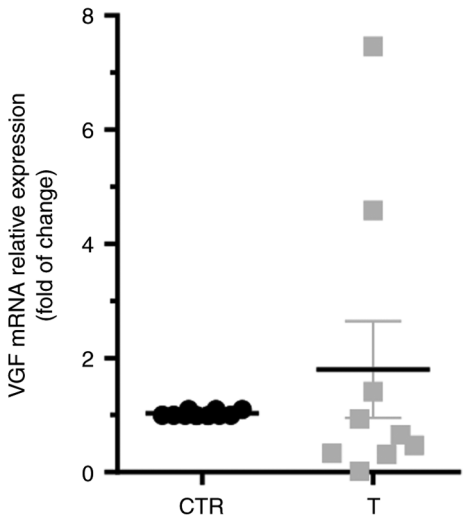

Figure 1. VGF mRNA analysis. The mRNA expression levels of VGF were detected by reverse transcription-quantitative PCR in tumor and normal adjacent tissues derived from a total of 11 patients with laryngeal squamous cell carcinoma. The results are expressed as fold of change. No significant differences were observed between the two groups (paired t-test). Data are expressed as the mean ± SD from three independent experiments. T, tumor; CTR, normal adjacent tissues.

at 4˚C. Membranes were then washed with PBS three times for 10 min and incubated with the secondary HRP-conjugated antibodies for 40 min at room temperature. After three washes in PBS, immunodetection of the reactive bands was revealed by chemiluminescence (ECL kit; Cytiva) and analyzed by iBright 1500 (Thermo Fisher Scientific Inc.). ImageJ v1.53a (National Institutes of Health) was used for densiometric analysis.

Immunofluorescence. Immunofluorescence analysis of FFPE samples was performed as described previously (28). Briefly, paraffin-embedded sections were dewaxed by two changes of xylene (5 min each) and hydrated in graded ethanol solutions (100, 90, and 70%, ethanol, for 2 min each). Sections were incubated in the antigen retrieval solution (10 mM sodium citrate, 0.05% Tween 20, pH 6.1) for 3x2 min and 4x30 sec into a microwave oven at 750 W. After cooling to room temperature slides were rinsed in PBS and blocked with 1% BSA in PBS for 1 h at room temperature. Samples were incubated at 4˚C overnight using the appropriate primary antibodies; washed three times in PBS/0.1% Tween 20; and incubated at room temperature with the appropriate secondary antibodies for 1 h. Slides were mounted with ProLong with DAPI (Thermo Fisher Scientific, Inc.) and examined by an epifluorescence microscope (Olympus BX53; Olympus Corporation) equipped with a SPOT RT3 camera. Images were merged using the image analysis software IAS 2000 (Delta Sistemi).

Blood collection. Peripheral blood samples of 5 ml were available from 5 of 15 LSCC patients included in the present study, and 5 age-matched healthy subjects. The samples were collected in BD Vacutainer Serum Separation Tubes (BD Biosciences) and centrifuged at 1,000 x g for 15 min at 4˚C to separate serum from plasma. Serum was then stored at -80˚C, until use.

Statistical analysis. All experiments were performed for at least three independent replicates. Data are presented as mean ± standard deviation Statistical analysis was performed using GraphPad Prism 9.4.1 software (GraphPad Software; Dotmatics). Data were analyzed using both the unpaired and paired t-test. $P<0.01$ was considered to indicate a statistically significant difference.

Results

Patients. A total of 15 patients with LSCC were enrolled in the present study. Among them, 11 patients provided tissue samples for WB and qPCR analysis, four patients for immunofluorescence staining and five patients for serum analysis. The clinical characteristics of patients with LSCC are listed in Table I.

