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Research article

Bioinformatic analysis reveals prognostic value and immunotherapy potential of Siglec-15 in laryngeal squamous cell carcinoma

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ABSTRACT

Background: Laryngeal squamous cell carcinoma (LSCC) is the ultimate common malignant head and neck cancer with dismal prognosis. The expression pattern and clinical significance of Siglec-15 (Sialic acid-binding immunoglobulin-like lectin 15) in LSCC are poorly understood. In order to lay the groundwork for future immune-related research on Siglec-15 in LSCC, we set out to study its expression and prognostic importance in the disease, as well as to use bioinformatics to investigate the immune features modulated by Siglec-15 in LSCC.

Methods: ① In order to get the gene expression profile and clinical data for TCGA head and neck cancer (TCGA-HNSC), you may access the relevant data from UCSC xena and use 110 cases of laryngeal cancer as a training set. Two datasets, GSE27020 and GSE25727, were obtained from the GEO databank and utilized as validation sets. These datasets include expression profiles and clinical information. The Siglec-15 gene and immune characteristics were analyzed by bioinformatics methods. ② Retrospectively collected routine paraffin specimens from patients with pathological diagnosis of squamous cell carcinoma from December 2012 to November 2015 in Sun Yat-sen Memorial Hospital and fresh frozen tissue of patients from June 2021 to March 2022. Immunohistochemistry method, immunofluorescence technique and real-time quantitative PCR was used to examine the difference of Siglec-15 appearance in LSCC tissue and adjacent tissue, and its correlation of prognosis, clinic pathological characteristics and CD8+T lymphocyte infiltration. Using human laryngeal cancer cell line (LCC), we studied the influence of Siglec-15 in cell proliferation and invasion.

Results: We identified Siglec-15 was upregulated in LSCC. The patients in Siglec-15 high expression group had a poor overall survival (OS) based on the clinical information from TGCA and 111 LSCC patients that hospitalized in Sun Yat-sen Memorial Hospital. The COX regression analysis indicated Siglec-15 as an independent predictor for poor prognosis of LSCC. Bioinformatic analysis suggested that the high expression of Siglec-15 shape an immune suppressive tumor microenvironment (TEM), leading to poor response to immunotherapy in LSCC. Siglec-15 enhanced cell invasion and proliferation, as we showed in vitro.

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Conclusion: Our study support Siglec-15 as a potential predictor for LSCC prognosis and an attractive target for LSCC immunotherapy.

1. Introduction

Laryngeal Cancer originating from the mucosal epithelium within the larynx stands as a prevalent form of head and neck malignancies globally. Within this spectrum, LSCC (laryngeal squamous cell carcinoma) emerges as the utmost frequently encountered subtype [1,2]. The occurrence of laryngeal cancers has increased by 12 % during the past 3 decades [3]. However, the 5-year survival rate of laryngeal cancer has not improved significantly in recent years despite the multimodal treatment [4].

Immunotherapy, which relies on medications that target immunological checkpoint genes (ICGs), has recently emerged as a prominent method for treating advanced laryngeal and other types of head and neck cancer [5]. Advanced melanomas have been treated using anti-PD-1 or PD-L1 immunotherapies, which do not kill cancer cells directly but rather to inhibit a pathway that protects tumor cells from immune system components [6,7]. Pembrolizumab and nivolumab are the two FDA-approved PD-1 blocking antibodies for treating recurrent or metastatic head and neck cancers [8,9]. However, due to the tumor heterogeneity, only a small percentage of patients are effective in PD-1/PD-L1 treatment [10]. Hence, immunotherapy for laryngeal cancer requires a novel target.

Siglec-15 is an immunoglobulin superfamily protein that is found in M2 macrophages, myeloid cells, dendritic cells and osteoclasts. It has the potential to cause immunosuppression in the TEM (tumor microenvironment) by persistently reducing T-cell activation [11–13]. Recent studies have shown that Siglec-15 is the primary immunosuppressive factor via a mechanism unrelated to the PD-1/PD-L1 pathway, it may provide a new promising immunotherapy target for cancer patients, who didn't have positive responses to PD1 or PD-L1 therapy [14–16]. Siglec-15 has emerged as a promising immunotherapy target for several kinds of tumors [17–19]. To date, the appearance and clinical importance of Siglec-15 in LSCC stay indistinct.

To summarize, there is an urgent need to explore suitable biomolecules to predict the prognosis of LSCC, identify effective populations for immunotherapy and serve as new targets for immunotherapy drugs. This research designed to investigate the expression characteristics, prognostic value of Siglec-15 in LSCC and its relationship to immunotherapy. To accomplish this, we initially obtained the gene expression profile of LSCC patients from public databases and conducted survival analysis by grouping based on the appearance of Siglec-15 to ascertain its prognostic value. Subsequently, we examined the connection among immune checkpoint gene expression and tumor immune infiltration and Siglec-15 expression levels. Enrichment analysis was employed to demonstrate the potential mechanisms of Siglec-15 in regulating the immune environment. Furthermore, we investigated the appearance levels of Siglec-15 in patients exhibiting different responses to immunotherapy, revealing its potential role in immunotherapy. We further validated the predictive value of Siglec-15 through qRT-PCR, immunohistochemistry, immunofluorescence, survival curves and Cox models in our own cohort. Additionally, cell experiments confirmed the impact of knocking out/overexpressing Siglec-15 on the proliferation and migration of laryngeal cancer cell line (LCC). Our research helped us learn more about the roles of Siglec-15 in LCSS and found that it might be a good target for immunotherapy.

2. Methods and materials

2.1. Dataset collection and analysis

Gene expression profiles of LCSS and normal larynx in TCGA databank were acquired from UCSC xena (https://xenabrowser.net/). The GSE27020 (n = 109) and GSE25727 (n = 56) datasets containing expression profiles of laryngeal carcinoma tissues were downloaded and annotated from the GEO (Gene Expression Omnibus) (https://www.ncbi.nlm.nih.gov/geo/) databank and applied as validation. Wilcoxon test was applied to compare the differences in Siglec-15 mRNA expression between LSCC tissues and Para tumorous tissues in the TCGA database. $P < 0.05$ was measured to be statistically different.

2.2. Kaplan-Meier survival analysis

Based on the median Siglec-15 expression, the patients were separated into Siglec-15 low and high group. Kaplan-Meier estimator was utilized to build the persistence function of each group. Log-rank test was applied to relate the survival curves created by the Kaplan-Meier estimator to assess the influence of Siglec-15 on overall survival (OS). The "survival" and "survminer" R programs were used to perform and display the survival analyses.

2.3. Estimation of immune infiltrating

The ESTIMATE method, which is part of the R package "estimate," was used to grade the tumor microenvironment based on the amount of immune and stromal cell infiltration. This was based on the particular gene appearance stages of these cells in LSCC, as measured by TCGA expression profiles [20].

2.4. Correlation analysis

The correlation of Siglec-15 with the expression of ICGs (Immune checkpoint genes), immune or stromal scores, proportion of specific immune cells and the GSVA score of specific pathways was analyzed using PCA (Pearson Correlation Analysis) and $P < 0.05$ and $|R| > 0.2$ were considered to be correlated in this exploratory study. The outcomes were visualized by R package "ggplot2".

2.5. Functional enrichment analysis

To carry out the differential gene expression analysis, the "limma" package was used. Genes that had an FDR < 0.05 and a $|\log2FC| > 1$ were considered to be DEGs. KEGG (Kyoto Encyclopedia of Genes and Genomes) and Gene Oncology (GO) enhancement analysis were executed for DEGs through R package "clusterProfiler" and the results were visualized by "ggplot2".

2.6. Gene sets and gene set variation analysis

In the present study, gene sets were attained from the Molecular Signatures Database (MSigDB). Then, GSVA (Gene Set Variation Analysis) was executed to derive the enrichment score of specific anti-cancer immune process and immunotherapy-related pathways in each certain sample using R package "GSVA". The Wilcoxon test was used to examine the differentiation of GSVA score in specific pathways between low and high Siglec-15 appearance groups.

2.7. Clinical specimens

LSCC and Para tumorous samples were collected from 111 patients diagnosed with LSCC from Sun Yat-sen Memorial Hospital. Clinical information was recorded from these patients and the clinical characteristics were summarized in Table 2. In addition, the Chi-Squared test was used to assess the relationship among low and high Siglec-15 expression and various categorical clinical characteristics of patients with LSCC. $P < 0.05$ was deliberated to be statistically diverse.

2.8. RNA extraction and quantitative real-time PCR (qRT-PCR)

We followed the directions supplied by Vazyme, a company based in Nanjing, China, to extract total RNA using their RNA Isolator Total RNA Extraction Reagent. After obtaining 500 ng of RNA from Takara in Shiga, Japan, the cDNA was reverse-transcribed using the Prime Script TM RT reagent Kit with gDNA Eraser. qRT-PCR was accomplished using TB Green® Premix Ex Taq™ II (Takara, Shiga, Japan). The following were the parameters used for qRT-PCR: Start at 95 °C for 30 s, then alternate between 60 °C for 30 s and 95 °C for 5 s for 40 repetitions. Triplicate runs of each reaction were initiated. The expression level was normalized using GAPDH. Utilizing the ΔΔCt technique, the results of the real-time PCR were examined. Primers orders were as follows: Siglec-15-forward: 5′-CGCGGATCGTCAACATCTC-3′; Siglec-15-reverse: 5′-GTTCGGCGGTCACTAGGTG-3'; GAPDH-forward: 5′-CGACCACTTTGT-CAAGCTCA-3′; GAPDH-reverse: 5′-AGGGGAGATTCAGTGTGGTG-3′.

