Exploration of the association between HIF3α mRNA and lncRNA MALAT1 in laryngeal squamous cell carcinoma by correlation analysis

SILVA GARO KYURKCHIYAN[1], GERGANA STANCHEVA[1], VERONIKA PETKOVA[1], YULIYAN HADZHIEV[2], VENERA DOBRIYANOVA[2], DIANA POPOVA[2], RADKA KANEVA[1] and TODOR MIROSLAVOV POPOV[2]

[1]Molecular Medicine Center, Department of Medical Chemistry and Biochemistry, Medical Faculty, Medical University, 1431 Sofia; [2]Department of Ear and Nose Treatment, UMHAT 'Tsaritsa Yoanna-ISUL', Medical University, 1537 Sofia, Bulgaria

Received August 24, 2023; Accepted March 28, 2024

DOI: 10.3892/ol.2024.14425

Abstract. Laryngeal squamous cell carcinoma (LSCC) is a significant global health burden, for which there has been limited evidence of improved survival rates. Although the roles of hypoxia-inducible factor (HIF)1α and HIF2α have been well documented in hypoxia, the involvement of HIF3α, particularly in LSCC, has been inadequately explored. The present study aimed to investigate the correlation between HIFα subunits and the hypoxia-related long noncoding RNAs (lncRNAs) MALAT1 and HOTAIR in 63 patients diagnosed with LSCC. Total RNA was extracted from fresh-frozen laryngeal tumor and adjacent normal tissues, and was subjected to reverse transcription-quantitative PCR for target detection. Statistical analyses were conducted using SPSS software, with significance set at $P<0.05$. The present study is the first, to the best of our knowledge, to report a positive moderate monotonic correlation (rs=0.347) and moderately strong positive linear correlation (r=0.630) between HIF3α mRNA and lncRNA MALAT1 in LSCC. Regression analysis revealed a direct association between 39.6% of both variables. Additionally, a positive correlation was observed between lncRNAs MALAT1 and HOTAIR (rs=0.353); HIF2α mRNA and lncRNA MALAT1 (rs=0.431); HIF3α mRNA and lncRNA HOTAIR (rs=0.279); and HIF3α mRNA and HIF2α mRNA (rs=0.285). Notably, a significant negative correlation (rs=-0.341) was detected between HIF3α mRNA and HIF1α mRNA, potentially indicative of the HIF switch or negative regulation. In addition, the present study investigated the association between HIFα subunits and the clinicopathological characteristics of patients. The results revealed a notable association between HIF1α transcript levels and the location of LSCC; specifically, subglottic tumors exhibited elevated HIF1α levels compared with glottic and supraglottic LSCC. Furthermore, a significant association was identified between HIF3α transcript levels and patient age (P=0.032) and positive family history (P=0.047). In conclusion, the present findings suggested a pivotal role for HIF3α in LSCC development, potentially involving direct regulation of lncRNA MALAT1. However, further research is warranted to elucidate its precise mechanisms.

Correspondence to: Dr Silva Garo Kyurkchiyan, Molecular Medicine Center, Department of Medical Chemistry and Biochemistry, Medical Faculty, Medical University, 2 Zdrave Street, 1431 Sofia, Bulgaria
E-mail: silva.kyurkchiyan@gmail.com

Key words: laryngeal cancer, laryngeal squamous cell carcinoma, HIF1α, HIF2α, HIF3α, long noncoding RNA, MALAT1, HOTAIR, expression, correlation

Introduction

Laryngeal carcinoma ranks as the second most prevalent type of head and neck cancer, following lip and oral cavity cancer. The Global Cancer Observatory reported 184,615 registered cases of laryngeal malignancies in 2020, with men exhibiting incidence and mortality rates of 3.6 and 1.9 per 100,000 (age-standardized rate), respectively, excluding non-melanoma skin cancer (1). Laryngeal squamous cell carcinoma (LSCC) constitutes the majority of laryngeal tumors, with tobacco smoking, heavy alcohol consumption and human papillomavirus infections identified as primary risk factors for its development; however, some authors dispute the latter in laryngeal cancer (2,3). Despite advances in cancer treatments and the evolution of personalized medicine in oncology, LSCC continues to pose a global health burden, with minimal improvements in survival rates over recent decades (4). Often diagnosed at advanced stages, LSCC significantly impacts the quality of life of affected individuals. Exploration of the molecular biology and genetics of LSCC remains crucial for improving current treatment protocols, which encompass surgery, radiotherapy, chemotherapy and/or EGFR-targeted therapy.

Active cell proliferation, a hallmark of cancer, enhances tumor growth and induces hypoxia due to reduced oxygen supply. Hypoxia, in turn, triggers changes in the expression levels of hypoxia-inducible factor (HIF)α subunits, leading to the formation of HIF transcriptional factors when

they bind to HIFβ subunits (5). These HIFs modulate the expression of genes associated with cell proliferation, angiogenesis, migration, invasion, cellular metabolism and cell survival (6). While HIF1α and HIF2α have been extensively studied and linked to the development of malignancies, the detailed investigation of HIF3α, particularly in the context of laryngeal cancer, remains limited (7). Dynamic changes in the levels of the three HIFα subunits in response to varying durations of hypoxia have been observed, with HIF1α activation occurring during acute hypoxia, followed by HIF2α, and subsequent expression of HIF3α during chronic hypoxia, a phenomenon known as the 'HIF switch'. This switch is purportedly regulated by non-coding RNAs (ncRNAs) (8,9).

