Cancer stem/progenitor signatures refine the classification of clear cell renal cell carcinoma with stratified prognosis and decreased immunotherapy efficacy

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Recent studies suggest that cancer stemness drives the acquired drug resistance process in cancer therapy. The complementary information provided by multi-omics data can help us to gain a deeper understanding of this process. This study aims to elucidate the impact of cancer stemness on the frontline treatment of clear cell renal cell carcinoma (ccRCC). Consensus clustering based on stem/progenitor signatures refined 3 subgroups in 1,730 tumor samples. We identified master regulons that regulate cancer stemness phenotypes, including key transcription factors, DNA methyltransferases, and promoter methylation probes. In addition, we compared the clinicopathological traits, genomic heterogeneity, cancer hallmarks, tumor microenvironment (TME), and oncological prognosis of the stemness subgroups. Cancer stemness was correlated with reduced efficiency of immune checkpoint blockade therapy. Cancer stemness profoundly affects the prognosis and treatment outcome of ccRCC by increasing genomic instability, tumor-associated malignant events, and immunosuppressive factors. For clinical application, we established and validated a 243-gene signature from stem/progenitor-related genes to distinguish anti-PD-1 outcomes. Overall, this presented study suggested that cancer stemness leads to adaptive resistance to anti-PD-1 treatment in CD8+ T-infiltrated ccRCC and provides a new reference for strategy development to further improve immunotherapy response rates.

INTRODUCTION

Approximately one-third of renal cell carcinoma (RCC) patients were in metastatic status at the time of diagnosis, and their 5-year survival rate decreased to 10%–20% compared with 70% for localized tumors.1 Although anti-angiogenic therapy and immune checkpoint blockade (ICB) therapy have achieved great strides, adaptive therapeutic resistance remains the main challenge that hinders further improvement of objective response rates. Substantial evidence has demonstrated that the presence of cancer stem cells (CSCs) with self-renewal properties plays a leading role in tumor metastasis and adaptive treatment resistance.2 Tumor cells acquire stem cell-like properties through WNT and EMT signaling and enter a slow cell cycle or semi-dormant state to confer the ability to persist. CSCs act as seeds to restore differentiation after long-term dormancy, which is the pathological basis for cancer relapse.3,4 Tumor cells under therapeutic stress, on the other hand, acquire stronger stemness characteristics through reversible epigenetic modifications and metabolic reprogramming to help them survive the crisis.3 Although CSC has been identified in several cancer types, including GBM, BLCA, BRCA, and PRAD, it was only recently that Fendler et al. isolated the CSC subpopulation in clear cell RCC (ccRCC) and described it as a cluster of CXCR4+MET+CD44+ cells.4 However, in ccRCC, the impact of stem-like cell clusters on oncological outcomes, particularly for frontline therapies, has not been established.

According to a pan-cancer investigation report, ccRCC is a highly inflamed tumor with the highest degree of T cell infiltration and cytolytic activity, as well as highly expressed immune checkpoint molecules such as programmed cell death 1 (PD-1) and PD ligand 1 (PD-L1).5,6 In contrast with the traditional perspective, a higher degree of CD8+ T infiltration seems to be associated with poorer oncological outcomes.7 However, no definitive correlation between baseline CD8+ T infiltration, PD-L1/PD-L1 expression levels, and ICB treatment responsiveness was found based on the reports of several clinical trials. Studies have reported that cancer stemness is correlated with immune cell exclusion from the tumor center, limiting anti-tumor immunity by inhibiting interferon-α/β signaling and...
up-regulating immune checkpoints. In addition, cancer stemness has also been reported to inhibit the expression of human immunogenic ERV, leading to the down-regulation of tumor immunogenicity. That is to say, the CSC theory holds the potential to provide new perspectives on tumor heterogeneity and acquired treatment resistance in ccRCC.

