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Serum hypoxia-inducible factor-2: A candidate prognostic biomarker for laryngeal cancer

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Abstract

Objectives: To determine the serum hypoxia-inducible factor-1, -2 and -3 (HIF-1, -2 and -3) levels in patients with laryngeal neoplasm, and to investigate their role in differential diagnosis, prediction of tumour characteristic and extension, and prognosis and survival.

Study Design: Prospective, cohort study at a tertiary referral centre.

Settings: The study was conducted in a tertiary medical centre.

Participants: Patients with benign, premalignant and malignant laryngeal neoplasms were included. Sixty-four patients with a laryngeal neoplasm were enrolled.

Main Outcome Measures: Serum HIF-1, -2 and -3 levels were measured from blood samples that were drawn before treatment, using ELISA.

Results: A statistically significant difference between benign (HIF-1, -2, -3:4046,1 pg/mL; 2581,5 pg/mL; 1321,0 pg/mL), premalignant (HIF-1, -2, -3:3630,3 pg/mL; 3229,7 pg/mL; 2549,8 pg/mL) and malignant (HIF-1, -2, -3:3576,7 pg/mL; 2595,8 pg/mL; 1106,3 pg/mL) laryngeal neoplasms was not detected when serum HIF-1, -2 and -3 levels were compared. However, high serum HIF-2 level adversely affected survival and locoregional control and had more than 7-fold increase in hazard ratio. Moreover, serum HIF-2 was an independent prognostic factor for 2-year overall, disease-free, distant metastasis-free survival and locoregional control.

Conclusion: This is the first clinical study in which the diagnostic, predictive and prognostic roles of hypoxia-related biomolecules were examined in laryngeal neoplasms. Hypoxia-inducible factor-2 is a prognostic factor in larynx cancer irrespective of treatment modality.

KEYWORDS
biomarker, hypoxia, hypoxia inducible factor, larynx cancer, prognosis, survival

1 | INTRODUCTION

The twenty-first century is described as the 'age of biomarker discovery' concerning early diagnosis and effective treatment of cancer.[1] However, the biomarker for head and neck cancers (HNCs) is inadequate. Currently, only Human papillomavirus and *p16* expression are available for oral and oropharyngeal cancers.[2] Recently, hypoxia-related biomolecules are candidate biomarkers for several cancers.

Hypoxia refers to the imbalance between the tissue's demand for oxygen and the supply of oxygen to the tissue. It orchestrates several processes such as tumoural progression and metabolism, chaotic angiogenesis, anoikis resistance and metastasis cascade.[3-6] Solid tumours are known to have dynamic hypoxic areas with heterogenic

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distributions.[7,8] Hypoxic areas may lead to unstable tumour behaviour, aggressivity, and an increase in tumour volume and trigger epithelial-mesenchymal transition (Figure 1A).[8,9] Moreover, hypoxia is one of the leading risk factors for treatment failure and mortality.[10-13] Chronic hypoxia and its effects on tumour biology were first introduced by Thomlinson and Gray who determined that 'diffusion restricted-hypoxia' occurred when a distance of 100 μm developed between cancer cells and capillary vessels.[14] Thereafter, Brown described the development of 'perfusion restricted-hypoxia'.[15] Acute hypoxia refers to the reduced oxygen perfusion caused by the intermittent, temporary and reversible occlusion occurring in the vessels that provide microcirculation; thereby fluctuations in oxygen supply may develop, which is called 'cycling hypoxia'. It is known that hypoxia progresses in a dynamic fashion in tumour microenvironment. In addition, anaemia generally increases the perfusion failure and heterogeneity in solid tumours. Thereby, it aggravates hypoxia and triggers the generation of hypoxic biomolecules at the microenvironment.[16]

Hypoxia may lead to activation of several signalling pathways and production of hypoxia-related biomolecules including hypoxia-inducible factors (HIFs), which are also referred to as the 'hypoxia sensors' (Figure 1B). These proteins are transcriptional complexes made of two heterodimers: HIF-α and-β.[17,18] To date, three different HIF isoforms have been described: HIF-1, -2 and -3. When oxygen level is reduced in a tumour microenvironment, hydroxylation of HIF does not occur; thereby HIF concentrations are increased. Thus, increase in HIF levels may alter the gene functions which affect cancer cell apoptosis, epithelial-mesenchymal transition, invasion capacity, angiogenic signalling and energy metabolism.[17-19] A systematic review reported that hypoxia-related biomarkers were frequently detected in patients with HNC and indicated poor prognosis.[20] Moreover, a recently published meta-analysis, which was particularly related to HIF expression, demonstrated that increased HIF-1α expression is associated with tumour size, stage, LNI and overall survival.[21]

In fact, histopathological examination of tumour biopsy samples is the gold standard for cancer diagnosis. However, biomolecules examined in biopsy samples may not reflect the tumour microenvironment due to tumour heterogeneity. Therefore, the biomarker role of these biomolecules is unreliable. Notably, Bredell et al showed significant limitations of biopsy-based hypoxic biomarkers in HNCs.[22] Moreover, tumour hypoxia is particularly related to the oxygen level of the tumour microenvironment which is affected by haemoglobin concentration. Therefore, the purpose of this study was to determine and evaluate the diagnostic, predictive and prognostic biomarker roles of serum HIF levels and anaemia in patients with laryngeal neoplasm.

2 | MATERIALS AND METHODS

An ethical approval for this prospective study was obtained from Ethics Committee of Dokuz Eylül University (Issue date/no: 09.05.2015/20478486-346).

KeyPoints

- Hypoxia orchestrates tumour biology and behaviour in tumour microenvironment and impacts on prognosis and survival of patients with cancer.
- Hypoxia-related biomolecules are promising biomarkers for several cancers.
- Hypoxia-inducible factor-1 and −3 are not diagnostic, predictive and prognostic biomarker for laryngeal cancer
- Serum HIF-2 level adversely affects survival and locoregional control in patients with laryngeal cancer.
- Serum HIF-2 is a candidate prognostic biomarker for laryngeal cancer.

2.1 | Patient selection and data collection

The study included patients who were diagnosed with:

(i) Benign neoplasm of the larynx (Group A).

(ii) Premalignant neoplasm of the larynx (mild or moderate dysplasia) (Group B).

(iii) Malignant neoplasm of the larynx (Group C) in Manisa Celal Bayar University between 2012 and 2017. The study exclusion criteria were: (i) presence of another primary cancer other than laryngeal cancer (LC), (ii) previous diagnosis and/or treatment for any cancer prior to the current diagnosis and (iii) rejection of treatment for LC. Sociodemographic data (age, sex, smoking state), haemoglobin (Hb) concentrations, tumour characteristics and stage, locoregional recurrence, distant metastasis and survival were recorded. Anaemia was defined according to World Health Organization (WHO) criteria as Hb≤13.0 gr/dL in males and Hb≤12.0 gr/dL in females. Additionally, WHO's 'smoking-adjusted anaemia' criteria were also applied.[23]

2.2 | Sample collection and storage

Blood samples were drawn from all patients before treatment. Blood samples were centrifuged at 1000 *g* for 5 minutes in a refrigerated centrifuge and treated with the protease inhibitor, and the serum portion was separated and stored at −80°C. Finally, measurements were performed using commercial ELISA assays.