The complexity of HIF3α is evident as >10 transcript variants have been identified (10). The presence of these variants adds a layer of intricacy to the function of HIF3α, with HIF3α1 and HIF3α9 bearing the closest sequence identity to HIF1α and HIF2α sequences. Moreover, tissue-specific expression and potential divergent roles in gene expression regulation have been suggested for HIF3α variant transcripts (10,11).

Long ncRNAs (lncRNAs) form a substantial category of RNA transcripts, >200 nucleotides long, and are recognized as potential molecular markers. lncRNAs serve as major regulators of cellular pathways, and have been implicated in cancer development and progression (12). Notably, the lncRNAs MALAT1 and HOTAIR have been extensively studied. Under hypoxic conditions in lung cancer, elevated levels of lncRNA HOTAIR have been associated with direct interaction with HIF1α, leading to increased proliferation, migration and invasion (13). Similarly, the lncRNA HOTAIR and HIF1α axis has been strongly linked to radioresistance in cervical cancer (14,15). Furthermore, lncRNA MALAT1, induced by hypoxia and regulated by HIF1α and HIF2α, has been shown to function as a sponge for microRNA (miR)-3064-5p in breast cancer, promoting cell proliferation, tumor growth and metastasis (16).

We have previously published results on the expression levels of the three HIFα subunits, and both lncRNAs MALAT1 and HOTAIR in LSCC (17,18). Building on this foundation, the present study aimed to explore the connection and relation of lncRNAs with major HIF genes in laryngeal carcinoma. In addition, the study aimed to investigate the association of HIF1α, HIF2α and HIF3α, with the clinicopathological features of patients with LSCC.

Materials and methods

Sample collection. The present study assessed samples from 63 patients diagnosed with LSCC. Tumor and adjacent normal tissue specimens within 3 cm from the border of the tumor were collected during surgical procedures conducted between the periods January 2015-June 2019 and November 2020-August 2023 at the Ear, Nose and Throat Department of UMHAT 'Tsaritsa Yoanna-ISUL' (Sofia, Bulgaria). Following surgical excision, the specimens were promptly frozen in liquid nitrogen (-196˚C) and transported to the Molecular Medicine Center Biobank (Sofia, Bulgaria) within 1-2 days. The tissue samples were subsequently stored at -80˚C until use or for longer durations. Written informed consent was obtained from each patient, which is kept at our repository at the Molecular Medicine Center and the Department of ENT, Medical University (Sofia, Bulgaria). The study was approved by the Ethics Committee of the Medical University of Sofia (approval nos. BK-297/14.04.2015; BK-373 11.04.2016; 7836/1 7.11.2020 and 4584/04.07.2023). None of the included patients had undergone radiotherapy or chemotherapy before surgery.

Histopathological and clinical characteristics. Additionally, from the same 63 patients with LSCC, formalin-fixed, paraffin-embedded tissue was utilized for to determine the histopathological characteristics of the samples. To fix the tissues 10% neutral buffered formalin for 24-48 h at room temperature was used. The section thickness used was 5-6 μm. The analysis included the gold standard for diagnosis of solid tumors. The histology results reported the type and origin of tissue samples, determination of cancer staging (how advanced is the cancer) and cancer grading (determining the aggressiveness of the cancer). Moreover, the presence or absence of lymph node metastasis and cell differentiation were examined histopathologically. The location of all malignant tumors (anatomical sites infiltrated by the tumor) were observed and determined during surgery. Along with histopathological characteristics, clinical features about sex, age, tobacco smoking, alcohol consumption, familial history and harmful environment were taken.

Total RNA extraction. Total RNA was isolated from fresh frozen normal and tumor tissue samples from each patient using the RNeasy Mini Kit (Qiagen GmbH), according to the manufacturer's protocol. The quality of the RNA was assessed by denaturing electrophoresis on a formaldehyde gel, while the concentration of RNA was determined using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Inc.).

Reverse transcription-quantitative PCR (RT-qPCR). For cDNA synthesis, 1 μg total RNA from each sample was used, employing the High-Capacity cDNA RT Kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) and the RT^2 First Strand Kit (Qiagen GmbH) in accordance with the respective manufacturer's recommendations. High-Capacity cDNA Kit provides improved detection of mRNAs and RT2 First Strand Kit is designed for lncRNA detection. The cDNA samples were subsequently processed for further analysis or stored at -20˚C until required. In the present study, the expression levels of HIF1α mRNA, HIF2α mRNA, HIF3α mRNA, were analyzed using 1X RotorGene SYBR Green PCR Mix (Qiagen GmbH), whereas lncRNA MALAT1 and lncRNA HOTAIR were analyzed using RT^2 SYBR Green Mastermix (Qiagen GmbH). Due to nature of lncRNAs the manufacturers suggested specific kits for the different aims. A laboratory optimization and analysis were performed of all included in the RT-qPCR techniques to validate the specificity of the reagents. The thermocycling conditions were as follows: Initial denaturation at 95˚C for 5 min, followed by 45 cycles of denaturation at 95˚C for 15 sec, primer annealing at 55˚C for 30 sec and synthesis with data acquisition at 72˚C for 30 sec. The 1X QuantiTect Primer Assays (Qiagen GmbH) were used for mRNA detection: Hs_HIF1A_1_SG (NM_001243084), Hs_EPAS1_1_SG (NM_001430),

