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RESEARCH

Prognostic significance of cytotoxic-T-lymphocytes to immunosuppressive lymphocytes ratio (CIL) in laryngeal squamous cell carcinoma

Duo Zhang[1] · Yu Heng[1] · Qiu-yan Jin[1] · Di Tang[1] · Xiao-ke Zhu[1] · Li-ming Lu[2] · Chun-ping Wu[1] · Lei Tao[1]

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Abstract

Immunoscore (IS), based on CD3/CD8, has been proposed to characterize the immune landscape of the tumor immune microenvironment and has demonstrated an association with the prognosis of laryngeal squamous cell carcinoma (LSCC). However, traditional IS does not include immunosuppressive cells. The purpose of this study is to evaluate the prognostic performance of cytotoxic-T-lymphocytes to immunosuppressive cells ratio (CIL) in laryngeal squamous cell carcinoma (LSCC) patients. Two cohorts were included in this study: The training cohort ($N=75$) consisted of tumor tissue microarrays from LSCC patients in our department, and the validation cohort ($N=116$) utilized bulk RNA-seq data from the TCGA database. Patients with high IS or CIL showed significantly prolonged overall survival and disease-free survival in both cohorts. Upon analyzing the relative contribution of each parameter, it was found that CIL exhibited the highest significance among the factors examined. It emerged as the strongest predictor of overall survival, emphasizing its crucial influence in determining the outcomes. The prognostic ability of IS-TCGA was similar to the original IS. Additionally, high CILM2-TCGA was associated with prolonged survival of patients with LSCC in the TCGA dataset. CIL, which is easier to construct than IS, proves to be reliable in predicting survival outcomes for patients with LSCC.

Keywords Immunoscore (IS) · The ratio of cytotoxic-T-lymphocytes to immunosuppressive cells (CIL) · Laryngeal squamous cell carcinoma (LSCC) · Immunosuppressive cells

Abbreviations

CIL	The ratio of cytotoxic-T-lymphocytes to immunosuppressive cells
HNSCC	Head and neck squamous cell carcinoma
ICIs	Immune checkpoint inhibitors
IS	Immunoscore
LSCC	Laryngeal squamous cell carcinoma
TI	Tumor inferior
TILs	Tumor-infiltrating lymphocytes
TS	Tumor stroma

Introduction

The tumor microenvironment comprises an extracellular matrix, mesenchymal cells, fibroblasts, and tumor-infiltrating lymphocytes (TILs). The balance between pro- and antitumor factors within the tumor microenvironment plays a crucial role in determining tumor progression and metastasis [1]. TILs can serve as biomarkers to predict survival in various types of cancer and can significantly impact the choice of first-line therapy, such as chemotherapy or surgery [2]. Notably, the prognostic significance of TILs in colorectal carcinoma (CRC) is even superior to AJCC/TNM staging [3]. Similar to gastrointestinal cancer, laryngeal squamous cell carcinoma (LSCC) is also an inflammation-induced tumor. In a previous study, we found a

Duo Zhang and Yu Heng have contributed equally to this work.

✉ Li-ming Lu
lulunew2003@163.com

✉ Chun-ping Wu
wcpeent@163.com

✉ Lei Tao
doctortaolei@163.com

[1] Department of Eye & ENT Hospital, Shanghai Key Clinical Disciplines of Otorhinolaryngology, Fudan University, 83 Fenyang Road, Shanghai 200031, People's Republic of China

[2] Shanghai Institute of Immunology, Shanghai Jiao Tong University School of Medicine, 280 South Chongqing Road, Shanghai 200025, People's Republic of China

positive correlation between the infiltration of CD3, CD4, and CD8 lymphocytes and the prognosis of LSCC patients [4].

Unfortunately, many LSCC patients are diagnosed at an advanced disease stage, leading to a poor prognosis despite various treatment options, including surgery, radiotherapy (RT), chemoradiotherapy (CRT), and chemotherapy. The 5-year survival rate for LSCC remains less than 50% due to factors like lymph node or distant metastases, resistance to RT or CRT, and local or regional recurrence [5]. Recently, immunotherapy, particularly immune checkpoint inhibitors (ICIs), has gained attention in tumor treatment, including head and neck squamous carcinoma (HNSCC) [6]. Consequently, immunotherapy, either alone or in combination with other treatments (radiotherapy and chemotherapy), may offer a new approach for LSCC treatment [7]. Cytotoxic-T-lymphocytes ($CD8^+$) are the main subset of T cells involved in immunotherapy to combat cancer [8]. However, the intended effect of immunotherapy can be weakened by the immunosuppressive environment created by immunosuppressive cells. Immunoscore (IS) primarily evaluates $CD3^+$ and $CD8^+$ T lymphocytes in the tumor and its invasive margin, and it has been validated as an independent prognostic factor in various types of tumors [9–11]. Yet, the role of $CD3^+$ lymphocytes in antitumor immunity remains controversial [12]. Moreover, IS does not account for immunosuppressive cells, such as regulatory T cells ($Foxp3^+$ Treg), M2 macrophages, and N2 neutrophils, which hinder an effective immune response against the carcinoma. Our previous study revealed that a high level of $CD206^+$ M2 infiltration and $CD66b^+$ neutrophils were correlated with poor prognosis in LSCC patients [13, 14]. Therefore, there is an urgent need to develop a new biomarker for more informative risk stratification, one that includes both $CD8^+$ T cells and immunosuppressive cells, also known as a modified immunoscore.