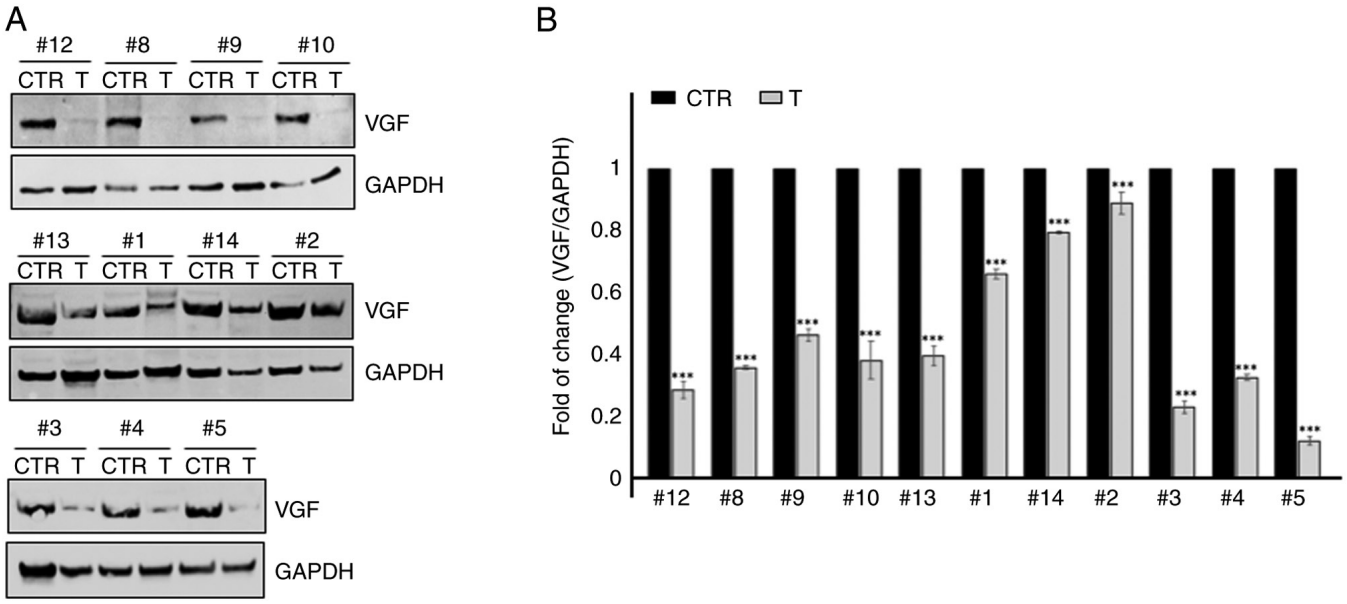

Figure 2. VGF protein expression levels in LSCC. (A) Representative western blot results from protein extracts derived from tumor or normal adjacent tissues of patients with LSCC are presented. The protein expression levels of VGF were determined in tissue samples derived from a total of 11 patients with LSCC. Equal amount of proteins was loaded per lane in SDS-PAGE and GAPDH served as a loading control. (B) Densitometric analysis of VGF expression, normalized to GAPDH expression, in tumor and normal adjacent tissues is presented. Data are expressed as the mean ± SD of three independent experiments. The results between the two groups were compared using paired t-test. Statistically significant differences are indicated by asterisks (***P<0.01). The densiometric analysis was carried out with ImageJ v1.53a software. LSCC, laryngeal squamous cell carcinoma; T, tumor; CTR, normal adjacent tissues.

Expression of VGF in LSCC tissues. To evaluate the expression profile of VGF in LSCC tissues, its mRNA and protein expression levels were detected by qPCR and WB, respectively (n=11 subjects for each assay). For each patient, the expression levels of VGF in LSCC tissues (T) were compared with those in normal adjacent tissues (CTR). As shown in Fig. 1, no significant differences were observed in the amount of VGF mRNA in tumor samples compared with CTR. In Fig. 2A, representative immunoblots obtained from 11 patients with LSCC are shown. WB revealed the presence of an immunoreactive band with a molecular weight of ~70 kDa, corresponding to human pro-VGF (19). Pro-VGF was mainly detected in CTR. WB was carried out using samples derived from 11 patients with LSCC and the expression levels of VGF were normalized to those of GAPDH. The results showed that pro-VGF was downregulated in tumor samples compared with CTR (Fig. 2B; P<0.01). In the current study, the expression levels of other pro-VGF-related peptides were not detected.