The development of bioinformatics tools to quantify the degree of tumor stemness has greatly facilitated the understanding of the presence of CSC in tumor tissue. Malta et al. developed a one-class logistic regression (OCLR) machine learning approach to quantify cancer stemness, called the "stemness index." using the gene expression and methylation profiles. Miranda et al. removed immune genes and proliferation-related genes from the published stem/progenitor signatures and obtained a curated signature of 109 genes. In this study, we identified 7 robust stem/progenitor quantification indicators specific to ccRCC from 28 published signatures through multi-cohort meta-analysis. We described 3 stemness subgroups with diverse oncological and ICB treatment outcomes in 1,730 tumor samples. We focused on the correlation of stemness subgroups with clinicopathological traits, tumor prognosis, genomic heterogeneity, tumor microenvironment, and master regulons controlling the stemness program activation, including transcription factors (TFs), DNA methyltransferase, methylation probes, and chromatin remodeling modifiers. In addition, this study also aimed to establish a stemness-related gene signature to distinguish ICB responsiveness for ccRCC patients.

RESULTS
Consensus clustering of cancer stem/progenitor signatures identified three stem/progenitor subgroups in ccRCC
CSCs exhibit great heterogeneity in tumor tissues, and there is a lack of tumor-specific markers for an accurate description of CSCs. Here we selected 12 datasets with normal and tumor samples (N = 1781) to identify ccRCC-specific stem/progenitor signatures. Cancer stemness was quantified by collected signatures and a comparison between tumor and normal samples was performed. We selected PNAS_Curated, Plurient, Hs_SC_Shats, Hs_SC_Palmer, Hs_ESC_Wong, Hs_ESC_Chia, and Hs_ESC_Bhattacharya because the enrichment scores of tumor samples were higher than normal samples in most of the datasets (Figure 1A). To further demonstrate the robustness of the seven selected gene sets, we merged datasets produced by the same platforms (TCGA, GPL6480, GPL570, GPL3921, and GPL10588) to perform a meta-analysis. As shown in Figure S1, the random forest model confirmed that the cancer stem/progenitor enrichment scores of the selected signatures were consistently higher than normal samples in the five meta datasets. To determine whether the identified stem/progenitor signatures reflect the potential presence of the CSC population, the correlation between the selected signatures and the identified CSC markers was evaluated. Figure 1B showed that the selected signatures exhibited a general positive correlation to CXCR4, MET, and CD44 expression in 1,946 tumor samples from 8 meta datasets. Note that MET was not detected and the correlation of six signatures to CXCR4 was negative in GPL17692. This meta dataset was excluded from subsequent analysis.

Unsupervised consensus clustering identified three stem/progenitor subgroups in Checkmate, and we termed them low-, moderate-, and high-stem/progenitor subgroups (Figures S2A–S2C). Meanwhile, validation in Javelin, TCGA-KIRC, E-MTAB-1980, and E-MTAB-3267 confirmed the stability of the classification results (Figures 1D and S2E–S2G). The ICB/anti-vascular treatment benefits, patient age, tumor Sarc/Rhab elements, and TCGA cluster differed significantly across the stem/progenitor subgroups in Checkmate and Javelin (Figures 1C and 1D). In TCGA-KIRC and E-MTAB-1980, the TNM stage, tumor grade, and immune phenotypes were unbalanced across stem/progenitor subgroups (Figures S2E and S2F).

Stem/progenitor subgroups were correlated with stratified prognosis and immunotherapy outcomes
In terms of oncologic outcomes, patient prognosis deteriorated significantly with elevated cancer stemness in TCGA-KIRC and E-MTAB-1980 (Figures 2A–2C). For anti-vascular therapy, no significant PFS benefit in patients treated with sunitinib (Figures 2D and 2E) and no significant overall survival (OS) benefit in patients treated with everolimus was observed, although they had shorter progression-free survival (PFS) in the high-stem/progenitor subgroup (Figures 2F and 2G). For ICB, we first compared the restricted mean PFS at 18 months and 60 months after nivolumab treatment was initiated. We found significantly accelerated tumor progression in the high-stem/progenitor subgroup within 18 months, while the difference was no longer significant when the observation period was extended to 60 months (Figure 2H). When comparing OS, the subgroups showed separate survival curves, and the OS time was significantly shorter in the high-stem/progenitor subgroup (Figure 2I). Next, we compared the survival differences in advanced-RCC patients treated with avelumab + axitinib. The restricted mean PFS within 12 months was significantly shorter in the high-stem/progenitor subgroup, while the PFS of the moderate-stem/progenitor subgroup within 24 months was significantly longer than the low and high-stem/progenitor subgroups (Figure 2J). Considering the delayed benefit of drug treatment, we found that the PFS benefit of the moderate-stem/progenitor subgroup occurred 3 months after treatment was initiated (Figure 2K). Last, we compared the objective response rates of sunitinib, everolimus, and nivolumab and found no significant difference across the stem/progenitor subgroups (Figure 2L). Additionally, the rates of PD-L1 positive samples assessed by the immunohistochemistry were significantly lower in the high-stem/progenitor subgroup (Figure 2M). These results suggest that evaluation of cancer stemness can distinguish the therapeutic efficacy of ICB rather than anti-vascular therapy in RCC.

