

Integrated multi-omics analysis reveals the remodeling characteristics of the immune microenvironment in mCRPC and the key regulatory role of BRAF

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Abstract

Objective

Metastatic Castrate Resistant Prostate Cancer (mCRPC), which is a major challenge in the treatment of prostate cancer (PCa). This study aims to integrate multi-omics data to identify key immune genes associated with mCRPC, reveal the remodeling characteristics of the immune microenvironment in mCRPC.

Methods

Immune-related genes were obtained from the GEO, ImmPort and GeneCards databases. A transcription factor-gene interaction network and a protein-protein interaction network were constructed. The CytoHubba plugin in Cytoscape was used for further screening. Functional enrichment analysis (GO and KEGG), clinical data analysis, and immune infiltration analysis were conducted on the identified hub genes. Finally, the DrugBank database was utilized to identify potential therapeutic agents targeting the hub genes.

Results

The 34 common genes were significantly enriched in immune-related pathways. Transcription factor analysis revealed close associations between core transcription factors like RELA, NFKB1, and SP1. Seven hub genes were ultimately identified. Among these, BRAF was upregulated in the mCRPC group and significantly correlated with prostate cancer T stage, Gleason score, and progression-free interval. Construction of a gene-miRNA interaction network revealed a close association between TNF and hsa-miR-143-3p. STAT1, CCL2, TNF, and IL-6 play important roles in the signaling pathways of tumor-associated macrophages (TAMs). Single-cell infiltration analysis demonstrates a significant association between CD1D and SH2D1A, both of which are linked to natural killer T cells (NKT cells).

Conclusion

TAMs and NKT cells play key roles in the transformation from localized prostate cancer (LPC) to mCRPC. The therapeutic implications of targeting BRAF overexpression in mCRPC warrant further exploration.

Introduction

Prostate cancer (PCa) is a malignant tumor that has a high incidence and mortality rate worldwide among men, posing a significant public health burden. According to 2020 global cancer statistics, there were over 1.41 million new cases of PCa, with an age-standardized incidence rate of 31 per 100,000

people. Additionally, there were over 375,000 deaths, corresponding to an overall mortality rate of 7.7 per 100,000 people[1]. In Asia, traditionally considered a low-incidence region, both incidence and mortality rates have risen rapidly over the past few decades. For example, in China, the age-standardized incidence rate of PCa increased by 95.2% between 1990 and 2019, which far exceeds the global increase of 13.2% [2, 3]. Castrate-resistant prostate cancer (CRPC) refers to a disease state in which, despite androgen deprivation therapy and serum testosterone levels maintained below 50 ng/dL or 1.7 nmol/dL (castrate levels), clinical or biochemical progression occurs. Biochemical progression is typically defined as a rise in prostate-specific antigen (PSA) levels by more than 25% above the nadir, with an absolute value above 2.0 ng/mL, confirmed in two consecutive measurements at least one week apart. Clinical progression is assessed using conventional imaging (such as CT and bone scans) based on Response Evaluation Criteria in Solid Tumors, identifying new metastatic lesions or enlargement of existing lesion[4]. Metastatic castrate-resistant prostate cancer (mCRPC) is considered the terminal stage of the disease, with limited treatment options and poor patient prognosis. A nationwide study of U.S. veterans showed that the age-adjusted incidence rate of mCRPC in 2022 was approximately 5 per 1,000 PCa patients. The median overall survival after diagnosis improved over time, from 1.23 years during 2005–2011 to 2.17 years during 2018–2024 [5], but this still represents a significant survival challenge. Additionally, the treatment of mCRPC is accompanied by substantial humanitarian and economic burdens, including high medical costs, reduced quality of life due to treatment-related adverse events, and loss of productivity [6].

The mechanisms underlying CRPC are extremely complex. The core driving factors and key mechanisms currently under extensive research are as follows: ¶ Even with serum testosterone at castrate levels, sustained amplification and overexpression of the androgen receptor (AR) signaling pathway remain central factors. The expression of androgen receptor splice variant 7 (AR-V7), the conversion of adrenal androgen precursors, alternative pathways, and the "backdoor pathway" that bypasses testosterone to produce dihydrotestosterone have been shown to play roles in intratumoral steroidogenesis [7]. ¶ Alterations in DNA damage repair pathway genes, such as BRCA1/2 and ATM mutations (present in about 20% of advanced patients), render these patients sensitive to PARP inhibitors[8]. ¶ Inactivation of tumor suppressor genes, such as PTEN deletion [9].

The tumor immune microenvironment (TIME) is a complex ecosystem formed by dynamic interactions among various cellular and non-cellular components. Its basic composition is far from a single entity but rather a collection including cancer cells, fibroblasts, myofibroblasts, endothelial cells, and various immune cells. The TIME in mCRPC typically exhibits an immunosuppressive state, characterized by sparse infiltration of effector T cells, enrichment of immunosuppressive cells, and active multiple immune evasion mechanisms, which constitute major obstacles to immunotherapy. This "immune cold phenotype" is one of the key reasons for the limited efficacy of traditional immune checkpoint inhibitors as monotherapy [10–12]. Existing research indicates that within this suppressive immune microenvironment, tumor-associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSCs) form the primary immunosuppressive axis [13]. These cells not only suppress the function of effector T cells by secreting immunosuppressive cytokines but also metabolically inhibit anti-tumor

immune responses by competitively depleting essential amino acids such as arginine [14]. Furthermore, regulatory T cells (Tregs) reinforce the local state of acquired immune tolerance through both contact-dependent and independent mechanisms [15]. This microenvironment dominated by innate and adaptive immunosuppressive cells makes it difficult for cytotoxic T lymphocytes (CTLs) to effectively infiltrate and activate, thereby forming physical and functional barriers to immune escape [11]. The expression patterns of immune checkpoint molecules in the mCRPC immune microenvironment are another important feature.

