Comprehensive analysis of signaling lymphocyte activation molecule family as a prognostic biomarker and correlation with immune infiltration in clear cell renal cell carcinoma

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Abstract. Clear cell renal cell carcinoma (ccRCC) is a common type of kidney cancer and accounts for 2-3% of all cancer cases. Furthermore, a growing number of immunotherapy approaches are being used in antitumor treatment. Signaling lymphocyte activation molecule family (SLAMF) members have been well studied in several cancers, whereas their roles in ccRCC have not been investigated. The present study comprehensively assessed the molecular mechanisms of SLAMF members in ccRCC, performed using The Cancer Genome Atlas database, with analysis of gene transcription, prognosis, biological function, clinical features, tumor-associated immune cells and the correlation with programmed cell death protein 1/programmed death-ligand 1 immune checkpoints. Simultaneously, the Tumor Immune Dysfunction and Exclusion algorithm was used to predict the efficacy of immune checkpoint blockade (ICB) therapy in patients with high and low SLAMF expression levels. The results demonstrated that all SLAMF members were highly expressed in ccRCC, and patients with high expression levels of SLAMF1, 4, 7 and 8 had a worse prognosis that those with low expression. SLAMF members were not only highly associated with immune activation but also with immunosuppressive agents. The level of immune cell infiltration was associated with the prognosis of patients with ccRCC with high SLAMF expression. Moreover, high ICB response rates were observed in patients with high expression levels of SMALF1 and 4. In summary, SLAMF members may serve as future potential biomarkers for predicting the prognosis of ccRCC and emerge as a novel immunotherapy target.

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Abbreviations: AUC, area under curve; BP, biological process; CAF, cancer-associated fibroblast; CAM, cell adhesion molecule; CC, cellular component; RCC, renal cell carcinoma; ccRCC, clear cell RCC; GEPIA, Gene Expression Profiling Interactive Analysis; GO, Gene Ontology; GSEA, gene set enrichment analysis; GTEx, Genotype-Tissue Expression; ICB, immune checkpoint blockade; ICIs, immune checkpoint inhibitors; KEGG, Kyoto Encyclopedia of Genes and Genomes; KIRC, kidney renal clear cell carcinoma; MF, molecular function; MHC, major histocompatibility complex; OS, overall survival; ROC, receiver operating characteristic; SCNA, somatic copy number alteration; SLAMF, signaling lymphocyte activation molecule family member; TAM, tumor-associated macrophage; TCGA, The Cancer Genome Atlas; TIDE, Tumor Immune Dysfunction and Exclusion; TME, tumor microenvironment

Key words: SLAMF, ccRCC, prognosis, immune checkpoints, immunotherapy

Introduction

Renal cell carcinoma (RCC) is one of the most common cancers in the world, ranking sixth and tenth in men and women, respectively (1). Clear cell (cc)RCC accounts for 75-80% of all instances of RCC, with the remaining % comprised of several non-cc cancer subtypes (2,3). The incidence of ccRCC, also known as kidney renal clear cell carcinoma (KIRC), has progressively increased in recent years, accounting for ~5% of adult malignant tumors (4,5). Therefore, its treatment and development warrant increased attention. Early ccRCC is often overlooked by patients due to its mild symptoms, such as fever and fatigue, with ~30% of patients already presenting with metastasis at the time of diagnosis (6-8). Although surgery can be used for tumor removal, ~25% of patients still experience relapse and metastasis (9). The 5-year survival rate is notably high at 90% for early ccRCC but drops to ~33.3% for advanced cases (10).

In recent years, the widespread use of immune checkpoint inhibitors (ICIs) in tumor therapy, especially for ccRCC, has marked revolutionary progress. Immunotherapy demonstrates a significant advantage over conventional chemotherapy, radiation therapy and standard tyrosine kinase inhibitor therapy, either alone or combined with other agents such as nivolumab plus ipilimumab or cabozantinib, pembrolizumab plus axitinib or lenvatinib, and avelumab plus axitinib (11). Additionally, several biological target molecules associated with the diagnosis and prognosis of RCC immunotherapy have been elucidated, including programmed cell death ligand 1 (PD-L1), tumor mutation burden and specific single gene mutations, such as Von Hippel-Lindau, polybromo 1, SET domain containing 2, breast cancer gene 1-associated protein 1 and lysine demethylase 5C. Moreover, DNA-damage repair gene alterations, including common mutations like checkpoint kinase 2, ataxia telangiectasia mutated kinase, MutS homolog 6 and MutY DNA glycosylase, are associated with immunotherapy (12). There are highly infiltrating immune cells in ccRCC, and it was one of the first cancers used in immunotherapy (13); however, the molecular mechanism of ccRCC development is still unclear. Furthermore, the results of a meta-analysis by Santoni *et al* (14) demonstrated that sex-related differences in the efficacy of ICIs remain an important but undiscussed aspect in cancer immunotherapy trials. In addition, clinical trials should emphasize assessment such as quality of life (15) and European Cancer Organization performance status (16) when evaluating the treatment effect of ICIs in RCC.

The signaling lymphocyte activation molecule family (SLAMF) is a group of receptors that are closely associated with the immune system. They are mainly expressed in hematopoietic cells and immune cells, and they have also been reported in cancer cells (17,18). The family has nine members, including SLAMF1 (CD150), SLAMF2 (CD48), SLAMF3 (CD299; Ly-9), SLAMF4 (CD244, 2B4), SLAMF5 (CD84), SLAMF6 (CD352; Ly108; NTB-A), SLAMF7 (CD319; CRACC; CS1), SLAMF8 (CD353; BLAME) and SLAMF9 (CD84H1; CD2F10; SF2001) (19). Increasing research results indicate that SLAMFs serve a crucial role in tumor immune regulation (18,20). SLAMF1 and SLAMF3 have been reported to be the key genes regulating immune infiltration in ovarian cancer (21). The activation of SLAMF5 upregulates PD-L1 expression and myeloid-derived suppressor cells formation, inhibiting T cell function (22). Reduced SLAMF7 in mouse models leads to decreased programmed cell death protein 1 (PD-1) in CD8^{+} T cells, reducing T cell failure and tumor progression (23). Additionally, SLAMF4 and SLAMF6 are associated with immune cell exhaustion (24,25). However, the molecular mechanism of SLAMFs in ccRCC remains insufficiently investigated.

In the present study, the expression of SLAMFs in ccRCC was analyzed on the basis of The Cancer Genome Atlas (TCGA) and Genotype-Tissue Expression (GTEx) data. The relationship between SLAMFs expression and prognosis in ccRCC was assessed using the Kaplan-Meier database. Finally, the Tumor Immune Estimation Resource (TIMER) was used to assess the correlation of SLAMFs with immune infiltration. The findings of the present study contribute to the understanding of the molecular mechanisms of SLAMFs in ccRCC development.

Materials and methods

mRNA expression levels of SLAMFs in cancers. TCGA (https://portal.gdc.cancer.gov/) is a comprehensive cancer analysis database that contains data, such as mRNA expression, micro mi(RNA) expression, patient clinical information and methylation. The GTEx portal (https://www.genome.gov/Funded-Programs-Projects/Genotype-Tissue-Expression-Project) serves as a database for transcriptome sequencing of human tissues (26). Data from the TCGA and GTEx databases were downloaded and processed for analysis in the present study (27). The mRNA levels of SLAMFs in 33 cancer and paracancerous tissues were assessed using R software (version 4.2.0; The R Foundation). Additionally, the transcription levels of SALMF1/4/7/8 in ccRCC cell lines were evaluated based on GSE20491 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE20491).

Analysis of SLAMFs with clinical characteristics. Gene Expression Profiling Interactive Analysis (GEPIA)2 (http://gepia2.cancer-pku.cn/#index) is a comprehensive web server designed for large-scale expression profiling and interactive analysis. It encompasses modules such as gene expression modules, prognostic modules and clinical features modules (28). The University of Alabama at Birmingham Cancer data analysis Portal (UALCAN; http://ualcan.path.uab.edu/index.html) is a dedicated portal for facilitating tumor subgroup gene expression and survival analyses (29). The correlations of SLAMFs with clinical features of ccRCC, such as cancer stage, ethnicity, sex, age, tumor grade and lymph node metastasis, were assessed using the GEPIA2 and UALCAN databases.

Analysis of diagnostic value and survival rate. Receiver operating characteristic (ROC) analysis, a widely accepted method for analyzing and comparing diagnostic accuracy (30), was used to assess the diagnostic value of SLAMFs in ccRCC. The area under the curve (AUC) from the ROC curves, indicative of the diagnostic value, was calculated using data from the TCGA database. To evaluate the impact of SLAMFs on survival in cancers, especially ccRCC, the Kaplan-Meier Plotter (http://kmplot.com/analysis) was used. This platform incorporates data on 30,000 genes (mRNA, miRNA and protein) and covers 21 types of tumors (31). The overall survival (OS) of SLAMFs in ccRCC was analyzed using the Kaplan-Meier Plotter with default settings, and the data set of kidney renal clear cell carcinoma (n=530) was used for this analysis.

Analysis of gene mutations and correlation. The cBioPortal website (https://www.cbioportal.org/) serves as a comprehensive database for assessing the genomic features of tumors. Currently, it stores genomic information, such as DNA copy number data, mRNA and miRNA expression data, non-synonymous mutations and protein level and phosphoprotein level data. Gene alterations of SLAMFs were evaluated using the cBioPortal portal (32,33). Furthermore, an analysis of the correlations and co-expression of genes with SLAMFs in ccRCC was performed using the TCGA database. Genes with a correlation coefficient >0.6 and P<0.001 were screened. The

intersection of co-expressed genes of SLAMFs was depicted using an online Venn diagram tool (http://bioinformatics. psb.ugent.be/webtools/Venn/). Subsequently, the top 20 co-expressed genes were selected, and their correlations to SLAMFs were plotted.

Gene enrichment analysis. The Gene Ontology (GO) database (http://geneontology.org/) categorizes protein function into three components based on its biological information: Biological process (BP), cellular component (CC) and molecular function (MF). Additionally, the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (https://www.genome. jp/kegg/) was used to assess the pathways associated with the genes. To evaluate the molecular mechanism of ccRCC, GO/KEGG analyses of the co-expressed genes related to SLAMFs were performed. The threshold for GO/KEGG analysis was P.adj<0.05, and the threshold for gene count with the default setting was >1. Furthermore, gene set enrichment analysis (GSEA) of the co-expression genes of SLAMFs was performed (34). The gene sets used for GSEA were obtained from the Molecular Signatures Database (https://www. gsea-msigdb.org/gsea/msigdb/index.jsp).

Analysis of immune cell infiltration and somatic copy number. TIMER (https://cistrome.shinyapps.io/timer) is a web server designed for the comprehensive analysis of tumor-infiltrating immune cells (TIICs) in several cancer types based on TCGA (35). The abundance of TIICs, including B cells, CD4^{+} T cells, CD8^{+} T cells, neutrophils, macrophages and dendritic cells, was evaluated using the TIMER portal. The estimate algorithm was used to analyze the immune score in the overall internal environment of ccRCC. Furthermore, the somatic copy number alterations (SCNA) module in TIMER was used to assess the relationship between SLAMFs SCNA and tumor immune-related cell infiltration levels (35). Additionally, the Tumor Immune Dysfunction and Exclusion (TIDE) score was used to assess the efficacy of immune checkpoint blockade (ICB) in ccRCC with high and low expression of SLAMF (4,9,17,21,36).

