

Exploration of the association between HIF3 α mRNA and lncRNA MALAT1 in laryngeal squamous cell carcinoma by correlation analysis

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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)1 α and HIF2 α have been well documented in hypoxia, the involvement of HIF3 α , particularly in LSCC, has been inadequately explored. The present study aimed to investigate the correlation between HIF α 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 ($r_s = 0.347$) and moderately strong positive linear correlation ($r = 0.630$) between HIF3 α 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 ($r_s = 0.353$); HIF2 α mRNA and lncRNA MALAT1 ($r_s = 0.431$); HIF3 α mRNA and lncRNA HOTAIR ($r_s = 0.279$); and HIF3 α mRNA and HIF2 α mRNA ($r_s = 0.285$). Notably, a significant negative correlation ($r_s = -0.341$) was detected between HIF3 α mRNA and HIF1 α mRNA, potentially indicative of the HIF switch or negative regulation. In addition, the present study investigated the

association between HIF α subunits and the clinicopathological characteristics of patients. The results revealed a notable association between HIF1 α transcript levels and the location of LSCC; specifically, subglottic tumors exhibited elevated HIF1 α levels compared with glottic and supraglottic LSCC. Furthermore, a significant association was identified between HIF3 α 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 HIF3 α in LSCC development, potentially involving direct regulation of lncRNA MALAT1. However, further research is warranted to elucidate its precise mechanisms.

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

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they bind to HIF β subunits (5). These HIFs modulate the expression of genes associated with cell proliferation, angiogenesis, migration, invasion, cellular metabolism and cell survival (6). While HIF1 α and HIF2 α have been extensively studied and linked to the development of malignancies, the detailed investigation of HIF3 α , particularly in the context of laryngeal cancer, remains limited (7). Dynamic changes in the levels of the three HIF α subunits in response to varying durations of hypoxia have been observed, with HIF1 α activation occurring during acute hypoxia, followed by HIF2 α , and subsequent expression of HIF3 α 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 HIF3 α is evident as >10 transcript variants have been identified (10). The presence of these variants adds a layer of intricacy to the function of HIF3 α , with HIF3 α 1 and HIF3 α 9 bearing the closest sequence identity to HIF1 α and HIF2 α sequences. Moreover, tissue-specific expression and potential divergent roles in gene expression regulation have been suggested for HIF3 α 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 HIF1 α , leading to increased proliferation, migration and invasion (13). Similarly, the lncRNA HOTAIR and HIF1 α axis has been strongly linked to radioresistance in cervical cancer (14,15). Furthermore, lncRNA MALAT1, induced by hypoxia and regulated by HIF1 α and HIF2 α , 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 HIF α 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 HIF1 α , HIF2 α and HIF3 α , 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 μ m. 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 μ g total RNA from each sample was used, employing the High-Capacity cDNA RT Kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) and the RT² 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 HIF1 α mRNA, HIF2 α mRNA, HIF3 α mRNA, were analyzed using 1X RotorGene SYBR Green PCR Mix (Qiagen GmbH), whereas lncRNA MALAT1 and lncRNA HOTAIR were analyzed using RT² 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 $2^{-\Delta\Delta C_q}$ 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 HIF α s (HIF1 α , HIF2 α and HIF3 α mRNA) and both lncRNAs (MALAT1 and HOTAIR) was observed in LSCC samples compared with in adjacent normal laryngeal specimens. Specifically, HIF1 α 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 HIF2 α 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, HIF3 α 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 HIF α expression levels and clinicopathological characteristics. The associations between the RQ data of the studied HIF1/2/3 α 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 HIF3 α mRNA expression levels, age and family history, and between HIF1 α mRNA levels and tumor localization. Notably, patients aged > 60 years exhibited significantly higher HIF3 α mRNA expression levels when compared with those ≤ 60 years ($P = 0.032$). Moreover, higher HIF3 α mRNA expression levels were observed in patients reporting a family history of cancer ($P = 0.047$). Additionally, HIF1 α mRNA expression levels demonstrated gradual elevation from supraglottis to subglottis tumor location ($P = 0.010$).

