

# Protein expression analysis for predicting recurrent laryngeal squamous cell carcinoma

SIWAPORN THANASAN<sup>1</sup>, KOMKRIT RUANGRITCHANKUL<sup>2</sup>, NAKARIN KITKUMTHORN<sup>3</sup>,  
SOMBOON KEELAWAT<sup>2</sup> and PATNARIN MAHATTANASAKUL<sup>4</sup>

<sup>1</sup>Department of Pathology; <sup>2</sup>Special Task Force for Activating Research, Department of Pathology, Faculty of Medicine, Chulalongkorn University, Bangkok 10330; <sup>3</sup>Department of Oral Biology, Faculty of Dentistry, Mahidol University, Bangkok 10400; <sup>4</sup>Department of Otolaryngology, Head and Neck Surgery, King Chulalongkorn Memorial Hospital, Thai Red Cross Society, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand

Received October 5, 2023; Accepted November 13, 2023

DOI: 10.3892/br.2023.1702

**Abstract.** The expression of a number of proteins plays a major role in predicting recurrent laryngeal squamous cell carcinoma (LSCC). Thus, the aim of the present study was to investigate the expression of 16 selected proteins as prognostic indicators for recurrent and non-recurrent LSCC. Samples from a total of 41 patients with LSCC were investigated by immunohistochemistry. Digital image analysis was performed, and various associated factors were calculated. Histo-score (H-score) and receiver operating characteristic curves were used to divide protein expression in high and low for predicting disease recurrence. Disease-free survival (DFS) curves, crude hazard ratios (HRs) and adjusted HRs were analyzed and compared. Significantly different H-scores were found between the recurrent and non-recurrent groups in terms of pRb and c-Met expression. pRb was expressed at high levels in recurrent LSCC, while c-Met was expressed at low levels. Patients with low pRb expression had a longer DFS than those with high pRb

expression (log-rank  $\chi^2$ , 5.161;  $P=0.023$ ). Patients with high c-Met expression had a longer DFS than those with low c-Met expression (log-rank  $\chi^2$ , 6.441;  $P=0.011$ ). Moreover, patients with high pRb expression and low c-Met expression had the shortest DFS (log-rank  $\chi^2$ , 11.827;  $P=0.008$ ). Differentiated histological factors had an impact on the risk of recurrence (Cox regression test; crude HR, 9.53; 95% confidence interval, 1.214-74.819;  $P=0.032$ ). The present study demonstrated that the grading of differentiated squamous cell carcinoma, pRb and c-Met expression are the most useful prognostic factors for the prediction of recurrent LSCC. These might be further applied as potential markers for clinical use.

## Introduction

Laryngeal cancer is one of the most common head and neck cancers. Based on the Global Burden of Disease study between 1990 and 2017, the incidence of laryngeal cancer increased by 58.67%, from 132,740 to 210,610 cases. The death- and disability-adjusted life-years were also increased by 33.84 and 25%, respectively (1). The most common diagnostic pathology is laryngeal squamous cell carcinoma (LSCC) (2). Treatment modality depends on various factors, including Tumor-Node-Metastasis (TNM) classification, and health and financial status (3). In early stage LSCC, three main options can be selected in single modality: Radiotherapy, transoral laser microsurgery and open partial laryngectomy. In advanced stage LSCC, combined modalities are applied; surgery and postoperative radiotherapy have long been the most common options (4). At present, numerous options have become available. Conservation laryngeal surgery and chemoradiotherapy are useful options as part of individualized treatment. The treatment modalities were considered based on host factor, tumor factor, surgeon factor, institutional factor, academic research center and financial factor. The outcome of every treatment approach can impact functional impairments, long-term morbidity and quality of life. Recurrent disease also reduces overall survival (OS) and disease-specific survival (DSS) (5).

Tumorigenesis is universally investigated. Tumor formation includes 10 major characteristics: Unlimited multiplication,

*Correspondence to:* Professor Somboon Keelawat, Special Task Force for Activating Research, Department of Pathology, Faculty of Medicine, Chulalongkorn University, 1873 Rama IV Road, Bangkok 10330, Thailand  
E-mail: trcskl@gmail.com

Dr Patnarin Mahattanasakul, Department of Otolaryngology, Head and Neck Surgery, King Chulalongkorn Memorial Hospital, Thai Red Cross Society, Faculty of Medicine, Chulalongkorn University, 1873 Rama IV Road, Bangkok 10330, Thailand  
E-mail: patnarinm@yahoo.com

**Abbreviations:** HR, hazard ratio; CI, confidence interval; DFS, disease-free survival; DSS, disease-specific survival; HGF/SF, hepatocyte growth factor/scatter factor; HNSCC, head and neck squamous cell carcinoma; H-score, histo-score; LSCC, laryngeal squamous cell carcinoma; OS, overall survival; ROC, receiver operating characteristic; SD, standard deviation

**Key words:** laryngeal squamous cell carcinoma, immunohistochemistry, prognostic indicator, pRb, c-Met

evasion from growth suppressors, promoting invasion and metastasis, resisting apoptosis, stimulating angiogenesis, maintaining proliferative signaling, elimination of cell energy limitation, evading immune destruction, genome instability and mutation, and tumor-enhanced inflammation (6,7). However, some clinical characteristics of tumor recurrence remain unknown. In clinical practice, the TNM classification does not indicate the responsiveness of treatment and tumor recurrence. The expression of a number of proteins can be used for prognosis. However, a standardized prognostic protein expression is not yet established to assist in decision-making (4,8,9). For example, epidermal growth factor (EGFR) is a member of the ErbB family of tyrosine kinase receptors. It is thought to play a major role in enhancing tumor growth, invasion and metastasis. Studies in LSCC show an association between high EGFR expression and poor prognosis, including OS (10,11). By contrast, other studies revealed that EGFR overexpression is associated with longer OS (12,13). Ki-67 is a nuclear protein which can be detected in all cell cycle phases except G0. It is associated with proliferation of normal and neoplastic cells. In immunohistochemistry, it is considered an important prognostic marker in various cancers including lung, brain, breast, prostate, esophagus and kidney cancer (14,15). Ki-67 in LSCC shows association between higher expression and advanced clinical stage, pathological characteristics, nodal metastasis and also shorter survival (16-19). By contrast, patients with LSCC and higher Ki-67 expression have notably improved response to radiotherapy compared with those patients with lower Ki-67 expression (20). The current study aimed to identify prognostic factors based on protein expression to predict recurrent LSCC.

## Materials and methods

**Ethical process.** All protocols in the current study were approved by the Institutional Review Board (IRB) of the Faculty of Medicine, Chulalongkorn University (approval no. IRB 889/63; certificate of approval, Faculty of Medicine, Chulalongkorn University no. 0158/2022; Bangkok, Thailand) which also covered any research conducted at King Chulalongkorn Memorial Hospital with the 1964 Declaration of Helsinki. Written informed consent was obtained from all individual participants included in the present study. Patients diagnosed with LSCC through histological confirmation by pathologists (K.R., N.K. and S.K.) were selected for inclusion in the present study. The clinical data of the patients were obtained from the Department of Otolaryngology, Head and Neck Surgery, King Chulalongkorn Memorial Hospital between January 2009 and December 2018. The medical records of a total of 268 patients with LSCC were assessed for inclusion in the present study. The exclusion criteria included: i) Supraglottic in origin (122 cases); ii) referral without paraffin block (37 cases); and iii) incomplete follow-up (68 cases). Follow-up was done every 2 months during year 1 after treatment, every 3 months in year 2 and every 6 months from year 3 to 5. The recurrence times were calculated from the last day of treatment completion to the date of recurrence. Finally, 41 patients were included in the present study. There were 15 cases of recurrent LSCC and 26 of non-recurrent LSCC (Fig. 1). The demographic data of the patients, including age, sex, staging, histological grading, median recurrence time and

rate were retrieved from the clinical chart records. TNM stage was categorized according to the 8th edition American Joint Committee on Cancer TNM Staging System (3).

