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Article

Evaluation of GDF15 Significance as a Biomarker in Laryngeal Squamous Cell Carcinoma

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Simple Summary: Laryngeal squamous cell carcinoma (LSCC) is a common head and neck cancer with limited survival rates, despite advances in treatment. Identifying reliable biomarkers could improve early diagnosis and treatment outcomes. Growth differentiation factor 15 (GDF15), a protein with roles in various cancers, was investigated in this study to determine its significance in LSCC. By analyzing tissue samples from patients and public datasets, we assessed whether GDF15 expression correlates with clinical factors and survival outcomes. Our findings suggest that GDF15 may serve as a prognostic biomarker in LSCC, potentially aiding in patient stratification and treatment planning. This research contributes to a better understanding of GDF15's role in cancer and highlights its potential utility in improving LSCC management. Future studies could build on these insights to explore GDF15's role as a therapeutic target.

Abstract: Background/Objectives: Laryngeal squamous cell carcinoma (LSCC) is a common malignancy with unsatisfactory survival rates, highlighting the need for reliable biomarkers to improve diagnosis and prognosis. Growth differentiation factor 15 (GDF15), a protein implicated in various cancers, has not been thoroughly investigated in LSCC. This study aimed to evaluate the significance of GDF15 expression in LSCC and its potential clinical implications. Methods: We analyzed archival tissue samples from 65 LSCC patients using immunohistochemistry and evaluated GDF15 expression profiles from The Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO) datasets. Statistical analyses included Kaplan-Meier survival analysis and Cox proportional hazards regression to assess the correlation between GDF15 expression, clinicopathological variables, and survival outcomes. Results: GDF15 expression showed no significant difference between tumor and adjacent normal tissues. However, in the tissue macroarray (TMA) cohort, high GDF15 expression was significantly associated with lower TNM stage and less advanced pT status. Kaplan-Meier analysis revealed that high GDF15 expression correlated with poorer overall survival in the TMA cohort but not in the TCGA or GEO datasets. Multivariable analysis identified GDF15 as an independent prognostic factor for disease-free survival in LSCC. Conclusions: Our findings suggest that GDF15 may serve as a prognostic biomarker in LSCC, particularly in early-stage disease. Further research is warranted to validate these results and explore GDF15's potential as a therapeutic target, contributing to improved patient management.

Keywords: laryngeal squamous cell carcinoma; GDF15; Tissue macroarrays; immunohistochemistry

1. Introduction

Laryngeal carcinoma remains the second most common malignancy of aerodigestive tract and makes up approximately 20% of head and neck cancers. It occurs almost five times more often in men than women. The vast majority of cases is squamous cell carcinoma, whose most significant risk factors are tobacco and alcohol consumption [1–3]. Patients with laryngeal squamous cell carcinoma (LSCC) are often diagnosed in the advanced stage of the disease and the 5-year survival rate oscillates around 65%. Although radiation and systemic therapy appeared as new treatment options, surgery still remains equal or necessary depending on stage and expansion of the disease [3]. Since survival remains unsatisfying, it remains urgent to identify specific biomarkers of the disease.

GDF15 (Growth Differentiation Factor 15) also known as MIC-1 (Macrophage Inhibitory Cytokine-1), PLAB (Placental Bone Morphogenetic Protein), P-TGF β (Placental Transforming Growth Factor Beta), PDF (Prostate-Derived Factor), HP00269 is a member of the TGF- β superfamily. The GDF15 gene is located on chromosome 19p12–13 and encodes a nuclear protein of 25 kDa. GDF15 is a stress responsive cytokine best known from its anorectic action [4,5]. However, this protein has vast biological activity, including roles in inflammation, cardiovascular diseases, and carcinogenesis. The role of GDF15 in carcinogenesis can be protumorigenic or antitumorigenic depending on the microenvironment, type and stage of cancer [6,7]. As a protumorigenic action we can count contribution to proliferation and survival of cancer cells, angiogenesis, metastasis and bone lysis. GDF15 presents protumorigenic action by affecting many signal pathways, for instance: ERK (extracellular signal-regulated kinases) pathway, ErbB2 (receptor tyrosine-protein kinase erbB-2), uPA (urokinase-type plasminogen activator), RhoE (Rho-related GTP-binding protein), catenin- δ 1 (ang. catenin-cadherin associated protein, δ 1), PI3K/AKT, MAPK. Additionally, GDF15 is a target gene for tumor suppressor protein p53 [6]. GDF15 is present in different tissues including kidneys, liver, muscles, fat tissue and macrophages and its expression increases in pregnancy, inflammation, cancers, heart and kidneys failure correlating with poor prognosis and weight loss [8,9]. Overexpression of GDF15 has also been observed in many cancers including breast, cervical, prostate, colorectal and oral squamous cell carcinoma [10–13]. Confirmed by many studies GDF15 is classified as a new potential cancer biomarker. However, the correlation between GDF15 and laryngeal squamous cell carcinoma remains unclear.

This study was conducted to investigate this correlation in order to improve diagnosis, prognosis and molecular target therapy.

