The Role of CXCL12-Mediated Aberrant Methylation and Tumor Microenvironment Remodeling in Carcinogenesis and Prognosis of Bladder Cancer

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Research Article

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Abstract

Background

Bladder cancer (BLCA) is the most common genitourinary tumor but lacks specific diagnostic biomarkers. Recent years have seen significant advances in the use and approval of immune checkpoint blockade (ICB) therapy to manage bladder cancer at advanced stages when platinum-based therapy has failed. The tumor microenvironment (TME) in bladder cancer is an essential player in patient's responsiveness to ICB therapy. Therefore, this manuscript explored the TME and identified CXCL12, a specific marker for inflammatory cancer associated fibroblasts(iCAFs), as potential molecular markers and therapeutic targets for bladder cancer.

Methods

We examined the gene expression profiles in the TCGA and GEO datasets to reveal the potential association of CXCL12 with the carcinogenesis and prognosis of bladder cancer. Methylation analysis of CXCL12 was performed using the UALCAN and MethSurv databases. The MCP-COUNTER, ESTIMATE, and TIDE algorithms were applied to estimate the TME components and predict immunotherapy responsiveness. An iCAFs signature was constructed using the ssGSEA algorithm. Bioinformatics analysis results were validated through immunohistochemistry of clinical samples. IMvigor210 cohort was used to validate bioinformatic predictions of therapeutic responsiveness to immune checkpoint inhibitors

Results

Our analysis revealed the potential association between aberrant promoter methylation of CXCL12 and bladder cancer carcinogenesis. CpG sites methylation of the CXCL12 gene body was associated with bladder cancer prognosis. Moreover, the expression level of CXCL12 exhibited a significant correlation with patients' pathological features and prognosis. Through gene enrichment analysis, CXCL12 was demonstrated to be associated with immune modulation and tumor microenvironment remodeling. The MCP-COUNTER and ESTIMATE algorithms verified significant correlations between CXCL12 and TME components, particularly CAFs, macrophages, and T cells. The TIDE algorithm provided evidence that T-cell clearance and dysfunction were more pronounced in bladder cancers characterized by high CXCL12 expression and high iCAFs scores, contributing to inferior responsiveness to ICB therapy. Patients who expressed high CXCL12 levels and had high iCAFs scores were likely to have less frequent FGFR3 mutation and a stromal-rich molecular subtype. Immunohistochemistry revealed that the close association of CXCL12 with iCAFs in bladder cancer potentially influenced the intratumoral infiltration of CD8 + T cells. CXCL12 expression in MIBC was increased significantly in NMIBC, which supports the bioinformatics analysis results. The IMvigor210 cohort confirmed the iCAFs score to be significantly associated with the responsiveness to immune checkpoint blockade therapy.

Conclusions

This work explores carcinogenesis and cancer-promoting roles of CXCL12 in bladder cancer. As a specific marker gene of iCAFs, CXCL12 potentially promotes bladder cancer progression by regulating the tumor microenvironment. Further exploration of the association between CXCL12 and iCAFs may unravel potential therapeutic targets for bladder precision medicine and improve the responsiveness of immune checkpoint blockade therapy.

Introduction

BLCA is a highly prevalent disease and is ranked eleventh among the most common cause of cancer-related deaths worldwide (1). In most cases, patients diagnosed with BLCA initially present with non-muscle invasive BLCA (NMIBC), managed through transurethral resection of bladder tumor (TURBT) with or without intravesical treatments, including Bacille Calmette-Guerin (BCG) immunotherapy (2). Although advancements in surgical techniques and multimodal therapy exist, the 5-year survival rates for muscle-invasive bladder cancer (MIBC) patients remain relatively low (3). Recent evidence indicates that several immune therapy strategies in human cancers, including immune checkpoint blockade (ICB), show good clinical activity (4). However, their effectiveness differs among patients.

The tumor microenvironment (TME) comprises immune and stromal components characterized by hypoxia, low extracellular pH, and high interstitial fluid pressure (5). Studies show that TME exhibits pronounced heterogeneity and is suggested to promote tumor growth (6). Recent evidence demonstrated a significant role for cancer-associated fibroblasts (CAFs) in the complex interaction between TME and tumor cells (7). Considerable research has revealed that CAFs play a pivotal role in tumor microenvironment remodeling, which influences patients' responsiveness to ICB therapy (8). There is evidence that CAFs critically support tumor progression, chemoresistance, metastasis (9), maintains cancer stem cells by producing growth factors, chemokines, and extracellular matrix (ECM) (10). CAFs secretes chemokines, including CXCL12, essential in recruiting CD8 + T cells into the tumor microenvironment (11). Studies have demonstrated that CAFs can attract and sequester CD8 + T cells in the extramural compartment (12), which is suggested to impair the effectiveness of ICB therapy.

With the rapid advancement in single-cell RNA sequencing, CAFs in solid human tumors are now classified into two subgroups, including myofibroblasts (myCAFs) and inflammatory fibroblasts (iCAFs) (13). These two CAFs subtypes have been described in disparate locations relative to the cancer cells. In particular, myCAFs are primarily located adjacent to cancer cells, whereas iCAFs are situated in the desmoplastic areas of the tumor, farther away from the cancer cells. Recent studies demonstrated that the prognosis-affecting function of CAFs might be primarily determined by iCAFs but not myCAFs, partly due to the strong paracrine characteristics of iCAFs. CXCL12, which is secreted explicitly by iCAFs, occupies an important position in iCAFs pathological processes(14). As such, more exploration of CXCL12 will deepen our understanding of the role of iCAFs in BLCA to uncover promising therapeutic targets for BLCA precision medicine and improve the effectiveness of ICB therapy.

Methods And Materials

Raw data acquisition

Data of transcriptome profiling and the corresponding clinical information were retrieved from the TCGA database (https://portal.gdc.cancer.gov/), including 408 patients. The method of data acquisition and application complied with the guidelines and policies of the TCGA database. The validation cohort (GES13507) was downloaded from the Gene expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/), including 165 primary BLCA patients and their corresponding clinical data. The IMvigor210 cohort was used to validate the relationship between iCAFs and ICB responsiveness(15).

Methylation analysis of CXCL12

The UALCAN database (http://ualcan.path.uab.edu/) was employed for CXCL12 methylation, from which gene promoter methylation data for each gene from the TCGA Infinium Human Methylation 450K Bead Chip arrays was obtained (16). MethSurv (https://biit.cs.ut.ee/methsurv/) was employed for multivariable survival analysis and to assess different CpG islands scattering (17). Moreover, the expression of three DNA methyltransferases (DNMT1, DNMT3A, and DNMT3B) was explored to establish the methylation level in different groups according to the TCGA database.

Survival analysis

Differences in the survival of patients were compared via the Kaplan-Meier (KM) survival analysis with the Log-rank test. Using the Log-rank test, we calculated the P-value and hazard ratio (HR) with a 95% confidence interval (CI). The R package "survival" and "survminer "was applied to plot the KM curves.

Screening the differentially expressed genes (DEGs) and functional analysis based on CXCL12 expression

TCGA BLCA patients were grouped into CXCL12 high and CXCL12 low groups based on the medium level of CXCL12. The Limma package of R software was applied to evaluate the DEGs between these two groups. DEGs were defined as genes with adjusted $P < 0.05$ and $|Log2 (Fold Change)| > 1$. Gene Ontology (GO)analysis on molecular function (MF), biological pathways (BP), and cellular components (CC) was performed for functional annotation. An analytical study of CXCL12 functions was achieved via Kyoto Encyclopedia of Genes and Genomes (KEGG) Enrichment Analysis The 'ClusterProfiler' package of R software was applied for GO function analysis of potential targets and KEGG pathway enrichment analysis. For Gene sets enrichment analysis (GSEA), we employed the GSEA program acquired from the Broad Institute (http://www.broadinstitute.org/gsea/index.jsp). The Hallmark v7.2, c2 Kegg, and c5 Go (BP, CC, MF) gene sets were used for GSEA analysis.

Estimating stromal cells and tumor infiltrated immune cells (TIICs)

The abundance of both the immune and stromal components, including CD8 T cells and macrophages CAFs, was assessed using the MCP-COUNTER (18) algorithm from the 'immunedeconv' (19) R software package. The R package 'estimate' was employed to evaluate TME components and tumor purity scores. Subsequently, scores of stromal and immune components were acquired. The ESTIMATE score (the sum of stromal and immune components) was negatively correlated with tumor purity.