Correlation analysis of SLAMFs with immunoinhibitory checkpoints. TIMER2.0 (http://timer.cistrome.org/) offers three modules, immunity, exploration and estimation, to assess the associations between immune infiltrates and genetic or clinical features based on the TCGA cohorts (37). In comparison with TIMER, TIMER 2.0 provides more detailed information on immune infiltrates cells. The present study used TIMER 2.0 to evaluate the correlation between three tumor-related immune cells [(M2, Treg and cancer-associated fibroblasts (CAFs)] and the expression of SLAMFs by three algorithms (EPIC, QUANTISEQ and CIBERSORT-ABS). Additionally, using R software (version 4.2.0), the correlations between PD-1, PD-L1, PD-L2 and SLAMF were assessed.

Correlation analysis of SLAMFs with immune-related molecules. Chemokine/chemokine receptors serve a crucial role in the development and homeostasis of the immune system, influencing several immune and inflammatory responses (38). Furthermore, chemokine/chemokine receptors are implicated in several essential steps of tumor metastasis, such as tumor

cell adhesion, vascular extravasations, metastatic colonization, angiogenesis and proliferation (39). In addition, major histocompatibility complex (MHC) molecules are key players in antigen presentation and the induction of immune responses (40). Therefore, an analysis was performed to assess the correlations between SLAMFs expression and chemokines/chemokine receptors, as well as with MHC molecules in ccRCC.

Effects of immune cells combined with SLAMFs on the prognosis of ccRCC. The Kaplan-Meier plotter website was used to analyze the impact of SLAMFs on the prognosis of ccRCC considering changes in the content of immune cells. Using the four types of immune cells available in the TIMER database (https://cistrome.shinyapps.io/timer) (35), namely B cells, CD4^{+} T cells, CD8^{+} T cells and macrophages, their effects on the prognosis of patients with ccRCC in relation to SLAMFs expression was assessed.

Cell culture and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The cell lines were purchased from Pricella (Procell Life Science & Technology Co., Ltd.), and the reverse transcription kit was purchased from Thermo Fisher Scientific, Inc. Human ccRCC 786-O and 769-P cell lines were cultured in an incubator (37˚C; 5% CO_2) in RPMI 1640 medium containing 10% fetal bovine serum (cat. no. C04001; ViaCell Inc.) and 1% penicillin/streptomycin (cat. no. C0222; Beyotime Institute of Biotechnology) for 2 days. To assess the expression levels of SLAMF1/4/7/8 in the ccRCC cell lines, cell clusters from 786-O and 769-P were extracted. Total RNA was extracted with RNAiso PLUS (cat. no. 9108; Takara Bio, Inc.), and then cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit (cat. no. K1622; Thermo Fisher Scientific, Inc.) according to the instructions provided by the manufacturer. qPCR was performed with UltraSYBR Mixture containing high ROX (cat. no. CW2602M; CoWin Biosciences) following the manufacturer's instructions. The mRNA expression of SLAMF members in ccRCC cell lines was assessed using the human kidney HK-2 cell line as control cells and 18S rRNA as an internal reference. The primers for RT-qPCR were designed by PrimerBank (The Massachusetts General Hospital; https://pga.mgh.harvard.edu/primerbank/index.html) (41) and the sequences are as follows: SLAMF1 (NM_003037), sense: 5'-GGAGAACAGTGTCGAGAACAAA-3' and antisense: 5'-CGTATCCCCAGGGTGAGATTC-3'; SLAMF4 (also known as CD244, NM_001166663), sense: 5'-TCGTGATTCTAAGCGCACTGT-3' and antisense: 5'-CAGGTTCTTGTGACGTGGGAG-3'; SLAMF7 (NM_021181), sense: 5'-GGCAGCTCACAGGGTCAG-3' and antisense: 5'-GGGTTGTGTTGAAGGTCCAGA-3'; SLAMF8 (NM_020125), sense: 5'-CTTCTCTGGGAAGATGCAGTG-3' and antisense: 5'-TTTCGCTGATGTTGGGGGC-3'; 18S rRNA, sense: 5'-GTAACCCGTTGAACCCCATT-3' and antisense: 5'-CCATCCAATCGGTAGTAGCG-3' (42). Relative mRNA expression was calculated using the comparative $2^{-\Delta\Delta Cq}$ method (43).

Statistical analysis. All analyses were performed using R software (version 4.2.0; The R Foundation; https://www.r-project. org/) (44), with graph making and statistical analysis using

the Xiantao Academic Platform (www.xiantaozi.com) and GraphPad (version 9.5.1). The Wilcoxon rank-sum test was used to analyze differences in expression in Figs. 1A, S1, 5E, 8B and 13. The paired t-test was used to analyze differences in Fig. 1C. One-way ANOVA was performed on the GEPIA website to analyze the differences in gene expression between pathological stage in Fig. 2A-I. One-way ANOVA with Tukey's honestly significant difference test was used for analysis of Fig. 5A-D and F. Spearman's rank was used for correlation analysis. pROC package (version 1.17.0.1) (https://cran.r-project.org/web/packages/pROC/index.html) was used for the ROC curve analysis, whilst the clusterprofiler package (version 3.14.3) was used for enrichment analysis and visualization (45). The estimate package (version 1.0.13) (https://rdrr.io/rforge/estimate/) was used to analyze the immune score (46). P<0.05 was considered to indicate a statistically significant difference.

Results

Increased transcription levels of SLAMFs in ccRCC. To compare the expression of the SLAMFs in tumor with normal tissues, the mRNA levels of the SLAMFs were assessed using TCGA and GTEx data. The results revealed significantly elevated expression of SLAMFs in esophageal carcinoma, glioblastoma multiforme, KIRC, low-grade glioma, pancreatic adenocarcinoma, skin cutaneous melanoma and stomach adenocarcinoma compared with corresponding normal tissues (Figs. 1A and SI). Additionally, SLAMFs (excluding SLAMF4) demonstrated significantly high expression in ovarian cancer and testicular cancer compared with normal tissue (Figs. 1A and S1). Moreover, the expression of SLAMFs in ccRCC was analyzed using both paired and unpaired methods. The results demonstrated that the mRNA expression level of SLAMFs in tumor tissues were significantly higher than those in normal tissues (Fig. 1B and C).

SLAMFs are highly associated with clinical features. To assess the association between SLAMFs with clinical characteristics, two online analysis tools were used. The results from the GEPIA2 database indicated that SLAMF3 and 5 demonstrated no statistically significant association with ccRCC staging; however, SLAMF1, 2, 4, 6, 7, 8 and 9 were significantly associated with the staging of ccRCC (Fig. 2A-I). Additionally, results from the UALCAN database indicated that SLAMFs were significantly associated with ethnicity, sex, age, tumor grade, ccRCC subtypes and nodal metastasis status. Compared with lower grade and lower age group, higher expression of SLAMFs was observed with significantly higher tumor grade and age. The ccB subtype in ccRCC demonstrated a significantly stronger association with SLAMF expression compared with the ccA subtype (SLAMF1, 3, 7, 8 and 9) (Table SI). These findings suggest that SLAMFs may contribute to the progression of ccRCC.

Prognosis of SLAMFs and associations among members. The diagnostic value of SLAMFs was assessed using ROC analysis, revealing high diagnostic values for SLAMF1-8 with corresponding AUC values of 0.820, 0.892, 0.884, 0.928,

0.924, 0.907, 0.913 and 0.920, respectively. By contrast, SLAMF9 demonstrated a relatively low diagnostic value, with an AUC value of 0.614 (Fig. 2J-L). Furthermore, to assess the effect of SLAMFs on the survival rate of patients with ccRCC, the effect of high and low expression of SLAMFs on OS were compared (Fig. 3). The results indicated that a high expression of SLAMF1, 4, 7, 8 and 9 was significantly associated with a worse prognosis in ccRCC compared with the low expression groups; however, the expression of SLAMF2, 3, 5 and 6 had no significant association with the prognosis of ccRCC. These findings suggest that certain members of SLAMFs, particularly SLAMF1, 4, 7, 8 and 9, may contribute to a poor prognosis in ccRCC.

Mutation frequency and related genes in SLAMFs. A comprehensive analysis of the mutation, copy number and structure of the SLAMFs was performed, revealing their primary involvement in missense mutations and amplifications (Fig. 4A). According to the analysis results, the main alteration of SLAMFs gene in ccRCC was amplification, that is, the increase in gene copy number, which was associated with an increase in SLAMFs gene expression. This was consistent with the results shown in Fig. 1. Since SLAMFs have a significant effect on the prognosis of patients with ccRCC, the co-expressed genes of SLAMF1, 4, 7, 8 and 9 were screened. A notably strong correlation was revealed for SLAMF1, 4, 7 and 8 (Fig. 4B). Subsequently, co-expressed genes were screened for each SLAMF in ccRCC, resulting in 463 co-expressed genes identified for SLAMF1, 452 for SLAMF4, 544 for SLAMF7, and 488 co-expressed genes for SLAMF8. In total, 288 co-expressed genes were identified for SLAMF1, 4, 7 and 8 (Fig. 4C). Additionally, the association between SLAMF1, 4, 7 and 8 expression levels with different tumor-node-metastasis staging and grading was analyzed (Fig. 5A-D). In addition, the expression of SLAMF1, 4, 7 and 8 in ccRCC cell lines (786-O and 769-P) was higher compared with that in a normal renal epithelial cell line (HK-2) (Fig. 5E and F). Furthermore, the top 20 genes that were among the 288 co-expressed genes associated with SLAMF1, 4, 7 and 8 were assessed (Fig. 6) (P<0.05).

