Role of Krüppel-like factor 4 and heat shock protein 27 in cancer of the larynx

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Abstract. Late detection and lack of standard treatment strategies in larynx cancer patients result in high levels of mortality and poor prognosis. Prognostic stratification of larynx cancer patients based on molecular prognostic tumor biomarkers may lead to more efficient clinical management. Krüppel-like factor 4 (KLF4) and Heat Shock Protein 27 (HSP27) have an important role in tumorigenesis and are considered promising candidate biomarkers for various types of cancer. However, their role in larynx carcinoma remains to be elucidated. The present study aimed to determine KLF4 and HSP27 expression profiles in laryngeal tumors. The protein and mRNA expression levels of KLF4 and HSP27 were evaluated by immunohistochemical and reverse transcription-polymerase chain reaction analyses in 44 larynx carcinoma samples and 21 normal tissue samples, and then correlated with clinical characteristics. A differential expression of KLF4 and HSP27 was observed between normal and tumor tissues. The protein and mRNA expression levels of KLF4 were significantly decreased in larynx squamous cell carcinoma (LSCC) compared with normal tissue, whereas HSP27 was significantly overexpressed in tumor tissues compared with normal tissues, at the protein and mRNA levels. KLF4 expression decreased gradually with tumor progression whereas HSP27 expression increased. A significant difference was observed between stages I and IV. KLF4 and HSP27 exhibit opposite functions and roles in the carcinogenic process of LSCC. Their role in laryngeal cancer initiation and progression emphasizes their use as potential future targets for prognosis and treatment. KLF4 and HSP27 expression levels may act as potential biomarkers in patients with cancer of the larynx.

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Introduction

Laryngeal squamous cell carcinoma (LSCC) is the second most common malignant neoplasm of head and neck squamous cell carcinoma. It is an aggressive malignancy associated with high rates of metastasis, recurrence and a low 5-year survival rate. It has a high incidence and primarily involves therapeutic failure especially for the advanced cases (1,2). Therefore, identifying novel potential prognostic markers may lead to an improved clinical management of patients with laryngeal cancer.

Krüppel-like factors (KLFs) are a family of DNA binding transcriptional regulators expressed in a wide variety of human tissues. These factors have diverse and essential functions in multiple cell processes, including proliferation, inflammation, differentiation, migration, pluripotency, maintenance of homeostasis, and apoptosis (3,4). KLF4 is a bifunctional transcription factor able to either activate or repress transcription using different mechanisms, depending on the target gene. Thus, depending on the cell type or cell context or cancer stage, KLF4 may act either as a tumor suppressor gene or as an oncogene. KLF4 is involved in cell cycle, where it can induce cycle arrest in some molecular contexts, while favoring proliferation in others (5). In colorectal and gastric cancers, KLF4 expression decreases at early stages due to different mechanisms including loss of heterozygosity, hypermethylation of the promoter and point mutations in the *KLF4* gene, and may be lost with tumor growth and progression (6-8). Moreover, it was found that KLF4 is downregulated at the mRNA and protein levels in several non-small cell lung carcinoma cell lines, partially due to promoter hypermethylation. The restoration of KLF4 expression inhibits the clone formation and induces a delay in *in vivo* tumor growth (9).

KLF4 is overexpressed in 70% of primary mammary cancers at the stage of ductal carcinoma, where it plays an oncogenic role. In addition, nuclear localization of KLF4 in ductal carcinoma predicts an unfavorable outcome (10,11). Previous findings showed the beneficial side of knocking down KLF4, as p53-dependent cell death is restored (12). In head and neck squamous cell carcinoma (HNSCC) tissues, persistent KLF4 expression predicts poor prognosis and confers aggressiveness (13). Recent studies showed that KLF4 is upregulated

in small cell lung carcinoma tissues and has a potential tumor-promoting role in this type of lung malignancy (14).

