

ORIGINAL RESEARCH ARTICLE

Sialic acid metabolism-related gene CTSA drives prostate cancer immunosuppression and tumor progression

Jiahao Lei^{1†}, Shuntian Gao^{2†}, Yao He^{3†}, Dongxin An¹, Rui Sun¹,
Lingwu Chen^{1*}, Ren Liu^{1*}, and Zongren Wang^{1*}

¹Department of Urology, The First Affiliated Hospital of Sun Yat-sen University, Guangzhou, Guangdong, China

²Institute of Precision Medicine, The First Affiliated Hospital of Sun Yat-sen University, Guangzhou, Guangdong, China

³Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou, Guangdong, China

[†]These authors contributed equally to this work.

*Corresponding authors:

Lingwu Chen
(chenlwu@mail.sysu.edu.cn)
Ren Liu
(liur227@mail.sysu.edu.cn)
Zongren Wang
(wangzr27@mail.sysu.edu.cn)

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Abstract

Prostate cancer (PCa) is the second most common cancer in males. Androgen deprivation therapy (ADT) serves as the primary non-surgical treatment approach. However, the frequent progression to castration-resistant PCa after ADT drives the need for new therapeutic modalities in PCa. In recent years, tumor immunotherapy has made significant progress in the treatment of many tumors, but clinically, only a small number of PCa patients benefit from immunotherapy because of cold immune microenvironment. Many studies have shown that sialic acid metabolism is related to the immunosuppressive tumor microenvironment (TME). Utilizing RNA sequencing data in The Cancer Genome Atlas (TCGA), we investigated the association between sialic acid metabolism-related genes and the carcinogenesis of PCa. Based on both transcriptomic and proteomics data, we discovered that the high expression of the cathepsin A (CTSA) is associated with the tumorigenesis and poor prognosis of PCa. Further pathway enrichment analysis revealed that CTSA is related to several immunity-associated pathways. We validated the role of CTSA in the TME of PCa using single-cell RNA sequencing. Through animal experiments and fluorescence staining, we demonstrated that CTSA contributes to the formation of immunosuppressive TME. Targeting CTSA provides a potential effective for PCa treatment and enhances immunotherapy efficacy.

Keywords: Sialic acid; Cathepsin A; Prostate cancer; Tumor microenvironment

1. Introduction

Prostate cancer (PCa) represents the second most common cancer in the global male population. Each year, over 1 million of new PCa cases are identified worldwide. Furthermore, PCa leads to more than 350,000 deaths annually. As a result, it has become one of the foremost contributors to cancer-related mortality among men.¹ Androgen deprivation therapy (ADT) is a cornerstone treatment for PCa, which is a hormone-sensitive malignancy.² Initial treatment with ADT induces remission in some PCa

patients, but progression to castration-resistant PCa remains common³. In recent years, immune checkpoint blockade therapy has emerged as a novel approach in multiple advanced malignancies. PCa, regarded as immune “cold” tumor, shows limited benefit from immune checkpoint inhibitors (ICIs).^{4,5} At present, the benefit of immunotherapy appears to be largely confined to PCa patients with mismatch repair deficiency (dMMR), a rare subtype with an estimated incidence of 0.4–2.8%.^{6,7} Recent finding highlights the tumor microenvironment (TME) as a critical determinant of immunotherapy efficacy in PCa.⁸ In the TME of PCa, while T cells and B cells exert anti-tumor immune functions, tumor-associated macrophages (TAMs) promote oncogenesis and metastatic spread through pro-inflammatory mechanisms and immune suppression.⁹ Furthermore, accumulating evidence identifies myeloid-derived suppressor cells (MDSCs) as major contributors to immune dysfunction in PCa.¹⁰ These various immune components collectively shape the dynamics of the TME and are instrumental in determining the clinical behavior of PCa.

Metabolic reprogramming of tumor cells is increasingly being identified as a critical driver of immune evasion.^{11,12} Sialic acid, a terminal modification molecule of cell-surface glycans, contributes to oncogenic progression in diverse cancers through its metabolic dysregulation.¹³ Studies have revealed that sialylation, mediated by sialic acid, can directly suppress the activation of T cells, natural killer cells and macrophages by either masking tumor antigens or engaging inhibitory receptors (*e.g.*, Siglecs) on immune cells.^{14–16} Moreover, desialylation has been proved to improve the efficacy of ICI therapy.¹⁴ Our previous work demonstrated that targeting *NANS*, a key gene in sialic acid metabolism, can reverse the immunosuppressive TME.¹⁷ However, whether other sialic acid metabolism-related genes can serve as potential therapeutic targets for PCa still requires further research.

Cathepsin A (CTSA), associated with sialic acid metabolism, is primarily located in lysosomes and involved in the degradation of various intracellular or extracellular matrices. CTSA interacts with neuraminidase-1 (NEU1, a sialidase), to form a complex *in vivo*, protecting it from degradation. Research suggests NEU1 may serve as a viable druggable target in cancer and immune-related diseases.¹⁸ Mutations or aberrant expression of CTSA are associated with occurrence of galactosialidosis.^{19,20} Existing research has established CTSA's involvement in the pathogenesis of multiple malignancies, including colorectal cancer, malignant melanoma, lung adenocarcinoma, and glioma.^{21–24} Notably, comparative analyses reveal marked CTSA upregulation in PCa tissues compared to benign controls, where increased expression serves as an

independent prognostic indicator.²⁵ However, systematic research on the functional role of CTSA in shaping the PCa's TME is lacking. Therefore, in-depth exploration of the role of the *CTSA* gene in PCa's TME not only helps to reveal new immune regulatory mechanisms but also provides potential targets for the immunotherapy of PCa.

In the current study, by integrating transcriptomic and proteomic data, we identified *CTSA* as a gene associated with the occurrence, development, and prognosis of PCa. Further pathway enrichment analysis revealed that *CTSA* is related to immune-related pathways. Single-cell sequencing data further confirmed the role of *CTSA* in the TME. Finally, through animal experiments and fluorescence staining, we demonstrated that *CTSA* plays a role in the formation of an immunosuppressive TME.