Identifying master regulons of the stemness regulatory network
Dynamic methylation, histone modification, DNA methylation, and altered biological behavior of TFs are known to be master regulons of stemness activation and maintenance. Chronic stress under drug treatment drives the formation of acquired resistance through
intrinsic cellular reprogramming to induce cancer stemness. Thus, elucidating the drivers of cancer stemness maintenance and plasticity will improve our understanding of acquired drug resistance. To determine the key TFs controlling the stemness program, ccRCC-specific TF target pairs were inferred by the ARACNe pipeline and enrichment analysis yielded significantly altered TFs across stemness subgroups (Table S2). When the regulatory pleiotropy effect was corrected, 148 master regulons were identified in the moderate- versus low-stem/progenitor subgroup, and 84 regulons were identified in the high- versus moderate-stem/progenitor subgroup (Table S3). The 37 sequentially regulated TFs were displayed in the upper panel of Figure 3A. Among them, FOXM1 and GATA2 are well known...
regulons that mediate stem cell processes in a variety of cancers. Enrichment analysis of these TFs showed that they were mainly involved in embryonic organ development, cell-cycle arrest, regionalization, and DNA binding transcription, suggesting their driving role in the stemness phenotype acquisition and transition (Figure S3A).

The histone modification marks influence chromatin accessibility and are frequently perturbed during tumorigenesis. It was found that H3K27me3 and H3K4me3 are bivalent histone methylation marks, and their delicate balance controls the maintenance of stemness and differentiation, whereas H3K27ac occurs at the same

Figure 2. Oncological outcomes of the stem/progenitor subgroups in multiple RCC cohorts

We compared the overall and pairwise survival differences in multiple cohorts using the Log rank test, and the p value was adjusted using the Bonferroni method. (A–C) Patient prognosis deteriorated significantly across stem/progenitor subtypes in TCGA-KIRC (A, B) and E-MTAB-1980 (C). (D and E) No significant survival difference was found in patients treated with sunitinib. (F and G) No significant OS benefit was observed in patients treated with everolimus, although the high-stem/progenitor subgroup had shorter PFS. (H and J) Curves of the restricted mean survival in patients receiving nivolumab and avelumab + axitinib therapies. (I) In patients treated with nivolumab, there was a difference in OS across the stem/progenitor subgroups. (K) The non-proportional risk model identified the delayed benefit of stem/progenitor subgroups after 3 months of avelumab + axitinib treatment initialization. (L) Bar plots of the objective response rates to sunitinib, everolimus, and nivolumab treatment. (M) Bar plot of PD-L1 positive samples in Javelin. The PD-L1 immunohistochemical analysis was performed using the Ventana assay. To compare the differences, the Fisher’s exact test was used. CR, complete response; PD, progressive disease; PR, partial response; SD, stable disease.
Figure 3. Master regulons that regulate the stem/progenitor phenotypes

(A) The heatmap displayed the regulon activity of 37 TFs (top), 19 chromatin remodeling modifiers (middle), and 21 DNA methylation modifiers (bottom). The activity for TFs was assessed by the ‘viper’ algorithm and the two-sided GSEA provided by ‘RTN’ assessed the modification activity of H3K27me3, H3K4me3, and H3K27ac labeling to explore their modified behavior patterns. To measure the DNA methylation modification, we collected 21 DNA methylation modification enzymes, including 4 writers, 3 erasers, and 14 readers. For chromatin modifiers, KDM6B, EP300, and CREBBP were significantly down-regulated in the high-stem/progenitor subgroup. For DNA methylation modifiers, MEC2P2, ZBTB4, and MBD1 were significantly down-regulated, while TET1 was significantly up-regulated (Figure S3B).