Localization of VGF in laryngeal tumor tissues. Immunofluorescence staining of tissues from primitive LSCC and LSCC with lymph node metastases was performed to evaluate the localization of VGF in SCC. Sections from FFPE laryngeal neuroendocrine carcinoma tissue samples served as a positive control. The neoplastic cells, as expected, were negative for vimentin. The cells immunoreactive for vimentin are 'stromal cells' (i.e., they are distributed among the nests) (29). As expected, a strong VGF immunoreactivity was observed in laryngeal neuroendocrine carcinoma tissues (Fig. 3A). By contrast, no immunoreactivity was obtained in LSCC tissues (Fig. 3B) and LSCC tissues with lymph node metastasis (Fig. 3C). However, a moderate immunoreactivity was detected around and within the SCC nests, co-localizing with vimentin, possibly representing tumor-(Fig. 3B) and nodal-related (Fig. 3C) T-lymphocytes (30,31). SCC tissues were also subjected to dual immunostaining for CD3 (T-lymphocytes marker) and vimentin (Fig. 3D). As expected, a CD3 immunostaining (green) was observed around and within the tumoral nests, co-localizing with vimentin (red; Fig. 3D), confirming the presence of T-lymphocytes. No immunoreactivity for VGF was observed in the epithelial lining of the larynx in sections from both LSCC and positive control tissues. However, unavailability of images of adjacent non-tumor tissue staining represents a limitation of the present study.

VGF levels in the serum of patients with LSCC. Since the protein expression levels of pro-VGF were decreased in tissues derived from patients with LSCC compared with CTR, the VGF content in the serum derived from a subgroup of five patients with LSCC were detected by WB. The serum levels of VGF in patients with LSCC were compared with those in age-matched healthy subjects. As shown in Fig. 4B, the levels of VGF-related peptide (~70 kDa) were significantly reduced in the serum from patients with LSCC compared with those in the serum of healthy donors (Fig. 4C; P<0.01).

Discussion

The present pilot study aimed to investigate the expression and the putative role, if any, of VGF in LSCC. Therefore, the expression profile of VGF-derived peptides in tumor tissues and serum of patients with LSCC was determined.

As expected, the results indicated that, at least in the larynx, SCC cells did not express VGF. This finding was consistent with that obtained in a previous study showing that only a very

