



# A Pan-Cancer Analysis Reveals CLEC5A as a Biomarker for Cancer Immunity and Prognosis

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**Background:** CLEC5A is a member of the C-type lectin superfamily. It can activate macrophages and lead to a series of immune-inflammation reactions. Previous studies reveal the role of CLEC5A in infection and inflammation diseases.

**Method:** We acquire and analyze data from The Cancer Genome Atlas (TCGA) database, Genotype-Tissue Expression (GTEx) database, and other comprehensive databases via GSCALite, cBioPortal, and TIMER 2.0 platforms or software. Single-cell sequencing analysis was performed for quantifying the tumor microenvironment of several types of cancers.

**Results:** CLEC5A is differentially expressed in a few cancer types, of which overexpression accompanies low overall survival of patients. DNA methylation mainly negatively correlates with CLEC5A expression. Moreover, CLEC5A is positively related to immune infiltration, including macrophages, cancer-associated fibroblasts (CAFs), and regulatory T cells (Tregs). Immune checkpoint genes are significantly associated with CLEC5A expression in diverse cancers. In addition, CLEC5A expression correlates with mismatch repair (MMR) in several cancers. Tumor mutation burden (TMB), microsatellite instability (MSI), and neoantigens show a positive association with CLEC5A expression in several cancers. Furthermore, CLEC5A in cancer correlates with signal transduction, the immune system, EMT, and apoptosis process. The drug sensitivity analysis screens out potential therapeutic agents associated with CLEC5A expression, including FR-180204, Tivozanib, OSI-930, Linifanib, AC220, VNLG/124, Bexarotene, omacetaxine mepesuccinate, narciclasine, leptomyacin B, PHA-793887, LRRK2-IN-1, and CR-1-31B.

**Conclusion:** CLEC5A overexpresses in multiple cancers in contrast to normal tissues, and high CLEC5A expression predicts poor prognosis of patients and immune infiltration. CLEC5A is a potential prognostic biomarker of diverse cancers and a target for anti-tumor therapy.

**Keywords:** pan-cancer, CLEC5A, prognostic biomarkers, cancer immune, immunotherapy

## INTRODUCTION

Cancer has the second mortality rate worldwide, and immunotherapy is one of the most promising cancer therapeutic strategies. Despite significant advances and successful application of immunotherapy across a wide range of human cancers, most patients had limited or no response to these therapies. Previous research indicated tumor mutation burden (TMB), the proportion of immune cell infiltration, and PD-L1/PD-L1 expression are widely recognized indicators for cancer immunotherapy response (1–4). However, these molecular markers have some limitations in predicting tumor immunotherapy response. Therefore, exploring more novel biomarkers to assess response to these immunotherapy approaches and helping develop a more effective treatment that ultimately improves clinical outcome for cancer patients is still urgently needed.

Human C-type lectin domain family 5 member A (CLEC5A), also known as myeloid DAP12-associating lectin-1 (MDL-1), is a II-type transmembrane protein and only expresses on the myeloid lineages including macrophages, monocytes, neutrophils, and dendritic cells (DCs) (5–7). As a pattern recognition receptor, CLEC5A transmits signals into cytoplasm through non-covalent binding to the adaptor protein DAP12 (8). Phosphorylation of DAP12 then initiates a Syk kinase-based signaling cascade resulting in macrophages activation and release of chemokines and proinflammation cytokines, including IL-6, TNF, CCL3, and CXCL8. No doubt that CLEC5A can trigger myeloid cell-related immune response and correlates with diverse infection and inflammatory diseases (9). CLEC5A has been proved to promote the production of high levels of pro-inflammatory cytokines and chemokines in flavivirus infections, especially dengue and Japanese encephalitis virus infections, and anti-CLEC5A mAb or CLEC5A inhibitors can reverse disease progression, suggesting that CLEC5A is a promising therapeutic target for flavivirus infections (10–13). In addition, similar in several autoimmune diseases, a high level of CLEC5A is found in active rheumatoid arthritis and CLEC5A activator increases proinflammation cytokines level (14). CLEC5A is closely related to the immune-inflammation process.

More recently, CLEC5A has emerged as a pivotal contributor to cancer development and progression (15–20). Aberrant high CLEC5A expression significantly correlates with decreased overall survival in high-grade serious ovarian cancer (HGSO), gastric cancer, and glioma (18–21). In gastric cancer, *CLEC5A* silencing or knockdown results in decreased cell growth, colony-formation capability, and tumor volume and weight, which is consistent with the findings in GBM (17, 19). Mechanistically,

CLEC5A correlates with the PI3K/Akt pathway in tumorigenesis (19). Moreover, CLEC5A expression in glioma positively correlates with immune score, but negatively correlates with tumor purity (21). Furthermore, CLEC5A expression in gliomas co-exists with more tumor-promoting leukocytes infiltration, especially M2 macrophages. However, the above findings are limited to finite cancer types, and more studies are essential to determine CLEC5A as a tumor prognostic biomarker and immunotherapy target.

This study systematically illustrated the CLEC5A profiles, including mRNA expression, methylation, mutation patterns, immune infiltration, correlation with signatures of interest, and patient's prognosis. The function enrichment analysis also identified the possible pathways that CLEC5A participates in tumorigenesis. In addition, we identified potential chemotherapeutic drugs targeting CLEC5A. In general, this study contributes to a comprehensive understanding of the critical role of CLEC5A in tumorigenesis and tumor immunity and provides candidate drugs based on CLEC5A as anti-tumor therapeutic targets.

## METHODS

### CLEC5A Expression Profiles

The gene expression data of *CLEC5A* in tumor and normal tissues and the patient's prognosis information were obtained from The Cancer Genome Atlas (TCGA, <http://cancergenome.nih.gov>) database and genotype-tissue expression (GTEx, <http://commonfund.nih.gov/GTEx/>). To make gene expression data more comparable between samples, fragments per kilobase million (FPKM) values were converted to transcript per kilobase million (TPM) values, normalized by Log2 conversion. The expression difference of *CLEC5A* in cancer and normal tissues was visualized.

The gene expression profile data of *CLEC5A* expression in cancer cell lines were acquired from the Cancer Cell Line Encyclopedia (CCLE) platform (<https://sites.broadinstitute.org/ccle/tools>) (22). The *CLEC5A* mRNA expression profile in various cancer cell lines was achieved from the Human Protein Atlas (HPA, <https://www.proteinatlas.org/>) website (23, 24).

### Survival Analysis

Kaplan-Meier Plotter was utilized to analyze the correlation between *CLEC5A* expression and patient survival in different cancers (25). The results of univariate Cox regression were summarized and presented by a forest plot.

## Single Cell Sequencing Analysis

Single-cell sequencing datasets of BLCA, BRCA, CHOL, COAD, GBM, HNSCC, KIRC, LIHC, OV, SKCM, and STAD were acquired from the Gene Expression Omnibus (GEO) database (<https://www.ncbi.nlm.nih.gov/geo/>). A single-cell sequencing dataset of LUAD was downloaded from the NCBI BioProject (PRJNA591860), and a single-cell sequencing dataset of PAAD, CRA001160, was downloaded from the Genome Sequence Archive (GSA) database (<https://ngdc.cncb.ac.cn/gsa/browse/CRA001160>). The detailed information on these GEO datasets and R packages for data integration and quality control are shown in **Table 1**. After data processing, principal component analysis (PCA) was performed. Tumor cells were identified using the R package *infercnv* and *copykat*. Immune cells and stromal cells were annotated based on specific markers. The UMAP function was used for visual dimension reduction, and *Vlnplot*, *Dimplot*, and *Featureplot* were used for visualization of *CLEC5A* expression.