An immunohistochemical technique was used to investigate the prognostic proteins between recurrent and non-recurrent LSCC. Antibodies were selected based on literature reviews and adapted from tumorigenesis (6). Cell cycle, proliferation signal maintenance, cell replication and survival, and cell-resistant apoptosis were considered in the experiments of the present study. Furthermore, the mechanism of recurrence was considered to be through angiogenesis stimulation, tumor invasion and metastasis, and evasion from growth suppressors. Finally, antibodies of Ki-67, pRb, cyclin D1, p16, c-Met, PI3K, HIF-1 $\alpha$ , VEGF,  $\beta$ -catenin, p53, RPA32, CD44, BAX, BAK, Bcl-xl and Bcl-2 were examined (Fig. S1).

**Immunohistochemistry.** Laryngeal tissue was fixed in 4% paraformaldehyde for 8 h at room temperature, then embedded in paraffin and sliced into 3- $\mu$ m thick sections. The sections were stained first with Mayer's hematoxylin (cat. no. C0303; Diapath S.p.A) for 5 min and then stained with eosin (cat. no. C0353; Diapath S.p.A) for 5 min at room temperature. For pRb, the sections were heated in a water bath at 95°C with Dako Target Retrieval Solution (Dako; Agilent Technologies, Inc.) for 20 min. After washing with Tris Buffered Saline, sections were incubated with mouse monoclonal pRb antibodies (1:500, cat. no. 9307S; Santa Cruz Biotechnology, Inc.) for 20 h at room temperature. Then, samples were incubated for 20 min at room temperature with EnVision reagent (Dako; Agilent Technologies, Inc.) and horseradish peroxidase. The visualization of the reaction was completed with 3,3'-Diaminobenzidine solution. After that the sections were stained with Hematoxylin II (cat. no. 790-2208; Roche Diagnostics, Ltd.) for 12 min at room temperature and Bluing Reagent for 4 min (cat. no. 760-2037; Roche Diagnostics, Ltd.) at room temperature. Antibody information can be found in Table S1. Positive controls were performed in each experiment. Negative controls were performed in the same condition without primary antibodies. Full slide images were reviewed and evaluated by three pathologists under a light microscope (BX45; Olympus Corporation; magnification, x40).

**Scoring.** The slides were evaluated by three pathologists (K.R., N.K. and S.K.) who were blinded from all clinical information. All pathologists were trained and evaluated by a Cohen's  $\kappa$  coefficient 0.97. All immunostaining slides were scanned using Aperio ScanScope XT (Leica Microsystems, Inc.). Image analysis was automatically scored using Aperio ImageScope (version 12.1.0.5029), and immunological expression was calculated by Nuclear ([https://tmalab.jhmi.edu/aperiou/userguides/IHC\\_Nuclear.pdf](https://tmalab.jhmi.edu/aperiou/userguides/IHC_Nuclear.pdf); version 9) and Membranous algorithms ([https://tmalab.jhmi.edu/aperiou/userguides/IHC\\_Membrane.pdf](https://tmalab.jhmi.edu/aperiou/userguides/IHC_Membrane.pdf); version 9). For H-score assessment, the staining intensity of malignant cells was categorized as 0 (negative), 1<sup>+</sup> (weak), 2<sup>+</sup> (moderate) and 3<sup>+</sup> (strong). Thereafter, the total number of cells in each field and the number of cells stained at each intensity were counted. The percentage of positive cells was calculated following the formula of the H-score and reported in range 0-300: H-score=(% of cells stained at 1<sup>+</sup> x 1) + (% of cells stained at 2<sup>+</sup> x 2) + (% of cells stained at 3<sup>+</sup> x 3).

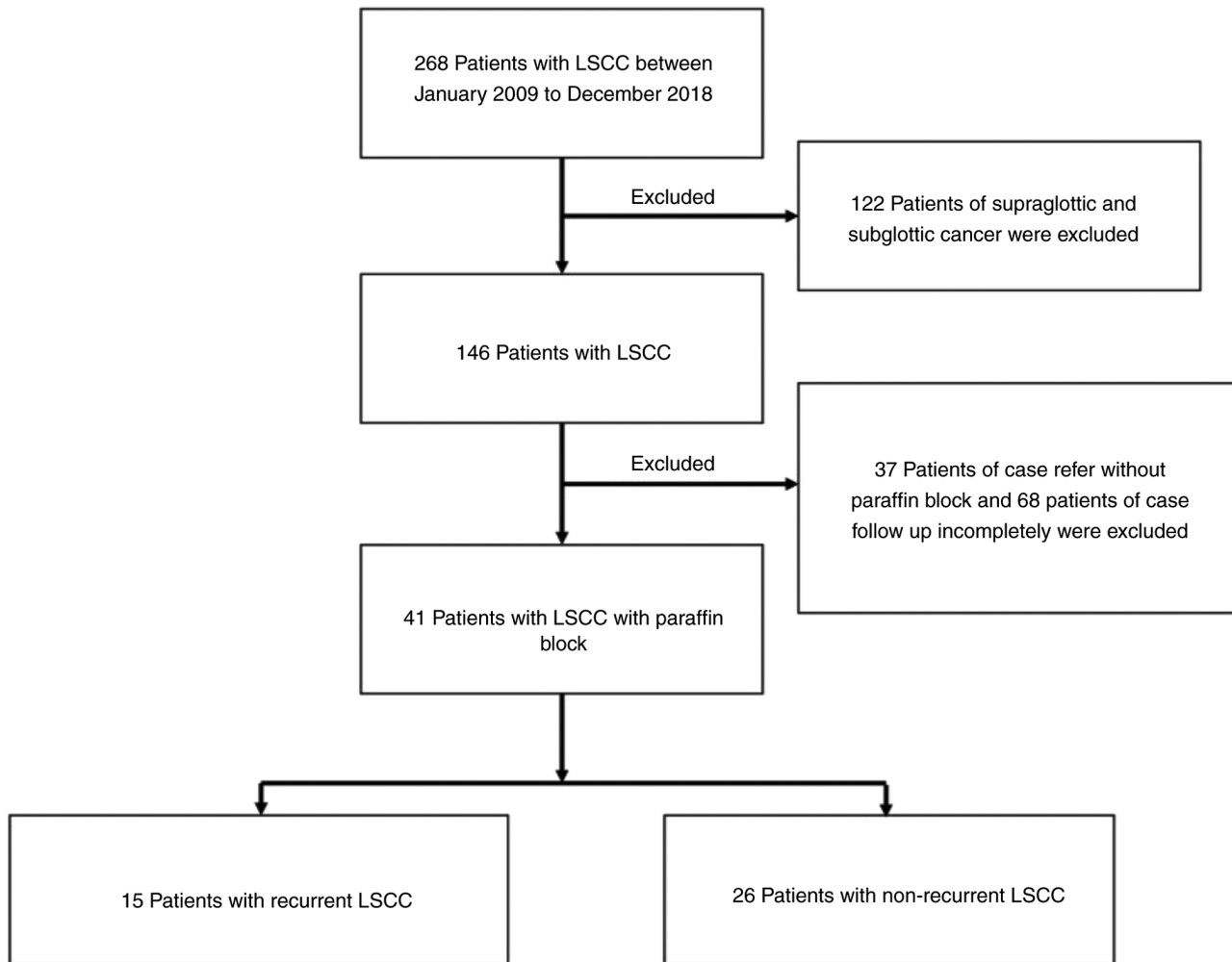


Figure 1. Flow chart of study selection. LSCC, laryngeal squamous cell carcinoma.