Correlation analysis between the expression levels of HIF α 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 ($r_s = 0.353$; $P < 0.004$); HIF2 α mRNA and lncRNA MALAT1 ($r_s = 0.431$; $P < 0.01$); HIF3 α mRNA and lncRNA MALAT1 ($r_s = 0.347$; $P = 0.005$); HIF3 α mRNA and lncRNA HOTAIR ($r_s = 0.279$; $P = 0.027$); and HIF3 α mRNA and HIF2 α mRNA ($r_s = 0.285$; $P < 0.025$). The only significantly negative correlation was detected between HIF3 α mRNA and HIF1 α mRNA ($r_s = -0.341$; $P = 0.006$). To further assess any linear relationship between the targets, Pearson's correlation analysis was performed, revealing a moderately strong positive linear dependence between HIF3 α 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 HIF3 α 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 HIF1/2/3 α 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 HIF α mRNAs and the hypoxia-related lncRNAs MALAT1 and HOTAIR in fresh-frozen LSCC samples, given

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

Feature	No. of patients (%)	RQ of HIF1 α , mean \pm SD (ranks)	RQ of HIF2 α , mean \pm SD (ranks)	RQ of HIF3 α , mean \pm SD (ranks)
Sex				
Female	2 (5)	2.82 \pm 2.29 (31.46)	0.91 \pm 0.70 (31.73)	0.68 \pm 1.72 (32.53)
Male	61 (95)	3.13 \pm 0.93 (42.83)	1.22 \pm 1.14 (37.50)	0.40 \pm 0.68 (21.33)
P-value		0.294	0.594	0.301
Age, years				
\leq 60	27 (43)	3.11 \pm 2.39 (35.00)	0.83 \pm 0.72 (29.97)	0.37 \pm 0.89 (17.72)
$>$ 60	36 (57)	2.50 \pm 1.43 (28.00)	1.03 \pm 0.71 (34.70)	0.99 \pm 2.30 (37.70)
P-value		0.134	0.311	0.032 ^a
Tumor stage				
T1/2	9 (14)	2.21 \pm 1.81 (21.44)	1.85 \pm 0.95 (24.56)	0.26 \pm 0.31 (29.33)
T3	23 (37)	2.64 \pm 1.29 (32.72)	1.10 \pm 0.69 (37.72)	0.69 \pm 2.18 (31.07)
T4	31 (49)	3.25 \pm 2.49 (34.53)	0.81 \pm 0.69 (29.92)	0.74 \pm 1.45 (33.47)
P-value		0.164	0.127	0.798
Nodal stage				
N0 negative	36 (48)	2.83 \pm 2.17 (31.47)	0.99 \pm 0.71 (33.55)	0.65 \pm 1.83 (30.17)
N1-3 positive	27 (52)	2.87 \pm 1.44 (33.70)	0.65 \pm 0.70 (27.03)	0.61 \pm 0.94 (37.87)
P-value		0.681	0.229	0.155
Tumor differentiation				
G1/well	25 (40)	2.67 \pm 1.34 (32.22)	1.10 \pm 0.94 (33.38)	0.83 \pm 2.32 (29.78)
G2/moderate	32 (51)	3.04 \pm 2.57 (32.08)	0.82 \pm 0.49 (33.52)	0.56 \pm 1.05 (34.77)
G3/poor	6 (9)	2.47 \pm 1.17 (30.67)	0.53 \pm 0.19 (18.17)	0.16 \pm 0.19 (26.50)
P-value		0.982	0.151	0.440
Tumor localization				
Supraglottis	18 (29)	2.32 \pm 1.32 (28.15)	0.95 \pm 0.73 (33.07)	0.69 \pm 1.95 (30.86)
Glottis	34 (54)	3.06 \pm 2.58 (31.71)	0.65 \pm 0.39 (26.97)	0.45 \pm 0.83 (34.18)
Subglottis	8 (12)	4.76 \pm 2.65 (50.00)	1.26 \pm 1.02 (39.13)	0.76 \pm 1.67 (31.94)
P-value		0.010 ^a	0.252	0.815
Family history				
No	35 (56)	2.90 \pm 1.90 (28.03)	0.88 \pm 0.66 (26.61)	0.51 \pm 1.72 (23.60)
Yes	17 (27)	2.71 \pm 2.33 (23.35)	0.98 \pm 0.85 (26.26)	0.91 \pm 1.60 (32.47)
Missing data	11 (17)			
P-value		0.297	0.938	0.047 ^a
Tobacco smoking				
No	3 (5)	5.01 \pm 4.72 (35.67)	0.81 \pm 0.71 (27.83)	1.54 \pm 2.60 (31.00)
\leq 20 cigarettes per day	21 (33)	2.38 \pm 1.10 (24.86)	1.10 \pm 0.74 (30.62)	0.30 \pm 0.71 (24.86)
21-40 cigarettes per day	19 (30)	2.59 \pm 1.22 (27.35)	0.90 \pm 0.72 (29.05)	1.10 \pm 2.46 (27.35)
\geq 41 cigarettes per day	13 (20)	3.46 \pm 3.05 (30.17)	0.64 \pm 0.68 (20.71)	0.17 \pm 0.27 (30.17)
Missing data	7 (11)			
P-value		0.632	0.145	0.241
Alcohol consumption				
No	9 (14)	3.71 \pm 2.91 (30.44)	0.85 \pm 0.81 (25.83)	0.61 \pm 1.48 (28.56)
$<$ 100 ml per day	15 (24)	2.68 \pm 0.90 (30.40)	0.97 \pm 0.70 (28.27)	0.46 \pm 2.52 (24.40)
100-200 ml per day	14 (22)	2.73 \pm 2.51 (22.32)	0.94 \pm 0.74 (26.91)	0.68 \pm 1.44 (25.06)
$>$ 200 ml per day	14 (22)	2.51 \pm 1.47 (24.41)	0.86 \pm 0.73 (24.00)	0.44 \pm 0.51 (29.91)
Missing data	11 (17)			
P-value		0.223	0.837	0.693
Environmental risks				
No	24 (38)	2.83 \pm 2.00 (27.52)	0.67 \pm 0.40 (23.02)	0.55 \pm 2.00 (24.10)
Yes	28 (44)	2.84 \pm 2.09 (25.63)	1.12 \pm 0.86 (29.48)	0.72 \pm 1.37 (28.55)
Missing data	11 (17)			
P-value		0.653	0.125	0.290