SLAMFs are associated with immune activation. GO/KEGG analysis was performed on 288 co-expressed genes associated with SLAMF1, 4, 7 and 8. The GO and BP analysis demonstrated significant associations between T cell activation, regulation of T cell activation, regulation of lymphocyte activation, leukocyte cell-cell adhesion and positive regulation of cell activation and SLAMF co-expression genes. CC analysis highlighted a significant association between T cell receptors, whilst MF analysis reveal a significant association with cytokines/cytokines receptor activity and SLAMF co-expression genes (P.adj<0.05; Fig. 7 and Table SII). KEGG pathway enrichment analysis demonstrated that SLAMFs co-expression genes were significantly associated with cell adhesion molecules (CAMs), hematopoietic cell and helper T 1, 2 and 17 immune cell differentiation, and T cell receptor signaling pathway (Fig. 7). In addition, KEGG enrichment analysis was performed using the top 20 co-expressed genes associated with SLAMF1, 4, 7 and 8 in (P.adj<0.05;
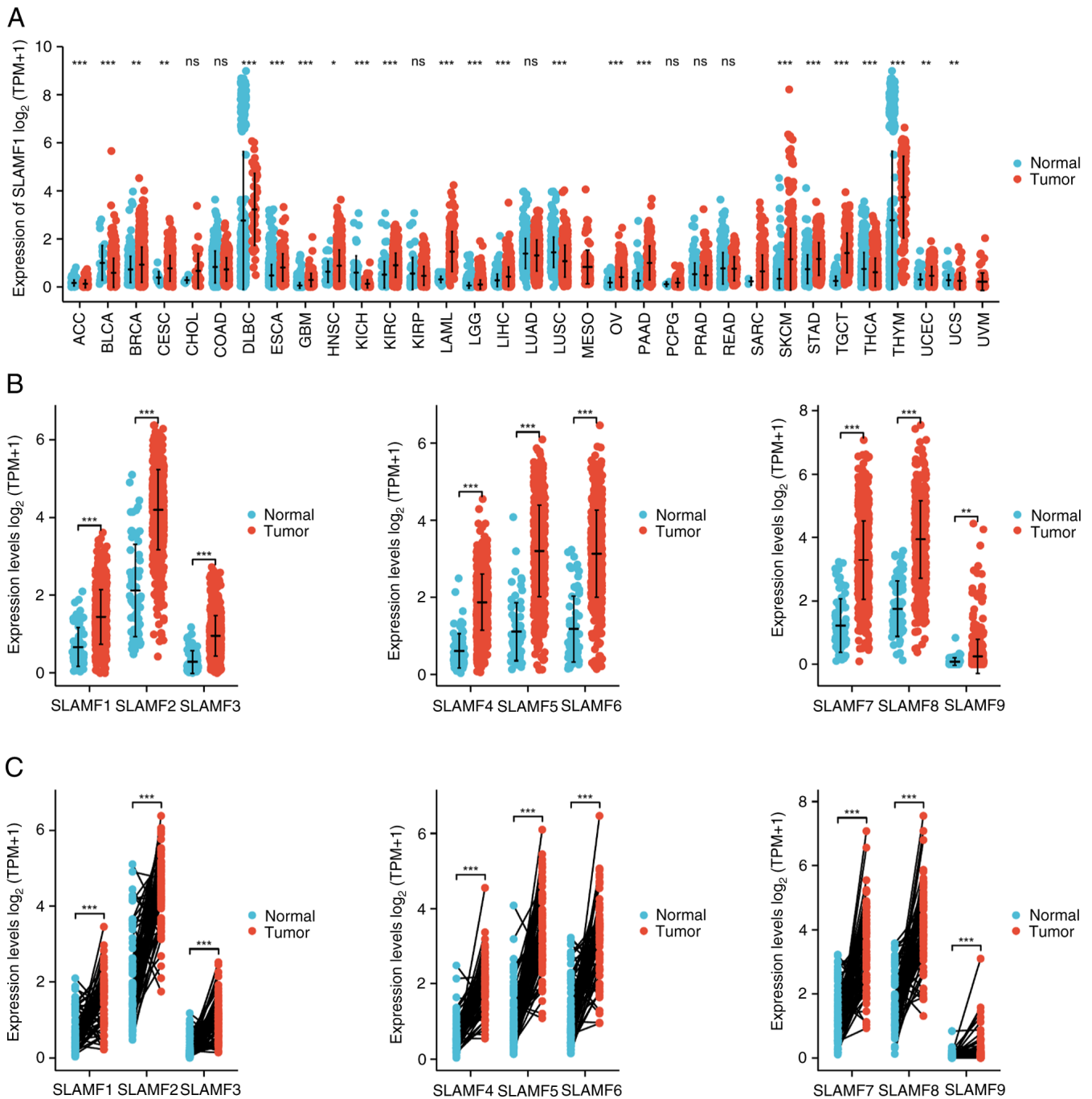
Figure 1. Expression of SLAMF in ccRCC and normal tissues. (A) Expression levels of SLAMF1 in 33 tumor and normal tissues. (B) Unpaired and (C) paired tissue expression of SLAMF family in ccRCC. $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$. SLAMF, signaling lymphocyte activation molecule family; ccRCC, clear cell renal cell carcinoma; TPM, transcript per million; ns, not significant.

Table SII). GSEA indicated involvement in pathways such as the Jak-stat signaling pathway, toll like receptor signaling pathway, B cell receptor signaling pathway and nod like receptor signaling pathway (Table SIII). These findings suggested that SLAMFs and their co-expressed genes may participate in the regulating of the immune system function and contribute to the development of ccRCC.

SLAMFs promote the infiltration of immune cells. The TIMER results indicated that SLAMF1, 4, 7 and 8 were positively correlated with infiltration levels of B cells, CD4+ T cells, CD8+ T cells, macrophages, neutrophils and dendritic cells in ccRCC (Fig. 8A). To further assess these findings, the correlations between SLAMF1, 4, 7 and 8 and immune cell gene markers were analyzed, including B cells, CD8+ T cells, T cells, monocytes, tumor-associated macrophages (TAMs), M1 macrophages, M2 macrophages and T cell exhaustion (Tables SIV-VII). The results demonstrated that SLAMF1, 4, 7 and 8 were significantly positively correlated with gene markers of immune cells. This suggests that SLAMFs can promote immune cell infiltration in the tumor microenvironment (TME). Analysis of SCNA revealed that arm-level deletion of SLAMF1, 4, 7 and 8 was significantly associated with decreased infiltration levels of B cells, CD4+ T cells,
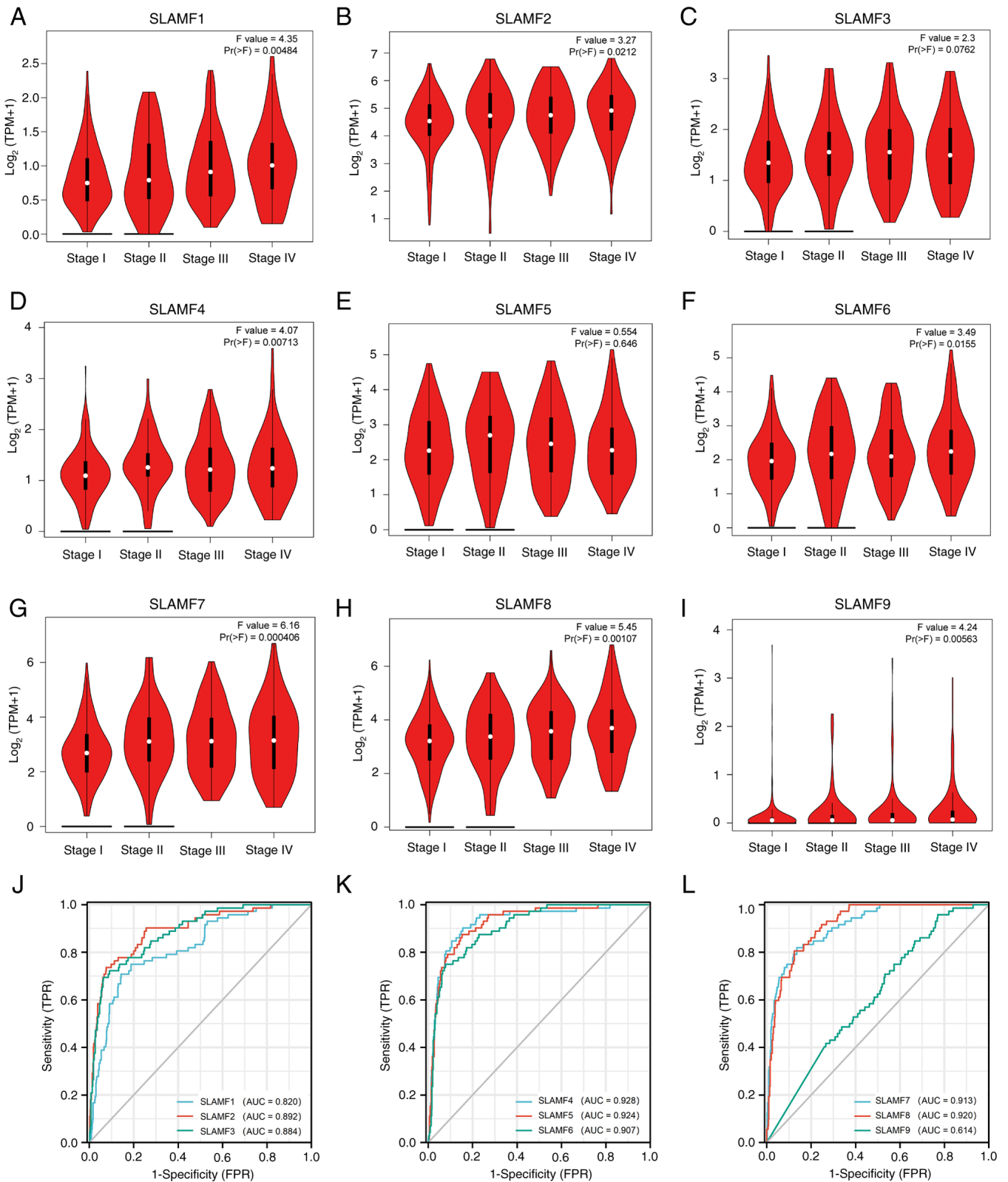
Figure 2. SLAMF expression in different tumor stages and the diagnostic value in ccRCC. Association between ccRCC stage and (A) SLAMF1, (B) SLAMF2, (C) SLAMF3, (D) SLAMF4, (E) SLAMF5, (F) SLAMF6, (G) SLAMF7, (H) SLAMF8 and (I) SLAMF9 expression, with $\log_2$(TPM+1) representing the gene transcription level. Diagnostic value of (J) SLAMF1-3, (K) SLAMF4-6 and (L) SLAMF7-9. SLAMF, signaling lymphocyte activation molecule family; ccRCC, clear cell renal cell carcinoma; TPM, transcript per million; FPR, false positive rate; TPR, true positive rate; AUC, area under the curve.

CD8+ T cells, macrophages, neutrophils and dendritic cells (Fig. 8B; P<0.001). Moreover, the level of immune infiltration in the ccRCC strongly and significantly correlated with the expression of SLAMF1, 4, 7 and 8 (Fig. 8C). In summary, the results indicate that SLAMF1, 4, 7 and 8 can promote immune cells infiltration in ccRCC.
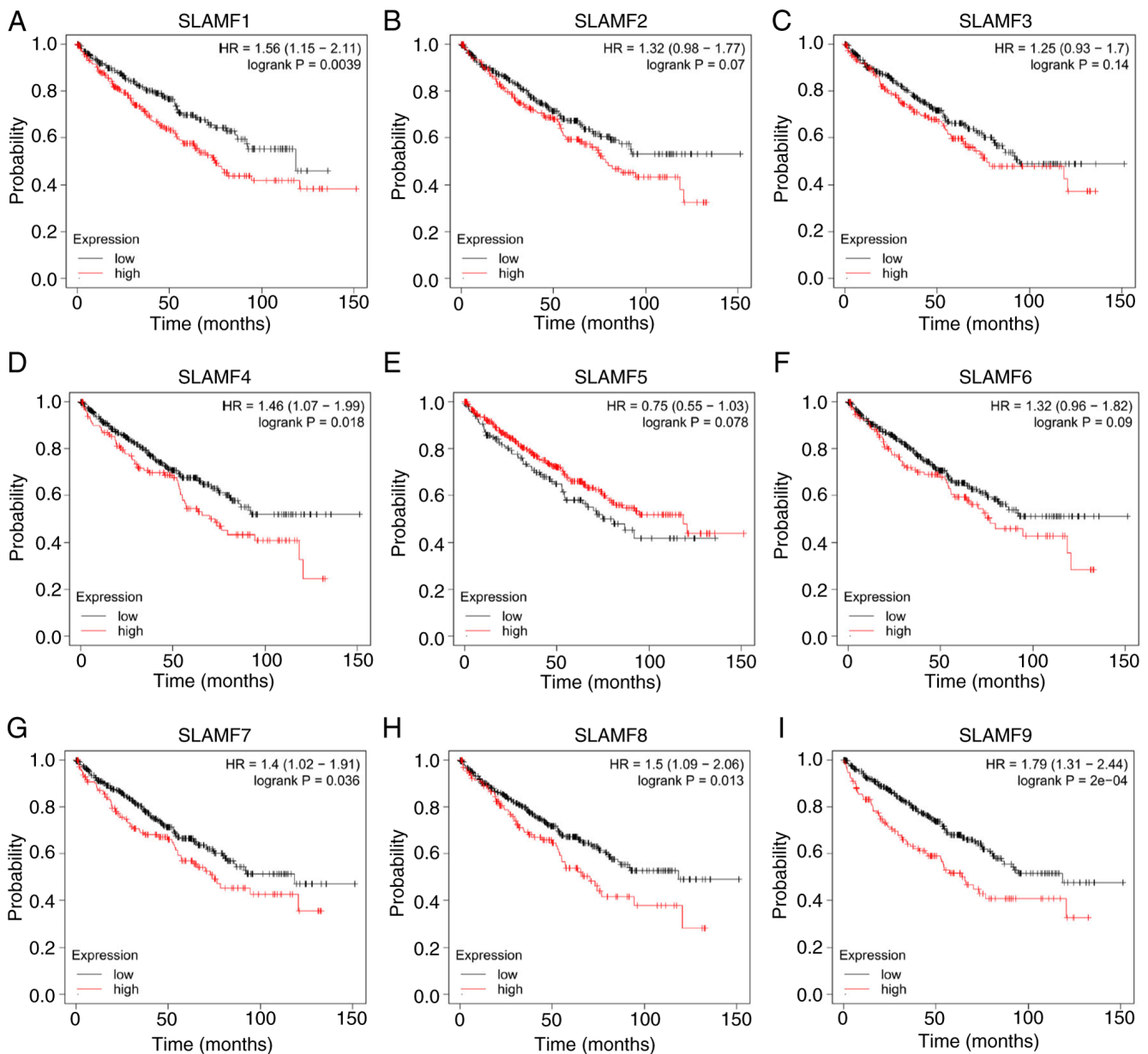
Figure 3. Effects of SLAMF expression on overall survival in patients with ccRCC. Association between the prognosis of patients with ccRCC and the high and low expression of (A) SLAMF1, (B) SLAMF2, (C) SLAMF3, (D) SLAMF4, (E) SLAMF5, (F) SLAMF6, (G) SLAMF7, (H) SLAMF8 and (I) SLAMF9. SLAMF, signaling lymphocyte activation molecule family; ccRCC, clear cell renal cell carcinoma; HR, hazard ratio.

