The CAFs Related Gene CALD1 Is a Prognostic Biomarker and Correlated With Immune Infiltration in Bladder Cancer

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Primary research

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Abstract

Background: Stromal components of the tumor microenvironment contribute to bladder cancer progression, and Cancer-Associated Fibroblasts (CAFs) were reported to play an important role. Accumulating pieces of evidence indicate that CAFs participate in the crosstalk with tumor cells and have a complex interaction network with immune components. Further study of the role of CAFs in the bladder cancer microenvironment and the search for possible specific markers are important for a deeper understanding of the roles of CAFs in bladder cancer progression and immunomodulation.

Methods: In the present study, we examined the abundance of CAFs in the TCGA and GEO datasets using the MCP-Counter algorithm. Additionally, the expression of genes related to CAFs was analyzed through the Weighted Gene Co-expression Network Analysis (WGCNA). The CIBERSORT and ESTIMATE algorithms were used for the correlation analysis between the key CAFs related gene and the tumor microenvironment components. Immunohistochemistry analysis in clinical samples was used to validate the results of bioinformatics analysis.

Results: The results showed that CAFs were closely associated with the progression and prognosis of bladder cancer. WGCNA also revealed that CALD1 was a key gene significantly associated with CAFs in bladder cancer. Moreover, the further in-depth analysis showed that CALD1 significantly affected the progression and prognosis of bladder cancer. The CIBERSORT and ESTIMATE algorithms significant correlations between CALD1 and the tumor microenvironment components, including CAFs, macrophages, T cells, and multiple immune checkpoint related genes. Finally, immunohistochemistry results of clinical samples' validated the strong association between CALD1, CAFs, and macrophages.

Conclusions: In the present study, we confirmed the cancer-promoting roles of CAFs in bladder cancer. Being a key gene associated with CAFs, CALD1 may promote bladder cancer progression by remodeling the tumor microenvironment. The bioinformatics methods, including the CIBERSORT, MCP-COUNTER and ESTIMATE algorithms, may provide important value for studying the tumor microenvironment.

Introduction

Bladder Cancer (BLCA) is one of the most common malignant tumors of the genitourinary system (1). Based on the invasion depth, BLCA can be divided into Non-muscle Invasive (NMIBC) and Muscle Invasive Bladder Cancer (MIBC). Notably, NMIBC has a better prognosis and more treatment options compared to MIBC (2). Besides, intravesical instillation of BCG was first reported to be effective in treating NMIBC by Morales et al. in 1976, with complete remission rates of up to 70% – 80% (3). This was the earliest application of immunotherapy for the management of BLCA and has been used up to date. Moreover, BLCA represents a growing number of cancers characterized by the infiltration of a significant number of immune cells in the TME (4, 5), making it suitable for immunotherapy.

Tumorigenesis and tumor development are complex processes affected by many factors. In addition to genetic changes and epigenetic defects, the tumor microenvironment (TME), which consists of cellular
and non-cellular components, also plays an essential regulatory role (6). Tumor stromal cells are the main cellular components of the TME. Furthermore, stromal cells in the TME, especially Cancer-Associated Fibroblasts (CAFs) (7), have multiple effects on cancer growth and maintenance.

CAFs interact with immune components by secreting various factors such as collagen, Matrix Metalloproteinases (MMPs), and chemokines (8). Additionally, Tumor-Infiltrating Immune Cells (TIICs) in the TME were critically associated with cancer outcomes. Out of all the TIICs, macrophages are always among the most abundant in the TME, including in BLCA (9). Moreover, existing evidence indicates that CAFs interact with the M2 macrophages, referred to as Tumor-Associated Macrophages (TAMs), promoting immunosuppression and inducing the occurrence and progression of cancers (10).

Furthermore, bioinformatics methods are now widely used in cancer research. Through high-throughput sequencing, mechanisms underlying pathological processes, including cancers, can be revealed by comparing different genes’ expression networks. Currently, the CIBERSORT (11) and MCP-COUNTER (12) algorithms for calculating the abundance of immune cells are widely used in studies of the TME. CAFs scores and relative levels of 22 TIICs can be computed using the MCP-COUNTER and CIBERSORT algorithms, which provide significant help in studying the relationship between CAFs and TIICs. Moreover, genes highly co-expressed in cancer can be identified through the Weighted Gene Co-expression Network Analysis (WGCNA)(13). Therefore, through these bioinformatics means, the present study uncovered and validated that Caldesmon 1 (CALD1), a key gene associated with CAFs, was crucial in regulating both the stromal and immune microenvironment of BLCA. Consequently, it may become a promising biomarker of BLCA progression.