Furthermore, we constructed RTN regulatory networks for these modifiers and inferred their biological activities by two-sided gene set enrichment analysis (GSEA). Similar to TFs, we found a gradually increased or decreased activity of these modifiers except for SETD1A, DNMT3B, MBDD3, and TET3 (Tables S5, S6, and Figure 3A), implying that the activation of the stemness program is a continuous biological process. In addition, univariate Cox analysis evaluated their correlation with nivolumab treatment prognosis, further emphasizing the biological significance of these epigenetic regulators (Figure S3C).
Identification of promoter region methylation probes in the regulation of stem/progenitor phenotypes

DNA methylation modification maintenance and erasure are epigenetic features that determine stem cell differentiation and fate. MethReg established 418 regulatory triplets in TCGA-KIRC on the 37 key TFs. Table S4 summarized 37 triplets after removing models with target genes not differentially expressed in stem/progenitor subgroups or TFs/probes that played no roles in target expression regulation. We visualized cg21796667-OTX1-NDC80 and cg10074775-PITX2-ZFHX4 to further elaborate the regulatory relationship (Figures 3B and 3C). For each triplet, the first row showed the overall association of gene expression with TF activity and promoter methylation levels. The second row takes the top 25% and bottom 25% methylated samples for hierarchical analysis to illustrate the trend of target gene expression with TF activity at different methylation levels. In the cg21796667-OTX1-NDC80 triplet (Figure 3B), the overall assessment showed that OTX1 independently activated NDC80 expression (activator, direct effect = 0.301, p < 2 × 10^-16), while the effect of cg21796667 did not reach a significant threshold (direct effect = -0.703, p = 0.17718). Thus, there was no synergistic effect between cg21796667 and OTX1 on the regulation of NDC80 (synergistic estimate = -0.0458, p = 0.31974). However, stratification analysis revealed a decreased activation of OTX1 on NDC80 in the high DNAm quartile (estimate value, 0.248 vs. 0.300), which was because cg21796667 methylation decreased the binding capacity of OTX1 (attenuating). In the cg10074775-PITX2-ZFHX4 triplet (Figure 3C), SFHX4 expression was affected by PITX2 and cg10074775 independently (direct effect: 0.731, -3.95). Meanwhile, the hierarchical analysis revealed that cg10074775 constituted a synergistic pair with PITX2 by attenuating the activation effect of PITX2 (estimate = -0.576, p = 3.45e-08). Gene expression analysis of NDC80 and ZFHX4 corroborated the reliability of the regulatory model (Figure S3D). Additionally, we analyzed the prognostic value of the methylation probes, and Kaplan-Meier curves showed significant survival differences between subgroups stratified by median expression values (Figures S3E and S3F).

Genomic heterogeneity analysis revealed that 9p21.3 loss led to stemness program activation in ccRCC

The genomic heterogeneity of the stem/progenitor subgroups was then depicted using gene mutation and copy number alterations at the arm and gene levels. Previous studies have found a general positive correlation between the stemness degree and TMB. Consistently, the high-stem/progenitor subgroup in ccRCC harbored higher non-synonymous TMB (Figures 4A, S4B, and S4C). Specifically, MUC16, BAP1, DNAH3, and VWF in TCGA-KIRC (Figure 4C), and SUGCT, NOS3, NSD1, STAR10, PIEZO1, ZNF292, FBXO11, TG, TRIO, SRCAP, and ZFHX4 in Checkmate (Figure 4D) were unbalanced across stem/progenitor subgroups. In Javelin, which included but was not limited to clear cell carcinoma (Figure S4A), we identified 26 differentially distributed high-frequency gene mutations (frequency >1%). However, no significant difference in neo-antigen load was observed (Figures S4D and S4E). Clinical studies have shown that neither ITH, TMB, nor tumor neo-antigens could predict ICB response in ccRCC, but several individual mutations, including PBRM1 and PTEN, have been linked to ICB benefit. These gene mutations were not differentially distributed in the stem/progenitor subgroups, implying that the stemness program does not affect ICB outcomes by carrying specific mutations.