## Methylation Profiles

GSCALite platform (<http://bioinfo.life.hust.edu.cn/web/GSCALite/>) was used to analyze differential methylation of *CLEC5A* in tumors and normal tissues across various TCGA cancer types (26). Subsequently, we used the same platform to explore the correlation between *CLEC5A* methylation and its expression in different cancers.

## Mutation Profiles

The copy number alteration (CNA) and mutation landscape of *CLEC5A* in pan-cancer was gained from cBioPortal (<http://www.cbioportal.org>) (27, 28). Thus, GSCALite was used to investigate the SNP frequencies and heterozygous/homozygous CNA of *CLEC5A*, *CD163*, *CD68*, *CD8A*, *CD8B*, and *MRC1* genes, as well as the correlation between CNA and mRNA expression of these genes.

## Immune-Related Characteristics

TIMER2.0 (<http://timer.cistrome.org/>) was utilized to analyze the relationship between *CLEC5A* expression and tumor-infiltration immune cells in tumors across various cancer types

(29). Tumor purity was indirectly evaluated with the ESTIMATE score, and the abundance of immune and stromal components in tumors was assessed by the immune score and the stromal score, respectively. Subsequently, we evaluated the correlation between *CLEC5A* expression and the markers for immune cell subsets, including CD8+ T cell, macrophage, T cell regulatory (Tregs), and cancer-associated fibroblast (CAF). A P-value less than 0.05 indicated a significant correlation. We evaluated the relationship between *CLEC5A* gene expression and immune checkpoint (ICP), TMB, microsatellite instability (MSI), and neoantigens using the Pearson correlation analysis. Pearson correlation analysis was also implemented to evaluate the correlation between *CLEC5A* gene expression and MMR genes (*MLH1*, *MSH2*, *MSH6*, *PMS2*, and *EPCAM*) and DNA methyltransferases (*DNMT1*, *DNMT2*, *DNMT3A*, and *DNMT3B*).

## Functional Enrichment Analysis

The proteins interacting with *CLEC5A* protein were analyzed with the STRING database (<https://string-db.org/>). The OPEN TARGET platform (<https://www.target-validation.org/>) was used to identify *CLEC5A* related diseases network (30). Gene set enrichment analysis (GSEA) of the Kyoto Encyclopedia of Genes and Genomes (KEGG) terms and HALLMARK terms was performed to describe the biological functions and pathways related to *CLEC5A*. Gene set variation analysis (GSVA) of gene ontology (GO) was also achieved to describe the immune pathways related to *CLEC5A*. The top KEGG and HALLMARK terms were listed, which  $|NES| > 1$ ,  $NOM\ p < 0.05$ , and  $FDR\ q < 0.25$  were set as the threshold for significantly enriched pathways. Moreover, the pathway activity module of the GSCALite platform was used to study the differences in genes expression between the activity groups (activation and inhibition) of cancer-related pathways in different cancer types.

## Immunofluorescence Staining

We obtained the tissue microarray from the Outdo Biotechcompany (HOrg-C110PT-01, Shanghai, China) and the ethics was approved. The primary antibodies were *CLEC5A* (Rabbit, Sigma-Aldrich, US), *CD68* (Rabbit, AiFang biological, China), *CD163* (Rabbit, Proteintech, China), *CD8* (Mouse, Proteintech, China). The secondary antibody was horseradish peroxidase-conjugated secondary antibody incubation (GB23301, GB23303, Servicebio, China), and the tyramide signal amplification was TSA [FITC-TSA, CY3-TSA, 594-TSA, and 647-TSA (Servicebio, China)]. Multispectral images were analyzed, and positive cells were quantified at single-cell levels by Caseviewer (CV 2.3, CV 2.0) and Panoramic viewer (PV 1.15.3) image analysis software.

## Immunotherapy Agents Response Prediction

*CLEC5A* expression levels were compared across tumor cell lines between pre- and post-cytokine treated samples and between pre- and post-ICB treated samples. The differences between groups were statistically evaluated by Wald test using DESeq2 (\* $FDR \leq 0.05$ , \*\* $FDR \leq 0.01$ , \*\*\* $FDR \leq 0.001$ ), and the comparison results were summarized in boxplots. Tumor

**TABLE 1** | The detailed information of these GEO datasets and R packages for data integration and quality control.

Cancer types	Datasets	R package
BLCA	GSE145137	Seurat
BRCA	GSE75688, GSE118389	
CHOL	GSE125449	
COAD	GSE81861	Seurat
GBM	GSE138794	
HNSCC	GSE103322	
KIRC	GSE121636, GSE171306	
LIHC	GSE125449	
OV	GSE118828	
SKCM	GSE72056	
STAD	GSE134520, GSE158631	harmony
LUAD	PRJNA591860	
PAAD	CRA001160	

models included were: mammary cancer: 4T1, E0771, EMT6, T11, KPB25L, p53-2225L, p53-2336R; colorectal carcinoma: CT26, MC38; gastric adenocarcinoma: YTN16; head and neck squamous cell carcinoma: MOC22; hepatocellular carcinoma: BNL-MEA; lung carcinoma: LLC; melanoma: B16, YUMM1.7, D3UV2, D4M.3A.3; sarcoma: 402230.

## Drug Sensitivity Prediction

GSCALite integrates over 750 small molecule drugs from the Genomics of Drug Sensitivity in Cancer (GDSC)/Therapeutics Response Portal (CTRP). We conducted the drug sensitivity analysis *via* GSCALite platform to explore the correlation between drug sensitivity and *CLEC5A* expression. Spearman correlation analysis was used to evaluate the correlation. Positive correlation indicated that tumor cells with high *CLEC5A* expression are prone to drug resistance, while negative correlation suggested that tumor cells with high *CLEC5A* expression are sensitive to drugs.

## Statistical Analysis

The statistical significance of differences between groups was analyzed by Student's *t* test, and the comparison among groups was analyzed by one-way ANOVA. *P*-value  $\leq 0.05$  indicated statistically significant.

## RESULTS

### CLEC5A Is Abnormally Expressed in Human Pan-Cancer and Serves as a Prognostic Biomarker

First, we comprehensively analyzed the *CLEC5A* expression in tumors and paired normal tissues in different cancers with TCGA and GTEx gene expression data. The results showed that *CLEC5A* mRNA expression was significantly up-regulated in 23 tumors (BLCA (bladder urothelial carcinoma), BRCA (breast invasive carcinoma), CESC (cervical squamous cell carcinoma and endocervical adenocarcinoma), CHOL (cholangiocarcinoma), COAD (colon adenocarcinoma), ESCA (esophageal carcinoma), GBM (glioblastoma), HNSC (head and neck squamous cell cancer), KIRC (kidney renal clear cell carcinoma), KIRP (kidney renal papillary cell carcinoma), LAML (acute myeloid leukemia), LGG (lower grade glioma), LIHC (liver hepatocellular carcinoma), LUAD (lung adenocarcinoma), OV (ovarian serous cystadenocarcinoma), PAAD (pancreatic adenocarcinoma), READ (rectum adenocarcinoma), SKCM (skin cutaneous melanoma), STAD (stomach adenocarcinoma), TGCT (testicular germ cell tumors), THCA (thyroid carcinoma), UCEC (uterine corpus endometrial carcinoma), and UCS (uterine carcinosarcoma)) and down-regulated in 4 tumors [ACC (adrenocortical carcinoma), KICH (kidney chromophobe), LUSC (lung squamous cell carcinoma), and PRAD (prostate adenocarcinoma)] (Figures 1A, B). In human tissues, *CLEC5A* mRNA expression was enriched in AML, B-cell, and T-cell of ALL, bile duct, and other tissues (Figure 1C). Moreover,

*CLEC5A* mRNA expression was highly expressed in immortal tumor cell lines, such as U937, HL-60, and NB-4 (Figure 1D). Therefore, *CLEC5A* differential expression in most cancer types indicates that *CLEC5A* may play a key role in carcinogenesis.