2. Materials and methods

2.1. Data collection

For the PCa analysis, we integrated three publicly available datasets (The Cancer Genome Atlas Prostate Adenocarcinoma [TCGA-PRAD], German Cancer Research Center [DFKZ], and Belfast cohorts, accessed December 1, 2023) containing biochemical recurrence-free survival (BCR) data with associated clinicopathological parameters. Pan-cancer gene expression data for tumor and normal tissues (*CHOL*, *LAML*, *KIRP*, *BLCA*, *ESCA*, *LIHC*, *PAAD*, *PCPG*, *SKCM*, and *STAD*) were obtained from TCGA (accessed December 1, 2023). In addition, single-cell RNA sequencing of 24 PCa patients was employed utilizing 10× Chromium Single-cell Platform (Lianchuan Biotech, Hangzhou, China). The proteomic analyses were conducted by Shanghai Zhongke New Life Biotechnology Co., Ltd (China) using mass spectrometry. PCa tissue samples were collected from patients undergoing radical prostatectomy at the First Affiliated Hospital of Sun Yat-sen University. None of the included subjects had received prior treatments. Another public single-cell transcriptomic data of normal prostate was obtained from GEO (GSE120716). The normal prostate specimens were obtained from 11 male organ donors aged 18–31 years.²⁶ The raw proteomic datasets from PCa cases in the First Affiliated Hospital of Sun Yat-sen University have been deposited in the iProX repository (<https://www.iprox.cn>) and are publicly accessible under the accession IDs PXD056748 and IPX0009562000.

2.2. Single-cell RNA sequencing data processing

Single-cell RNA sequencing was performed on 24 PCa specimens collected at our center. By applying quality-control filtering, we removed cells expressing fewer than 200 genes or more than 5,000 genes, as well as those with UMI counts below 300. Cells exhibiting mitochondrial

gene expression exceeding 15% of total endogenous gene expression were also excluded. The raw data underwent log2 normalization through the `NormalizeData` function, followed by data scaling using the `ScaleData` function. To identify highly variable genes, we employed the `FindVariableFeatures` function (`selection.method = "vst"`), which selected the top 2,000 genes based on standardized variance.

Cell-type-specific marker genes used for cell annotation are listed in Table A1.

2.3. Cell culture

Cells were cultured as outlined in a previous study.²⁷ The PCa cell line RM1 was acquired from the American Type Culture Collection (Manassas, MD, USA). RM1 cells were cultured at 37°C in RPMI-1640 medium (Procell, Wuhan, China) supplemented with 10% fetal bovine serum (Procell, Wuhan, China) in a humidified incubator with 5% CO₂. Cells were detached by 0.25% trypsin-EDTA solution (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) when they were at 70–80% confluency.

2.4. Mouse experiments

The experimental protocol received formal approval from the IEC for Clinical Research and Animal Trials of the First Affiliated Hospital of Sun Yat-sen University (Approval No. 2024-054), adhering strictly to the institution's ethical standards for biomedical research. For this study, we utilized two murine models: immunocompetent C57BL/6 mice and immunodeficient Balb/c-nude mice, both sourced from the Sun Yat-sen University Animal Experiment Center. Healthy 4-week-old male C57BL/6 ($n = 18$) and Balb/c-nude mice ($n = 18$), each weighing 20–25 g, were used in the animal experiments. Animals displaying evidence of disease or physiological abnormalities at baseline were excluded from the study.

A suspension containing 5×10^6 RM1 cells in phosphate-buffered saline (PBS) was administered through subcutaneous injection into the left axillary region of C57BL/6 and Balb/c-nude mice. To minimize selection bias, randomization was performed after the baseline measurements were taken. The *in vivo* experiments were randomly allocated into three groups (6 mice/group) (Figure 1A). Tumor size and murine body weight were assessed at 3-day intervals. When tumor volumes reached approximately 100–200 mm³, each group received a subcutaneous injection of 50 µL of physiological saline, an intratumoral injection of CTSA small interfering RNA (siRNA)#1, and an intratumoral injection of CTSA siRNA#2, respectively. The concentration of siRNA (0.05 µg/µL) was the same. Samples were injected every

2 days for a total of seven treatments. The investigators who administered the injection and measurement were blinded to the group allocation throughout the experiment. The experimental injection was prepared by an independent researcher and coded to conceal group identity. To prepare the si-CTSA knockdown vector (from Jima, Shanghai, China), the siRNA sequence targeting the CTSA gene was designed and chemically synthesized (detailed sequences are listed in Table A2). The following formula was applied to calculate tumor volumes: $V = L \times W^2 \times 0.5$. Tumor-bearing mice were sacrificed 2 weeks after intratumoral injection or when tumor volume exceeded 1500 mm³.

2.5. Multiplex immunohistochemistry (mIHC)

For immunohistochemical (IHC) analysis, 4-µm-thick sections were trimmed off formalin-fixed, paraffin-embedded tumor tissue blocks using a microtome. To ensure optimal staining quality, sections were first dewaxed by immersion in xylene (2 × 5 min) to remove paraffin, followed by rehydration in a graded ethanol series (100%, 95%, 85% and 75% 5 min each) and final rinsing in distilled water. Subsequently, tissues were post-fixed in 10% neutral buffered formalin for 10 min to preserve antigen integrity. To enable spatially resolved protein profiling, mIHC was performed using the PANO 7-plex IHC Kit (Panovue, Beijing, China; Cat# 0004100100), which utilizes tyramide signal amplification (TSA) technology for high-sensitivity multi-target detection on a single slide. Before staining, antigen retrieval was conducted by microwave heating (95°C, 15 min) in citrate buffer or Tris-EDTA buffer, depending on antibody requirements. Non-specific binding sites were blocked with 10% goat serum in PBS for 10 min at room temperature. Primary antibodies (listed in Table A3), diluted in 1% goat serum, were applied to sections and incubated at 37°C for 30 min in a humidified chamber. After washing, HRP-conjugated secondary antibodies were added for 15 min, followed by TSA fluorophore conjugation to enhance signal specificity. Cell nuclei were labeled with 4',6-diamidino-2-phenylindole (DAPI) for 5 min. Slides were mounted with anti-fade medium to prevent photobleaching. High-resolution whole-slide imaging was performed using the TissueFAXS platform (TissueGnostics, Vienna, Austria). Quantitative analysis of marker co-expression and spatial distribution was conducted using TissueFAXS Spectra software.