2. Materials and Methods

Patients and Tissue Material

The study was conducted on archival formalin-fixed, paraffin-embedded (FFPE) tissue samples derived between 2016 to 2020 from 65 patients hospitalized and diagnosed with laryngeal squamous cell carcinoma at the Department of Otolaryngology, Oncological Laryngology and Maxillofacial Surgery, University Hospital No. 2, Bydgoszcz, Poland and the Department of Clinical Pathomorphology, Collegium Medicum in Bydgoszcz, Nicolaus Copernicus University in Toruń, Poland. The institutional tissue macroarray cohort was thereafter referred to as 'TMA cohort'. All tumors were reclassified in accordance with the standardized TNM 8th edition classification of the American Joint Committee on Cancer (AJCC) criteria. The study group involved 65 laryngeal squamous cell carcinoma samples, while the control group included 21 samples of normal tissue adjacent to the tumor (NAT). Patients were excluded when they had a second primary carcinoma or tumor in situ (Tis), as well as when no cancer cells were detected by immunohistochemical staining, or if patients received prior treatment. Clinicopathological data of 65 patients with laryngeal squamous cell carcinoma is presented in Table 1. The study protocol was approved by the Bioethics Committee at Collegium Medicum in Bydgoszcz of Nicolaus Copernicus University in Toruń KB 58/2022 and performed according to the guidelines of the Declaration of Helsinki.

Table 1. Association of GDF15 with clinicopathological variables in the TMA cohort.

Variable	n (%)	GFD15 expression		p value
		low	high	
Age (years)				
≤ 60	17 (26.15)	7 (41.18)	10 (58.82)	>0.999
> 60	48 (73.85)	21 (43.75)	27 (56.25)	
Gender				
Male	55 (84.62)	22 (40.00)	33 (60.00)	0.306
Female	10 (15.38)	6 (60.00)	4 (40.00)	
Tumor grade				
G1	16 (24.62)	6 (37.50)	10 (62.50)	0.854
G2	45 (69.23)	20 (44.44)	25 (55.56)	
G3	4 (6.15)	2 (50.00)	2 (50.00)	
pT status				
T1	32 (49.23)	8 (25.00)	24 (75.00)	0.02
T2	23 (35.39)	14 (60.87)	9 (39.13)	
T3-T4	10 (15.38)	6 (60.00)	4 (40.00)	
pN status				
N0	52 (80.00)	20 (38.46)	32 (61.54)	0.210
N1-N2	13 (20.00)	8 (61.54)	5 (38.46)	
Resection margin				
negative	50 (76.92)	23 (46.00)	27 (54.00)	0.554
positive	15 (23.08)	5 (33.33)	10 (66.67)	

TNM stage				
I	30 (46.15)	7 (23.33)	23 (76.67)	0.019
II	17 (26.15)	11 (64.71)	6 (35.29)	
III	7 (10.77)	3 (42.86)	4 (57.14)	
IV	11 (16.92)	7 (63.64)	4 (36.36)	
Tumor location				
Glottis	34 (52.31)	9 (26.47)	25 (73.53)	0.001
Supraglottis	10 (15.39)	3 (30.00)	8 (70.00)	
Subglottis+others	21 (32.31)	16 (76.19)	5 (23.81)	
Smoking status				
Non-smoker	22 (33.85)	8 (36.36)	14 (63.64)	0.335
Current smoker	36 (55.38)	19 (52.78)	17 (47.22)	
Ex-smoker	4 (6.15)	1 (25.00)	3 (75.00)	
N/A	3 (4.62)	-	-	

'others' includes overlapping lesion of larynx; N/A not available.

Tissue Macroarrays

Tissue macroarrays (TMA) were constructed as previously described [14]. In brief, representative areas within the tissue samples, containing at least 80% of tumor cells, were identified under the microscope using archival tissue sections stained with hematoxylin and eosin (H&E). Then, fragments of FFPE tissue specimens were removed from every donor block and inserted into recipient blocks. The prepared macroarrays blocks were routinely sectioned at 3 μm thickness using a rotary microtome (Accu-Cut® SRMTM200, Sakura Finetek, Torrance, CA, USA) and placed on high-adhesive glass slides (Superfrost Plus; MenzelGlasser, Germany) afterward.

Immunohistochemistry

The sections (3 μm thick) were subjected to IHC staining according to the previously described protocol [15]. Immunohistochemistry was carried out by BenchMark® Ultra automatic staining machine (Roche Diagnostics/Ventana Medical Systems, Tucson, AZ, USA) with Ventana UltraView DAB Detection Kit (Ventana Medical Systems, Tucson, AZ, USA). GDF15 was detected by using anti-GDF15 rabbit polyclonal antibody (cat. no: HPA011191, Sigma-Aldrich, St. Louis, MO, USA) in a 1:150 dilution for 32 min. Positive and negative controls were set for each test.

Evaluation of Immunohistochemical Reactions

The immunohistochemical expression of GDF15 protein was evaluated by two independent pathologists using a modified immunoreactive score of Remmele and Stegner (IRS) [16]. The staining score (0-12) was determined by multiplying the percentage of positively stained cells/tissue area (0: 0%; 1: 1-5%; 2: 6-25%; 3: 26-75%; 4: 76-100%) and the expression intensity (0: negative, 1: weak, 2: moderate, 3: strong). The analysis was conducted at 20× original objective magnification in three randomly selected fields of view, and the final staining score was presented as a mean of three results. To determine “high” or “low” expression levels of GDF15 protein, the final IRS scores (0-12) were dichotomized based on the cut-off point (IRS < 4, low expression; IRS ≥ 4, high expression) established with the Evaluate Cutpoints software [17]. Of note, the median expression level of GDF15 protein in tumor tissue was 4 (IRQ, 3-5.835).