Methods And Materials

The source of data

The gene expression quantification data for transcriptome profiling included 430 samples (19 normal samples and 411 tumor samples). All the data was downloaded from the TCGA database (https://portal.gdc.cancer.gov/). Moreover, the gene expression profiling dataset (GES13507) was downloaded from the Gene expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/), including 165 primary BLCA samples, 23 recurrent non-muscle invasive tumor tissues, 58 normal looking bladder mucosae surrounding Cancer, and the corresponding clinical data.

Calculation of CAFs scores

The MCP-counter algorithm (12, 14), provided by TIMER 2.0 (http://timer.cistrome.org/), was used to calculate the CAFs score of patients recruited from both the TCGA and GEO databases.

Estimation of the tumor microenvironment

The ESTIMATE algorithm in the estimate package of the R software (version 4.0.2) was used. Three scoring forms, namely immune score, stromal score and ESTIMATE score, were positively correlated with
the proportion of immune and stromal components as well as the sum of both. Therefore, it is indicated that the higher the score, the more significant the ratio of the corresponding parts in the TME.

**Screening for differentially expressed genes (DEGs)**

To screen for DEGs in both the TCGA and GEO cohorts, we divided the patients into two groups (High and low CAFs) according to the medium level of the CAFs score. After that, analysis of differential gene expression between these two groups was performed using the Bioconductor *limma* package of the R software (version 4.0.2). Genes with $p \leq 0.01$ and $|\log FC| \geq 1$ were considered to be significantly differentially expressed.

**WGCNA for the determination of key genes**

The Weighted Gene Correlation Network Analysis (WGCNA) R-package was used for co-expression network analysis. CAFs related genes were selected from the most significant modules related to high levels of CAFs. Key genes were obtained from the intersection of CAFs related genes and DEGs in both the TCGA and GEO cohorts.

**GO and KEGG enrichment analysis**

The "clusterprofiler" R package was used to perform GO and KEGG enrichment analyses for hub genes. GO terms or KEGG pathways with corrected P-values $\leq 0.05$ were considered to be significantly enriched in the hub genes.

**Gene Set Enrichment Analysis (GSEA)**

The Hallmark and C2 Kegg gene sets v7.2 were used for GESA, which was performed using the GSEA software (version 4.1.0) obtained from the Broad Institute. Gene sets with NOM $p < 0.05$ and False Discovery Rate (FDR) $q < 0.05$ were considered to be significant.

**Survival Analysis**

Survival analysis was conducted using the "survival" and "survminer" packages of the R software. Additionally, the Kaplan-Meier method with the best cut-off value was used to draw the survival curves. P values from the log-rank test that was less than 0.05 were considered to be significant.

**TIICs Profile**

The R software's CIBERSORT algorithm was used to determine the profile of TIICs (including 22 immune cells) in all tumor samples of the TCGA cohort. 235 patients with $p < 0.05$ were selected for subsequent analysis.

**Immunohistochemistry analysis**

Here, we used ACTA2, a marker of CAFs, and CD206, a marker of macrophage M2, to discuss the relation between CALD1, CAFs, and macrophage M2. The expression of CALD1 (1:250, Abcam, ab32330), ACTA2 (1:250, Abcam, ab7817), and CD206 (1:4000, Abcam,ab252921) in tumor tissues was detected using the
BenchMark GX automatic immunohistochemical staining system (Roche, Switzerland). After deparaffinization, the tissue sections were incubated with primary antibody for 32 minutes. Biotinylated anti-IgG antibody and horseradish peroxidase were used to show positive expression areas. Hematoxylin was used for counterstaining and Bluing Reagent for post counterstaining.

Statistics analysis

Univariate Cox regression was performed using the "survival" package of the R-software. Genes with p values of less than 0.05 were considered to be related to survival. Moreover, survival-related hub genes were identified from the intersection of survival-related genes in the TCGA and GEO cohorts. Correlation analysis was conducted through the Pearson correlation. Gene expression was transformed by log2 (FPKM + 1). Differential analysis between the high and low CALD1 expression groups was then conducted through the Wilcoxon test. The Kruskal test estimated statistical significance for variables of more than two groups. P-value < 0.05 was considered significant.