HIF, hypoxia-inducible factor; lncRNA, long noncoding RNA; R², coefficient of determination; RQ, relative quantification.

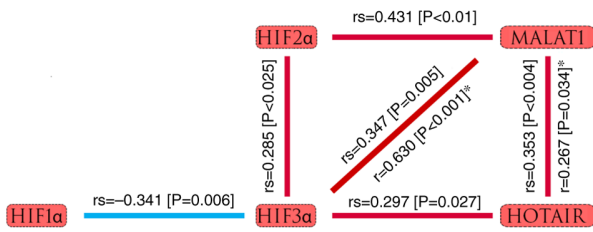


Figure 1. Network representing the significant associations between studied targets, according to Spearman's correlation analysis. Correlation coefficient and P-values are shown. The red lines indicate a positive correlation, whereas the blue line represents a negative correlation. * $P < 0.05$ (Pearson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

their dysregulation in cancer (20). The Spearman's correlation analysis highlighted moderate dependence between HIF3 α and both HIF1 α and HIF2 α , as well as between lncRNA MALAT1 and HOTAIR. Furthermore, a moderately strong positive tendency was observed for a linear relationship between HIF3 α 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 HIF2 α 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 HIF3 α 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 HIF3 α mRNA/lncRNA MALAT1/miR-429 axis as a target for therapeutic investigation in patients with LSCC and with elevated HIF3 α 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 HIF3 α 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 HIF α 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 HIF α expression (24,25).

Furthermore, the present study identified a positive association between HIF3 α 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 HIF3 α 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 HIF3 α mRNA expression.

An examination of the expression of all three HIF α mRNA levels in comparison to laryngeal tumor localization yielded novel insights. A significant gradual expression of HIF1 α between supraglottic, glottic and subglottic laryngeal tumors was observed, with subglottic tumors exhibiting higher HIF1 α 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 HIF1 α mRNA and protein levels, and the mechanisms of action in LSCC (27,28). Given that tumor size is associated with increased expression levels of HIF1 α , 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 HIF1 α and HIF3 α 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 HIF3 α and HIF2 α mRNAs, consistent with previous research suggesting a potential target gene relationship between HIF3 α and HIF2 α (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 HIF2 α and HIF3 α transcript levels.