2.9. Immunohistochemistry

Immunohistochemistry was accomplished on 5 μm paraffin-embedded sections as previously described [21]. Briefly, xylene was used for deparaffinization, and a graded series of ethanol was used to rehydrate the sections. After a 10-min microwaving session and subsequent cooling to ambient temperature, the antigen was extracted from a 0.01 M sodium citrate buffer with a pH of 6.0. The sections were exposed to 3 % H_2O_2 for a duration of 10 min to inhibit the activity of the endogenous horseradish peroxidase (HRP). The sections were treated with the anti-Siglec-15 antibody after being blocked with 5 % BSA (1:1000, SAB3500654, Sigma, and St. Louis, MO) at 4 °C overnight. After rinsing with PBS, the slices were incubated with goat anti-rabbit IgG antibodies that were biotin-labeled for 30 min. They were then incubated with streptavidin-HRP complex for another 30 min. The positive signals were detected using the DAB Horseradish Peroxidase Colour Development Kit (P0202, Beyotime, Shanghai, China), which appeared as a reddish-brown color. Hematoxylin was used as a counterstain on the sections, and Image J was used for image analysis.

2.10. Immunofluorescence

Xylene was used to deparaffinized 5 μm slices that were embedded in paraffin, and a graded series of ethanol was used to rehydrate them. Sodium citrate buffer (0.01 M) with a pH of 6.0 was used to microwave the antigen for 10 min. After soaking for an hour in 5 % goat serum, the sections were treated with anti-CD8 or control overnight at 4 °C (1:500, 55397) or anti-Siglec-15 (1:500, SAB3500654, Sigma, St. Louis, MO) antibodies. Following a PBS wash, the sections were left to incubate at room temperature with the secondary antibody for 1 h. Afterwards, 4, 6-diamidino-2-phenylindole dihydrochloride (DAPI) was used as a counterstain.

2.11. Cell culture and transfection

The human LCC (laryngeal cancer cell) line was sustained in RPMI-640 medium containing 15 % FBS penicillin-streptomycin and 5 % CO_2 with raised temperature at 37 °C. ShSiglec-15 and pLVX-Siglec-15 were constructed by Shanghai HaoGe biotechnology

company (Shanghai, China). LCC were transfected with shSiglec-15 and pLVX-Siglec-15 using Lipofectamine 2000.

2.12. Cell counting kit-8 (CCK-8) assay

The CCK-8 test was used to determine the degree of cell proliferation. In conclusion, after transfection with shSiglec-15 and pLVX-Siglec-15 for 24, 48 and 72 h, the cells were cultured for 4 h at 37 °C. Each well was supplemented with 10 μL of the CCK-8 solution at every time point.

2.13. Transwell invasion assay

A mixture of 1 × 105 cells and 150 μg of Matrigel (BD Biosciences Clontech, Palo Alto, CA) were added to the top chamber. The attractant was a medium comprising 15 % FBS, which was introduced to the bottom chambers. After that, the cells were left to incubate for 24 h at 37 °C. Then, any cells that had not transferred were carefully removed from the top chamber using cotton swabs. Fixing and staining the migrating cells in a mixture of 20 % methanol and 0.1 % crystal violet allowed them to be perceived using an inverted microscope (Olympus, Tokyo, Japan). The quantification of five fields that were randomly chosen was then counted.

2.14. Statistical analysis

Error bars indicate ±SEM and significance was calculated using an independent student t-test if not otherwise stated, with $P < 0.05$ considered significant. SPSS 25.0 and R software were used for the statistical analysis. Analysis of the relationship between the independent variables was conducted using estimated HR (Hazard Ratio), univariate and multivariate Cox regression models. For cell culture, at least $n = 4$ independent biological replicates were examined for each experiment.

3. Results

3.1. Association of Siglec-15 with overall survival in LSCC

Based on the TCGA database, we found that appearance level of Siglec-15 in LSCC was considerably greater than the corresponding normal tissues (Fig. 1A). Based on the median value for the total dataset, patients in TCGA were separated into groups with low and high appearance of Siglec-15. This was done to investigate, if there was a connection among Siglec-15 expression and the prognoses of LSCC patients. According to Kaplan-Meier investigation, patients whose Siglec-15 expression was high had a substantially lower overall survival (OS) compared to those whose expression was low (29.7 ± 28.9 months *vs.* 34.9 ± 32.2 months, P = 0.030, Fig. 1B).

3.2. The effect of Siglec-15 on the immune infiltration of LSCC

The ESTIMATE method was used to obtain the immunological, stromal and ESTIMATE scores from the TGCA database. The Pearson correlation coefficient results displayed that the Siglec-15 expression was completely connected with immune score (R = 0.38, P = 4.95E-05), stromal score (R = 0.47, P = 2.58E-07) and ESTIMATE score (R = 0.38, P = 4.95E-07) (Fig. 2A), suggesting that the greater level of Siglec-15 was linked to the higher infiltration degree of stromal and immune cells in LSCC. To examine the connection among Siglec-15 and sixteen different kinds of immune cells, the Pearson correlation test was used. The expression of Siglec-15 indicated a

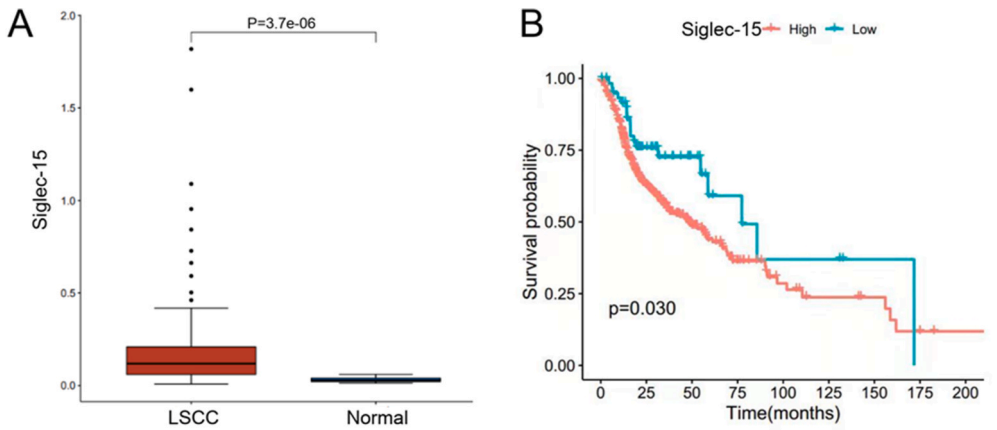

Fig. 1. The expression level of Siglec-15 is higher in LSCC. (A) Siglec-15 mRNA levels in LSCC tissues and normal larynx tissues in TCGA ($P < 0.001$, Wilcoxon test). (B) Kaplan-Meier curves of OS in patients with LSCC grouped by the Siglec-15 mRNA expression. LSCC, Laryngeal Squamous Cell Carcinoma; TCGA, The Cancer Genome Atlas Program; OS, overall survival.

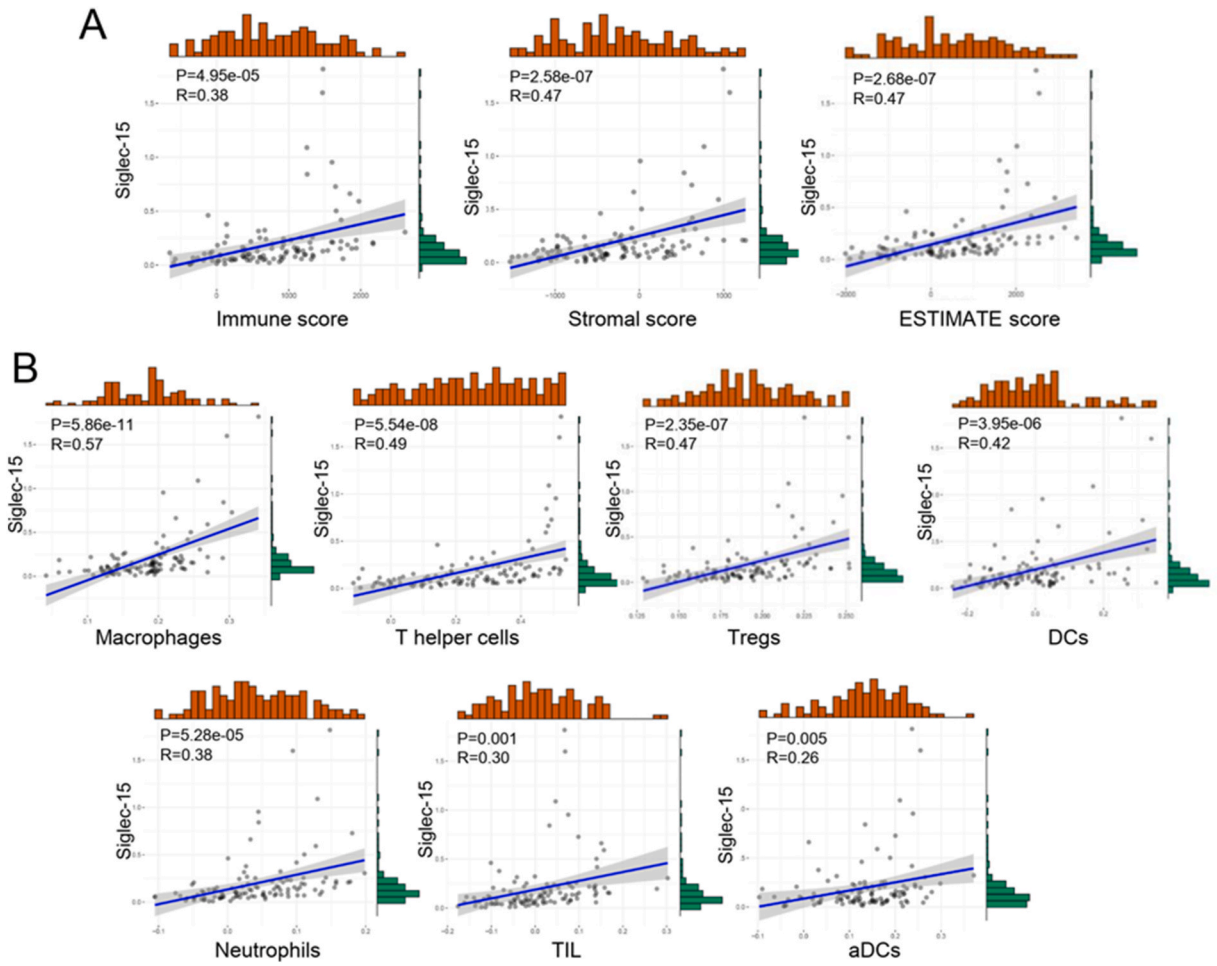

Fig. 2. Correction of Siglec-15 with immune infiltration in LSCC. (A) Pearson correlation analyses demonstrating the positive association between Siglec-15 and immune, stromal and estimate scores based on TCGA database data. (B) Scatter plot illustrating the correlation between Siglec-15 and seven distinct immune cells. TCGA, The Cancer Genome Atlas Program.

positive association with 7 immune cell types, containing macrophages, Dendritic cells (DCs), T helper cells, Regulatory T cells (Tregs), Neutrophils, Tumor-infiltrating lymphocytes (TIL) and active DCs (aDCs) (Fig. 2B). Taken together, high expression of Siglec-15 altered the TME by promoting the immune infiltration of LSCC.