This study conducted bioinformatics analyses on microarray datasets obtained from public databases. Focusing on immune regulation, the study integrated multi-dimensional data, including genomics and transcriptomics, to identify key immune regulatory genes driving the transition of PCa from localized to metastatic and castration-resistant states. This study aims to facilitate future in-depth exploration of their molecular functions, regulatory networks, and the identification of mCRPC-related immunotherapeutic targets, thereby providing new research directions for clinical drug development.

Materials and Methods

1. Data Acquisition

Samples of benign prostate tissue (BPH), localized prostate cancer (LPC), and mCRPC were obtained from the GSE 35988 dataset (platform GPL6480) in the GEO database (<https://www.ncbi.nlm.nih.gov>) using the keywords "castration-resistant prostate cancer" and "human samples" [16]. This dataset includes 12 normal prostate tissue samples, 49 castration-sensitive prostate cancer tissue samples, and 27 castration-resistant prostate cancer tissue samples. A total of 3,118 immune-related genes (IMGs) were retrieved from the ImmPort database (<https://www.immport.org/>) [17]. Additionally, 176 immune regulation-related genes (IR) were retrieved from the GeneCards database (<https://www.genecards.org/>) using the keyword "immune regulation (IR)" [18]. For sample datasets obtained from public databases, patient informed consent and approval from an ethics committee are not required.

2. Analysis of DEGs

Using R (version 4.2.1), data were cleaned and analyzed using UMAP via the UMAP package. The analysis results were visualized using the ggplot2 package to generate the UMAP analysis plot for the GSE 35988 (GPL6480) dataset. DEGs were analyzed using the GEO2R tool for differential analysis results, visualized with ggplot2 (version 3.4.4) in the form of a gradient volcano plot, with conditions set at $P < 0.05$ and $|\text{LogFC}| > 1$. Finally, genes with missing data and duplicate genes were removed to obtain the Differential Genes (DEGs) dataset [19].

3. Intersection Genes and Their Functional Enrichment

A Venn diagram was constructed using disease genes from DEGs, IR, and IMGs to analyze the overlapping elements among the groups, referred to as common genes. The results were visualized using the ggplot2 and VennDiagram packages in R. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were conducted for the common gene[20, 21]. Some immune-related Molecular Functions, Biological Processes, and Cellular Components were visualized as bubble plots. Some immune-related pathways were visualized using chord diagrams, with images generated by the ggplot2 package.

4. Construction of the Interaction Network Between Common Genes and Transcription

Transcription factors are major trans-acting factors involved in transcriptional regulation. Transcription factors related to common genes were obtained from the TRRUST database (<https://www.grnpedia.org/trrust>), and those with $P < 0.05$ were included in the study [22]. Cytoscape V3.10.3 was used to visualize the collected genes and their transcription factors. The Analysis Network function and the Betweenness Centrality algorithm were employed to arrange the color blocks and fonts of each component from small to large based on relevance, transparency from semi-transparent to opaque, and color from light to dark. The strength of associations between nodes was visualized by varying the line color from light to dark, providing a more intuitive display of important common genes and strongly associated transcription factors.

5. Construction of the Protein-Protein Interaction (PPI) Network for Common Genes and Acquisition of Hub Genes

The 34 aforementioned common genes were input into the STRING 12 database (<https://cn.string-db.org/>) using "Homo sapiens" as the search keyword, and the results were imported into Cytoscape software for visual analysis (23). The Analysis Network function and the Degree algorithm were used to order the nodes by size (small to large) and color intensity (light to dark). The connections between nodes were arranged from light to dark using the EdgeBetweenness algorithm. Subsequently, the Bottleneck and Betweenness algorithms in Cytoscape Hubba were each used to screen the top 10 hub genes, and the intersection was taken to present the final hub genes in the form of a Venn diagram [24]. Expression profiles of the 7 hub genes were generated, and statistical analysis was performed using the stats and car packages, with data visualization conducted using the ggplot2 package.

6. Enrichment Analysis of Hub Genes, Hub Gene-miRNA Interaction Network Diagram, and Clinical Significance of the BRAF

Molecule GO and KEGG enrichment analyses were performed on the finally screened Hub genes, and results related to immune regulation were selected and visualized using the ggplot2 package. Simultaneously, the interaction relationships between hub genes and miRNAs were explored through the

NetworkAnalyst website (<https://www.networkanalyst.ca/>), using miRNA data from "miRTarBase v9.0". Conditions were set as: score > 0.90 and 3' untranslated region (UTR) as the target gene binding region, with Degree > 1, and the results were imported into Cytoscape for visualization. The "edgebetweenness" algorithm was applied to arrange the strength of connections between nodes from light to dark, while node sizes were arranged based on the Degree values downloaded from the website [25]. RNAseq data from the TCGA-PRAD (prostate cancer) project processed by the STAR pipeline were downloaded and organized from the TCGA database (<https://portal.gdc.cancer.gov>), extracting FPKM format data and clinical data. Normal samples and those without clinical information were removed. The Wilcoxon rank sum test was used for statistical analysis to determine the relationship between BRAF expression and PCa T stage and Gleason score, with data visualized using the ggplot2 package [26]. The survival package was used to test the proportional hazards assumption and perform Cox regression; results were visualized with the survminer and ggplot2 packages to evaluate the impact of high versus low BRAF expression on progression-free interval.

7. Immune Infiltration Analysis and Exploration of Single-Cell Infiltration of Hub Genes

The Wilcoxon rank sum test was used for statistical analysis of the GSE 35988 (platform GPL6480) data to compare immune cell infiltration between the LPC and mCRPC groups, with data visualized using the ggplot2 package. The Spearman method was used for correlation analysis of the proportion of immune cells in samples for the hub genes, and the results were visualized using a heatmap.

Using TISCH2 (<https://tisch.compbio.cn/>), metastatic prostate cancer datasets GSE141445 (bone metastasis samples), GSE143791 (mCRPC samples), and GSE137829 (CRPC samples) were identified. The 7 hub genes were input into the "database" module for single-cell infiltration analysis, and UMAP plots were generated using the median to display the infiltration of the 7 hub genes in metastatic (castration-resistant) prostate cancer [27].