In this study, we investigated CD3, CD4, CD8, Foxp3, CD206, CD68, CXCR4, and CD66b through digital pathology, focusing on the main subset of TILs. The ratio of cytotoxic-T-lymphocytes to immunosuppressive lymphocytes (CILs) along with IS were used to assess the immune microenvironment of LSCC. CIL includes CILM2N2 (the ratio of CD8 to the sum of CD8, M2 macrophages, and N2 neutrophils), CILM2 (the ratio of CD8 to the sum of CD8 and M2 macrophages), and CILN2 (the ratio of CD8 to the sum of CD8 and N2 neutrophils). The primary aim of this article is to evaluate the role of CIL in predicting LSCC survival.

Methods

Patients, study design, and tissue microarray (TMA) construction

This study comprises two cohorts. In the training cohort, LSCC cases were collected from the Otolaryngology Head and Neck Surgery Department of the Eye, Ear, Nose, and Throat Hospital between 2014 and 2018. The LSCC cohort included TMA cores from 80 patients, with 5 patients excluded due to loss during follow-up. Tumor recurrence was defined as either local recurrence or distant metastases. The study was ethically approved by the Ethics Boards of the Eye and ENT Hospital of Fudan University, and it was conducted in adherence to the principles of the Declaration of Helsinki (No. KJ2008-01). Informed consent was obtained from all participants. Supplementary Table S1 provides baseline clinical characteristics of the LSCC patients included in the TMA analysis.

In the validation cohort, RNA-seq data and clinical information of 116 LSCC sample and 12 noncancerous adjacent tissue samples were obtained from the TCGA database (https://portal.gdc.cancer.gov), which includes transcriptome information and clinical data. The clinical information for these 116 LSCC patients comprises survival time, TNM classification, stage, age, and gender.

Immunohistochemistry (IHC) and IS construction

Sections of 4–5 μm thickness were cut from each selected block of formalin-fixed, paraffin-embedded TMA tissue (Wuhan Biosci Biotechnology Co., Ltd., Wuhan, China). The staining protocol was established as previously reported [4, 14]. $CD3^+$, $CD8^+$, $CD4^+$, and $Foxp3^+$ staining were imaged using an Aperio digital slide scanner (Leica Biosystems) and analyzed with QuPath v. 0.2.3 (Queen's University, Belfast, Northern Ireland) to determine the number of positive cells on the TMA. Two board-certified pathologists confirmed the definitions of tumor inferior (TI) and tumor stroma (TS) areas through performing hematoxylin and eosin (HE) staining. Tumor-infiltrating lymphocytes (TILs) were calculated by summing individual cells from both the tumor interior (TI) and the tumor stroma (TS). The cutoff value for each TIL type, which separated patients into two groups (low or high infiltration), was determined using the Youden index. Based on the infiltration of $CD3^+$ and $CD8^+$ T cells, the mean of the four percentiles (two markers and two regions) was calculated and converted into an immunoscoring (IS) system. Specifically, the IS categories are as follows: 1) IS = 0 indicates low infiltration of both $CD3^+$ and $CD8^+$ T

cells; 2) IS = 1 indicates high density of at least one marker in one region (TI or TS); 3) IS = 2–4 indicates a total score for each region with high density [15].

Immunofluorescence staining and CIL construction

For TMA analysis, anti-CD68 primary antibody (1:200, 76,437, clone D4B9C, CST) or goat monoclonal anti-CD206 (1:100, AF2534, R&D Systems) were incubated overnight at 4 °C. Following three 10-min washes with PBS, the sections were incubated in the dark for 60 min with different fluorochrome-conjugated secondary antibodies. After washing, the slides were mounted using Vectashield HardSet mounting medium, with 4′, 6-diamidino-2-phenylindole (1:200, DAPI; Solarbio, C0060). Co-expression of both $CD206^+$ and $CD68^+$ was considered M2 macrophages, while co-expression of both $CD66b^+$ (1:200, 305,102, clone G10F5, BioLegend) and $CXCR4^+$ (ab124824, UMB2, Abcam) was considered N2 neutrophils. QuPath was used to evaluate cell detection through nuclei (DAPI), considering cytoplasmic fluorescence of each cell with a mean intensity greater than 100 as positive.

CILM2N2 was computed as the ratio of $CD8^+$ cell density to the sum of $CD8^+$, M2 ($CD206^+CD68^+$), and N2 ($CD66b^+CXCR4^+$). CILM2 was computed as the ratio of $CD8^+$ cell density to the sum of $CD8^+$ and M2 ($CD206^+CD68^+$). Similarly, CILN2 was computed as the ratio of $CD8^+$ cell density to the sum of $CD8^+$ and N2 ($CD66b^+CXCR4^+$).

