ONCOIMMUNOLOGY
2020, VOL. 9, NO. 1, e1807291 (11 pages)
https://doi.org/10.1080/2162402X.2020.1807291

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ORIGINAL RESEARCH

Expression signature, prognosis value, and immune characteristics of Siglec-15 identified by pan-cancer analysis

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ABSTRACT

Sialic acid-binding immunoglobulin-like lectin 15 (Siglec-15) is considered a novel anti-tumor target comparable to programmed cell death 1 ligand 1(PD-L1). However, little is known about Siglec-15. Our study aims to understand its expression signature, prognosis value, immune infiltration pattern, and biological function using multi-omic bioinformatics from public databases and verify them in lung cancer patients. Integrated analysis of The Cancer Genome Atlas and Genotype-Tissue Expression portals showed Siglec-15 was overexpressed across cancers. Genetic and epigenetic alteration analysis was performed using cBioportal and UALCAN, showed Siglec-15 was regulated at the genetic and epigenetic levels. Survival estimated using Kaplan–Meier plotter indicated high Siglec-15 expression correlated with favorable or unfavorable outcomes depending on the different type and subtype of cancer. Components of immune microenvironment were analyzed using CIBERSORT, and the correlation between immune cells and Siglec-15 was found to be distinct across cancer types. Based on Gene Set Enrichment Analysis, Siglec-15 was implicated in pathways involved in immunity, metabolism, cancer, and infectious diseases. Lung cancer patients with positive Siglec-15 expression showed significantly short survival rates in progression-free survival concomitant with reduced infiltration of CD20 + B, and dendritic cells by immunohistochemistry. Quantitative real-time PCR results indicated the overexpression of Siglec-15 was correlated with activation of the chemokine signaling pathway. In conclusion, Siglec-15 could serve as a vital prognostic biomarker and play an immune-regulatory role in tumors. These results provide us with clues to better understand Siglec-15 from the perspective of bioinformatics and highlight the importance of Siglec-15 in many types of cancer.

ARTICLE HISTORY
Received 16 February 2020
Revised 30 July 2020
Accepted 31 July 2020

KEYWORDS
Siglec-15; prognosis; cancer Immunity; pathway; multi-omics Bioinformatics

Introduction

Tumor cells and the tumor microenvironment (TME) can secrete or express various signaling molecules, that act on immune checkpoints expressed in immune cells to suppress immune responses. The immune escape mechanisms in the TME are highly heterogeneous, and this inherent genomic instability helps cancer cells avoid cytotoxic or targeted therapy in metastatic tumors.[1] The approval of ipilimumab in 2011 as cancer immunotherapy has opened a new chapter in anti-cancer therapy.[2] Immunotherapy as a fourth method of cancer treatment after surgery, radiotherapy, and chemotherapy is turning the dream of a clinical cure for advanced tumor patients into reality.[3–7] Surprisingly, neoadjuvant immunotherapy appears to rewrite the current status of treatment for early cancer.[8] Blocking programmed cell death-1 (PD-1)/PD-1 ligand 1 (PD-L1) is widely regarded as a successful strategy in normalization cancer immunotherapy.[9,10] However, treatment using anti-PD-1/PD-L1 pathway is not applicable to all patients.[11,12] Finding new targets to make up for the lack of PD-1/PD-L1 antibodies is imperative.

Siglecs are a family of sialic acid-binding immunoglobulin-like lectins that specifically recognize sialylated glycans and regulate immune cell function.[13] In recent years, an increasing number of Siglec members have been found to play a crucial role in tumor immunosuppression.[14–16] Siglec-7 and Siglec-9 expressed on the surface of natural killer (NK) cells and interacting with sialoglycans on cancer cells inhibited NK cells cytolytic capacity.[17,18] Siglec-9 on T cells and tumor-associated macrophages (TAMs) also play a role in "immune checkpoints" leading to immune evasion.[15] The glycoprotein CD24 is a novel "do not eat me" signal that prevents cancer cells from phagocytosis by binding to Siglec-10 on macrophages.[14] Siglec-15, originally classified into Siglecs family as a type I transmembrane protein. It is well conserved through vertebrate evolution and is mainly expressed on a subset of myeloid lineage cells.[19] Siglec-15 was found to be overexpressed in giant cell tumors of the bone and regulates bone remodeling and osteoclast differentiation through interaction with DAP12.[20–24] Siglec-15 expressed on TAMs may contribute to tumor immunosuppression by cooperating with

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DAP12 and Syk to increase TGF-β secretion.[25] Chen et al. first revealed that Siglec-15 showed high homology and similar domain composition with B7 family members, which have the ability to sustainably suppress T-cell responses. The expression of Siglec-15 is mutually exclusive to that of PD-L1.[26] Siglec-15 is considered a new promising target for immune normalization independent of the PD-1/PD-L1 pathway. Targeting Siglec-15 may be an effective alternative therapy for patients who do not respond to PD-1/PD-L1 antibodies.[26–28] A humanized monoclonal antibody against Siglec-15 is currently being evaluated in patients with advanced or metastatic solid tumors in phase I clinical trials.[29]

This study was conducted to comprehensively analyze Siglec-15 mRNA expression signature, genetic and epigenetic characteristics, prognostic value, correlation with tumor-infiltrating immune cells, and associated pathways using next-generation sequencing (NGS) data from public platforms,[30] and we further performed immunohistochemistry (IHC) and real-time quantitative PCR (RT-qPCR) to verify its association with TME and signaling pathway. Our research aims to provide more information to better understand the significance of Siglec-15 in various cancers.

Materials and methods

The cancer genome atlas (TCGA)

TCGA is a web-based, freely accessible database, which has generated large amounts of NGS data with a landscape of more than 11,000 tumors across 33 cancer types until 2018. It provided gene expression, methylation, copy number variation data sets, and clinical data.[30,31]

Genotype-tissue expression (GTEx)

GTEx provides gene expression data from 53 normal tissue sites across nearly 1000 people by RNA sequencing, which is publicly available. RNA-seq data including GTEx and TCGA data were derived from UCSC Xena data hubs (http://xena.ucsc.edu/) for a pan-cancer differential expression of Siglec-15. TCGA and GTEx data were integrated using R version 3.5.3 and limma package.

cBioPortal

The cBioPortal for Cancer Genomics (http://www.cbioportal.org) is a repository of cancer genomics datasets.[32,33] We investigated the copy number alterations (CNA) and mutations of Siglec-15 in different cancers.

UALCAN

The UALCAN network (http://ualcan.path.uab.edu)[34] was used to analyze DNA methylation levels of the Siglec-15 promoter between tumor and normal tissues of lung adenocarcinoma (LUAD), cervical squamous cell carcinoma and endocervical adenocarcinoma (CESC), colon adenocarcinoma (COAD), kidney renal papillary cell carcinoma (KIRP), rectum adenocarcinoma (READ), kidney renal clear cell carcinoma (KIRC), and prostate adenocarcinoma (PRAD). Data were collected and shown as violin plots created using R version 3.5.3 and ggplot2 package.