8. Drug Prediction for Hub Genes

DrugBank (<https://go.drugbank.com>) has now developed into a "gold standard" knowledge resource for drugs, drug targets, and related drug information, currently updated to version 6.0 [28]. Drugs related to the hub genes that have been confirmed to have pharmacological effects were selected and included in the table.

Results

1. Analysis of the LPC group and mCRPC group to generate DEGs

Expression information from 12 BPH group samples, 49 LPC group samples, and 27 mCRPC group samples was obtained from the GSE 35988 (platform GPL6480) dataset, and UMAP analysis was performed (Fig. 1A). Differences were observed between the LPC and mCRPC group datasets. Using $|\log FC| \geq 1$ and $p < 0.05$ as thresholds, a total of 4,306 DEGs were identified. Among them, 2,869 were upregulated (left side of the figure) and 2,180 were downregulated (right side), as visualized in a gradient volcano plot (Fig. 1B).

2. Screening of common genes and functional enrichment

A total of 176 IR-related genes were obtained from the GeneCards database, 3,118 IMGs were retrieved from the ImmPort database, and 4,306 genes were derived from the DEGs. Further screening for genes common to all three databases, as shown in a Venn diagram, yielded 34 common genes (Fig. 1C). GO and KEGG enrichment analysis of these 34 common genes revealed that they were primarily enriched in immune-related pathways such as Cytokine-cytokine receptor interaction, IL-17 signaling pathway, TNF signaling pathway, Natural killer cell mediated cytotoxicity, among others (Fig. 1D). GO enrichment results indicated that the common genes involved in immune regulation mainly included signaling receptor activator activity (GO:0030546), CXCR chemokine receptor binding (GO:0045236) in the Molecular Function (MF) module; membrane raft (GO:0045121), external side of plasma membrane (GO:0009897) in the Cellular Component (CC) module; and regulation of leukocyte cell-cell adhesion (GO:1903037), leukocyte cell-cell adhesion (GO:0007159), regulation of leukocyte proliferation (GO:0070663) in the Biological Process (BP) module (Fig. 1E). This suggests that these common genes are involved in the progression from the LPC state to the mCRPC state.

3. Analysis of transcription factors related to common genes

To further explore the relationship between common genes and transcription factors, Cytoscape V3.10.3 was used to visualize each factor (Fig. 2A). The outer triangles represent transcription factors (53 in total), and the inner circles represent the 34 common genes. As shown in the figure, PTGS2, IL1B, IL6, VEGFA, and TNF have the closest relationships with transcription factors, while RELA, NFkB1, and SP1 are the top three core trans-acting elements, respectively. Notably, purple lines represent the closest connections between genes and transcription factors.

4. Establishment of the PPI network and screening of hub genes

In the STRING 12 analysis platform, Cytoscape software was used to construct a PPI network map (Fig. 2B). As shown in the figure, TNF, IL6, and CCL2 are the hub genes in the PPI network. The interactions between UNC12D and SH2D1A, and between ITCH and BRAF are the strongest. The top 10 hub genes obtained through the Betweenness algorithm in Cytoscape Hubba are BRAF, TNF, STAT1, IL6, SH2D1A, CD1D, CCL2, CD28, ICOS, FASLG; selecting the Bottleneck algorithm yielded the top 10 hub

genes as STAT1, CCL2, BRAF, CD1D, IL1B, TNF, IL6, SH2D1A, TIGIT, CCR5. Therefore, the final hub genes obtained are STAT1, CCL2, BRAF, CD1D, TNF, IL6, SH2D1A (Fig. 2C).

Based on the boxplot comparison, the 7 hub genes were statistically significant in both the LPC group and the mCRPC group ($P < 0.001$). Among them, BRAF was upregulated in the mCRPC group, whereas the other six genes were downregulated (Fig. 2D).

5. Functional enrichment of hub genes, Gene-miRNA network diagram, and correlation study between BRAF and PCa clinicopathological features

Functional enrichment of the hub genes showed that the 7 hub genes play important roles in key nodes (pathways) such as positive regulation of leukocyte mediated immunity, receptor signaling pathway via STAT, and C-type lectin receptor signaling pathway (Fig. 3A). In the Gene-miRNA interaction diagram, according to the Degree algorithm, the hub genes with the most intersections is IL-6. According to the edge betweenness algorithm, IL6 with hsa-miR-98-5p, hsa-miR-335-5p, hsa-miR-124-3p are important hub pathways, as is TNF with hsa-miR-143-3p (Fig. 3B).

BRAF expression was elevated in PCa cells, especially in the mCRPC group, and was closely related to T stage and Gleason score. Furthermore, the PFI (Progress Free Interval) of the high BRAF expression group was significantly lower than that of the low expression group (Figs. 3C-E).

6. Exploration of immune infiltration and single-cell infiltration of hub genes in metastatic (castration-resistant) prostate cancer

Based on ssGSEA analysis of the GSE 35988 expression profile, significant differences were observed in the infiltration of multiple immune cell types between the LPC group and the mCRPC group (Fig. 4A). Among them, B cells, iDC cells, Macrophages, Mast cells, T cells, T helper cells, TFH, Tgd, Th1 cells, Th2 cells, and Tcm showed statistically significant downregulation, while NK CD56 bright cells and Th17 cells showed statistically significant upregulation. The expression of CCL2 and CD1D showed significant positive correlations with iDC cells, Macrophages, Mast cells, T cells, T helper cells, and Th1 cells. The expression of TNF, SHD1A, STAT1, and IL-6 showed positive correlations with iDC cells, Macrophages, T cells, T helper cells, and Th1 cells. In contrast, the infiltration pattern of BRAF in immune cells was more complex, showing significant negative correlations with iDC cells, Macrophages, Mast cells, T cells, TFH, T helper cells, and Th1 cells, but positive correlations with NK CD56 bright cells, NK cells, and TH17 cells (Fig. 4B).