Results

1. Abundance of CAFs is a poor prognostic factor associated with the progression of BLCA.

We first calculated the abundance of CAFs in the TCGA cohort using the MCP-COUNTER algorithm. The results indicated that CAFs were more abundant than any other cell types in the tumor microenvironment (Figure 1A) and had a significant correlation with the stromal score (R=0.73), immune score (R=0.37) as well as the ESTIMATE score (R=0.59) (Figure 1B). Moreover, high levels of CAFs were significantly associated with low survival in BLCA patients, as shown in Figure 1C (p=0.003). Furthermore, we compared the effects of different cell types in the microenvironment on the clinical characteristics of BLCA. Notably, CAFs were found to have a significant impact on BLCA grade as shown in Figure 1D (p=0.001), stage as highlighted in Figure 1E (p=0.001), T classification as indicated by Figure 1F (p=0.001), and lymph node metastasis as shown in Figure 1G (p=0.001). These results, therefore, suggested that the abundance of CAFs supported the progression of BLCA. These results were further verified by analyzing the role of CAFs in the GEO BLCA cohort. Similar results were obtained since CAFs were highly abundant in the TME, showed a strong correlation with the ESTIMATE score (Figure 2A), and lowered the Overall Survival (OS) of BLCA patients (Figure 2B). Moreover, CAFs were closely associated with the stage of BLCA in patients and lymph node metastasis (Figure 2C), which strongly validated the findings from TCGA. Although CAFs had no significant effect on distant metastasis in BLCA in both cohorts due to the limited number of M1 patients, the study still observed a trend in which CAFs promoted distant metastasis. Therefore, the above results suggested that the abundance of CAFs is a poor prognostic factor and enhances the progression of BLCA.

2. Identification of 74 hub genes related to CAFs as well as their enriched functions and pathways.

We further categorized patients into the high and low CAFs groups then screened for DEGs in the TCGA and GEO cohorts. A total of 555 and 187 genes were differentially expressed between the low and high
CAFs groups in the TCGA and GEO cohorts. The heatmap shows the top 50 DEGs in Figures 3A and B. Moreover, WCGNA was applied to screen for modules that had the most significant association with levels of CAFs in both the TCGA (Figure 3C) and GEO cohorts (Figure 3D). The yellow module in the TCGA cohort showed the most significant association with a correlation level of 0.8, while the correlation between gene significance (GS) and module membership (MM) was 0.96. Similarly, the correlation between the blue module and the abundance of CAFs was shown to be 0.52, while GS and MM's correlation was 0.56.

Additionally, the intersection of DEGs and genes in the most related modules identified 74 hub genes (Figure 4A). Go functional analysis and KEGG enrichment analysis indicated that these genes were crucial in functions related to remodeling of the extracellular matrix. Notably, the following GO terms were enriched; extra matrix organization, collagen-containing extracellular matrix and extracellular matrix structure constituent, et al. (Figure 4B). On the other hand, the following KEGG pathways were enriched; focal adhesion, ECM-receptor interaction, et al. (Figure 4C).

3. Identification of three key genes related to CAFs in BLCA.

Univariate cox regression was first conducted on both the TCGA and GEO cohorts based on the expression of hub genes. The results showed that 10 and 22 genes, respectively, were significantly related to patients' survival with p values less than 0.05. The genes' p values and hazard ratios are shown in forest plots separately (Figure 4D and 4E). The intersection of survival-related hub genes in TCGA and GEO identified CALD1, COL18A1 and TNC as the three key genes related to CAFs and further influenced OS in BLCA (Figure 4F). Notably, all these genes were significantly correlated with markers of CAFs, including; ACTA2 (α-SMA), MFAP5, MMP2, PDGFRB, VIM, FN1, FAP, FOXF1 and ZEB1 (Figure 4G)(15). Additionally, TNC was reported to be a biomarker of CAFs (16) and is a well known independent risk factor for BLCA (17). COL18A1 was previously reported to be involved in a 12-gene progression score significantly associated with progression(18). CALD1 was also defined as a poor prognostic factor in BLCA (19). In the present study, we selected CALD1 for further analysis. GESA analysis through the hallmarks gene sets confirmed that CALD1 was positively involved in pathways related to epithelium to mesenchymal transition and hypoxia, which are crucial for inducing immunosuppression of the TME (Figure 4H). Besides, GSEA of KEGG gene sets indicated that CALD1 was involved in multiple microenvironment remodeling pathways such as adhesion molecules cams, ECM receptor interaction and focal adhesion. It was also enriched in immune-related pathways, including the chemokine signaling pathway and cytokine-cytokine receptor interaction (Figure 4I).