Heat shock proteins (HSPs) are highly conserved molecular chaperones with principal roles in protein homeostasis, transport processes and signal transduction. Recently, heat shock proteins, found to be overexpressed in a wide range of malignancies, have been considered as promising candidate biomarkers for some cancers (15-17). Heat shock protein 27 (HSP27) is a molecular chaperone highly expressed in aggressive cancers, where it is involved in numerous pro-tumorigenic signaling pathways (18,19). Overexpression of HSP27 was observed across different types of cancer including breast, ovarian, prostate, bladder, gastric, and oral squamous cell carcinoma and many others (18,20). Its overexpression contributes to cancer progression via different mechanisms, and its anti-apoptotic and pro-survival activities play crucial roles in tumorigenesis. HSP27 increases proliferation by facilitating cell cycle progression and enhances migration and invasion via several mechanisms (21,22). Additionally, high levels of HSP27 have been associated with poor prognosis and chemo- and radioresistance in various cancers including prostate, breast, head and neck and lung cancer (21,23-27). HSP27 is now considered an attractive therapeutic target for cancer treatment. *In vitro* and *in vivo* studies have shown that the downregulation of HSP27 protein expression using antisense oligonucleotides or siRNA contributes in the reduction of tumor progression, induction of apoptosis and tumor sensitization to treatment (27-29). The strategy of HSP27 gene inhibition, using Apatorsen (OGX-427) a 2'-methoxyethyl-modified antisense oligonucleotide, has shown a promising therapeutic effect in clinical application. A phase I dose-escalation study showed a good tolerance of Apatorsen, associated with a decrease in tumor markers and in circulating tumor cells and a stable measurable disease in patients with castration-resistant prostate, breast, ovary, lung, and bladder cancer (30).

Only a few studies have investigated the potential role and the profile of mRNA and protein expression of HSP27 in laryngeal squamous cell carcinoma tissues (31,32). Nevertheless, no studies have assessed the role and the expression of KLF4 in laryngeal cancer tissues. In this study, we examined the KLF4 and HSP27 mRNA and protein levels, by RT-PCR and immunohistochemical analyses, respectively, in laryngeal tumors (n=44) and normal tissues (n=21). We also evaluated the combinational clinical significance of KLF4 and HSP27 expression for the diagnosis or prognosis and treatment decision-making in laryngeal cancers.

Materials and methods

Sample collection. Forty-four formalin-fixed paraffin-embedded larynx carcinoma samples and 21 normal tissue samples were collected from the Department of Pathological Anatomy of the Notre Dame de Secours University Hospital (Byblos, Lebanon) and the National Institute of Pathology (Baabda, Lebanon). Following surgical removal, all the tissue samples were fixed in formalin and embedded in paraffin prior to sectioning for histological, immunohistochemical and gene expression analyses. The cancer tissue samples were graded independently by a pathologist and histologically classified. Epidemiological and clinical data were collected from patient records and registries (Table I). This study was approved by the Institutional Review Board of the Notre Dame de Secours University Hospital.

Table I. Patient characteristics.

Characteristics	No. (%)
Total Subjects	65
Normal tissues	21 (32.3)
Tumor tissues	44 (67.7)
Sex	
Male	83.1
Female	16.9
Age median (range), years	65 (47-88)
Stage	
I	9 (20.5)
II	11 (25)
III	6 (13.6)
IV	18 (40.9)

Table II. Results of KLF4 immunostaining in normal and tumor tissues (cases per intensity of expression).

Protein expression	Normal tissue (%)	Tumor tissue (%)	P-value (normal vs. tumor)
0	3 (14.3)	27 (61.4)	<0.001
1+	14 (66.7)	7 (15.9)	<0.001
2+	4 (19)	7 (15.9)	>0.05
3+	0 (0)	3 (6.8)	>0.05

Immunohistochemistry and immunoscoring. Sections of paraffin-embedded tissue specimens (4 μm) were subjected to immunostaining using the Ventana automated stainer (BenchMark XT; Roche Diagnostics GmbH, Mannheim, Germany) at the National Institute of Pathology (Baabda, Lebanon). The tissue sections were deparaffinized using xylene, rehydrated through graded ethanols and equilibrated in phosphate-buffered saline before undergoing antigen retrieval. The endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide for 5 min. The tissue sections were subsequently incubated with the primary mouse monoclonal antibodies at a dilution of 1:200 for 1 h at room temperature: anti-KLF4 antibody (SAB5300069; clone 1E6), and anti-HSP27 antibody (SAB3701437; clone G3.1) (both from Sigma-Aldrich, St. Louis, MO, USA). The appropriate secondary antibody was horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG (A9044; Sigma-Aldrich) at a dilution of 1:200 for 1 h at room temperature. The HRP detection was achieved with 3,3'-diaminobenzidine substrate (Sigma-Aldrich) and counterstained with hematoxylin.