Analysis of TCGA RNA data: enhancing immunologiclal profiling in laryngeal squamous cell carcinoma

The IS-TCGA was derived by calculating the average of expression CD8A and CD3E, following a previously reported methodology [16]. To assess the landscape of tumor-infiltrating lymphocytes (TILs) in TCGA-LSCC, the CIBERSORT algorithm, utilizing R software (Version 458, https://www.r-project.org/), was employed. Additionally, CILM2-TCGA was determined as the ratio of $CD8^+$ cell density to the combined $CD8^+$, M0, and M2 cell population.

Statistical analysis: evaluating predictive accuracy and survival outcomes

To assess the predictive accuracy of CIL, IS, and clinicpathological parameters, integrated area under the ROC curve (iAUC) was computed with 500 × bootstrap resampling through R Studio (Version 2022.02.2). Overall survival (OS) and disease-free survival (DFS) curves were generated using the Kaplan–Meier method with SPSS (version 25.0) and GraphPad Prism (version 8.0), respectively. The risk of OS and DFS was analyzed through univariate and multivariate analyses using the log-rank (Mantel–Cox) test and Cox proportional hazard models, respectively. Additionally, the hazard ratio (HR) with 95% confidence intervals (CIs) was determined through univariate Cox regression.

We determined the relative importance of parameters in estimating survival risk using a multivariable Cox proportional hazards model. In this analysis, we considered clinical factors, CIL, and IS as co-variables for the 'cph' function from the 'rms' R package. By applying the 'anova' function to the 'chp' object, we obtained a matrix of predictors that reflects the variables' importance in the model, assessed by the Wald Chi-square (χ^2) statistic, as previous reported [16]. The specific code is provided in the supplementary materials.

Associations between TIL density, SIRI, and recorded clinicopathological characteristics were examined using χ^2 tests for categorical variables. A *P*-value of 0.05 (two-tailed) was considered statistically significant.

Result

Evaluating the prognostic effect of TILs in LSCC patients from the training cohort

To investigate the tumor immune microenvironment of LSCC, we utilized immunohistochemistry (IHC) and immunofluorescence (IF) to analyze adoptive ($CD3^+$/$CD4^+$/$CD8^+$/$Foxp3^+$ T lymphocytes) and innate (M2 macrophages/N2 neutrophils) immune cells, as shown in Fig 1. Subsequently, the prognostic impact of tumor-infiltrating lymphocytes (TILs) was assessed using Kaplan–Meier survival analysis. Our findings revealed that a high density of $CD8^+$ T lymphocytes in both the tumor stroma (TS) and tumor inferior (TI) significantly correlated with prolonged overall survival (OS) and disease-free survival (DFS). Patients with high density of $CD3^+$ or $CD4^+$ T lymphocytes also exhibited improved outcomes, although the results were not statistically significant (Supplementary Fig S1 and S2). In addition, there was no significant difference in overall survival (OS) between the high and low expression groups of Foxp3. The forest plot demonstrated a significant positive association between the density of $CD8^+$ T lymphocytes and OS (HR=0.39, 95%CI 0.19–0.82, p=0.0013, Fig. 2A) and DFS (HR=0.37, 95%CI 0.18–0.57, p=0.006, Supplementary Fig. S3). Furthermore, the density of $CD206^+CD68^+$ macrophages (M2) and $CD66b^+CXCR4^+$ neutrophils (N2) showed a negative association with OS (M2: HR=4.3, 95%CI 1.8–10, p=0.00012; N2: HR=3.2, 95%CI 1.6-6.2, p=0.00073) and DFS (M2: HR=1.8, 95%CI 1.0-3.28, p=0.836; N2: HR=1.8, 95%CI 0.9-3.4, p=0.0454). Furthermore, patients