Evaluating T cell exclusion and dysfunction and predicting ICB treatment reactiveness

The TIDE algorithm was used to predict potential ICB response (20). TIDE integrates various gene expression markers to analyze two distinct mechanisms of tumor immune escape, tumor-infiltrating cytotoxic T lymphocyte (CTL) dysfunction and CTL exclusion, by immunosuppressive factors. A higher TIDE score denotes poorer efficacy of immune checkpoint blocking therapy (ICB).

Single-sample gene set enrichment analysis (ssGSEA)

The abundance of iCAFs was explored via the ssGSEA(21) in the R Bioconductor package Gene Set Variation Analysis (GSVA, v.3.5). The ssGSEA algorithm is a rank-based method defining a score representing the degree of absolute enrichment of a particular gene set in each sample. We calculated the iCAFs abundance using iCAFs markers, including PDGFRA, CXCL12, CFD, DPT, LMNA, AGTR1, HAS1, CXCL1, CXCL2, CCL2, IL6, and IL8 (22).

Gene mutation analysis

The somatic mutation information was retrieved from the TCGA database and visualized using the R package "maftools." the waterfall plot showed mutation data of each gene. Specific mutation types were annotated by different colors at the bottom left of the waterfall plot.

Bladder cancer molecular subtyping

The molecular subtype of BLCA was retrieved from two previously published articles, which classified BLCA into five (22) and six (23) subtypes based on the molecular expression level, mutation information, and degree of immune infiltration.

Sample collection and IHC analysis

Thirty post-operative BLCA sections from 2017–2020 were recruited for IHC analysis with the approval of the institutional ethics committee. Patients' clinical information was listed in the following table (Table 1). CXCL12, PDGFRA and CD8A expressions in bladder cancer sections were detected using the Benchmark GX automatic multifunctional immunohistochemical staining system (Roche, Switzerland) with Opti View DAB Detection Kit (Ventana, USA) following the manufacturer's protocol. A horseradish peroxidase-labeled secondary antibody visualized the primary antibodies. Hematoxylin was applied for counterstaining and Bluing Reagent for post counterstaining. Pathological sections from 30 patients with bladder cancer were included in this study. We examined the expression of CXCL12, PDGFRA and CD8A in 3 consecutive slices in each section. We selected three most typical areas to discuss the association of

stromal and intra-tumor CD8 + T cell infiltration with the expression of CXCL12 and PDGFRA. ImageJ (version 1.50) was used to measure the stromal and intratumoral area and T cell counts.

Table 1
Clinical information for BLCA patients with IHC analysis

Characteristics	Age		Gender		T stage		N stage		M stage		Grade	
	≤ 65	> 65	Male	Female	Ta-T1	T2-T4	N-	N+	M-	M+	High	Low
Number	14	16	20	10	19	11	29	1	28	2	25	5

Results

Aberrant promoter methylation of CXCL12 is potentially associated with BLCA carcinogenesis

CXCL12 expression was explored across tumor types in the TCGA database, followed by paired differential analysis (tumor vs. normal tissue). CXCL12 expression was further validated in the GEO dataset (GSE13507). Significantly lower expression of CXCL12 was reported in tumor tissues among various tumor types, including BLCA ($p < 0.001$) (Fig. 1A-C). Moreover, to establish the potential correlation of low expression of CXCL12 in BLCA with its promoter methylation, the promoter methylation of CXCL12 in BLCA was analyzed in the UCLCAN database ($p < 0.001$). Intriguingly, the CXCL12 promoter methylation was significantly higher in bladder tumors compared to normal bladder tissue. Furthermore, significantly higher expression of CXCL12-related promoter methylases, including DNMT1 ($p < 0.001$), DNMT3A ($p < 0.001$), and DNMT3B ($p < 0.001$) were found in tumors compared with normal tissues (Fig. 1D). These results provided evidence of aberrant methylation of the CXCL12 promoter during bladder cancer development. The contribution of decreased CXCL12 expression levels in bladder cancer diagnosis was explored using the receiver operating characteristic curve (ROC). The results demonstrated that decreased CXCL12 expression allowed for highly accurate BLCA diagnosis in both TCGA (AUC = 0.906) and GEO datasets (AUC = 0.930) (Fig. 1E). These data strongly suggest that CXCL12 is valuable in the diagnosis and prediction of BLCA pathogenesis.

Survival-related CpG sites in the body area of the CXCL12 gene are associated with BLCA patients' pathological features

CXCL12 methylation was explored at different CpG sites (Fig. 2A). Survival analysis of the various methylation sites revealed four CpG methylations that were potentially associated with BLCA prognosis. Notably, all these four methylation sites related to survival were in the gene body of CXCL12 (Fig. 2B). Furthermore, we evaluated the methylation levels of these four methylation sites in BLCA patients with

different pathological features. Results demonstrated that the methylation levels of these survival-related CpG sites tended to increase with BLCA progression (Fig. 2C). These findings provide convincing evidence that methylation of the CpG site in the body area of the CXCL12 gene was strongly associated with bladder cancer pathological features and could ultimately impair the overall survival (OS) of bladder cancer patients.

CXCL12 expression level is significantly associated with BLCA prognosis and clinical features

The influence of CXCL12 expression levels on the prognosis and clinical characteristics of BLCA was explored by analyzing the difference in OS between the $CXCL12^{high}$ and $CXCL12^{low}$ groups. The OS of patients in the $CXCL12^{high}$ expression group was significantly lower than that of the $CXCL12^{low}$ group ($p = 0.005$), suggesting that high CXCL12 expression was an unfavorable factor for BLCA prognosis (Fig. 3A). Further exploration of the CXCL12 expression levels between patients with different clinical statuses demonstrated significantly higher CXCL12 expression in BLCA patients with higher tumor grade ($p < 0.001$), advanced clinical stage ($p < 0.001$), lymph node metastasis ($p < 0.01$) (Fig. 3B-3G). Single-gene logistics regression analysis supported the view that CXCL12 expression level was a detrimental indicator of the clinicopathological features of BLCA (Fig. 3H).

CXCL12 plays multiple regulatory functions in the tumor microenvironment

Analysis of the DEGs between $CXCL12^{high}$ and $CXCL12^{low}$ groups in the TCGA BLCA cohort revealed 757 upregulated and 56 down-regulated DEGs (Fig. 4A). GO and KEGG enrichment analysis revealed the significant involvement of CXCL12 in KEGG pathways, including Cytokine-cytokine interaction, cell adhesion cams and ECM-receptor interaction, and GO functions, including extracellular structure organization and extracellular matrix organization (Fig. 4B). GSEA suggested high enrichment of CXCL12 in hallmark gene sets, including epithelial to mesenchymal transition, angiogenesis, and hypoxia. While it was also indicated that CXCL12 was essentially involved in GO gene sets encompassing immune receptor activity, cytokine binding, chemokine receptor binding, and macrophage activation. Lastly, the KEGG gene sets demonstrated that CXCL12 was associated with pathways covering chemokine and T cell receptor signaling (Fig. 4C). These results indicated an essential role for CXCL12 in TME remodeling.