**Statistical analysis.** All pathologists were trained and evaluated by a Cohen's  $\kappa$  coefficient ( $\kappa$  index) 0.97. Data analysis was performed using SPSS (version 23.0; IBM Corp.) and GraphPad Prism (version 9.0; Dotmatics). The age, sex, stage grouping, treatment modalities, histological grading and lymphatic involvement of the patients were reported. Significant factors to the number of recurrent LSCC were analyzed using Pearson's  $\chi^2$ -test. Differences in H-score between the recurrent and non-recurrent groups were tested using the Mann-Whitney U test. Then, a receiver operating characteristic (ROC) curve was analyzed to extract the optimum cut-off point to distinguish high and low immunological expression. OS was analyzed and disease-free survival (DFS) curves were plotted using the Kaplan-Meier method and compared using the log-rank test. Crude HR and adjusted HR were calculated using the Cox regression test. The significance of tests was evaluated at 95% confidence interval (CI).  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

Patients were divided into two groups based on clinical information: The recurrent LSCC and the non-recurrent LSCC group. Overall, the median OS of the patients in the

current study was 7.55 years. The median DFS was 5.95 years. Patients with recurrent LSCC had a shorter OS than those with non-recurrent LSCC (median, 4.73 vs. 9.14 years;  $P = 0.003$ ). The 5-year DFS after treatment was 57.6%. Table I shows the frequency of recurrent and non-recurrent LSCC in relation to various parameters. A significant association was found between histological grading (21) and number of patients with recurrent LSCC (Pearson's  $\chi^2$  test, 4.374;  $P = 0.036$ ).

For immunohistochemistry, immunological expression was detected in the nucleus of tumors: Ki67, pRb, RPA32, cyclin D1, p53 and HIF-1 $\alpha$ . Stained cytoplasm was detected in BAX, BAK, Bcl-x1, c-Met, PI3K,  $\beta$ -catenin, p16 and CD44. Weakly stained cytoplasm was also identified in VEGF and Bcl-2. Table SII shows the comparison of the median H-scores of 16 proteins between the recurrent and non-recurrent groups using the Mann-Whitney U test. A significantly different H-score was found between the recurrent group and the non-recurrent group in terms of pRb ( $P = 0.0014$ ) and c-Met expression ( $P = 0.0012$ ). The median H-scores of pRb were 194.90 and 98.80 for recurrent and non-recurrent LSCC, respectively (Mann-Whitney U test,  $P = 0.014$ ; Fig. 2A-C), and there was a high expression in recurrent LSCC. The median H-scores of c-Met were 103.60 and 178.20 for recurrent and non-recurrent LSCC, respectively (Mann-Whitney U test;  $P = 0.0012$ ). The

Table I. Clinical and pathological parameters of total LSCC (n=41), recurrent LSCC (n=15) and non-recurrent LSCC (n=26).

Characteristics	Total LSCC, n	Recurrent LSCC, n (%)	Non-recurrent LSCC, n (%)	P-value
Age, years				0.322
≤60	12	3 (20.00)	9 (34.60)	
>60	29	12 (80.00)	17 (65.40)	
Sex				N/A
Male	41	15 (100.00)	26 (100)	
Female	0	0 (0.00)	0 (0.00)	
Smoking				0.827
Never	7	2 (18.20)	5 (21.70)	
Current	17	5 (45.50)	12 (52.20)	
Former	10	4 (36.40)	6 (26.10)	
No data available	7			
Alcohol				0.953
Never	7	2 (18.20)	5 (22.70)	
Current	20	7 (63.60)	13 (59.10)	
Former	6	2 (18.20)	4 (18.20)	
No data available	8			
Stage				0.215
Early	7	4 (26.70)	3 (11.50)	
Advance	34	11 (73.30)	23 (88.50)	
Treatment				0.983
Definite radiation	7	3 (20.00)	4 (15.40)	
Concurrent chemo radiotherapy	6	2 (13.30)	4 (15.40)	
Radical resection	3	1 (6.70)	2 (7.70)	
Post operation radiation	25	9 (60.00)	16 (61.50)	
Histological grade				0.036 <sup>a</sup>
Well-differentiated	25	6 (40.00)	19 (73.10)	
Moderately-differentiated	16	9 (60.00)	7 (26.90)	
Poorly-differentiated	0	0 (0.00)	0 (0.00)	
Lymph node status				0.548
Negative	27	9 (60.00)	18 (69.20)	
Positive	14	6 (40.00)	8 (30.80)	
Anterior commissure invasion				0.446
Negative	16	7 (46.70)	9 (34.60)	
Positive	25	8 (53.30)	17 (65.40)	

<sup>a</sup>P<0.05. P-value calculated using the  $\chi^2$ -test. LSCC, laryngeal squamous cell carcinoma; N/A, not applicable.

expression levels of c-Met were lower in recurrent LSCC than in non-recurrent LSCC (Fig. 3A-C). A ROC curve was plotted from the sensitivity and specificity of pRb and c-Met. The optimum cut-off point of pRb to distinguish recurrent LSCC was 125.8, which demonstrated a sensitivity and specificity of 80.00 and 61.54%, respectively (Fig. 4A). For c-Met, the optimum cut-off point was 166.9, which demonstrated sensitivity and specificity of 85.71 and 61%, respectively (Fig. 4B).

All patients were followed up until recurrence or until December 2018. Kaplan-Meier survival analysis for DFS was completed, and results showed that patients with well-differentiated LSCC had a longer DFS time compared with that of patients with moderate-differentiation, with DFS

times of 6.95 and 4.32 years, respectively (log-rank  $\chi^2=5.268$ ; P=0.022). For the expression of all proteins, data showed that patients with low pRb expression had a significantly longer DFS time (7.83 years) compared with those with high pRb expression (4.34 years; log-rank  $\chi^2=5.161$ ; P=0.023; Fig. 5A). By contrast, patients with high c-Met expression had a longer DFS (7.98 years) compared with those with low c-Met expression (4.54 years; log-rank  $\chi^2=6.441$ ; P=0.011; Fig. 5B). Moreover, patients with high pRb and low c-Met expression had a short DFS compared with high pRb and high c-Met, low pRb and high c-Met and low pRb and low c-Met with DFS 2.40 years compared with patients with low pRb and high c-Met expression (log-rank  $\chi^2=11.827$ ; P=0.008; Fig. 5C).