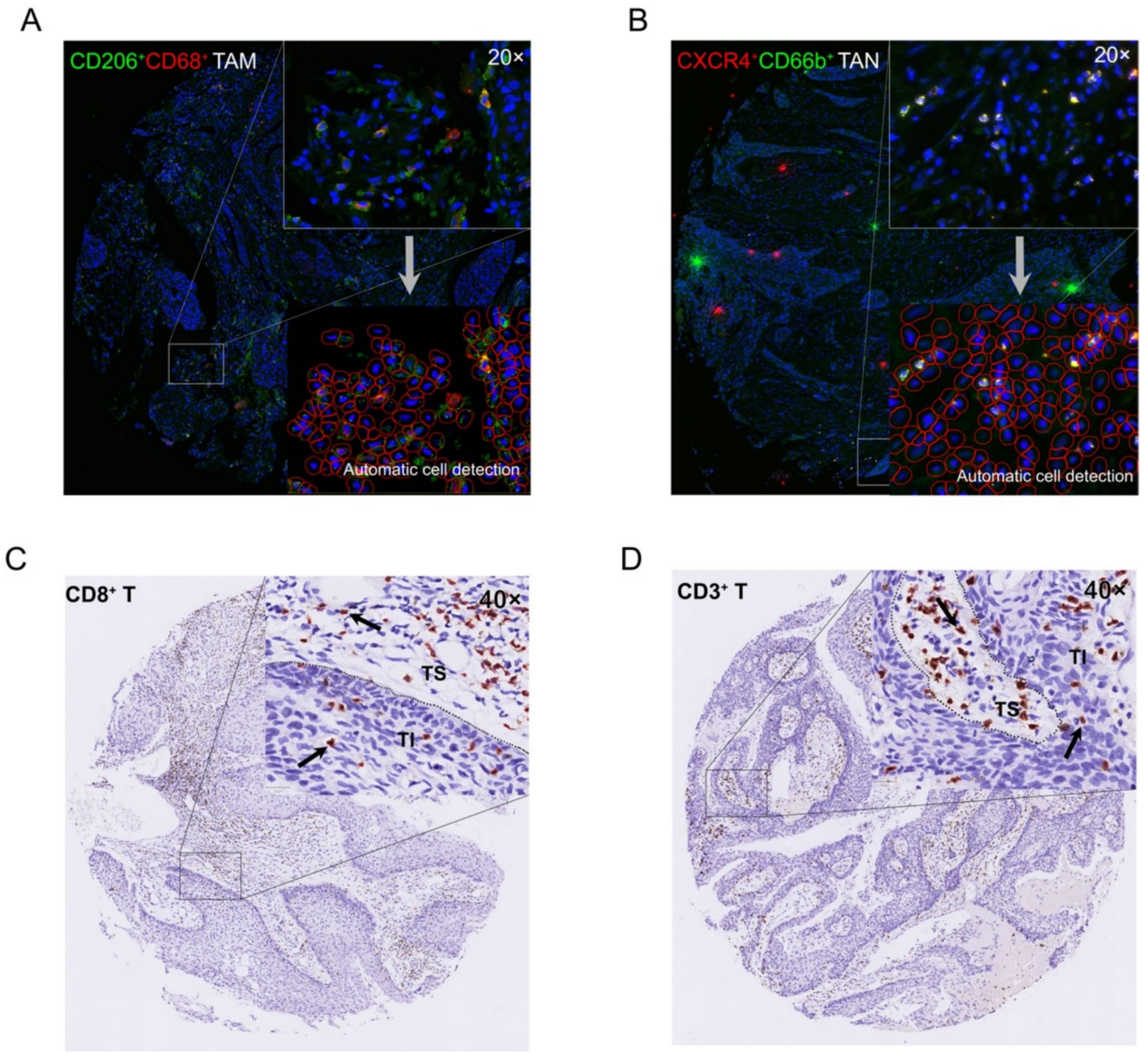

Fig. 1 Tumor-infiltrating lymphocytes automatically detected by QuPath software. Using the QuPath software, we automatically detected tumor-infiltrating lymphocytes (TILs) based on cytoplasmic fluorescence (red and green) with a mean intensity greater than 100, which was considered positive. Co-expression of $CD206^+$ and $CD68^+$ was identified as M2 macrophages (**A**), while co-expression of $CD66b^+$ and $CXCR4^+$ was designated as N2 neutrophils (**B**). Positive cell detection (CD3/CD8) was analyzed through immunohistochemistry (IHC) using QuPath. The tumor tissue was divided into two regions: tumor inferior (TI) and tumor stroma (TS). Figurative examples of CD8 (**C**) and CD3 (**D**) expression in LSCC are shown for reference

with low CILM2N2 (the ratio of CD8 to the sum of CD8, M2, and N2) had a lower OS rate at 24 months (37.5%) compared to those with high CILM2N2 (55.9%), showing an unadjusted HR of 0.2 (95%CI 0.097–0.43, $p<0.0001$). Similarly, patients with a low IS (based on $CD3^+/CD8^+$ infiltration in the tumor and its invasive margin) had a lower OS rate at 24 months (39.4%) compared to those with a high IS (61.9%), showing an unadjusted HR of 0.53 (95%CI 0.39–0.73, $p<0.0001$).

IS and CILN2: two independent risk factors for the prognosis of LSCC in the training cohort

Univariate analysis revealed that IS, CILM2N2, CILN2, and CILM2 were significantly associated with both overall survival (OS) and disease-free survival (DFS). In a multivariable Cox model adjusted for differentiation, T stage, N stage, and clinical stage (Table 1), IS and CILN2 emerged as two independent predictors for LSCC prognosis. Additionally,