Association of the SLAMFs with immunoinhibitors. A total of three algorithms were used (EPIC, QUANTISEQ and CIBERSORT-ABS) based on the TIMER database to analyze the correlations between the infiltration levels of M2 macrophages, Treg cells and CAFs in ccRCC and the expression of SLAMFs (Fig. 9). The infiltration levels of M2 macrophages and Treg cells were significantly positively correlated with the expressions of SLAMF1, 4, 7 and 8. Meanwhile, the infiltration level of CAFs was only significantly correlated with the expression of SLAMF1 and 8. Nevertheless, the overall trend indicates a notable upregulation of tumor-related immune cells with increased expression of SLAMF members. Additionally, PD-1 is a crucial immunosuppressive molecule (47), and the results of the present study demonstrated that the expression of SLAMF1, 4, 7 and 8 was significantly positively correlated with the expression of PD-1 and its ligands PD-L1 and PD-L2

in ccRCC (Fig. 10). These findings suggest that SLAMF may inhibit the tumor-killing effect of immune cells via PD-1 and tumor-associated immune cells.

Association between chemokines/chemokine receptors with SLAMFs. Based on the results of the functional analysis of SLAMFs, the association between cytokines and SLAMFs were further assessed. The findings revealed that the SLAMF1, 4, 7 and 8 were generally positively associated with chemokines/chemokine receptors in ccRCC (Fig. 11A and B). Moreover, C-C motif chemokine ligand (CCL)4, CCL5, C-X-C motif chemokine ligand (CXCL)9, CXCL10, CXCL11, X-C motif chemokine ligand (XCL)1, XCL2, C-C motif chemokine receptor (CCR)1, CCR2, CCR4, CCR5, CCR8, C-X-C motif chemokine receptor (CXCR)3, CXCR6 and X-C motif chemokine receptor
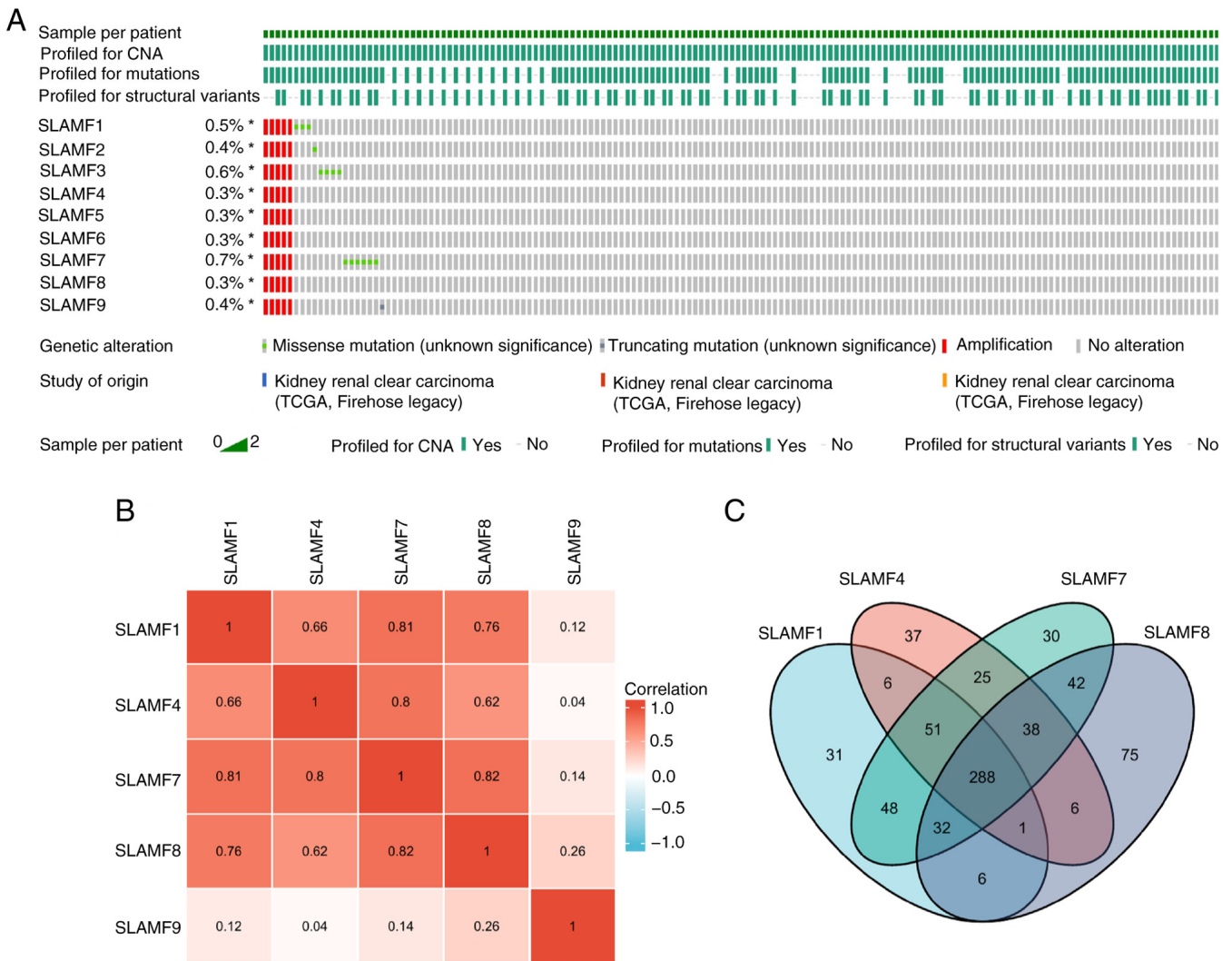
Figure 4. Gene alterations and associations among SLAMF members. (A) Alterations of SLAMF mutation, copy number and structure. (B) Correlations among SLAMF1, 4, 7, 8 and 9. (C) Co-expressed genes of SLAMF1, 4, 7 and 8. SLAMF, signaling lymphocyte activation molecule family; TCGA, The Cancer Genome Atlas; CNA, copy number alterations.

1 were significantly correlated with SLAMF1, 4, 7 and 8 exhibited (r>0.5; P<0.001). As the main molecules in antigen presentation, MHCs were also demonstrated to be significantly positively correlated with SLAMF1, 4, 7 and 8 (P<0.001; Fig. 11C) (48). These results suggest that SLAMFs may promote immune cell infiltration in ccRCC through interactions with chemokines and MHCs.

Increased level of immune cell infiltration affects the prognosis of SLAMFs in ccRCC. A prognosis analysis of SLAMFs were performed in combination with B cells, CD4+ T cells, CD8+ T cells and macrophages in ccRCC (Fig. 12). Forest plots containing hazard ratio (HR) and P-values were used to present the results. The results showed that, in the group with decreased B cell infiltration levels, ccRCC patients with high expression of SLAMF4 and 7 had a better prognosis than those with low expression of SLAMFs. Patients with ccRCC with high expression of SLAMF1, 4, 7 and 8 had a worse prognosis than those with low expression of SLAMFs in the group with enriched CD4+ T cell infiltration levels, whilst those with high expression of SLAMF1,