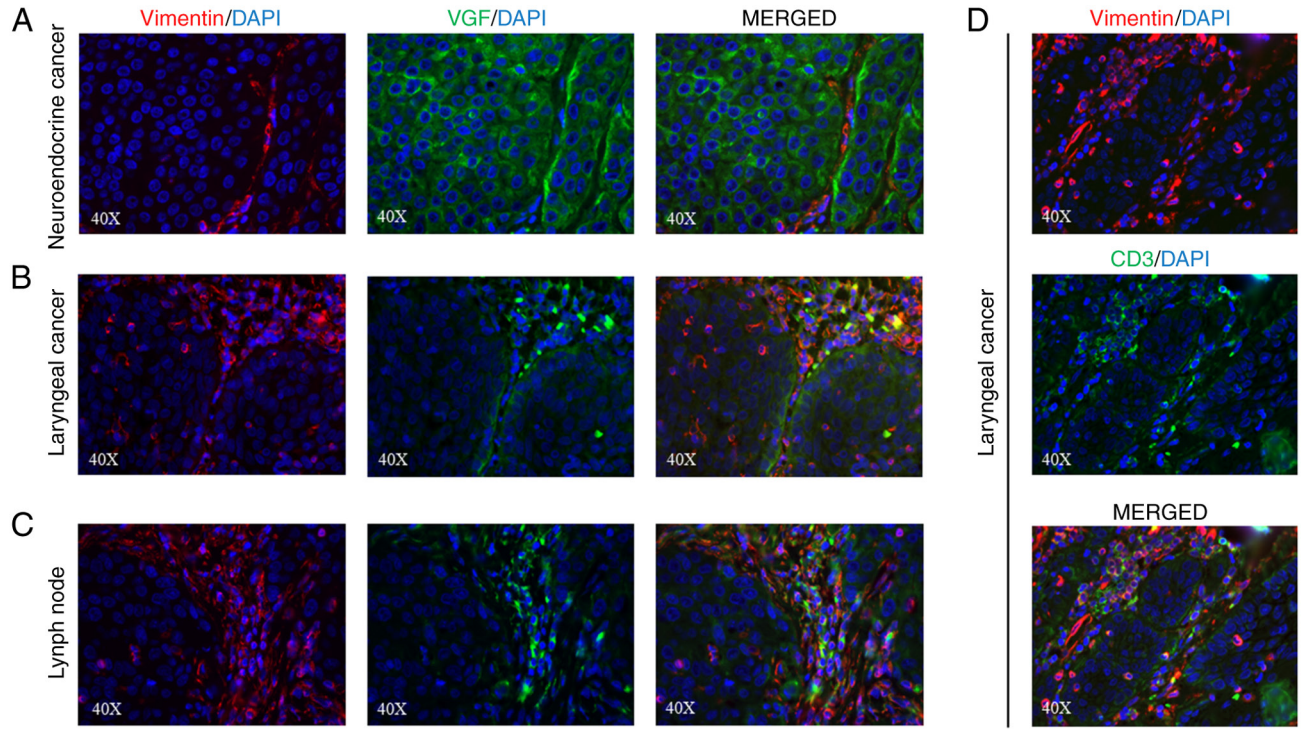

Figure 3. VGF localization in LSCC. Representative images of immunofluorescence assays are shown. De-paraffinized sections of (A) neuroendocrine carcinoma and (B) primitive and (C) metastatic LSCC were subjected to dual immunostaining for VGF (green) and vimentin (red). (D) Primitive LSCC was also subjected to dual immunostaining for CD3 (green) and vimentin (red). Nuclei were stained with DAPI (blue). Images were acquired at a magnification of 40x, as indicated. LSCC, laryngeal squamous cell carcinoma; T, tumor; CTR, normal adjacent tissues; CD3, cluster of differentiation 3.