Overall survival (OS) was defined as the time from date of curative surgery to the time of death from any cause. Disease free survival was defined as the time from date of curative surgery to the time of relapse or death. Overall survival (OS) and disease-free survival (DFS) data were censored if patients were alive at the last follow-up date (11 January 2023). The median follow-up time (calculated by the reverse Kaplan-Meier method) for OS and DFS was 49 and 46 months, respectively.

Extraction of RNA-Sequencing TCGA Data and Clinical Information

RNA-sequencing data (DESeq2 normalized counts) for GDF15 expression in 116 LSCC tissues and 12 adjacent tissues were downloaded from The Cancer Genome Atlas (TCGA) using the University of California Santa Cruz (UCSC) Xena Browser (<https://xenabrowser.net/>, accessed on 10 February 2023), whereas corresponding clinical information was retrieved from cBioPortal (<https://www.cbioportal.org>, accessed on 10 February 2023). The clinical endpoints were OS and DFS. The former was defined as the time from date of curative surgery to the time of death from any cause, while the latter as the time from date of curative surgery to the time of recurrence or progression. Overall survival and DFS data were available for 116 (100%) and 87 (75%) patients, respectively. GDF15 expression levels were categorized into low-level and high-level groups based on the optimal cut-off values determined with the Evaluate Cutpoints software [18]. The cut-point values for OS and DFS were 9.731 (< 9.731, low expression; ≥ 9.731, high expression) and 7.217 (< 7.217, low expression; ≥ 7.217, high expression), respectively. The median OS and DFS times for the TCGA cohort were 65 and 76 months, respectively.

GEO Data Collection

The expression levels of GDF15 were extracted from the Gene Expression Omnibus (GEO) database through the ShinyGEO web-based application (<http://gdancik.github.io/shinyGEO/>, accessed on 13 March 2023) [18]. Five GEO datasets for LSCC: GSE25727 [19], GSE27020 [20], GSE59102 [21], GSE117973 [22,23], and GSE51985 [24] were included. If several probes were mapped to the GDF15, the mean value was utilized as the final expression of this gene.

Statistical Analysis

All statistical analyses were performed using the GraphPad Prism (version 8.0, GraphPad Software, San Diego, CA, USA), SPSS (version 28.0, IBM Corporation, Armonk, NY, USA) or Rstudio (version 1.3.1093) software packages. Data distribution was determined with the Shapiro-Wilk test and the appropriate parametric or nonparametric statistical tests were then applied. The Kaplan-Meier method and log-rank test was used to depict and compare unadjusted survival curves for OS and DFS. Univariable and multivariable Cox proportional hazards regression analyses were conducted to explore the predictors of survival by estimating the hazard ratio (HR) and 95% confidence interval (CI). A backward elimination procedure built all multivariable models, with a significance level of $p < 0.2$ to enter the model and $p \leq 0.05$ to stay. Proportional hazard assumption was verified by testing for significant interactions when each variable was entered as a time-based

covariate. In the TCGA cohort, multiple imputation of missing variables for the Cox proportional hazards models was used to adjust for the bias of missing data, but the variables with amounts of missing data over 20% were not considered for the model. It was assumed that the missing data of predictor variables occurred at random. Combined estimates were obtained from 10 imputed datasets. Associations were considered statistically significant if the two-sided p-value was < 0.05.

3. Results

Comparison of GDF15 Expression Between Tumor Tissue and Non-Tumor Tissue in TMA and Publicly Available Cohorts

There was no statistically significant difference in GDF15 expression between tumor tissue and non-tumor tissue at both protein and mRNA expression levels (Figure 1). Boxplot graphs in Figure 1 illustrate GDF15 expression levels in tumor tissue versus histologically normal tissue across various cohorts, including the institutional TMA, TCGA, and gene expression data from the GEO.

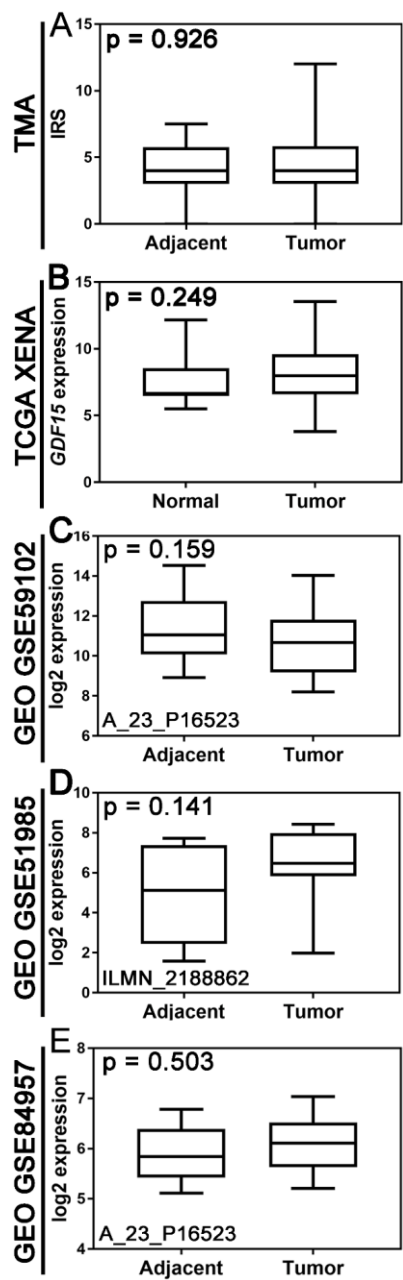


Figure 1. Expression of GDF15 in laryngeal squamous cell carcinoma compared to non-cancerous control tissue. Boxplot graphs of (A) GDF15 expression in tumor tissue (n = 65) and histologically normal tissue adjacent to the