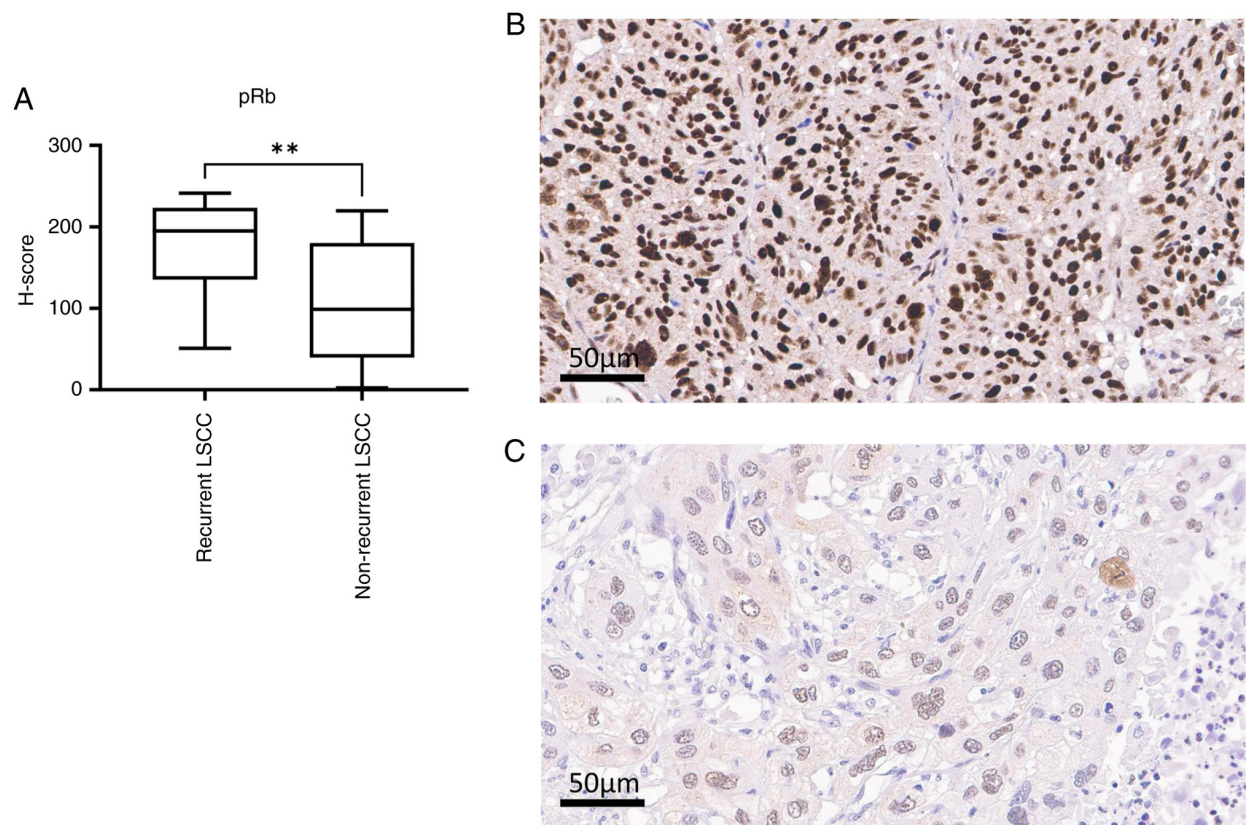


Figure 2. (A) Median of H-score of pRb expression between recurrent and non-recurrent LSCC. Immunohistochemical staining of pRb in LSCC, (B) High pRb expression (magnification, x40) and (C) low pRb expression (magnification, x40). \*\*P=0.014, Mann-Whitney U test, VS non-recurrent LSCC. LSCC, laryngeal squamous cell carcinoma; H-score, histoscore.

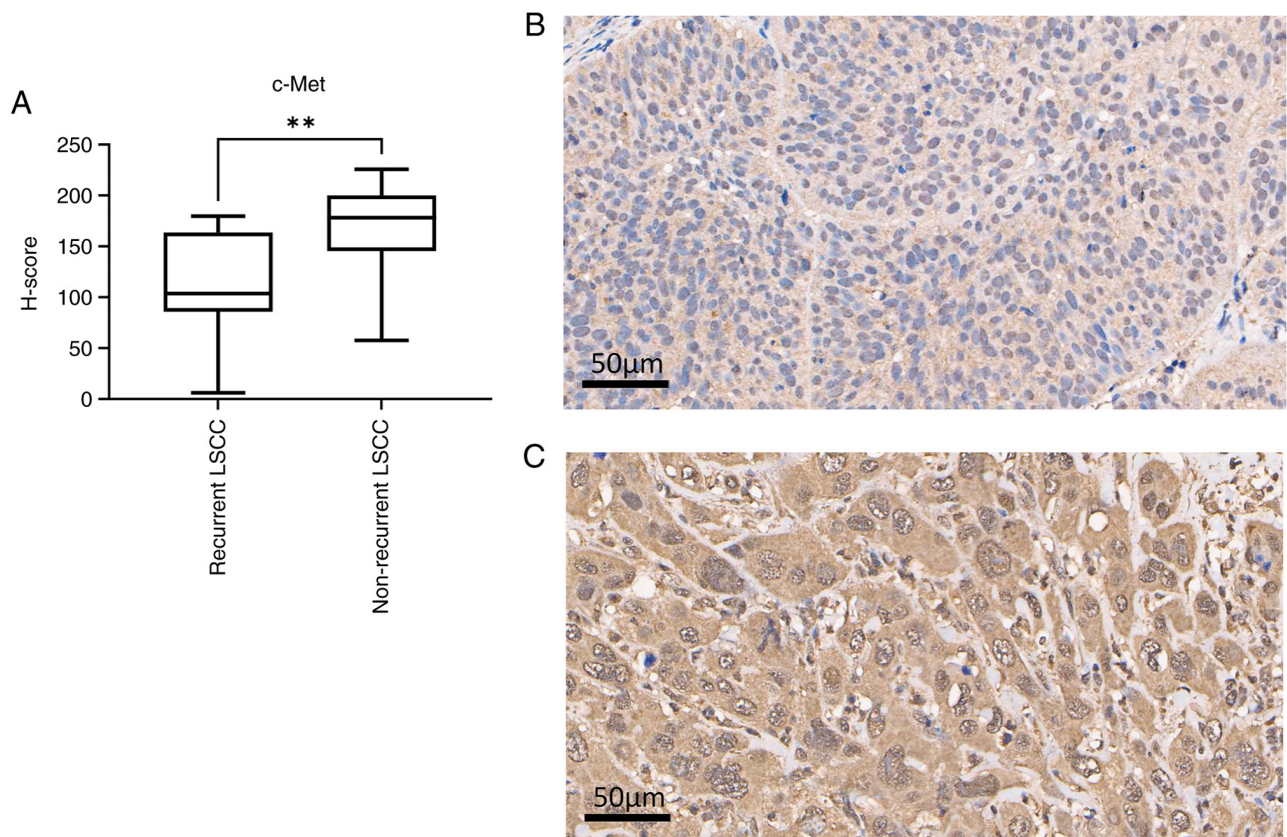


Figure 3. (A) Median of H-score of c-Met expression between recurrent and non-recurrent LSCC. Immunohistochemical staining of c-Met in LSCC; (B) Low pRb expression (magnification, x40) and (C) high pRb expression (magnification, x40). \*\*P=0.0012, Mann-Whitney U test, VS non-recurrent LSCC.

Table II. Association between pathological factors and median recurrence time of total LSCC (n=41), recurrent LSCC (n=15) and non-recurrent LSCC (n=26).