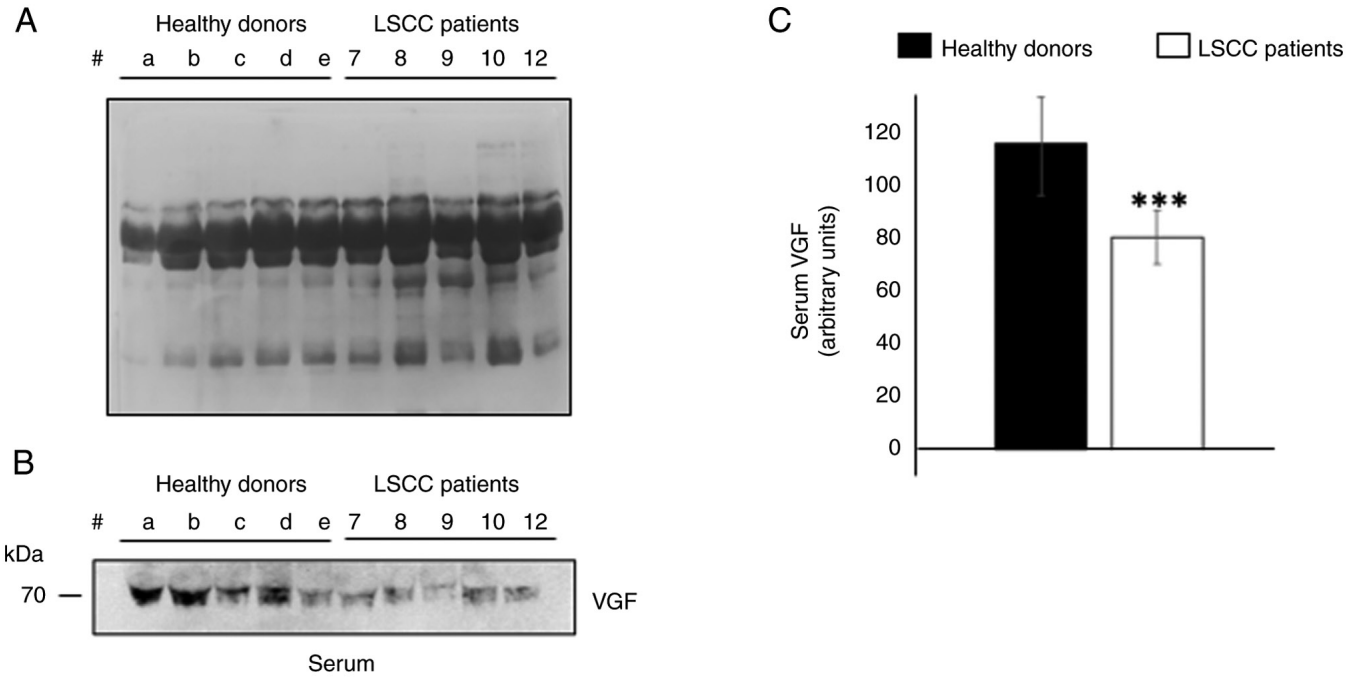

Figure 4. VGF levels in the serum of patients with LSCC. (A) The loading of serum was verified following the reversible staining of the membrane with Ponceau S. (B) Representative western blot results using serum samples from five patients with LSCC and five age-matched healthy donors are shown. Equal amount of serum was loaded per lane in SDS-PAGE. (C) Densitometric analysis of VGF immunoreactivity in the serum of patients with LSCC compared with healthy donors. Data are expressed as the mean ± SD of three independent experiments. The differences between two groups were compared using unpaired t-test. Statistically significant differences are indicated by asterisks (***P<0.01). The densiometric analysis was carried out with ImageJ v1.53a software. LSCC, laryngeal squamous cell carcinoma.

small fraction of head and neck SCCs could express neuroendocrine markers (12).

VGF is an active neuroendocrine regulatory polypeptide, mainly expressed in the human hypothalamus, in the medial

and lateral frontal gyrus and in several neuroendocrine tissues, including the pituitary gland and various gastrointestinal and pancreatic neuroendocrine cells (19). In addition to nerve growth factor, several stimuli can induce VGF expression, such as cell depolarization, growth factors, IL-6, insulin and cyclic adenosine monophosphate (19).

The VGF gene encodes a precursor protein, namely pro-VGF, with a molecular weight of ~70 kDa, which in humans consists of 615 amino acids (19). Pro-VGF is then processed by pro-protein convertases (PC1/3 or PC2), resulting in a series of VGF-related peptide fragments, which are stored in dense core granules and secreted via regulated pathways (32). It has been reported that several low molecular weight VGF-encoded peptides, covering ~20% of the pro-VGF sequence, including TLQP-21, TLQP-62 and AQEE-30, with total lengths of 21, 62 and 30 amino acids, respectively, exhibit several biological functions (15,33,34). Processing at different sites or under diverse conditions can result in different acting end products. However, the significance of VGF remains currently poorly understood (19).