Two investigators (G.A. and E.H.) independently scored the slides in a blinded manner. A quantitative score was performed

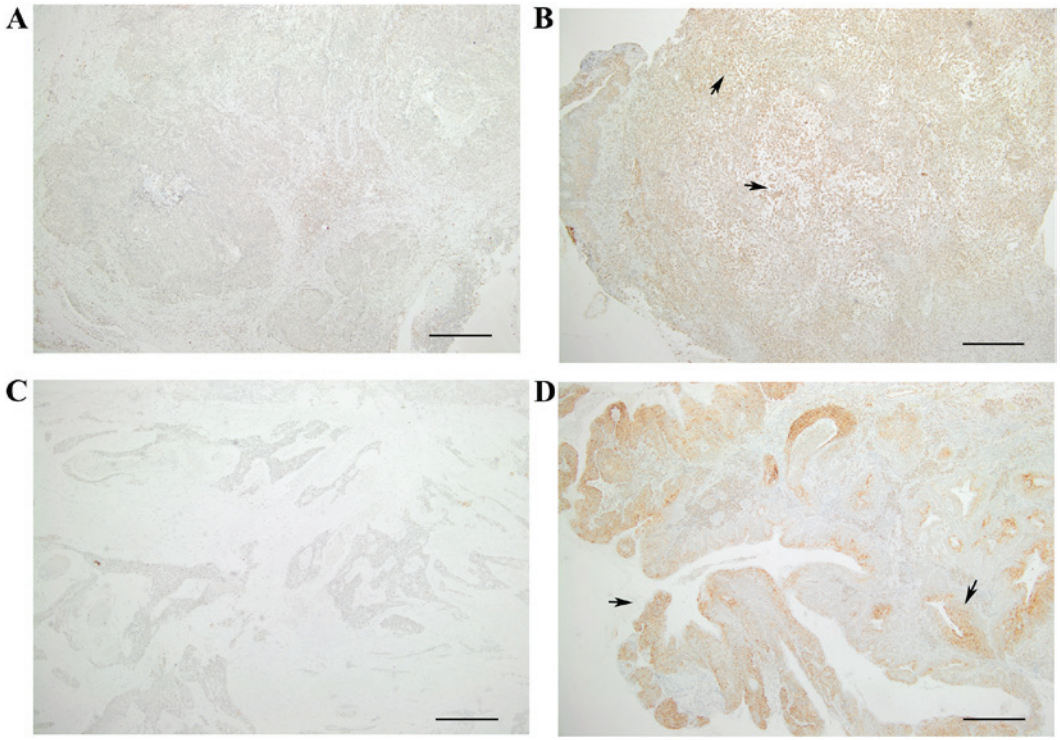

Figure 1. Immunohistochemical analysis of Krüppel-like factor 4 and Heat Shock Protein 27 in larynx tissues. Representative tumor tissue sections with (A) negative KLF4 expression, (B) intense positive KLF4 expression in tumor cells (arrow), (C) negative HSP27 expression, and (D) intense positive HSP27 expression at basal layer (arrow). Magnification x40; scale bar, 52 μm.

by adding the score of the staining area and the score of staining intensity for each case to assess the expression levels of KLF4 and HSP27. The quantitative score was estimated by calculating the percentage of immunopositive cells as follows: 0, no staining of cells in any microscopic fields; 1+, <30% of tissue stained positive; 2+, between 30 and 60% stained positive; and 3+, >60% stained positive. The intensity of staining was scored by evaluating the average staining intensity of the positive cells: 0, no staining; 1+, mild staining; 2+, moderate staining; and 3+, intense staining .

RNA extraction and reverse transcriptase-quantitative PCR analysis. Total RNA extraction from formalin-fixed paraffin-embedded tissue sections (20 μm) was performed using GenElute™ FFPE RNA Purification kit (RNB400; Sigma-Aldrich) according to the manufacturer's instructions. RNA (2 μg) was reverse transcribed using iScript™ cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA). Reverse transcriptase-quantitative PCR (RT-qPCR) was performed in a CFX96™ Real-Time System using iQ™ SYBR® Green Supermix (Bio-Rad). The primer sequences used in RT-PCR were: HSP27 reverse, 5'-TCGAAGGTGACTGGGATGGT-3' and forward, 5'-CCCCCATGCCCAAGCTA-3'; KLF4 reverse, 5'-ATGTGTAAGGCGAGGTGGTC-3' and forward, 5'-ACC CACACAGGTGAGAAACC-3'; GAPDH reverse, 5'-TGGTGG TCCAGGGGTCTTAC-3' and forward, 5 '-TTGCCCTCAACG ACCAGTTT-3' (Sigma-Aldrich). The *GAPDH* gene was used as an internal control for the relative mRNA amount. All the experiments were performed in triplicate and normalized to GAPDH mRNA expression. The relative RNA level was automatically calculated with the ΔΔCq method.