Variables	Total patients with LSCC, n	Patients with recurrent LSCC, n	DFS (95% CI)	Person-years of Observation	Incident rate/100 person years	P-value
Histological grade						0.022 <sup>a</sup>
Well-differentiated	25	6	6.95 (5.375-8.520)	86.64	6.92	
Moderately- differentiated	16	9	4.32 (2.319-6.325)	35.4	25.42	
Poorly-differentiated	0	0	0.00	0.00	0.00	
pRb expression						0.023 <sup>a</sup>
Low	19	3	7.83 (6.345-9.312)	63.26	4.74	
High	22	12	4.34 (2.655-6.017)	58.79	20.41	
c-Met expression						0.011 <sup>a</sup>
Low	23	13	4.54 (2.806-6.273)	62.86	19.09	
High	18	2	7.98 (6.729-9.234)	58.78	3.40	
Combined markers						0.008 <sup>a</sup>
Low pRb and high c-Met	11	1	6.26 (5.095-7.427)	29.67	3.37	
Low pRb and low c-Met	8	2	7.08 (4.474-9.694)	33.59	5.95	
High pRb and high c-Met	7	1	7.75 (5.591-99.909)	29.10	3.43	
High pRb and low c-Met	15	11	2.40 (1.291-3.519)	29.27	34.16	

<sup>a</sup>P<0.05. P-value calculated using the Kaplan-Meier test. LSCC, laryngeal squamous cell carcinoma.

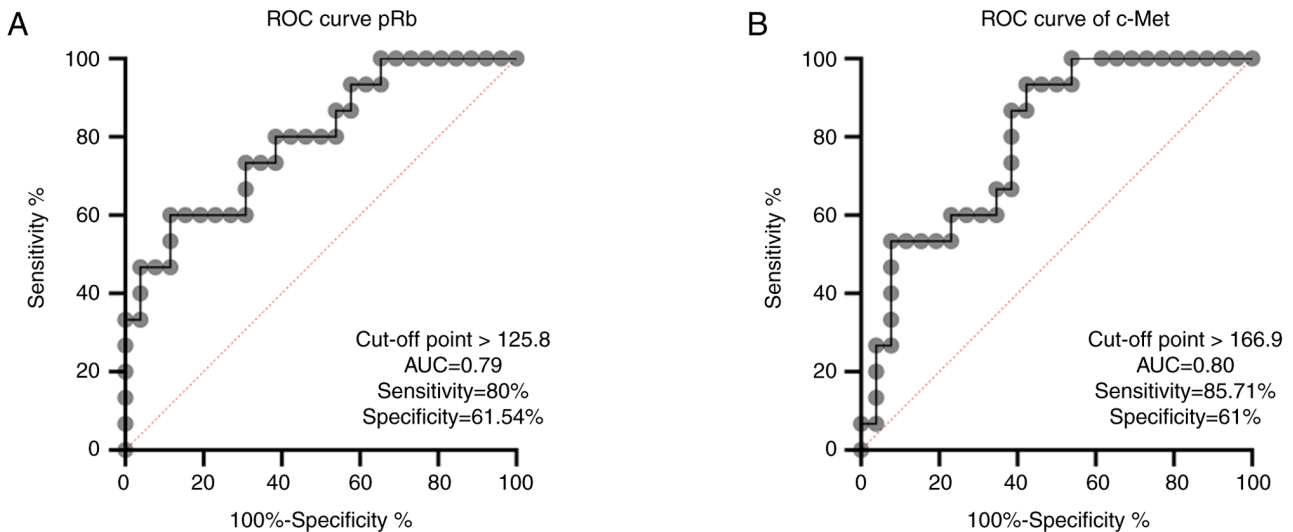


Figure 4. ROC curve of (A) pRb and (B) c-Met expression. ROC, receiver operating curve; AUC, area under curve.

The incidence rate of recurrent LSCC in well-differentiated LSCC was 6.92 cases per 100 person-years, while that in moderately-differentiated LSCC was 25.42 cases per 100 person-years. For the combined high pRb expression and low c-Met expression, the incidence rate of recurrent LSCC was 34.16 cases per 100 person-years, more than that of other combined settings (Table II).

Several factors were significantly associated with the risk of recurrence and differentiation, including the expression of both pRb and c-Met. There was a significant increase in patients with moderately-differentiated LSCC compared

with those with well-differentiated LSCC (Cox regression test; crude HR, 3.16; 95% CI, 1.12-8.92; P=0.030). In terms of protein expression, the risk of recurrence increased in high pRb and low c-Met expression compared with that in low pRb and high c-Met expression (Cox regression test; crude HR, 9.53; 95% CI, 1.214-74.819; P=0.032).

The adjusted HR model analysis revealed the same results. Histological grading was an independent prognostic factor for recurrent LSCC (Cox regression test; adjusted HR, 4.19; 95% CI, 1.321-13.268; P=0.015). High pRb and low c-Met expression were independent prognostic factors for recurrent LSCC

Table III. Crude HR and adjusted HR analysis of factors associated with recurrent LSCC.

Variables	Crude HR (95% CI)	P-value	Adjusted HR	P-value
Histological grade				
Well-differentiated	1		1	
Moderately-differentiated	3.16 (1.213-3.980)	0.030 <sup>a</sup>	4.19 (1.321-13.268)	0.015 <sup>a</sup>
Poorly-differentiated	N/A	N/A	N/A	N/A
Combined markers				
Low pRb and high c-Met	1		1	
Low pRb and low c-Met	2.54 (0.230-28.112)	0.447	1.41 (0.123-16.260)	0.781
High pRb and high c-Met	1.38 (0.086-22.109)	0.82	0.80 (0.048-13.153)	0.875
High pRb and low c-Met	9.53 (1.214-74.819)	0.032 <sup>a</sup>	8.73 (1.094-69.638)	0.041 <sup>a</sup>

<sup>a</sup>P<0.05. P-value calculated using the Cox regression test. LSCC, laryngeal squamous cell carcinoma; N/A, not applicable; HR, hazard ratio.