2.6. Statistical analysis

Data analysis was conducted using R programming environment (v4.2.3) and GraphPad Prism (v9.5.0). The bioinformatics methodology via R program refers to previous research.²⁸ Appropriate statistical tests were selected based on data characteristics: continuous variables

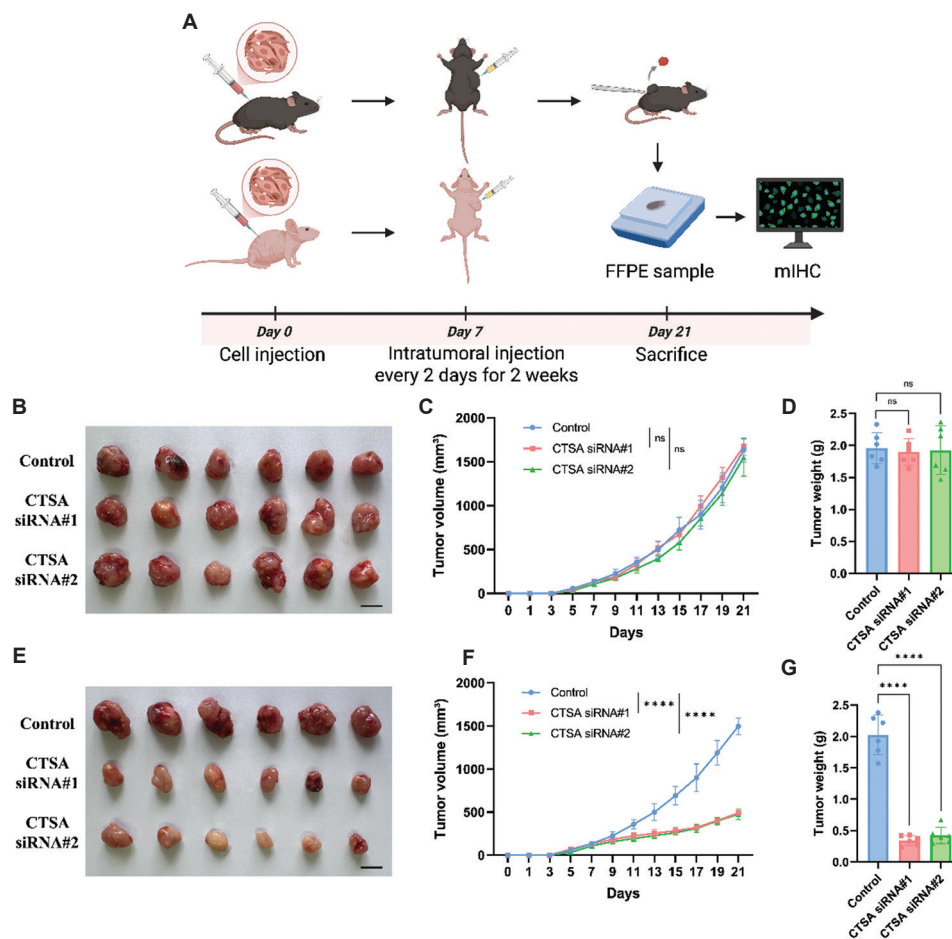


Figure 1. Targeting CTSA enhances the anti-tumor effect. (A) Flowchart of the animal experiments. Created with BioRender. Lei, J. (2025) <https://BioRender.com/aeb7l7w> (Agreement No. GO294ZS16G). (B) Gross appearance of the Balb/c-nude mice tumors. (C) Tumor growth curves of each group in Balb/c-nude mice. Scale bar: 1 cm. (D) Tumor weight of each group in Balb/c-nude mice. (E) Gross appearance of the C57BL/6 mice tumors. Scale bar: 1 cm. (F) Tumor growth curves of each group in C57BL/6 mice. (G) Tumor weight of each group in C57BL/6 mice. Student's *t*-test was used for data analysis in this experiment. $n = 6$; **** $p < 0.0001$.

Abbreviations: CTSA: Cathepsin A; FFPE: Formalin-fixed, paraffin-embedded; mIHC: Multiplex immunohistochemistry.

were compared using paired or unpaired *t*-tests, while categorical variables were analyzed by chi-square tests or Fisher's exact test. Survival outcomes were assessed using the Kaplan–Meier test (survival package). Differential expression analysis was conducted using DESeq2 and limma packages. Pathway enrichment and single-sample gene set enrichment analysis (ssGSEA) were performed using clusterProfiler and GSVA packages, respectively. Single-cell RNA sequencing data were processed using the Seurat packages. Statistical significance was defined as $p < 0.05$ (significant) and $p < 0.01$ (highly significant).

3. Results

3.1. CTSA correlates with poor prognosis in PCa

According to our preliminary research findings, sialic acid metabolic pathways appear significantly associated with

the initiation and metastatic spread of PCa. Therefore, we investigated the expression of all sialic acid metabolism-related genes in the TCGA data for PCa and discovered that CTSA, GNE, NANS, NEU3, SLC17A5, ST6GALNAC1 and ST8SIA5 were overexpressed in PCa tissues compared to prostate benign tissues ($p < 0.05$). Among these, CTSA exhibited one of the most statistically significant differences between tumor and normal tissues ($p < 0.0001$) (Figure 2A). Further analysis revealed that CTSA was also overexpressed in tumor tissues of CHOL, LAML, ESCA, LIHC, PAAD, SKCM, and STAD compared to normal tissues (Figure 2B), indicating that CTSA serves as a key regulator in both cancer initiation and development.

We then employed survival analysis to explore the association between CTSA expression levels in tumor tissues and patient prognosis across different datasets. In

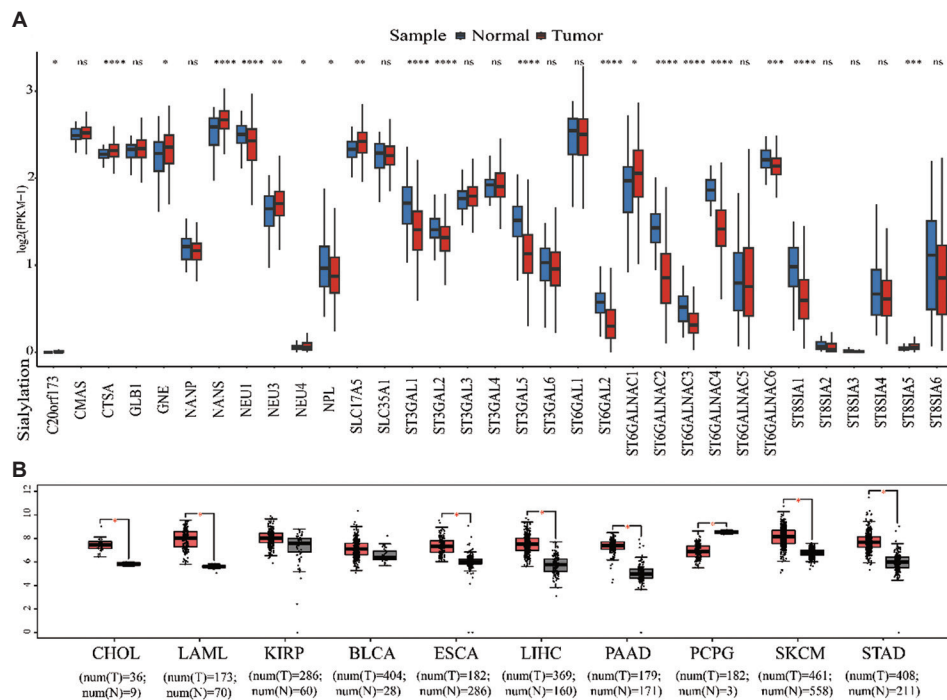


Figure 2. CTSA appears to significantly contribute to PCa tumorigenesis among the sialic acid metabolism-related genes. (A) Expression of sialic acid metabolism-related genes in tumor versus benign tissues, based on the TCGA-PRAD dataset. (B) CTSA expression in tumor versus normal tissues, based on the pan-cancer data in the TCGA dataset. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

Abbreviations: CTSA: Cathepsin A; PCa: Prostate cancer.