CXCL12 is correlated with multiple immune checkpoint-related genes, TIICs, and stromal cells

The correlation of CXCL12 with the stromal and immune microenvironment was explored by analyzing the association between CXCL12 and eight immune checkpoint-related genes, including PDCD1, PDCD1LG2, CD274, CTLA4, TIGIT, LAG3, HAVCR2, and SIGLEC15. Results demonstrated that CXCL12 expression was positively correlated with PDCD1 ($R = 0.450$, $p < 0.001$), PDCD1LG2 ($R = 0.600$, $p < 0.001$), CD274 ($R = 0.300$, $p < 0.001$), CTLA4 ($R = 0.430$, $p < 0.001$), TIGIT ($R = 0.440$, $p < 0.001$), LAG3 ($R = 0.400$, p

< 0.001), and HAVCR2 (R = 0.600, p < 0.001) and negatively correlated with SIGLEC15 (R=-0.260, p < 0.001) (Fig. 5A,5B). Subsequently, using the MCP-COUNTER algorithm, we explored the abundance of immune and stromal cells, including CD8 + T cells, macrophages, and CAFs. The Spearman correlation test showed a positive correlation of CXCL12 with CD8 + T cells(R = 0.222, p < 0.001), macrophages (R = 0.272, p < 0.001), and CAFs (R = 0.646, p < 0.001) (Fig. 5C-D). The ESTIMATE algorithm disclosed a profound association of CXCL12 with both the stromal score (R = 0.430, p < 0.001) and the immune score (R = 0.330, p < 0.001) (Fig. 5E-5F). CXCL12, as a CAFs secreted protein, potentially attracts T cells to the stromal area, thereby contributing to T cell exclusion and dysfunction. In this view, the TIDE algorithm was employed to evaluate the T cell dysfunction and exclusion level. Moreover, we performed correlation analysis between T cell exclusion and dysfunction with the expression level of CXCL12. Results revealed that CXCL12 was significantly positively correlated with T cell dysfunction and exclusion, suggesting a substantial role for CXCL12 in T cell depletion in the tumor immune response. Further, the response of a patient with high and low CXCL12 expression levels to ICB treatment was explored using the TIDE algorithm. Correspondingly, patients expressing high CXCL12 levels tended to exhibit impaired responses to ICB therapy (p < 0.001) (Fig. 5G-5H). These findings provide more evidence on the significant contribution of CXCL12 in the interaction of stromal with immune components. Such an association causes immune suppression in the TME, impacting the patient's response to ICB therapy.

Construction of iCAFs-based signature by ssGSEA

The effect of iCAFs on BLCA was investigated using the iCAFs signature in BLCA patients, constructed by ssGSEA algorithm based on the specific markers of iCAFs. Subsequently, we explored the relationship between iCAFs and the prognosis and clinical features of TCGA BLCA patients. The OS of patients with high iCAFs scores was significantly lower than that in patients with low iCAFs scores (p = 0.005). Meanwhile, in different clinical stages based on TNM classification, the iCAFs score of patients increased with tumor up-staging (Fig. 6A). Furthermore, the prognostic role of the iCAFs score (p = 0.002) was validated in GEO datasets (Fig. 6B). The correlation analysis provided more evidence on the significant association of iCAFs score with CXCL12, T cells and macrophages (Fig. 6C-D). Using the ssGSEA algorithm, we further identified significant involvement of iCAFs in multiple immune-related functions, covering CCR, checkpoint, inflammation-promoting and T cell regulation (Fig. 6E-F).

iCAFs score is correlated with FGFR3 mutation, molecular subtype, and immune therapy responsiveness

Correlation analysis between iCAFs scores and gene mutations revealed a significantly higher frequency of TP53 mutations (p = 0.005)and markedly lower frequency of FGFR3 mutations (p < 0.001) in the $iCAFs^{high}$ group (Fig. 7A) (Table 2). Patients in the TP53 mutation and the wild-type groups exhibited no significant difference in CXCL12 expression and iCAFs scores. Contrarily, significantly lower CXCL12 levels and iCAFs scores were reported in patients in the FGFR3 mutation group compared to those in the FGFR3 wild-type group (Fig. 7B). Comparison of the iCAFs scores between patients with different molecular subtypes revealed high concordance between the iCAFs scores and the characteristics of each subtype. Notably, the iCAFs scores were significantly higher in the fibroblast-rich molecular subtypes,

such as stromal infiltrated, but markedly lower in the luminal papillary, which earned the least stromal components (Fig. 7C). Correlation analysis between the TIDE and iCAFs scores showed a strong correlation between iCAFs scores and T cell dysfunction and exclusion ($R = 0.620$, $p < 0.001$). By ICB responsiveness prediction of TIDE algorithm, we also found that iCAFs score significantly influenced ICB responsiveness BLCA patients ($p < 0.001$) (Fig. 7D).

Table 2
Difference of gene mutations with significance between iCAFs high and low groups of TCGA BLCA patients.

Gene	iCAFs high-wild	iCAFs High-mutation	iCAFs Low-wild	iCAFs Low-mutation	p-value
FGFR3	190(96.45%)	7(3.55%)	158(77.45%)	46(22.55%)	< 0.001
TP53	103(52.28%)	94(47.72%)	136(66.67%)	68(33.33%)	0.005

CXCL12 is closely associated with iCAFs, CD8 + T cells exclusion, and clinical-pathological features

IHC assay demonstrated that tumors with significant CD8 + T-cell intra-tumoral infiltration usually exhibited negative CXCL12 and PDGFRA expression in the stromal components (Fig. 8A). Contrarily, stromal CXCL12- and PDGFRA-positive tumors showed high CD8 + T-cell infiltration in the stromal compartment accompanied by low intra-tumoral infiltration (Fig. 8B). These results suggest that the expression of stromal PDGFRA and CXCL12, namely the presence of iCAFs, played an essential role in T-cell clearance. Subsequent examination of the intensity of CXCL12 expression in bladder cancers of different clinical stages suggested broad and robust positive expressions of CXCL12 in MIBC and weak expressions of CXCL12 in NMIBC (Fig. 8C). We further quantified and validated the CD8 + T cell were excluded and sequestered in the stromal compartments where iCAFs were located (stromal areas with positive PDGFRA and CXCL12 expression). Finally, Sequencing results from the immunotherapy cohort IMvigor210 validate a significant association between ssGSEA-constructed iCAFs scores and patient responsiveness to immunotherapy ($p = 0.02$) (Fig. 8E).

Discussion

The prevalence and incidence of BLCA are seeing an unprecedented rise worldwide (23). BLCA can be classified into NMIBC and MIBC based on tumor invasion depth. MIBC is a lethal type, which warrants definitive treatment. It is approximated that only half of MIBC patients undergo definitive treatment, including surgery rate for fewer than 20% (24). The ease of metastasis in MIBC may explain the low surgical rate in patients with MIBC. Many patients with MIBC show metastases at diagnosis, therefore, are thus deprived of surgery. However, this group of patients is managed using a combination of medical therapies, including platinum-based chemotherapy. For many years, researchers are yet to make significant progress in the treatment of metastatic BLCA, especially after the failure of platinum-based

chemotherapy. Recently, the advent of immune checkpoint inhibitors has provided new treatment options for patients with advanced BLCA. Several clinical pieces of evidence have validated the remarkable effectiveness of immune checkpoint inhibitors in the management of advanced BLCA. In this view, immune checkpoint inhibitors are now guideline-recommended therapy for advanced BLCA that has failed prior chemotherapy. Nevertheless, immune checkpoint inhibitors still face problems of low responsiveness and frequent adverse effects, which warrants urgent exploration of the strategies to increase the responsiveness of BLCA immunotherapy.

As the tumor cells begin to proliferate and invade, they initiate microenvironment remodeling by activating resident fibroblasts, which replace the adipocyte-rich stroma with CAFs (25). The contribution of the microenvironment to tumor progression is underpinned by autocrine and paracrine signaling, in which the secretome of CAFs and cancer cells plays a pivotal role (26). Recent advancement in single-cell RNA sequencing significantly enriches our understanding of the heterogeneity of the TME. There is a common view that the CAFs can be classified into subgroups, including iCAFs and myCAFs. The iCAFs, which earned profound secreting features and specifically expressed PDGFRA and CXCL12, are now considered to impact BLCA patients' prognosis essentially. Through single-cell RNA sequencing, iCAFs have been shown to exert essential functions in recruiting immune cells into the tumor microenvironment (14). Recent evidence also supports that the direct interactions between CAFs and T cells, mediated via coincident upregulation and engagement of PD-1 on T cells, drive T cell dysfunction and death within tumors (27). Besides, CAFs are critical players in shaping a tumor-permissive and immunosuppressive TME as they preferentially induce the tumor-promoting function of TAMs. The recruitment and pro-tumoral activation of both cell types are significantly induced via a combination of heterogeneous CAFs and TAMs through reciprocal interaction. Accordingly, this accelerates tumor progression and proves that therapies targeting both TAMs and CAFs, or those targeting the cell-cell interaction between TAMs and CAFs improve anti-tumor therapeutic efficacy (28).