2.3 | The examination of serum hypoxia-related biomolecules

The samples were assayed in the laboratory of Institute of Oncology, Dokuz Eylül University using *Human HIF-1α*, *Human HIF-2α (EPAS1: Endothelial PAS domain-containing protein 1)* and *Human HIF-3α ELISA kit*, in accordance with the manufacturer's instructions.

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For the calculation of serum HIF-1, -2 and -3 levels; (Relative OD450) = (Sample OD450 value)-(Control OD450 value). Standard curve can be drawn as corresponding concentration of standard solution (X) plotted against relative OD450 value of each standard solution (Y). HIF-1, -2 and -3 concentrations in the samples were calculated using the curve.

2.4 | Statistical analysis

All study data were entered in SPSS for Mac software. Normality assessment was performed. The study sample showed normal distribution for HIF-2 and non-normal distribution for HIF-1 and -3.[24] Therefore, analyses related to HIF-2 were made using parametric tests and analyses for the others were performed using non-parametric tests.

Overall, disease-free and distant metastasis-free survival and locoregional control of the cases in Group C were calculated using Kaplan-Meier test. The cut-off values for serum HIF-1, -2 and -3 levels were determined with receiver operating characteristic (ROC) analysis. In case the sensitivity and specificity values were not acceptably high, median values were determined and accepted as the cut-off value for that variable. Log-rank test was used to compare the survival. Additionally, hazard ratios of the variables were calculated using Cox regression test. Multivariate analysis was applied to the variables that were found to have effect on survival and locoregional control in order to determine the independent prognostic factors. A *P*-value of less than .05 was considered statistically significant.

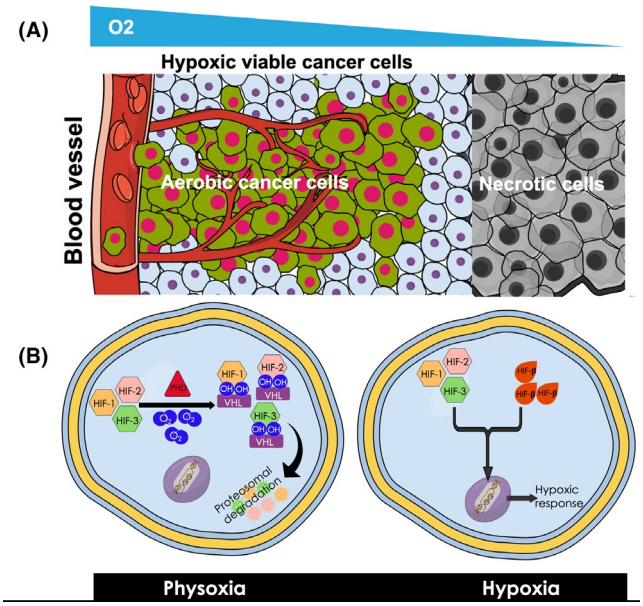


FIGURE 1 The illustration of cancer cells affected by hypoxia at the tumour microenvironment (A) and the mechanism of hypoxia-inducible factors in physoxia and hypoxia (B) HIF, Hypoxia-inducible factor; OH, Hydroxyl radical; PHD, Prolyl hydroxylase; VHL, von Hippel Lindau protein

3 | RESULTS

3.1 | Sociodemographic and clinicopathological data

Sixty-four patients were enrolled into the study. The distribution of all cases according to study groups:

(i) Group A: 12 cases [10 (83.3%) males, 2 (16.7%) females]
(ii) Group B: 8 cases [7 (87.5%) males, 1 (12.5%) female]
(iii) Group C: 44 cases [41 (93.2%) males, 3 (6.8%) females].

The mean age of study group was 59.5 (36.0-86.0) years. The mean smoking intensity in Group C was 56.2 (1-134) packs/year. Among these cases, anaemia was detected in 13 cases, while smoking-adjusted anaemia was detected in 16 cases. The glottic, supraglottic and transglottic LCs were 28.1%, 20.3% and 20.3%, respectively. The majority of LCs were moderately differentiated (27, 61.4%). According to T-stages of study group, 20.5% were carcinoma in situ/T1, 11.4% were T2, 29.5% were T3 and 38.6% were T4. Sixteen patients had cervical lymph node involvement (LNI). Most of the study group had advanced stage larynx cancer (31, 70.5%). Twenty-seven patients were treated by non-surgical treatment modalities and seventeen patients were treated by surgery with/without adjuvant treatment.

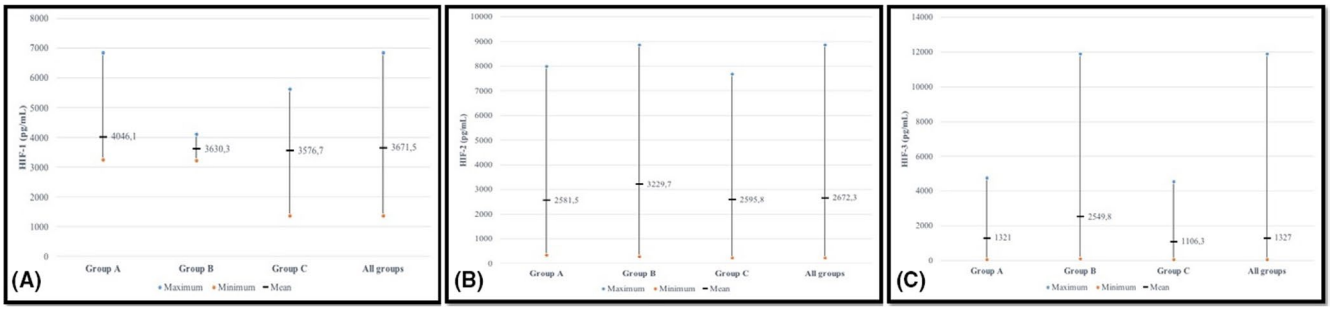


FIGURE 2 The mean, minimum and maximum values and comparative analysis between groups in terms of serum HIF-1, -2 and -3 levels ($P = .241$, $P = .765$ and $P = .465$) (A-C)

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TABLE 1 Predictive analysis according to serum HIF-1, −2 and −3 levels