4. Correlation between CALD1, OS, and clinical characteristics in the TCGA BLCA cohort and its involvement in the modulation of the TME

In the TCGA BLCA cohort, CALD1 was shown to markedly impact BLCA patients' OS since there was a significant difference between the high and low CALD1 expression groups (p<0.001). Additionally, the predictive value of CALD1 in cancer progression was confirmed through the ROC curve with an AUC of 0.679. Moreover, the expression levels of CALD1 differed significantly between different stages, T and N
classifications (Figure 5A). Furthermore, the study observed a trend of increasing CALD1 levels with cancer metastasis, although no statistical significance was obtained. We further compared CAFs, macrophages and ESTIMATE scores between the high and low CALD1 expression groups. Results showed that the high CALD1 group had significantly higher CAFs, macrophages, stromal, immune, and ESTIMATE scores than the low CALD1 group (Figure 5B,5C). These results, therefore, indicated that CALD1 is a detrimental factor in the progression of BLCA. The findings also confirmed that CALD1 was involved in modulating both stromal and immune microenvironment, which was possibly achieved through CAFs and macrophages.

5. Involvement of CALD1 in the regulation of TIICs and the immune checkpoint pathway.

The CIBERSORT algorithm was further used to validate the effect of CALD1 on TIICs in BLCA. The proportion of each TCGA BLCA patient's infiltrated immune subsets was analyzed using the CIBERSORT algorithm (Figure 6A). Notably, correlation analysis showed that CALD1 was positively associated with macrophages (M0, M2) and negatively related to CD8+ T cells (Figure 6C). A comparison of the TIICs levels between the high and low expression of CALD1 also confirmed an elevated level of macrophages (M0, M2) and decreased CD8 + T cells in the high CALD1 expression group (Figure 6D). Consequently, the study further examined whether CALD1 was correlated with immune checkpoints such as PD-L1, which was also crucial in predicting immunotherapy efficacy in BLCA. Immune-checkpoint related genes, including CTLA-4, LGALS9 (GAL9), LAG-3, PDCD1 (PD-1), PDCD1LG2 (PD-L2), CD274 (PD-L1), TIGIT and HAVCR2 (TIM-3) were therefore selected for further analysis. Interestingly, almost all the genes (CTLA-4, LAG-3, PD1, PDL2, PDL1, TIGIT and TIM-3) were up-regulated in patients with high expression of CALD1 (Figure 6E, F). These results, therefore, highlighted the role of CALD1 in regulating TIICs and immune checkpoint pathways.

6. Validation of the immune regulatory role of CALD1 in the GEO cohort

To validate the results from the TCGA cohort, we further analyzed the effect of CALD1 on BLCA patients in the GEO cohort. The results showed that high expression of CALD1 was significantly associated with a shorter OS. Moreover, the ROC curve revealed that CALD1 had an AUC of 0.730 in predicting localized BLCA progression to metastatic BLCA. Significant differences in the expression levels of CALD1 were also observed between stage as well as T and N classification (Figure 7A). Moreover, up-regulated CAFs, macrophages and ESTIMATE scores were shown in the high CALD1 expression group compared to the other category (Figure 7B, C). Furthermore, significantly higher CTLA4, LAG3, PDL1, PDL2 and TIM3 expression were observed in patients with high expression of CALD1 (Figure 7D, E), further confirming the role of CALD1 in regulating immune checkpoints.

7. Expression of CALD1 in clinical specimens in the validation cohort.

40 BLCA patients with different grades, stage and TNM classification, were recruited to validate the above results. Expression levels of CALD1 were examined in pathological sections after clinical treatment with TURBT or radical cystectomy. The results revealed high expression levels of CALD1 in patients with a
higher grade (Figure 8A) and stage (Figure 8B). Moreover, high co-expression was found between CALD1, ACTA2, and CD206 in the tumor stroma, especially in patients with advanced BLCA. These results confirmed the association of CALD1 with CAFs and macrophages, which may further lead to the progression of BLCA.

**Discussion**

BLCA is among the cancers characterized by infiltration of abundant immune cells in the TME, confirmed by BCG's treatment efficiency. Moreover, recent advances in immune checkpoint inhibitor therapy for BLCA further demonstrate that BLCA is profoundly regulated by tumor immunity (20).