In the present study, the constructed iCAFs signature represented the relative abundance of iCAFs in each patient. Our analysis demonstrated that the iCAF abundance was an unfavorable prognostic factor for BLCA, encompassing significant influences on TME components. Furthermore, a significant negative association between iCAFs scores and mutations in FGFR3 was reported. Previous studies suggested that mutations of FGFR3 were more common in low-stage urothelial cancers. Similarly, our results laterally confirmed a positive correlation between iCAFs scores and tumor stage.

CXCL12 is now considered explicitly expressed by iCAFs in the TME (29). Studies have reported CAFs-driven modulatory effects of CXCL12 in immune suppression status in several cancer types (30, 31). However, reports on expression patterns and immune-modulating functions of CXCL12 in BLCA are unknown. The present work explored the roles of CXCL12 in BLCA through multi-omics bioinformatics analysis along with immunohistochemistry. We reported three exclusive characteristics of CXCL12 in BLCA: (i) the expression level of CXCL12 in BLCA tissues was significantly lower than that in normal tissues, and the decrease in the expression level of CXCL12 was highly accurate in the diagnosis of BLCA; (ii): methylation of CXCL12 has distinct effects on bladder cancer, with methylation of promoters tending

to play a role in bladder carcinogenesis and methylation of the CpG sites in the gene body playing a vital role in bladder cancer prognosis. (iii) the expression of CXCL12 in bladder cancer increased with increasing tumor stage. Although these findings may seem contradictory, they implied intricate regulatory processes. Of note, the significant decrease in CXCL12 expression levels in tumors was potentially related to the hypermethylation of its promoter in tumors. While the increase in CXCL12 expression levels in tumors with tumor staging could be ascribed to several mechanisms, including gene body methylation(32) and increased tumor heterogeneity(33). However, we only found that CXCL12 gene body methylation was associated with the prognosis of bladder cancer patients, but not with CXCL12 expression levels. In view of the current evidence, we believe that the accumulation of CAFs during tumor progression may be one of the important reasons for the increase in CXCL12 expression.

Within the tumor patients, it is no doubt that CXCL12 is an adverse prognostic factor. Gene enrichment analysis suggested a significant role of CXCL12 in multiple TME modulating processes, including ECM organization, cytokine to cytokine interaction, epithelial to mesenchymal transition, hypoxia, angiogenesis, T cell receptors signaling pathway, and macrophage activation. Moreover, correlation analysis revealed that CXCL12 was significantly associated with the immune checkpoint-related gene expression, CD8 + T cells, macrophages infiltration, and the abundance CAFs. These results were consistent with the previous evidence that CXCL12, secreted by CAFs, was involved in regulating CD8 + T cells and macrophages, resulting in immunosuppression in the TME, thus promoting tumor progression(34). Using the TIDE algorithm, the impact of CXCL12 on T cell dysfunction and exclusion was revealed, and high CXCL12 expression was proved to influence the responsiveness of BLCA patients to ICB therapy.

Finally, by exploring the expression of CXCL12, PDGFRA, and CD8A in BLCA specimens, it was demonstrated that PDGFRA and CXCL12 were highly co-expressed in tumor stromal components. Meanwhile, a significant increase of CD8 + T-cell infiltration was reported in the stromal region. In contrast, a substantial decrease of intratumoral CD8 + T-cell infiltration was revealed in tumors positive for PDGFRA and CXCL12. Contrarily, intratumoral CD8 + T-cell infiltration was significantly elevated in tumor tissues negative for PDGFRA and CXCL12 expression. These findings mirrored the exclusive effect of CXCL12 and iCAFs on CD8 + T cells. In addition, CXCL12 protein was found globally expressed in MIBCs, whereas only a small subset of NMIBC expressed CXCL12. Moreover, the IMvigor210 cohort further validated that the iCAFs may impact patients' responsiveness to ICB therapy. These findings affirmed the results of bioinformatics analysis, strongly suggesting that CXCL12 and iCAFs play a part in the immunosuppression of BLCA and are potentially relevant factors in BLCA progression.

Although the present study revealed remarkable findings, a few limitations cannot be ignored. First, our research is mainly based on bioinformatics analysis, though Chen K et al. had confirmed the interaction of $PDGFRA^{+}CXCL12^{+}$ iCAFs with various immune cells in the TME (14). In this view, experimental data are still needed to verify the specific roles of CXCL12 methylation and TME remodeling in BLCA. Secondly, the number of clinical samples analyzed was limited; therefore, a clinical cohort of larger samples is

needed to verify the accuracy of decreased CXCL12 levels in diagnosing BLCA and the effect of iCAFs and CXCL12 on how bladder cancer patients respond to immunotherapy.

In conclusion, this work demonstrates the effect of CXCL12 on the occurrence and development of BLCA at multiple levels through systematic multi-omics bioinformatics analysis along with immunohistochemical verification. Additionally, iCAFs and CXCL12 significantly impact the immunotherapy of BLCA and the prognosis of BLCA patients. The treatment targeting iCAFs may provide new ideas for advancing BLCA treatment in the future to improve patient's responsiveness to existing ICB treatments.

Declarations

Ethics approval and consent to participate

30 BLCA spacemen were collected from Suzhou Kowloon Hospital, with patients' informed consent and the approval of the Medical Ethics Committee of Suzhou Kowloon Hospital

Consent for publication

All authors read and approved the publication of the final manuscript.

Availability of data and materials

All datasets generated in the present study can be found in onlinedatabases. The names of databases and accessionnumber(s) are provided in the article.All the data can be obtained from the corresponding author upon reasonable request.

Competing interests

The authors declare that there are no conflicting interests.

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Authors' contributions

Du YiHeng and Cao Jin contributed equally to this work. Du YiHeng designed the study and assigned the conceptualization and methodology. Cai XiaoWei provided the materials for this study. Wang Yi and Yu Jiang did the data analysis and interpretation. Cao Jin and Du YiHeng wrote the original draft and the data curation. Wang XiZhi and Xue BoXin provided administrative support. Cao Jinand Jiang Xiang did the immunohistochemistry analysis.