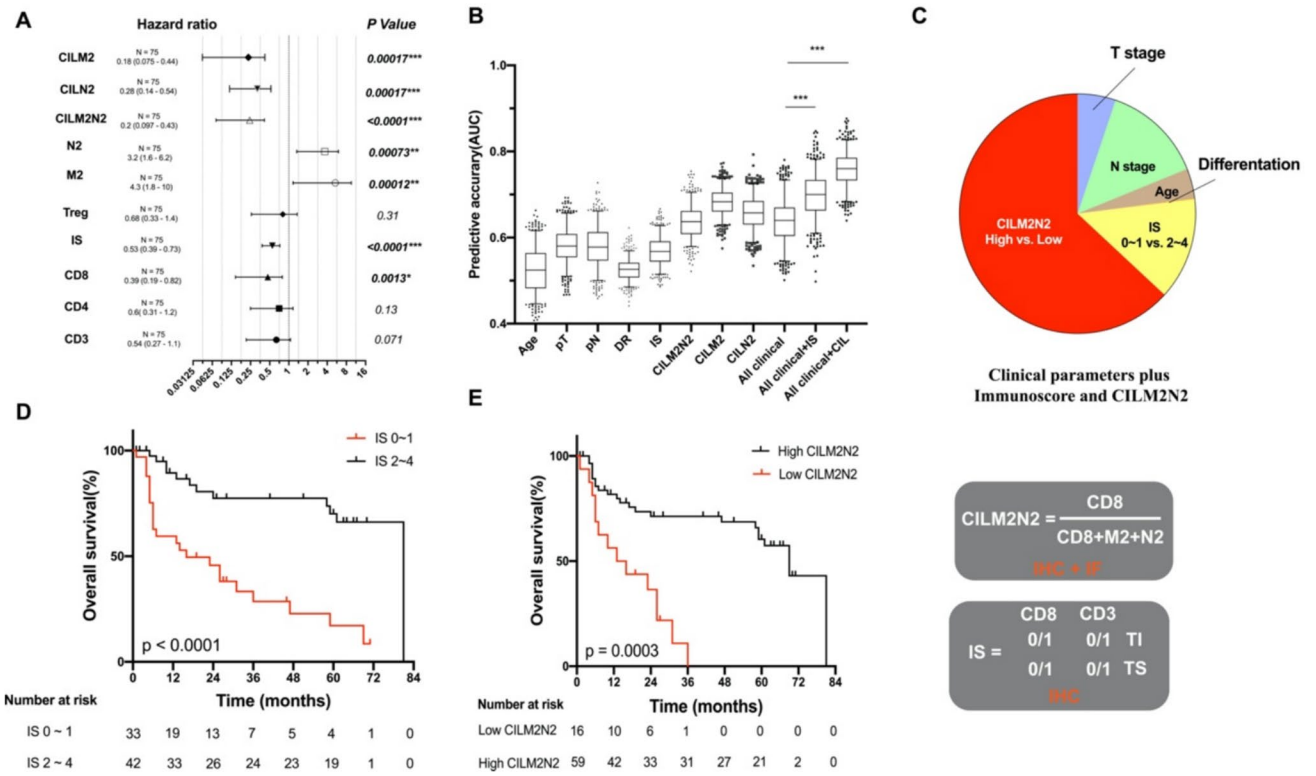

Fig. 2 Prognostic performance of IS and CIL in LSCC compared with clinic-pathological parameters. **A** Forest plot displaying the univariable association of tumor-infiltrating lymphocytes with overall survival (OS) in LSCC patients. Hazard ratios, 95% confidence intervals, and *p*-values (*$p<0.050$, **$p<0.001$, ***$p<0.0001$) are presented. **B** The predictive accuracy of IS, CILM2N2, CILM2, CILN2, and clinic-pathological parameters for overall survival based on the area under the curve (AUC) with 500 × bootstrap resampling. The horizontal lines represent the mean value of AUC, while the scatter points indicate the estimated values. The edge of the box represents the 95% confidence interval, and the whiskers depict the maximum and minimum values. **C** Relative importance of each risk parameter to survival risk using the χ^2 proportion test for clinical parameters plus immunoscore and CIL. **D** Kaplan–Meier survival analysis of IS, illustrating the impact on overall survival (OS), **E** Kaplan–Meier survival analysis of CILM2N2, demonstrating the association with overall survival (OS)

recurrence at 2 years was observed in 60% of patients with low IS (0–1) and 38% of patients with high IS (2–4). Similarly, recurrence at 2 years occurred in 65% of patients with low CILM2N2 and 43% of patients with high CILM2N2 (Supplementary Fig. S3). We further conducted an integrated AUC (iAUC) analysis to assess the predictive ability of CIL (Fig. 2B) compared to IS and other well-established clinical risk factors. The analysis demonstrated that CILM2N2 exhibited superior predictive ability for overall survival compared to IS and was comparable to the combination of all clinical variables (median AUC for CIL = 0.636; median AUC for all clinical variables = 0.638; median AUC for IS = 0.568). Upon analyzing the relative contribution of each parameter to predict overall survival, we made a fascinating discovery. Among the clinical parameters examined, CILM2N2 exhibited significantly higher importance, emerging as the most powerful predictor. This finding highlights the pivotal role of CIL in determining survival outcomes, surpassing all other variables studied (Fig. 2C). Using the Kaplan–Meier method, we observed that patients with high IS or CILM2N2 experienced a significantly prolonged OS (Fig. 2D, E).