Statistical analysis. Statistical analyses were performed using GraphPad Prism 5. The χ^2 test, paired t-test, and Fisher test were used to compare the protein and mRNA expression level of KLF4 and HSP27 between tumors and normal tissues and between the different tumor stages. P<0.05 was considered to indicate statistically significant differences (P<0.05, P<0.01, P<0.001).

Results

Patient characteristics. Table I shows the characteristics of the patients. The median age of patients was 65 years and 83.1% of the patients were male. According to the TNM staging system, 20.5% were stage I (n=9), 25% were stage II (n=11), 13.6% were stage III (n=6), and 40.9% were stage IV (n=18).

Expression levels of KLF4. The immunohistochemical analysis showed that KLF4 was expressed in the nucleus of tumor and normal cells. Representative findings of the immunohistochemical staining are shown in Fig. 1A and B. A significant difference in KLF4 protein expression was observed between normal and cancer tissues (P<0.001) (Table II). KLF4 expression was significantly lower in tumor tissues compared to normal tissues. The profile of KLF4 protein expression in each tumor stage is shown in Table III. No significant difference of expression was observed between stages. The protein expression of KLF4 in stage I is similar to that in normal tissues. It decreases in stages II and III, and was mostly downregulated in stage IV tumors (Table III). To determine whether this decrease of the protein expression occurred at the transcriptional level, the KLF4 mRNA levels were evaluated

Table III. Profile of KLF4 protein expression in the tumor stages (cases per intensity of expression).

	KLF4 protein expression, n (%)			
Stage (n)	0 (no staining)	1+ (mild staining)	2+ (moderate staining)	3+ (intense staining)
I (9)	4 (44.5)	2 (22.2)	1 (11.1)	2 (22.2)
II (11)	5 (45.4)	3 (27.3)	3 (27.3)	0
III (6)	3 (50.0)	1 (16.7)	2 (33.3)	0
IV (18)	15 (83.2)	1 (5.6)	1 (5.6)	1 (5.6)

Table IV. Results of HSP27 immunostaining in normal and tumor tissues (cases per intensity of expression).

Protein expression	Normal tissue (%)	Tumor tissue (%)	P-value (normal vs. tumor)
0	5 (23.8)	0 (0)	<0.001
1+	15 (71.4)	1 (2.3)	>0.05
2+	1 (4.8)	19 (43.2)	<0.001
3+	0 (0)	24 (54.5)	<0.001

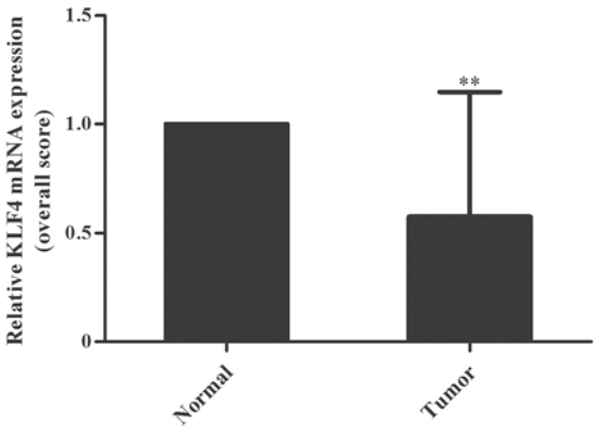

Figure 2. KLF4 mRNA expression in normal and tumor larynx tissues. KLF4 mRNA expression levels were significantly decreased in larynx tumor tissues compared with normal tissues (**P=0.0058).

in normal and cancer tissues by quantitative real-time PCR analysis. A significant lower KLF4 mRNA copy numbers were found in tumor tissues compared to normal tissues (P=0.0058), in the same manner as for the protein expression (Fig. 2). These results showed that KLF4 expression was downregulated in laryngeal tumors not only at the protein level but also at the RNA level.