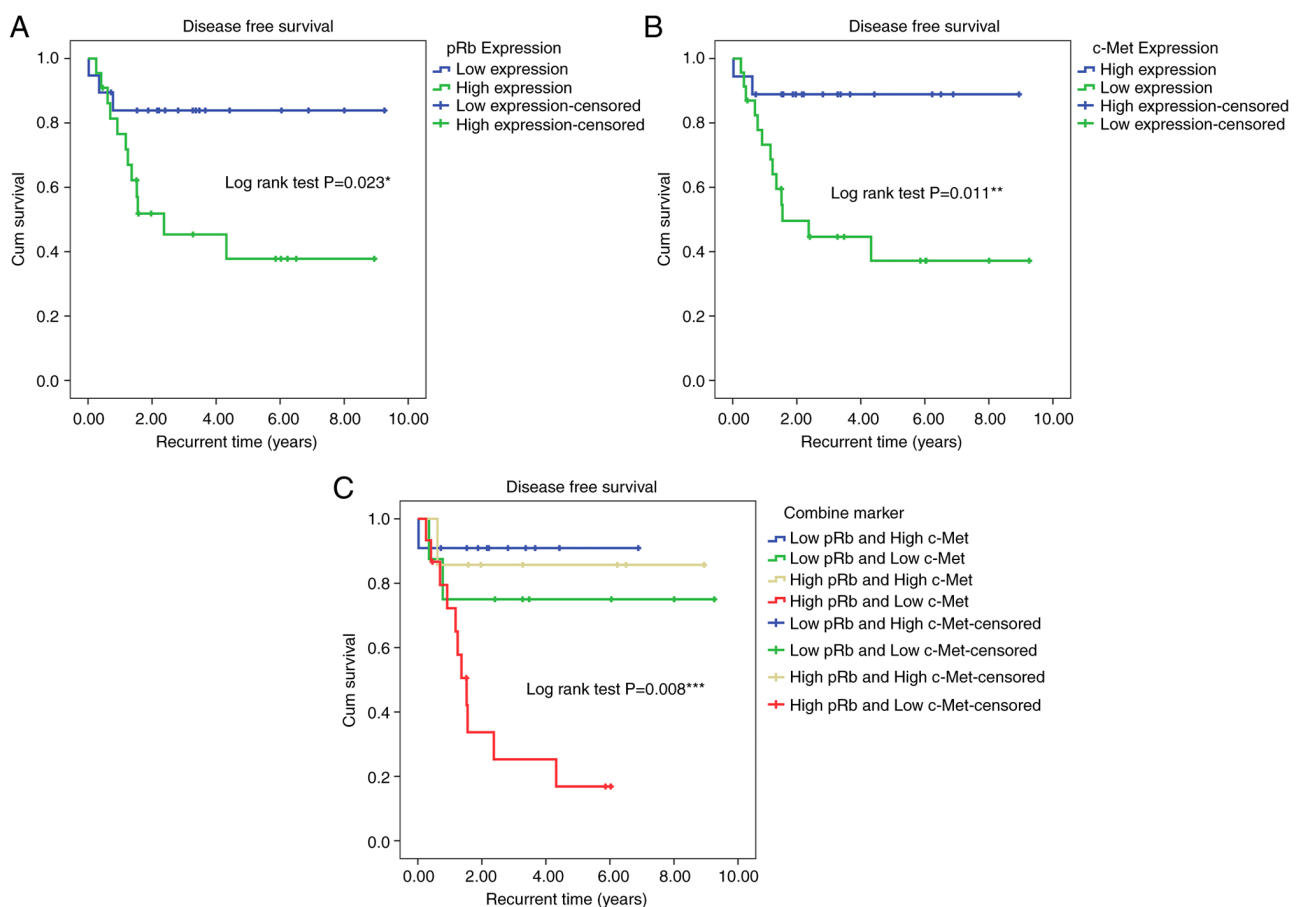


Figure 5. Kaplan-Meier survival analysis for disease-free survival and expression of (A) pRb, (B) c-Met and (C) combined marker. (\*\*\*\*\*) P<0.05, log-rank  $\chi^2$ . Censored refers to the time of observation.

(Cox regression test; adjusted HR, 8.73; 95% CI, 1.094-69.638; P=0.041; Table III).

## Discussion

In clinical practice, it is challenging to identify a prognostic factor that can predict the treatment outcome of LSCC. TNM staging is not a precise and accurate marker for prognostic outcomes (15,22). Patients with identical TNM staging may

have a variable clinical course, response to treatment and prognosis.

In the current study, a significant association between histological grading and recurrent LSCC and DFS was found. The present study is consistent with previous studies; Wang *et al* (23) found an association between well-differentiated LSCC and OS. A previous study on 998 patients with LSCC showed that patients with well- to moderately-differentiated LSCC had notably improved survival outcomes, including DSS, DFS and

OS, than patients with poorly-differentiated LSCC; that study included all supraglottic, glottic and subglottic LSCC (24). Meanwhile, the present study focused only on glottic cancer. It was considered that supraglottic and subglottic LSCC might affect the prognosis. Chen *et al* (25) studied 110 patients with LSCC, and there were 55 patients with well-differentiated and poorly-differentiated LSCC, including all subsites. Patients with well-differentiated LSCC were found to have significantly different OS (HR, 0.18; 95% CI, 0.07-0.46;  $P=0.001$ ), DSS (HR, 0.16; 95% CI, 0.05-0.45;  $P<0.001$ ) and DFS (HR, 0.17; 95% CI, 0.07-0.41;  $P=0.003$ ) than patients with poorly-differentiated LSCC. Another study on all subsites of 250 patients with early glottic LSCC found that the risk of mortality of patients with poor-differentiation was 1.45-fold more than that of patients with a well-differentiated tumor (HR, 2.45; 95% CI, 1.19-5.40;  $P=0.01$ ) (26). By contrast, Piccirillo *et al* (27) studied 196 LSCC cases and found no statistical significance between histological grading and symptom duration, and survival rate.

The location of tumors at the anterior commissure was frequently discussed as a prognostic factor. In the present study, tumor location was not significantly associated with recurrent LSCC. By contrast, numerous previous studies showed significance in terms of local control and recurrence (28-35). However, due to the small sample size of the present study, the significance of local control and recurrence rate was not determined.

In immunohistochemistry, the expression of 16 proteins was investigated in accordance with tumorigenesis. The results showed a statistically significant expression only in pRb and c-Met. pRb is a protein product of the RB tumor suppressor gene. It controls the cell cycle, preserving genetic integrity and mediating cell differentiation (36). It plays a role in the negative control of the cell cycle and tumor progression. It works at the G1 checkpoint for block entrance of S-phase and inhibits cell progression. The loss of pRb function may lead to cell cycle dysregulation and malignant transformation. Similarly to a previous study, a high pRb expression showed higher recurrent LSCC and lower DFS (37). Moreover, Mizokami *et al* (38) found that the loss of pRb expression was associated with invasive tumor behavior, such as high T classification or histological grade, which could predict disease relapse. Similarly, Lee *et al* (39) found that a low pRb expression could be notable in predicting recurrence and 3-year DFS.

c-Met, a mesenchymal-epithelial transition factor, is a transmembrane receptor of tyrosine kinase found on the surface of various epithelial cells. Hepatocyte growth factor/scatter factor (HGF/SF) is a common ligand to c-Met receptors (40,41). A disruption of HGF/c-Met signaling can cause uncontrolled proliferation, motility, invasion and angiogenesis that could lead to head and neck squamous cell carcinoma (HNSCC) (42). The present study obtained results different from those of previous meta-analyses (43-45). The present study was shown that a lower c-Met expression had higher recurrence and shorter DFS. Crossing over with previous studies, a higher c-Met expression had a predisposition for tumor recurrence, and was associated with shorter OS and DFS (43-45). Immunohistochemistry results were also considered. The small sample size and the different clones of primary antibodies might affect the results.

Results showed that pRb and c-Met are co-expressed in HNSCC. They play a major role in the cell cycle and

tumorigenesis. They could lead to uncontrolled cell cycle, cell proliferation, invasion, angiogenesis, affecting tumor recurrence and survival. However, their precise associated functions are not revealed. The small sample size and different clones of primary antibodies used in the present study might affect the difference. Moreover, the variant molecular and subsequent cellular alterations might impact the results. Further large-scale studies including multiple cohorts should be performed.

In clinical practice, it will be useful to define the risk of recurrent LSCC based on pRb and c-Met expression from routine pathological reports. Currently, there are small molecule inhibitors and monoclonal antibodies of c-Met in various trials. One of them is crizotinib which was examined in a phase I cohort study of gastroesophageal cancer to check the responses to Met-amplified metastatic disease (46). In the part of pRb, it works with mechanism of the cyclin-dependent kinase 4 and 6 (CDK4/6) inhibitors. The United States Food and Drug Administration has approved three agents: Palbociclib, ribociclib and abemaciclib for the treatment of advanced breast cancer in combination with endocrine therapy (47). In locally advanced HNSCC, there is currently no clinical use of CDK4/6 inhibitors concurrently with radiotherapy (48). Further research could provide the potential for targeted therapeutic agents in the future.