	HIF-1		HIF-2		HIF-3	
	Mean ± SD (pg/mL)	*P*	Mean ± SD (pg/mL)	*P*	Mean ± SD (pg/mL)	*P*
Groups						
Group A (n = 12)	4046.1 ± 923.9	.24	2581.6 ± 2411.5	.26	1321.0 ± 1447.8	.46
Group B (n = 8)	2630.3 ± 259.8		3229.7 ± 3040.1		2549.8 ± 3883.3	
Group C (n = 44)	3576.7 ± 925.6		2595.8 ± 2120.9		1106.3 ± 1374.5	
Gender						
Female (n = 6)	4287.9 ± 1295.8	.24	2481.9 ± 2843.6	.83	1475.6 ± 1260.2	.58
Male (n = 58)	3607.7 ± 815.2		2692.0 ± 2233.1		1311.7 ± 1946.1	
Age						
<65 years (n = 45)	3712.8 ± 884.9	.81	2557.0 ± 2322.1	.53	1459.0 ± 2084.3	.31
>65 years (n = 19)	3573.6 ± 884.5		2945.5 ± 2181.4		1014.5 ± 1294.7	
Smoking intensity						
<50 packs years (n = 21)	3653.5 ± 885.8	.73	2535.7 ± 2161.2	.84	1158.9 ± 1449.9	.86
>50 packs years (n = 23)	3492.7 ± 982.2		2661.5 ± 2127.1		1219.4 ± 1198.5	
Anaemia						
Absent (n = 31)	3538.6 ± 919.4	.71	2511.0 ± 2013.4	.68	1078.2 ± 1282.6	.93
Present (n = 13)	3667.8 ± 971.4		2797.8 ± 2234.9		1173.4 ± 1627.9	
Smoking adjusted anaemia						
Absent (n = 28)	3543.8 ± 962.8	.88	2612.6 ± 2173.3	.94	1062.6 ± 1306.2	.90
Present (n = 16)	3634.5 ± 884.3		2566.3 ± 2095.6		1182.9 ± 1528.0	
Tumour differentiation						
Carcinoma in situ/Well differentiated (n = 11)	3415.3 ± 1097.6	.85	2966.3 ± 2657.0	.17	866.3 ± 1213.0	.71
Moderately differentiated (n = 27)	3617.4 ± 960.6		2167.1 ± 1855.0		1180.7 ± 1528.7	
Poor differentiated (n = 6)	3690.0 ± 280.8		3845.3 ± 1871.7		1212.0 ± 991.3	
Tumour location						
Supraglottic (n = 13)	3518.6 ± 892.6	.78	2045.1 ± 2013.5	.44	1305.5 ± 1627.8	.87
Glottic (n = 18)	3648.9 ± 765.7		3038.7 ± 2344.4		916.7 ± 1219.0	
Transglottic (n = 13)	3534.9 ± 1195.5		2533.2 ± 1910.6		1169.7 ± 1383.3	
Tumour extension						
Glottic/Supraglottic (n = 31)	3594.9 ± 1195.5	.95	2622.0 ± 2232.7	.90	1079.8 ± 1392.9	.87
Transglottic (n = 13)	3534.9 ± 1195.5		2533.2 ± 1910.6		1169.7 ± 1392.9	
T-stage						
Carcinoma in situ/T1+T2 (n = 14)	3587.9 ± 748.7	.74	2923.6 ± 2465.2	.49	1025.8 ± 1428.9	.74
T3 + T4 (n = 30)	3571.6 ± 1009.4		2442.8 ± 1967.1		1143.9 ± 1371.6	
Thyroid cartilage involvement						
Absent (n = 27)	3633.7 ± 894.5	.69	2754.9 ± 2333.9	.53	967.0 ± 1240.4	.33
Present (n = 17)	3486.2 ± 993.9		2343.0 ± 1768.0		1327.6 ± 1578.7	
Lymph node involvement						
N0 (n = 28)	3710.8 ± 1062.2	.09	2667.5 ± 2045.8	.77	1052.1 ± 1326.3	.86
N+ (n = 16)	3342.2 ± 576.9		2470.2 ± 2309.7		1201.2 ± 1494.7	
Extranodal involvement						
Absent (n = 9)	3524.9 ± 1323.7	.54	2886.3 ± 2279.0	.29	1073.9 ± 1126.0	.35
Present (n = 7)	3480.3 ± 585.1		1762.2 ± 1611.2		1459.0 ± 1644.2	

(Continues)

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TABLE 1 (Continued)

	HIF-1		HIF-2		HIF-3	
	Mean ± SD (pg/mL)	*P*	Mean ± SD (pg/mL)	*P*	Mean ± SD (pg/mL)	*P*
Stage						
Early stage (n = 13)	3579.1 ± 778.6	.79	2567.4 ± 2158.4	.95	1100.8 ± 1108.8	.83
Advanced stage (n = 31)	3575.8 ± 992.7		2607.7 ± 2140.9		1108.8 ± 1362.7	
Treatment modality						
Surgery (n = 17)	3514.5 ± 1003.2	.84	2300.6 ± 2007.9	.47	1399.9 ± 1451.2	.59
Non-surgical (n = 27)	3615.9 ± 890.8		2781.6 ± 2205.8		921.5 ± 1317.8	

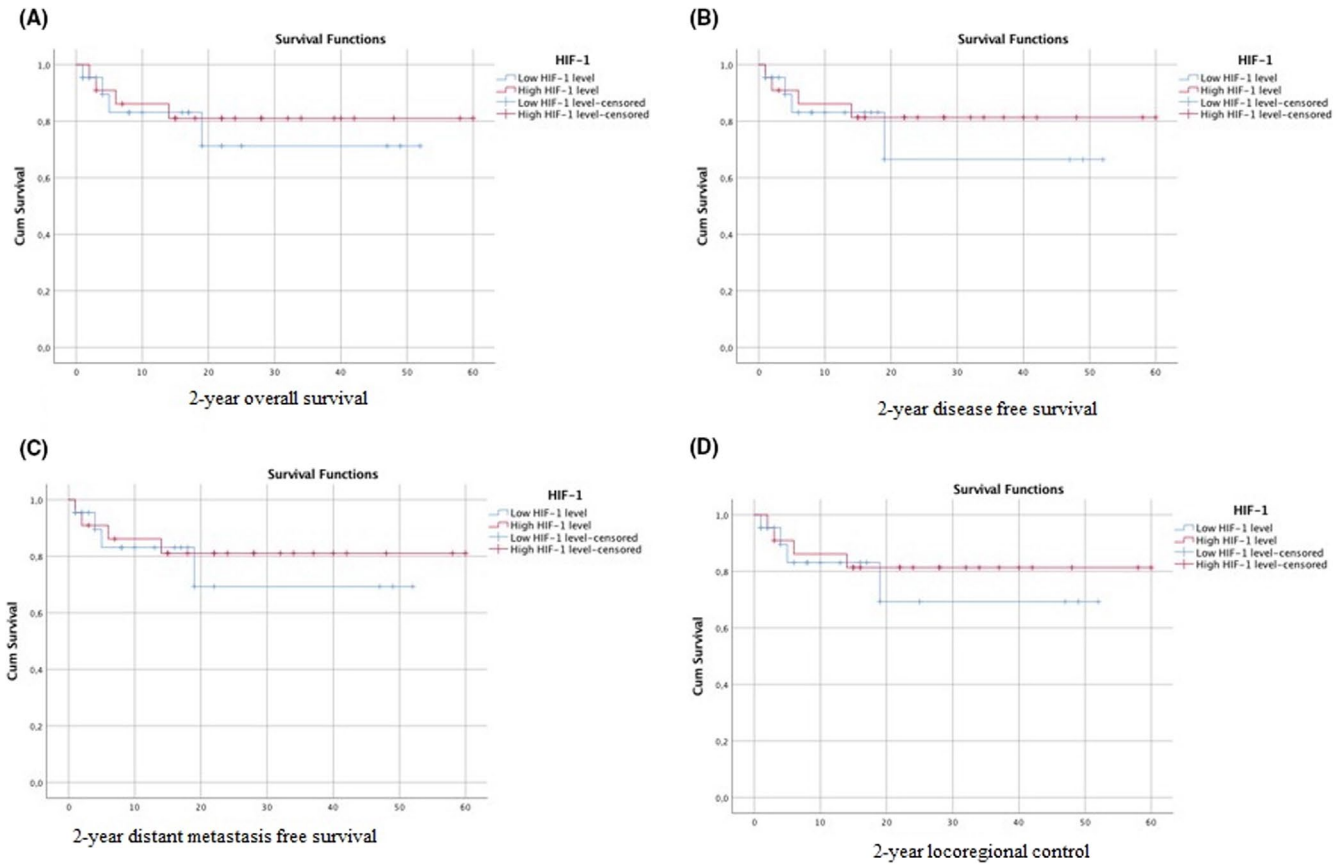


FIGURE 3 Log-rank analysis of 2-year overall survival (A), disease-free survival (B), distant metastasis-free survival (C), locoregional control (D) according to serum HIF-1 levels