Expression levels of HSP27. The immunohistochemical staining showed that HSP27 is mainly expressed in the cytoplasm at a significant difference of intensities between normal and tumor sections. Examples of the immunohistochemical staining for HSP27 are shown in the Fig. 1C and D. As shown in Table IV, HSP27 was significantly overexpressed in tumor tissues compared to normal tissues (P<0.001). A significant gradual increase in the HSP27 protein expression was observed from stage I to stage IV (Table V) (P=0.0039). These results were confirmed by quantitative real-time PCR analysis showing that HSP27 was also up-regulated at RNA level in larynx cancers (Fig. 3). The HSP27 copy numbers were significantly higher in cancer tissue sections compared to normal tissue sections (P=0.0115).

Factors associated with KLF4 and HSP27 expression levels. In order to determine any correlation of KLF4 and HSP27 expression levels with age and gender, a statistical analysis was performed. KLF4 and HSP27 expression levels were not found to be significantly associated with age or gender.

Discussion

Laryngeal carcinoma is the second most common malignancy among head and neck tumors. Although the clinical outcome of laryngeal carcinoma has gradually improved, the prognosis of this tumor remains poor. A better understanding of the molecular mechanisms and key molecules driving laryngeal carcinogenesis may aid in the identification of novel predictive and prognostic biomarkers, and in the development of novel treatment strategies for this cancer. The role of KLF4 and HSP27 as possible biomarkers and therapy targets has been extensively investigated in various types of cancer (33,34). However, the aberrant expression of these proteins in laryngeal squamous cell carcinoma (LSCC) is poorly understood. In the present study, the expression profile and the potential role of these proteins as possible biomarkers of LSCC were investigated.

In this study, we examined the expression of KLF4 and HSP27 in a series of human laryngeal tumors and normal tissues. The expression profile of these proteins indicated that they are significantly regulated in LSCC. The protein and mRNA expression levels of KLF4 were significantly decreased in LSCC compared to those in normal tissue, while HSP27 was significantly overexpressed in tumor compared to normal tissues, at the protein and mRNA levels. Regarding tumor stages, the expression of the two proteins varies in an opposite manner. The KLF4 expression decreases gradually with tumor progression suggesting that KLF4 expression is lost as the tumor progresses, while HSP27 expression increases with stages, showing a significant difference between stages I and IV. These findings suggest that KLF4 and HSP27 may be opposite functions and roles in the carcinogenic process of LSCC. KLF4 seems to play a tumor suppressing role in

Table V. Profile of HSP27 protein expression in the tumor stages (cases per intensity of expression).

	Hsp27 protein expression, n (%)			
Stage (n)	0 (no staining)	1+ (mild staining)	2+ (moderate staining)	3+ (intense staining)
I (9)	0	1 (11.1)	6 (66.7)	2 (22.2)
II (11)	0	0	5 (45.5)	6 (54.5)
III (6)	0	0	4 (66.7)	2 (33.3)
IV (18)	0	0	4 (22.2)	14 (77.8)

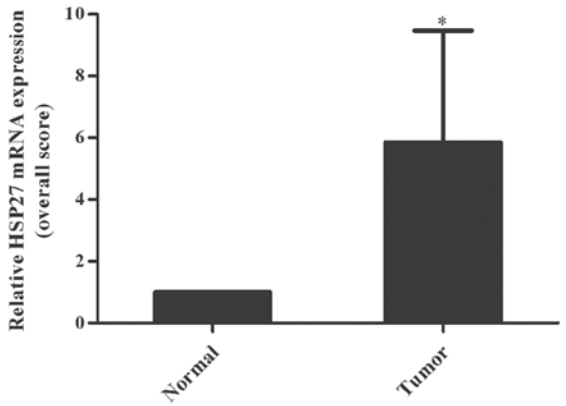

Figure 3. HSP27 mRNA expression levels in normal and tumor larynx tissues. HSP27 mRNA expression levels were significantly increased in larynx tumor tissues compared with normal tissues (*P=0.0115).