Hs_HIF3A_1_SG (NM_152795), Hs_VEGFA_1_SG (NM_001025366), whereas the RT2 lncRNA qPCR assays for human MALAT1 (NR_002819) and human HOTAIR (NR_003716) (Qiagen GmbH) were used for lncRNA detection. Each sample underwent triplicate examination and corresponding no-template controls were assessed. β-actin (Hs_ACTB_1_SG, NM_001101; QuantiTect Primer Assay; Qiagen GmbH) was employed as the reference gene for normalization. The relative expression of each gene in the tumor samples was determined using the $2^{-\Delta\Delta Cq}$ method, with the calculation of relative quantification (RQ) (19). An RQ ≥2 was defined as upregulation, RQ <0.5 as downregulation, and RQ values between 1.99 and 0.5 indicated no change in expression.

Statistical analysis. IBM SPSS Statistics v.23 (IBM, Corp.) was used for all statistical analyses. The expression levels of the studied targets were evaluated in 63 laryngeal tumor and adjacent normal laryngeal squamous cell tissue specimens. Normality was assessed using the Kolmogorov-Smirnov test, after which appropriate non-parametric tests, including Mann-Whitney U test, Kruskal-Wallis test and Spearman's rank correlation analysis were employed. Additionally, in non-parametric tests, ranks were calculated and were compared in statistical analysis. Additionally, Pearson's correlation analysis and coefficient of determination were utilized for the analysis of linear dependence between variables. P<0.05 was considered to indicate a statistically significant difference.

Results

Study cohort. The present study included 63 patients, comprising 2 women and 61 men, all of whom were diagnosed with LSCC. The mean age of the patient group was 61 years (age range, 41-84 years). The clinicopathological characteristics of the patients and the expression levels of the explored targets are summarized in Table I.

Relative expression in laryngeal tumor samples. Significant dysregulation of the three HIFαs (HIF1α, HIF2α and HIF3α mRNA) and both lncRNAs (MALAT1 and HOTAIR) was observed in LSCC samples compared with in adjacent normal laryngeal specimens. Specifically, HIF1α mRNA was found to be upregulated in 44 out of 63 patients (69.84%) and normally expressed in the remaining 19 patients (30.16%). Additionally, upregulation of HIF2α mRNA was detected in 7 patients (11.11%), while the expression was almost silenced in 24 patients, with the remaining 32 patients exhibiting normal expression levels. Notably, HIF3α mRNA was predominantly downregulated in the majority of patients (48 patients, 76.19%), while 10 patients (15.87%) exhibited normal levels of expression and 5 patients (7.94%) demonstrated significant upregulation. Furthermore, lncRNA MALAT1 showed downregulation in 35 patients (55.56%) and upregulation in 4 patients (6.35%), whereas it was found to have normal expression in the remaining 24 patients with LSCC (38.09%). On the other hand, lncRNA HOTAIR exhibited upregulation in 35 patients with LSCC (55.56%), downregulation in 16 patients (25.40%), and normal expression in 12 patients (19.05%). Comprehensive details of the relative expression levels of the studied targets are published separately in previous original articles by our team (12,13).

Association between HIFα expression levels and clinicopathological characteristics. The associations between the RQ data of the studied HIF1/2/3α mRNAs and the clinicopathological characteristics of patients with LSCC are presented in Table I, along with the results of non-parametric tests (Mann-Whitney U and Kruskal-Wallis tests). Statistically significant associations were demonstrated between HIF3α mRNA expression levels, age and family history, and between HIF1α mRNA levels and tumor localization. Notably, patients aged >60 years exhibited significantly higher HIF3α mRNA expression levels when compared with those ≤60 years (P=0.032). Moreover, higher HIF3α mRNA expression levels were observed in patients reporting a family history of cancer (P=0.047). Additionally, HIF1α mRNA expression levels demonstrated gradual elevation from supraglottis to subglottis tumor location (P=0.010).