TCGA datasets, PCa patients with high CTSA expression exhibited lower relapse-free survival (RFS) ($p = 0.024$, HR = 1.21 [1.02, 1.64]) (Figure 3A). In DFKZ datasets, PCa patients with high CTSA expression exhibited lower RFS ($p = 8.32 \times 10^{-3}$, HR = 3.21 [1.44, 7.15]) (Figure 3B). In Belfast datasets, PCa patients with high CTSA expression exhibited lower RFS ($p = 0.049$, HR = 1.70 [1.01, 2.87]) (Figure 3C). This finding was further validated in our center's proteomics dataset ($p = 0.04$, HR = 2.00 [1.04, 3.85]) (Figure 3D). Collectively, elevated CTSA expression was associated with poorer prognosis of PCa patients.

Finally, we assessed associations of CTSA expression with clinical characteristics in the TCGA transcriptomic data and our center's proteomics cohort (Table 1). In the TCGA cohort, high CTSA expression was significantly associated with higher pT stage ($p = 0.0008$) and higher pN stage ($p = 0.0098$). While no statistically significant difference in CTSA expression was observed using a Gleason score of 7 as the cutoff, a notable trend was found wherein high CTSA expression was more frequently associated with Gleason scores of 8 or above ($p = 0.0175$). High CTSA expression of PCa patients is prone to experience biochemical recurrence ($p = 0.011$). In proteomics data of our center, similarly, high CTSA expression was significantly linked to biochemical recurrence ($p = 0.03$). However, probably due to the small sample size, there was

no significant difference in pT stage, pN stage, Gleason score, and prostate-specific antigen (PSA) levels between patients with high expression and low expression ($p > 0.05$).

3.2. CTSA promotes the immunosuppressive TME

To investigate the potential mechanisms by which CTSA contributes to PCa progression, we stratified PCa patients from the TCGA database into high- and low-expression groups. Differential gene expression analysis of tumor tissues was performed between two groups (Figure 4A). Gene ontology pathway enrichment analysis of the differentially expressed genes indicated that the expression of CTSA may be related to the immune and inflammatory pathway (Figure 4B). To further verify the hypothesis, we performed immune cell infiltration scoring analysis via ssGSEA on patients in the high-expression group and the low-expression group. The results demonstrated significant differences in the scores of immune cells (Figure 4C). Specifically, we found that scores of inhibitory immune cells infiltrating tumors were significantly higher in patients with high CTSA expression than those in the low-expression group, such as macrophages, regulatory T cells (Tregs) and MDSCs. Taken together, these results indicate that CTSA-driven PCa advancement is significantly influenced by the tumor immune microenvironment.

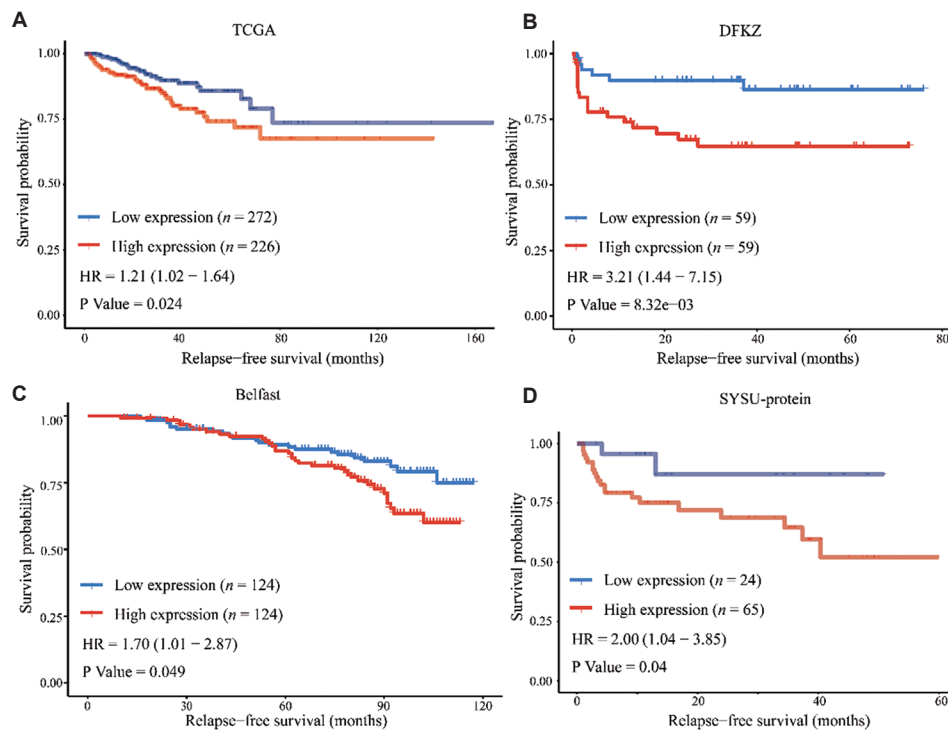


Figure 3. High CTSA expression serves as a negative prognostic indicator for PCa patients. (A) Kaplan–Meier survival analysis based on CTSA RNA expression in the TCGA-PRAD cohort. (B) Kaplan–Meier survival analysis based on CTSA RNA expression in the DFKZ cohort. (C) Kaplan–Meier survival analysis based on CTSA RNA expression in the Belfast cohort. (D) Kaplan–Meier survival analysis based on CTSA protein expression in the Sun Yat-sen University (SYSU) cohort.

Abbreviations: CTSA: Cathepsin A; PCa: Prostate cancer.

To investigate CTSA's involvement in the PCa immune microenvironment, we performed single-cell RNA sequencing on 24 radical prostatectomy specimens collected at our center. After performing quality-control step and removing low-quality cells, a total of 158,545 cells were selected for further analysis. Following UMAP-based dimensionality reduction, the cellular populations segregated into 10 discrete clusters representing unique cell types, including epithelium, endothelium, myeloid, fibroblast, natural killer, B, mast, proliferative, and plasma cells (Figure 5A). We found that CTSA was predominantly expressed in myeloid cells, proliferating cells, and plasma cells (Figure 5B and C).