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Figures

Figure 1

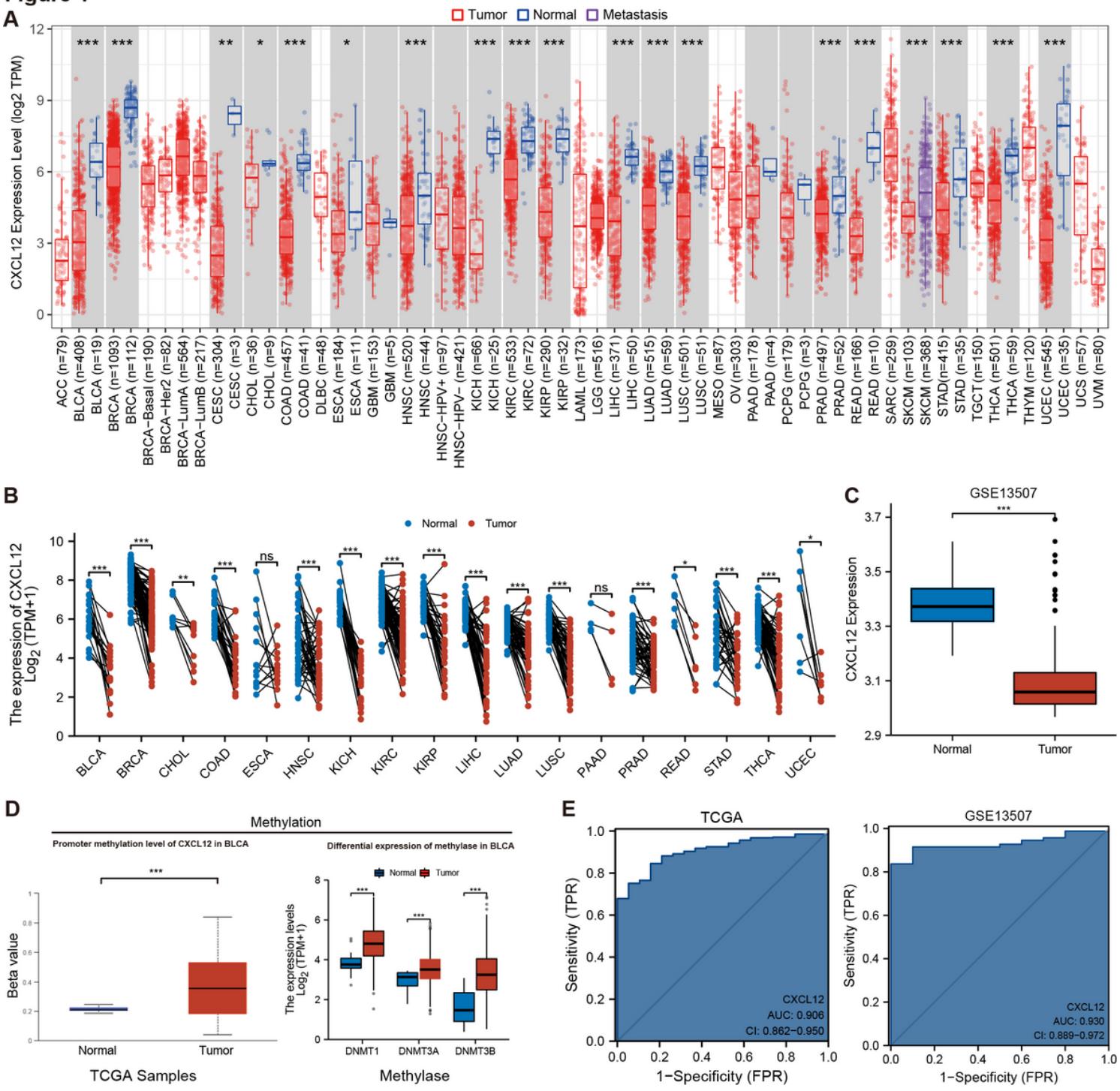

Figure 1

Expression and promoter methylation patterns of CXCL12 A.The expression of CXCL12 wassignificantly lower in multiple cancer types, including bladder cancer (p<0.001). B. CXCL12 expressionbetweenpaired normal and tumor tissues in TCGA patients. C. Comparison of CXCL12 expression between tumor and normal tissues in the GEO cohort (p<0.001). D. Promoter methylation levels of CXCL12 between normal and tumor patients from the TCGA BLCA cohort (p<0.001).Three methylases, including DNMT1,DNMT3A

and DNMT3B, were significantly upregulated in bladder cancer tissues (p<0.001). E. ROC curves showing the high accuracy of CXCL12 in predicting bladder cancer prognosis. *** p<0.001, **p<0.01, *p<0.05

Figure 2

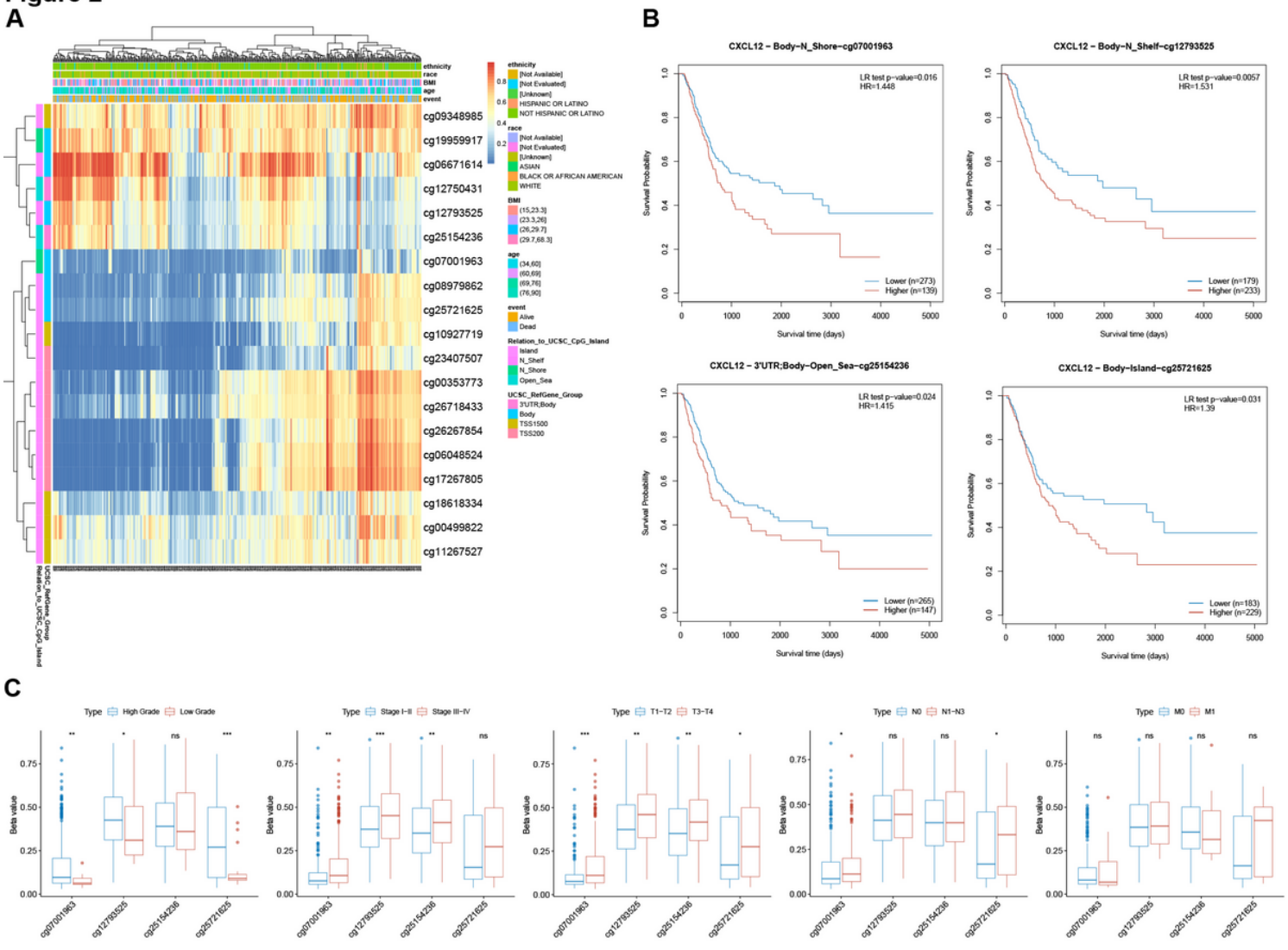

Figure 2

Analysis of methylation pattern of CXCL12 CpG sites A. The distribution of different CpG sites methylation status in TCGA BLCA patients. B. Survival-related methylationCpG sites in the CXCL12 gene body. C. The four survival-related CpG sites were highly methylated in tumors with higher grade, tumor stage, T and N classifications. *** p<0.001, **p<0.01, *p<0.05