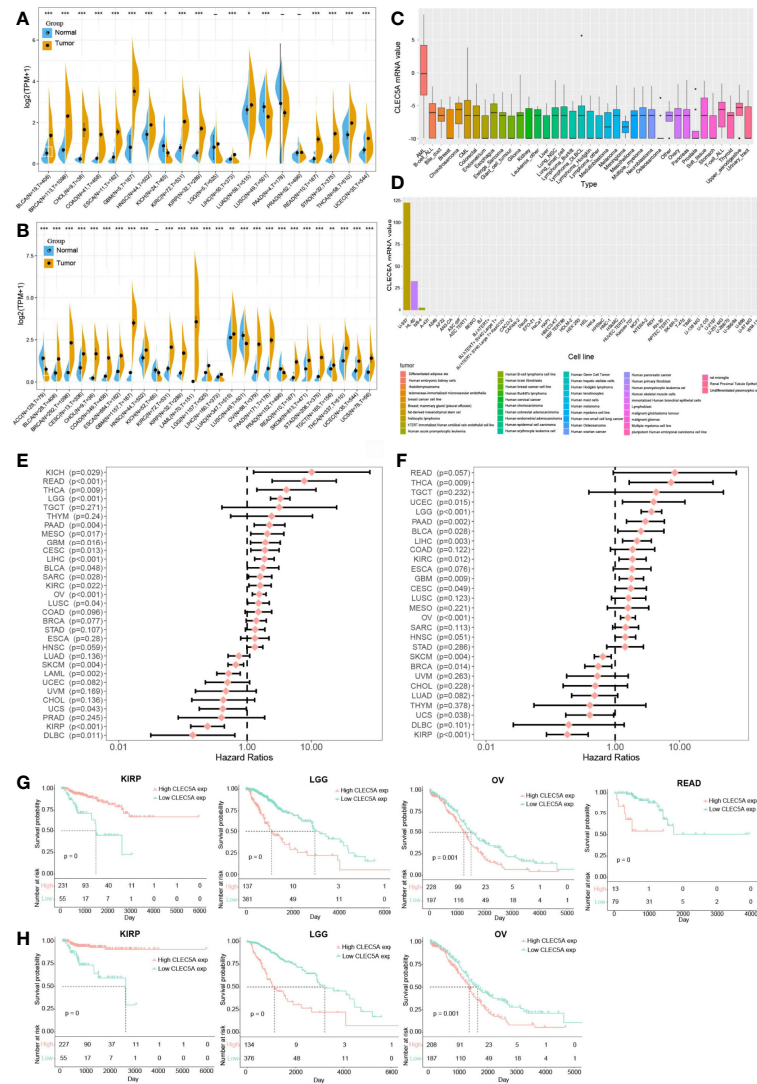
We next explored the correlation between *CLEC5A* expression and the prognosis of patients with pan-cancer. By univariate Cox regression analysis of 32 cancer types, we found that high *CLEC5A* expression indicated poor OS and DFS in patients with THCA, LGG, PAAD, GBM, CESC, LICH, KIRC, and OV but predicted better OS and DFS in patients with SKCM, UCS, and KIRP (Figures 1E, F). In the TCGA dataset, *CLEC5A* high expression was correlated with poor OS in patients with LGG, OV, and READ, and *CLEC5A* high expression was also associated with poor DFS in patients with LGG and OV (Figures 1G, H). In the TCGA dataset, *CLEC5A* high expression was correlated with better OS and DFS in patients with KIRP. Moreover, the expression of *CLEC5A* was also negatively associated with the survival of patients with LIHC, LUAD, and PAAD in GSE39582, GSE13213, and GSE57495, respectively (Supplementary Figure S1).

### Single Cells Analysis of CLEC5A in Human Cancers

To clarify *CLEC5A* mRNA expression in different cell types in pan-cancer, we obtained single-cell RNA sequencing data of BLCA, BRCA, CHOL, COAD, GBM, HNSCC, KIRC, LIHC, LUAD, OV, PAAD, SKCM, and STAD for analysis. The results showed that macrophages exhibited higher *CLEC5A* expression than other cell types in BLCA, COAD, GBM, PAAD, and SKCM. The *CLEC5A* expression in M2 macrophages in BRCA and HNSCC was higher than that in other cell types, while the *CLEC5A* expression in M1 macrophages and cancer cells in OV was higher than that in different types (Figures 2A–C and Supplementary Figures 2, 3). In addition, the ligand-receptor interactions in cells with high *CLEC5A* expression and 4 categories of intercellular communication (cytokine, growth factor, checkpoint, and other) were identified (Figures 2D–H). Obviously, strong interactions among neoplastic, T cells, M1 macrophages, and M2 macrophages were observed in growth factor analysis (Figure 2F). These results indicated that *CLEC5A* widely existed in tumor cells and macrophages in the tumor microenvironment, and suggested that *CLEC5A* may influence tumor progression and prognosis by participating in the tumor microenvironment and tumor immunity regulation.

### The Methylation Analysis of CLEC5A in Human Cancers

Research has confirmed that DNA methylation is closely related to changes in gene expression in cancers (31). Thus, the DNA methylation differences of *CLEC5A* and immune-related gene (*MRC1*, *CD163*, *CD8A*, *CD8B*, *CD68*) between tumors and normal tissues in various cancers were evaluated with the GSCALite platform. The results indicated that *CLEC5A* methylation was significantly down-regulated in UCEC, LIHC, KIRC, KIRP (kidney renal papillary cell carcinoma), LUAD, HNSC, BRCA, BLCA, and PRAD (prostate adenocarcinoma)