tumor (n = 21) of the institutional tissue macroarray (TMA) cohort based on immunohistochemistry (IHC); (B) GDF15 expression levels in tumor tissue (n = 116) and normal solid tissue (n = 11) based on RNA-Seq data from The Cancer Genome Atlas (TCGA) and Genotype-Tissue Expression (GTEx) databases, respectively, downloaded from the UCSC Xena Browser. (C) GDF15 expression in tumor tissue (n = 29) and histologically normal tissue adjacent to the tumor (n = 13) based on the gene chip data derived from the Gene Expression Omnibus (GEO) database through the ShinyGEO web-based tool. The GSE number and probe information are shown. (D) GDF15 expression in tumor tissue (n = 10) and corresponding adjacent non-neoplastic tissue (n = 10) based on the gene chip data derived from the Gene Expression Omnibus (GEO) database through the ShinyGEO web-based tool. The GSE number and probe information are shown. (E) GDF15 expression in tumor tissue (n = 9) and corresponding adjacent non-neoplastic tissue (n = 9) based on the gene chip data derived from the Gene Expression Omnibus (GEO) database through the ShinyGEO web-based tool. The GSE number and probe information are shown.

At the protein level, GDF15 expression, assessed by immunohistochemistry (IHC), revealed primarily cytoplasmic staining at any level of expression intensity in tumor tissues and non-tumor tissues adjacent to the tumor. Representative IHC images demonstrate the variation in GDF15 staining intensity between tumor tissues (Figure 2). However, tumor tissues showed a more heterogeneous and often stronger staining pattern compared to the adjacent normal tissues. Additionally, the staining pattern observed in the tumor tissues exhibited significant heterogeneity, with variations in intensity across different regions of the same sample.

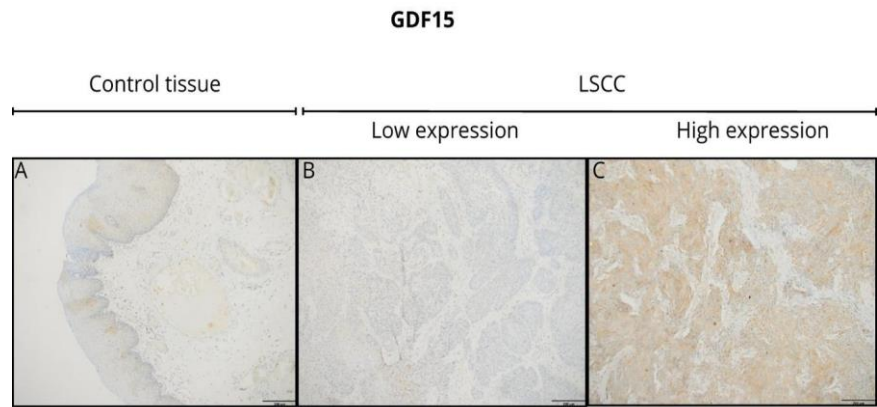


Figure 2. Representative images of immunohistochemical expression of GDF15 in laryngeal squamous cell carcinoma (LSCC) (B-C) and histologically normal tissue adjacent to the tumor (control tissue) (A). Original magnification 10x.

Association Between GDF15 Expression and Clinicopathological Variables in TMA and TCGA Cohorts

After data dichotomization according to the established cut-off values, the TMA cohort included 28 (43.08%) samples with low and 37 (56.92%) samples with high GDF15 expression and the TCGA cohort included 89 (76.72%) samples with low and 27 (23.28%) samples with high gene expression.

In the TMA LSCC cohort (Table 1), high GDF15 expression was significantly associated to less advanced pT stages (p=0.02). Moreover, high GDF15 expression was significantly associated with lower TNM stage (p=0.019). A statistically significant relationship was also demonstrated between GDF15 expression and the location of the primary tumor (p=0.001), with higher expression being associated with the location of glottis. No other associations existed between GDF15 expression and investigated clinicopathological parameters.

In the TCGA cohort, there were no statistically significant differences in clinicopathological variables depending on GDF15 expression status (Table 2). However, the association between GDF15 expression and N status was of borderline statistical significance (p = 0.047; Table 2).

Table 2. Association of *GDF15* with clinicopathological variables in the TCGA cohort.

Variable	n (%)	GDF15 expression		p value
		Low	High	
Age (years)				
< 60	40 (34.48)	33 (82.50)	7 (17.50)	0.358
≥ 60	76 (65.52)	56 (73.68)	20 (26.32)	
Gender				
Male	96 (82.76)	74 (77.08)	22 (22.92)	0.780
Female	20 (17.24)	15 (75.00)	5 (25.00)	
Tumor grade				
G1	8 (6.90)	7 (87.50)	1 (12.50)	0.111
G2	71 (61.21)	50 (70.42)	21 (29.58)	
G3-G4	33 (28.45)	29 (87.88)	4 (12.12)	
N/A	4 (3.45)	-	-	
T status				
T1	7 (6.03)	5 (71.43)	2 (28.57)	0.966
T2	20 (17.24)	15 (75.00)	5 (25.00)	
T3	33 (28.45)	25 (75.76)	8 (24.24)	
T4	56 (48.28)	44 (78.57)	12 (21.43)	
N status				
N0	52 (44.83)	35 (67.31)	17 (32.69)	0.047
N1-N2	63 (54.31)	53 (84.13)	10 (15.87)	
N/A	1 (0.86)	-	-	