Figure 3

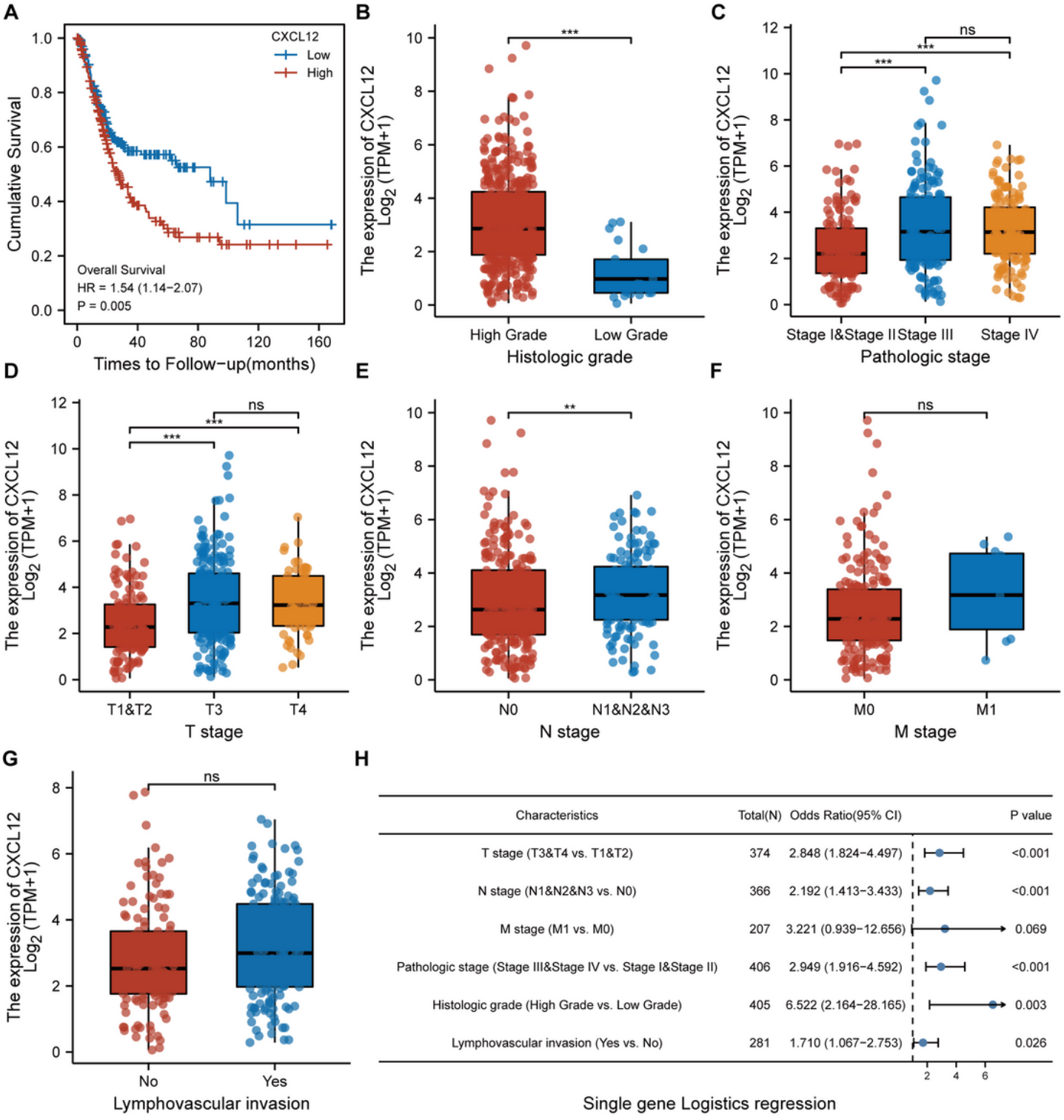

Figure 3

The impact of CXCL12 on BLCA survival and clinical features. A. Patients with high CXCL12 expression tended to have a relatively lower OS (p=0.005). B-G. CXCL12 expression was upregulated in patients with relatively advanced bladder cancers. H. The single-gene logistics regression indicated that CXCL12 expression was correlated with BLCA pathological features, including grade (p=0.003), stage (p<0.001), T

classification (p<0.001), N classification (p<0.001)and lymphovascular invasion (p=0.026). *** p<0.001, **p<0.01

Figure 4

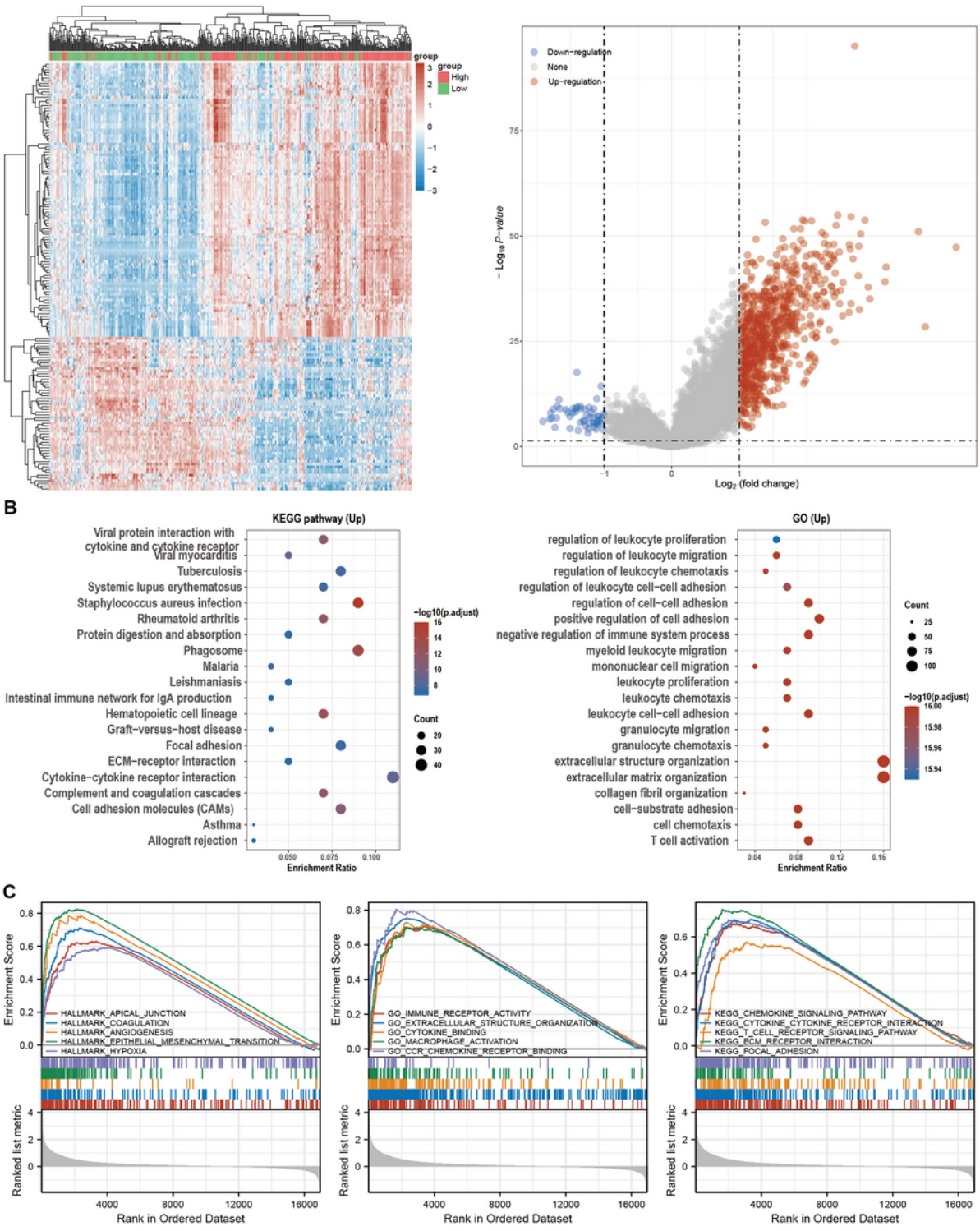

Figure 4

Functional annotation of CXCL12 by GO, KEGG and GSEA. A. A heatmap showing the top 50 upregulated and downregulated DEGs between high- and low-CXCL12 expression groups, anda volcano plot showing the upregulated and downregulated DEGs. B. KEGG and GO enrichment analyses showed that CXCL12

was related to multiple ECM remodeling and immune-related processes. C. GSEA, GO, and KEGG analyses revealed that CXCL12 was involved in remodeling stromal and immune components.