3.2 | The distribution of the patients regarding serum HIF-1, -2 and -3 levels

The mean, minimum and maximum levels of HIF-1, -2 and -3 are presented in Figure 2A-C. ROC analysis in patients with larynx cancer yielded a cut-off value of HIF-2 level for predicting LC as 2915.4 pg/mL (75.0% sensitivity and 72.2% specificity). Since no cut-off value for other variables (HIF-1 and -3) provided acceptably high sensitivity and specificity; median levels of these variables were accepted as their cut-off values. Thus, patients were stratified according to HIF-1 and -3 levels by taking cut-off values as 3573.8 pg/mL and 332.4 pg/mL, respectively.

3.3 | The assessment of the predictive roles of serum HIF-1, -2 and -3 levels over sociodemographic and clinicopathological data

The distribution of HIF-1, -2 and -3 levels according to study groups, gender, age, smoking intensity, presence of anaemia, tumour location, tumour differentiation and stage, thyroid cartilage involvement, LNI and treatment are presented in Table 1. When the correlation between HIF-1, -2 and -3 with smoking (packs/year) and age was examined, an inverse weak-moderate correlation was found between age and HIF-3 level ($r = -0.248$; $P = .05$).

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3.4 | Locoregional recurrence, distant metastasis and survival in patients with malignant laryngeal neoplasm

The mean duration of follow-up in patients with malignant laryngeal neoplasm was 20.2 (1-60) years. Thirty-six cases (81.8%) were alive, and 8 (18.2%) were decreased. In this group, 8 cases developed local and 6 cases developed regional recurrence, whereas distant metastasis was detected in 5 cases. In Group C, survival analysis demonstrated that 2-year overall survival, disease-free survival, distant metastasis-free survival and locoregional control were 77.5%, 77.1%, 77.4% and 77.2%, respectively.

3.5 | The assessment of the prognostic role of sociodemographic, clinicopathological and hypoxia-related factors using log-rank analysis

The prognostic roles of age, smoking intensity, anaemia, smoking-adjusted anaemia, tumour differentiation and location, T-Stage, thyroid cartilage involvement, LNI, stage, treatment modality and serum HIF-1, -2 and -3 levels on 2-year overall, disease-free, distant metastasis-free survival and locoregional control in patients with malignant laryngeal neoplasm are presented in Figures 3-5 and Table 2.

3.6 | The asssessment of the risk factors on survival using Cox regression analysis and multivariate hazard regression analysis

Cox regression analysis was performed to assess the adverse effects of sociodemographic, clinicopathological and hypoxic factors on survival outcomes in patients with LC (Table 3). Accordingly, the presence of anaemia (HR: 4.55, 95% CI: 1.08-19.17), the presence of smoking-adjusted anaemia (HR: 6.03, 95% CI: 1.21-30.07), transglottic tumour (HR: 6.24, 95% CI: 1.11-30.55) and elevated HIF-2 level (HR: 5.40, 95%CI: 1.09-26.76) were associated with negative impact on survival.

Anaemia, smoking-adjusted anaemia, tumour location and serum HIF-2 level were found statistically significant in bivariate analyses; therefore they were selected for multivariate hazard regression analysis. Thereby, serum HIF-2 (2-year overall survival: HR: 8.59, 95% CI: 1.38-53.19, $P = .021$; 2-year disease-free survival: HR: 7.76, 95% CI: 1.26-47.78, $P = .027$; 2-year distant metastasis-free survival: HR: 7.77, 95% CI: 1.26-47.87, $P = .027$; 2-year locoregional control: HR: 8.57, 95% CI: 1.38-53.04, $P = .021$) and tumour location (2-year overall survival: HR: 15.34, 95% CI: 2.70-87.11, $P = .002$; 2-year disease-free survival: HR: 13.97, 95% CI: 2.50-78.22, $P = .003$; 2-year distant metastasis-free survival: HR: 14.00, 95% CI: 2.50-78.34, $P = .003$; 2-year locoregional control: HR: 15.30, 95% CI:

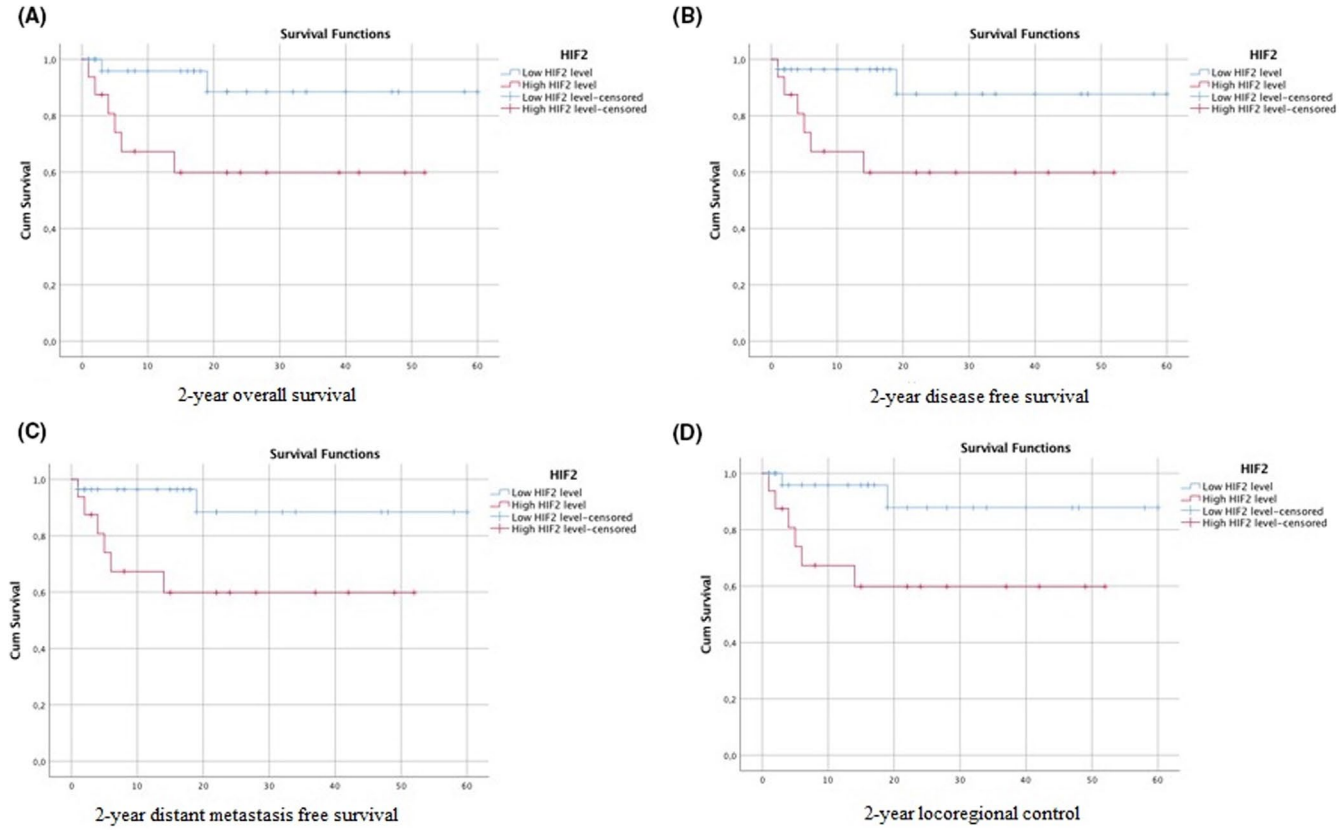


FIGURE 4 Log-rank analysis of 2-year overall survival (A), disease-free survival (B), distant metastasis-free survival (C), locoregional control (D) according to serum HIF-2 levels

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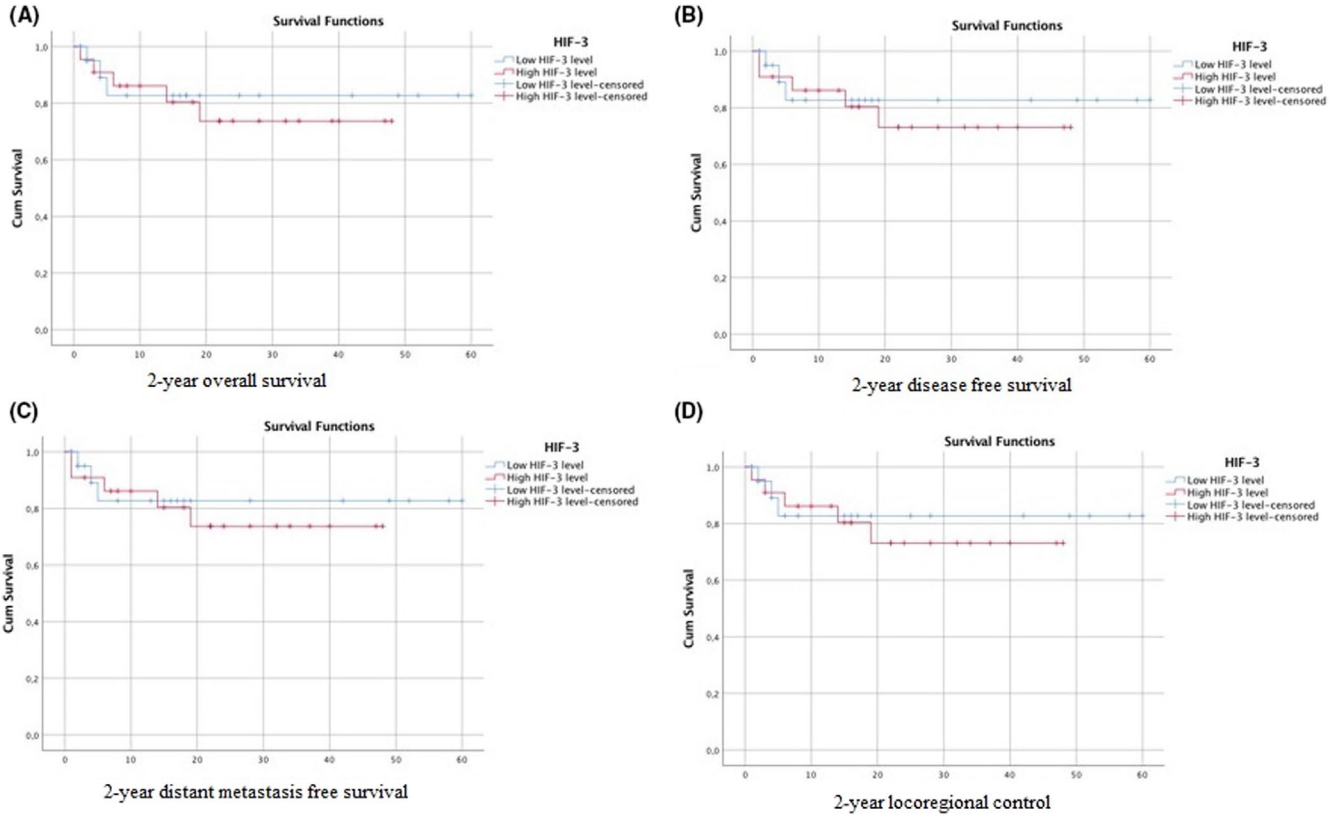


FIGURE 5 Log-rank analysis of 2-year overall survival (A), disease-free survival (B), distant metastasis-free survival (C), locoregional control (D) according to serum HIF-3 levels

2.69-86.90, $P = .002$) were determined as independent prognostic factors regarding all oncological outcomes.

4 | DISCUSSION

To the best of our knowledge, this is the first clinical study in which the diagnostic, predictive and prognostic roles of anaemia, hypoxemia and serum HIF-1, -2 and -3 were comprehensively examined in laryngeal neoplasms. Approximately 30%-40% of patients with cancer have anaemia that adversely affects treatment success and survival.[25,26] Caro et al reported that anaemia had the greatest negative impact on survival in patients with HNC, causing an approximately 75% increase in the mortality risk.[27] Our previous meta-analysis also showed that anaemia is the most important risk factor for radiotherapy failure in patients with early-stage glottic LC.[28] Of note, approximately 80% of patients with HNC may develop mild-to-severe anaemia that has a negative impact on treatment tolerance and causes treatment failure.[29] Moreover, smoking may aggravate anaemia in patients with lung and HNCs. Therefore, WHO recommends evaluating 'smoking-adjusted anaemia'.[23] Our survival analysis showed that anaemia and smoking-adjusted anaemia were adverse prognostic factors in terms of 2-year overall, disease-free and distant metastasis-free survival and locoregional control (Table 2). Additionally, anaemia and smoking-adjusted anaemia increased the hazard risk by 5- and 6-fold, respectively (Table 3). However, neither anaemia nor smoking-adjusted anaemia was found to be an independent prognostic factor. Therefore, we believe that anaemia/smoking-adjusted anaemia should be evaluated with hypoxia-related biomarkers.