Differently TIL infiltration in TCGA (validation cohort) and prognostic value of IS-TCGA and CIL-TCGA

The transcriptome data of 116 LSCC patients were obtained from the TCGA database (https://portal.gdc.cancer.gov). Using the CIBERSORT algorithm, we quantified the landscape of tumor-infiltrating lymphocytes (TILs) in LSCC, revealing higher percentages of M0, M2, and $CD8^+$ T lymphocytes in tumor tissue compared to adjacent tissue (Fig. 3A). Next, we analyzed the prognostic ability of TILs (Supplementary Fig. S4). High density of $CD8^+$ T cells, M1 macrophages, B cells, NK cells, neutrophils, and Treg was associated with better overall survival (OS), while poor OS was associated with M0 macrophages, M2 macrophages, $CD4^+$ T cells. Notably, a positive correlation was found between Treg and $CD8^+$ T cells through spearman rank text

Table 1 Univariate and multivariate analysis of IS and CIL adjust for clinical risk factors

	Overall survival		Disease-free survival	
	HR (95% CI)	*p* value	HR (95% CI)	*p* value
Unadjusted stratified Cox model				
IS based on CD3 and CD8	0.259 (0.128–0.527)	**< 0.0001*****	0.339 (0.179–0.642)	**0.001****
CILM2N2	0.207 (0.098–0.437)	**< 0.0001*****	0.315 (0.160–0.622)	**0.001****
CILM2	0.212 (0.100–0.450)	**< 0.0001*****	0.382 (0.200–0.732)	**0.004****
CILN2	0.278 (0.142–0.544)	**< 0.0001*****	0.375(0.190–0.671)	**0.001****
pT stage (T1-2 versus T3-4)	0.6204(0.279–1.378)	0.2409	0.7432(0.308–1.794)	0.509
pN stage (N0 versus N +)	0.6374(0.325–1.251)	0.1905	0.7298(0.360–1.480)	0.385
Clinical stage (I-II versus III-IV)	0.5031(0.192–1.321)	0.1632	0.3948(0.141–1.107)	0.077
Differentiation	2.073(0.900–4.773)	0.8680	2.962(1.206–7.271)	0.0180
Multivariable stratified Cox model adjusted for clinical risk factors				
pT stage (T1-2 versus T3-4)	1.398 (0.375–5.209)	0.618	0.610 (0.182–2.043)	0.423
pN stage (N0 versus N +)	1.678(0.784–3.594)	0.182	1.125(0.564–2.241)	0.738
Clinical stage (I-II versus III-IV)	0.223 (0.025–1.962)	0.176	0.086(0.011–0.659)	**0.018***
Differentiation	1.339 (0.342–5.242)	0.675	0.413 (0.090–1.891)	0.254
IS based on CD3 and CD8	0.349 (0.129–0.946)	**0.039***	0.407 (0.181–0.918)	**0.030***
CILM2N2	1.703 (0.489–5.931)	0.403	1.322(0.450–3.879)	0.612
CILM2	0.523 (0.184–1.482)	0.222	1.242(0.518–2.981)	0.627
CILN2	0.219 (0.062–0.767)	**0.018***	0.242(0.086–0.682)	**0.007****

IS, immunoscore; CILM2N2, the ratio of CD8 to the sum of CD8, M2, and N2; CILM2, the ratio of CD8 to the sum of CD8 and M2; CILN2, the ratio of CD8 to the sum of CD8 and N2; Statistical significance was defined as $p < 0.05$; *$p < 0.050$, **$p < 0.001$, ***$p < 0.0001$

($R = 0.3612$, $p < 0.0001$). Conversely, $CD8^+$ T cells were negatively correlated with tumor-associated neutrophils (TAN) or tumor-associated macrophages (TAM) (R for TAN −0.1515, $p = 0.1048$; R for TAM −0.4105, $p < 0.0001$) (Supplementary Fig. S5). Although the original IS could not be directly calculated from the transcriptome data (as the CIBERSORT algorithm cannot analyze $CD3^+$ T cell infiltration), the IS-like data were extrapolated from the average gene expression of CD8A and CD3E, as previously reported [16]. The prognostic ability of IS-TCGA was found to be similar to the original IS. Patients with high IS-TCGA exhibited significantly prolonged OS compared to those with low IS-TCGA (HR for high versus low, 0.3253, 95%CI 0.1555–0.6804, $p = 0.0029$) (Fig. 3D). To account for the inability to distinguish between N1 and N2 neutrophils from bulk RNA data in TCGA, we observed a high similarity between M0 and M2 macrophages from cor-heatmap (M0/M2 = 0.19, M0/M1 = 0.08, M1/M2 = −0.09, Fig. 3B). Thus, the sum of M0 and M2 was considered as the main immunosuppressive cell infiltrated in LSCC-TCGA. Consistent with the original CILM2-TMA (the ratio of CD8 to the sum of CD8 and M2), CILM2-TCGA also showed good prognostic value for the overall survival of LSCC (Fig. 3C). Finally, the predictive accuracy of CILM2-TCGA was evaluated using the integrated AUC. The ability of CILM2 to predict overall survival was found to be similar to IS and the combination of all clinical variables (median AUC for CILM2 = 0.571; median AUC for all clinical variables = 0.598; median AUC for IS = 0.574) (Fig. 3E).