Kaplan–Meier plotter

Using the Kaplan–Meier plotter (http://kmplot.com/analysis/),[35] we analyzed the prognostic values of Siglec-15 mRNA expression in different cancers mainly from TCGA (RNA-seq) and Gene Expression Omnibus (GEO) (microarray). We computed the log-rank p-value and HR with 95% confidence intervals. A log p-value <0.05 was considered statistically significant. The significant survival data were collected and shown as forest plots created using R version 3.5.3 and survival package.

TISIDB

TISIDB is a website for gene- and tumor-immune interaction (http://cis.hku.hk/TISIDB/index.php).[36] It was used to analyze Siglec-15 gene expression in different immune subtypes, including C1 (wound healing), C2 (IFN-γ dominant), C3 (inflammatory), C4 (lymphocyte deplete), C5 (immunologically quiet), and C6 (TGF-β dominant) subtypes; and Siglec-15 gene expression in different molecular subtypes tumor samples from TCGA.

CIBERSORT

CIBERSORT calculates the putative proportion of immune cell fraction from gene expression profiles.[37] To estimate relative abundance of tumor-infiltrating immune cells in tumor mass from TCGA and GEO, we used a reference set with 22 sorted immune cell subtypes (LM22) by online analytical platform CIBERSORT (https://cibersort.stanford.edu/).

Gene set enrichment analysis (GSEA)

We downloaded the RNA-seq data from TCGA portal (https://portal.gdc.cancer.gov/). We have chosen the Siglec-15 and its co-expression genes to perform GSEA to discover potential pathways by Kyoto Encyclopedia of Genes and Genomes (KEGG) and gene ontology (GO) terms affected by Siglec-15 using R version 3.5.3 and clusterProfiler, ggplot2, enrichplot, and org.Hs.eg.db packages. GO analysis was classified into three categories: biological process (BP), cellular component (CC), and molecular function (MF). Results with a normalized enrichment score (NES) ≥1.0 and false discovery rate (FDR) adjusted p-value <0.25 were considered statistically significant.

Patients and tissue samples

Data from 103 patients who had received radical (R0) resection with histological diagnosis of LUAD at the Tianjin Medical University Cancer Institute and Hospital, China, from January 2013 to May 2013, were retrospectively collected. All patients underwent R0 resection and lymph node dissection. The lymph node and lung cancer stages were categorized according to the eighth edition of the International Association for the Study of Lung Cancer TNM staging system.

Histological analysis was reviewed by a pathologist after H&E staining. The 2 mm tissue cores are chosen as representative tumor parts on tissue microarray (TMA), obtained from non-necrotic areas in the primary tumors using a needle. The use of patient information and tissues was sanctioned by the Ethics Committee of the Tianjin Medical University Cancer Institute and Hospital.

Immunohistochemical staining and evaluation

After deparaffinized, rehydrated, and antigen retrieval (Siglec-15, citrate buffer pH 6.0; CD4, CD8, CD20, CD11 c, FoxP3, CD68, CD163, EDTA 9.0), TMA slides were blocked with 3% hydrogen peroxide and 5% goat serum. After being incubated overnight at 4°C with primary antibodies, slides were incubated with enhancer and secondary antibody for half an hour at room temperature. A DAB Substrate Kit was used for chromogenic reaction. Finally, the sections were counterstained with hematoxylin, then dehydrated, cleared, and evaluated. Primary antibodies include anti-Siglec-15 antibody (PA5-72765, Thermo Fisher Scientific, USA, 1:100), anti-CD4 antibody (ab133616, Abcam, USA, 1:200), anti-CD8 antibody (SP16, Thermo Fisher Scientific, USA, 1:200), anti-CD20 antibody (EP459Y, Abcam, USA, 1:200), anti-CD68 antibody (KP1, Thermo Fisher Scientific, USA, 1:100), anti-CD163 antibody (10D6, Thermo Fisher Scientific, USA, 1:100), anti-FoxP3 antibody (236A/E7, Abcam, USA, 1:100), and anti-CD11c antibody (EP1347Y, Abcam, USA, 1:200)

Immunostaining was evaluated under light microscopy at 400× magnification by two independent pathologists. Siglec-15 was observed in the membrane and/or cytoplasm of the tumor cells and immune cells. In our study, we identified positive Siglec-15 expression on tumor cells as membrane staining. The absolute number of immune cells was counted manually. The total number of stained immune cells (in the central tumor and peritumoral stroma) was included in the analyses.

Statistical analysis

We summarized patient characteristics through descriptive statistics. Overall survival (OS) was defined as the time interval between the date of surgery and date of death. Progression-free survival (PFS) was defined as the time interval between the date of surgery and date of progression. Survival analyses were conducted by Kaplan–Meier curves (p-values from Log-rank test) by R version 3.5.3 and survival package. Hazard ratios (HR) were calculated using R package. All statistics in Tables 1–3 were two-sided and analyzed through SPSS statistical software (version 24.0.0). Statistical significance was defined as $p < .05$.

RT-qPCR

Total RNA was isolated from patients with LUAD by Trizol and converted into cDNA by reverse transcription (GoScript™ Reverse Transcription System, USA). The mRNA expression of Siglec-15, immune-related genes, and pathway-related genes were assayed by RT-qPCR. Siglec-15 primers purchased from Sino Biological Inc. RT-qPCR primers of other genes are provided in Supplementary Table S1.

Table 1. Patient clinical parameters and their association with Siglec-15 expression.

		Siglec-15		
Clinical parameters	n (%)	Positive	Negative	*p*-Value
T classification				
T1	55(53.4)	6	49	0.149
T2	39(37.9)	10	29	
T3+ T4	9(8.8)	1	8	
N classification				
N0	62(60.2)	9	53	0.303
N1	6(5.8)	0	6	
N2	35(34.0)	8	27	
Clinical stage				
I	48(46.6)	6	42	0.275
II	18(17.5)	2	16	
III	37(35.9)	9	28	
Smoking index				
<400	70(68.0)	13	57	0.572
≥400	33(32.0)	4	29	
Age (years)				
≤60	58(56.3)	10	48	0.819
>60	45(43.7)	7	38	
Gender				
Males	42(40.8)	5	37	0.419
Females	61(59.2)	12	49	

Results

Siglec-15 mRNA expression in human cancers

We integrated tumor samples from TCGA and normal samples from GTEx databases to identify Siglec-15 mRNA expression characteristics. Collectively, Siglec-15 was upregulated across most cancer types, such as breast invasive carcinoma (BRCA), cholangiocarcinoma (CHOL), COAD, esophageal carcinoma (ESCA), glioblastoma multiforme (GBM), head and neck squamous cell carcinoma (HNSC), kidney chromophobe (KICH), acute myeloid leukemia (LAML), brain lower-grade glioma (LGG), liver hepatocellular carcinoma (LIHC), LUAD, ovarian serous cystadenocarcinoma (OV), pancreatic adenocarcinoma (PAAD), READ, skin cutaneous melanoma (SKCM), stomach adenocarcinoma (STAD), thyroid carcinoma (THCA), uterine corpus endometrial carcinoma (UCEC), and uterine carcinosarcoma (UCS) (Figure 1a). When only tumor and adjacent nearby tissues in TCGA were included, Siglec-15 was found to be upregulated in COAD, HNSC, KICH, KIRP, LUAD, READ, the metastatic tissue of SKCM, THCA, and UCEC. Siglec-15 was downregulated in PRAD via the TIMER database (Supplementary Figure S1a). Using Oncomine, Siglec-15 was found to be overexpressed in bladder cancer, brain, and central nervous system (CNS) cancer, breast cancer, cervical cancer, colorectal cancer, kidney cancer, leukemia, lymphoma, ovarian cancer, and sarcoma but was underexpressed in gastric and pancreatic cancers (Supplementary Figure S1b).