Hypoxia-inducible factors are the principal mediators of the hypoxic process. Heikal et al reported the sample collection and storage conditions to optimise an assay for HIF-1α in vitro and in vivo models. Moreover, they demonstrated the release kinetics of HIF-1α. Thereby, they emphasised that plasma HIF can be a candidate biomarker for tissue hypoxia in hypoxia-related conditions such as coronary artery diseases, stroke and restenosis.[30] In the present study, the biomarker roles of serum HIF-1, -2 and -3 were investigated in benign, premalignant and malignant laryngeal neoplasms. We were unable to detect diagnostic or predictive biomarker roles of HIFs (Figure 2A-C and Table 1). Our literature survey demonstrated that there is no other clinical study in which serum HIF levels in benign, premalignant, or malignant laryngeal neoplasms were investigated. However, Popov et al performed a comparative analysis of total mRNA levels of hypoxia-associated biomolecules in fresh normal and tumour tissue samples obtained from patients with LC.[31] They found that HIF-1 was upregulated in a significant majority (69.8%) of the study population, whereas overexpression of HIF-2 (11.1%) and HIF-3 (7.9%) was observed in a fewer number of patients. The authors emphasised that their findings indicated the opposite of 'HIF switch', even though they were unable to explain this contradiction. Indeed, HIF-1 is a biomolecule that responds to hypoxia initially. However,

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TABLE 2 Log rank survival analysis of sociodemographic, clinicopathologic and hypoxia-related factors*

Variable	2-years overall survival		2-years disease free survival		2-years distant metastasis free survival		2-years locoregional control	
	%	*P*	%	*P*	%	*P*	%	*P*
Age								
<65 y	86.0	.24	86.0	.25	86.0	.24	86.0	.27
>65 y	65.2		65.6		65.2		65.2	
Smoking intensity								
<50 pack (y)	77.9	.85	76.7	.86	77.9	.84	77.4	.85
>50 pack (y)	77.9		77.8		77.8		77.4	
Anemia								
Absent	89.3	.02	89.8	.02	89.8	.02	89.3	.02
Present	46.7		41.5		41.5		46.7	
Smoking adjusted anemia								
Absent	92.2	.01	92.2	.01	92.2	.01	92.2	.01
Present	51.2		48.1		48.1		51.3	
Tumor differentiation								
CiS/Well	100.0	.17	100.0	.18	100.0	.17	100.0	.18
Moderately	69.7		68.7		68.7		69.4	
Poor	66.7		66.7		66.7		66.7	
Tumor location								
Supraglottic	90.0	.02	90.0	.02	90.0	.02	90.0	.02
Glottic	87.7		87.7		87.7		87.7	
Transglottic	40.1		40.8		40.8		40.1	
Tumor extension								
Glottic/Supraglottic	89.1	.005	89.0	.006	89.0	.005	89.0	.005
Transglottic	40.1		40.8		40.1		40.1	
T-stage								
CiS/T1+T2	92.3	.16	92.3	.16	92.3	.16	92.3	.17
T3+T4	70.0		69.5		69.5		69.7	
Thyroid cartilage involvement								
Absent	88.2	.10	88.2	.09	88.2	.09	88.2	.10
Present	56.7		54.9		54.9		56.7	
Lymph node involvement								
Absent	74.4	.65	74.0	.64	74.4	.65	73.8	.64
Present	85.9		85.9		85.9		85.9	
Stage								
Early stage	91.7	.20	91.7	.20	91.7	.19	91.7	.21
Advanced stage	70.9		70.3		70.3		70.6	
Treatment modality								
Surgery	86.2	.45	86.9	.46	86.9	.47	86.2	.43
Non-surgical	72.0		71.1		71.8		71.1	
Serum HIF-1 level								
Low	76.7	.67	66.5	.63	69.2	.67	69.2	.62
High	86.1		81.3		81.1		81.3	
Serum HIF-2 level								
Low	88.5	.02	87.7	.02	88.4	.02	87.8	.02
High	59.8		59.8		59.8		59.8	
Serum HIF-3 level								
Low	82.7	.68	82.7	.66	82.7	.67	82.7	.68
High	73.7		73.1		73.7		73.1	

*statistically significant variables designated in bold.

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TABLE 3 Cox regression analysis of sociodemographic, clinicopathologic and hypoxia-related factors*

		2-years overall survival			2-years disease free survival			2-years distant metastasis free survival			2-years locoregional control		
Characteristic	**Variable**	**HR**	**%95 CI**	***P***	**HR**	**%95 CI**	***P***	**HR**	**%95 CI**	***P***	**HR**	**%95 CI**	***P***
Age	<65 years vs >65 years	2.28	0.54-9.58	.26	2.23	0.53-9.38	.27	2.30	0.55-9.65	.25	2.20	0.52-9.24	.28
Smoking intensity	<50 packs years vs >50 packs years	1.14	0.28-4.58	.85	1.13	0.28-4.54	.86	1.14	0.28-4.60	.84	1.14	0.28-4.57	.85
Anemia	Absent vs Present	4.55	1.08-19.17	.04	4.69	1.11-19.86	.03	4.73	1.11-20.07	.03	4.48	1.06-18.86	.04
Smoking adjusted anemia	Absent vs Present	6.03	1.21-30.07	.03	6.26	1.25-31.31	.02	6.33	1.26-31.71	.02	5.92	1.19-29.52	.03
Tumor location	Glottic vs Supraglottic	0.82	0.07-9.06	.87	0.84	0.08-9.24	.88	0.85	0.08-9.39	.89	0.82	0.07-9.09	.87
	Glottic vs Transglottic	5.82	1.11-30.55	.04	5.68	1.08-29.81	.04	5.76	1.09-30.29	.04	5.74	1.09-30.11	.04
Tumor extension	Glottic/ Supraglottic vs Transglottic	6.24	1.46-26.61	.01	6.05	1.42-25.74	.01	6.10	1.43-25.99	.01	6.15	1.45-26.18	.01
Tumor differentiation	Well+Moderately vs Poor	2.21	0.45-10.98	.33	2.20	0.44-10.98	.33	2.23	0.45-11.07	.33	2.18	0.44-10.83	.34
Stage	Early stage vs Advanced stage	3.56	0.44-29.00	.24	3.53	0.43-28.80	.24	3.61	0.44-29.44	.23	3.51	0.43-28.62	.24
T-stage	CiS/T1+T2 vs T3+T4	3.97	0.49-32.33	.20	3.94	0.48-32.12	.20	4.02	0.49-32.76	.19	3.93	0.48-32.00	.20
Thyroid cartilage involvement	Absent vs Present	3.14	0.75-13.19	.12	3.20	0.76-13.45	.11	3.23	0.77-13.60	.11	3.09	0.73-12.96	.12
Lymph node involvement	Absent vs Present	0.69	0.14-3.46	0.66	0.69	0.14-3.43	.65	0.69	0.14-3.46	.65	0.68	0.14-3.41	.64
Treatment modality	Surgery vs Non-surgerical	1.83	0.37-9.12	.46	1.81	0.36-9.03	.47	1.79	0.36-8.90	.47	1.87	0.38-9.32	.44
Serum HIF-1 level	Low vs High	0.74	0.18-2.99	.68	0.71	0.17-2.91	.64	0.74	0.18-3.01	.68	0.70	0.17-6.56	.62
Serum HIF-2 level	Low vs High	5.40	1.09-26.76	.04	5.23	1.06-25.96	.04	5.29	1.07-26.23	.04	5.30	1.07-26.29	.04
Serum HIF-3 level	Low vs High	1.34	0.32-5.64	.69	1.37	0.32-5.75	.67	1.35	0.32-5.69	.68	1.34	0.32-5.63	.69

*statistically significant variables designated in bold.