As seen in Figures C-E, in the three metastatic (castration-resistant) prostate cancer samples GSE141445, GSE143791, and GSE137829, BRAF and STAT1 genes were expressed in malignant tumor cells and infiltrating immune cells; TNF was mainly found in macrophages (Mono/Macro cells), CD8 + T cells (mainly CD8Tex cells), and mast cells (Mast cells); CCL2 was mainly present in malignant tumor cells, fibroblasts (Fibroblasts, including myofibroblasts (MyoFibroblasts)), and endothelial cells

(Endothelial cells); CD1D was mainly present in macrophages (Mono/Macro cells) and dendritic cells (DCs); SH2D1A was almost entirely found in CD8Tex cells and NK cells; IL6 was found in macrophages (Mono/Macro cells), fibroblasts (Fibroblasts, including myofibroblasts (MyoFibroblasts)), and endothelial cells (Endothelial cells), although the infiltration levels were generally low.

7. Prediction of potential therapeutic drugs targeting hub genes

We searched the DrugBank database to identify potential drugs targeting the hub genes, facilitating further analysis of the LPC transformation to mCRCP. Dabrafenib and other inhibitors are available for BRAF. Mimosine is an inhibitor of CCL2. The regulatory landscape for TNF is more complex because multiple inhibitors, agonists, and neutralizers targeting TNF have been approved. All drugs targeting IL-6 are antagonists (Table 1).

Table 1
Analysis of potential drugs targeting hub genes

GENE	NAME	GROUP	ACTIONS	Accession Number
BRAF	Dabrafenib	approved,investigational	inhibitor	DB08912
BRAF	Encorafenib	approved,investigational	inhibitor	DB11718
BRAF	PLX-4720	experimental	inhibitor	DB06999
BRAF	Regorafenib	approved,investigational	inhibitor	DB08896
BRAF	Ripretinib	approved,investigational	inhibitor	DB14840
BRAF	Sorafenib	approved,investigational	inhibitor	DB00398
BRAF	Vemurafenib	approved,investigational	inhibitor	DB08881
CCL2	Mimosine	experimental	inhibitor	DB01055
TNF	Adalimumab	approved,investigational	inhibitor/antibody	DB00051
TNF/IL6	Foreskin keratinocyte (neonatal)	approved	agonist	DB10772
TNF	Certolizumab pegol	approved,investigational	neutralizer	DB08904
TNF	Etanercept	approved,investigational	inhibitor/antibody	DB00005
TNF	Glycyrrhizic acid	approved,investigational	antagonist	DB13751
TNF	Golimumab	approved,investigational	antibody	DB06674
TNF	Infliximab	approved,investigational	inhibitor	DB00065
TNF	Pomalidomide	approved,investigational	inhibitor	DB08910
IL6	Siltuximab	approved,investigational	antagonist/antibody	DB09036
IL6	Olokizumab	experimental	antagonist	DB13127

Discussion

Analysis of the GSE 35988 expression profile revealed a total of 4,306 differentially expressed genes (DEGs) between the mCRPC and LPC groups, with 2,869 upregulated and 2,180 downregulated. By integrating the ImmPort and GeneCards databases, 34 common genes were identified. These 34 common genes were primarily enriched in pathways such as cytokine-cytokine receptor interaction, IL-17 signaling pathway, TNF signaling pathway, and regulation of leukocyte cell-cell adhesion. T helper 17 (Th17) cells are a specialized subset of CD4 + T cells capable of simultaneously expressing the cytokines IL-17 and TNF- α . Studies have shown that Th17 cells secreting IL-17 and TNF- α are

significantly enriched in colon and PCa tissues. IL-17 and TNF- α can upregulate PD-L1 expression, primarily through the induction of the NF- κ B signaling pathway [29, 30]. TNF- α synergizes with FSH to upregulate the expression of vascular cell adhesion protein 1 (VCAM-1) via two parallel pathways: cAMP/PKA/CREB/c-Jun and PI3K/AKT/GSK-3 β /GATA-6, thereby enhancing the inflammatory response in castrated mice and leading to vascular atherosclerosis [31].

Regarding transcription factors and common genes, increasing evidence suggests that the NF- κ B pathway is involved in PCa progression, and this study appears to confirm this view. NF- κ B is a family of transcription factors involved in various physiological and pathological processes, with its most common form being a heterodimer of the p50 (product of the NF- κ B1 gene) and p65 (RELA) subunits. The p65 subunit possesses a transcriptional activation domain and is involved in cell proliferation, metastasis, and angiogenesis. The NF- κ B pathway can promote PCa progression and castration resistance [32]. Prostaglandin-endoperoxide synthase 2 (PTGS 2) catalyzes the rate-limiting step in prostaglandin biosynthesis. It can interact with its receptors to inhibit downstream signaling, thereby promoting cancer cell proliferation, survival, angiogenesis, and metastasis [33]. Furthermore, studies have found that PTGS2 can activate the AKT/NF- κ B pathway to promote PCa cell survival and proliferation. miR-124-3p can directly and complementarily bind to its target PTGS2, reducing PTGS2 expression and thereby inhibiting the aforementioned processes. miR-124-3p can also interact with IL-6, leading to disease progression in LPC patients (see below) [34]. VEGF inhibits the function and migration capacity of CD8 + T cells expressing VEGFR2. MYC and p53 can synergistically induce VEGF expression. VEGFR2 activation significantly increases the efficiency of Tregs and myeloid-derived suppressor cells while reducing the antigen-presenting capacity of macrophages and dendritic cells, thereby reducing interactions between antigen-presenting cells and T cells [35].