We then analyzed mRNA expression patterns of Siglec-15 in different clinical stages and molecular subtypes. Siglec-15 expression varied significantly in different clinical stages of BRCA, THCA, COAD, SKCM, KIRP, LIHC, and bladder urothelial carcinoma (BLCA) (Figure 1b). Siglec-15 expression in different molecular subtypes of BRCA, KIRP, pheochromocytoma and paraganglioma (PCPG), SKCM, and ESCA was significantly different (Figure 1c). No association was found between Siglec-15 expression and cancer stage, or molecular subtype in other cancers (Supplementary Figure S1c, S1d).

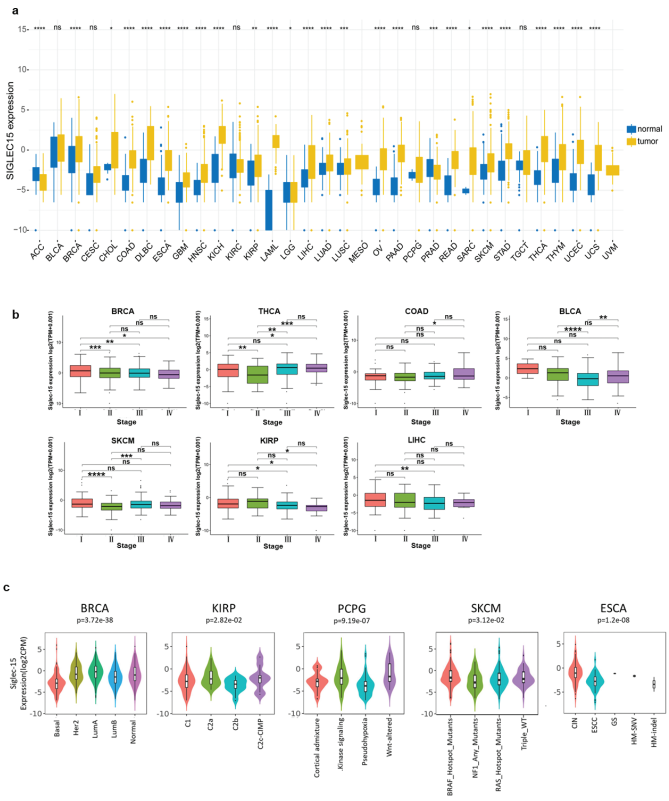

Figure 1. The transcription levels of Siglec-15 in human cancers. (a) The mRNA expression of Siglec-15 between tumor and normal tissues was assessed using tissues from TCGA and GTEx. (b) Association of Siglec-15 mRNA expression and different pathological stages in patients with different cancers from TCGA. (c) The expression of Siglec-15 in different molecular subtypes of cancers via TISIDB. (. *p*-value ≤ 0.1; **p*-value ≤ 0.05; ***p*-value ≤ 0.01; *** *p*-value ≤ 0.001; Siglec-15 gene symbol is SIGLEC15).

CNA and DNA methylation alterations of Siglec-15 across different human cancers

Genetic and epigenetic changes play a critical role in regulating cancer development and immune tolerance. Genetic alterations of Siglec-15 were further explored using cBioPortal. We found that patients with high Siglec-15 expression were accompanied by gene alterations in OV, HNSC, sarcoma (SARC), UCEC, BLCA, BRCA, SKCM, and lymphoid neoplasm diffuse large B-cell lymphoma (DLBC) (Figure 2a, Supplementary Figure S2a). The trends in Siglec-15 genetic alteration were consistent with its mRNA levels in these cancers. To explore gene expression variation contributed by CNA, we analyzed the relationship between gene expression and relative linear copy number values. The results revealed a positive correlation between Siglec-15 expression and CNA in COAD, HNSC, LUSC, OV, STAD, testicular germ cell tumors (TGCT), and UCEC, but a negative correlation was observed in SARC and PAAD (Figure 2b). No association was found between Siglec-15 expression and CNA in other cancers (Supplementary Figure S2b). However, expression alterations of Siglec-15 cannot be reasonably explained by CNA in other cancers, including LUAD, CESC, COAD, KIRP, READ, and KIRC. We then determined whether epigenetic mechanisms played a role in regulating Siglec-15 mRNA expression. DNA methylation is an epigenetic mechanism that can control gene expression without incurring any change to the genomic sequence.[38] To link promoter DNA methylation levels to

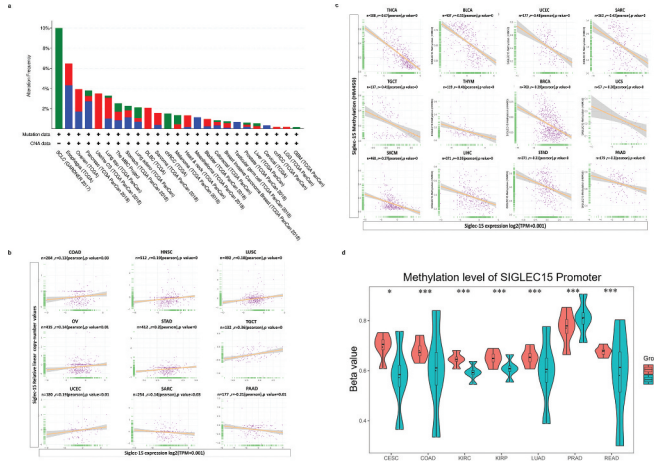

Figure 2. CNA and DNA methylation of Siglec-15 in human cancers. (a) CNA and mutation frequency data of Siglec-15 in different cancer studies were accessed from cBioPortal. (b) The relationship between Siglec-15 expression and relative liner copy-number values from TCGA. (c) Negative correlations between DNA methylation and mRNA expression of Siglec-15 were shown from TCGA. (d) DNA-methylation beta values ranging from 0 (unmethylated) to 1 (fully methylated) were determined by UALCAN. (hypermethylation [beta-value: 0.7–0.5] or hypo-methylation [beta-value: 0.3–0.25]).