3.3. Correlation analysis of Siglec-15 expression and ICGs

We next investigated the relation between Siglec-15 expression and ICGs using Pearson correlation test. Based on the TCGA database, 31 ICGs associated with Siglec-15 expression were identified in LSCC (Table 1). Positive correlations are observed between Siglec-15 expression level and B7-1 (CD80) in the TCGA, GSE27020, and GSE25727 datasets, as well as cytotoxic T-lymphocyte-associated antigen 4 (CTLA4) in the TCGA and GSE27020 datasets (Fig. 3). Suggesting it played an immunosuppressive role in laryngeal cancer through immunomodulation.

Table 1
31 ICGs associated with Siglec-15 expression.

Immune checkpoint genes
HAVCR2 CD80 CD86 TNFSF14 TNFSF4 LGALS9 TNFRSF9 CD28 CD226 HLA-DMB ICOS TIGIT CD274 HLA-DOA CTLA4 HLA-DQA1 HLA-DPB1 HLA-DPA1 HLA-DRA PDCD1LG2 HLA-DRB1 SIRPA CD70 HLA-DMA TNFRSF14 BTN2A2 ADORA2A TNFRSF4 BTNL9 BTN2A1 CD209

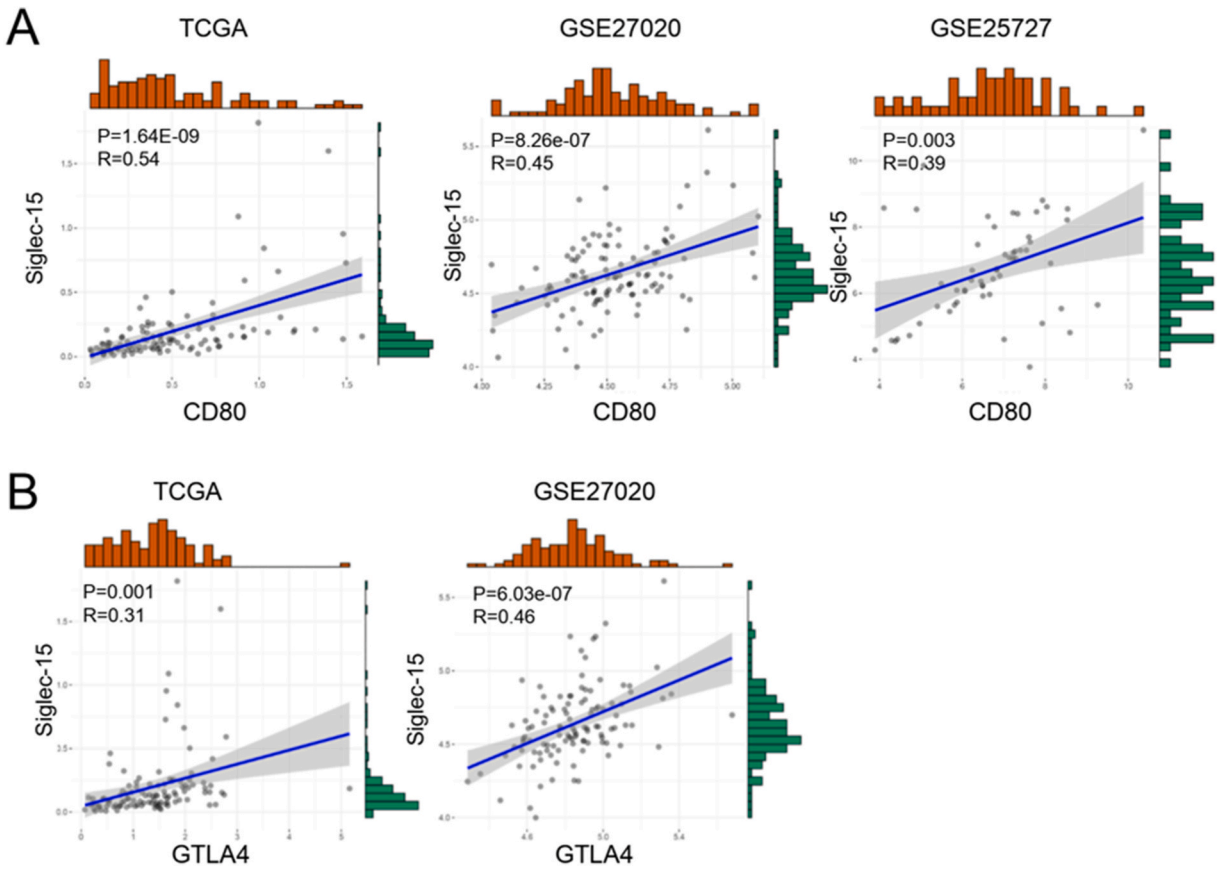

Fig. 3. Association of Siglec-15 with CD80 and CTLA4 in LSCC. (A, B) Pearson correlation analysis showing the positive association between Siglec-15 expression and CD80 (A) and CTLA4 expression (B) based on indicated databases.

3.4. Functional enrichment analysis of Siglec-15 in LSCC

We found 101 DEGs by comparing the gene expression patterns of the Siglec-15 low expression group with the high expression group. This will help us learn more about Siglec-15's roles in LSCC. Annotations based on GO functions showed that upregulated DEGs were primarily involved in improving the following areas: Biological Processes (lymphocyte activation regulation), Cellular Component (MHC class II protein complex), Immune receptor activity, Molecular Function (peptide antigen binding) and Cellular Component (clathrin-coated endocytic vesicle membranes) (Fig. 4A). In addition, DEGs were very abundant in cell adhesion molecules, Th1 and Th2 cell differentiation, and Th17 cell differentiation, according to the KEGG pathway enrichment analysis shown in Fig. 4B. These outcomes suggested that the DEGs were closely associated with immune-related functions and tumor-associated pathways. Therefore, we next compared the appearance levels of 122 immunomodulatory among Siglec-15 low expression and high expression groups. Heat map showed 16 immunomodulatory genes were significantly different indicated in Fig. 5A.

Using the ssGSEA algorithm, we obtained the anti-cancer immune process matrix score and immunotherapy-related pathway enrichment value for individual patients in the TCGA databank. The Pearson correlation investigation exhibited that the Siglec-15 expression was connected with multiple anti-cancer immune process and immunotherapy-related pathway (Fig. 5B). The immunotherapy-related pathways included the FGFR3 co-expressed gene pathway ($P = 0.018$, $R = -0.22$), the spliceosome pathway ($P = 0.031$, $R = -0.20$) and the hypoxia pathway ($P = 0.0044$, $R = 0.27$). The anti-cancer immune process includes immune initiation and activation ($P = 2.12E\text{-}06$, $R = -0.43$), CD4 + T cell recruitment ($P = 0.020$, $R = 0.22$), macrophage recruitment ($P = 0.010$, $R = 0.24$) and Treg cell recruitment ($P = 0.0029$, $R = 0.28$).

3.5. The effect of Siglec-15 on immunotherapy in LSCC

The functional enrichment analysis showed that Siglec-15 is connected to a number of immunotherapy-related pathways. We then used the Wilcoxon test to look at the differences in the immunotherapy-related pathway enrichment score among low and high appearance groups of Siglec-15. This showed how Siglec-15 affects immunotherapy. Fig. 6A shows that the low Siglec-15 subgroup was considerably more enriched in the EGFR ligand route, the FGFR3 co-expressed gene pathway and the pyrimidine metabolism pathway, while the high Siglec-15 subgroup was more enriched in the interferon (IFN) pathway.

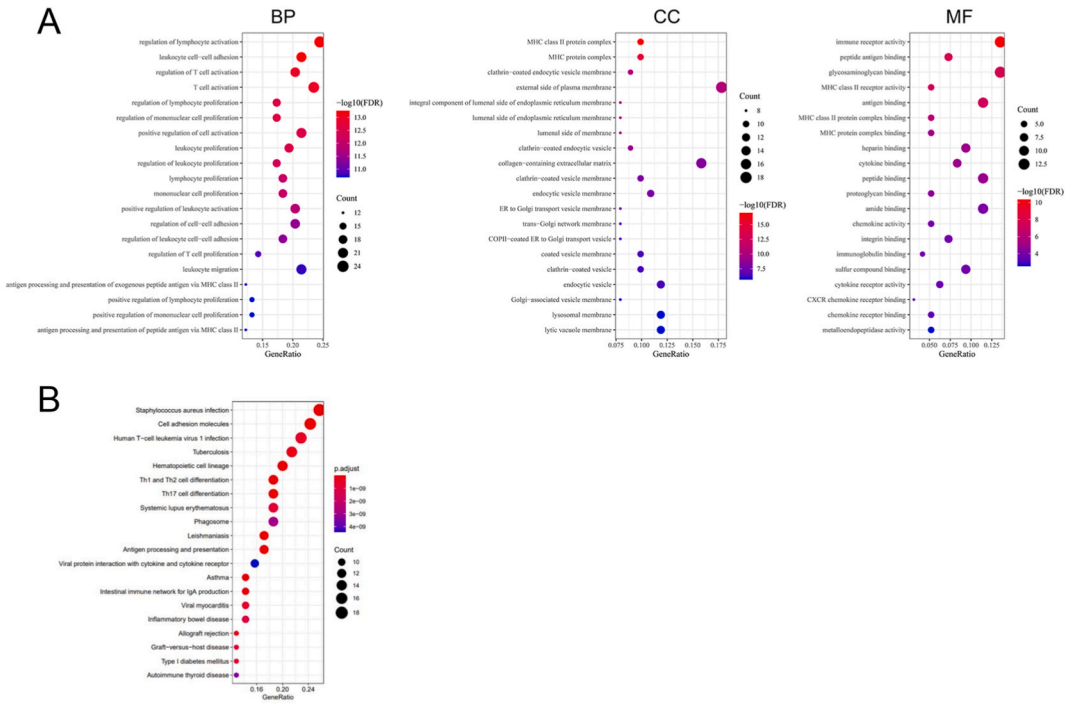

Fig. 4. GO and KEGG pathways enrichment analysis. (A, B) Bubble plot demonstrating GO (A) and KEGG pathways (B) analysis of DEGs between Sigles-15 high expression group and Sigles-15 low expression group. GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; DEGs, differentially expressed genes; BP, biological process; CC, cellular component; MF, molecular function.