In the current study, the degree of differentiation of SCCA is an important clinicopathological factor in predicting recurrent LSCC. The immunohistochemistry of the biological markers pRb, and c-Met is the most useful prognostic factor for distinguishing recurrent from non-recurrent disease. The present study showed that the combined upregulated pRb expression and downregulated c-Met expression is useful in predicting recurrent LSCC. The assessment of pRb and c-Met expression should be considered in clinical practice.

## Acknowledgements

The authors would like to thank the Immunohistochemistry Unit of the Department of Pathology, Faculty of Medicine, Chulalongkorn University and King Chulalongkorn Memorial Hospital for their assistance in the pathological process.

## Funding

The present study was funded by The Graduate School, Chulalongkorn University to commemorate the 90th Anniversary of Chulalongkorn University (Rachadapisek Sompote Fund, grant no. GCUGR1125643036M). The funding body did not interfere with the design of the study and collection, analysis, or interpretation of data, and in writing the manuscript.

## Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Authors' contributions

ST, KR, NK, SK and PM conceptualized the study. ST, KR, NK, SK and PM carried out data curation. ST, NK and PM



completed formal analysis. ST and SK acquired funding. ST, KR, NK, SK and PM completed the investigation. ST, NK and PM developed methodology used. ST, NK and PM carried out project administration. ST, KR, NK, SK and PM provided resources. ST, KR, NK and SK used software. NK, SK and PM supervised the study. ST, NK and PM validated the data. SK and PM visualized the data. ST and PM wrote the original draft. ST, KR, NK, SK and PM reviewed and edited the manuscript. ST, NK, SK and P.M. confirm the authenticity of all the raw data. All authors have read and approved the final version of the manuscript.

### Ethics approval and consent to participate

All protocols used in the present study were approved by the IRB of the Faculty of Medicine, Chulalongkorn University (approval no. IRB 889/63; COA-MDCU no. 0158/2022, Bangkok, Thailand) which also covered any study conducted in the King Chulalongkorn Memorial Hospital in accordance with the Declaration of Helsinki. All participants provided written informed consent.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

### References

- Deng Y, Wang M, Zhou L, Zheng Y, Li N, Tian T, Zhai Z, Yang S, Hao Q, Wu Y, *et al*: Global burden of larynx cancer, 1990-2017: Estimates from the global burden of disease 2017 study. *Aging (Albany NY)* 12: 2545-2583, 2020.
- Morshed K, Skomra D, Korobowicz E, Szymański M, Polz-Dacewicz M and Gołabek W: An immunohistochemical study of cyclin D1 protein expression in laryngeal squamous cell carcinoma. *Acta Otolaryngol* 127: 760-769, 2007.
- American Joint Committee on Cancer, Amin MB, Edge SB, Schilsky RL and Gaspar LE: *AJCC cancer staging manual*. Springer, New York, NY, 2017.
- Bradford CR, Ferlito A, Devaney KO, Mäkitie AA and Rinaldo A: Prognostic factors in laryngeal squamous cell carcinoma. *Laryngoscope Invest Otolaryngol* 5: 74-81, 2020.
- Brandstorp-Boesen J, Sørum Falk R, Boysen M and Brøndbo K: Impact of stage, management and recurrence on survival rates in laryngeal cancer. *PLoS One* 12: e0179371, 2017.
- Hanahan D and Weinberg RA: Hallmarks of cancer: The next generation. *Cell* 144: 646-674, 2011.
- Wang M, Zhao J, Zhang L, Wei F, Lian Y, Wu Y, Gong Z, Zhang S, Zhou J, Cao K, *et al*: Role of tumor microenvironment in tumorigenesis. *J Cancer* 8: 761-773, 2017.
- Leesutipornchai T, Ratchataswan T, Vivatvakin S, Ruangritchankul K, Keelawat S, Kerekhanjanarong V, Bongsebandhu-Phubhakdi S and Mahattanasakul P: EGFR cut-off point for prognostic impact in laryngeal squamous cell carcinoma. *Acta Otolaryngol* 140: 610-614, 2020.
- Vivatvakin S, Ratchataswan T, Leesutipornchai T, Ruangritchankul K, Keelawat S, Mahattanasakul P and Bongsebandhu-Phubhakdi S: MCM-2, Ki-67, and EGFR downregulated expression levels in advanced stage laryngeal squamous cell carcinoma. *Sci Rep* 11: 14607, 2021.
- Almadori G, Cadoni G, Galli J, Ferrandina G, Scambia G, Exarchakos G, Paludetti G and Ottaviani F: Epidermal growth factor receptor expression in primary laryngeal cancer: An independent prognostic factor of neck node relapse. *Int J Cancer* 84: 188-191, 1999.
- Maurizi M, Almadori G, Ferrandina G, Distefano M, Romanini ME, Cadoni G, Benedetti-Panici P, Paludetti G, Scambia G and Mancuso S: Prognostic significance of epidermal growth factor receptor in laryngeal squamous cell carcinoma. *Br J Cancer* 74: 1253-1257, 1996.
- Furuta Y, Takasu T, Asai T, Yoshimura S, Tokuchi F, Shinohara T, Nagashima K and Inuyama Y: Clinical significance of the epidermal growth factor receptor gene in squamous cell carcinomas of the nasal cavities and paranasal sinuses. *Cancer* 69: 358-362, 1992.
- Marioni G, Staffieri A, Bertolin A, Giacomelli L, D'Alessandro E, Ottaviano G, Accordi D, Stramare R, de Filippis C and Blandamura S: Laryngeal carcinoma lymph node metastasis and disease-free survival correlate with MASPIN nuclear expression but not with EGFR expression: A series of 108 cases. *Eur Arch Otorhinolaryngol* 267: 1103-1110, 2010.
- Cavaliere M, Bisogno A, Scarpa A, D'Urso A, Marra P, Colacurcio V, De Luca P, Ralli M, Cassandro E and Cassandro C: Biomarkers of laryngeal squamous cell carcinoma: A review. *Ann Diagn Pathol* 54: 151787, 2021.
- Gioacchini FM, Alicandri-Ciufelli M, Magliulo G, Rubini C, Presutti L and Re M: The clinical relevance of Ki-67 expression in laryngeal squamous cell carcinoma. *Eur Arch Otorhinolaryngol* 272: 1569-1576, 2015.
- Cai K, Luo Y, Li L and Liu Y: Expression and significance of MCM2, Ki-67 and Rb protein in laryngeal squamous cell carcinomas. *Lin Chuang Er Bi Yan Hou Tou Jing Wai Ke Za Zhi* 26: 425-428, 2012 (In Chinese).
- Re M, Zizzi A, Ferrante L, Stramazzotti D, Goteri G, Gioacchini FM, Olivieri F, Magliulo G and Rubini C: p63 and Ki-67 immunostainings in laryngeal squamous cell carcinoma are related to survival. *Eur Arch Otorhinolaryngol* 271: 1641-1651, 2014.
- Nowinska K, Chmielewska M, Piotrowska A, Pula B, Pastuszewski W, Krecicki T, Podhorska-Okołow M, Zabel M and Dziegiel P: Correlation between levels of expression of minichromosome maintenance proteins, Ki-67 proliferation antigen and metallothionein I/II in laryngeal squamous cell cancer. *Int J Oncol* 48: 635-645, 2016.
- Krecicki T, Jeleń M, Zalesska-Krecicka M and Szkudlarek T: Ki-67 immunostaining and prognosis in laryngeal cancer. *Clin Otolaryngol Allied Sci* 23: 539-542, 1998.
- Kropveld A, Slootweg PJ, Blankenstein MA, Terhaard CH and Hordijk GJ: Ki-67 and p53 in T2 laryngeal cancer. *Laryngoscope* 108: 1548-1552, 1998.
- Roland NJ, Caslin AW, Nash J and Stell PM: Value of grading squamous cell carcinoma of the head and neck. *Head Neck* 14: 224-229, 1992.
- Cordes C, Münzel AK, Rudolph P, Hoffmann M, Leuschner I and Gottschlich S: Immunohistochemical staining of Ki-67 using the monoclonal antibody Ki-s11 is a prognostic indicator for laryngeal squamous cell carcinoma. *Anticancer Res* 29: 1459-1465, 2009.
- Wang N, Huang X and Cheng J: BIRC5 promotes cancer progression and predicts prognosis in laryngeal squamous cell carcinoma. *PeerJ* 10: e12871, 2022.
- Zhu Y, Shi X, Zhu X, Diao W and Chen X: Association between pathological differentiation and survival outcomes of patients with laryngeal squamous cell carcinoma. *Eur Arch Otorhinolaryngol* 279: 4595-4604, 2022.
- Chen P, Yu W, Huang J, Xu H, Li G, Chen X and Huang Z: Matched-pair analysis of survival in patients with poorly differentiated versus well-differentiated glottic squamous cell carcinoma. *Oncotarget* 8: 14770-14776, 2017.
- Fararouei M, Daneshi N, Mohammadianpanah M, Reza Tabatabaei H, Zare-Bandamiri M and Dianatinasab M: Factors predicting survival in patients with early stage laryngeal cancer: A cohort study between 2000 to 2015. *J BUON* 22: 996-1003, 2017.
- Piccirillo JF, Wells CK, Sasaki CT and Feinstein AR: New clinical severity staging system for cancer of the larynx. Five-year survival rates. *Ann Otol Rhinol Laryngol* 103: 83-92, 1994.
- Kitani Y, Kubota A, Furukawa M and Sato K: Prognostic factors for local control in patients receiving radiation therapy for early glottic cancer: Anterior commissure involvement and effect of chemoradiotherapy. *Eur Arch Otorhinolaryngol* 273: 1011-1017, 2016.
- Chone CT, Yonehara E, Martins JE, Altemani A and Crespo AN: Importance of anterior commissure in recurrence of early glottic cancer after laser endoscopic resection. *Arch Otolaryngol Head Neck Surg* 133: 882-887, 2007.