Discussion

Immunoscore (IS) is derived from the combined infiltration of $CD3^+$ and $CD8^+$ T cells in both tumor inferior (TI) and tumor stroma (TS) [15]. It has been established as a crucial prognostic factor in various tumor types [17–19], particularly in inflammation-induced tumors like colorectal carcinoma (CRC) and laryngeal squamous cell carcinoma (LSCC). $CD8^+$ T cells, also known as cytotoxic T cells (CTLs), play a vital role in antitumor immunity. On the other hand, $CD3^+$ TILs encompass various T lymphocytes, including both antitumor CTLs and immunosuppressive T cells like regulatory T cells (Treg). As a result, the predictive value of $CD3^+$ TILs is not as significant as that of $CD8^+$ TILs [12]. Hence, IS provides a general assessment of lymphocyte infiltration in tumor specimens without distinguishing based on the function of T cells, and it may not fully capture the immunogenicity of the tumor microenvironment. In this study, our aim is to evaluate whether enhancing the predictive accuracy of IS by incorporating

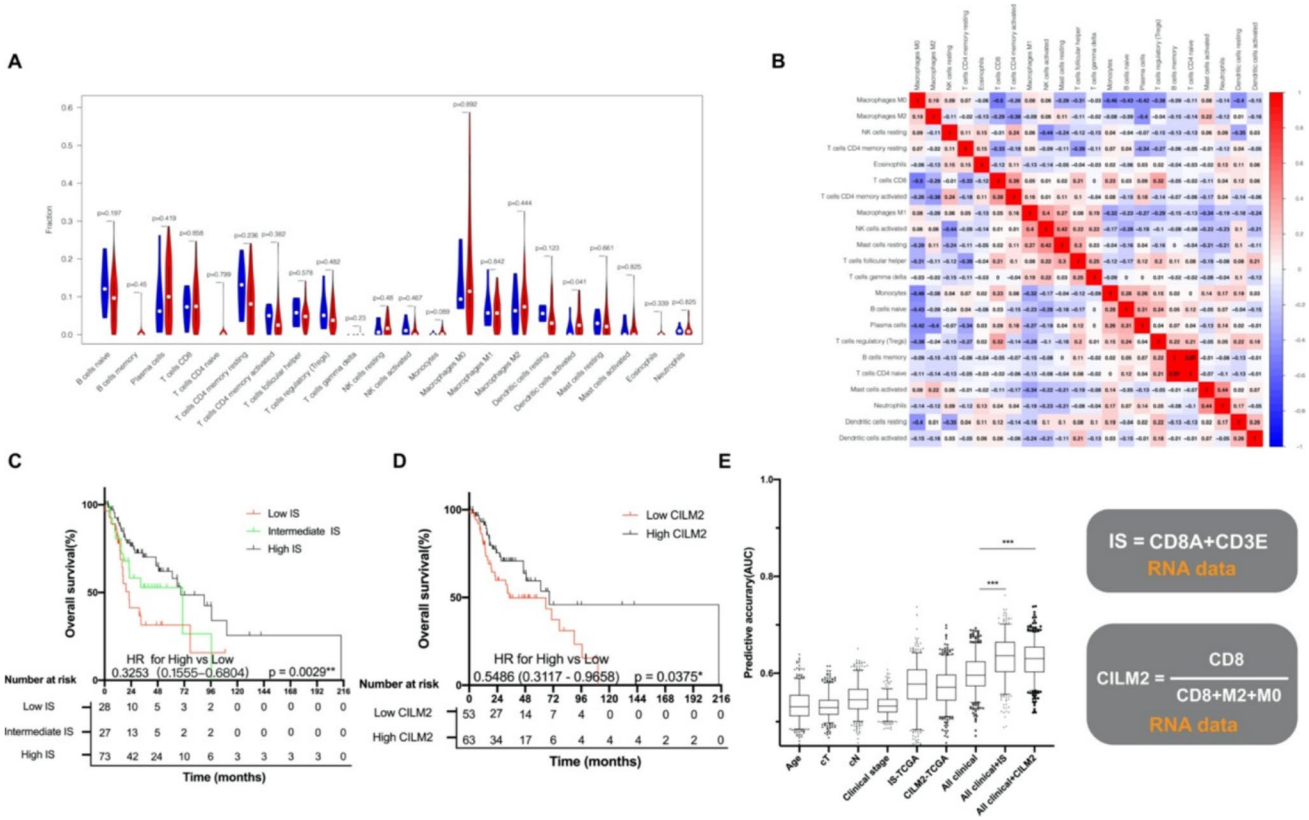

Fig. 3 The prognostic value of IS and CILM2 in LSCC-TCGA. **A** Vioplot showing the proportions of tumor-infiltrating lymphocytes (TILs) in TCGA-LSCC (Blue: normal tissue; Red: tumor tissue). **B** Cor-heatmap illustrating all 22 immune cell types in the TCGA cohort. **C** Kaplan–Meier analysis of overall survival (OS) based on CILM2 in LSCC-TCGA. **D** Kaplan–Meier analysis of overall survival (OS) based on IS in LSCC-TCGA. **E** Predictive accuracy of IS, CILM2, and clinic-pathological parameters for overall survival (OS) based on the area under the curve (AUC) with 500 × bootstrap resampling. The horizontal lines represent the mean value of AUC, while the scatter points indicate the estimated values. The edge of the box represents the 95% confidence interval, and the whiskers depict the maximum and minimum values

immunosuppressive cells could lead to more informative and comprehensive risk stratification.