Siglec-15 expression, we conducted a correlation analysis between DNA methylation states and Siglec-15 expression (Figure 2c). A significant negative correlation was found between DNA methylation and Siglec-15 expression in THCA, BLCA, UCEC, SARC, TGCT, thymoma (THYM), BRCA, UCS, SKCM, LIHC, STAD, PAAD ($-1 < \text{Pearson r} < -0.3$, Figure 2c), and in CESC, COAD, KICH, KIRP, LUAD, PCPG, PRAD, and READ ($-0.3 < \text{Pearson r} < -0.1$, Supplementary Figure S3a). No association was found between Siglec-15 expression and DNA methylation in other cancers (Supplementary Figure S3b). We then investigated the differential promoter DNA methylation status of Siglec-15 between cancer and adjacent normal tissues by using UALCAN (Figure 2d). Siglec-15 had lower DNA methylation levels in LUAD, CESC, COAD, KIRP, READ, and KIRC tissues than in adjacent normal tissues. Therefore, the abnormal increase of Siglec-15 mRNA expression in some cancers is likely a result of both genetic alterations and lower DNA methylations levels.

Correlation analysis between mRNA expression of Siglec-15 and prognostic value

After exploring the characteristics of Siglec-15 expression at the DNA and mRNA levels, we investigated the effect of abnormal Siglec-15 mRNA expression on prognosis by using Kaplan–Meier plotter. Our results showed that Siglec-15 had different prognostic values in different types of cancer (Figure 3). In TCGA data, upregulated Siglec-15 expression was found to be associated with longer OS in BLCA, BRCA, HNSC, THCA, and UCEC and with longer relapse-free survival (RFS) in BRCA, LIHC, OV, and UCEC (Figure 3a). By contrast, upregulated Siglec-15 expression was associated with shorter OS in KIRC, PAAD, and SARC, and with shorter RFS in PAAD and SARC (Figure 3b). Siglec-15 did not show prognostic values for other cancers (Supplementary Figure S4). In addition to TCGA, we analyzed ChIP and RNAseq data from GEO and other projects

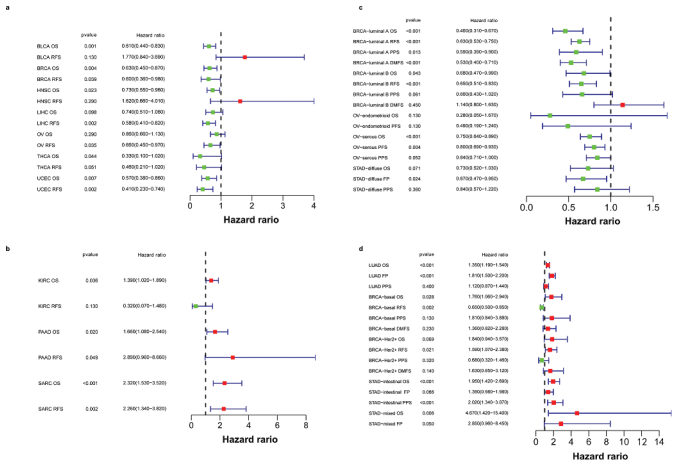

Figure 3. The prognostic significance of Siglec-15 assessed by Kaplan-Meier analysis. (a) High Siglec-15 mRNA expression correlated with good prognosis in BLCA, BRCA, HNSC, LIHC, OV, THCA, and UCEC from TCGA (HR < 1, *p*-value <0.05). (b) High Siglec-15 mRNA expression correlated with bad prognosis in KIRC, PAAD, and SARC from TCGA (HR > 1, *p*-value <0.05). (c) High Siglec-15 mRNA expression correlated with good prognosis in BRCA-luminal A, BRCA-luminal B, and OV-serous, and STAD-diffuse from GEO and other projects (HR < 1, *p*-value <0.05). (d) High Siglec-15 mRNA expression correlated with bad prognosis in LUAD, BRCA-Basal, BRCA-Her2, STAD-intestinal, and STAD-mixed from GEO and other projects (HR < 1, *p*-value <0.05).

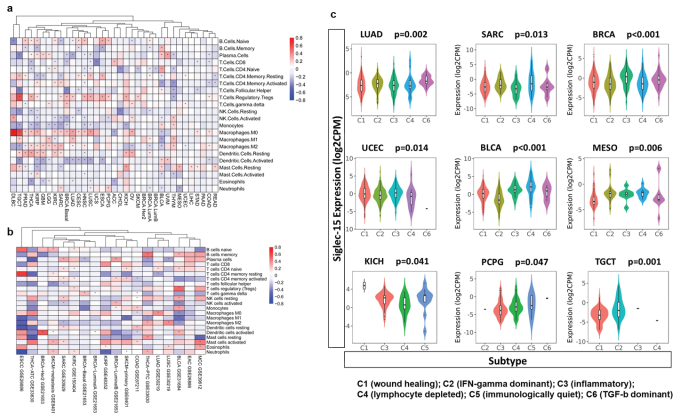

Figure 4. Correlation analysis between Siglec-15 expression and tumor-infiltrating immune cell. (a) Correlation analysis of Siglec-15 mRNA expression with 22 types of immune cells were investigated across cancers from TCGA by CIBERSORT (*p-value ≤ 0.05, |Spearman r| > 0.2). (b) Correlation analysis of Siglec-15 mRNA expression with 22 types of immune cells were investigated across cancers from GEO by CIBERSORT. (c) Siglec-15 mRNA expression in different immune subtypes in LUAD, SARC, BRCA, UCEC, BLCA, MESO, KICH, PCPG, and TGCT via TISIDB.

to acquire survival information in different cancer subtypes. We found that high mRNA expression of Siglec-15 indicated a better prognosis for BRCA-luminal A, BRCA-luminal B, OV and STAD-diffuse (Figure 3c). High Siglec-15 expression indicated good OS, RFS, post-progression survival (PPS), and distant metastasis-free survival (DMFS) in BRCA-luminal A; good OS and RFS in BRCA-luminal B; good OS and PFS in OV-serous; and good first-progression (FP) in STAD-diffuse. High Siglec-15 expression predicted worse outcomes in LUAD, BRCA-basal, BRCA-Her2+ ,and STAD (Figure 3d). Furthermore, high Siglec-15 expression was associated with poor OS and FP in LUAD; poor OS in BRCA-basal; poor RFS in BRCA-Her2+; poor OS and PPS in STAD-intestinal; and poor OS in STAD-mixed. Unexpectedly, high Siglec-15 expression predicted good RFS in BRCA-basal. Siglec-15 expression had the contrasting prognostic significance in different BRCA or STAD subtypes, which may be owing to its different immune infiltration properties. Siglec-15 mRNA expression was not significantly associated with the prognosis in other cancers from the Kaplan-Meier plotter (Supplementary Figure S5).