In terms of chromosomal instability, we found that copy number amplification and deletion accumulated rapidly with stemness activation (Figure 4B). When we focused on all unbalanced distributions of CNA segments, we found that almost all alterations more frequently occurred in the high-stem/progenitor subgroup, except for segments from 5q regions, which conversely much less frequently occurred in the high-stem/progenitor subgroup (Figures 4C and 4E). We were delighted to find that 9p21.3 loss of heterozygosity, which was reported to be an inducer of anti-PD-1 resistance in CD8 T infiltrated ccRCC, more frequently occurred with stemness degree increased (Figures 4C and 4E). Human chromosome 9p21.3 is susceptible to inactivation during cellular immortalization, and tumor suppressor genes CDKN2A/B in this region have been identified to be associated with tumor metastasis and worse survival. A recent study reported that homozygous deletion of 9p21.3 (loss of heterozygosity [LOH]) leads to pan-cancer ICB resistance through the down-regulation of CDKN2A/MTAP expression. We found more frequent copy number deletions of CDKN2A/B and DMRTA1/MTAP in the high-stem/progenitor subgroup (Figure 4C). However, CDKN2A/MTAP expression was elevated in the high-stem/progenitor subgroups (Figure S4F). In addition, we noted that the 9q34.3 segment, which was reported to be closely correlated with enhanced CD8+ T infiltration, was more frequently lost in the high-stem/progenitor subgroup in Checkmate (Figure 4E). The expression levels of immune-related molecules encoded by these differentially altered fragments, including immunoproteasomes, antigen presentation-related proteins, HLA class II alleles, and histone lysine methyltransferases, were also investigated (Figure S4F). We divided the Checkmate-nivolumab arm into complete response/partial response versus stable disease/progressive disease groups and compared all genes encoded on 9p21.3 (Figure S4G). To our surprise, only IFNA21 was differentially expressed in the comparison, and no significant survival difference was observed between the high- and low-expression subgroups (Figures S4H and S4I). After that, we divided patients into 9p21.3 loss mutant (MUT) and wild-type groups for GSEA analysis (Figures 4F–4H), which revealed activation of stromal features such as hypoxia, angiogenesis, WNT, EMT, transforming growth factor (TGF) signaling, and pluripotent stem cell induction pathways in the 9p21.3 loss MUT group. These results suggest that the activation of the stemness program is a potential cause of resistance to anti-PD-1 therapy in ccRCC.

Molecular and immune characteristics of the stem/progenitor subtypes

We characterized the cancer hallmarks of the stem/progenitor phenotype (Figure 5A) in the Checkmate cohort and found that cell cycle-related events such as DNA repair, MYC targets, G2M checkpoints, E2F targets, and KRAS signaling were significantly up-regulated in the high-stem/progenitor subgroup, indicating the presence of cancer...
progenitor cells. In contrast, metabolic processes, such as bile acid metabolism, fatty acid metabolism, cholesterol metabolism, and oxidative phosphorylation, were found to be more active in the low-stem/progenitor subgroup. We then looked at the immune infiltration landscape of the Checkmate and Javelin cohorts (Figure 5A). We found more abundant infiltrated CD4+/CD8+ T cells, Tfh, Th1, activated dendritic cells (DCs), and macrophages in the high-stem/progenitor subgroup. In addition, we compared the cytotoxic activity of the subgroups as it reflected the substantial effect of the cytotoxic CD8+ T cells. We found that antigen-presenting molecules (HLA-A, B, C, B2M), apoptotic effector molecules (CASP8), cytolytic activity, and immune checkpoints (PD-1, PD-L1, CTLA4, LAG3) were significantly increased as cancer stemness was activated. However, assessment of CD8+ T cell density in the tumor core and infiltrating margins by immunohistochemical staining (Javelin) or immunofluorescence (Checkmate) showed no significant differences in ccRCC, despite significant differences being present in RCC (Figures 5C–5F). In addition, cancer-associated fibroblasts (CAFs), immune suppression, immune exhaustion, and immune exclusion-related signals were more active in the high-stem/progenitor subgroup (Figures 5G–5J). Given that