Correlation analysis between the expression levels of HIFα mRNA and the lncRNAs MALAT1 and HOTAIR. A correlation analysis was conducted to investigate the strength and degree of dependency among the included targets in samples from the 63 patients with LSCC. Spearman's rank correlation analysis was applied, revealing statistically significant weak and moderate monotonic dependencies between various targets. Positive correlations were observed between the lncRNAs MALAT1 and HOTAIR (rs=0.353; P<0.004); HIF2α mRNA and lncRNA MALAT1 (rs=0.431; P<0.01); HIF3α mRNA and lncRNA MALAT1 (rs=0.347; P=0.005); HIF3α mRNA and lncRNA HOTAIR (rs=0.279; P=0.027); and HIF3α mRNA and HIF2α mRNA (rs=0.285; P<0.025). The only significantly negative correlation was detected between HIF3α mRNA and HIF1α mRNA (rs=-0.341; P=0.006). To further assess any linear relationship between the targets, Pearson's correlation analysis was performed, revealing a moderately strong positive linear dependence between HIF3α mRNA and lncRNA MALAT1 (r=0.630; P<0.001) and a weak linear dependence between both lncRNAs, MALAT1 and HOTAIR (r=0.267; P=0.034). The results indicated that 39.6% of the HIF3α and MALAT1 variables could be predicted to be linearly dependent, with the rest of the coefficient of determinations (R^2) at 7.2% or lower. Fig. 1 displays a small network representing the significant dependence between the studied targets, illustrating positive and negative correlations. Additionally, scatter plots with fitted regression lines and R^2 values are presented in Fig. 2 to visually represent the relationships between significantly correlated targets.

Discussion

The results of the present study indicated marked dysregulation in the expression levels of HIF1/2/3α mRNAs, and the lncRNAs MALAT1 and HOTAIR, in the studied group of patient samples, suggesting their potential involvement in laryngeal carcinogenesis. The primary focus of the present study was to assess the correlations between the three investigated HIFα mRNAs and the hypoxia-related lncRNAs MALAT1 and HOTAIR in fresh-frozen LSCC samples, given

Table I. Associations between HIF1/2/3α transcripts levels and patient clinicopathological features.

Feature	No. of patients (%)	RQ of HIF1α, mean ± SD (ranks)	RQ of HIF2α, mean ± SD (ranks)	RQ of HIF3α, mean ± SD (ranks)
Sex				
Female	2 (5)	2.82±2.29 (31.46)	0.91±0.70 (31.73)	0.68±1.72 (32.53)
Male	61 (95)	3.13±0.93 (42.83)	1.22±1.14 (37.50)	0.40±0.68 (21.33)
P-value		0.294	0.594	0.301
Age, years				
≤60	27 (43)	3.11±2.39 (35.00)	0.83±0.72 (29.97)	0.37±0.89 (17.72)
>60	36 (57)	2.50±1.43 (28.00)	1.03±0.71 (34.70)	0.99±2.30 (37.70)
P-value		0.134	0.311	0.032[a]
Tumor stage				
T1/2	9 (14)	2.21±1.81 (21.44)	1.85±0.95 (24.56)	0.26±0.31 (29.33)
T3	23 (37)	2.64±1.29 (32.72)	1.10±0.69 (37.72)	0.69±2.18 (31.07)
T4	31 (49)	3.25±2.49 (34.53)	0.81±0.69 (29.92)	0.74±1.45 (33.47)
P-value		0.164	0.127	0.798
Nodal stage				
N0 negative	36 (48)	2.83±2.17 (31,47)	0.99±0.71 (33.55)	0.65±1.83 (30.17)
N1-3 positive	27 (52)	2.87±1.44 (33,70)	0.65± 0.70 (27.03)	0.61±0.94 (37.87)
P-value		0.681	0.229	0.155
Tumor differentiation				
G1/well	25 (40)	2.67±1.34 (32.22)	1.10±0.94 (33.38)	0.83±2.32 (29.78)
G2/moderate	32 (51)	3.04±2.57 (32.08)	0.82±0.49 (33.52)	0.56±1.05 (34.77)
G3/poor	6 (9)	2.47±1.17 (30.67)	0.53±0.19 (18.17)	0.16±0.19 (26.50)
P-value		0.982	0.151	0.440
Tumor localization				
Supraglottis	18 (29)	2.32±1.32 (28.15)	0.95±0.73 (33.07)	0.69±1.95 (30.86)
Glottis	34 (54)	3.06±2.58 (31.71)	0.65±0.39 (26.97)	0.45±0.83 (34.18)
Subglottis	8 (12)	4.76±2.65 (50.00)	1.26±1.02 (39.13)	0.76±1.67 (31.94)
P-value		0.010[a]	0.252	0.815
Family history				
No	35 (56)	2.90±1.90 (28.03)	0.88±0.66 (26.61)	0.51±1.72 (23.60)
Yes	17 (27)	2.71±2.33 (23.35)	0.98±0.85 (26.26)	0.91±1.60 (32.47)
Missing data	11 (17)			
P-value		0.297	0.938	0.047[a]
Tobacco smoking				
No	3 (5)	5.01±4.72 (35.67)	0.81±0.71 (27.83)	1.54±2.60 (31.00)
≤20 cigarettes per day	21 (33)	2.38±1.10 (24.86)	1.10±0.74 (30.62)	0.30±0.71 (24.86)
21-40 cigarettes per day	19 (30)	2.59±1.22 (27.35)	0.90±0.72 (29.05)	1.10±2.46 (27.35)
≥41 cigarettes per day	13 (20)	3.46±3.05 (30.17)	0.64±0.68 (20.71)	0.17±0.27 (30.17)
Missing data	7 (11)			
P-value		0.632	0.145	0.241
Alcohol consumption				
No	9 (14)	3.71±2.91 (30.44)	0.85±0.81 (25.83)	0.61±1.48 (28.56)
<100 ml per day	15 (24)	2.68±0.90 (30.40)	0.97±0.70 (28.27)	0.46±2.52 (24.40)
100-200 ml per day	14 (22)	2.73±2.51 (22.32)	0.94±0.74 (26.91)	0.68±1.44 (25.06)
>200 ml per day	14 (22)	2.51±1.47 (24.41)	0.86±0.73 (24.00)	0.44±0.51 (29.91)
Missing data	11 (17)			
P-value		0.223	0.837	0.693
Environmental risks				
No	24 (38)	2.83±2.00 (27.52)	0.67±0.40 (23.02)	0.55±2.00 (24.10)
Yes	28 (44)	2.84±2.09 (25.63)	1.12±0.86 (29.48)	0.72±1.37 (28.55)
Missing data	11 (17)			
P-value		0.653	0.125	0.290