4 and 7 had better prognosis in the group with decreased CD4+ T cell infiltration levels. In the group with enriched CD8+ T cell infiltration levels, patients with ccRCC had a worse prognosis with high expression of SLAMF1, 7 and 8 than those with low expression of SLAMFs, whilst those with high expression of SLAMF7 had a better prognosis in the group with decreased CD8+ T cells infiltration levels. In the group with enriched macrophage infiltration levels, patients with ccRCC with high expression of SLAMF1, 4, 7 and 8 had a worse prognosis, whereas those with high expression of SLAMF4 and 7 had a better prognosis in the group with decreased macrophages infiltration levels. These results suggest that SLAMFs may impact the prognosis of patients through the level of immune cell infiltration in ccRCC. Furthermore, the high-expression SLAMF4 and 7 groups had significantly lower TIDE scores, compared with the low-expression groups (Fig. 13). The TIDE score was inversely proportional to ICB efficacy, indicating that a high expression of SLAMF4 and 7 was associated with an improved response to ICB therapy in patients with ccRCC.
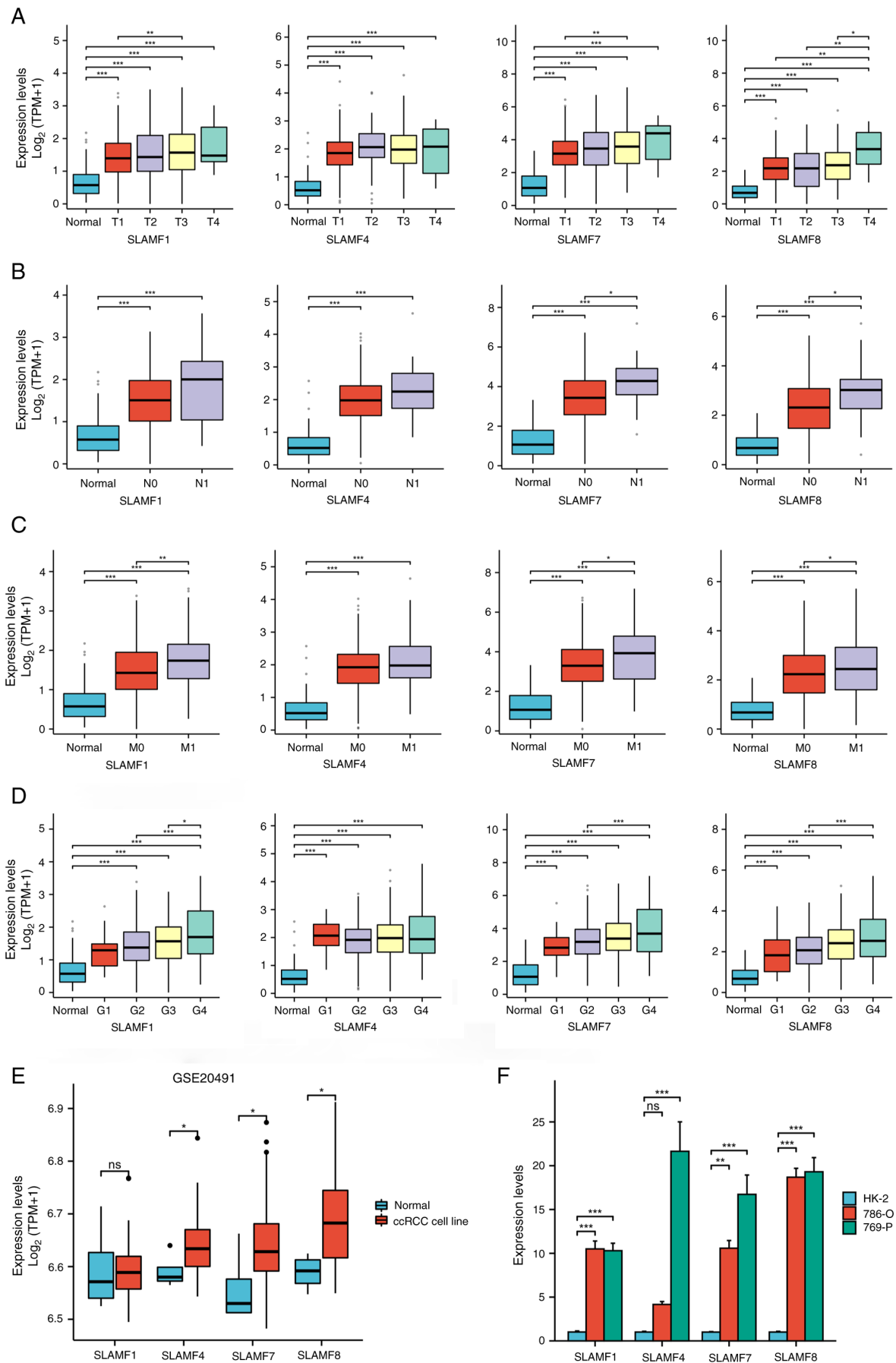
Figure 5. Expression of SLAMF members in different tumor stages and cell lines. Associations between SLAMF1, 4, 7 and 8 expression levels with different (A) T, (B) N and (C) M staging, and (D) grading. Differential expression of SLAMF1, 4, 7 and 8 in (E) normal and ccRCC cell lines in GSE20491, and (F) normal (HK-2) and ccRCC cell lines (786-O and 769-P). *P<0.05; **P<0.01; ***P<0.001. SLAMF, signaling lymphocyte activation molecule family; T, tumor; N, node; M, metastasis; G, grade; ccRCC, clear cell renal cell carcinoma; TPM, transcript per million; ns, not significant.
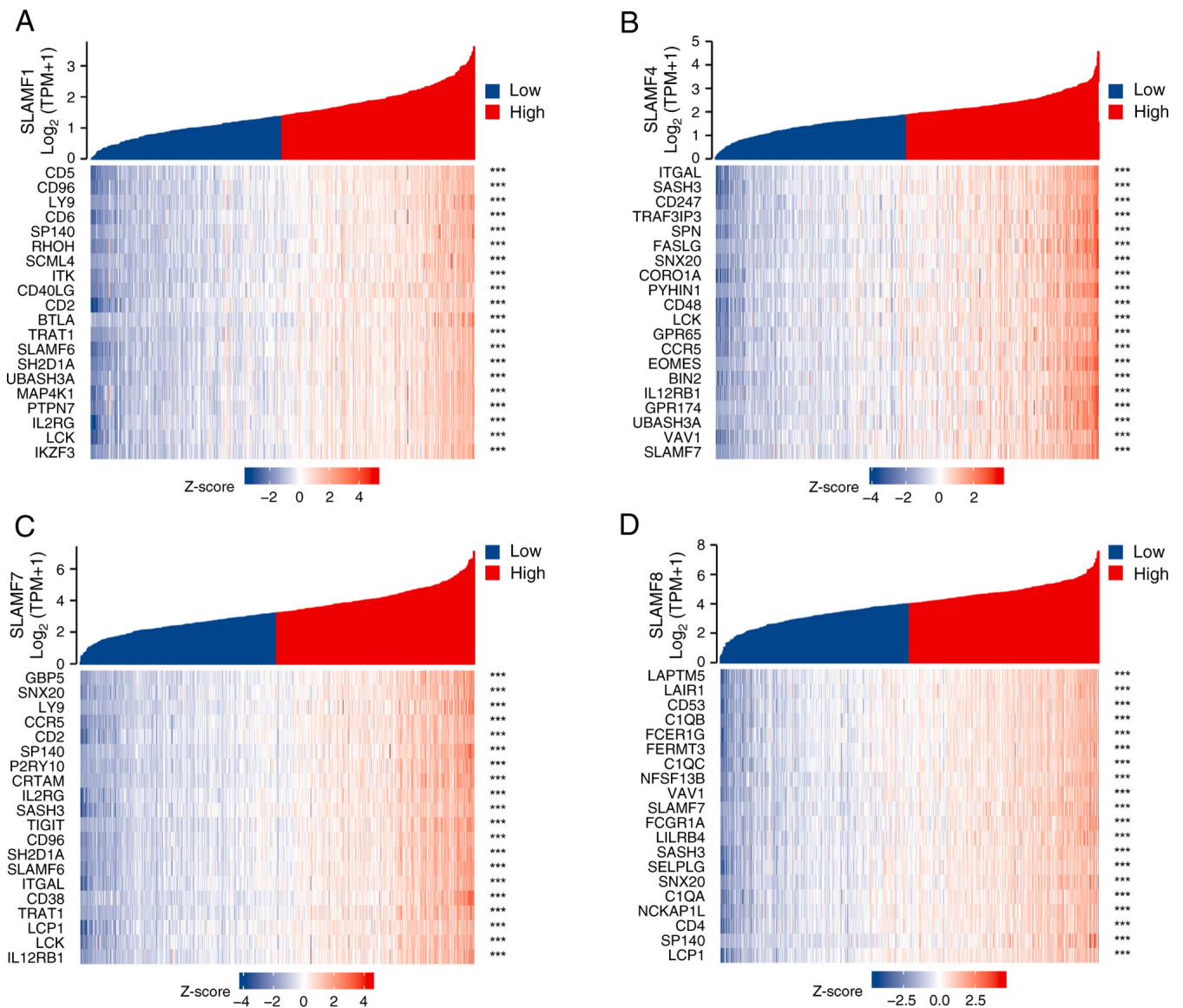
Figure 6. Association between the top 20 co-expressed genes in clear cell renal cell carcinoma with SLAMF members. (A) SLAMF1, (B) SLAMF4, (C) SLAMF7 and (D) SLAMF8. ***P<0.001. SLAMF, signaling lymphocyte activation molecule family; TPM, transcript per million.

Discussion

With the advent of the era of immune-targeted tumor therapy, an increasing number of SLAMF members, which are associated with the immune system, have been extensively studied in several tumors, including head and neck squamous cell carcinoma, gastric cancer, colorectal cancer, ovarian cancer and chronic lymphocytic leukemia (25,49-53). However, research on the role of SLAMF members in ccRCC has been limited. In the present study, a flow chart of the present research is shown in Fig. 14. In addition, it was demonstrated that SLAMF members were differentially expressed in several cancers relative to normal tissues, and notably, the expression of nine SLAMF members was upregulated in ccRCC, providing a compelling rationale for further investigation. Moreover, the high expression of SLAMF was associated with cancer stage, tumor grade and ccRCC subtype. The results also indicated that a high expression of SLAMF members was associated with poor prognosis in patients with ccRCC.

Previous studies reported that SLAMF members strongly bind SLAMF-associated proteins (SAP). They have also identified mutations in SAP in patients with X-linked lymphoproliferative immunodeficiency disease, and reported that reduced SAP exerts inhibitory effects on SLAMF members (54,55). These findings suggest that SLAMF has a regulatory role in the immune system. In the field of cancer, SLAMF members have been reported to have an inhibitory effect on immune responses in the TME, contributing to the immune escape of tumors (50). However, the molecular mechanism of SLAMF members in ccRCC has been rarely studied.