The present study provided information on the complex interactions between HIF α 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 HIF3 α 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 HIF3 α , and LSCC tumor location and HIF1 α expression, underscoring the complex interplay between clinicopathological characteristics and HIF α 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

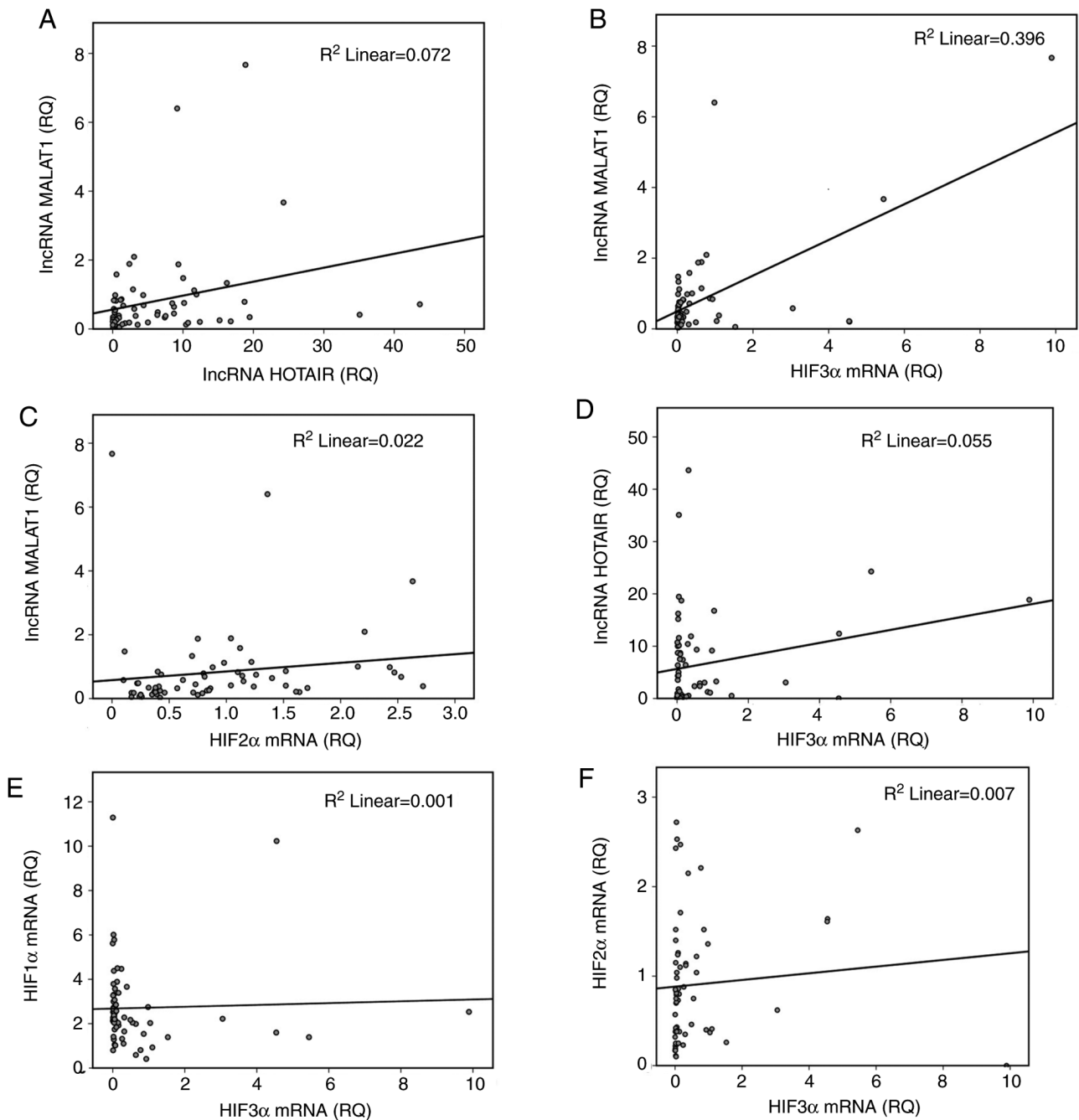


Figure 2. Scatter plots between significantly correlated targets (Spearman's correlation analysis). Fitted regression line and R^2 were added to each graph. (A) lncRNA MALAT1 and lncRNA HOTAIR; (B) lncRNA MALAT1 and HIF3 α mRNA; (C) lncRNA MALAT1 and HIF2 α mRNA; (D) lncRNA HOTAIR and HIF3 α mRNA; (E) HIF1 α mRNA and HIF3 α mRNA; (F) HIF2 α mRNA and HIF3 α 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.

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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.

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