Relationship between mRNA expression of Siglec-15 and the tumor-immune microenvironment

After defining the prognostic value of Siglec-15, we explored the relationship between Siglec-15 and tumor-infiltrating immune cells in cancers (Figure 4a). First, we assessed components of the immune microenvironment across 33 cancers from TCGA by CIBERSORT algorithm. The clustering heatmap according to the relationship between Siglec-15 and immune cells showed a positive correlation between Siglec-15 and regulatory T cells (Tregs) in several cancers, especially ESCA (Spearman r = 0.31, p = .0002) and TGCT (Spearman r = 0.59, p = 3.77E-15). Most correlations with macrophages were positive. However, Siglec-15 was negatively correlated with macrophages in BLCA, and adrenocortical carcinoma (ACC). Because of its function in suppressing T cells, we focused on the relationship between Siglec-15 and CD8 + T cells. Siglec-15 was positively associated with CD8 + T cells in ACC and negatively associated with CD8 + T cells in BRCA-basal, THCA, and UCS. It is essential to note that the relationship between Siglec-15 and immune cells represents diverse functions in different breast cancer subtypes. In BRCA-basal, Siglec-15 was negatively correlated with follicular helper T cells (Spearman r = −0.37, p = 5.74E-07), activated NK cells, activated dendritic cells (DCs), and CD8 + T cells. However, Siglec-15 was not correlated with immune cells in the other BRCA subtypes. Additionally, no significant correlation was found between the composition of immune cells and Siglec-15 in COAD, LIHC, PAAD, READ, STAD, THCA, and UCEC.

We also used multiple databases from GEO to analyze the relationship between Siglec-15 and the immune microenvironment for further validation (Figure 4b). Siglec-15 was positively associated with memory B cells and negatively macrophage (M0, M1) in BLCA; positively with γδT cells in BRCA-Basal; positively with macrophage (M0) in LUAD, which were consistent with the results in TCGA. We found that Siglec-15 was negatively associated with activated NK cells and positively associated with activated DCs and Tregs in BLCA in GSE31684. Siglec-15 was positively associated with naive B cells, neutrophils, and activated mast cells and negatively associated with follicular helper T cells in metastatic SKCM in GSE8401 compared to the associations in primary SKCM. Combining the results in TCGA and GSE30219 for LUAD and LUSC resulted a similar clustering pattern. This included a positive relationship with macrophages and a negative relationship with activated DCs. In BRCA-basal, Siglec-15 showed a significantly positive correlation with γδT cells in both TCGA and GSE21653. These results suggest opposite correlation patterns in esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC) in GSE26886; however, the results were not significant. This is possibly owing to the

limited sample size (ESCC n = 9; EAC n = 21). Additional details can be found in Supplementary Table S2.

Using molecular typing of immune subtypes,[39] we investigated Siglec-15 mRNA expression in different immune subtypes. A significant difference in Siglec-15 expression characteristics was observed across C1 (wound healing), C2 (IFN-γ dominant), C3 (inflammatory), C4 (lymphocyte deplete), C5 (immunologically quiet), and C6 (TGF-β dominant) subtypes in LUAD, SARC, BRCA, UCEC, BLCA, mesothelioma (MESO), KICH, PCPG, and TGCT (Figure 4c). No significant difference was observed for other cancers (Supplementary Figure S5). The highest level of Siglec-15 expression was observed in subtype C6 in LUAD, C4 in SARC, C3 in BRCA and UCEC. Differential expression of Siglec-15 in different immune subtypes may partially explain why Siglec-15 played contrasting roles in the prognosis of various cancers.

Significant pathways influenced by Siglec-15

To further investigate the potential functions of Siglec-15, we performed KEGG and GO GSEA across many cancer types (Figure 5a). The heatmap showed a clear clustering pattern by KEGG analysis (NES ≥ 1.0, FDR < 0.25). Immune-related pathways were highly enriched in a large fraction of cancers, such as TGCT, SARC, HNSC, PRAD, STAD, LGG, THCA, LUSC, GBM, KIRP, LUAD, and SKCM. Siglec-15 was significantly involved in activation of the "chemokine signaling pathway," "JAK-STAT signaling pathway," "NF-kappa B signaling pathway," "toll-like receptor signaling pathway," "T cell receptor signaling pathway," "natural killer cell-mediated cytotoxicity," "B cell receptor signaling pathway," and "TNF signaling pathway." It also influenced several cancer-related pathways, including the "PI3 K-Akt signaling pathway," "MAPK signaling pathway," "VEGF signaling pathway," and "apoptosis."

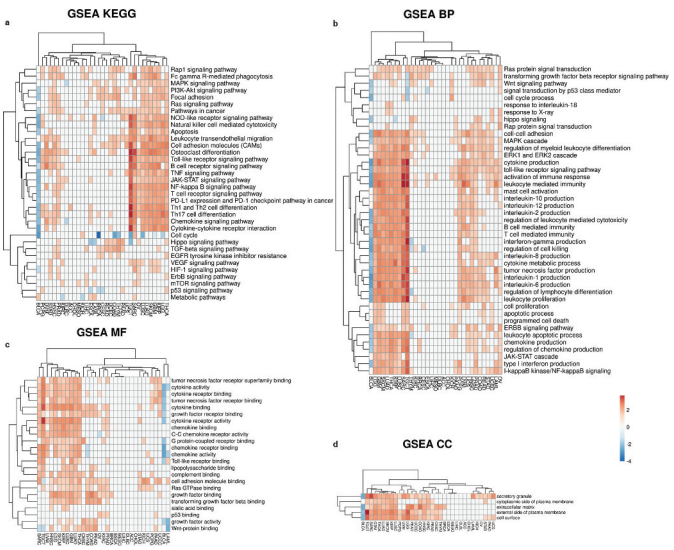

Figure 5. Significant pathways influenced by Siglec-15. (a) Relationships between Siglec-15 and KEGG pathways in cancers from TCGA analyzed by GSEA. (b) Relationships between Siglec-15 and GO BP terms in cancers from TCGA analyzed by GSEA (c) Relationships between Siglec-15 and GO MF terms in cancers from TCGA analyzed by GSEA. (d) Relationships between Siglec-15 and GO CC terms in cancers from TCGA analyzed by GSEA. (NES ≥ 1.0, FDR <0.25)

Siglec-15 affected "metabolic pathways," "mTOR signaling pathway" and "HIF-1 signaling pathway" in several cancers. It is interesting to note that Siglec-15 suppressed most pathways mentioned above, while some pathways such as "metabolic pathways" were activated in BLCA. Additional details can be found in Supplementary Table S3.

GO analysis indicated that Siglec-15 had an effect on immune-related BP, CC, and MF in most cancers. In particular, "cytokine production," "cytokine metabolic process," "MAPK cascade," "ERK1 and ERK2 cascade," "ERBB signaling pathway," "JAK-STAT cascade," and "toll-like receptor signaling pathway" in GO BP terms were found to be active in LGG, GBM, SKCM, LUAD, THCA, KIRP, and TGCT (Figure 5b). In GO MF terms, cytokine-related MFs were enriched in SARC, TGCT, UVM, HNSC, LGG, SKCM, KIRP, LUSC, GBM, LUAD, and THCA (Figure 5c). In the CC category, "external side of plasma membrane," "cytoplasmic side of plasma membrane," "cell surface," "secretory granule," and "extracellular matrix" were the main enriched terms (Figure 5d).