Based on data from TCGA and GTEx, the present study comprehensively explored the biological role of SLAMF in ccRCC for the first time, to the best of our knowledge, including: SLAMF expression and gene changes, clinical characteristics and diagnostic significance, correlation of immune cell infiltration, and prediction of immune checkpoint inhibitor efficacy. The present study lays a theoretical foundation for the future research of SLAMF in ccRCC, especially in immunotherapy.
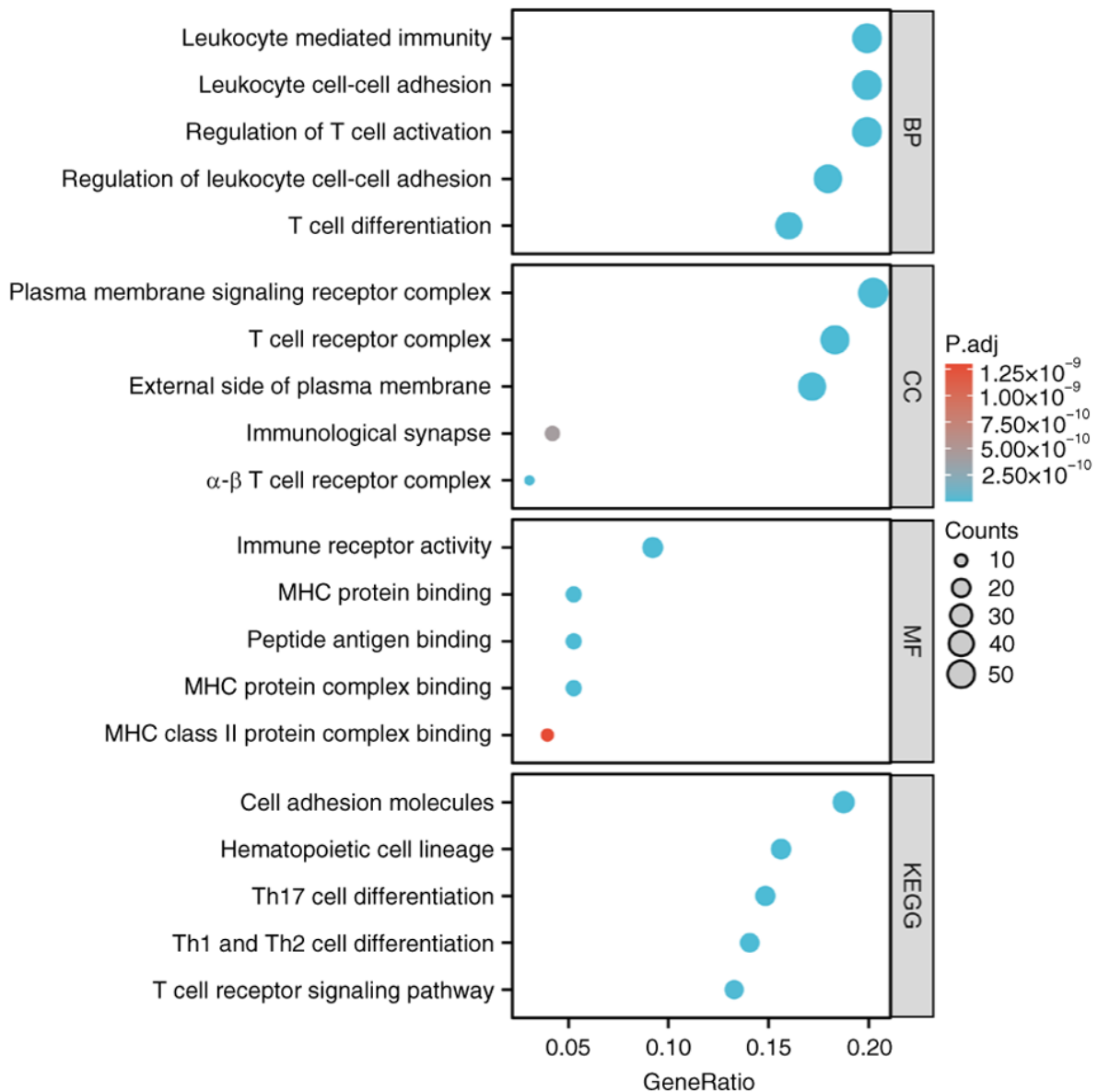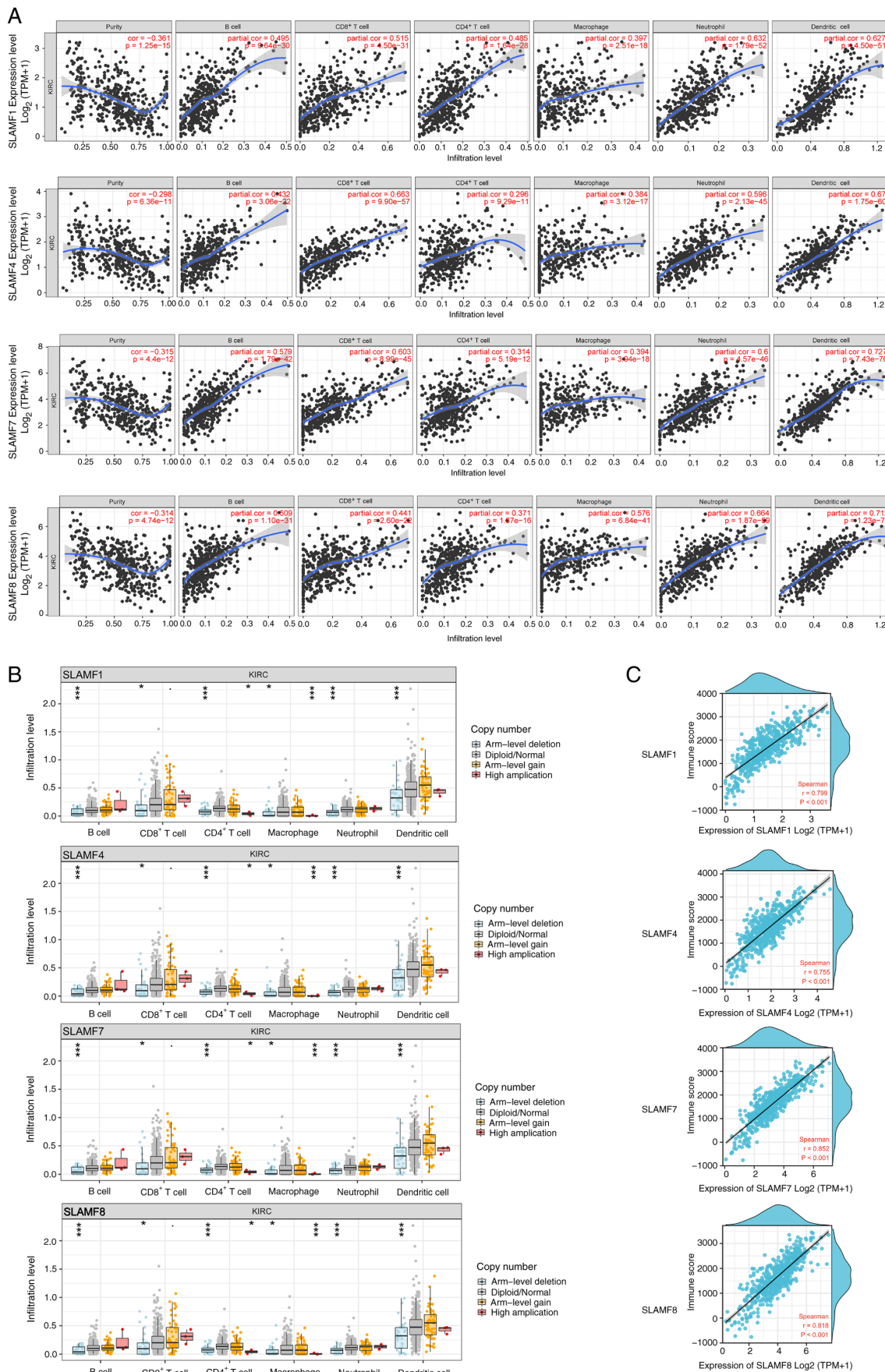
Figure 7. Enrichment analysis of signaling lymphocyte activation molecule family co-expressed genes, with the five most significantly items enriched in BP, CC, MF and KEGG gene sets for co-expressed genes. BP, biological process; CC, cellular component; MG, molecular function; KEGG, Kyoto Encyclopedia of Genes and Genomes; MHC, major histocompatibility complex; Th, T helper.

The present study demonstrated that the molecular mechanisms of SLAMF members are associated with immunity, as expected. Furthermore, the pathway enrichment of SLAMF members was associated with CAMs and leukocyte cell-cell adhesion. Cancer migration is a process wherein cancer cells are shed from the primary tumor site and metastasize to distant sites, in which CAMs serve an essential role in this process (56). The metastasis of ccRCC is associated with a poor prognosis for patients with cancer (57), and blocking the metastasis of ccRCC may improve the prognosis. The high expression of SLAMF members may be a factor contributing to ccRCC metastasis and there is evidence that SLAMF members are involved in several physiological and pathological processes, including the regulation of immune responses (58).

In the present study, the high expression of SLAMF members was highly correlated with several immune cells, a finding which is consistent with previous results. There is

evidence to suggest that SLAMF1 serves a co-stimulatory role in the activation and differentiation of T and B cells (19), the formation of germinal centers, and antibody production (59,60). Moreover, SLAMF1 is markedly upregulated in CD8^{+} T cells compared with CD4^{+} T cells (61). However, in tumor cells, a high expression of SLAMF1 can promote the growth and survival of tumor cells. There are reports that SLAMF1 can mediate the survival and proliferation of tumor cells through the PI3K/Akt signaling pathway, and participate in regulating cell metabolism, proliferation and survival (62,63). SLAMF4 serves a complex role in cancer immunity, acting as an inhibitory immune checkpoint and 'don't eat me' receptor on macrophages, hindering tumor cell phagocytosis (64). In CD8^{+} T cells, particularly in cancers like head and neck squamous cell carcinoma, SLAMF4 expression is notably increased, especially in exhausted T cells expressing other co-inhibitory receptors like PD-1 (50,65). Immunogenic peptides from

Figure 8. Immune infiltration analysis of SLAMF members. (A) Correlation between the expression of SLAMF members and the immune infiltration level. (B) Association between the somatic copy number alterations of SLAMF members and immune cell infiltration level in clear cell renal cell carcinoma. (C) Correlation analysis between SLAMF membership and immunity scores. SLAMF1: r=0.799, P<0.001. SLAMF4: r=0.755, P<0.001. SLAMF7: r=0.852, P<0.001. SLAMF8, r=0.818, P<0.001. *P<0.05; ***P<0.001. SLAMF, signaling lymphocyte activation molecule family; TPM, transcript per million; cor, correlation; KIRC, kidney renal clear cell carcinoma.
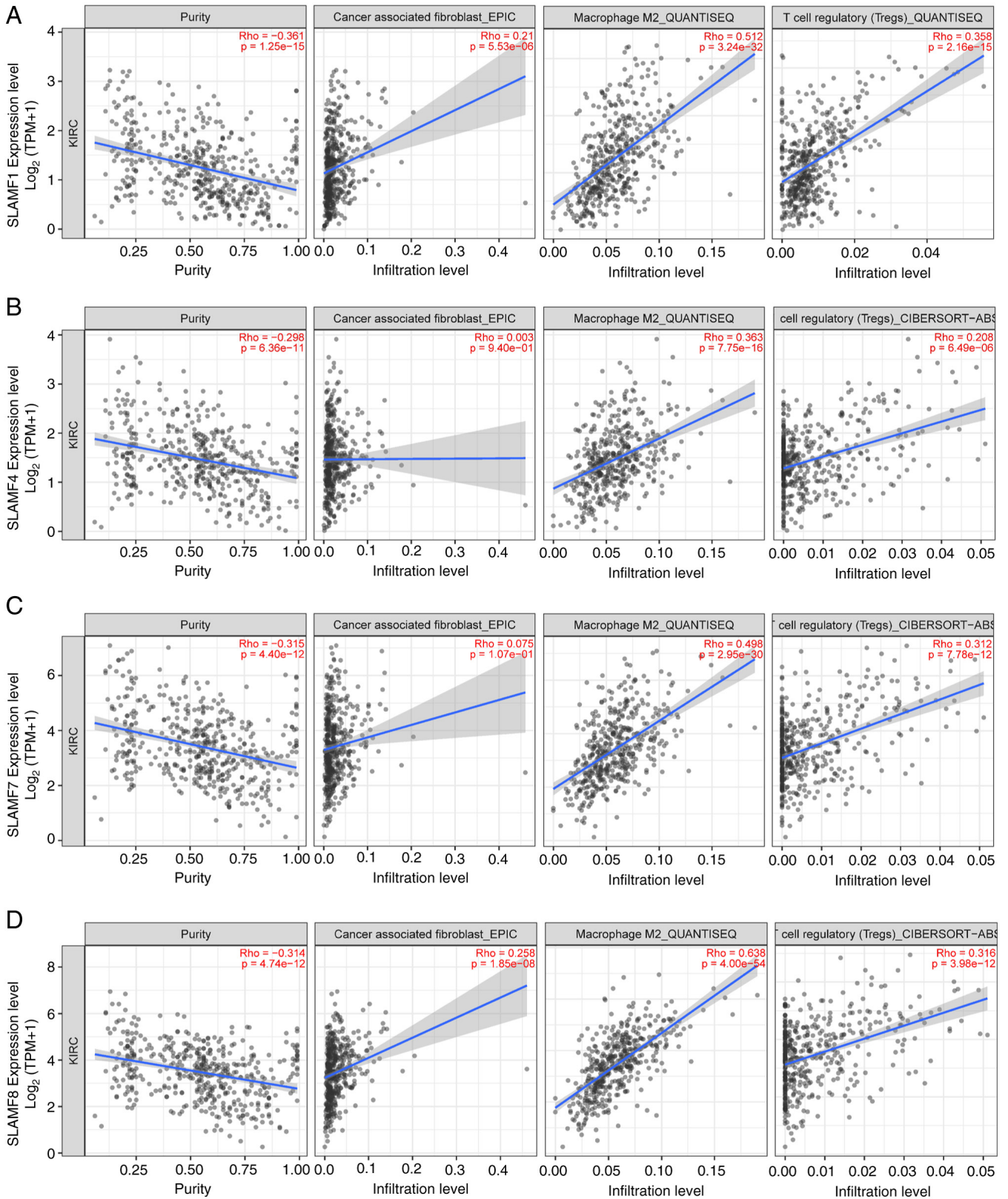
Figure 9. Correlation between tumor-associated immune cells and SLAMF members in clear cell renal cell carcinoma. Correlation between (A) SLAMF1, (B) SLAMF4, (C) SLAMF7 and (D) SLAMF8 expression with cancer-associated fibroblasts, M2 macrophages and Treg infiltration levels. SLAMF, signaling lymphocyte activation molecule family; TPM, transcript per million; KIRC, kidney renal clear cell carcinoma.