TNM stage				
I-II	15 (12.93)	10 (66.67)	5 (33.33)	
III	19 (16.38)	13 (68.42)	6 (31.58)	0.327
IV	82 (70.69)	66 (80.49)	16 (19.51)	
LVI				
No	42 (36.21)	33 (78.57)	9 (21.43)	
Yes	36 (31.03)	28 (77.78)	8 (22.22)	>0.999
N/A	38 (32.76)	-	-	
Alcohol co.				
No	39 (33.62)	33 (84.62)	6 (15.38)	
Yes	75 (64.66)	55 (73.33)	20 (26.67)	0.240
N/A	2 (1.72)	-	-	

N/A – not available; LVI – lymphovascular invasion.

Relationships to Survival

In Kaplan-Meier survival analysis of the TMA cohort, high GDF15 expression was significantly associated with a median overall survival (OS) time compared with low GDF15 (not reached vs. not reached; log-rank $p=0.004$; Figure 3A), but disease-free survival (DFS) time in this group was not significantly (not reached vs. not reached; log-rank $p=0.188$; Figure 3B). High GDF15 expression was not significantly associated with worse OS and DFS in the TCGA Xena and GEO cohorts (Figures 3C-F).

The area under the prognostic ROC curve for GDF15 as a prognostic marker for the OS in the TMA cohort was 0.71 and the asymptotic $p=0.004$ (Figure S2).