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HIF-2 becomes the main biomolecule when chronic hypoxia occurs. Therefore, the transformation in hypoxia-related biomolecules is defined as HIF switch.[32] Holmquist-Mengelbier et al showed that while HIF-1 played an active role in short-term and intensively hypoxic/anoxic environments, HIF-2 played an active role in long-term and relatively milder hypoxic environments.[33,34] In addition, cancer cells might acquire aggressive properties in the physoxic environment due to HIF-2 activation and dedifferentiated with increased chaotic vascularisation which is referred as 'pseudohypoxia phenotype'.[35-37] In an experimental study performed by same group, it was shown that the intracellular domain of the stem cell marker CD44, a stem cell biomarker for HNCs, was released in hypoxic conditions and caused marked increase in HIF target gene activation by binding only to HIF-2.[38,39] Our data indicated that serum HIF-2 level might be a candidate prognostic biomarker in LC. We found that survival and locoregional control was adversely affected in patients with elevated HIF-2 levels (Figure 4). Moreover, Cox regression analysis showed that elevated serum HIF-2 levels were associated with more than a 7-fold increase in the hazard ratio (Table 3). Multivariate analysis of several risk factors showed that HIF-2 was an independent prognostic factor for LC. Similarly, Koukourakis et al performed immunohistochemical examination in tumour specimens of patients with HNC and reported that HIF-2 expression was an independent prognostic factor.[40] In addition, they found that HIF-1α overexpression was associated with radio +chemotherapy failure and decreased local control and overall survival. In the literature, there is a controversy about the predictive and prognostic role of HIF-1α expression in LC. Douglas et al[42] were unable to find any predictive or prognostic role of HIF-1α expression in advanced stage laryngopharyngeal cancer.[41] They also examined tumour samples of patients with early-stage glottic LC that received radiotherapy and reported that HIF-1α expression had no prognostic role regarding locoregional control. In contrast, Kwon et al[43] examined tumour samples of 45 patients with early-stage LC and concluded that despite a relationship between HIF-1α overexpression and residual tumour risk and locoregional control; it was not a prognostic biomarker for locoregional recurrence. Another study showed that HIF-1α overexpression was associated with adverse local control and overall survival in patients who underwent radiotherapy due to early-stage glottic LC.[44] However, same study group also reported that HIF-1α overexpression did not have any prognostic role in early-stage supraglottic LC.[45] Notably, we were unable to find the diagnostic, predictive, or prognostic role of HIF-1α in LC.

In this study, we were unable to determine a statistically significant difference between benign, premalignant and malignant laryngeal neoplasms when serum HIF-1, -2 and -3 levels were compared. As Fang et al[46] demonstrated HIFs can also be expressed in benign laryngeal neoplasms. Therefore, the diagnostic biomarker role of HIFs is unclear.

In biomarkers, the selection of sample type (tissue, plasma, serum, saliva, urine, etc) is of utmost importance. Apparently, every sample has its unique advantages and disadvantages. As a matter of fact, histopathological examination of tumour biopsy samples is the gold standard for cancer diagnosis. However, due to the heterogeneity in solid tumours, biomolecules examined in biopsy samples do not reflect the whole tumour microenvironment. Therefore, the biomarker role of these biomolecules is greatly compromised. Furthermore, Bredell et al[22] showed important limitations of biopsy-based hypoxic biomarkers in head and neck cancers: (a) biopsies are unable to reflect the tumour microcirculation and perfusion as they are generally obtained from the marginal parts of the tumour microenvironment; (b) acute and chronic hypoxic regions are not reflected due to the heterogeneous metabolic activity in the solid tumours; (c) tumour hypoxia shows regional changes because it progresses in a dynamic process; and (d) hypoxia has a unique behaviour irrespective of tumour size. However, it is noteworthy that this is a preliminary study with limited sample size. Therefore, the results of this study need to be confirmed by large-scale clinical studies.

5 | CONCLUSION

Our study demonstrated that the assessment of serum HIF-2 levels might be a useful method for the prediction of survival and locoregional control in LC. Thus, serum HIF-2 might be a candidate prognostic biomarker for patients with LC irrespective of stage and treatment modality.

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CONFLİCT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR CONTRİBUTİONS

The authors confirm contribution to the paper as follows: Study conception and design: Dr Görkem Eskiizmir, Dr Hülya Ellidokuz, Dr Yasemin Başpınar. Data collection: Dr Görkem Eskiizmir, Dr Gizem Çalıbaşı Koçal, Tuğba Uysal. Analysis and interpretation of results: Dr Görkem Eskiizmir, Dr Hülya Ellidokuz, Dr Yasemin Başpınar, Dr Gizem Çalıbaşı Koçal, Tuğba Uysal. Draft manuscript preparation: Dr Görkem Eskiizmir, Dr Hülya Ellidokuz, Dr Yasemin Başpınar, Dr Gizem Çalıbaşı Koçal, Tuğba Uysal. All authors reviewed the results and approved the final version of the manuscript.

ETHİCAL APPROVAL

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

DATA AVAİLABİLİTY STATEMENT

Data available on request due to privacy/ethical restrictions.