30. Hakeem AH, Tubachi J and Pradhan SA: Significance of anterior commissure involvement in early glottic squamous cell carcinoma treated with trans-oral CO<sub>2</sub> laser microsurgery. *Laryngoscope* 123: 1912-1917, 2013.
31. Zouhair A, Azria D, Coucke P, Matzinger O, Bron L, Moeckli R, Do HP, Mirimanoff RO and Ozsahin M: Decreased local control following radiation therapy alone in early-stage glottic carcinoma with anterior commissure extension. *Strahlenther Onkol* 180: 84-90, 2004.
32. Le QT, Fu KK, Kroll S, Ryu JK, Quivey JM, Meyler TS, Krieg RM and Phillips TL: Influence of fraction size, total dose, and overall time on local control of T1-T2 glottic carcinoma. *Int J Radiat Oncol Biol Phys* 39: 115-126, 1997.
33. Bron LP, Soldati D, Zouhair A, Ozsahin M, Brossard E, Monnier P and Pasche P: Treatment of early stage squamous-cell carcinoma of the glottic larynx: Endoscopic surgery or cricohyoidoepiglottomy versus radiotherapy. *Head Neck* 23: 823-829, 2001.
34. Allegra E, Saita V, Azzolina A, De Natale M, Bianco MR, Modica DM and Garozzo A: Impact of the anterior commissure involvement on the survival of early glottic cancer treated with cricohyoidoepiglottomy: A retrospective study. *Cancer Manag Res* 10: 5553-5558, 2018.
35. Mannelli G, Comini LV, Santoro R, Bettiol A, Vannacci A, Desideri I, Bonomo P and Piazza C: T1 glottic cancer: Does anterior commissure involvement worsen prognosis? *Cancers (Basel)* 12: 1485, 2020.
36. Dimaras H and Gallie BL: Retinoblastoma protein, biological and clinical functions. In: *Encyclopedia of Cancer*. Schwab M (ed). Springer, Berlin, pp3277-3280, 2011.
37. Morshed K, Korobowicz E, Skomra D, Szymanski M, PolzDacewicz M, Gołabek W and Śmolen A: Immunohistochemical study of retinoblastoma protein expression in laryngeal squamous cell carcinoma according to low and high overexpression. *Bulletin of the Veterinary Institute in Pulawy* 52: 675-681, 2008.
38. Mizokami H, Sawatsubashi M, Tokunaga O and Shin T: Loss of retinoblastoma protein expression in laryngeal squamous cell carcinoma. *Mod Pathol* 12: 47-53, 1999.
39. Lee LA, Fang TJ, Li HY, Huang CG, Chen TC, Liao CT, Kang CJ, Chang KP and Yen TC: Low expression of pRB predicts disease relapse in early glottic cancer treated with transoral laser microsurgery. *Laryngoscope* 129: E220-E226, 2019.
40. Raj S, Kesari KK, Kumar A, Rath B, Sharma A, Gupta PK, Jha SK, Jha NK, Slama P, Roychoudhury S and Kumar D: Molecular mechanism(s) of regulation(s) of c-MET/HGF signaling in head and neck cancer. *Mol Cancer* 21: 31, 2022.
41. Zhang Y, Xia M, Jin K, Wang S, Wei H, Fan C, Wu Y, Li X, Li X, Li G, *et al*: Function of the c-Met receptor tyrosine kinase in carcinogenesis and associated therapeutic opportunities. *Mol Cancer* 17: 45, 2018.
42. Rothenberger NJ and Stabile LP: Hepatocyte Growth Factor/c-Met signaling in head and neck cancer and implications for treatment. *Cancers (Basel)* 9: 39, 2017.
43. Li L, Sun Z, Huang X, Li X, Sun L, Zhang L, Zhang X, Ye L, Yuan J, Mao L and Li G: Role of c-Met expression on prognosis of head and neck cancer: A literature review and meta-analysis. *Head Neck* 41: 1999-2006, 2019.
44. Jiang M, Zhang H, Xiao H, Zhang Z, Que D, Luo J, Li J, Mao B, Chen Y, Lan M, *et al*: High expression of c-Met and EGFR is associated with poor survival of patients with glottic laryngeal squamous cell carcinoma. *Oncol Lett* 15: 931-939, 2018.
45. Szturz P, Budíková M, Vermorken JB, Horová I, Gál B, Raymond E, de Gramont A and Faivre S: Prognostic value of c-MET in head and neck cancer: A systematic review and meta-analysis of aggregate data. *Oral Oncol* 74: 68-76, 2017.
46. Mo HN and Liu P: Targeting MET in cancer therapy. *Chronic Dis Transl Med* 3: 148-153, 2017.
47. Shah M, Nunes MR and Stearns V: CDK4/6 inhibitors: Game changers in the management of hormone receptor-positive advanced breast cancer? *Oncology (Williston Park)* 32: 216-222, 2018.
48. Ngamphaiboon N, Chairoungdua A, Dajsakdipon T and Jiarpinittun C: Evolving role of novel radiosensitizers and immune checkpoint inhibitors in (chemo)radiotherapy of locally advanced head and neck squamous cell carcinoma. *Oral Oncol* 145: 106520, 2023.



Copyright © 2023 Thanasan et al. This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.