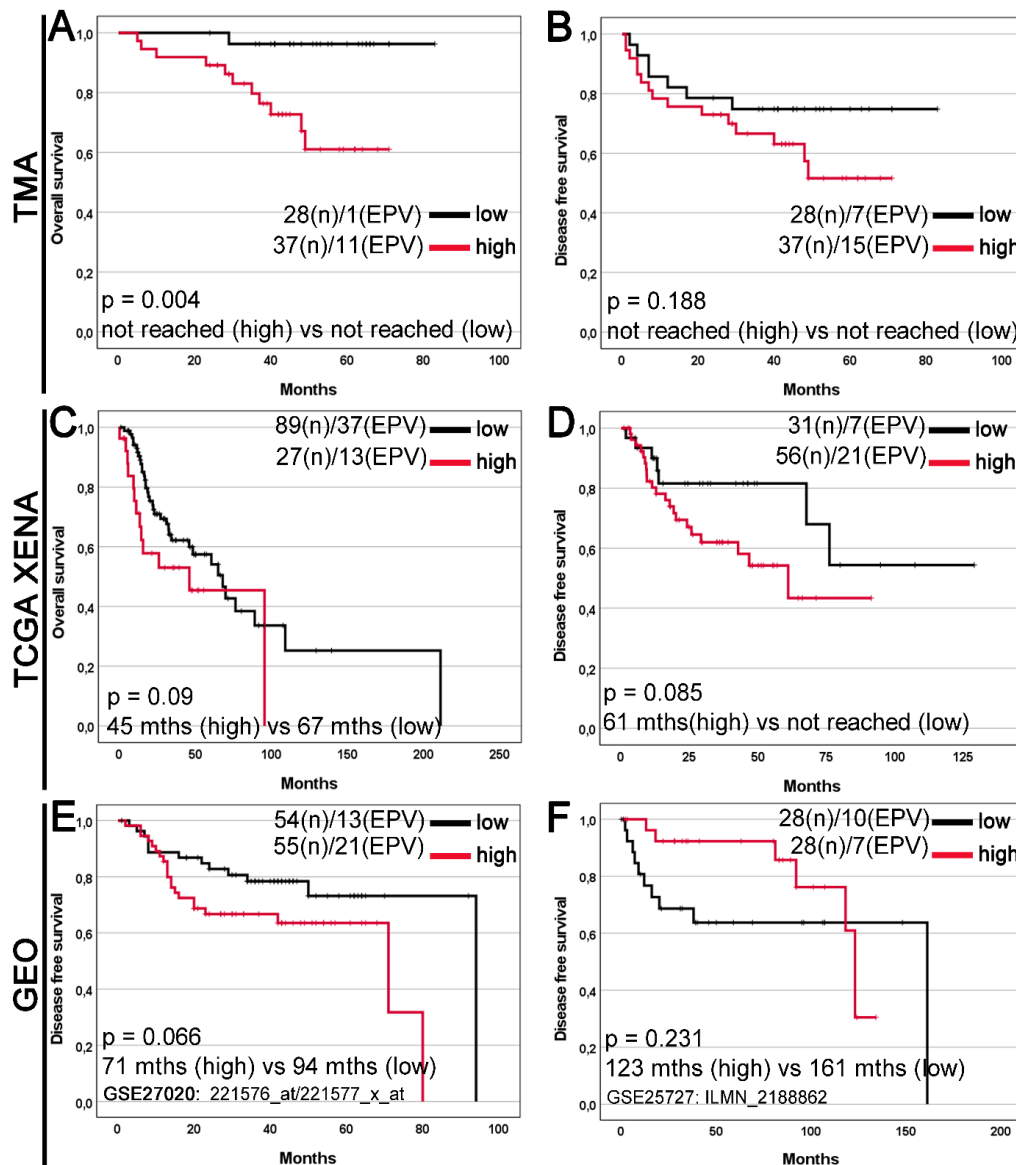


Figure 3. Kaplan-Meier curves for the overall survival (A, C) and disease free survival (B, D-F) of laryngeal squamous cell carcinoma patients stratified by GDF15 expression. The survival curves were plotted based on the (A, B) immunohistochemistry data for the tissue microarray (TMA) cohort; gene expression levels for (C, D) The Cancer Genome Atlas (TCGA) dataset downloaded from the UCSC Xena Browser; (E, F) the Gene Expression Omnibus (GEO) datasets downloaded from the ShinyGEO web-based tool (the GSE number and probe information are shown). Cases were divided into two expression groups (low and high) according to (A-D) the optimal cutoff point determined by the Evaluate Cutpoints software; (E, F) the median expression. The results are shown with median OS or DFS (in months; abbr. mths) for high and low expression groups, as well as with p-value from the log-rank test. The number of cases (n) and events (EPV) in low and high expression groups is displayed.

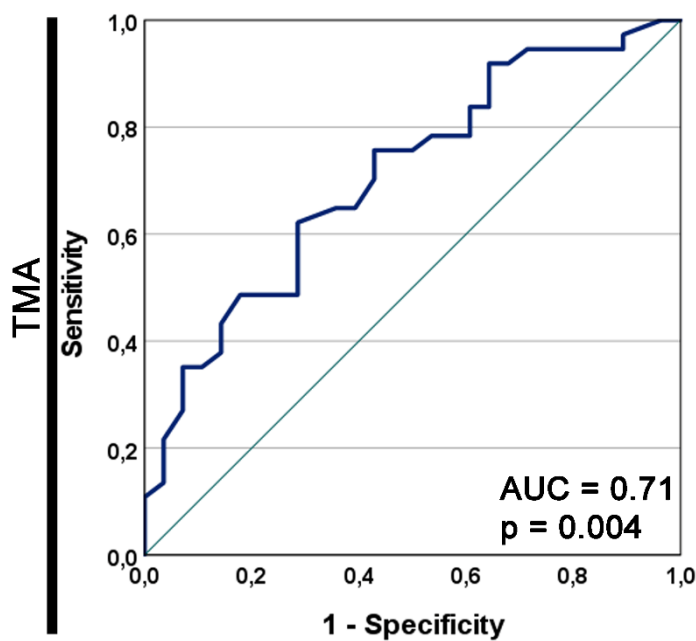


Figure S2. The receiver operating characteristic (ROC) curve for GDF15 as a prognostic marker for the overall survival in the tissue macroarray (TMA) cohort. The asymptotic p value of the ROC curve is shown. AUC, area under the ROC curve.

As presented in Table 3, a univariate analysis of DFS in the TMA cohort indicated statistically significant univariable hazard ratios (HRs) were found for resection margins positive (HR 2.52, 95% CI 1.05–6.04, p=0.038) and pT2 stage (HR 2.65, 95% CI 1.03-6.86, p=0.044). Next, in the multivariate analysis confirmed high GDF15 expression, stage II, stage III as an independent predictor DFS LSCC patients (GDF15 HR 2.98, 95% CI 1.16-7.65, p=0.023; TNM stage (p=0.022) included: TNM stage II HR 4.64, 95% CI 1.53-14.13, p=0.007; TNM stage III-IV HR 3.13, 95% CI 1.06-9.27, p=0.04).

Table 3. Univariable and multivariable Cox analysis of disease-free survival in the TMA cohort (n = 65).

Variable	n/EPV	Univariable analysis				Multivariable model*			
		HR	95% CI		p	HR	95% CI		p
			L	U			L	U	
Disease free survival									
GDF15 (high vs low)	37/15_28/7	1.81	0.74	4.43	0.197	2.98	1.16	7.65	0.023
Age (≥ 59 vs < 59)	48/18_17/4	2.53	0.75	8.54	0.136	-	-	-	-
Sex (male vs female)	55/20_10/2	2.07	0.48	8.85	0.329	-	-	-	-
Tumor grade (G2-G3 vs G1)	49/21_16/1	-	-	-	-	-	-	-	-
T stage					0.131				
T1	32/7			REF.				-	
				-				-	

				-				-	
				-				-	
T2 stage vs REF.	23/11	2.65	1.03	6.86	0.044	-	-	-	-
T3-T4 stage vs REF.	10/4	1.75	0.51	5.98	0.376	-	-	-	-
pN (N1-N2 vs N0)	13/6_52/16	1.36	0.53	3.49	0.518	-	-	-	-
TNM stage					0.128				0.022
				REF.				REF.	
I	30/6			-				-	
				-				-	
				-				-	
II vs REF.	17/8	2.92	1.01	8.43	0.048	4.64	1.53	14.13	0.007
III-IV vs REF.	18/8	2.23	0.77	6.44	0.138	3.13	1.06	9.27	0.04
Margins (positive vs negative)	15/8_50/14	2.52	1.05	6.04	0.038	-	-	-	-
Tumor location (glottis vs others)	34/8_31/14	1.98	0.83	4.71	0.125	-	-	-	-

Abbreviations: TMA – tissue macroarray; HR – hazard ratio, REF. – reference; n – case number; EPV, events per variable, L – lower limit of the 95% confidence interval (CI), U – upper limit of the 95% confidence interval (CI). The group called ‘others’ includes supraglottic cancer, subglottic cancer, as well as overlapping lesion of larynx. HR is shown for the subgroup marked in bold. *Final result of a multivariable Cox analysis with backward elimination of the nonsignificant variables (with removal criterion of $p > 0.05$). The initial model included all univariate predictors ($p < 0.2$): GDF15, age, resection margin, tumor location, and tumor stage. ‘Grade’ was not introduced into the models due to insufficient events per G1 subgroup. T stage has not entered the multivariable model due to its collinearity with the tumor stage.