Activating BRAF mutations are most common in melanoma and papillary thyroid carcinoma. The overall prevalence of activating BRAF alterations in PCa is 3.3%. The most common BRAF mutation is a valine-to-glutamic acid substitution at position 600 (V600E), with alterations primarily consisting of class II mutations and rearrangements [36, 37]. In this study, high BRAF gene expression was significantly associated with high Gleason scores, advanced T stages, and shorter progression-free intervals (PFI). Studies have confirmed the role of amplified BRAF in increasing Gleason scores and reducing overall survival (OS) and failure-free survival [38, 39], indicating that the BRAF gene plays an important role in PCa progression. RAF family protein kinases play a central role in mediating MEK/ERK oncogenic signaling pathways. ITCH has been shown to be a versatile ubiquitin E3 ligase that promotes K48-, K29-, K33-, and K63-linked polyubiquitination on different substrates. Qing et al. [40] demonstrated that endogenous BRAF is predominantly modified by K27-linked ubiquitin chains. ITCH interacts with BRAF via its kinase domain, promoting ubiquitination of all three RAF isoforms, thereby driving MAPK activation in melanoma cells and promoting tumorigenesis. However, research on the ITCH-mediated BRAF ubiquitination process in PCa, especially mCRPC, is limited and holds promise as a new research target. Regarding immune infiltration, research on BRAF has mostly focused on melanoma, particularly dendritic cells (DC) and T cells. Protein phosphatase 2A (PP2A) is essential for T cell development and function. Ablation of the PP2A catalytic subunit alpha (PPP2CA) in double-positive thymocytes leads to

a significant increase in apoptosis, resulting in abnormal thymocyte development. The catalytic subunit beta isoform (PPP2CB) shares 97% sequence similarity with PPP2CA. Unlike the critical role of PPP2CA in thymocyte development, PPP2CB deletion has little impact on normal T cell development. Furthermore, PPP2CB deletion results in augmented T-cell activation induced by phorbol 12-myristate 13-acetate (PMA) plus ionomycin without affecting T cell receptor (TCR) or cytokine-induced T-cell responses. PPP2CB deletion enhances the activation of the PI3K/Akt/mTOR signaling pathway stimulated by PMA plus ionomycin [41]. Currently, drugs targeting BRAF are mainly applied in melanoma, such as PLX4032 and Vemurafenib. Notably, multiple studies have reported that the SND1-BRAF fusion gene is a new therapeutic target for advanced PCa patients. Patients treated with trametinib (a MEK inhibitor) have seen their PSA levels drop from over 900 ng/mL to below 0.6 ng/mL [42–44].

This study found that during the evolution from LPC to mCRPC, the transformation of the immune microenvironment primarily occurs in the macrophage activation process centered on TNF, IL-6, CCL2, and STAT, as well as in NK cells (including invariant natural killer T (iNKT) cells) centered on SH2D1A and CD1D. TNF, IL-6, CCL2, and STAT are closely linked in the process of macrophage polarization. In bone metastasis samples, TAMs promote PCa proliferation in bone tissue. TNF- α and IL-1 secreted by macrophages are strong stimulators of IL-6 production by tumor cells. Reducing IL-6 expression significantly decreased bone tumor size, osteolysis, and the incidence of lymph node metastasis [45]. Metastatic tumor cells stimulate osteoblasts, which in turn stimulate osteoclasts via the TNF family member receptor activator of nuclear factor kappa-B ligand (RANKL). Activated osteoclasts degrade the bone matrix by producing strong acids and proteases. Transforming growth factor-beta (TGF- β) released by osteoclasts and other growth factors stored in the bone matrix further stimulate tumor cells, a process termed the "vicious cycle" of bone metastatic tumors [46]. Additionally, Han et al. indicated that IL-6 stimulates M2 macrophage polarization and is associated with PCa cell development. M1 macrophages have pro-inflammatory properties. M1 macrophages typically exhibit anti-tumor properties by secreting pro-inflammatory cytokines such as interferon-gamma (IFN- γ) and tumor necrosis factor-alpha (TNF- α), thereby enhancing the immune response. The gene expression of these inflammatory factors is regulated by STAT1. Phosphorylation and activation of STAT1 mediate the skewing of macrophages towards the M1 phenotype. In contrast, M2 macrophages are associated with tissue repair and immunosuppression. In the nucleus, the IL-6-triggered NF- κ B/STAT3/JAK signaling pathway activates STAT3. STAT3 can bind to specific DNA sequences, activating the transcription of multiple genes related to M2 polarization. Activation of these factors can significantly enhance CRPC signaling [47–49], a process that may be related to the simultaneous loss of Pten and p53 [50]. Specific JAK-STAT small molecule inhibitors (ruxolitinib and fludarabine) have shown effective efficacy in the treatment of castration-resistant PCa [51]. This process is reflected in the GO enrichment analysis in this paper.

Furthermore, IL-6 has a similar effect on NK cells. Xu et al. [52] found that IL-6 acts by upregulating PD-L1 and downregulating NK group 2D (NKG2D) ligand levels in CRPC cells, with its downstream signal transduction primarily through JAK-Stat3. In this study, IL-6 was found in macrophages (Mono/Macro cells), fibroblasts (MyoFibroblasts), and endothelial cells in mCRPC, but the degree of infiltration was not high, which is highly consistent with the findings of Yu et al. [53]. Their research suggests that CRPC

cells themselves do not express IL-6 mRNA. IL-6 mRNA expression is almost exclusively limited to cells in the stromal compartment (such as endothelial cells, fibroblasts, smooth muscle cells, and infiltrating macrophages). These cells influence the signaling pathways of PCa cells in a paracrine manner, thereby affecting tumor progression.

CCL2, as a potent chemokine, binds to the CCR2 receptor on the macrophage surface, activating downstream signaling pathways such as PI3K/Akt, thereby influencing macrophage polarization (primarily towards M2). miR-124-3p acts as a post-transcriptional regulator of CCL2, binding to the 3' UTR of CCL2 and inhibiting its expression. This regulation may inhibit the M2 polarization process of TAMs [54, 55]. After anti-androgen therapy, AR signaling blockade induces LPC cells to secrete CCL2, which activates the cancer cells themselves in an autocrine manner, leading to transformation into CRPC. Therefore, PCa cells themselves express CCL2, which is consistent with the findings of this study. There is a positive feedback effect between CCL2 and STAT3, and it is also positively correlated with PTGS2 upregulation. Aoyama et al.'s research also confirmed the inhibitory effect of miR-124-3p on CCL2 [56–58]. Additionally, CCL2 may directly or indirectly (through a macrophage-dependent manner) induce tumor neovascularization, with the indirect process related to the aforementioned VEGF [59]. SPDEF, a SAM pointed domain-containing ETS transcription factor, can inhibit CCL2 and PCa epithelial-mesenchymal transition mediated by AR signaling, which may become a new research direction [60].