Tumor cells and the microenvironment are a whole functional unit where the cells are regarded as seeds while the microenvironment in which they are located is considered the soil (21). Therefore, tumor cells and their microenvironment interact with each other and evolve together to promote tumors. Moreover, the TME is a complex integrated system divided into the immune microenvironment and non-immune microenvironment. Stromal components dominated the non-immune microenvironment (22), especially CAFs (23). There have been numerous studies on the crosstalk between stromal and immune components of the TME (24, 25). Notably, macrophages are usually the most abundant TIICs in the tumor microenvironment, including in BLCA (26). Macrophages consist of two groups with different phenotypes, namely M1 and M2. Macrophages M2 are associated with immunosuppressive functions, angiogenesis, and the extracellular matrix's degradation, contributing to cancer migration and metastasis (27).

Numerous studies have demonstrated that the interaction between CAFs and macrophages can further promote the progression of cancer. For instance, Mazur et al. reported that CAFs could increase macrophages' adhesive ability and promote cancer invasion and metastasis (28). Additionally, Betul et al. showed that CAFs could recruit and differentiate monocytes into M2 macrophages and exert their immunosuppressive role through the PD-1 axis (29). The macrophages could also reversely modulate the status of CAFs. Zhang et al. demonstrated that macrophages could turn umbilical cord mesenchymal stem cells into CAFs, further promoting cancer progression through Epithelial-mesenchymal Transition (EMT) (30). The above evidence demonstrates that CAFs are involved in tumor immunomodulation and promoted tumor progression by interacting with macrophages. In the present study, CAFs were shown to be abundant in BLCA. Furthermore, our results also indicated that CAFs conferred significant adverse effects on the progression of BLCA and OS. We also confirmed a close relationship between CAFs and macrophages. Therefore, combined with existing evidence, the interaction between these two cell types can significantly promote the progression of BLCA. Targeting these two cell types may be a potential strategy for the treatment of BLCA.

On the other hand, we identified three key genes related to CAFs by WCGNA and take CALD1 for further study. Previous researches have demonstrated that CALD1 was involved in cell proliferation and migration through actin cytoskeleton recombination (19). It was also recognized as a tumor-specific splicing variant in colon, bladder, and prostate tissue samples. The mis-splicing of CALD1 is an
independent epigenetic event that is related to the destruction of the tight junctions between epithelial
cells, hence altering the stiffness of the extracellular matrix and promoting cancer invasion and
metastasis (31). Studies also confirmed CALD1 to be a risk factor for the progression of BLCA, but its role
with regard to CAFs and immune regulation in BLCA is yet to be reported.

In this study, the vital role of CALD1 in the progression of BLCA was demonstrated in three independent
cohorts. As a key gene associated with CAFs, CALD1 showed strong correlations with stromal and
immune scores, suggesting its dual regulation of stromal and immune components. Also, CALD1
exhibited essential involvements in the processes modulating the TME, including EMT (32), hypoxia (33),
extracellular matrix remodeling (34) and cytokine regulation (35), as revealed by GSEA. Moreover, CALD1
represented a positive correlation with M2 macrophages and a negative correlation with CD8+ T cells.
Also, high correlations between the expression levels of CALD1 and multiple immune checkpoint genes
were observed. These results highlighted the critical function of CALD1 in inducing immunosuppression
in BLCA. The increased expression of CALD1 may be correlated with high expression of macrophage M2
and checkpoints and the depletion of T cell CD8. In the clinical validation cohort, we well confirmed the
co-expression of CALD1, ACTA2 and CD206 through immunohistochemistry, which demonstrated the
close correlation between CALD1, CAFs and macrophages. At the same time, in tumor specimens with
different stages and depth of tumor invasion, we found significantly differentially expressed CALD1 level,
which further indicated that CALD1 has the potential to be used as a marker of BLCA progression.

From our study, we confirmed that CALD1 is a risk factor in the progression of BLCA. It was also approved
for the first time that CALD1 regulated the tumor microenvironment associating with CAFs and
macrophages. Also, our results clearly demonstrate the importance of bioinformatics analysis in cancer
researches. Through the bioinformatics means such as WCGNA, CIBERSORT and MCP-counter, combined
with clinical verification, we can get information that other means cannot achieve. It is believed that with
the continuous progress of the algorithms, bioinformatics can provide more significant help for clinical
diagnosis and treatment.

Despite the insightful findings, limitations still exist in our study. First, relationships among CALD1, CAFs,
macrophages and immunosuppression were only verified by correlation analysis. Further verification
from in vitro and in vivo experiments are required for exploring the exact mechanisms. Second, the
potential of CALD1 to become a specific marker of CAFs in BLCA still need validation since we did not
discuss its expression in normal fibroblasts. Last, although significant co-expression was found between
CALD1, ACTA2 and CD206 in BLCA sections, a more extensive validation cohort is still necessary to avoid
the selection bias.