In unadjusted Cox proportional hazards regression in the TCGA cohort, age at diagnosis (HR 2.04, 95% CI 1.10-3.81, $p=0.025$), sex (HR 0.28 95% CI 0.14-0.54, $p=0.0002$), tumor grade ($p=0.012$) was significantly associated with OS (Table 4). Multivariable regression analysis confirmed sex and tumor grade as an independent predictor of OS LSCC patients (sex HR 0.30 95% CI 0.15-0.59, $p=0.0006$; tumor grade $p=0.017$). Next univariable and multivariable Cox analysis of DFS in the TCGA cohort, N stage was significantly associated with DFS (OS and DFS HR 2.69, 95% CI 1.14-6.35, $p=0.024$).

Table 4. Univariable and multivariable Cox analysis of overall survival (n = 116) and disease-free survival (n =87) in the TCGA cohort.

Variable	n/EPV	Univariable analysis			Multivariable model 1*			
		HR	95% CI		p	HR	95% CI	
			L	U			L	U

Overall survival									
GDF15 (high vs low)	27/13_89/37	1.73	0.91	3.28	0.095	-	-	-	-
Age (≥ 69 vs < 69)	24/15_92/35	2.04	1.10	3.81	0.025	-	-	-	-
Sex (male vs female)	96/38_20/12	0.28	0.14	0.54	0.0002	0.30	0.15	0.59	0.0006
Tumor grade					0.012				0.017
							REF.		
G1	11/4		REF.				-		
							-		
G2 vs REF.	72/35	3.25	0.98	10.8	0.054	4.83	1.14	20.43	0.032
G3-G4 vs REF.	33/11	1.29	0.36	4.63	0.699	2.13	0.47	9.70	0.326
T stage					0.651				
			REF.						
			-				-		
T1-T2	27/12		-				-		
			-				-		
			-				-		
T3 vs REF.	33/15	1.15	0.53	2.50	0.731	-	-	-	-
T4 vs REF.	56/23	0.84	0.41	1.75	0.646	-	-	-	-
N stage (N1-N3 vs N0)	63/32_53/18	1.54	0.85	2.80	0.152	-	-	-	-
TNM stage (III-IV vs I-II)	101/44_15/6	0.79	0.33	1.88	0.595	-	-	-	-

Multivariable model 2**									
Disease free survival									
GDF15 (high vs low)	56/21_31/7	2.14	0.88	5.20	0.092	-	-	-	-
Age (> 60 vs ≤ 60)	48/13_39/15	0.53	0.25	1.12	0.095	-	-	-	-
Sex (male vs female)	76/25_11/3	0.78	0.23	2.60	0.680	-	-	-	-
Tumor grade (G3-G4 vs G1-G2)	30/10_57/18	0.84	0.37	1.90	0.677	-	-	-	-
T stage (T3-T4 vs T1-T2)	68/24_19/4	1.41	0.48	4.10	0.533	-	-	-	-
N stage (N1-N3 vs N0)	48/21_39/7	2.69	1.14	6.35	0.024	2.69	1.14	6.35	0.024
TNM stage (III-IV vs I-II) ¹	78/28_9/0	-	-	-	-	-	-	-	-

Abbreviations: TCGA – The Cancer Genome Atlas; HR – hazard ratio; REF. – reference; n – case number; EPV, events per variable, L – lower limit of the 95% confidence interval (CI), U – upper limit of the 95% confidence interval (CI). HR is shown for the subgroup marked in bold. *Final result of a multivariable Cox model 1 with backward elimination of the nonsignificant variables (with removal criterion of $p > 0.05$). The initial model included all univariate predictors ($p < 0.2$): *GDF15*, age, sex, tumor grade, and N stage. ** Final result of a multivariable Cox model 2 with backward elimination of the nonsignificant variables (with removal criterion of $p > 0.05$). The initial model included all univariate predictors ($p < 0.2$): *GDF15*, age, and N stage. ¹TNM stage was not introduced into the models due to insufficient events per stage I-II subgroup.

4. Discussion

Growth differentiation factor 15 (GDF15) is a peptide hormone belonging to the transforming growth factor beta (TGF β) superfamily. Under physiological conditions, it is present in various human tissues, including the placenta, liver, prostate, bladder, kidneys, colon, and endometrium, although in small quantities. This protein has pleiotropic effects, which has led to research aimed at demonstrating its role in the formation and development of malignant tumors as well as its role in the occurrence of cancer metastasis. Previous studies on GDF15 as a marker for malignant tumors and metastasis diagnosis prompted us to investigate its expression in patients with laryngeal squamous cell carcinoma. To our knowledge, our study is the first to evaluate GDF15 expression in patients with laryngeal cancer.