HIF, hypoxia-inducible factor; lncRNA, long noncoding RNA; R^2, coefficient of determination; RQ, relative quantification.

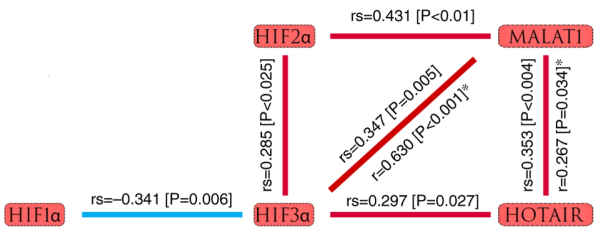

Figure 1. Network representing the significant associations between studied targets, according to Spearman's correlation analysis. Correlation coefficient and P-values are shown. The red lines indicate a positive correlation, whereas the blue line represents a negative correlation. *P<0.05 (Pearson correlation coefficient). HIF, hypoxia-inducible factor.

their dysregulation in cancer (20). The Spearman's correlation analysis highlighted moderate dependence between HIF3α and both HIF1α and HIF2α, as well as between lncRNA MALAT1 and HOTAIR. Furthermore, a moderately strong positive tendency was observed for a linear relationship between HIF3α and lncRNA MALAT1 via Pearson's correlation analysis, indicating that a proportion of the variables could be linearly dependent. Additionally, the findings revealed a positive correlation between HIF2α and lncRNA MALAT1, which is consistent with other research (16) indicating a link between hypoxia-induced upregulation of both molecules, and their regulatory roles in metastasis and invasion. Moreover, positive associations between lncRNA MALAT1 and HIF3α transcripts have been linked to the involvement of miR-429 in cancer development (9,21-23), highlighting the complexity of the interplay between these factors in malignant transformation. Such findings underscore the potential of the HIF3α mRNA/lncRNA MALAT1/miR-429 axis as a target for therapeutic investigation in patients with LSCC and with elevated HIF3α levels. To the best of the authors' knowledge, the present study is the first to demonstrate such significant associations between lncRNAs and the major key players of angiogenesis, the HIFs.

Given that laryngeal cancer primarily develops over a protracted period and is less common in young individuals, it is notable that the present results revealed a significant association between HIF3α mRNA expression levels and age. This age-associated association may be linked to the impact of chronic hypoxia on the long-term development of laryngeal cancer. However, while the changes in HIFα expression between age groups are often contradictory across different studies, the present findings indicate the need for a more comprehensive understanding of the relationship between age and HIFα expression (24,25).

Furthermore, the present study identified a positive association between HIF3α mRNA expression levels and a positive family history of cancer. Given the major etiological factors for LSCC development in Europe (i.e., tobacco smoking and alcohol consumption), a positive family history represents a critical risk factor in malignancies. The positive family history may be related with inheritance of genetic predispositions to laryngeal cancer development or tobacco smoking addiction. The combination of both positive familial history and tobacco smoking fold increases the risk of laryngeal malignancy. The present study suggested that inherited oncological co-factors may modulate HIF3α mRNA expression, potentially contributing to an aggressive phenotype closely associated with increased HIF expression. Notably, mutations in the von Hippel-Lindau gene, a key regulator of the multiprotein complex responsible for HIF molecule degradation, have been implicated in the increased expression of HIFs in hereditary cancer (26). To the best of the authors' knowledge, the present study is the first to investigate a significant association between age, family history and HIF3α mRNA expression.

An examination of the expression of all three HIFα mRNA levels in comparison to laryngeal tumor localization yielded novel insights. A significant gradual expression of HIF1α between supraglottic, glottic and subglottic laryngeal tumors was observed, with subglottic tumors exhibiting higher HIF1α transcript levels in comparison to supraglottic and glottic tumors. While other studies have produced varying results, the present findings highlight the critical need for in-depth investigations into the role of both HIF1α mRNA and protein levels, and the mechanisms of action in LSCC (27,28). Given that tumor size is associated with increased expression levels of HIF1α, it could be hypothesized that relatively asymptomatic subglottic carcinoma may exhibit a larger extent in the same tumor stage patient subgroups, thereby demonstrating a heightened expression profile (29).