Deichaite et al. [61] observed that IL-6 is strongly expressed in localized disease and downregulated in metastatic disease, which aligns with the conclusions of this study. Primary localized disease is characterized by M1-dominated local inflammation and adaptive immunity (regulated by STAT1), while metastatic disease features an M2-dominated immunosuppressive microenvironment and a shift toward the resolution of inflammation and tissue repair (regulated by STAT3). Studies have shown that samples from patients with high Gleason scores exhibit significant accumulation of M2-like TAMs [62]. In the conclusions of this paper, STAT1 expression is higher in the LPC group compared to BPH, but significantly decreases during the transformation from LPC to mCRPC. Regarding therapeutic approaches, TAMs reprogrammed using the glutamine antagonist JHU 083 can exhibit increased tumor cell phagocytosis, reduced pro-angiogenic capacity, and can induce apoptosis in PCa cells in vitro [63]. Toll-like receptor 2 ligand peptidoglycan (PGN)-activated DCs induce a strong host immune response, and PGN-treated antigen-sensitized DCs also enhance the function of CD8 + T cells mediated by IFN- γ and TNF- α , thereby increasing cytotoxic T lymphocyte (CTL) function [64]. Additionally, research by Wang et al. [65] suggests that adoptive allogeneic natural killer (NK) cell therapy offers new insights for treating CRPC. The conclusion section of the paper mentions that drugs targeting TNF and IL6 are more commonly used in other diseases (such as skin diseases, autoimmune diseases, hematological diseases, etc.), with fewer studies in PCa patients, but this also provides new ideas for PCa cytokine therapy.

Research on the SH2D1A gene has primarily focused on its mutations causing X-linked lymphoproliferative disease (XLP1). The SH2D1A gene encodes the SLAM-associated protein (SAP), an adaptor molecule involved in the signal transduction of SLAM family immune cell receptors. Its

mutations alter CD4 + T cell function, reduce CD8 + T cell cytotoxicity, decrease NK cell (including NKT cell) cytotoxicity, and lead to reduced antibody production and impaired memory B cell generation. SAP binds to the intracellular domains of SLAM family signaling receptors and supports the activation or inhibition of immune cell signaling. XLP1 is strongly associated with Epstein-Barr virus (EBV), and EBV infection significantly increases the probability of patients developing hemophagocytic lymphohistiocytosis (HLH) [66–68]. Studies have found that CD4 and CD8 T cells lacking SAP have a reduced ability to differentiate into IL-17-producing Th17 and cytotoxic cells [69]. Additionally, in the absence of SAP, invariant natural killer T (iNKT) cell precursors cannot differentiate through the double-positive stage of thymocyte development. This is because the homotypic interaction between iNKT cell precursors and CD1d-expressing cortical thymocytes, mediated by SAP/Fyn signaling downstream of SLAM and Ly108, is necessary for mature NKT cells. Therefore, in XLP patients, mature NKT cells are almost absent [70, 71]. In this study, the SH2D1A gene is also downregulated in mCRPC patients and is highly expressed in inactive CD8 + T cells and some NK cells, which is consistent with previous research. We propose the possibility that similar mutations in the SH2D1A gene also occur during the transformation from LPC to mCRPC. Many previous studies have confirmed that EBV plays a role in various tumor diseases, where T cell expansion is a key component of EBV immunity. In PCa, EBV-positive samples exhibit higher Gleason scores, perineural invasion (PNI), and lymphocyte infiltration [72, 73]. Furthermore, the latest Mendelian randomization analysis suggests that genetically predicted Epstein-Barr virus (EBV) EA-D antibody (Early Antigen-Diffuse Antibody) and EBNA-1 antibody (Epstein-Barr Nuclear Antigen 1) are highly correlated with PCa onset [74].

As mentioned earlier, the CD1d molecule plays a key role in the maturation of iNKT cells. iNKT cells recognize lipid antigens presented by the monomorphic MHC-like molecule CD1d, with a relatively well-established antigen being alpha-galactosylceramide (α-GalCer). In vitro experiments show that the presence of this antigen can enhance the efficacy of granulocyte-macrophage colony-stimulating factor (GM-CSF) tumor vaccines [75]. Additionally, artificial adjuvant vector cell (aAVC) therapy can induce multiple antigen-specific CD8 + T cells, providing preventive and therapeutic effects against tumor development [76]. Unlike MHC-restricted T cells, iNKT cells exhibit an effector memory phenotype independent of foreign antigen exposure and are acquired in the thymus through a unique developmental pathway (as described earlier). The lack of iNKT cells leads to the emergence of aggressive tumors and significantly reduces animal survival. Transgenic adenocarcinoma mice lacking iNKT cells develop tolerance to tumor-associated antigens at a relatively rapid rate. iNKT cells can directly exert cytotoxicity against CD1d+ tumor cell targets; they are not involved in controlling the induction of CTL tolerance or in anti-tumor effector T cell responses. iNKT cells also have antigen-presenting functions, and their migration and infiltration in PCa cells in vitro are mediated by CCL2 [77, 78]. Furthermore, iNKT cells regulate macrophages through the synergistic effects of CD1d, Fas, and CD40, reducing tumor angiogenesis, promoting selective killing of M2-like macrophages, and enhancing the survival of M1-like macrophages, thereby promoting tumor immunity [62]. In this study, the CD1d molecule shows a significant decrease during the transformation from LPC to mCRPC, which is consistent with previous research findings. In the GSE143791 (mCRPC samples) dataset, it mainly infiltrates DCs and

macrophages, with minor infiltration in NK cells. Unfortunately, none of the three datasets—GSE141445, GSE143791, and GSE137829—performed single-cell infiltration analysis specifically for iNKT cells.