Rindi *et al* (35) demonstrated that the expression of VGF-related peptides, such as that of pro-VGF, in human neuroendocrine cells could promote endocrine hyperplasia and neoplasia, depending on the cell type-specific processing of pro-VGF. This finding was further supported by the finding that 88/102 endocrine tumors tested were positive for the expression of VGF peptides, thus indicating that VGF could mark an active/proliferative state in response to specific stimuli (35). The increased expression and release of VGF-related fragment peptides have been also verified in large-cell neuroendocrine carcinoma of the lungs (21,36) and in breast cancer with neuroendocrine features (20). The present study also demonstrated that, in addition to CGA, VGF was also upregulated in neuroendocrine carcinomas of the larynx, thus suggesting that VGF could be considered as a potential novel biomarker for neuroendocrine tumors.

However, emerging evidence has also suggested that VGF exhibits different roles, as it possesses a protective effect on non-endocrine tumors, such as breast (22), testicular (23) and ovarian cancer (24). Therefore, it was hypothesized that the abnormal expression and processing of VGF could depend on tumor type.

RT-qPCR analysis revealed that the mRNA expression levels of VGF were comparable in LSCC tissues compared with the adjacent non-tumor tissues. By contrast, WB showed that the protein expression levels of pro-VGF were significantly reduced in LSCC tissues compared with CTR tissues. No other lower molecular weight bands were present in the membrane, at least not under the experimental conditions of the present study.

In addition, immunofluorescence assays verified the weak VGF immunoreactivity in primary LSCC and LSCC with lymph node metastasis. Notably, VGF immunoreactivity was observed in vimentin-positive cells within the stromal tissue in tumor samples, possibly corresponding to T-lymphocytes (30,31). Pro-VGF levels were also notably reduced in the serum of patients with LSCC compared with healthy donors, thus indicating that VGF could be downregulated both locally and systemically through post-transcriptional mechanisms.

The present results, obtained in the serum of a limited number of LSCC patients using WB, should be confirmed by quantifying the level of VGF in a larger number of patients, possibly using a more selective analysis, such as an ELISA test.

The current study also aimed to uncover the meaning of VGF downregulation in tissues and serum derived from patients with LSCC, as the significance of VGF precursor in the diagnosis or prognosis of patients is worth further exploration. Indeed, additional *in vivo* and *in vitro* experiments could confirm these preliminary findings and support evidence of abnormal expression and processing of VGF in this type of tumor. By using cellular models of human laryngeal carcinoma, it is hoped to provide further evidence of the VGF implication in cancer-relevant behaviors.

Given the role of VGF in regulating energy homeostasis and metabolism, it was hypothesized that VGF depletion in various types of tumor, such as LSCC, could promote the proliferation and spread of neoplastic cells. Indeed, previous studies show that VGF knockout mice are hyperactive and hypermetabolic (37). Hypermetabolism is a well-known feature of cancer, which allows tumor cells to undergo uncontrolled cell division and proliferation (38).

Growing evidence has also supported the significance of enhanced metabolism and thus energy production in the tumor microenvironment. The above effect negatively affects the availability of nutrients to immune cells, and more particular in tumor-invasive T cells, which also require a high metabolic energy status to function efficiently. Therefore, immune cells should compete with cancer cells for the available energy resources (39). However, whether VGF downregulation in SCC also serves a significant role in other sites either within or outside the head and neck region should be further investigated.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

MRa contributed to conception and design, responsible for supervision, funding acquisition and writing the original draft of the manuscript. CS was responsible for supervision and writing the original draft of the manuscript and performed the formal analysis. MV was responsible for supervision and performign formal analysis. AnC, AlC, MC, RL, RP and AG were responsible for investigation, writing, reviewing and editing; ER, MRi and EP were responsible for formal analysis and data curation. FG and DM were responsible for formal analysis, writing, reviewing and editing. MRa and CS confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board (or Ethics Committee) of Sapienza University and Policlinico Umberto I, Rome, Italy; approval number: 6129. Informed consent was obtained from all subjects involved in the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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