Notably, the only negative correlation was detected between HIF1α and HIF3α transcripts, a finding in line with previous studies highlighting a regulatory relationship between these two factors in different types of cancer (30,31). Moreover, the present study identified a significant positive correlation between HIF3α and HIF2α mRNAs, consistent with previous research suggesting a potential target gene relationship between HIF3α and HIF2α (32,33). The observed positive correlation may reflect a specific cellular mechanism involving alternative pathways in laryngeal cancer development, underscoring the need for further detailed investigation to clarify the monotonic correlation between HIF2α and HIF3α transcript levels.

The present study provided information on the complex interactions between HIFα subunits and hypoxia-related lncRNAs in the context of LSCC, uncovering novel associations and dependencies. The findings call for further in-depth investigations to elucidate the precise mechanisms underlying these correlations and their implications for targeted therapeutic approaches in LSCC. A major limitation of the present study includes the need for additional methodology that would confirm findings, such as immunohistochemistry and *in situ* hybridization.

In conclusion, the present study may improve the understanding of laryngeal cancer by demonstrating strong associations between lncRNAs and the major key players of angiogenesis, i.e. HIF isoforms. The analysis revealed a significant direct correlation between HIF3α mRNA and lncRNA MALAT1, indicating a potential axis involved in cancer cell progression under chronic hypoxia. Furthermore, the present findings highlighted novel associations between age, family history and HIF3α, and LSCC tumor location and HIF1α expression, underscoring the complex interplay between clinicopathological characteristics and HIFα forms. These results emphasize the need for further investigations to fully elucidate the intricate molecular and genetic mechanisms underlying LSCC development. The present study may