Comparing the Siglec-15 expression levels in the clinical reactions of LSCC patients getting immunotherapy was needed to learn more about how Siglec-15 expression levels affect the clinical benefit of immunotherapy for patients. Because of a lack of immunotherapy group data, the fact that the gene profiles of immunotherapy for head and neck squamous cancer are very similar [22]. We obtained gene expression profiles of patients with uroepithelial carcinoma treated with anti-PD-L1 immunotherapy and compared the appearance levels of Siglec-15 in dissimilar clinical responses to immunotherapy, containing Stable Disease, partial response, progressive disease and complete response. We observed that Siglec-15 expression was considerably greater in PD and SD than in CR and Siglec-15 expression was utmost in the PD group and lowermost in the CR group (Fig. 6B). Collectively, our outcomes show that increased Siglec-15 expression is associated with a lower immune response (SD or even PD).

3.6. The expression of Siglec-15 in LSCC

The data for this study came from 111 patients who were diagnosed with LSCC between 2012 and 2016 from Sun Yat-sen University Memorial Hospital. The data include 106 males and 5 females, with age distribution ranging from 29 to 80 years old and a median age of 61 years. As stated in the AJCC (American Joint Committee on Cancer) TNM scheme, 31 cases were tumor (T) 1, 30 cases were T2, 36 cases were T3 and 14 cases were T4; 89 cases were node (N) 0, 2 cases were N1, 19 cases were N2, and 1 case was N3. No metastasis patient was found. There were 31 cases in clinical phase I, 25 cases in clinical phase II, 28 cases in clinical phase III and 27 cases in clinical phase IV. According to the degree of pathological differentiation, these patients with LSCC were divided into three groups. The groups including well-differentiated squamous carcinoma (53 cases), moderately differentiated squamous carcinoma (54 cases) and poor-differentiated squamous carcinoma (4 cases). The median duration of follow-up was 68 months, and all patients had full clinical records. The OS rate at 5 years was 78.4 %. These cases had no other treatment such as radiotherapy or chemotherapy before surgery and only received surgery.

As shown in Fig. 7A, the qRT-PCR findings demonstrated that the expression level of Siglec-15 mRNA in LSCC was much higher than in paratumor. After that, Immunohistochemistry was used to categories LSCC patients into two groups according to the expression of Siglec-15. The majority of Siglec-15 expression was seen in the cytoplasm of the cells, according to immunohistochemistry pictures shown in Fig. 7B. In a study of 111 LSCC patients, 61 (55.0 %) showed high Siglec-15 expression, while 50 (45.0 %) showed low expression indicated in Fig. 7B.

3.7. Association of Siglec-15 with patient prognosis in LSCC

We then analyzed the correlation among clinical characteristics and Siglec-15 expression levels in patients with LSCC through Chi-Squared test. As indicates in Table 2, there was no significant association among high and low Siglec-15 expression and age, sex,

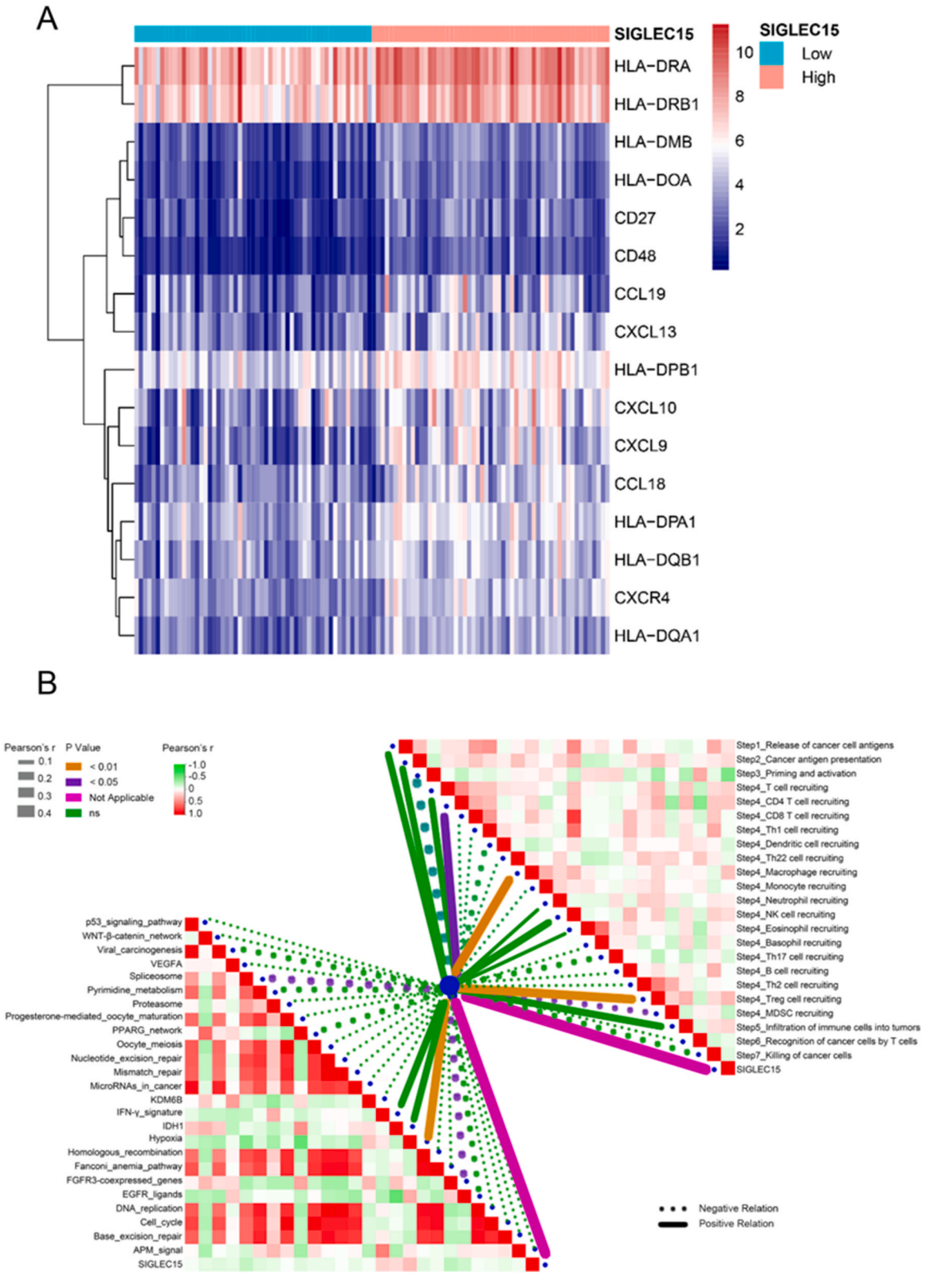

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Fig. 5. Correction of Siglec-15 with immunomodulatory genes in LSCC. (A) Heatmap of 16 immunomodulatory genes that was significantly different between Siglec-15 high expression group and Siglec-15 low expression group. (B) The Pearson correlation analysis showing the correlation of Siglec-15 expression with multiple anti-cancer immune process and immunotherapy-related pathways based on GSVA score for individual patients in the TCGA database. GSVA, gene set variation analysis; TCGA, The Cancer Genome Atlas Program.

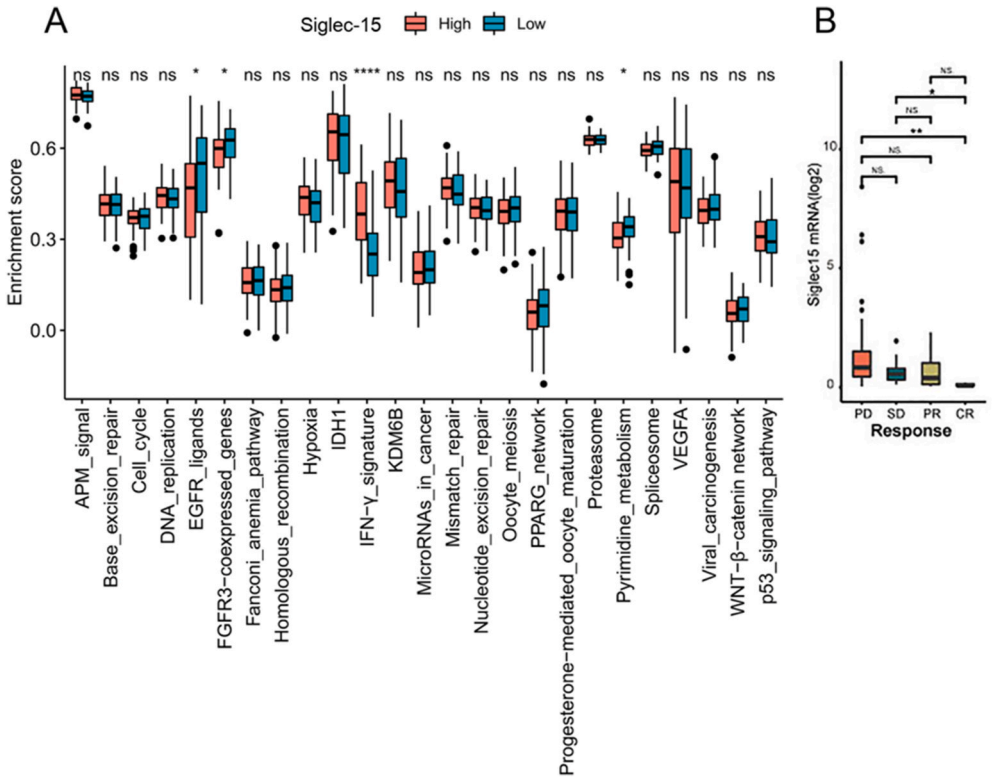

Fig. 6. The effect of Siglec-15 on immunotherapy. (A) Wilcoxon test showing the difference of immunotherapy-related pathway enrichment score between Siglec-15 low expression group and high expression group. (B) mRNA expression levels of Siglec-15 in patients with different clinical responses to immunotherapy for uroepithelial carcinoma. PD: progressive disease; SD: stable disease; PR: partial response; CR: complete response.