In addition, we explored CTSA's potential immunomodulatory functions in benign prostate tissue. By analyzing single-cell RNA sequencing data from 11 normal prostate epithelial tissue specimens, UMAP dimensionality reduction classified the cells into nine distinct cell types, including basal epithelia, club epithelia, endothelia, fibroblast, hillock epithelia, leukocyte, luminal epithelia, neuroendocrine epithelia, and smooth muscle (Figure 6A). We observed that CTSA was mainly expressed in leukocytes (Figure 6B and C).

3.3. Targeting CTSA enhances anti-tumor immunity

To elucidate CTSA's immunoregulatory functions in PCa development and TME remodeling, we conducted *in vivo* experiments in immunodeficient (Balb/c-nude) and immunocompetent (C57BL/6) mice. Figure 1A shows the flowchart of animal experiments. We found that there were no significant differences in the volume and weight of the tumors among the control group, siRNA#1, and siRNA#2 groups, which indicated intratumoral siRNA-mediated knockdown of CTSA did not affect PCa progression in immunodeficient mice (Figures 1B–D). However, in immunocompetent mice, knockdown of CTSA significantly suppressed tumor growth ($p < 0.0001$) (Figures 1E–G), confirming that CTSA regulates tumor progression through immune-related pathways.

Next, we performed mIHC on paraffin sections from C57BL/6 mice in the three groups (Figure 7). Compared to the control group, CTSA fluorescence was significantly weaker in the knockdown group, validating successful model establishment. Following the downregulation of CTSA expression, an increase in CD8⁺ T cell infiltration within tumors was observed, accompanied by a reduction in myeloid cell infiltration. These preliminary findings

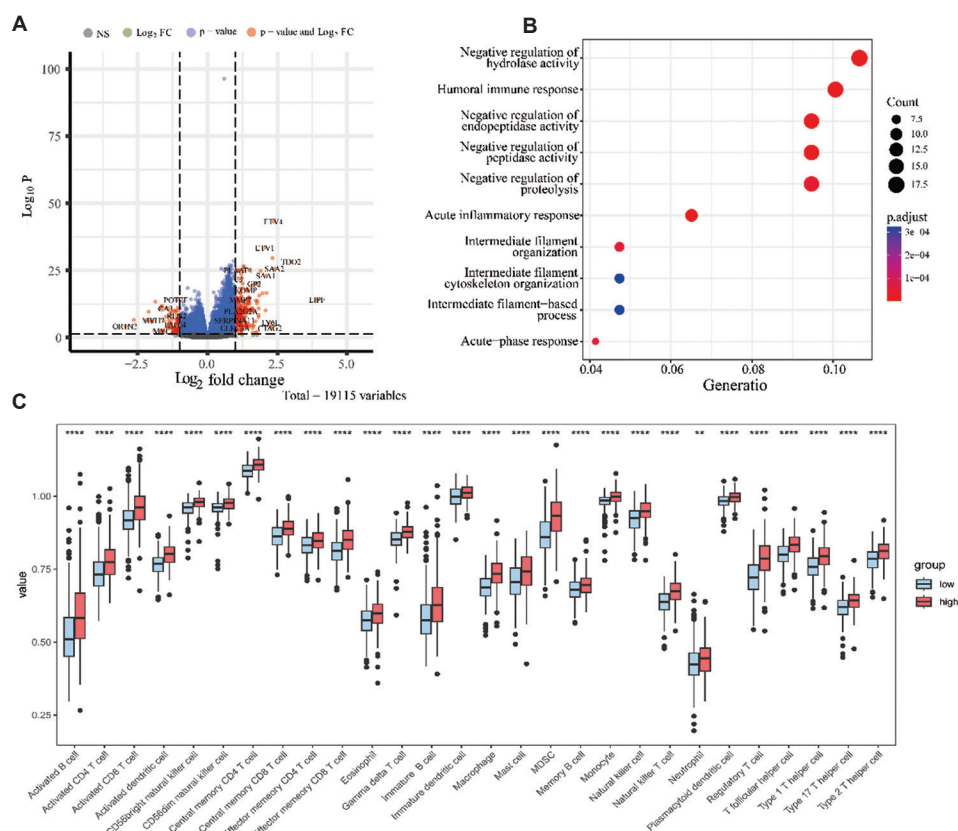


Figure 4. Comparative transcriptomic profiling of high-CTSA versus low-CTSA expression groups in the TCGA-PRAD dataset. (A) Volcano plot visualization of differentially expressed genes (DEGs) between CTSA expression subgroups. (B) Gene ontology pathway analyses of differential genes between high-CTSA and low-CTSA expression groups. (C) Boxplots showing the infiltration status of 28 immune cells in high-CTSA and low-CTSA expression groups calculated by single sample gene set enrichment analysis (ssGSEA). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$. Abbreviations: CTSA: Cathepsin A; MDSC: Myeloid-derived suppressor cell.

demonstrate that CTSA can increase the recruitment of myeloid cells, particularly MDSCs, thereby diminishing CD8⁺ T cell infiltration and fostering the formation of an immunosuppressive TME.

4. Discussion

PCa represents a leading cause of cancer-related morbidity and mortality in the global male population. Despite recent advancements in surgery, radiotherapy, endocrine therapy, and targeted therapy, patients with advanced PCa continue to face treatment resistance and disease progression. The formation of immunosuppressive TME may limit the efficacy of immunotherapy and promote tumor immune evasion. This study identified CTSA as a cancer-promoting gene associated with PCa from a pool of sialic acid metabolism-related genes. Through transcriptomic and proteomic data, our findings revealed a significant association between CTSA upregulation and aggressive tumor behavior, which may be caused by immune-related pathways. Single-cell RNA sequencing

analysis showed that CTSA plays a role in forming an immunosuppressive TME. Further animal experiments demonstrated that knocking down CTSA can inhibit tumor growth in immunocompetent mice and reverse the immunosuppressive TME.