LSCC, while HSP27 seems to play an oncogenic role and its overexpression may contribute in the initiation of the disease and its progression and aggressiveness. The downregulation of KLF4 in LSCC may be associated with promoter hypermethylation, a loss of heterozygosity of the KLF4 locus, or with point mutations in the coding region. Epigenetic control and gene network may play a role in the decrease of KLF4 levels in laryngeal cancers. The decrease in its expression in stage IV was associated with increased tumor differentiation and aggressiveness. The mechanisms underlying this downregulation require elucidation by future studies.

The aberrant expression of KLF4 has been reported in various forms of cancer. However, no previous studies have examined the expression level of KLF4 in laryngeal carcinoma. To the best of our knowledge, the present study is the first to provide a preliminary description of the profile of expression of KLF4 and its potential role in laryngeal carcinogenesis. This is the first study showing a significant difference in KLF4 protein and mRNA expression levels between normal and cancerous laryngeal tissues. Our findings were consistent with those reported by different studies showing a tumor suppressor role of KLF4 in several types of cancer, including gastric and colon cancers (6-8), bladder cancer (35), esophageal cancer (36), and non small cell lung carcinoma (14,37). Of note, in head and neck squamous cell carcinoma malignancies, KLF4 can exert different roles in different types. In oral squamous cell carcinoma, the expression of KLF4 increases at the early stages of the disease where it plays an oncogenic role (38). However, in our study on LSCC, opposite results were obtained. The expression of KLF4 appears to exert a dual effect depending on the cell context and gene network. Our actual *in vitro* study aims to determine the underlying mechanisms and the potential factors that regulate the gene or the protein expression of KLF4.

The expression level and role of HSP27 have been considerably investigated by several studies that reported a higher expression level of this protein in tumors from various origins, associated with tumor aggressiveness and poor survival of patients. HSP27 is clearly involved in the tumorigeneis process, tumor resistance and progression, and metastasis. It is considered as a promising therapeutic target (39). Its gene inhibition using antisense oligonucleotide showed a promising therapeutic effect in clinical application (30). HSP27 has been identified as a candidate biomarker. Some studies have explored its potential prognostic value and its role in predicting the adequate therapy (16). However, studies regarding the role of HSP27 in larynx cancer are limited (31,32). In the present study, the expression of HSP27 increases significantly in larynx tumor tissues compared with normal tissues. This expression is correlated to tumor stages, confirming the role of this protein in oncogenic transformation and tumor progression. The level of HSP27 seems to be associated with the level of tumor differentiation. These findings are consistent with recent studies investigating HSP27 expression in cancer and elucidating its prognostic role.

Our results were not consistent with those of Xu *et al*, that showed an absence of significant difference in HSP27 protein expression between laryngeal carcinoma and normal controls (32). This discrepancy may be due to the marginally larger tumor sample collection in our study, potentially contributing to more relevant results, and to the different method used to evaluate HSP27 expression. Our observations were also not consistent with those of Kaigorodova *et al* who demonstrated a high nuclear expression of the phosphorylated and unphosphorylated forms of HSP27 in the biopsies of patients with lymph node metastases (31). However, the cytoplasmic expression of HSP27 in these patients did not differ statistically, and in their study, they did not evaluate the mRNA expression level of HSP27 in their larynx tissues.

Since HSP27 is upregulated from early stages of the disease and KLF4 is downregulated progressively and specially in advanced stages, this implies that HSP27 might suppress the expression of KLF4 probably through indirect mechanisms. In fact, a study showed that HSP27 interacts with SP1 in the brain (40) which is a transcription factor of the KLF/SP family that can activate the transcription of KLF4 (41,42). This

interaction was shown to potentiate the transcription activity of SP1, and had a cytoprotective role for the neurons (40). In laryngeal carcinoma, HSP27 could interact with SP1 and thus preventing SP1 from activating the transcription of KLF4. Our actual *in vitro* study aims to elucidate the presence of this link between these two proteins and the underlying mechanisms and factors or protein partners implied in this regulation. To date, neither KLF4 nor HSP27 have been extensively studied in laryngeal cancer. This is the first study that investigated the expression and role of KLF4 in larynx cancer, and that showed a potential association between KLF4 and HSP27 in this type of cancer. However, due to the lack of patient survival data, we were unable to investigate any correlation between immunohistochemical and RTPCR findings and patient survival.

Understanding the molecular mechanisms underlying the pathogenesis of larynx cancer is required to achieve better patient outcomes. The role of HSP27 and KLF4 in larynx cancer initiation and progression highlights their use as potential future targets for prognosis and treatment.

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