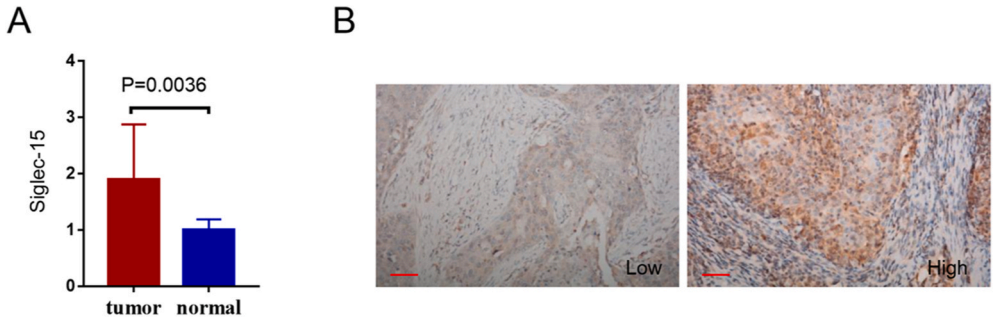

Fig. 7. The expression of Siglec-15 is increased in LSCC. (A, B) qRT-PCR (B) and Immunochemistry (A) analyses of Siglec-15 expression in our LSCC cohort. LSCC, Laryngeal Squamous Cell Carcinoma.

clinical stage, pathological rank, N stage and T stage ($P > 0.05$), which recommended that the Siglec-15 expression did not correlate with the clinical features of patients with LSCC.

Based on immunohistochemistry data, 111 cases of LSCC were split into two groups: the high expression group (61 cases, or 55 %) and the low expression group (50 cases, or 45 %). The OS rate was much lower in low appearance group of Siglec-15 compared to the Siglec-15 high expression group, according to the Kaplan-Meier analysis (66.0 ± 7.8 months *vs.* 90.7 ± 5.9 months, $P = 0.011$, Fig. 8A) and suggesting a poor OS rate for LSCC patients with Siglec-15 high expression.

Table 2
Clinical characteristics of patients with LSCC.

Variables	Cases	High expression (61 case)	Low expression (50 case)	Statistics	P
Age				0.087	0.849†
≤60	56 (50.5)	30 (27.0)	26 (23.5)		
> 60	55 (49.5)	31 (27.9)	24 (21.6)		
Sex				0.479	0.489*
Male	106 (95.5)	57 (51.4)	49 (44. 1)		
Female	5 (4.5)	4 (3.6)	1 (0.9)		
Clinical stage				2.599	0. 129†
I+II	56 (50.5)	35 (31.6)	21 (18.9)		
III+IV	55 (49.5)	26 (23.4)	29 (26. 1)		
Pathological grade				0. 195	1.000[6]
Well	53 (47.7)	29 (26. 1)	24 (21.6)		
Moderately	54 (48.6)	30 (27.0)	24 (21.6)		
Poor	4 (3.6)	2 (1.8)	2 (1.8)		
T stage				1.870	0. 186†
T1+T2	59 (53.2)	36 (32.5)	23 (20.7)		
T3+T4	52 (46.8)	25 (22.5)	27 (24.3)		
N stage				0.002	1.000†
0	89 (80.2)	49 (44.2)	40 (36.0)		
N1+N2+N3	22 (19.8)	12 (10.8)	10 (9.0)		

A Cox regression model was used to examine Siglec-15's prognostic value for LSCC patients' prognoses. The univariate Cox regression result showing in Table 3 suggested that the factors affecting the survival prognosis included clinical phase ($P = 0.011$), T part ($P = 0.020$), N part ($P = 0.003$) and Siglec-15 expression ($P = 0.0115$, and there was no statistical significance in age, gender and pathological grading (all $P > 0.05$).

Afterwards, we used multivariate Cox regression analysis to find the single factor that had the most significant impact on the clinical outcome of LSCC cases. Considering the strong connection among clinical phase and T-stage and N-stage, clinical phase was excluded for multivariate Cox regression analysis. As shows in Table 4, N-stage ($P = 0.036$) and Siglec-15 ($P = 0.002$) appearance were independent factors affecting prognosis of LSCC patients.

Higher $CD8^+$ T cell infiltration is associated with improved immunotherapy results and prognosis in patients [23]. We studied the protein appearance of Siglec-15 and CD8 in 80 LSCC case (40 early-stage patients and 40 late-stage patients) by immunofluorescence. Immunofluorescence images and quantification of fluorescence intensity presented an important negative connection among Siglec-15 and CD8 protein levels ($P < 0.0001$, $R = 0.2807$, Fig. 8B and C).

3.8. Functional characterization of Siglec-15 in LCC cells

To investigate the roles of Siglec-15 in LSCC, viral-based shRNAs of shSiglec-15 and lentiviral vector of pLVX-SIGLEC15 were transfected to LCC in vitro, respectively. qRT-PCR results showed that shSiglec-15 transfected LCCs had substantially reduced Siglec-15 mRNA expression, while it was significantly increased in LCC transfected pLVX-SIGLEC15 (Fig. 9A and B). Knockout of Siglec-15 significantly inhibited the proliferation of LCC, while overexpression of Siglec-15 promoted the proliferation (Fig. 9C and D). Using transwell invasion assay, we observed that silence of Siglec-15 reduced the migration ability of LCC, while overexpression of Siglec-15 enhanced cell migration (Fig. 9E and F). Overall, Siglec-15 downregulation reduced LCC cell invasion and proliferation in *vitro*.

4. Discussion

20 % of head and neck cancers are laryngeal cancers and most of them are LSCCs. The incidence of laryngeal cancer has been increasing at a rate of about 1/10,000 per year in recent years. However, the progression mechanisms of laryngeal cancer are still poorly understood [24]. Despite the rapid advances in tumor diagnostic techniques and treatment strategies, the prognosis for laryngeal cancer has not significantly improved [25]. Nivolumab and pembrolizumab are examples of ICIs that have transformed the way advanced laryngeal cancer is treated [8,9]. They are only effective in a minority of laryngeal cancer and are limited in patients with frequent or metastatic laryngeal cancer. Consequently, it is critical to detect the predictive biomarkers for checkpoint inhibitor-based immunotherapy for LSCC.

Compared to solid tumors, LSCC has a high concentration of immune cells, and the patterns of this infiltration can influence laryngeal cancer patients' disease prognoses and the therapeutic effectiveness of immunotherapy [26–28]. However, studies on the effect of immune cell infiltration on immunotherapy and prognosis of laryngeal cancer are still limited. Based on our study, Siglec-15 may be a useful biomarker for predicting the outcome of LSCC. We used the TCGA database to find that Siglec-15 was highly expressed in LSCC. The qRT-PCR result showed the evidence of the upregulating Siglec-15 in LSCC. Tumor and mesenchymal cell membranes and cytoplasm were the primary sites of its expression. Afterwards, we used immunohistochemistry to categorize the LSCC data into two sets, one for each level of Siglec-15 expression. Kaplan-Meier investigation exposed that patients in Siglec-15 great expression group had a poor OS. Based on the COX regression, Siglec-15 was identified as an autonomous factor that associated with the prognosis of LSCC.