In our studies, we have demonstrated that the lack of statistically significant difference in GDF15 expression between tumor tissue and normal tissue adjacent to the tumor may suggest similar levels of activity of this protein in both types of tissue. However, it is important to consider the potential differences in cellular composition and microenvironment present in these two types of tissues, which could influence the interpretation of these results. The associations between GDF15 expression and clinical variables, such as T feature and TNM stage, exhibited variations between the tissue macroarray (TMA) and The Cancer Genome Atlas (TCGA) cohorts, as indicated by the data dichotomization and analysis presented earlier. The TMA cohort assesses protein expression levels, whereas the TCGA or GEO cohorts measure the molecular level, that is, gene/mRNA expression levels. In the TMA cohort, consisting of 28 samples with low gene expression and 37 samples with high gene expression, the frequency of high and low GDF15 protein expression significantly differed in relation to the T feature according to pT status and TNM stage. Notably, patients in stage I demonstrated a higher prevalence of high GDF15 protein expression compared to low expression levels. Conversely, no statistically significant differences in clinicopathological variables were observed in the TCGA cohort. Significant association between high GDF15 expression and poorer overall survival in laryngeal squamous cell carcinoma patients, predominantly observed in the tissue macroarray (TMA) dataset, may suggest the potential prognostic value of this protein. However, the lack of significant differences in disease-free survival across all datasets necessitates further analysis considering additional prognostic factors. Highlighting trends approaching statistical significance in DFS overall survival observed in TCGA and GEO datasets suggests the potential broader significance of GDF15 expression in cancer prognosis. Analyzing these trends indicates that higher levels of GDF15 may be associated with worse survival outcomes, which could have significant clinical implications.

These findings underscore the complex interplay of GDF15 and highlight the importance of comprehensive investigations to elucidate its role as a prognostic marker in various cancer types. However, further investigation and confirmation of these results are necessary to precisely delineate the role of GDF15 as a prognostic biomarker and its potential application in clinical practice.

Growth differentiation factor 15 (GDF15) exhibits a diverse array of physiological and pathological effects across multiple contexts. Elevated serum concentrations of GDF15 have been associated with various malignancies, indicating its potential utility as a diagnostic biomarker for neoplastic diseases [7,25]. Clinical studies have indicated its involvement in promoting metastasis [4,25]. Moreover, GDF15 expression has been correlated with decreased survival in cancer patients,

particularly evident in laryngeal squamous cell carcinoma, highlighting its potential prognostic value [4]. Additionally, the concentration of this marker has been shown to be significant in colorectal [26,27], gastric [28,29], pancreatic [30,31], breast [32,33], and prostate cancers [34,35]. However, to better understand the role of GDF15 as a prognostic biomarker, further research is needed, considering methodological limitations and other prognostic factors. Despite these limitations, these results suggest that GDF15 could be an important prognostic factor in various types of cancer, with significant clinical implications for cancer treatment and patient care.

Our study's discussion extends to the broader implications of GDF15 in cancer-related symptoms, particularly anorexia and cachexia. GDF15 has been implicated in tumor-induced anorexia and cachexia, as evidenced by its association with weight loss and its ability to induce loss of fat and lean tissue mass [36]. Transgenic mice overexpressing human GDF15 exhibited reduced body weight, suggesting its role in metabolic dysregulation [37]. Further studies demonstrated that tumor-derived GDF15 contributes to weight loss and cachexia in mice, with treatment using GDF15 antibodies reversing these effects [38]. Additionally, serum analysis indicated a correlation between GDF15 and weight loss in various cancers. Recent findings highlight the circadian rhythm-dependent secretion of GDF15 by muscles, suggesting its pathological role in systemic energy metabolism regulation [39]. Potential therapeutic utilization of GDF15 neutralization using monoclonal antibodies to alleviate cancer-associated anorexia and metabolic dysfunctions is also under investigation [40]. Further research is necessary to elucidate the precise mechanisms underlying GDF15-induced metabolic dysregulation and its therapeutic implications in cancer treatment.

This study has several limitations that must be acknowledged. Firstly, the cohort size was very small, which reduces the statistical power of our findings. Secondly, the low number of events per variable precluded us from performing Cox proportional hazards analysis in relation to overall survival time. These limitations may affect the interpretation of the results and underscore the need for caution in drawing definitive conclusions. Future studies with larger cohorts and more robust event data are necessary to validate our findings and to provide more comprehensive insights into the role of GDF15 as a prognostic biomarker in laryngeal squamous cell carcinoma.

5. Conclusions

The study results showed no significant difference in GDF15 expression between tumor and adjacent normal tissue. However, variability in GDF15 expression was observed concerning clinical variables, exclusively in the TMA cohort. Elevated GDF15 levels were significantly associated with poorer overall survival in laryngeal squamous cell carcinoma patients, suggesting its potential prognostic value. Trends approaching statistical significance in overall survival across various datasets highlight its significance as a prognostic marker. Further research is warranted to better understand GDF15's role as a potential diagnostic biomarker and its clinical implications in different cancer types.

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Abbreviations

The following abbreviations are used in this manuscript:

AJCC – American Joint Committee on Cancer

DFS – Disease-Free Survival

FFPE – Formalin-Fixed, Paraffin-Embedded

GDF15 – Growth Differentiation Factor 15

GEO – Gene Expression Omnibus

GTEX – Genotype-Tissue Expression

HR – Hazard Ratio

IHC – Immunohistochemistry

IRS – Immunoreactive Score

LSCC – Laryngeal Squamous Cell Carcinoma

N/A – Not Available

OS – Overall Survival

ROC – Receiver Operating Characteristic

TCGA – The Cancer Genome Atlas

TGF- β – Transforming Growth Factor Beta

TMA – Tissue Macroarray

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