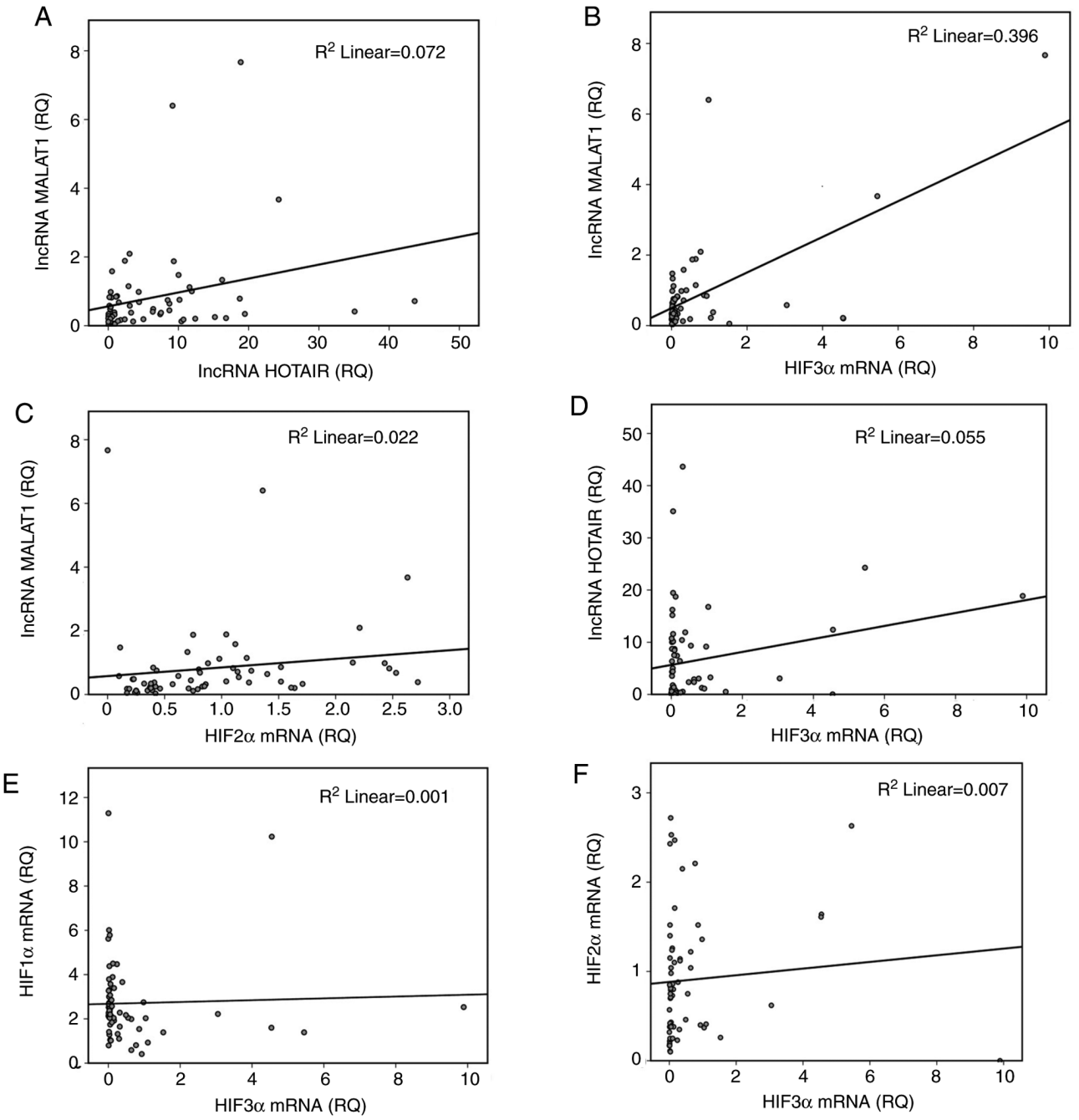

Figure 2. Scatter plots between significantly correlated targets (Spearman's correlation analysis). Fitted regression line and R^2 were added to each graph. (A) lncRNA MALAT1 and lncRNA HOTAIR; (B) lncRNA MALAT1 and HIF3α mRNA; (C) lncRNA MALAT1 and HIF2α mRNA; (D) lncRNA HOTAIR and HIF3α mRNA; (E) HIF1α mRNA and HIF3α mRNA; (F) HIF2α mRNA and HIF3α mRNA. HIF, hypoxia-inducible factor; lncRNA, long noncoding RNA; R^2, coefficient of determination; RQ, relative quantification.

stimulate new projects essential for advancing the comprehension of laryngeal cancer biology and potentially informing the development of targeted therapeutic approaches.

Acknowledgements

Not applicable.

Funding

The present study was financed by the European Union-Next Generation EU, through the National Recovery and Resilience Plan of the Republic of Bulgaria (project no. BG-RRP-2.004-0004-C01, group 3.1.5).

Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

SGK, GS and TMP designed the study. TMP, YH and VD performed the surgery treatments of all enrolled in the