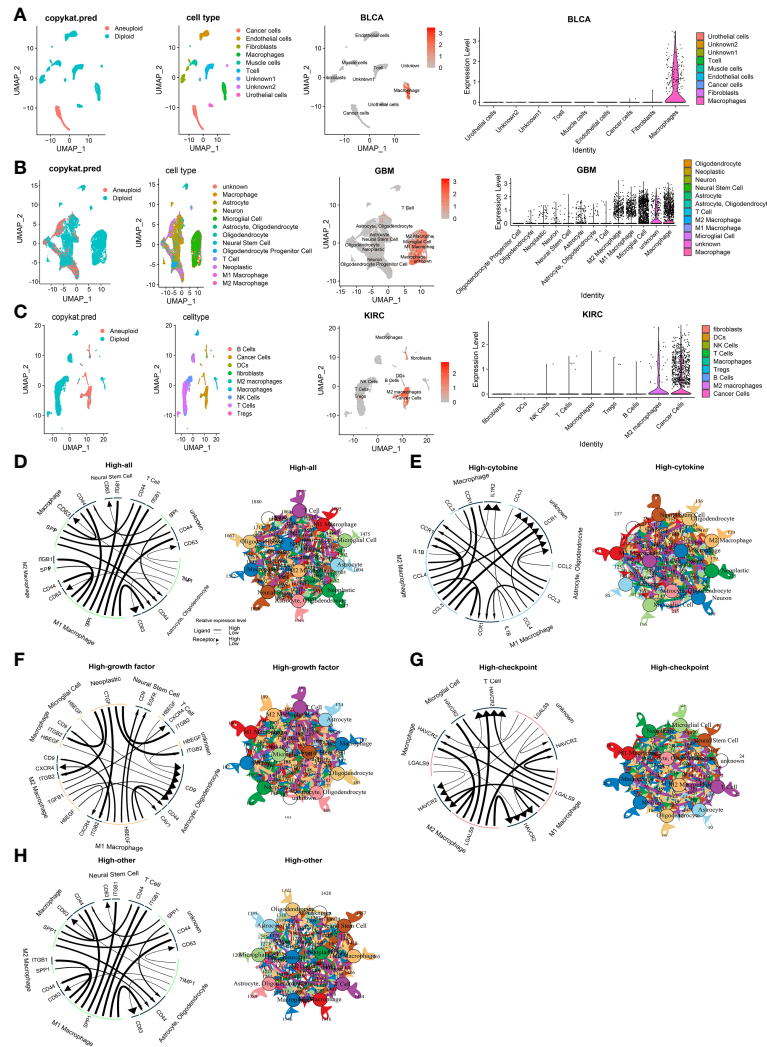


**FIGURE 1 |** *CLEC5A* is abnormally expressed in human pan-cancer and serves as a prognostic biomarker. **(A)** *CLEC5A* expression in cancers and normal tissues from TCGA database. **(B)** *CLEC5A* expression in cancers and normal tissues combining the GTEx database and the TCGA database ( $*P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$ ). **(C)** *CLEC5A* expression in tumor cells from the CCLE database. **(D)** *CLEC5A* expression in tumor cells from the HPA website. **(E)** Forest plots showed the correlation between *CLEC5A* expression and patient’s overall survival (OS) in different cancers. **(F)** Forest plots showed the correlation between *CLEC5A* expression and patient’s disease-free survival (DFS) in different cancers. **(G)** Kaplan-Meier curves for patient’s OS stratified by different expression levels of *CLEC5A* in KIRP, LGG, OV, and READ. **(H)** Kaplan-Meier curves for patient’s DFS stratified by different expression levels of *CLEC5A* in KIRP, LGG, and OV.

(Figure 3A). Next, we evaluated the correlation between the DNA methylation and mRNA expression of *CLEC5A* and immune-related gene (*MRC1*, *CD163*, *CD8A*, *CD8B*, *CD68*) in pan-cancer. The results exhibited that the *CLEC5A* mRNA expression was mainly negatively correlated with its DNA methylation in most tumors but only positively correlated with its DNA methylation in only some tumors, such as SKCM (cutaneous skin melanoma), TGCT (testicular germ cell tumors), LIHC and HNSC (Figure 3B). Moreover, except for *MRC1*, macrophage biomarker genes (*CD163*, *CD68*) and T cells biomarker genes (*CD8A*, *CD8B*) also mainly showed a negative correlation with their DNA methylation (Figure 3B).

### The Mutation Landscape of *CLEC5A* in Human Cancers

To study the mutation landscape of *CLEC5A* in human cancers, the cBioPortal tool was utilized to analyze the *CLEC5A* alteration (mutation and CNAs) frequency in 32 TCGA cancers types. The results showed that the alteration frequency of *CLEC5A* varies with tumor type. As shown in Figure 3C, *CLEC5A* was found to have a high alteration frequency of nearly 8% in both SKCM and OV, but no related alteration was found in the other 10 cancer types (CSCC, CHOL, DLBC, KICH, MESO, PCPG, TGCT, THYM, THCA, and UVM). Moreover, in the four alteration types (mutation, amplification, deletion, and multiple changes)



**FIGURE 2** | Single cells analysis of *CLEC5A* in human cancers. (A–C) Composition and distribution of single cells and *CLEC5A* expression in single cells in BLCA, GBM, KIRC. Each cell type is ranked by its average expression value. (D) The overall cellular communication pattern regarding high *CLEC5A* expression in GBM. (E) The cytokine-associated cellular communication pattern regarding *CLEC5A* expression in GBM. (F) The growth factor-associated cellular communication pattern regarding *CLEC5A* expression in GBM. (G) The checkpoint-associated cellular communication pattern regarding *CLEC5A* expression in GBM. (H) The other-associated cellular communication pattern regarding *CLEC5A* expression in GBM.

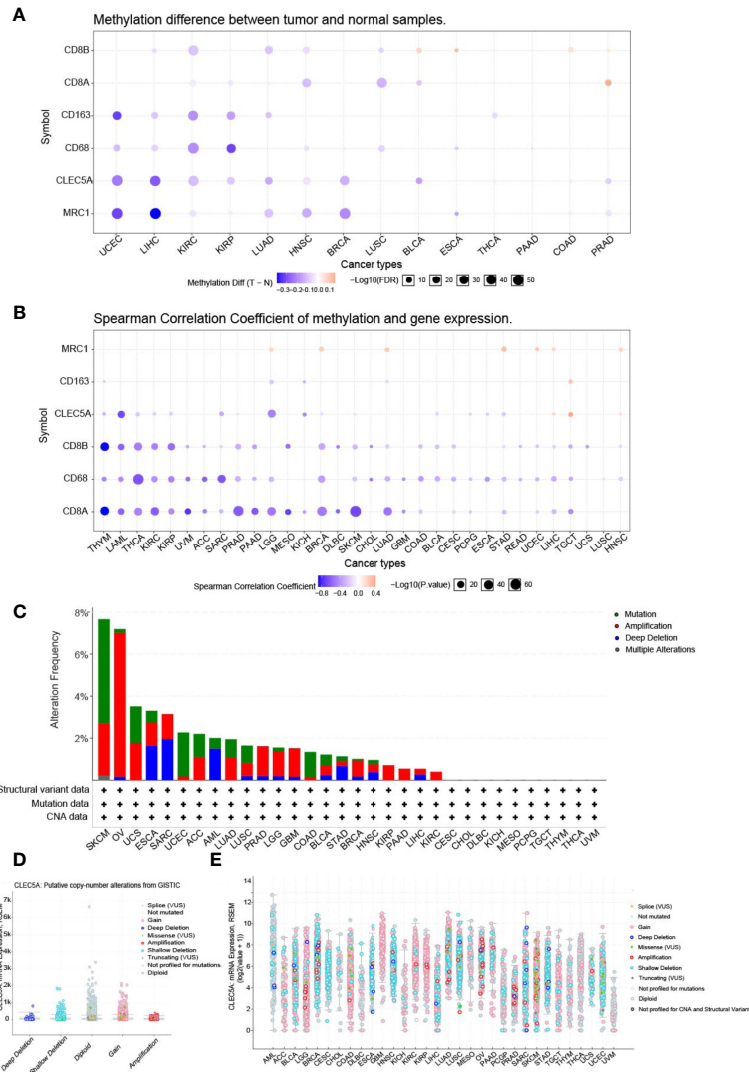
of *CLEC5A*, copy-number alteration (CNA, including deletion and amplification) frequency was the highest, followed by mutation and considerable alteration. Then, we explored the correlation between the putative CNA of *CLEC5A* and its gene expression in tumors. The putative CNA of *CLEC5A* in pan-cancer were shown in (Figures 3D, E).