Conclusion

This study focuses on the immune regulatory mechanisms of mCRPC. It aims to screen immune-related hub genes that play a key role in the transformation from LPC to mCRPC and to explore their clinical significance and potential therapeutic value. By integrating multi-omics data with bioinformatic analysis, we systematically identified seven hub genes closely associated with disease progression and performed an in-depth analysis of their roles in remodeling the immune microenvironment, clinical prognosis, and drug response. This article emphasizes the roles of these seven hub genes in TAMs, iNKT cells, and their involvement in additional immune-related processes during the development of mCRPC. This study is the first to propose the BRAF gene as a potential therapeutic target, providing a theoretical basis and direction for developing or repurposing existing drugs to treat mCRPC.

This study has some limitations. For example, it did not validate the specific regulatory mechanisms of these genes in the immune microenvironment, particularly the molecular pathways involved in the interaction between tumor cells and immune cells. Further functional validation and mechanistic exploration through in vitro and in vivo experiments are needed, and future research could further deepen the study of the functional mechanisms of these hub genes. Although this study predicted potential therapeutic drugs targeting genes such as BRAF, CCL2, TNF, and IL6, these predictions have not yet been experimentally validated. Subsequent studies could be based on the screened hub genes to conduct drug sensitivity experiments and animal model studies. They could also evaluate the efficacy of monotherapy or combination therapies targeting these genes in the treatment of CRPC and explore their synergistic effects with existing therapies (such as immune checkpoint inhibitors).

Declarations

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All the authors have approved the final manuscript.

Conflicts of Interest The authors declare no conflicts of interest.

Author Contribution

H.L.design, data collection, data analysis, manuscript writing, Q. Z.manuscript revision, manuscript writing. C .T.data analysis, funding acquisition, manuscript writing. X. G.manuscript revision, and

supervision. X .W. data analysis, manuscript writing.All authors reviewed the manuscript.

Data Availability

These data were derived from the following resources available in the public domain:1.Gene Expression Omnibus database(GEO database) :<https://www.ncbi.nlm.nih.gov/GSE35988> dataset:Accession: GSE35988 ID: 200035988 <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE35988>platform GPL6480:Accession: GPL6480 ID: 100006480<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GPL6480>GSE141445: Accession: GSE141445 ID: 200141445<https://www.ncbi.nlm.nih.gov/gds/?term=GSE141445%5BAccession%5DGSE143791>:Accession: GSE143791 ID: 200143791<https://www.ncbi.nlm.nih.gov/gds/?term=GSE143791>[Accession]GSE137829 Accession: GSE137829 ID: 200137829<https://www.ncbi.nlm.nih.gov/gds/?term=GSE137829+%5BAccession%5D2>.2.The Cancer Genome Atlas Program(TCGA) (<https://portal.gdc.cancer.gov>)3.TRRUST database (version 2) (<https://www.grnpedia.org/rrust>)4.STRING database (version 12) (<https://cn.string-db.org/>)5.NetworkAnalyst website (<https://www.networkanalyst.ca/>)6.TISCH2 (<https://tisch.compbio.cn/>)7.DrugBank (version6) (<https://go.drugbank.com>)