Preliminary experimental verification of Siglec-15 signature in LUAD

To verify the above results, we focused on Siglec-15 expression, prognostic value, relationship with immune cells, and associated pathways in LUAD and conducted in vitro experiment using tissues samples collected from the Tianjin Medical University Cancer Institute and Hospital. We first assessed the expression of Siglec-15 by 20 matched-pair LUAD tissue samples by RT-qPCR. The results showed Siglec-15 was significantly upregulated in primary tumor tissues (p = .0266) (Figure 6a). As mentioned above, Siglec-15 was associated with activation of the "chemokine signaling pathway" and "cytokine-cytokine receptor interaction" in KEGG and GO terms "cytokine production" and "regulation of cytokine production" in LUAD (Figure 6b). We focused on the "chemokine signaling pathway" (Supplementary Figure S6a) to verify it by RT-qPCR using 20 LUAD tissue samples (Figure 6c). Expressions of genes belonging to chemokine signaling pathway (CXCR3, GNAI2, RAC2, and MMP9) and the downstream PI3 K-AKT signaling pathway (AKT3, LPAR6, and CDK6) showed a trend toward positive association with Siglec-15, although this was not significant. And PI3 K was significantly positively associated with Siglec-15 expression (Pearson r = 0.54, p = .01). We divided the cancer tissues into two groups (Siglec-15 high and Siglec-15 low) and evaluated mRNA expression levels of key genes in the pathways (Supplementary Figure S6b). PI3 K was highly expressed in the Siglec-15 high group (p < .05). Other genes showed a trend toward higher expression in Siglec-15 high group, although this was not significant.

Simultaneously, we performed IHC to evaluate the expression and prognosis value of Siglec-15 in a cohort of 103 LUAD specimens. The patient demographics and relationship between Siglec-15 expression and clinicopathological factors are shown in Table 1. Siglec-15 positive expression was not associated with tumor size, lymphatic metastasis, TNM stage, smoking index, age, and gender by the Fisher's exact test. A total of 17 (16.5%) patients had positive Siglec-15 staining

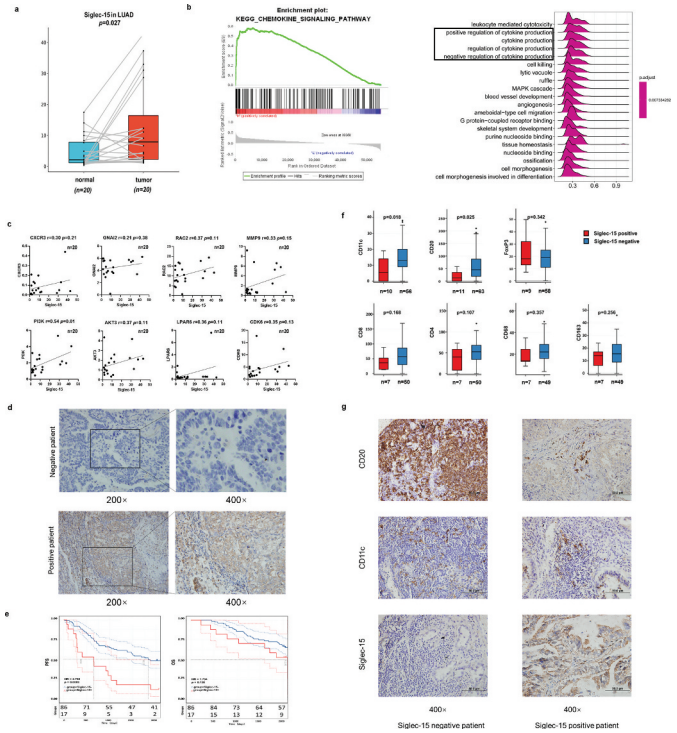

Figure 6. Preliminary experimental verification of Siglec-15 signature in LUAD. (a) Siglec-15 expression on 20 paired LUAD tissues and adjacent normal tissues by RT-qPCR. (b) "Chemokine signaling pathway" associated with Siglec-15 in LUAD. "Cytokine production" and "regulation of cytokine production" included in the TOP20 GO terms in LUAD (NES ≥ 1.5, FDR <0.05). (c) The association between the key genes in chemokine signaling pathway and Siglec-15 was detected by RT-qPCR in 20 LUAD tissue samples (r: Pearson's correlation coefficient). (d) Representative negative and positive samples were taken at 200x and 400x magnification for IHC staining of Siglec-15. (e) Survival analysis of patients stratified into positive group (red) and negative group (blue) according to the membrane staining of Siglec-15 by IHC in a cohort of 103 LUAD specimens. (f) The relative levels of immune cells infiltration investigated for Siglec-15 negative and positive samples. (g) Immunohistochemical staining of DCs (CD11 c) and B cells (CD20) in Siglec-15 positive and negative group (400x).

Table 2. Univariate survival analysis of clinical parameters and Siglec-15 expression with PFS and OS in patients with LUAD.

		PFS		OS	
Clinical parameters	n	Hazard ratio (95%CI)	*p*-Value	Hazard ratio (95%CI)	*p*-Value
T classification					
T1	56	2.346	**0.001**	2.566	**0.003**
T2+ T3+ T4	47	(1.393–3.952)		(1.375–4.789)	
N classification					
N0	62	2.450	**<0.001**	2.889	**<0.001**
N1+ N2	41	(1.434–4.187)		(1.505–5.544)	
Clinical stage					
I+II	66	3.380	**<0.001**	3.050	**<0.001**
III	37	(2.031–5.625)		(1.581–5.887)	
Smoking index					
<400	70	1.427	0.218	1.120	0.742
≥400	33	(0.839–2.427)		(0.580–2.162)	
Age (years)					
≤60	58	1.112	0.679	0.8703	0.656
>60	45	(0.670–1.845)		(0.471–1.610)	
Gender					
Males	42	0.838	0.501	1.074	0.821
Females	61	(0.504–1.393)		(0.575–2.008)	
Siglec-15					
Positive	17	2.755	**<0.001**	1.734	0.138
Negative	86	(1.219 – 6.229)		(0.717–4.195)	

Note. Bold font indicates $p < 0.05$.

Table 3. Univariate survival analysis of Siglec-15 expression in subgroups with different clinical parameters.