Conclusion

In conclusion, this study confirmed the pro-tumor function of CAFs and identified CAFs-related genes in
BLCA through WCGNA and screening for DEGs in both the TCGA and GEO cohorts. Moreover, CALD1 was
recognized as one of the key genes related to CAFs and outcomes in BLCA. Further analyses showed that
CALD1 played a vital role in regulating the TME of BLCA. Furthermore, CIBERSORT and correlation analysis confirmed that CALD1 was related to the infiltration level of multiple immune cells in the TME, especially macrophages M2 and CD8 T cells. It was also shown that high expression of CALD1 might lead to an increased level of immune checkpoint-related genes, including PDL1. Therefore, CALD1 may be associated with the immunosuppression status of TME in BLCA, which further leads to tumor progression. Further studies on CALD1 may provide insights into the immune network in BLCA and offer new targets for cancer treatment.

**Declarations**

**Ethics approval and consent to participate**

40 BLCA spacemen were collected from Shanghai First People's Hospital, with patients' informed consent and the approval of the Medical Ethics Committee of Shanghai First People's Hospital (approval number: 2020KY143).

**Consent for publication**

All authors read and approved the final manuscript.

**Availability of data and materials**

The datasets used and analyzed during the current study are available from Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) and The Cancer Genome Atlas (portal.gdc.cancer.gov).

The results of the immunohistochemistry will be available when it is necessary.

**Competing interests**

The authors declare that there are no conflicting interests.

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**Authors' contributions**

YiHeng Du and Xiang Jiang have equal contributions to this study. YiHeng Du and HaiTao Liu designed the whole study. YiHeng Du and Bo Wang conducted the statistical analysis. Xiang Jiang and Jin Cao did the immunohistochemistry analysis. YiHeng Du and Xiang Jiang draft the manuscript. Yi Wang and Jiang Yu made the relevant edits to the manuscript. XiZhi Wang and HaiTao Liu revised the manuscript.
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Figures
Figure 5

CALD1 regulated the TME and promoted the progression of BLCA. A. Kaplan-Meier curves of OS between high and low CALD1 group using Log-Rank test in TCGA BLCA cohort. ROC curve demonstrated the accuracy of CALD1 in predicting cancer progression, with an AUC of 0.679. The expression of CALD1 was significantly elevated with cancer progression. Three different bar plots showed the expression level of CALD1 in different tumor stages, T and N classification, respectively. B. CAFs (p<0.001), macrophages (p<0.001), stromal score (p<0.001), immune score (p<0.001) and ESTIMATE score (p<0.001) in the CALD1 high expression group were significantly higher than those in the CALD1 high expression group. C. Close relationships of CALD1 with CAFs (R=0.88, p<0.001), Macrophages (R=0.58, p<0.001), stromal score (R=0.84, p<0.001), immune score (R=0.51, p<0.001) and ESTIMATE score (R=0.72, p<0.001) were observed in the TCGA cohort.
Figure 7

Validation of the TME regulating, Oncogenic promoting and immune checkpoint associating role of CALD1 in the GEO cohort. A. Kaplan-Meier curve validated the difference of OS between high and low CALD1 expression BLCA Patients in the GEO cohort, with a P-value <0.001. The AUC value of the ROC curve for CALD1 prediction of tumor progression in the GEO cohort was 0.73. A significantly higher level of CALD1 was also detected in patients with higher grade, stage, T and N classification in the GEO cohort. B-C. CAFs (p<0.001), macrophage (p<0.001), stromal score (p<0.001), immune score (p<0.001) and ESTIMATE score (p<0.001) were significantly different between the high and low CALD1 expression
groups, and their expression level was also strongly correlated with CALD1 (CAFs: $R=0.86, p<0.001$; macrophage: $R=0.47, p<0.001$; stromal score: $R=0.72, p<0.001$; immune score: $R=0.51, p<0.001$; stromal score: $R=0.81, p<0.001$) D-E. Close relationships were validated between CALD1 and immune checkpoint related genes, including CTLA4 ($p<0.001$, $R=0.46$), GAL9 ($p<0.05$, $R=-0.3$), LAG3 ($p<0.001$, $R=0.31$), PD-L2 ($p<0.001$, $R=0.28$) and TIM3 ($p<0.001$, $R=0.38$) in the GEO cohort.