### **CLEC5A CNVs, SNV, and Alternative Splicing in Human Cancers**

Copy number variation (CNV) module of the GSCALite platform provided heterozygous and homozygous CNV profiles in 33 cancer types. The pie chart showed that the heterozygous CNVs of all 6 genes (*CD68*, *MRC1*, *CD8A*, *CD8B*, *CD163*, and *CLEC5A*) was more frequent in various

cancers (Figure 4A). *CLEC5A* homozygous amplification only existed in OV and SKCM, and no homozygous deletion was found (Figure 4B). In most tumors, *CLEC5A* heterozygous amplification was standard, and *CD68* heterozygous deletion was dominant (Figure 4C). Additionally, the correlation between CNV and gene expression was investigated. The result showed that *CLEC5A* expression was positively correlated with CNV only in LGG and negatively correlated with CNV in PAAD, THYM, and COAD. In contrast, *CD68* expression was strongly and positively correlated with CNV in most cancer types (Figure 4D). Therefore, the CNV seems to have limited influence on *CLEC5A* expression.

Single nucleotide variation (SNV) is a common gene mutation associated with gene expression or function.

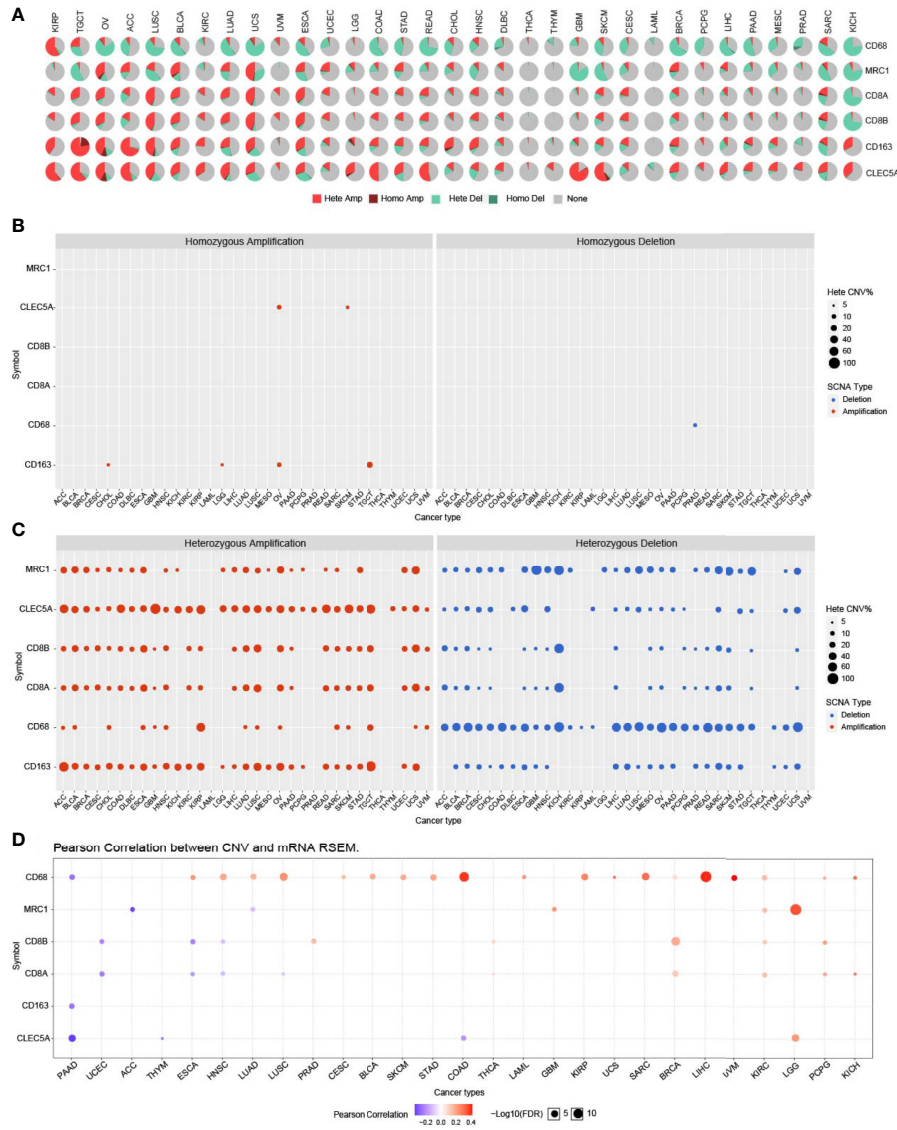


**FIGURE 3** | The methylation analysis and mutation landscape of *CLEC5A* in human cancers. **(A)** DNA methylation differences of *CLEC5A* between tumors and normal tissues in various cancers with GSCALite platform. **(B)** Correlation between the DNA methylation and mRNA expression of *CLEC5A* in 32 cancer types. **(C)** *CLEC5A* alteration (mutation and CNAs) frequency in 32 TCGA cancer types. **(D, E)** Correlation between the putative copy-number alteration of *CLEC5A* and its expression in tumors.

GSCALite was utilized to identify the SNV frequency of all 6 genes (*CD68*, *MRC1*, *CD8A*, *CD8B*, *CD163*, and *CLEC5A*) in various cancers. As the waterfall plots exhibited, the SNV frequency of *CD163* was 68%, and that of *CLEC5A* was 12%, with missense mutation accounting for the majority (**Supplementary Figure S4A**). *CLEC5A* SNV frequency was higher in SKCM, COAD, BRCA, LUAD and LGG. Besides, we obtained a mutation pattern of *CLEC5A* in various cancers using the cBioportal tools, and the results coincided with the waterfall plots obtained by GSCALite (**Supplementary Figure S4B**).

Alternative splicing represents a robust method regulate gene expression and protein complexity at the mRNA level. A mutation frequently occurs in *CLEC5A*, and it is located at alternative splicing sites; we used OncoSplicing to explore the

association between *CLEC5A* and alternative splicing (32). In **Supplementary Figure S5A**, PanPlot displayed the percent spliced in (PSI) distribution of alternative splicing events across different TCGA cancers and GTEx tissues. Differential alternative splicing analyses showed a significant difference in alternative splicing events between TCGA tumor tissue and GTEx normal tissue in LUSC and LUAD but no significant differences in alternative splicing events between TCGA tumor tissue and adjacent normal tissue (**Supplementary Figure S5B**). In addition, the PanCox plot showed that based on the median and optimal PSI cutoffs, the PSI distribution of *CLEC5A* alternative splicing events was significantly negatively correlated with progression-free survival (PFS) in HNSC, KIRC, BRCA and positively associated with PFS in PAAD and



**FIGURE 4 |** *CLEC5A* CNVs in human cancers. **(A)** CNV profiles of *CLEC5A*, *CD68*, *CD163*, *MRC1*, *CD8A*, and *CD8B* in 33 cancer types with the GSCALite platform. Hete Amp, heterozygous amplification; Hete Del, heterozygous deletion; Homo Amp, homozygous amplification; Homo Del, homozygous deletion. **(B)** In pan-cancer, homozygous amplification or deletion CNV of *CLEC5A*, *CD68*, *CD163*, *MRC1*, *CD8A*, and *CD8B*. Blue bubbles represent deletion and red bubbles represent amplification, whereas the size of bubbles refers to the percentage of CNV. **(C)** In pan-cancer, heterozygous amplification or deletion CNV of *CLEC5A*, *CD68*, *CD163*, *MRC1*, *CD8A*, and *CD8B*. **(D)** Correlation between CNV and mRNA expression of *CLEC5A*, *CD68*, *CD163*, *MRC1*, *CD8A*, and *CD8B* in pan-cancer. Blue bubbles represent a negative correlation, whereas red bubbles represent a positive correlation, and the darker the color, the higher the correlation. The size of each bubble means statistical significance.