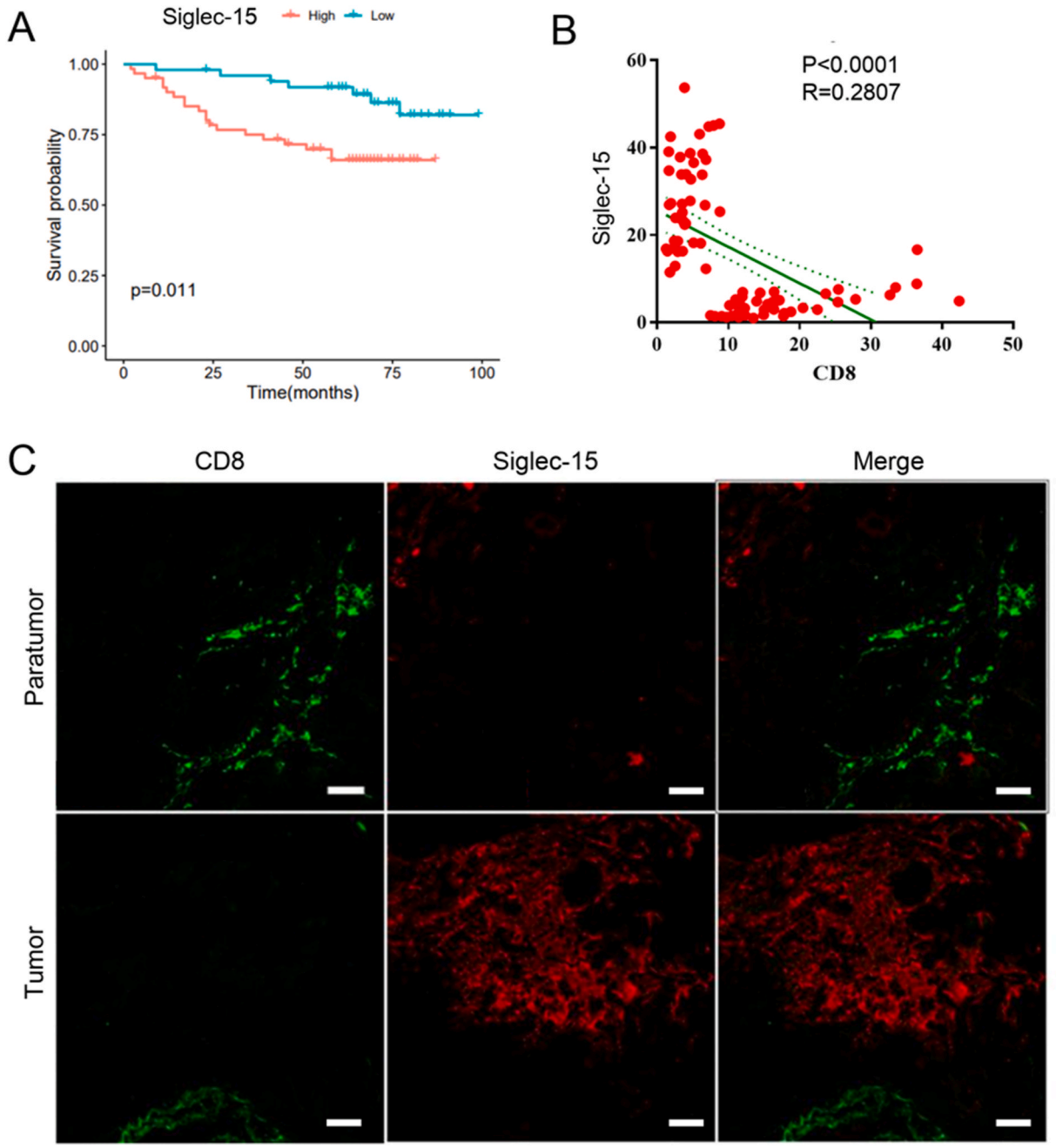

Fig. 8. Association of Siglec-15 with survival probability and CD8 in LSCC. (A) Kaplan-Meier analysis showing the survival probability of patients in Siglec-15 high expression and Siglec-15 low expression. (B, C) Quantification of fluorescence intensity and immunofluorescence images exhibiting a significant negative correlation between Siglec-15 and CD8 protein levels. Scale: 20 μm.

LSCC is classified to those tumors with strong immunogenicity [29]. Herein, we observed a favorable connection among the Siglec-15 expression level and the stromal score, immunological score and ESTIMATE score of LSCC. These findings demonstrated that Siglec-15 promoted of stromal and immune cell infiltration. TME consists of immune and non-immune stromal components, which are closely associated with tumor growth [30]. Next, we identified that Siglec-15 was positive related to 7 immune cell types, including macrophages, T helper cells, Tregs, Neutrophils, DCs, TIL and aDCs. It is reported that Siglec-15 is mostly stated in macrophages and DCs [31,32]. Normally, Siglec-15 is weakly expressed in macrophage. However, M-CSF (Macrophage Colony-Stimulating Factor) induces the appearance of Siglec-15 in tumor-associated macrophages in response to tumor or inflammatory factor stimulation, remodeling the tumor microenvironment and promoting tumor metastasis and poor prognosis [33,34]. Macrophages are an important component of TME immune cells that promote tumorigenesis, progression, metastasis and have acquired a lot of interest as potential

Table 3
Univariate Cox results of independent variables to LSCC.

Variables	HR (95 % CI)	P
Age	0.938 (0.441–1.995)	0.867
Sex	0.819 (0. 111–6.041)	0.844
Clinical stage	2.911 (1.273–6.657)	0.011
Pathological grade	0.789 (0.396–1.572)	0.500
T stage	2.580 (1.159–5.748)	0.020
Siglec-15 expression	2.927 (1.234–6.943)	0.015
N stage	3.353 (1.527–7.366)	0.003

Table 4
Multivariate Cox results of independent variables to LSCC.

Variables	HR (95 % CI)	P
Siglec-15 expression	3.920 (1.622–9.474)	0.002
T stage	2.284 (0.897–5.811)	0.083
N stage	2.661 (1.065–6.648)	0.036

therapeutic targets [35]. Tregs, which are generated by T helper cell activation and recruited by TME, suppress immune responses, perpetuating an immunosuppressive microenvironment and promoting tumorigenesis and progression [36,37]. Neutrophils in the TME might facilitate cancer progression through secretion of proteases [38]. IC (Immune checkpoint) pathways are very important for keeping the immune system in balance and stopping disease. Nevertheless, the tumor microenvironment (TME) of many cancers often displays an increase in ICs and their ligands. Accordingly, effective anti-tumor responses might be initiated by blocking the interaction between ICs and their ligands [39,40]. It has been shown that some tumors may be effectively treated with one of many newly discovered immune checkpoint inhibitors (ICIs) [40]. One negative regulator of T-cell immune activity is the CTLA4 immune checkpoint. Upregulated CTLA4 competes with CD28 for binding of B7 molecules such as CD80, limiting the T cell responses and survival [41,42]. Our data suggested that the appearance of Siglec-15 had a positive correction with the expression of CTLA4 and CD80 in LSCC, indicating that Siglec-15 could suppress the immune system through upregulating the expression of CTLA4 and CD80. In order to delve further into the functions of Siglec-15 in LSCC, we analyzed the gene expression patterns of LSCCs with high and low Siglec-15 expression. Improved control of lymphocyte activation, leukocyte cell, and cell adhesion was seen in the DEGs. The cellular component of T cells, the MHC class II protein complex, the MHC protein complex and the Biological Processes section on T cell instruction. Furthermore, we also identified 16 immunomodulatory genes were significantly different between Siglec-15 low expressed LSCC and Siglec-15 high expressed LSCC. The MHC proteins HLA-DRA, HLA-DRB1, HLA-DPB1, HLA-DMB, HLA-DOA, HLA-DPA1, HLA-DQB1 and DQA1 are involved in antigen recognition during the immune response and the chemokines (CCL19, CXCL13, CXCL10, CXCL9 and CCL18) and chemokine receptors (CXCR4) can promote the recruitment of myeloid-derived suppressor cells, leading to immune tolerance or immune escape. These results indicated Siglec-15 promotes immune cell infiltration to exert immunosuppressive effects, which might be associated with various chemokines and chemokine receptors in LSCC.

We employed the Wilcoxon test to analyze the differences of immunotherapy-related pathway, enrichment score among Siglec-15 low expression and high appearance groups and observed that the immunotherapy-related pathway including EGFR ligand pathway, FGFR3 co-expressed gene pathway and pyrimidine metabolism pathway were considerably enhanced in the low Siglec-15 subgroup, while IFN pathway was considerably enhanced in the high Siglec-15 subgroup. The different mechanisms underlying primary and acquired immunotherapy resistance are associated with IFN signaling [43].

This study systematically explored the predictive part of Siglec-15 in LSCC prognosis and its implications in immunotherapy for the first time. It confirmed that the appearance level of Siglec-15 is an independent factor influencing LSCC prognosis. Focusing on the emerging theme of immunotherapy, it provided novel insights into identifying sensitive populations for LSCC immunotherapy and selecting immunotherapeutic targets, which display promising applications. We employed various research methods including Bio-informatic analyses, cellular experiments and clinical samples to verify the results from multiple angles. Besides, the prospective mechanism that Siglec-15 affects the prognosis of LSCC and the response to immunotherapy by regulating immune infiltration and immune checkpoint gene expression was in-depth discovered.

In spite of the significant outcomes of our research, they exist some limitations. First, the reliance on a single laryngeal cancer cell line for in vitro experiments might present a limited representation of the heterogeneity in LSCC, although this cell line is widely used and well characterized. Second, clinical samples and corresponding patient data in our research derived from a single medical center, potentially introducing a bias in demographic characteristics. This limitation might constrain the generalizability of our findings to a broader population of LSCC patients. Despite collaborative validation with transcriptome data from the TCGA database, future studies are needed to validate these findings across diverse patient populations from multiple centers to ensure broader applicability. In general, our study lays a foundation for understanding the predictive value and potential immunotherapeutic implications of Siglec-15 in LSCC. Addressing these limitations through collaborative efforts and larger cohort studies could enhance the translational impact of our findings.