SLAMF7 antigens activate specific cytotoxic T lymphocyte clones against multiple myeloma (66). In mouse models lacking SLAMF7, tumor growth is slower, and CD8^{+} T cells express lower levels of PD-1 (23). SLAMF8 is expressed in anaplastic large-cell lymphoma cell lines and serves a role in oncogenic signaling pathways, with knockdown of SLAMF8 associated with a reduction in cell proliferation and an increase in apoptosis (67). High SLAMF8 expression is associated with a worse
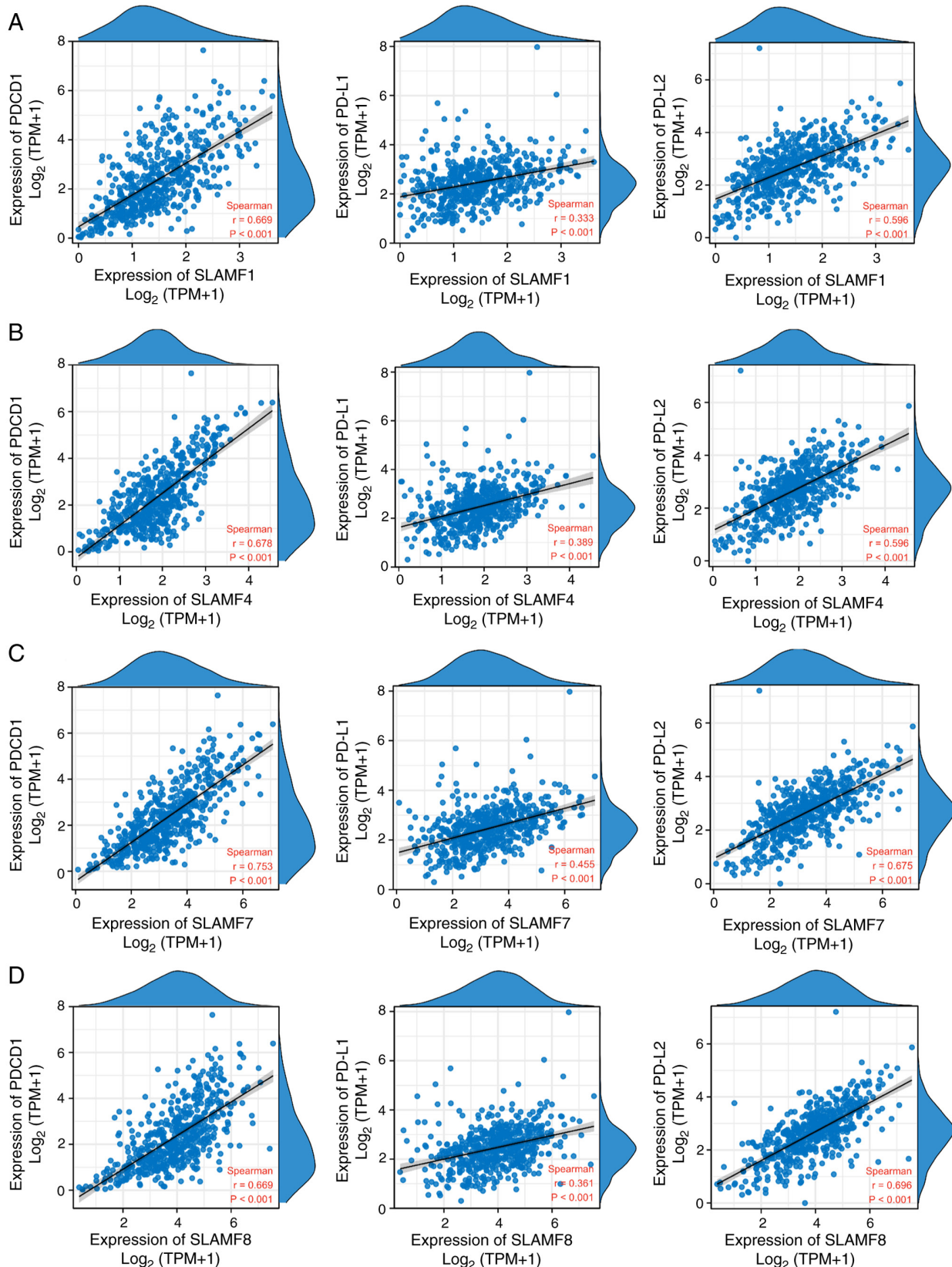
Figure 10. Correlation between SLAMF members and PDCD1/PD-L1 immune checkpoints. Correlation between (A) SLAMF1, (B) SLAMF4, (C) SLAMF7 and (D) SLAMF8 expression and PDCD1, PD-L1 and PD-L2 expression. SLAMF, signaling lymphocyte activation molecule family; PDCD1, programmed cell death 1; PD-L, programmed death-ligand; TPM, transcript per million.

prognosis in glioma, including reduced OS and chemotherapy resistance (68), but it is correlated with improved efficacy of anti-PD-1 immunotherapy in gastrointestinal cancers (69).

In the present study, based on the gene mutations of SLAMF members, it was demonstrated that the arm-level deletion of SLAMF members was associated with the level of immune cell
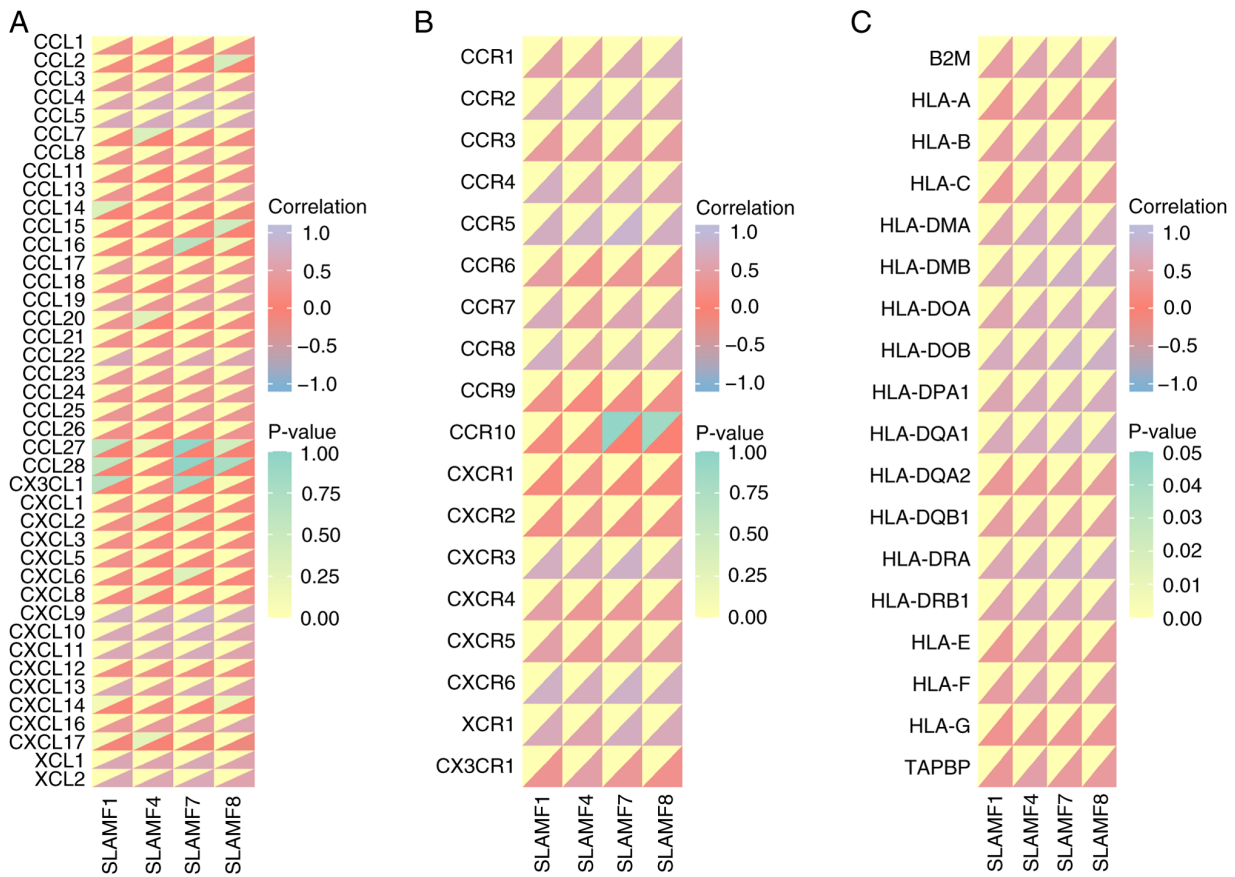
Figure 11. Correlation of SLAMF members with immune-related molecules. Correlation between SLAMF1, SLAMF4, SLAMF7, SLAMF8 and (A) chemokines, (B) chemokine receptors and (C) major histocompatibility complex molecules. SLMAF, signaling lymphocyte activation molecule family; CCL, chemokine (C-C motif) ligand; CXCL, chemokine (C-X-C motif) ligand; CX3CL, chemokine (C-X3-C motif) ligand; XCL, chemokine (X-C motif) ligand; HLA, human leukocyte antigen; B2M, β-2 microglobulin; TABPB, transporter associated with antigen processing binding protein.
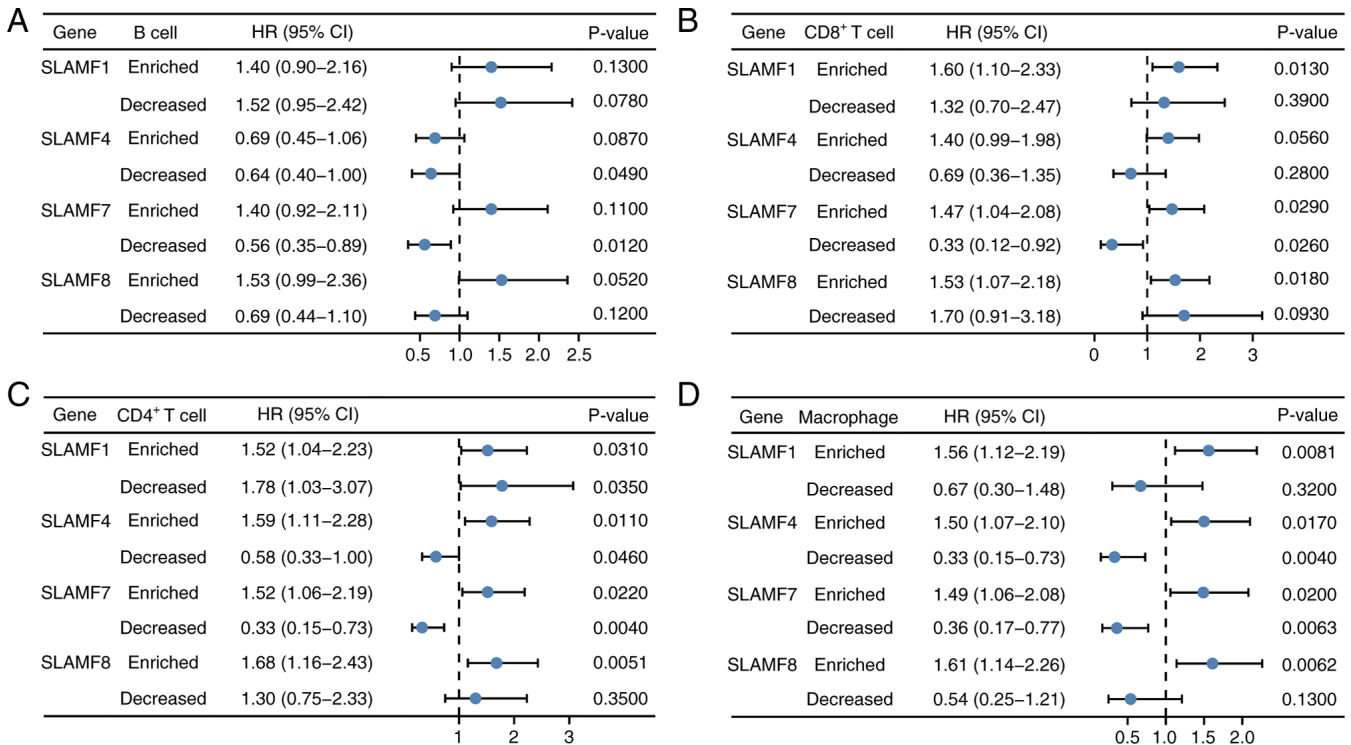