LUAD, while negatively correlated with OS in LUSC, LUAD, HNSC and positively associated with OS in PAAD (Supplementary Figures 5C, D), indicating the existence of survival-associated *CLEC5A* alternative splicing events.

### CLEC5A Expression Correlates With Immune Cells Infiltration in Human Cancers

To identify the role of *CLEC5A* in the immune infiltration process, we evaluated the correlation between *CLEC5A*

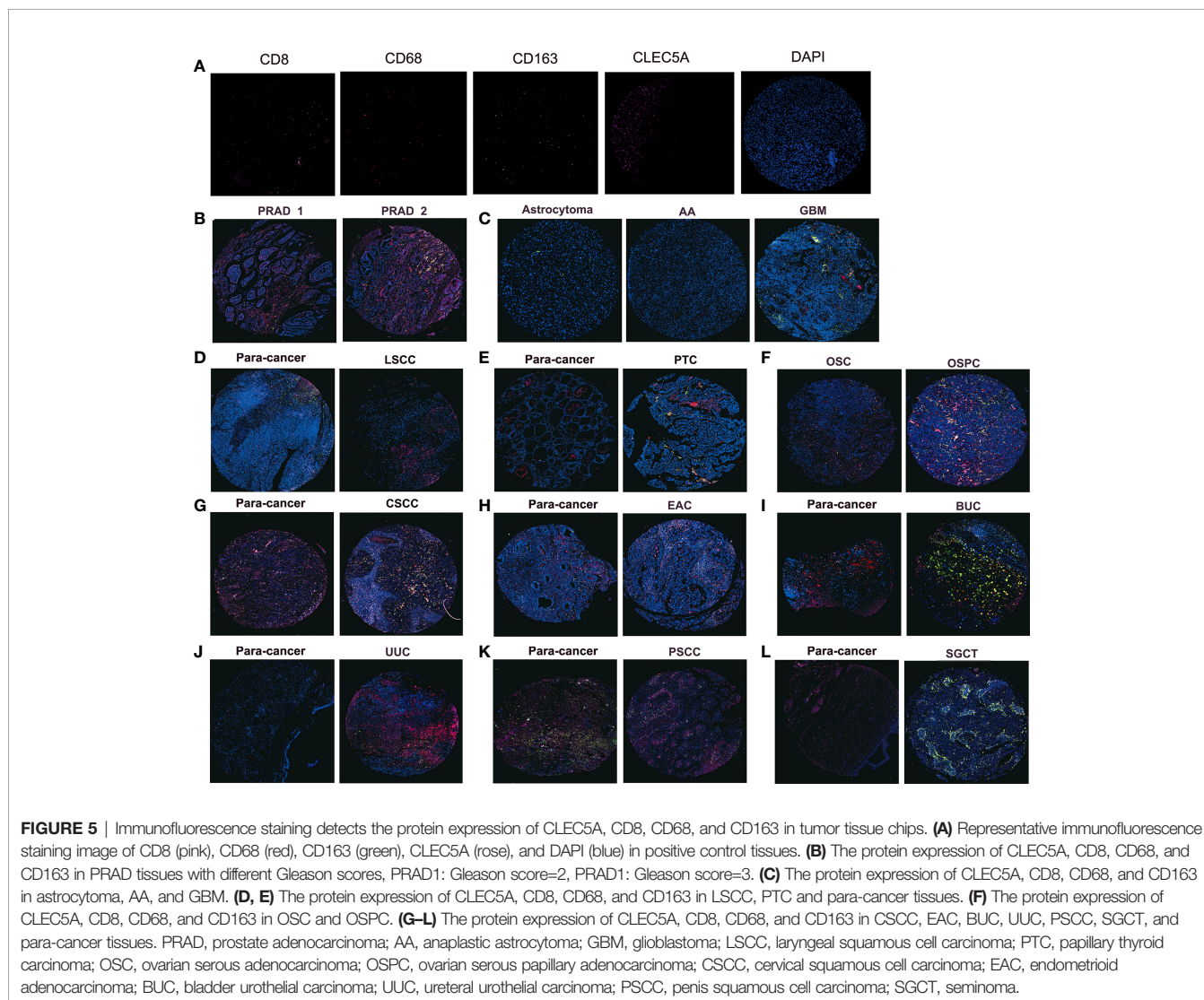
expression and immune and stromal components and tumor purity in pan-cancer. The ESTIMATE score indirectly reflected the tumor purity, and immune and stromal scores represented the immune and stromal components that indirectly reflected tumor purity. Supplementary Figures 6A–C exhibited the top 6 cancer types with a high immune score, ESTIMATE scores, and stromal scores. *CLEC5A* expression was positively correlated with an immune score in BLCA, COAD, KICH, IGG, OV, and THCA. Meanwhile, *CLEC5A* expression was positively correlated with stromal components abundance in COAD, DLBC, KICH, LUSC,



PAAD, and READ. In addition, ESTIMATE score showed a positive correlation with *CLEC5A* expression in BLCA, COAD, KICH, LUSC, OV, and READ, with  $r^2 > 0.6$ . Tumor progression is intimately related to the alteration of immune infiltration and TME composition in cancers. The above results indicated that *CLEC5A* was highly involved in immune infiltration and diverse TME components formation in the above cancers.

Furthermore, we explored the relationship between *CLEC5A* expression and specific immune cells infiltration in human cancer. The results showed that *CLEC5A* expression was positively correlated with the immune infiltration levels of macrophages, cancer associated fibroblast, and Tregs, and negatively correlated with MDSC abundance (**Supplementary Figure S6D**). Notably, we performed immunofluorescence staining on tumor tissue chips, and CD8 positive T cells were labeled with pink fluorescence, CD68 positive macrophages were labeled with red fluorescence, CD163 positive macrophages were labeled with green fluorescence, *CLEC5A* was labeled with rose-red fluorescence, and DAPI was

used to counterstain the nucleus (**Figure 5A–L**). In PRAD (prostate adenocarcinoma) tissues, *CLEC5A* expression was increased to accompany the increase of the Gleason score and the infiltration of macrophages, M2-type macrophages, and T cells also increased (**Figure 5B**). In GBM, *CLEC5A* was higher than that in astrocytoma and anaplastic astrocytoma, accompanied by an increase in M2-type macrophage infiltration (**Figure 5C**). *CLEC5A* expression was significantly higher in LSCC (laryngeal squamous cell carcinoma), PTC (papillary thyroid carcinoma) and UUC (ureteral urothelial carcinoma) than in para-cancer tissues, and was positively correlated with the infiltration proportion of macrophages (**Figures 5D, E, J**). Compared with OSC (ovarian serous adenocarcinoma), *CLEC5A* expression was higher in OSPC (ovarian serous papillary adenocarcinoma) and was significantly correlated with the increased infiltration of macrophages, M2-type macrophages, and T cell (**Figure 5F**). These results suggested that *CLEC5A* may be involved in the immune infiltration process and play an essential role in the immune-tumor interaction.





correlated with EPCAM in CESC, COAD, KIRC, LGG, LUAD, LUSC, PADD, and THCA (**Figure 6B**). MSI, TMB, and neoantigens are all intimately related to tumor initiation and progression, and they can independently predict the efficacy of tumor immunotherapy. Next, we conducted the correlation analysis between *CLEC5A* expression and MSI, TBM, and neoantigens. The results showed that *CLEC5A* expression was significantly positively correlated with MSI in LGG, COAD, PRAD, UCEC, THYM, SARC, STAD, and SKCM, but negatively correlated with MSI in LUSC (**Figure 6C**). In addition, positive correlation was observed between *CLEC5A* expression and TMB in COAD, READ, SARC, and UCEC, whereas negative correlation was found between *CLEC5A* expression and TMB in HNSC, LUAD, and LUSC (**Figure 6D**). As for neoantigen, it was positively correlated with *CLEC5A* expression in KIRP, UCEC, COAD, and STAD but negatively correlated with neoantigen in HNSC (**Supplementary Figure S7**). These results indicated that *CLEC5A* expression might influence tumor initiation and play a vital role in predicting the efficacy of tumor immunotherapy.