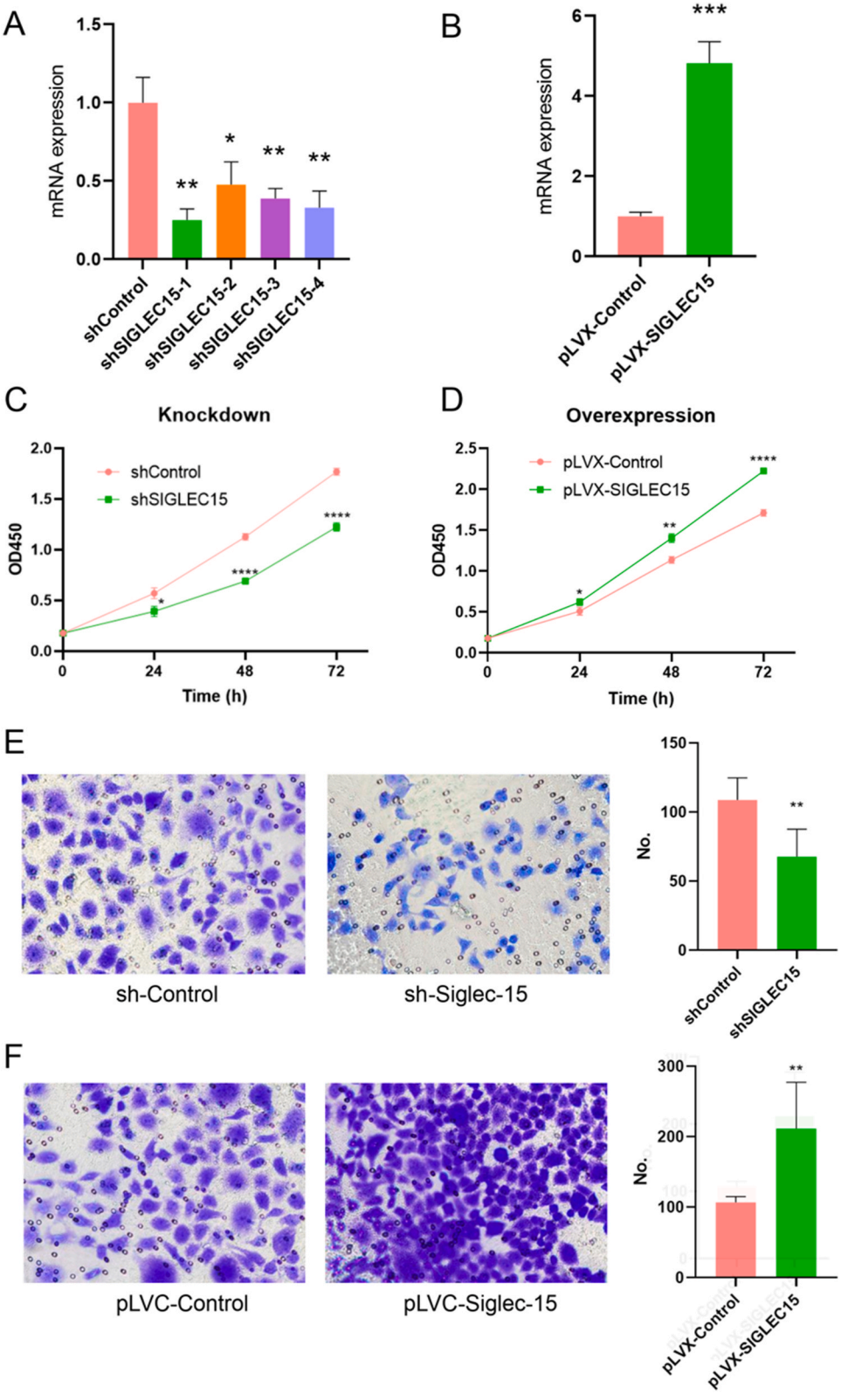

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Fig. 9. The effects of Siglec-15 on cell proliferation and invasion in vitro. (A, B) qRT-PCR showing the mRNA expression of Sigles-15 in LCC transfected with shSiglec-15 (A) and pLVC-Siglec-15 (B) respectively. (C, D) Cell proliferation was assessed by CCK8 assay. (E, F) Cell invasion was examined using transwell cell invasion assay. *P < 0.05, **P < 0.01, ***P < 0.001 between control and indicated group. LCC, laryngeal cancer cell line.

5. Conclusions

The findings demonstrated that LSCC tissues expressed Siglec-15 at a higher level than paratumor tissues, and that a high level of Siglec-15 appearance was significantly connected with a worse diagnosis for LSCC patients. According to a Bioinformatics research, immunomodulation of the tumor microenvironment (TME) in LSCC may contribute to poor immunotherapy response when Siglec-15 expression is high. Our study provides perceptions into the part of Siglec-15 in LSCC. The results recognize Siglec-15 as a robust immune-suppressive factor in LSCC and highlight its potential as an immunotherapeutic target for LSCC.

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Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Xiaoting Chen: Writing – review & editing, Writing – original draft, Visualization, Validation, Investigation, Formal analysis, Data curation. **Qian Cai:** Writing – review & editing, Writing – original draft, Visualization, Validation, Investigation, Formal analysis, Data curation. **Kaiyi Wong:** Resources, Methodology, Data curation. **Ximing Shen:** Software, Formal analysis. **Zhong Guan:** Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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List of abbreviations

HPV	Human papillomavirus
EBV	Epstein-Barr virus
PD-L1	programmed cell death ligand 1
TME	tumor microenvironment
OS	overall survival
ICGs	immune checkpoint genes
KEGG	Kyoto Encyclopedia of Genes and Genomes
qRT-PCR	quantitative real-time PCR
CCK-8	Cell counting kit-8
HR	hazard ratio
Tregs	Regulatory T cells
DCs	Dendritic cells
TIL	Tumor-infiltrating lymphocytes
aDCs	active DCs
CTLA4	cytotoxic T-lymphocyte–associated antigen 4
DEGs	differential expression genes
PD	progressive disease
SD	stable disease
PR	partial response

CR	complete response
AJCC	American Joint Committee on Cancer
M-CSF	macrophage colony-stimulating factor
IC	Immune checkpoint
ICIs	immune checkpoint inhibitors