Consistent with previous studies, elevated CTSA expression levels were observed in PCa specimens and showed association with adverse clinical outcomes. Knockdown of CTSA in PCa cell lines significantly reduced colony formation and was accompanied by decreased protein levels of Cdk2 and Cyclin B1, suggesting impaired proliferation and invasion capabilities.²⁵ This confirms the pro-tumorigenic role of CTSA. However, results obtained from experiments with nude mice were different from those in the *in vitro* studies. This discrepancy may be attributed to the absence of *in vivo* microenvironment (such as stromal cells, angiogenesis, extracellular matrix, *etc.*) in *in vitro* experiments, and the antiproliferative effects might have been diluted in long-term *in vivo* studies. Our study primarily focused on the impact of CTSA on immune

Table 1. Association of CTSA expression with clinical variables in PCa cohorts

Clinical features	CTSA expression in TCGA cohort				CTSA expression in SYSU protein cohort			
	n	High	Low	p	n	High	Low	p
Age				0.864				0.748
≤65 years	354	165	189		31	22	9	
>65 years	144	61	83		58	43	15	
pT				0.0008*				0.495
≤T2	189	67	122		57	43	14	
>T2	302	154	148		32	22	10	
pN				0.0098*				0.669
N0	346	146	200		82	59	23	
N1	79	46	33		7	6	1	
Gleason score								
≤7	292	127	165		56	38	18	
>7	206	99	107	0.3136	33	27	6	0.152
8 [#]	64	20	44	0.0716	9	7	2	0.5495
>8 [#]	142	79	63	0.0175*	24	20	4	0.1554
PSA levels				-				0.777
≤12 ng/mL	-	-	-		28	21	7	
>12 ng/mL	-	-	-		61	44	17	
PSA failure				0.011*				0.03*
Negative	435	188	247		67	45	22	
Positive	63	38	25		22	20	2	

Notes: *Compared with the Gleason score ≤7 group. Statistical significance was tested by chi-square test or Fisher's exact test. "PSA Failure" refers to biochemical recurrence or failure after treatment, typically indicating a rise in PSA levels following therapy. * indicates $p < 0.05$.

Abbreviations: CTSA: Cathepsin A; PCa: Prostate cancer; PSA: Prostate-specific antigen.

cells and its role in the TME. In addition, quantitative assessment demonstrated statistically significant variations in tumor-infiltrating immune cell scores when stratifying PCa by CTSA expression, highlighting the importance of CTSA in TME. Moreover, silencing CTSA can enhance CD8⁺ T cell recruitment and diminish immunosuppressive cells. Targeting CTSA holds promise for reversing the immunosuppressive TME, and its combination with ICIs may enhance therapeutic efficacy.

CTSA also plays an important role in the occurrence and development of other malignant tumors. CTSA protein is highly expressed in ductal breast carcinoma. Patients with higher CTSA expression exhibit clinicopathological features and poorer prognosis, characterized by a greater tendency to invasive recurrence and progression, which may be driven by immune-related factors.²⁹ In colorectal cancer, CTSA inhibition mediated by miR-106b-56p

significantly attenuates the migration and invasion abilities of colorectal cancer cells.²¹ CTSA was also found to exhibit significantly higher activity in primary malignant melanoma lesions compared to normal pigmented nevi. Furthermore, CTSA may be involved in the malignant transformation and metastasis of melanoma.²² It has been reported that the mRNA expression level of CTSA in lung adenocarcinoma tissues was significantly higher than that in normal lung tissues, and CTSA knockout reduced the migration and invasion capabilities of lung adenocarcinoma cells.²³ Similar results were also observed in hepatocellular carcinoma, and it was found that CTSA may mainly participate in biological processes such as lysosomal organization and negative regulation of fibroblast proliferation in hepatocellular carcinoma.³⁰

The immunosuppressive microenvironment is typically characterized by the infiltration of immunosuppressive cells such as Tregs, MDSCs and TAMs, as well as the high expression of immune checkpoint molecules like PD-1/PD-L1 and CTLA-4. Together, these components inhibit effective anti-tumor immunity and facilitate immune escape. Our study preliminarily demonstrated that CTSA knockdown leads to reduced infiltration of myeloid cells, particularly MDSCs. MDSCs are associated with resistance to immunotherapy in PCa³¹ and can drive the progression of castration-resistant PCa by secreting interleukin-23 (IL-23), which activates the pSTAT3/RORγ axis, thereby promoting androgen receptor signaling and expression of its downstream targets.³² We also found that high CTSA-expressing PCa exhibited enriched Treg populations and macrophage accumulation within the TME. Study has shown the number of Tregs in the peripheral blood mononuclear cells (PBMC) of PCa patients is significantly higher than healthy individuals. It has also been reported that the Tregs in patients exhibit stronger inhibitory effects compared to those in normal individuals.³³ TAMs, which exhibit an M2-polarized phenotype, facilitate tumor growth, neovascularization, and immune suppression in the TME.³⁴ An increased presence of M2-like macrophages in both epithelial and stromal regions correlates statistically with a poorer prognosis in PCa patients.³⁵ Furthermore, during the progression of PCa, Tregs and M2-like macrophages can mutually stimulate each other through the secretion of cytokines, thereby creating an immunosuppressive TME.³⁶ Therefore, the expression of CTSA is a potential driver of the formation of an immunosuppressive TME formation, and targeting CTSA can reduce the infiltration of immunosuppressive cells, thereby enhancing tumor immunity. In addition to PCa, CTSA may also contribute to the formation of an immunosuppressive TME in other tumors. In glioma, CTSA expression was also significantly positively correlated with TAMs infiltration, aligning with our results.²⁴

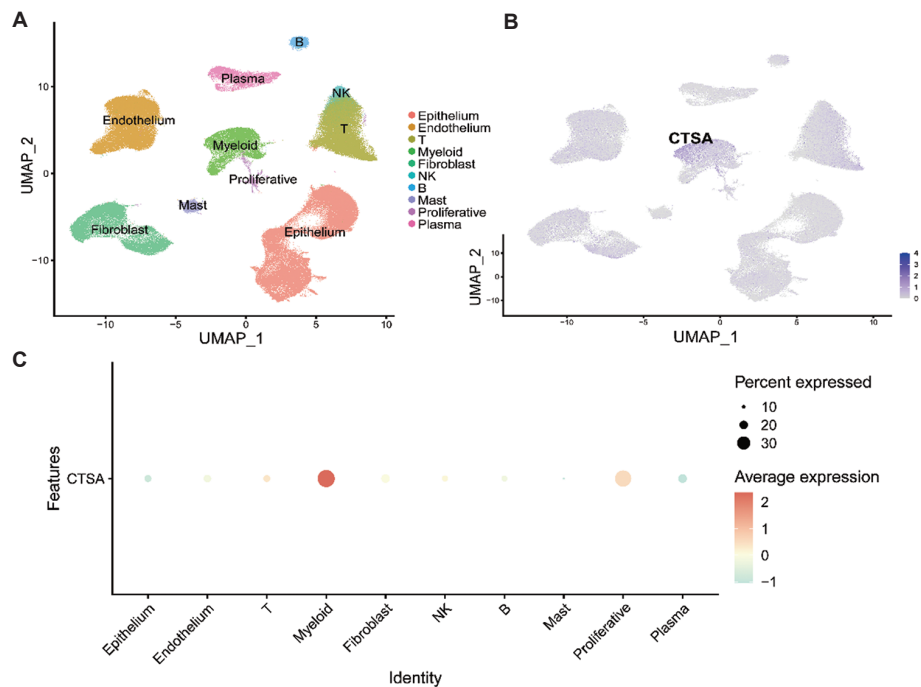


Figure 5. Single-cell RNA sequencing analysis reveals predominant cell types and expression of CTSA in PCa. (A) UMAP plots of cells clusters and cell types in PCa. (B) The distribution and expressions of CTSA across different cell types (C) Bubble plot showing expression levels of CTSA for each cell type. Abbreviations: CTSA: Cathepsin A; NK: Natural killer; PCa: Prostate cancer; UMAP: Uniform Manifold Approximation and Projection.