		PFS		OS	
Clinical parameters	n	Hazard ratio (95% CI)	*p*-Value	Hazard ratio (95% CI)	*p*-Value
T classification					
T1	56	1.761(0.471–6.590)	0.290	0.538(0.112–2.591)	0.542
T2+ T3+ T4	47	2.962(1.120–7.833)	**0.001**	2.060(0.753–5.634)	0.080
N classification					
N0	62	3.156(0.895–11.120)	**0.005**	2.026(0.497–8.260)	0.207
N1+ N2	41	2.125(0.773–5.843)	0.053	1.431(0.474–4.318)	0.472
Clinical stage					
I+ II	66	2.788(0.758–10.25)	**0.018**	1.432(0.350–5.858)	0.566
III	37	1.980(0.778–5.042)	0.073	1.534(0.538–4.374)	0.361
Smoking index					
<400	70	2.488(1.036–5.973)	**0.005**	1.738(0.635–4.754)	0.196
≥400	33	3.221(0.453–22.90)	0.052	1.625(0.266–9.941)	0.527
Age (years)					
≤60	58	2.941(1.001–8.639)	**0.003**	2.167(0.666–7.046)	0.100
>60	45	2.552(0.728–8.943)	**0.034**	1.140(0.315–4.127)	0.834
Gender					
males	42	5.040(0.803–31.63)	**<0.001**	1.723(0.379–7.845)	0.386
females	61	2.065(0.842–5.063)	**0.043**	1.742(0.586–5.180)	0.231

Note. Bold font indicates $p < 0.05$.

and were classified as either membrane positive or membrane negative (Table 1). Images of representative negative and positive samples were taken at 200× and 400× magnification (Figure 6d). T classification, N classification, and clinical stage were risk factors used to determine OS and PFS (Table 2). The survival curves revealed that patients with positive expression of Siglec-15 had an unfavorable PFS (HR = 2.755, p = .0003). No significant difference in OS was observed between Siglec-15 positive and negative patients (HR = 1.734, p = .138) (Figure 6e).

Moreover, we investigated the prognostic value of Siglec-15 in selective patient subgroups of LUAD classified by clinicopathological factors (Table 3). Siglec-15 correlated with poor prognosis for PFS in patients with larger tumor size (T2+ T3 + T4), without lymph node metastasis (N0), earlier clinical stage (I+ II), and a smoking index less than 400. Positive Siglec-15 expression showed greater significant prognostic value in patients aged ≤60 y (PFS HR = 2.941, p = .003) than >60 y (PFS HR = 2.552, p = .034). Positive Siglec-15 expression indicated poorer PFS in male patients (HR = 5.04, p < .001) than in female patients (HR = 2.065, p = .043). No significant difference in OS was observed. We found that Siglec-15 was a poor prognostic predictor in our LUAD cohort, which is generally consistent with our survival analysis of the database, especially in GEO.

We perform IHC to evaluate the relative levels of immune cells infiltration of Siglec-15 in a cohort of 88 LUAD specimens with Siglec-15 negative and positive patients (Figure 6f, 6g, Supplementary Figure S6). The patient clinicopathological characteristics and Siglec-15 expression are shown in Table S4. The results showed that infiltration of B cells and DCs was stronger in Siglec-15 negative patients than in Siglec-15 positive patients (p < .05). However, there was no difference in the infiltration of macrophages and T cells (CD68 p = .36, CD163 p = .26, CD4 p = .11, CD8 p = .17, and FoxP3 p = .34). We found that Siglec-15 expression was negatively related to B cells and DCs, which is consistent with the results

from TCGA. However, Siglec-15 expression was not associated with macrophages and Tregs, which showed positive association with Siglec-15 in TCGA (Supplementary Figure S6d). Then, we further assessed the expression of these genes by RT-qPCR. Siglec-15 was positively associated with CD68 (r = 0.37, p = .11), CD163 (r = 0.50, p = .02), and FoxP3 (r = 0.58, p = .01) in 20 LUAD tissues.

Discussion

Tumor immunotherapy has greatly changed the paradigm in the management of tumor patients. Immune-checkpoint blockade therapy has improved survival in patients with advanced melanoma, non-small-cell lung cancer (NSCLC), and other cancers.[40] However, the anti-tumor effect of a single-agent PD-1 inhibitor is limited, with an effective response rate of less than 30%.[3,29,41,42] Chen et al. discovered a close relationship between Siglec-15 and the B7 family by protein sequence analysis.[26] Although several binding partners on tumor cells have been identified for Siglec-15,[43–45] the ligand on T cells for Siglec-15 is unclear. The role of suppressing T cells by Siglec-15 remains to be further investigated.

Glycosylation pattern modification on tumor cells and bacteria to escape immunity has been known for decades;[46] however, little progress has been made in treatment strategies targeting glycosylation. Sialylation is one of the most common types of modifications in glycosylation. Siglecs expressed on tumor or immune cells bind to sialoglycans and play a differential immunoregulatory role.[47] In recent years, several studies have suggested that Siglecs are responsible for immune cell activation, proliferation, and apoptosis. Meanwhile, Siglecs have also been implicated in immune tolerance regulation and may play an essential role in autoimmune and autoinflammatory diseases and tumorigenesis.[16] Therefore, the Siglecs family of proteins have received increasing attention in tumor immunity.[14–17,26] An increasing number of Siglecs- or sialoglycan-targeted therapeutic drugs have been developed and may represent a potential novel immune-checkpoint. Targeting Siglec-15 may be an effective alternative therapy for patients that do not respond to PD-1/PD-L1 antibodies.

Genetic and epigenetic changes play a critical role in regulating cancer development and immune tolerance. For example, mutant PD-L1 with structural variations leads to aberrant PD-L1 expression and immunosuppression.[48,49] JAK2/PD-L1 /PD-L2 (9p24.1) amplifications can also bring about constitutive overexpression of PD-L1 and a significant response to checkpoint inhibitors.[50–52] Different mechanisms for regulating PD-L1 expression may reflect the varied roles on different cellular localization and cell types. Preliminary analysis suggested that Siglec-15 expression was genetically and epigenetically regulated through CNA and promoter methylation. In addition to a role in immune regulation, GSEA revealed that Siglec-15 was associated with oncogenic pathways (e.g. MAPK, PI3 K-Akt, Hippo, p53, and apoptosis). Whether Siglec-15 works as an oncogene and the exact mechanisms which regulated Siglec-15 remains to be elucidated.

Many of the genes are found to play different roles through different mechanisms in different cancers.[53–56] Similarly, Siglec-15 has variable prognostic significance in diverse cancers, and even in different subtypes of the same cancer. It is well accepted that tumor heterogeneity is a major challenge in cancer treatment.[57–59] Siglec-15 may play a role in inhibiting tumorigenesis and development in "cold tumors" like BRCA-luminal A/B. In "hot tumors" like BRCA-basal, Siglec-15 appears to exert an immunosuppressive function by regulating immune-related pathways. In some small sample phase II studies and retrospective studies, the single-agent efficacy of PD-1 or ipilimumab only helped individual patients with certain subtypes achieve tumor remission.[60] Siglec-15 overexpression in immune desert tumor, SARC, was involved in immune-related pathways and predicted poor prognosis. If the immune microenvironment can be converted from "cold" to "hot," anti-Siglec-15 monoclonal antibody may play a more critical role than PD-1/PD-L1 in SARC. In addition, Siglec-15 expression was the highest in the C6 (TGF-β dominant) immune subtype in LUAD, which has a worse prognosis among the six immune subtypes with the highest TGF-β signature.[39] It is worth noting that Siglec-15 associated pathways and functions in BLCA were totally different from other cancers. These phenomena may be attributed to inter-/intra-tumor heterogeneity and unique immunophenotyping, which presents new challenges for Siglec-15 studies.