References

[1] A.A.M. Koroulakis, Laryngeal Cancer. StatPearls [Internet]. Treasure Island (FL), StatPearls Publishing, 2022.
[2] R.L. Siegel, K.D. Miller, H.E. Fuchs, A. Jemal, Cancer statistics, 2021, CA. Cancer J. Clin. 71 (2021) 7–33.
[3] R. Nocini, G. Molteni, C. Mattiuzzi, G. Lippi, Updates on larynx cancer epidemiology, Chin. J. Cancer Res. 32 (2020) 18–25.
[4] J. Han, B.D. Sumer, The changing demographics and treatment of larynx cancer, Ann. Surg Oncol. 28 (2021) 6927–6928.
[5] M. Tagliabue, F. Maffini, C. Fumagalli, S. Gandini, D. Lepanto, F. Corso, S. Cacciola, A. Ranghiero, A. Rappa, D. Vacirca, M. Cossu Rocca, D. Alterio, E. Guerini Rocco, A. Cattaneo, F. Chu, S. Zorzi, G. Curigliano, S. Chiocca, M. Barberis, G. Viale, M. Ansarin, A role for the immune system in advanced laryngeal cancer, Sci. Rep. 10 (2020) 18327.
[6] B. Burtness, K.J. Harrington, R. Greil, D. Soulières, M. Tahara, G. de Castro, A. Psyrri, N. Basté, P. Neupane, Bratland Å, T. Fuereder, B.G.M. Hughes, R. Mesía, N. Ngamphaiboon, T. Rordorf, W.Z. Wan Ishak, R.L. Hong, R. González Mendoza, A. Roy, Y. Zhang, B. Gumuscu, J.D. Cheng, F. Jin, D. Rischin, G. Lerzo, M. Tatangelo, M. Varela, J.J. Zarba, M. Boyer, H. Gan, B. Gao, B. Hughes, G. Mallesara, A. Taylor, M. Burian, C.H. Barrios, D.O. de Castro Junior, G. Castro, F. A. Franke, G. Girotto, I.P.F. Lima, U.R. Nicolau, G.D.J. Pinto, L. Santos, A.P. Victorino, N. Chua, F. Couture, R. Gregg, A. Hansen, J. Hilton, J. McCarthy, D. Soulieres, R. Ascui, P. Gonzalez, L. Villanueva, M. Torregroza, A. Zambrano, P. Holeckova, Z. Kral, B. Melichar, J. Prausova, M. Vosmik, M. Andersen, N. Gyldenkerne, H. Jurgens, K. Putnik, P. Reinikainen, V. Gruenwald, S. Laban, G. Aravantinos, I. Boukovinas, V. Georgoulias, D. Kwong, Y. Al-Farhat, T. Csoszi, J. Erfan, G. Horvai, L. Landherr, E. Remenar, A. Ruzsa, J. Szota, S. Billan, I. Gluck, O. Gutfeld, A. Popovtzer, M. Benasso, S. Bui, V. Ferrari, L. Licitra, F. Nole, T. Fujii, Y. Fujimoto, N. Hanai, H. Hara, K. Matsumoto, K. Mitsugi, N. Monden, M. Nakayama, K. Okami, N. Oridate, K. Shiga, Y. Shimizu, M. Sugasawa, M. Takahashi, S. Takahashi, K. Tanaka, T. Ueda, H. Yamaguchi, T. Yamazaki, R. Yasumatsu, T. Yokota, T. Yoshizaki, I. Kudaba, Z. Stara, S.K. Cheah, J. Aguilar Ponce, R. Gonzalez Mendoza, C. Hernandez Hernandez, F. Medina Soto, J. Buter, A. Hoeben, S. Oosting, K. Suijkerbuijk, A. Bratland, M. Brydoey, R. Alvarez, L. Mas, P. Caguioa, J. Querol, E.E. Regala, M.B. Tamayo, E.M. Villegas, A. Kawecki, A. Karpenko, A. Klochikhin, A. Smolin, O. Zarubenkov, B.C. Goh, G. Cohen, J. du Toit, C. Jordaan, G. Landers, P. Ruff, W. Szpak, N. Tabane, I. Brana, L. Iglesias Docampo, J. Lavernia, R. Mesia, E. Abel, V. Muratidu, N. Nielsen, V. Cristina, S. Rothschild, H.M. Wang, M.H. Yang, S.P. Yeh, C.J. Yen, N. Soparattanapaisarn, V. Sriuranpong, S. Aksoy, I. Cicin, M. Ekenel, H. Harputluoglu, O. Ozyilkan, S. Agarwala, H. Ali, R. Alter, D. Anderson, J. Bruce, N. Campbell, M. Conde, J. Deeken, W. Edenfield, L. Feldman, E. Gaughan, B. Goueli, B. Halmos, U. Hegde, B. Hunis, R. Jotte, A. Karnad, S. Khan, N. Laudi, D. Laux, D. Martincic, S. McCune, D. McGaughey, K. Misiukiewicz, D. Mulford, E. Nadler, J. Nunnink, J. Ohr, M. O'Malley, B. Patson, D. Paul, E. Popa, S. Powell, R. Redman, V. Rella, C. Rocha Lima, A. Sivapiragasam, Y. Su, A. Sukari, S. Wong, E. Yilmaz, J. Yorio, Pembrolizumab alone or with chemotherapy versus cetuximab with chemotherapy for recurrent or metastatic squamous cell carcinoma of the head and neck (KEYNOTE-048): a randomised, open-label, phase 3 study, Lancet 394 (2019) 1915–1928.
[7] A. Botticelli, A. Cirillo, L. Strigari, F. Valentini, B. Cerbelli, S. Scagnoli, E. Cerbelli, I.G. Zizzari, C Della Rocca, G. D'Amati, A. Polimeni, M. Nuti, M.C. Merlano, S. Mezi, P. Marchetti, Anti–PD-1 and anti–PD-L1 in head and neck cancer: a network meta-analysis, Front. Immunol. 12 (2021) 705096.
[8] R. Pei, Y. Shi, S. Lv, T. Dai, F. Zhang, S. Liu, B. Wu, Nivolumab vs pembrolizumab for treatment of us patients with platinum-refractory recurrent or metastatic head and neck squamous cell carcinoma: a network meta-analysis and cost-effectiveness analysis, JAMA Netw. Open 4 (2021) e218065.
[9] P. Cui, R. Li, Z. Huang, Z. Wu, H. Tao, S. Zhang, Y. Hu, Comparative effectiveness of pembrolizumab vs. nivolumab in patients with recurrent or advanced NSCLC, Sci. Rep. 10 (2020) 13160.
[10] M. de Miguel, E. Calvo, Clinical challenges of immune checkpoint inhibitors, Cancer Cell 38 (2020) 326–333.
[11] H. Läubli, A. Varki, Sialic acid–binding immunoglobulin-like lectins (Siglecs) detect self-associated molecular patterns to regulate immune responses, Cell. Mol. Life Sci. 77 (2020) 593–605.
[12] G.D. Brown, P.R. Crocker, Lectin receptors expressed on myeloid cells, Microbiol. Spectr. 4 (2016).
[13] G. Murugesan, V.G. Correia, A.S. Palma, W. Chai, C. Li, T. Feizi, E. Martin, B. Laux, A. Franz, K. Fuchs, B. Weigle, P.R. Crocker, Siglec-15 recognition of sialoglycans on tumor cell lines can occur independently of sialyl Tn antigen expression, Glycobiology 31 (2021) 44–54.
[14] X. Chen, S. Mo, Y. Zhang, H. Ma, Z. Lu, S. Yu, J. Chen, Analysis of a novel immune checkpoint, Siglec-15, in pancreatic ductal adenocarcinoma, J. Pathol. Clin. Res. 8 (2022) 268–278.
[15] X. Ren, Immunosuppressive checkpoint Siglec-15: a vital new piece of the cancer immunotherapy jigsaw puzzle, Cancer Biol. Med. 16 (2019) 205–210.
[16] B. Li, B. Zhang, X. Wang, Z. Zeng, Z. Huang, L. Zhang, F. Wei, X. Ren, L. Yang, Expression signature, prognosis value, and immune characteristics of Siglec-15 identified by pan-cancer analysis, OncoImmunology 9 (2020) 1807291.
[17] M. Fan, G. Zhang, M. Xie, X. Liu, Q.W.L. Zhang, Siglec-15 as a new perspective therapy target in human giant cell tumor of bone, Curr. Oncol. 29 (2022) 7655–7671.
[18] M.W.L. Quirino, M.C. Pereira, M. de Fátima Deodato de Souza, I. da Rocha Pitta, A.F. da Silva Filho, M.S. de Souza Albuquerque, A.P. de Barros Albuquerque, M. R. Martins, M.G. da Rocha Pitta, M.J.B. de Melo Rêgo, Immunopositivity for Siglec-15 in gastric cancer and its association with clinical and pathological parameters, Eur. J. Histochem. 65 (2021) 57–63.
[19] J. Wang, J. Sun, L.N. Liu, D.B. Flies, X. Nie, M. Toki, J. Zhang, C. Song, M. Zarr, X. Zhou, X. Han, K.A. Archer, T. O'Neill, R.S. Herbst, A.N. Boto, M.F. Sanmamed, S. Langermann, D.L. Rimm, L. Chen, Siglec-15 as an immune suppressor and potential target for normalization cancer immunotherapy, Nat. Med. 25 (2019) 656–666.
[20] K. Yoshihara, M. Shahmoradgoli, E. Martínez, R. Vegesna, H. Kim, W. Torres-Garcia, V. Treviño, H. Shen, P.W. Laird, D.A. Levine, S.L. Carter, G. Getz, K. Stemke-Hale, G.B. Mills, R.G.W. Verhaak, Inferring tumour purity and stromal and immune cell admixture from expression data, Nat. Commun. 4 (2013) 2612.
[21] S.Y. Li, Z. Song, M.J. Song, J.W. Qin, M.L. Zhao, Z.M. Yang, Impaired receptivity and decidualization in DHEA-induced PCOS mice, Sci. Rep. 6 (2016) 38134.
[22] X. Zhang, M. Shi, T. Chen, B. Zhang, Characterization of the immune cell infiltration landscape in head and neck squamous cell carcinoma to aid immunotherapy, Mol. Ther. Nucleic Acids 22 (2020) 298–309.
[23] G.J. Hanna, P. Lizotte, M. Cavanaugh, F.C. Kuo, P. Shivdasani, A. Frieden, N.G. Chau, J.D. Schoenfeld, J.H. Lorch, R. Uppaluri, L.E. MacConaill, R.I. Haddad, Frameshift events predict anti-PD-1/L1 response in head and neck cancer, JCI insight 3 (2018) e98811.
[24] L. Wang, W. Ren, X. Zhou, W. Sheng, J. Wang, Pleomorphic liposarcoma: a clinicopathological, immunohistochemical and molecular cytogenetic study of 32 additional cases, Pathol. Int. 63 (2013) 523–531.
[25] E. Du, A.L. Mazul, D. Farquhar, P. Brennan, D. Anantharaman, B. Abedi-Ardekani, M.C. Weissler, D.N. Hayes, A.F. Olshan, J.P. Zevallos, Long-term survival in head and neck cancer: impact of site, stage, smoking, and human papillomavirus status, Laryngoscope 129 (2019) 2506–2513.
[26] M.J.M. Gooden, G.H. De Bock, N. Leffers, T. Daemen, H.W. Nijman, The prognostic influence of tumour-infiltrating lymphocytes in cancer: a systematic review with meta-analysis, Br. J. Cancer 105 (2011) 93–103.
[27] F. Caponigro, F. Ionna, G.D.V. Scarpati, F. Longo, R. Addeo, R. Manzo, P. Muto, S. Pisconti, L. Leopaldi, F. Perri, Translational research: a future strategy for managing squamous cell carcinoma of the head and neck? Anti Cancer Agents Med. Chem. 18 (2018) 1220–1227.

[28] F. Perri, F. Ionna, F. Longo, G. Della Vittoria Scarpati, C. De Angelis, A. Ottaiano, G. Botti, F. Caponigro, Immune response against head and neck cancer: biological mechanisms and implication on therapy, Transl. Oncol. 13 (2020) 262–274.
[29] D. Bruni, H.K. Angell, J. Galon, The immune contexture and Immunoscore in cancer prognosis and therapeutic efficacy, Nat. Rev. Cancer 20 (2020) 662–680.
[30] A. Labani-Motlagh, M. Ashja-Mahdavi, A. Loskog, The tumor microenvironment: a milieu hindering and obstructing antitumor immune responses, Front. Immunol. 11 (2020) 940.
[31] T. Angata, Y. Tabuchi, K. Nakamura, M. Nakamura, Siglec-15: an immune system Siglec conserved throughout vertebrate evolution, Glycobiology 17 (2007) 838–846.
[32] R. Takamiya, K. Ohtsubo, S. Takamatsu, N. Taniguchi, T. Angata, The interaction between Siglec-15 and tumor-associated sialyl-Tn antigen enhances TGF-β secretion from monocytes/macrophages through the DAP12-Syk pathway, Glycobiology 23 (2013) 178–187.
[33] F. Xu, H. Zhao, J. Li, H. Jiang, Mucin-type sialyl-Tn antigen is associated with PD-L1 expression and predicts poor clinical prognosis in breast cancer, Gland Surg. 10 (2021) 2159–2169.
[34] A. Bärenwaldt, H. Läubli, The sialoglycan-Siglec glyco-immune checkpoint–a target for improving innate and adaptive anti-cancer immunity, Expert Opin. Ther. Targets 23 (2019) 839–853.
[35] J. Zhou, Z. Tang, S. Gao, C. Li, Y. Feng, X. Zhou, Tumor-associated macrophages: recent insights and therapies, Front. Oncol. 10 (2020) 188.
[36] Y. Takeuchi, H. Nishikawa, Roles of regulatory T cells in cancer immunity, Int. Immunol. 28 (2016) 401–409.
[37] A. Tanaka, S. Sakaguchi, Regulatory T cells in cancer immunotherapy, Cell Res. 27 (2017) 109–118.
[38] L. Wu, S. Saxena, R.K. Singh, Neutrophils in the Tumor Microenvironment (2020) 1–20.
[39] S.M. Toor, V. Sasidharan Nair, J. Decock, E. Elkord, Immune checkpoints in the tumor microenvironment, Semin. Cancer Biol. 65 (2020) 1–12.
[40] Y. Zhang, J. Zheng, Functions of Immune Checkpoint Molecules beyond Immune Evasion (2020) 201–226.
[41] E.I. Buchbinder, A. Desai, CTLA-4 and PD-1 pathways similarities, differences, and implications of their inhibition, Am. J. Clin. Oncol.: Cancer Clin. Trials 39 (2016) 98–106.
[42] P. Lane, Regulation of T and B cell responses by modulating interactions between CD28/CTLA4 and their ligands, CD80 and CD86, Ann. N. Y. Acad. Sci. 815 (1997) 392–400.
[43] M. von Locquenghien, C. Rozalén, T. Celià-Terrassa, Interferons in cancer immunoediting: sculpting metastasis and immunotherapy response, J. Clin. Invest. 131 (2021) e143296.