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Figures

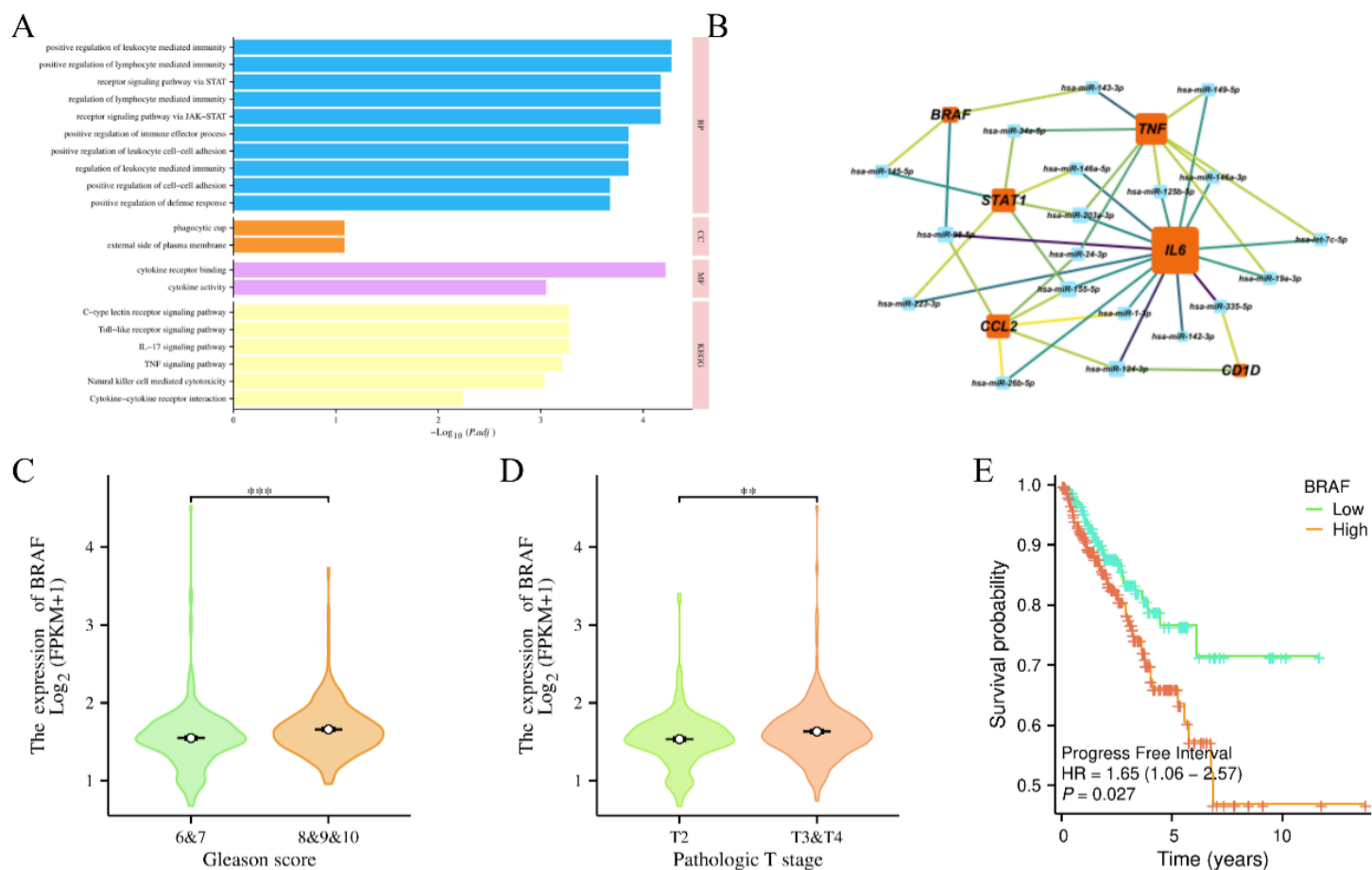


Figure 3

Immune infiltration and validation of hub genes in metastatic (castration-resistant) prostate cancer datasets. A-B. sssGSEA analysis of hub genes in the GSE35988 dataset (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$). C-E. Single-cell infiltration maps of the validation datasets GSE141445, GSE143791, and GSE137829.

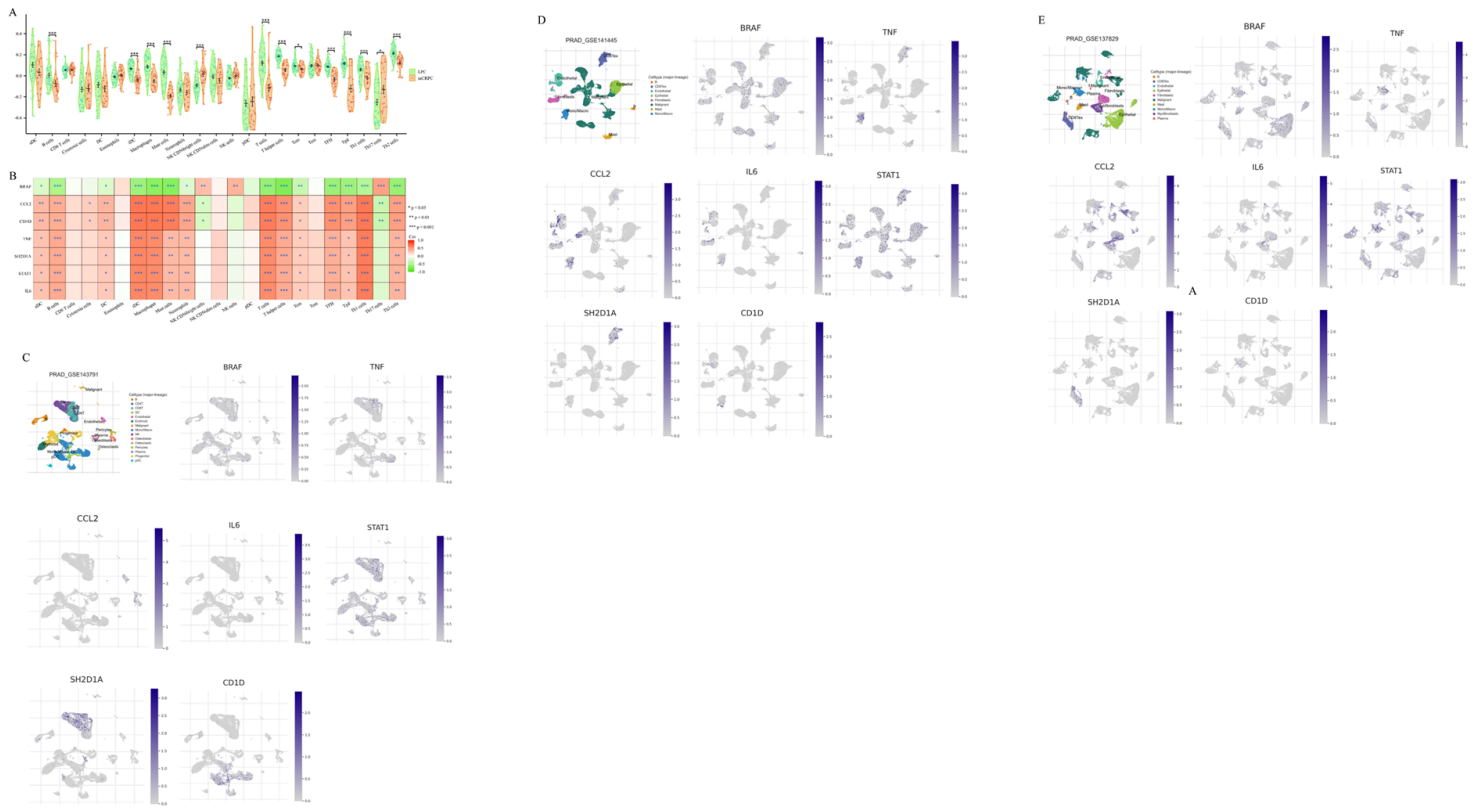


Figure 4

Overview of common gene and transcription factor interactions, protein-protein interaction networks, hub gene screening, and group-wise comparisons of hub genes. A. 34 common genes and their related transcription factors, with darker colors representing common transcription factors and common genes; darker connecting lines indicate closer relationships between genes and transcription factors. B. Protein-protein interaction (PPI) network of common genes, in which darker nodes represent genes with high connectivity, and darker edges indicate stronger molecular associations. C. Identification of the top 10 hub genes via Bottleneck and Betweenness algorithms; the Venn diagram illustrates the intersection of these sets, yielding 7 final hub genes. D. Comparative analysis of hub gene expression across the BPH, LPC, and mCRPC groups (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).