The tumor immunosuppressive microenvironment, comprising immunosuppressive cells and cytokines, plays a crucial role in antitumor immunity. Apart from regulatory T cells (Treg), several innate immune cells also contribute significantly to immunosuppression. Among these, tumor-associated macrophages (TAMs) represent the most abundant innate immune cells in tumors, with two main functional subtypes: M1 and M2. M1 macrophages exhibit low expression of CD206 and high expression of MHCII. They secrete TNF-α, iNOS, and express co-stimulatory molecules CD86 and CD40, thereby exerting antitumor immune responses. In contrast, M2 macrophages display low MHCII and high CD68 and CD206 expression, releasing immunosuppressive factors such as vascular endothelial growth factor (VEGF), arginase 1 (Arg-1), IL-10, and TGF-β [20]. Consequently, M2 macrophages promote tumor angiogenesis, proliferation, and migration. Similarly, tumor-associated neutrophils (TANs) can be divided into N1 (antitumor type) and N2 (pro-tumor type) subtypes. N2 neutrophils are characterized by overexpression of VEGF and CXCR4, promoting tumor proliferation by facilitating tumor angiogenesis, invasion, and metastasis [21]. Both M2 macrophages and N2 neutrophils have been reported to exhibit negative predictive value for patient survival outcomes [22–25].

Compared with IS, CIL was constructed by the combination of $CD8^+$ T and immunosuppressive cells. $Foxp3^+$ T cells, also known as regulatory T cells (Treg), were proved to be associated with unfavorable prognosis of many tumors [26–29]. However, the prognostic value of Treg in colorectal carcinoma (CRC) remains controversial, with some studies showing a better prognosis [30, 31]. Since Treg did not exhibit a poor predictive effect on LSCC (in both cohorts) similar to CRC (an inflammation-induced tumor type), this study only includes M2 and N2 as immunosuppressive cells. As a result, CIL includes CILM2N2 (the ratio of CD8 to the sum of CD8, M2 macrophages, and N2 neutrophils), CILM2 (the ratio of CD8 to the sum of CD8 and M2 macrophages), and CILN2 (the ratio of CD8 to the sum of CD8 and N2

neutrophils). Moreover, CIL does not require artificial separation between tumor inferior (TI) and tumor stroma (TS), making it easier to construct compared to IS. In this study, we compared the predictive value of two markers representing the tumor immune microenvironment, IS and CIL, in patients with LSCC. Both high IS and CIL were found to be associated with better prognosis of LSCC in TMA. Upon analyzing the relative contribution, CILM2N2 demonstrated the highest importance among the clinical parameters studied, emerging as the most powerful predictor of overall survival, highlighting its pivotal role in determining outcomes. Although CILM2N2 is not an independent predictive factor in multifactorial analysis, this may be due to insufficient case numbers in this study. Additionally, bioinformatics analysis of bulk RNA-sequencing (RNA-seq) data from LSCC-TCGA also showed that patients with high IS-TCGA or CILM2-TCGA had significantly prolonged overall survival. These findings suggest that RNA-seq-based immunological evaluation may align with TIL (IHC- and IF-based) for predicting LSCC outcomes. Several clinical trials targeting tumor-associated neutrophils (TANs) by inhibiting chemokines (CXCL8/CXCL5) or its receptors (CXCR1/CXCR2) have demonstrated relief in antitumor resistance [32, 33]. Moreover, combining immune checkpoint inhibitors (ICIs) with M2 blockers has shown improved immunotherapy response rates [34]. This suggests that CIL may have a crucial predictive effect on the prognosis of immunotherapy.

The present study has certain limitations. Firstly, the validation cohort was derived from bulk RNA-seq data obtained from the TCGA database. Unlike the training cohort, where IS-TCGA or CIL-TCGA was constructed through IHC or IF, the validation cohort was constructed using the CIBERSORT algorithm. This means that the distinction between N1 and N2 neutrophils was not feasible with bulk RNA data from TCGA. Secondly, the predictive value of CIL needs further confirmation through multicenter and large-scale studies to establish its robustness and generalizability.

In summary, the CIL, which is constructed by combining $CD8^+$ T cells and immunosuppressive cells (M2 and N2) and relies on digital analysis and artificial intelligence, has demonstrated significant prognostic value for LSCC. The study findings suggest that CIL can effectively predict the prognosis of LSCC patients and potentially serve as a valuable tool for patient risk stratification and treatment decisions.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00262-025-04008-0.

Author contributions Li-ming Lu, Chun-ping Wu, Lei Tao conceived and designed the work. Duo Zhang, Di Tang enrolled patients and collected clinical data. Qiu-Yan Jin, Yu Heng, Xiao-ke Zhu performed the experiments. Duo Zhang wrote the manuscript. All authors made contributions to the article, participated in critical manuscript revisions, and gave approval for the submitted version. All individuals involved in this study are recognized as co-authors.

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Data availability The datasets generated during and/or analyzed during the current study are available in the TCGA database (https://portal.gdc.cancer.gov).

Declarations

Conflict of interest The authors declare no competing interests.

Consent to participate Informed consent was obtained from all individual participants included in the study.

Ethical approval This study was approved by ethics boards of the Eye and ENT Hospital of Fudan University and was conducted in line with the principles of the Declaration of Helsinki (No. KJ2008-01).

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