Numerous studies have found that abnormal DNA methylation catalyzed by DNMTs is involved in the occurrence and progression of various cancers (37, 38). We investigated the correlation between *CLEC5A* expression and four essential DNMTs expressions in pan-cancer. As shown in **Figure 6E**, *CLEC5A* expression was significantly correlated with the expression of *DNMT* signatures in more than 20 tumors. The result suggested *CLEC5A* may respond to oncogenesis by influencing DNA methylation.

## Enrichment Analysis of *CLEC5A*-Related Biological Functions in Pan-Cancer

The OPENTARGET platform was used to identify *CLEC5A*-related diseases network. The results showed that *CLEC5A* is highly associated with nervous system diseases, immune system diseases, infectious diseases, and musculoskeletal or musculoskeletal or connective tissue diseases (**Figure 7A**). To study the molecular mechanism of *CLEC5A* in tumorigenesis, we used the STRING tool to predict *CLEC5A*-binding proteins and conducted functional enrichment analysis for *CLEC5A*-related genes using GSEA. As shown in **Figure 7B**, we obtained 10 *CLEC5A*-binding proteins, including *TREM2*, *SIGLEC1*, *FCER1G*, *SYK*, *HCST*, *SIRPB1*, *TREM1*, *TYROBP*, *TSPAN6*, and *MGAM*. KEGG enrichment analysis showed that the top three enriched terms related to the *CLEC5A* expression included Leishmania infection, leukocyte trans-endothelial migration, and hematopoietic cell lineage (**Figure 7C**). KEGG enrichment analysis revealed that *CLEC5A*-related partners in pan-cancer were involved in B cell receptor signaling, T cell receptor signaling, and JAK-STAT signaling (**Supplementary Figure S8A**). HALLMARK enrichment analysis found that the top three enriched terms related to the *CLEC5A* expression included IL6-JAK-STAT3 signaling, inflammatory response, and KRAS signaling up (**Figure 7D** and **Supplementary Figure S8B**). Moreover, GSVA of GO terms related to *CLEC5A* expression was performed in pan-cancer (**Figure 7E**).

*CLEC5A* significantly correlated with activation and migration of immune infiltrating cells such as T cells, macrophages, and mast cells. All these suggested that *CLEC5A* participates in tumorigenesis by impacting the immune response.

Then, GSCALite was utilized to investigate the impact of the gene set on tumor pathway activity. The pie chart showed that *CLEC5A* had an activation effect on the EMT pathway and an inhibitory effect on DNA damage response and the hormone AR pathway. All 6 genes could activate EMT, apoptosis, and hormone ER pathway and inhibit the hormone AR pathway (**Figure 7F**). In addition, the percentage of cancers in which mRNA expression of 6 specific genes (*CD68*, *MRC1*, *CD8A*, *CD8B*, *CD163*, and *CLEC5A*) had a potential impact on pathway activity were also summarized in **Figure 7G**. *CLEC5A* mediated activation of the EMT signaling pathway in 35% of cancers, while *CLEC5A* mediated inhibition of DNA damage response and hormone AR pathway in 18% and 25% of cancers, respectively.

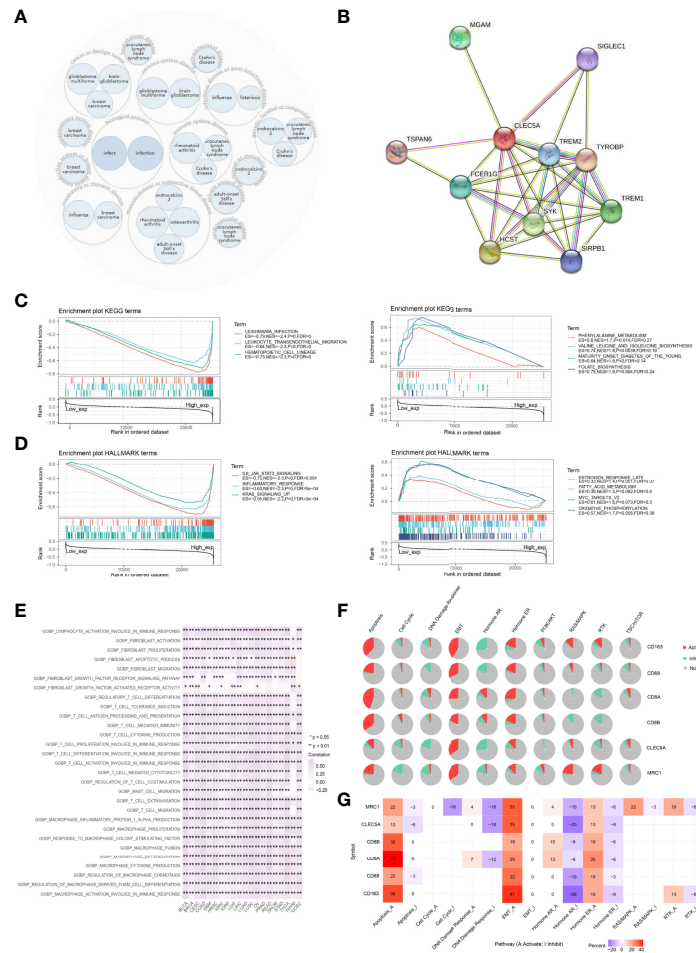
## Immunotherapy Agent Response Associated With *CLEC5A* Expression

To investigate the correlation between *CLEC5A* expression in tumor cells and their sensitivity to immunotherapy agents, *CLEC5A* expression levels across tumor cell lines were compared between pre- and post-cytokine-treated samples. As shown in **Figure 8A**, IFN $\gamma$  treatment significantly down-regulated *CLEC5A* expression in MOC2 cells, while IFN $\gamma$ , IFN $\beta$ , TNF $\alpha$ , and TGF $\beta$ 1 treatment did not change substantially *CLEC5A* expression in 4T1, B16, CT26, E0771, EMT6, KPC, LLC, MC38, MOC1, MOC22, Panc02, and Renca cells. We then compared the *CLEC5A* expression level across different tumor cell lines between pre- and post-ICB treatment and responders and non-responders. *CLEC5A* expression was significantly increased in B16 cells and T11 cells that responded to anti-CTLA4 and anti-PD-1 treatment. In addition, *CLEC5A* expression was increased in MOC22 cells that responded to anti-PD-1 therapy, and *CLEC5A* expression was increased in EMT6 that responded to anti-PDL-1 treatment. In contrast, *CLEC5A* expression was significantly decreased in CT26 cells that responded to anti-PD-1 therapy (**Figure 8B**).