present study patients, collected the tissue samples as appropriate and information about clinicopathological features of the patients. SGK, GS and VP performed the laboratory experiments and analyzed the data. SGK and GS confirm the authenticity of all the raw data. SGK drafted the manuscript. SGK, DP, RK contributed to the data interpretation and discussion. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Written informed consent was obtained from each patient, which is kept at the repository at the Molecular Medicine Center and the Department of ENT, Medical University, Sofia, and the current study was approved by the Ethics Committee of Medical University of Sofia (approval nos. BK-297/14.04.2015; BK-373 11.04.2016; 7836/17.11.2020 and 4584/04.07.2023).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A and Bray F: Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 71: 209-249, 2021.
2. Nocini R, Molteni G, Mattiuzzi C and Lippi G: Updates on larynx cancer epidemiology. Chin J Cancer Res 32: 18-25, 2020.
3. Liberale C, Soloperto D, Marchioni A, Monzani D and Sacchetto L: Updates on larynx cancer: Risk factors and oncogenesis. Int J Mol Sci 24: 12913, 2023.
4. Li MM, Zhao S, Eskander A, Rygalski C, Brock G, Parikh AS, Haring CT, Swendseid B, Zhan KY, Bradford CR, *et al*: Stage migration and survival trends in laryngeal cancer. Ann Surg Oncol 28: 7300-7309, 2021.
5. Satija S, Kaur H, Tambuwala MM, Sharma P, Vyas M, Khurana N, Sharma N, Bakshi HA, Charbe NB, Zacconi FC, *et al*: Hypoxia-Inducible Factor (HIF): Fuel for cancer progression. Curr Mol Pharmacol 14: 321-332, 2021.
6. Sebestyén A, Kopper L, Dankó T and Tímár J: Hypoxia signaling in cancer: From basics to clinical practice. Pathol Oncol Res 27: 1609802, 2021.
7. Jaskiewicz M, Moszynska A, Serocki M, Króliczewski J, Bartoszewska S, Collawn JF and Bartoszewski R: Hypoxia-inducible factor (HIF)-3a2 serves as an endothelial cell fate executor during chronic hypoxia. EXCLI J 21: 454-469, 2022.
8. Serocki M, Bartoszewska S, Janaszak-Jasiecka A, Ochocka RJ, Collawn JF and Bartoszewski R: miRNAs regulate the HIF switch during hypoxia: A novel therapeutic target. Angiogenesis 21: 183-202, 2018.
9. Janaszak-Jasiecka A, Bartoszewska S, Kochan K, Piotrowski A, Kalinowski L, Kamysz W, Ochocka RJ, Bartoszewski R and Collawn JF: miR-429 regulates the transition between hypoxia-inducible factor (HIF)1A and HIF3A expression in human endothelial cells. Sci Rep 6: 22775, 2016.
10. Duan C: Hypoxia-inducible factor 3 biology: complexities and emerging themes. Am J Physiol Cell Physiol 310: C260-C269, 2016.
11. Tanimato K: Genetics of the hypoxia-inducible factors in human cancers. Exp Cell Res 356: 166-172, 2017.
12. St Laurent G, Wahlestedt C and Kapranov P: The Landscape of long noncoding RNA classification. Trends Genet 31: 239-251, 2015.
13. Zhou C, Ye L, Jiang C, Bai J, Chi Y and Zhang H: Long noncoding RNA HOTAIR, a hypoxia-inducible factor-1α activated driver of malignancy, enhances hypoxic cancer cell proliferation, migration, and invasion in non-small cell lung cancer. Tumour Biol 36: 9179-9188, 2015.
14. Li N, Meng DD, Gao L, Xu Y, Liu PJ, Tian YW, Yi ZY, Zhang Y, Tie XJ and Xu ZQ: Overexpression of HOTAIR leads to radioresistance of human cervical cancer via promoting HIF-1α expression. Radiat Oncol 13: 210, 2018.
15. Kabakov AE and Yakimova AO: Hypoxia-Induced cancer cell responses driving radioresistance of hypoxic tumors: Approaches to targeting and radiosensitizing. Cancers (Basel) 13: 1102, 2021.
16. Shih CH, Chuang LL, Tsai MH, Chen LH, Chuang EY, Lu TP and Lai LC: Hypoxia-Induced MALAT1 promotes the proliferation and migration of breast cancer cells by sponging MiR-3064-5p. Front Oncol 11: 658151, 2021.
17. Garo Kyurkchiyan S, Miroslavov Popov T, Stancheva G, Rangachev J, Ivanov Mitev V, Petrova Popova D and Petrova Kaneva R: Novel insights into laryngeal squamous cell carcinoma from association study of aberrantly expressed miRNAs, lncRNAs and clinical features in Bulgarian patients. J BUON 25: 357-366, 2020.
18. Popov TM, Goranova T, Stancheva G, Kaneva R, Dikov T, Chalakov I, Rangachev J, Konov D, Todorov S, Stoyanov O and Mitev V: Relative quantitative expression of hypoxia-inducible factor-1α, -2α and -3α, and vascular endothelial growth factor A in laryngeal carcinoma. Oncol Lett 9: 2879-2885, 2015.
19. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25: 402-408, 2001.
20. Aiello A, Bacci L, Re A, Ripoli C, Pierconti F, Pinto F, Masetti R, Grassi C, Gaetano C, Bassi PF, *et al*: MALAT1 and HOTAIR Long Non-Coding RNAs play opposite role in estrogen-mediated transcriptional regulation in prostate cancer cells. Sci Rep 6: 38414, 2016.
21. Xiao H, Zhu Q and Zhou J: Long non-coding RNA MALAT1 interaction with miR-429 regulates the proliferation and EMT of lung adenocarcinoma cells through RhoA. Int J Clin Exp Pathol 12: 419-430, 2019.
22. Liu X, Zhao W and Wang X: Inhibition of long non-coding RNA MALAT1 elevates microRNA-429 to suppress the progression of hypopharyngeal squamous cell carcinoma by reducing ZEB1. Life Sci 262: 118480, 2020.
23. Fang H, Li HF, He MH, Yan JY, Yang M, Zhang FX, Wang RR, Wang QY and Zhang JP: Long non-coding RNA MALAT1 sponges microRNA-429 to regulate apoptosis of hippocampal neurons in hypoxic-ischemic brain damage by regulating WNT1. Brain Res Bull 152: 1-10, 2019.
24. Dzhalilova DS and Makarova OV: The role of hypoxia-inducible factor in the mechanisms of aging. Biochemistry (Mosc) 87: 995-1014, 2022.
25. Ebersole JL, Novak MJ, Orraca L, Martinez-Gonzalez J, Kirakodu S, Chen KC, Stromberg A and Gonzalez OA: Hypoxia-inducible transcription factors, HIF1A and HIF2A, increase in aging mucosal tissues. Immunology 154: 452-464, 2018.
26. Shanbhogue KP, Prasad AS, Ucisik-Keser FE, Katabathina VS and Morani AC: Hereditary ovarian tumour syndromes: Current update on genetics and imaging. Clin Radiol 76: 313.e15-313.e26, 2021.
27. Wu XH, Chen SP, Mao JY, Ji XX, Yao HT and Zhou SH: Expression and significance of hypoxia-inducible factor-1α and glucose transporter-1 in laryngeal carcinoma. Oncol Lett 5: 261-266, 2013.
28. Singh P, Rajput M and Pandey M: Tumor hypoxia and role of hypoxia-inducible factor in oral cancer. World J Surg Oncol 22: 18, 2024.
29. Bronikowska I, Swietochowska E, Morawski R, Sowa P and Czecior E: Concentration of hypoxia-inducible factor-1, glucose transporter 1 and vascular endothelial growth factor in tissue samples and serum in patients with primary laryngeal carcinoma. Acta Otolaryngol 142: 532-536, 2022.
30. Forristal CE, Wright KL, Hanley NA, Oreffo RO and Houghton FD: Hypoxia inducible factors regulate pluripotency and proliferation in human embryonic stem cells cultured at reduced oxygen tensions. Reproduction 139: 85-97, 2010.

31. Xu Z, Lv H, Wang Y, Hu C, Chen S, Du Y, Shi C and Cheng X: HAND2-AS1 inhibits gastric adenocarcinoma cells proliferation and aerobic glycolysis via miRNAs Sponge. Cancer Manag Res 12: 3053-3068, 2020.
32. Liu P, Fang X, Song Y, Jiang JX, He QJ and Liu XJ: Expression of hypoxia-inducible factor 3α in hepatocellular carcinoma and its association with other hypoxia-inducible factors. Exp Ther Med 11: 2470-2476, 2016.
33. Kirschner KM, Kelterborn S, Stehr H, Penzlin JLT, Jacobi CLJ, Endesfelder S, Sieg M, Kruppa J, Dame C and Sciesielski LK: Adaptation of the oxygen sensing system during lung development. Oxid Med Cell Longev 2022: 9714669, 2022.