Figure 12. Effect of SLAMF members on ccRCC prognosis may be related to the level of immune cell infiltration (B cells, CD4+ T cells, CD8+ T cells and macrophages). Effect of (A) B cells, (B) CD8+ T cells, (C) CD4+ T cells and (D) macrophages on the prognosis of patients with ccRCC with high expression of SLAMF1, SLAMF4, SLAMF7 and SLAMF8. SLAMF, signaling lymphocyte activation molecule family; HR, hazard ratio; CI, confidence interval.
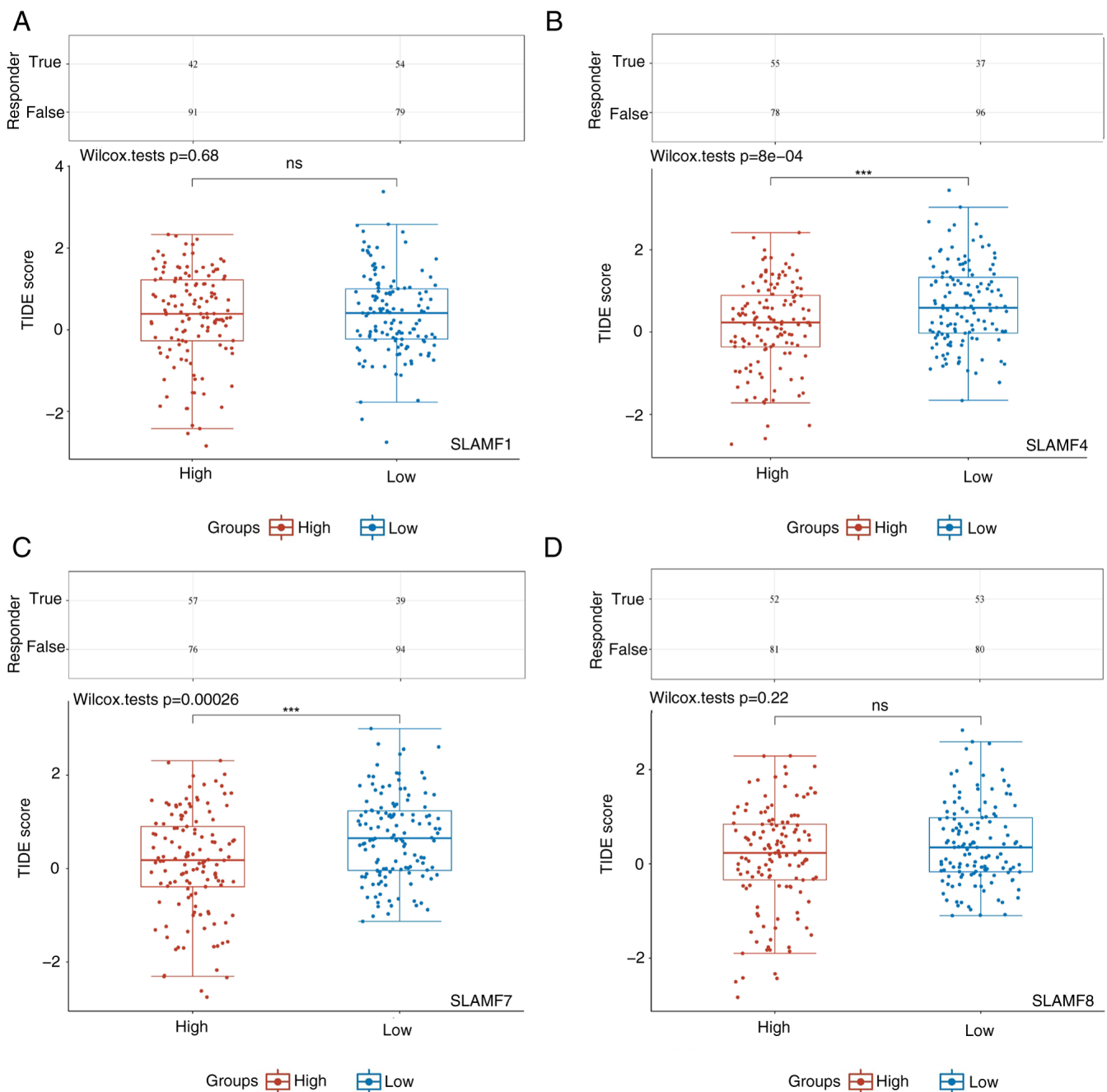
Figure 13. Immunotherapy prediction based on TIDE scores. Evaluation of ICB efficacy based on TIDE algorithm in patients with high and low expression of (A) SLAMF1 (P=0.68), (B) SLAMF4 (P=8x10^{-4}), (C) SLAMF7 (P=0.00026) and (D) SLAMF8 (P=0.22). Red represents the top 25% of high-expression samples and blue represents the top 25% of low-expression samples. TIDE score was inversely proportional to ICB efficacy. ***P<0.001. TIDE, Tumor Immune Dysfunction and Exclusion; SLAMF, signaling lymphocyte activation molecule family; ns, not significant; ICB, immune checkpoint blockade.

infiltration in tumors. Furthermore, the effect of the expression of SLAMF members on the prognosis of patients with ccRCC was regulated by immune cells. Specifically, enriched immune cells in patients with ccRCC and high SLAMF expression were associated with a worse prognosis relative to decreased immune cells. Although immune cells resisted tumor cells under normal conditions, the prognosis of ccRCC was poor. Therefore, we hypothesize that tumor cells may inhibit immune cells through several mechanisms to achieve immune escape. The results of the present study demonstrated that the expression of SLAMF was generally positively correlated with the infiltration of CAFs, M2 macrophages and Tregs cells, that is, high expression

of SLAMF can promote the infiltration of these cells in the TME. Existing studies had reported that immunosuppressive suppressor cell subsets are transferred to the TME through the secretion of cytokines (including chemokines and interleukins) by CAFs (48,70). Interferon-γ secretion by CD8$^+$ T cells would be inhibited by Tregs, which are immunosuppressive regulatory molecules (71). M2 macrophages, which function as tumor-associated immune macrophages, have also been reported to promote cancer progression in many cancers (72,73). Tregs, CAFs and M2 cells differ from normal immune cells, exerting a negative effect on the immune response. The present study demonstrated that certain SLAMF members inhibited
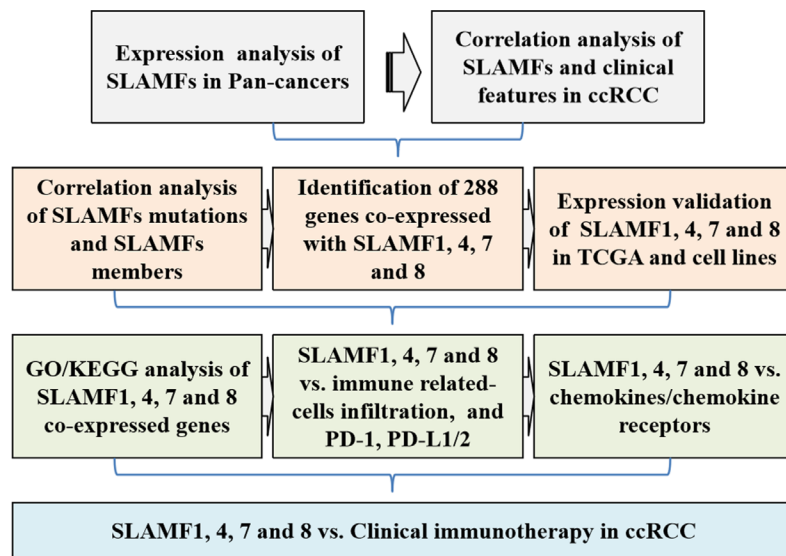
Figure 14. Flow chart of the study design. SLAMF, signaling lymphocyte activation molecule family; ccRCC, clear cell renal cell carcinoma; TCGA, The Cancer Genome Atlas; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; PD-1, programmed cell death protein 1; PD-L, programmed death-ligand.

tumor-related immune cell function by promoting the infiltration of tumor immune negative regulatory cells, such as CAFs, M2 macrophages and Tregs cells.

Moreover, PD-1 immunotherapy has made considerable progress in the treatment of several cancers (74). PD-1 mainly binds to two ligands, PD-L1 and PD-L2, participating in the activation of the PD-1 signaling pathway. The activation of the PD-1 signaling pathway can inhibit the activity of T cells, and prevent dendritic cells (DCs) from activating T cells. Therefore, anti-PD-1 treatment is highly beneficial for tumor treatment (75). In the present study, SLAMF members were positively correlated with PD-1 and its ligands PD-L1/2.

Chemokines serve a crucial role in guiding the migration of immune cells. Recent studies have demonstrated that chemokine receptors induce CD8^{+} T cells and DCs to migrate to the TME by binding to ligands (76). However, Tregs and TAMs are also induced to migrate to the TME, leading to immune tolerance (76). Additionally, chemokines promote tumor progression through several mechanisms, including angiogenesis and metastasis (77). In the present study, the expression of chemokines/chemokine receptors and SLAMF members were positively correlated. Past research has reported that the CCL17/CCL22-CCR4 axis migrated Tregs to the TME; CXCL9/10/11/12-CXCR3/CXCR4 axis migrated CD8^{+} T cells to the TME; and the CCL2/CCR2 axis migrated TAM to the TME (76). CXCL13^{+}CD8^{+} T cells could not only exhaust immune cells but also damage the function of CD8^{+} T cells (78). Furthermore, CCL4 and CCL5 have been reported participate in the proliferation, metastasis and invasion of ccRCC cells (79,80). These findings indicate that although immune cells migrate to the TME, they may inhibit immune function to promote immune escape via chemokines.

In summary, through the analysis of the biological function of SLAMF members in ccRCC, it was demonstrated that SLAMF1, 4, 7 and 8 may serve an important role in ccRCC: They may promote the progression of ccRCC through immune-related pathways and may become new immunotherapeutic targets in the future. The current study has certain limitations that need to be addressed. First, the results are mainly based on bioinformatic analysis of public datasets, and further experimental validation, especially *in vivo*, as well as validation of clinical samples, are needed to confirm the findings. Second, the expression of SLAMF members, especially SLAMF1, 4, 7 and 8, maybe used as clinical prognostic biomarkers of ccRCC, but the appropriate threshold of their expression levels need to be determined, and the accuracy, feasibility and clinical practicability of expression detection needs to be fully demonstrated in a larger patient population. Third, more clinical trials and population data are needed to further validate the expression of SLAMF members and the efficacy of ICI therapy. Nevertheless, the present study provides valuable insight into the potential role of SLAMF members as prognostic biomarkers for ccRCC and promotes further research into their clinical relevance and therapeutic potential.

Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

HW and NS conceived and coordinated the study. HW and NS confirm the authenticity of all the raw data. KC and NS wrote the manuscript. KC, LZ and YF analyzed the data, and made

the figures and tables. ZW, WD, JL, WS and PS processed the data. WS and HW reviewed and revised the article. All authors contributed to interpretation of data, manuscript revision and critical discussion. All authors contributed to the article and have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing of interests

The authors declare that they have no competing interests.

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