Next, we evaluated the correlation between *CLEC5A* expression and the response outcomes and OS of the human immunotherapy cohort. The results showed that *CLEC5A* alone had an AUC of > 0.5 in 14 of the 25 immunotherapy cohorts (**Figure 8C**). Moreover, *CLEC5A* showed a higher predictive value than TMB, T. Clonality, and B. However, *CLEC5A* had lower predictive value scores than the CD274 score (AUC > 0.5 in 21 immunotherapy cohorts), CD8 score (AUC > 0.5 in 18 immunotherapy cohorts), IFNG score (AUC > 0.5 in 17 immunotherapy cohorts), and Merck 18 score (AUC > 0.5 in 18 immunotherapy cohorts). Above all, *CLEC5A* expression can function as an effective biomarker for predicting immunotherapy response.

## Identify Potential Therapeutic Agents Associated With *CLEC5A* Expression

According to the gene expression, survival analysis, mutation analysis, and immune infiltration analysis of *CLEC5A* in pan-cancer, we confirmed that *CLEC5A* might play an essential role



**FIGURE 7 |** Functional annotation of *CLEC5A* in pan-cancer. **(A)** *CLEC5A* related diseases network. **(B)** *CLEC5A*-binding proteins were obtained using the STRING tool. **(C)** KEGG enrichment analysis related to *CLEC5A* expression using GSEA. **(D)** HALLMARK enrichment analysis related to *CLEC5A* expression using GSEA. **(E)** GSEA of GO terms associated with *CLEC5A* expression in pan-cancer. **(F)** The pathway activity module of GSCALite was used to investigate the impact of the gene set (*CLEC5A*, *CD68*, *CD163*, *MRC1*, *CD8A*, and *CD8B*) on tumor pathway activity in pan-cancer. **(G)** The percentage of cancers in which mRNA expression of 6 specific genes (*CLEC5A*, *CD68*, *CD163*, *MRC1*, *CD8A*, and *CD8B*) had a potential impact on pathway activity.

in tumorigenesis and tumor immunity, suggesting its enormous potential as a tumor therapeutic target. To identify potential therapeutic agents associated with *CLEC5A* expression, we conducted a Spearman correlation analysis between *CLEC5A* expression and drug sensitivity (*IC50*) via the GSCALite website. The results indicated that *CLEC5A* expression was negatively correlated with *IC50* of small molecule drugs such as FR-180204, Tivozanib, OSII-930, Linifanib, AC220, VNLG/124, Bexarotene, Omacetaxine mepesuccinate, narclaslaine, leptomyacin B, PHA-793887, LRRK2-IN-1, and CR-1-31B, but positively associated with *IC50* of JW-55 (**Supplementary Figure S9**).

## DISCUSSION

Pan-cancer analysis can reveal the similarities and differences of tumors, which provide theoretical support for cancer prevention,

therapeutic target design, and potential therapeutic drug screening (39). Emerging publications indicated that *CLEC5A* plays a vital role in immune-inflammation response covering macrophage activation, proinflammation cytokines releasing, and neutrophil extracellular trap (NET), and *CLEC5A* high expression appears in diverse diseases, especially tumors (40–43). Although *CLEC5A* has been studied in glioma, gastric cancer, breast cancer, and ovarian cancer (15–20), its roles in pan-cancer remain unclear. In the present study, we performed a comprehensive bioinformatics analysis to reveal the abnormal expression, DNA methylation, alteration, and immune landscape in pan-cancer, which vary significantly among different cancers. We found that gene mutations and methylation levels of *CLEC5A* can lead to abnormally high *CLEC5A* expression, which is significantly associated with increased immune infiltration of macrophages, CAF, and Treg and poor prognosis of various cancers. In addition, a significant association exists



with *DNMT1*, *DNMT2*, *DNMT3*, and *DNMT4* in more than 20 tumors. These results suggested that aberrantly overexpressed CLEC5A plays a vital role in tumorigenesis, which may be related to MMR gene level and DNA methylation.

Tumor immune microenvironment (TIME), an essential part of TME, intimately correlates with tumor immunity accounting for tumor progression (46–48). Immune cells are the main components of TIME (49, 50). Macrophages belong to innate immunity, which is the first defense line for anti-tumor immunity (51–54). The different activation states of macrophages (M1, M2) contribute to anti-tumor and pro-tumor effects, respectively (55, 56). A previous study has revealed CLEC5A as an M2 biomarker. We conducted an immune infiltration analysis. The results implicated that *CLEC5A* expression was positively correlated with immune infiltration levels of macrophages, CAF, and Tregs, whereas it is negatively correlated with MDSC in most cancer types. Tumor purity, a key index reflecting the condition of TME, was indirectly evaluated by the ESTIMATE score, and the distribution of immune and stromal components in TME was reviewed by the immune score and stromal score (57). The present study showed that *CLEC5A* expression positively correlates with an immune score in BLCA, COAD, KICH, LGG, OV, and THCA, with the ESTIMATE score in BLCA, COAD, KICH, LUSC, OV, and READ. These results indicated that CLEC5A might be involved in tumor immunity regulation by mediating immune infiltration. In addition, more than 30 immune checkpoints were positively correlated with *CLEC5A* expression in LGG, OV, and PAAD. The strong correlation between CLEC5A and immune checkpoints suggested that CLEC5A might be an ideal tumor immunotherapeutic target and play specific roles in tumor immunotherapy response and outcome.

It is no doubt that CLEC5A participates in tumorigenesis; the underlying mechanism remains unclear. We implemented the functional enrichment analysis to identify the biological roles of CLEC5A in pan-cancer. The results showed that in addition to activation of EMT and apoptosis and the inhibition of DNA damage response, CLEC5A high expression was mainly related to leukocyte trans-endothelial migration, inflammatory response, and two signaling pathways (IL6-JAK-STAT3 and KRAS signaling). It implicated that CLEC5A is related to tumor initiation and metastasis. Previous studies on dengue virus infection have revealed the role of CLEC5A in disease development and the efficacy of CLEC5A silencing or inhibition in disease treatment (58, 59). Whether CLEC5A can be a target for cancer treatment still needs more experimental exploration. In addition, we revealed potential therapeutic agents by analyzing the correlation between CLEC5A expression and drug sensitivity, which verified the role of CLEC5A as a potential target for tumor treatment.

Even though the pan-cancer analysis covers comprehensive information, this study is still limited. Most results were based on data analysis, and more experimental verification is essential to

provide solid evidence. Previous studies have shown that anti-CLEC5A mAb can be applied to treat viral infection with good clinical efficacy. However, whether it can be used as a tumor treatment drug remains to be studied further.

## CONCLUSIONS

In summary, we proved that CLEC5A expression is associated with immune infiltration and affects immunotherapy sensitivity patient's prognosis in pan-cancer, suggesting that CLEC5A can serve as a biomarker for tumor immunity and prognosis of a potential promising anti-tumor therapeutic target.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

## AUTHOR CONTRIBUTIONS

WW performed the data analysis and drafted the figures. S-YC wrote the manuscript. ZZL revised the manuscript. RC, QC, and W-JZ conceived and designed the study. Z-PW, ZL, JZ, PL, JY, and LZ supervised the study. All authors approved the submitted version of the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2022.831542/full#supplementary-material>

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