Figure 5

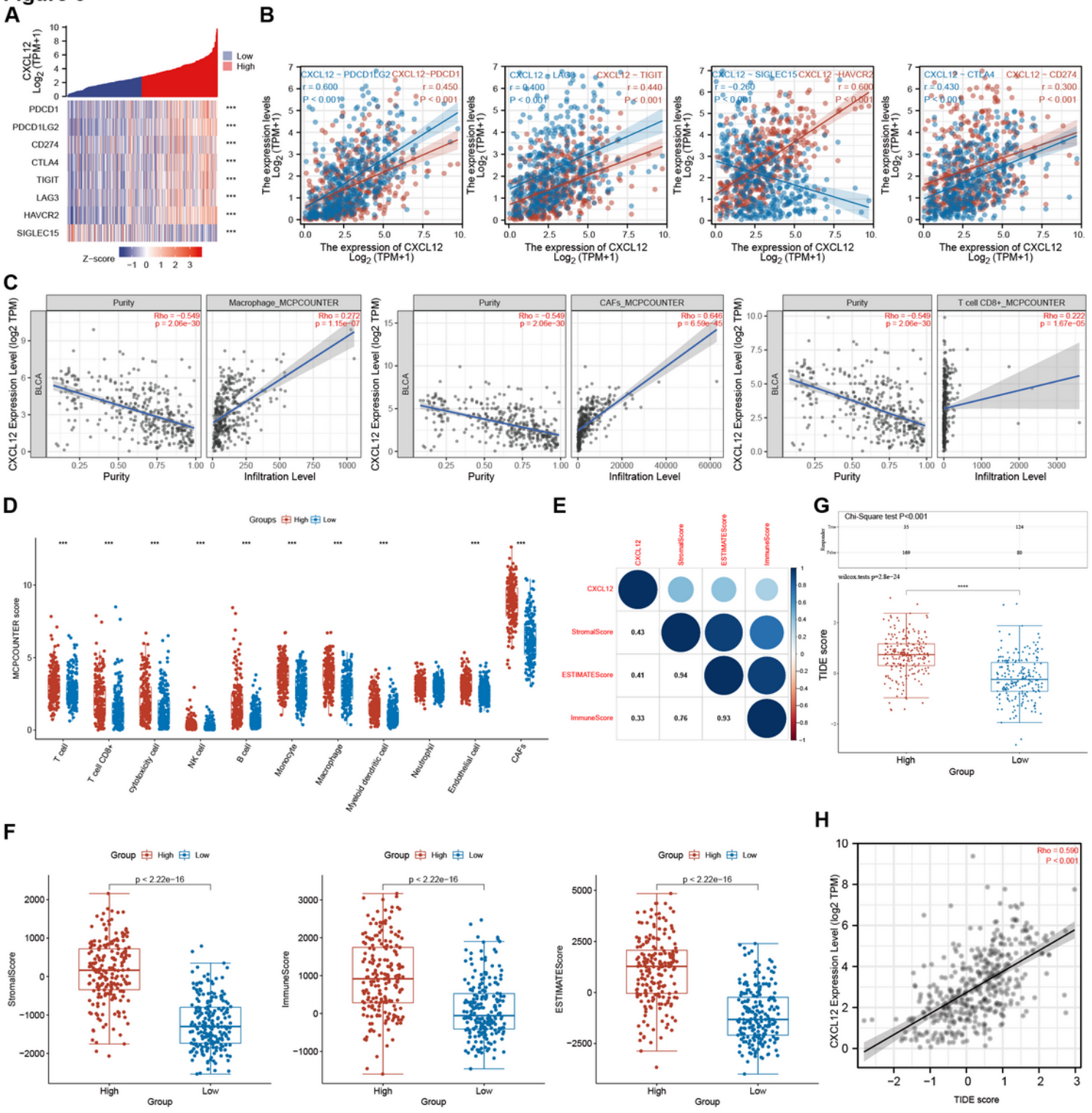

Figure 5

CXCL12 correlateswith multiple TME components that influence patients' responsiveness to ICB therapy. A-B. CXCL12 is positively correlated with immune checkpoint genes, including PDCD1, PDCD1LG2, CD274, CTLA4, TIGIT, LAG3, and HAVCR2, but negatively related to SIGLEC15. C-D. The MCP-COUNTER

algorithm indicates that CXCL12 expression levelis positively associated with macrophages, CD8 T cell infiltration, and CAFs abundance in the TME. E-F. The estimate algorithm shows a positive correlation between CXCL12 expression and estimate scores(Stromal, Immune, and ESTIMATE scores). G-H. The TIDE algorithm demonstrated that CXCL12 expression was positively related to the exclusion and dysfunction of T cells, thereby influencingthe responsiveness of BLCA patients to ICB therapy. **** p<0.0001, ***p<0.001

Figure 6

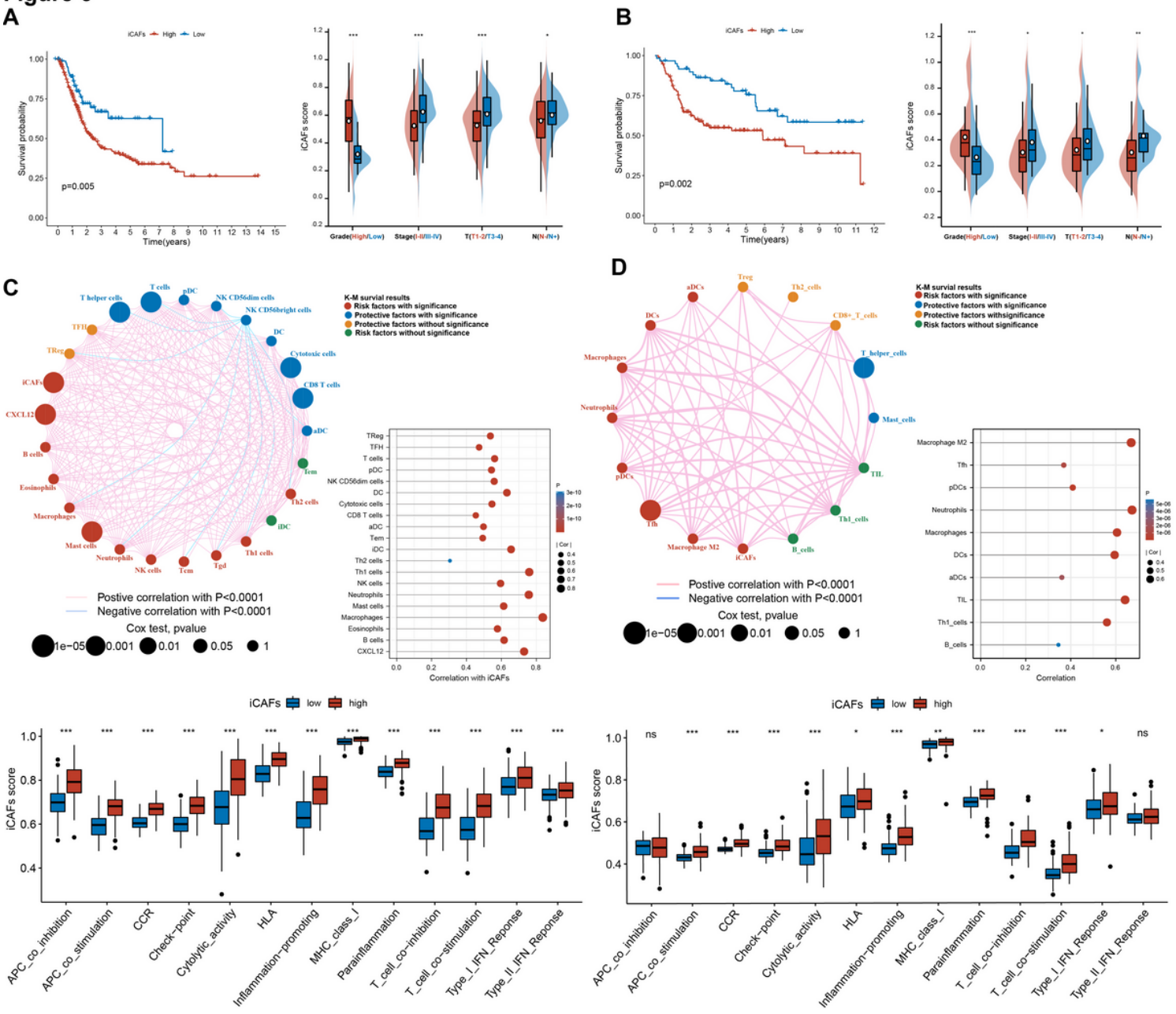

Figure 6

iCAFs score predicted poor prognosis of BLCA patients. A-B. iCAFs score was associated withpoor OS of patients with BLCA in the TCGA and GEO cohorts, with higher iCAFs scores recorded in advanced BLCA. C-D. iCAFs scores were positively associated with the expression of CXCL12, T cells and macrophage

infiltration. E-F. iCAFs were involved in multiple immune-related functions, including CCR, checkpoint, inflammation-promoting and T cell regulation. *** p<0.001, **p<0.01, *p<0.05