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REFERENCES

1. Lampri ES, Chondrogiannis G, Ioachim E, et al. Biomarkers of head and neck cancer, tools or a gordian knot? *Int J Clin Exp Med*. 2015;8:10340-10357.
2. Cramer JD, Hicks KE, Rademaker AW, Patel UA, Samant S. Validation of the eighth edition American Joint Committee on Cancer staging system for human papillomavirus-associated oropharyngeal cancer. *Head Neck*. 2018;40:457-466.
3. Quail DF, Joyce JA. Microenvironmental regulation of tumor progression and metastasis. *Nat Med*. 2013;19(11):1423-1437.
4. Dvorak HF, Weaver VM, Tlsty TD, Bergers G. Tumor microenvironment and progression. *J Surg Oncol*. 2011;103:468-474.
5. Catalano V, Turdo A, Di Franco S, et al. Tumor and its microenvironment: a synergistic interplay. *Semin Cancer Biol*. 2013;23:522-532.
6. Paoli P, Giannoni E, Chiarugi P. Anoikis molecular pathways and its role in cancer progression. *Biochim Biophys Acta*. 2013;1833:3481-3498.
7. Vaupel P. Tumor microenvironmental physiology and its implications for radiation oncology. *Semin Radiat Oncol*. 2004;14:198-206.
8. Kim Y, Lin Q, Glazer PM, Yun Z. Hypoxic tumor microenvironment and cancer cell differentiation. *Curr Mol Med*. 2009;9:425-434.
9. Muz B, de la Puente P, Azab F, Azab AK. The role of hypoxia in cancer progression, angiogenesis, metastasis, and resistance to therapy. *Hypoxia (Auckl)*. 2015;3:83-92.
10. Bussink J, Kaanders JH, van der Kogel AJ. Tumor hypoxia at the micro-regional level: clinical relevance and predictive value of exogenous and endogenous hypoxic cell markers. *Radiother Oncol*. 2003;67:3-15.
11. Vaupel P, Mayer A. Hypoxia in cancer: significance and impact on clinical outcome. *Cancer Metastasis Rev*. 2007;26(2):225-239.
12. Hoogsteen IJ, Marres HAM, Bussink J, van der Kogel AJ, Kaanders JH. Tumor microenvironment in head and neck squamous cell carcinomas: predictive value and clinical relevance of hypoxic markers. A review. *Head Neck*. 2007;29:591-604.
13. Curry JM, Sprandio J, Cognetti D, et al. Tumor microenvironment in head and neck squamous cell carcinoma. *Semin Oncol*. 2014;41(2):217-234.
14. Thomlinson RH, Gray LH. The histological structure of some human lung cancers and the possible implications for radiotherapy. *Br J Cancer*. 1955;9(4):539-549.
15. Brown JM. Evidence for acutely hypoxic cells in mouse tumours, and a possible mechanism of reoxygenation. *Br J Radiol*. 1979;52:650-656.
16. Eskiizmir G. Tumor microenvironment in head and neck squamous cell carcinomas. *Turk Arch Otorhinolaryngol*. 2015;53(3):120-127.
17. Semenza GL. HIF-1, O(2), and the 3 PHDs: how animal cells signal hypoxia to the nucleus. *Cell*. 2001;107:1-3.
18. Semenza GL. Hypoxia-inducible factors in physiology and medicine. *Cell*. 2012;148:399-408.
19. Weidemann A, Johnson RS. Biology of HIF-1alpha. *Cell Death Differ*. 2008;15:621-627.
20. Swartz JE, Pothen AJ, Stegeman I, Willems SM, Grolman W. Clinical implications of hypoxia biomarker expression in head and neck squamous cell carcinoma: a systematic review. *Cancer Med*. 2015;4(7):1101-1116.
21. Zhou J, Huang S, Wang L, et al. Clinical and prognostic significance of HIF-1α overexpression in oral squamous cell carcinoma: a meta-analysis. *World J Surg Oncol*. 2017;15(1):104.
22. Bredell MG, Ernst J, El-Kochairi I, et al. Current relevance of hypoxia in head and neck cancer. *Oncotarget*. 2016;31:50781-50804.
23. http://www.who.int/vmnis/indicators/haemoglobin.pdf
24. Tabachnick BG, Fidell LS. *Fidell Using Multivariate Statistics* (6th ed.). Boston, MA: Pearson; 2013.
25. Ludwig H, Fritz E. Anemia in cancer patients. *Semin Oncol*. 1998;25(Suppl 7):2-6.
26. Harrison L, Shasha D, Shiaova L, White C, Ramdeen B, Portenoy R. Prevalence of anemia in cancer patients undergoing radiation therapy. *Semin Oncol*. 2001;28:54-59.
27. Caro JJ, Salas M, Ward A, Goss G. Anemia as an independent prognostic factor for survival in patients with cancer: a systemic, quantitative review. *Cancer*. 2001;91(12):2214-2221.
28. Eskiizmir G, Baskın Y, Yalçın F, Ellidokuz H, Ferris RL. Risk factors for radiation failure in early-stage glottic carcinoma: a systematic review and meta-analysis. *Oral Oncol*. 2016;62:90-100.
29. Tanvetyanon T, Choudhury AM. Severity, risk factors, and physician practices in the management of anemia during concurrent chemoradiation for head and neck carcinoma. *Cancer*. 2006;106(7):1554-1559.
30. Heikal L, Ghezzi P, Mengozzi M, Ferns G. Assessment of HIF-1α expression and release following endothelial injury in-vitro and in-vivo. *Mol Med*. 2018;24:22.
31. Popov TM, Goranova T, Stancheva G, et al. Relative quantitative expression of hypoxia-inducible factor-1α, -2α and -3α, and vascular endothelial growth factor A in laryngeal carcinoma. *Oncol Lett*. 2015;9:2879-2885.
32. Koh MY, Powis G. Passing the baton: the HIF switch. *Trends Biochem Sci*. 2012;37(9):364-372.
33. Holmquist-Mengelbier L, Fredlund E, Löfstedt T, et al. Recruitment of HIF-1alpha and HIF-2alpha to common target genes is differentially regulated in neuroblastoma: HIF-2alpha promotes an aggressive phenotype. *Cancer Cell*. 2006;10:413-423.
34. Löfstedt T, Fredlund E, Holmquist-Mengelbier L, et al. Hypoxia inducible factor-2alpha in cancer. *Cell Cycle*. 2007;6:919-926.
35. Pietras A, Gisselsson D, Ora I, et al. High levels of HIF-2alpha highlight an immature neural crest-like neuroblastoma cell cohort located in a perivascular niche. *J Pathol*. 2008;214:482-488.
36. Pietras A, Hansford LM, Johnsson AS, et al. HIF-2alpha maintains an undifferentiated state in neural crest-like human neuroblastoma tumor-initiating cells. *Proc Natl Acad Sci USA*. 2009;106:16805-16810.
37. Pietras A, Johnsson AS, Pahlman S. The HIF-2alpha-driven pseudo-hypoxic phenotype in tumor aggressiveness, differentiation, and vascularization. *Curr Top Microbiol Immunol*. 2010;345:1-20.
38. Chen J, Zhou J, Lu J, et al. Significance of CD44 expression in head and neck cancer: a systemic review and meta-analysis. *BMC Cancer*. 2014;14:15.
39. Johansson E, Grassi ES, Pantazaopoulou V, et al. CD44 Interacts with HIF-2α to Modulate the Hypoxic Phenotype of Perinecrotic and Perivascular Glioma Cells. *Cell Rep*. 2017;20:1641-1653.
40. Koukourakis MI, Giatromanolaki A, Sivridis E. Hypoxia-inducible factor (HIF1A and HIF2A), angiogenesis, and chemoradiotherapy outcome of squamous cell head-and-neck cancer. *Int J Radiat Oncol Biol Phys*. 2002;53(5):1192-1202.
41. Bernstein JM, Andrews TD, Slevin NJ, West CM, Homer JJ. Prognostic value of hypoxia-associated markers in advanced larynx and hypopharynx squamous cell carcinoma. *Laryngoscope*. 2015;125:E8-15.
42. Douglas CM, Bernstein JM, Ormston VE, et al. Lack of prognostic effect of Carbonic Anhydrase-9, Hypoxia Inducible Factor-1α and Bcl-2 in 286 patients with Early Squamous Cell Carcinoma of the Glottic Larynx Treated with Radiotherapy. *Clin Oncol*. 2013;25:59-65.
43. Kwon OJ, Park JJ, Ko GH. HIF-1α and CA-IX as predictors of locoregional control for determining the optimal treatment modality for early-stage laryngeal carcinoma. *Head Neck*. 2015;37:505-510.
44. Schrijvers ML, van der Laan BF, de Bock GH, et al. Overexpression of intrinsic hypoxia markers HIF1alpha and CA-IX predict for local

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recurrence in stage T1-T2 glottic laryngeal carcinoma treated with radiotherapy. *Int J Radiat Oncol Biol Phys*. 2008;72:161-169.
45. Wachters JE, Schrijvers ML, Slagter-Menkema L, et al. Prognostic significance of HIF-1a, CA-IX, and OPN in T1-T2 laryngeal carcinoma treated with radiotherapy. *Laryngoscope*. 2013;123(9):2154-2160.
46. Fang R, Jiang JJ, Smith BL, Wu D. Expression of hypoxia inducible factor-1α and vascular endothelia growth factor in vocal polyps. *Laryngoscope*. 2013;123(9):2184-2188.

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