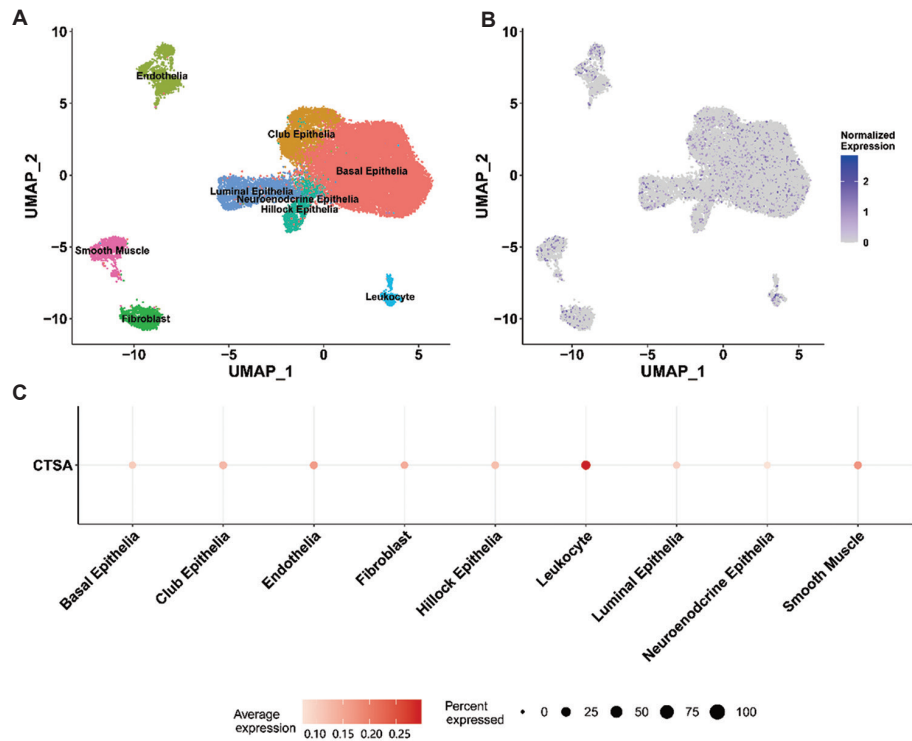


Figure 6. Single-cell RNA sequencing analysis reveals predominant cell types and expression of CTSA in the normal prostate. Created in http://bioinfo.jialab-ucr.org/PCaDB/#shiny-tab-tab_home. (A) UMAP plots of cells clusters and cell types in normal prostate. (B) The distribution and expression of CTSA across different cell types. (C) Bubble plot showing expression levels of CTSA for each cell type. Abbreviations: CTSA: Cathepsin A; UMAP: Uniform manifold approximation and projection.

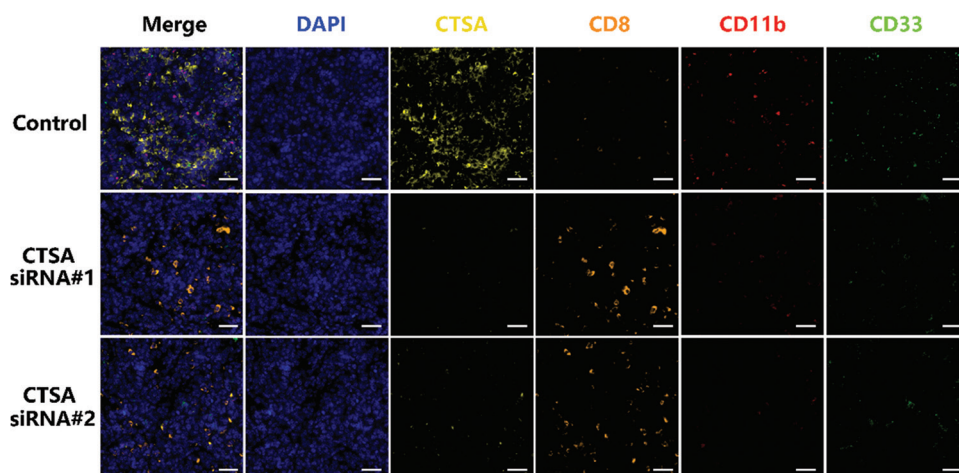


Figure 7. Multiplex Immunohistochemistry analyses of CTSA (yellow signal), CD8 (orange signal), CD11b (red signal), and CD33 (green signal) in the mouse subcutaneous tumor tissues. The nuclei of cells were stained by DAPI (blue signal). Scale bar: 50 μ m.

Previous studies have shown that sialyltransferase inhibitors can neutralize the acidic TME, activate CD8⁺ T cells, and suppress tumor growth and metastasis.³⁷ Cao *et al.*¹⁶ demonstrated that S3gal3 facilitates the formation of an immunosuppressive TME in ovarian cancer, which is characterized by an abundance of tumor-promoting macrophages and reduced infiltration of CD8⁺ T cells. Besides, the researchers also found that blocking sialylation can enhance anti-tumor immunity and improve the response of ovarian cancer to ICIs therapy.¹⁶ Our preliminary research revealed that NANS deficiency reduces sialic acid synthesis and tumor cell sialylation, decreases M2 macrophage infiltration, increases CD8⁺ T cell infiltration, reverses the immunosuppressive TME, and inhibits tumor growth.¹⁷ Our study further confirmed the relationship between sialic acid metabolism and immunity. Nevertheless, future studies are warranted to explore whether knocking down CTSA has a synergistic effect with immunotherapy.