Figure 7

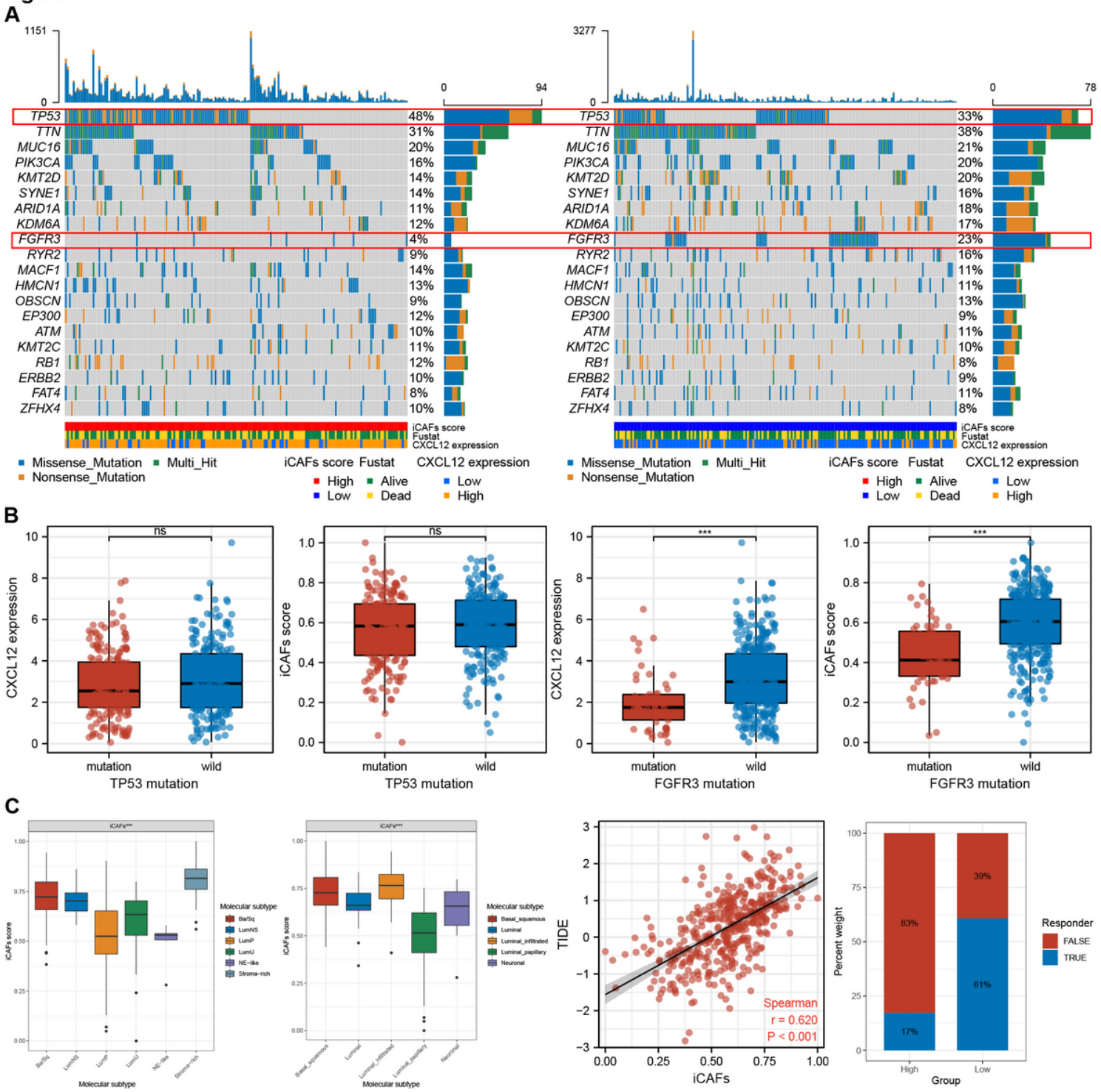

Figure 7

The correlations of iCAFs scores with gene mutation, molecular subtype and ICB responsiveness. A-B. The iCAFs score negatively correlated with the mutation frequency of FGFR3. C. iCAFs score was significantly upregulated in BLCA with stromal rich molecular subtype. D. The TIDE algorithm

indicatedsignificantlylower responsiveness in high iCAFs patients, possibly due to the dysfunction and exclusion of T cells. *** p<0.001

Figure 8

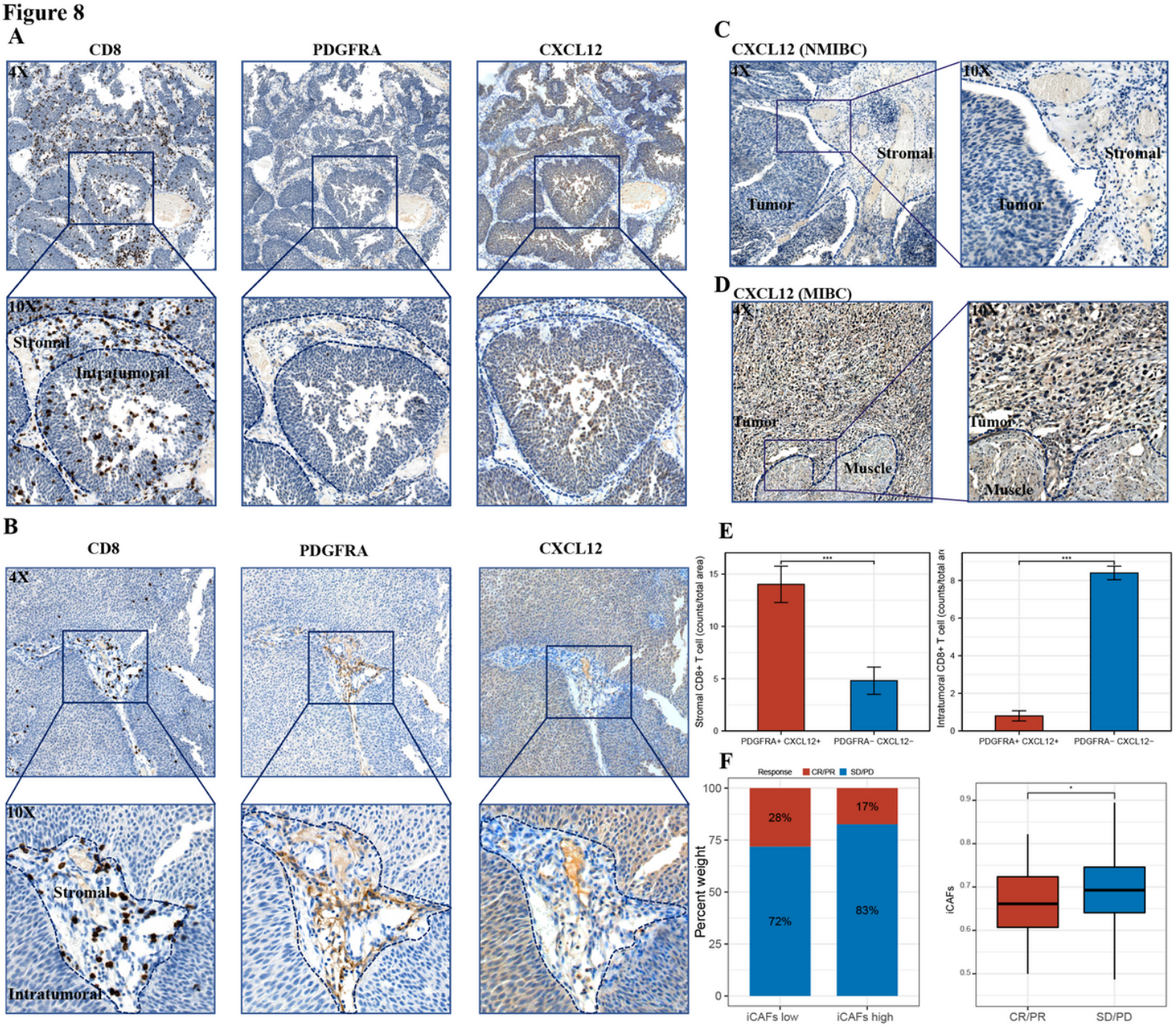

Figure 8

IHC verification of the association of CXCL12 and iCAFs with CD8 T cells infiltration. A.Intratumoral CD8 T cells were observed in tumors with negative stromal CXCL12 and PDGFRA expression B.Significant infiltration of CD8 T cells in the stromal components of CXCL12 and PDGFRA positive tumors, highlighting the exclusion of CD8 T cells by iCAFs. C. CXCL12 expression was highly upregulated in advanced BLCA. D. Statistics analysis indicated a significant decrease of intratumoral CD8 T cells in iCAFs positive tumors. E. The IMvigor210 cohort confirmed the iCAFs score was correlated with patients' responsiveness to ICB therapy. *** p<0.001, *p<0.05