Siglecs on macrophages and DCs could modulate toll-like receptor-induced cytokine responses.[45,61,62]The pathway analysis also suggested that Siglec15 was related to activation of the chemokine signaling pathway. CXC chemokine receptor CXCR3 is mainly expressed in Treg, B cells, and tumor cells.[63,64] There is an increasing trend for CXCR3 and MMP9 in patients with overexpressed Siglec-15, which may recruit Treg.[65] Our data analysis demonstrated that Siglec-15 was significantly associated with Tregs infiltration in cancers from TCGA and positively associated with FoxP3 in LUAD tissues by RT-qPCR. This may result in a specific $Treg^{high}Siglec15^{high}$ TME. These results indicate that Siglec-15 may exist in an "immune-excluded" environment, which is consistent with more Tregs and higher TGF-β signaling.[66] Therefore, application of anti-Siglec-15 antibody after other therapeutic interventions may hold promise.

There were some differences between the public data analysis and experimental verification by IHC and RT-qPCR. This phenomenon may be due to that we currently focused on Siglec-15 expression on the membrane of tumor cells and used tissue microarray in the assessment of the complex TME. Firstly, Siglec-15 is primarily expressed on macrophage and tumor cells. We cannot exclude the simultaneous expression Siglec-15 in the membrane and cytoplasm. Differences in Siglec-15 expression in macrophage and tumor cells remain unclear. Secondly, TME is widely known for its complexity and variability caused by spatial and temporal heterogeneity.[57] In addition to the number of immune cells, the distribution of immune cells in the central core, tumor stroma, and peripheral areas[57] contributes to different immune infiltration patterns in the same tissue. Thirdly, the number of patients in our validation cohort was limited to 103 patients. Recurrent patients received different treatment options, which may explain this phenomenon. In one word, more samples and more detailed analysis in specific subgroups are needed to conduct a more accurate and robust evaluation. In addition, our results are based on NGS analysis, which are derived from bulk cells and limited our analysis on

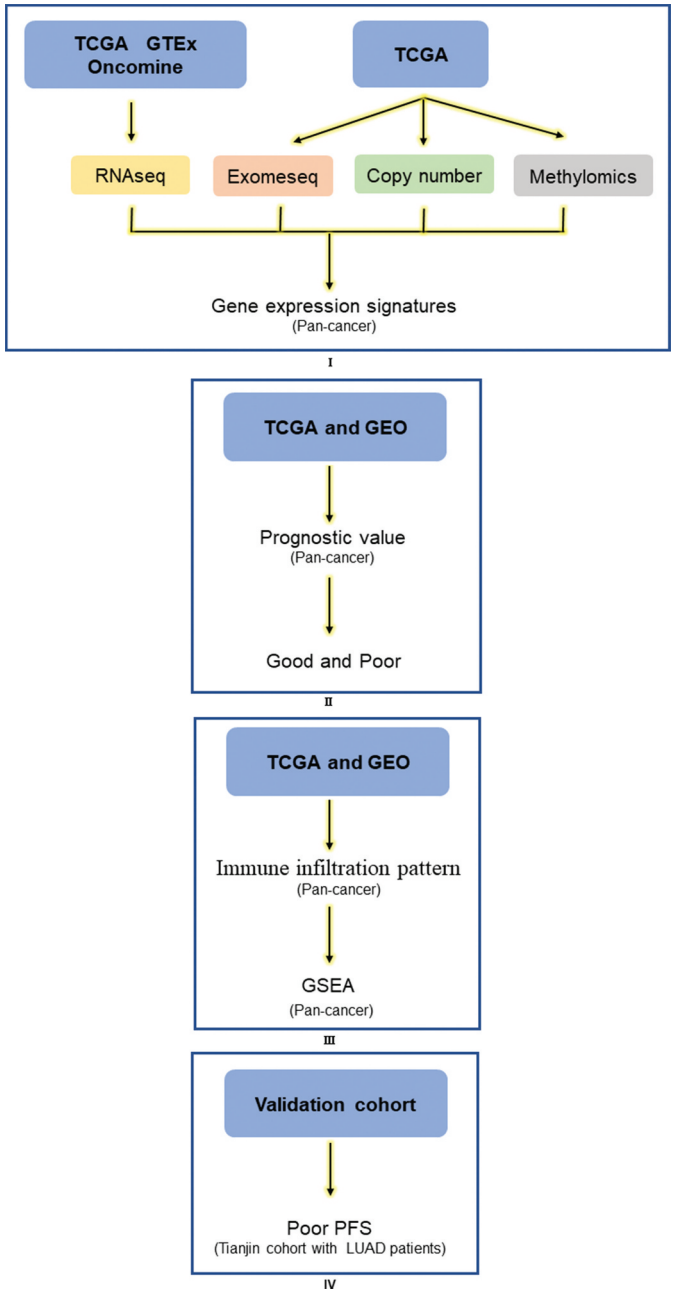

Figure 7. Analysis explanation with a detailed flow chart of this study. The study comprises 4 parts: Ⅰ Siglec-15 mRNA expression characteristics investigated by TCGA, GTEx, and Ocomine; Ⅱ Siglec-15 prognostic value landscape in TCGA and GEO; Ⅲ Tumor infiltrating pattern and associated pathways about Siglec-15 in TCGA and GEO; Ⅳ Experiment verification in LUAD patients.

accurate differentiation of immune cells and tumor cells. Therefore, single-cell RNA sequencing of sorted immune cells are needed to provide more exact information. Although the experimental results in LUAD samples were generally consistent with data analysis in TCGA and GEO, additional experiments are required to confirm the significance of Siglec-15 in other cancers.

In summary, we investigated Siglec-15 mRNA expression characteristics, prognostic value landscape, relationship with tumor-infiltrating immune cells, and associated pathways in various cancers from the perspective of multi-omic bioinformatics (Figure 7). We further verified that Siglec-15 expression was associated with poor prognosis and an inhibitory microenvironment in patients with LUAD. These results validated the importance of Siglec-15 expression in cancer prognosis and treatment and identified significant areas for further exploration and confirmation.

Acknowledgments

We would like to acknowledge tumor tissue banking facility of the Tianjin Medical University Cancer Institute and Hospital for providing us tissues from patients with LUAD.

Funding

This work was supported by a grant from the National Natural Science Foundation of China [No.81974246 & 81572265];National Key Technology R&D Program [No.2018YFC1313400];Key Projects of Tianjin Health Industry [No.15KG145].

Declaration of interest statement

The authors declare no potential conflicts of interest.

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