In addition, progression to castration-resistant PCa is also a significant factor contributing to poor prognosis in PCa. Moreover, the majority of patients often develop bone metastasis when progressing to castration-resistant PCa. A previous study has found that sialyltransferase inhibitor can reverse enzalutamide resistance in PCa cells,³⁸ demonstrating the association between sialylation modification and ADT treatment resistance. Hodgson *et al.*³⁹ demonstrated that alpha-2,6-sialyltransferase (ST6GAL1) can promote the development of M2-like macrophages by remodeling the pre-metastatic bone microenvironment, thus leading to the occurrence of PCa bone metastasis. Inhibiting sialylation can suppress the spread of tumor cells to bones. Emerging evidence implicates sialylation can not only cause resistance to ADT

but also correlate with chemoresistance across multiple cancer types.⁴⁰ Duarte *et al.*⁴¹ indicated that the sialylation modification of the ErbB2 receptor in gastric cancer leads to trastuzumab resistance. Smithson *et al.*⁴² discovered that ST6GAL-1-mediated sialylation confers therapeutic resistance to chemoradiation in rectal cancer cell lines by inhibiting apoptosis. Chakraborty *et al.*⁴³ found that knockdown of ST6GAL-1 enhances gemcitabine-induced DNA damage, and ST6GAL-1 is considered a potential target for reversing gemcitabine resistance in pancreatic cancer.

While our findings provide valuable insights, several limitations should be acknowledged. First, although we employed rigorous statistical methods to mitigate potential confounding factors, the retrospective nature of this study may still introduce selection bias, as is common with analyses of existing clinical datasets. Prospective validation and patient histopathology in independent cohorts would strengthen our conclusions. Second, our analysis of immune cell infiltration is based on transcriptomic data and a single algorithm (ssGSEA). Although this approach is widely used, the lack of cross-validation with alternative methods (*e.g.*, CIBERSORT) or other validation cohorts is a recognized limitation. Third, the biological relevance of our subcutaneous xenograft model is constrained by its anatomical and immunological differences from the human prostate TME. Specifically, the use of immunodeficient mice precludes the study of adaptive immune components, including prostate-associated macrophages (*e.g.*, MAC-MT cells)⁴⁴ and tumor-infiltrating lymphocytes, which play critical roles in human PCa progression. While this model provides insights into innate immune responses, future studies should incorporate orthotopic or humanized

mouse models to better recapitulate the complex immune landscape of PCa. Finally, our investigation primarily concentrated on CTSA-mediated modulation of macrophage polarization, leaving its potential effects on other immune cell populations, such as natural killer cells and dendritic cells, unexplored. Additionally, it is important to note that our study did not elucidate the precise mechanism by which CTSA influences the immune landscape—an area that remains poorly explored in the current literature. Further research is needed to determine whether CTSA influences immune checkpoint pathways, cytokine secretion, or metabolic reprogramming in tumor-associated immune cells. Elucidating these mechanisms could reveal novel therapeutic targets for PCa immunotherapy.

5. Conclusion

Our study demonstrates that elevated CTSA expression is significantly associated with poorer prognosis in PCa, suggesting its potential role as a prognostic biomarker. Further mechanistic investigations revealed that high CTSA expression contributes to the establishment of an immunosuppressive TME, marked by abundant infiltration of MDSCs and sparse cytotoxic T cells. These findings highlight CTSA as a key regulator of immune evasion in PCa, providing a plausible explanation for its association with aggressive disease progression. Given the critical role of CTSA in shaping an immunosuppressive TME, targeting CTSA may represent a promising therapeutic strategy to reverse immune suppression and enhance the efficacy of existing immunotherapies. Future studies should explore the molecular mechanisms by which CTSA modulates immune cell function, as well as evaluate the therapeutic potential of CTSA inhibition in combination with immune checkpoint blockade or other emerging immunotherapeutic approaches. In addition, clinical validation in larger, independent cohorts is warranted to confirm the prognostic and predictive value of CTSA in PCa.

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Conflict of interest

The authors declare that they have no competing interests.

Author contributions

Conceptualization: Zongren Wang, Jiahao Lei

Data curation: Yao He, Dongxin An

Formal analysis: Jiahao Lei

Funding acquisition: Zongren Wang, Ren Liu, Lingwu Chen

Investigation: Jiahao Lei, Shuntian Gao

Methodology: Shuntian Gao

Project administration: Jiahao Lei, Shuntian Gao

Software: Yao He, Dongxin An

Resources: Rui Sun

Supervision: Zongren Wang, Ren Liu, Lingwu Chen

Validation: Yao He, Rui Sun

Visualization: Shuntian Gao, Yao He

Writing—original draft: Jiahao Lei

Writing—review & editing: Dongxin An, Rui Sun

Ethics approval and consent to participate

This study was approved by the Ethics Committees of the First Affiliated Hospital of Sun Yat-sen University (2023-778). This animal trial was approved by the Experimental Animals Ethics Committee of the Experimental Animal Center of Sun Yat-sen University (2024-054).

Consent for publication

Not applicable.

Availability of data

Public data used in this study is available through the TCGA Research Network portal (<https://portal.gdc.cancer.gov/>), cbiportal (<https://www.cbiportal.org/>), and GEO (<http://www.ncbi.nlm.nih.gov/geo/>). Compiled public data are available from the corresponding authors upon reasonable request.

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Appendix

Table A1. Cell-type-specific marker genes used for cell annotation in single-cell RNA sequencing analysis

Cell	Markers
Epithelium	<i>EPCAM, AR, KLK2, KLK3, KLK4, TMPRSS2, MSMB, KRT18, KRT8</i>
Endothelium	<i>PECAM1, VWF, EHD3</i>
T cell	<i>PTPRC, CD3E, CD8A, CD4</i>
Myeloid cell	<i>PTPRC, CD163, CD14, CD68, HLA-DQB1, HLA-DRB1, APOE, C1QA, C1QB</i>
Fibroblast	<i>FAP, DCN, POSTN, ACTA2, VIM, COL1A1, PDGFRB, LUM</i>
Natural killer cell	<i>NKG7, NCR1, FGFBP2, GNLY, FCGR3A, FCGR3B, NCAM1</i>
B cell	<i>CD19, MS4A1, CD79A</i>
Mast cell	<i>MS4A2, TPSAB1</i>
Proliferative cell	<i>MKI67, TOP2A, STMN1</i>
Plasma cell	<i>SDC1, IGHG1, MZB1</i>

Table A2. siRNA sequences

siRNA	Sequences (5'–3')
CTSA siRNA#1	UUGUACUCCGAAAGAGGC
CTSA siRNA#2	UUGUAGAUGUUGAGGCCAG

Table A3. Primary antibodies used in this study

Name (species)	Catalog number	Clone number	Company	Application
CTSA (Rabbit)	15020-1-AP	Polyclonal	Proteintech	mIHC
CD8 (Rabbit)	PTM-7132	JMMR- 3466-VH	PTMab	mIHC
CD11b (Rabbit)	DF2911	Polyclonal	Affinity	mIHC
CD33 (Rabbit)	DF6789	Polyclonal	Affinity	mIHC

Abbreviation: mIHC: Multiplex immunohistochemistry.