Figure 4. Genomic heterogeneity of the stem/progenitor subgroups
(A) TMB was higher in the high-stem/progenitor subgroup. The Kruskal-Wallis test compared the overall difference. (B) Fraction genome gain and loss were presented as bar charts of mean values with standard errors. The Kruskal-Wallis test. (C) The landscape of the TCGA-KIRC gene mutation (frequency >5%), arm-level, and gene-level CNAs. (D) Selected differentially mutated genes in Checkmate (frequency >5%). (E) The selected differential CNA in Checkmate. The frequency difference was compared using Fisher’s exact test. F-H EMT, WNT, angiogenesis, TGF-β signaling, and PSC signaling were found to be up-regulated in 9p21.3 loss samples in Checkmate (F and G) and TCGA-KIRC (H), while RIG-I-like receptor signaling was down-regulated (G).
lymphocyte-depleted and TGF-dominant subtypes were more frequently enriched in the high-stem/progenitor subgroup in TCGA-KIRC, these findings indicated the dominative role of "immunosuppressive effects" in the high-stem/progenitor subgroup.

Stemness-related genes are correlated with ICB benefits and are differently expressed in the CSC cluster

We examined the correlation between the 6,507 stemness-related differentially expressed genes (DEGs) (high- vs. low-stem/progenitor subgroups) and anti-PD-1 treatment benefits in Checkmate to see if they could distinguish ICB outcomes. 34 genes positively correlated with a clinical benefit and 209 genes negatively correlated with clinical benefit with a p value of less than 0.05 were retained (Table S7). These genes were enriched in the cell cycle G2/M checkpoint regulation process in gene ontology (GO) annotation (Figure 6A). We looked at the single-cell transcriptome of 17,665 cells from 7 ccRCC tumor samples according to the published ccRCC cell marker,20–23 the cells were manually identified as 11 cell types (Figures 6B and 6C). Forty-eight of the stemness-related genes marked the following cell types: ccRCC...
A sub-cluster of all sub-clusters was ranked by CytoTRACE, and it was found that analysis was performed on ccRCC cells and the dedifferentiation level sub-clusters (Figure S5A). It was further found that the well recognized and ITGB1, ITGAV, ccRCC stem cell markers, including CXCR4, CD44, PROM1, ENG, and MRPL32 (Figure 6F). The results of univariate Cox regression further revealed that BICC1 was a risk factor for both PFS and OS after nivolumab treatment (Figure 6G). Afterward, the hazard score was generated and mapped to the single cell level, and it was found to be significantly correlated with the CytoTRACE dedifferentiation rank (Figure 6H) (Spearman’s R = 0.34, p < 2.2 x 10^-16). Additionally, CSCs hold substantially higher hazard scores than the non-CSCs (Figure 6I).

Stemness-related gene signature was able to distinguish ICB responsiveness

Significant PFS and OS benefits were observed in patients in the low score group when treated with nivolumab (Figures 7A and 7B). Significantly higher hazard scores were observed in the PD and NCB groups (Figures 7C and 7D), with a predictive power of 0.766 (Figure 7E). In mechanism, the hazard score was negatively correlated with immune infiltration (Figure 7F). Primary validation was performed in a mouse model consisting of 24 samples (Figure 7G and GSE117358), and the
Figure 7. Stemness-related gene signature is able to distinguish the anti-PD-1 outcomes in ccRCC

(A and B) Survival curves of the stratified hazard score groups in Checkmate. (C and D) The hazard scores in the clinical benefit and objective response subgroups of Checkmate. (E and H) receiving operating characteristic (ROC) curves of the hazard score’s predictive power to ICB treatment outcome in Checkmate (E) and GSE117358 (H). (F) The heat map displayed the Pearson’s correlation coefficients of the hazard score with immune cell infiltration levels. (G) The hazard score between nivolumab-resistant and non-responders in GSE117358. (I) The TIDE algorithm predicts the response fraction to anti-PD-1/CTLA4 between subgroups stratified by the cohort-specific real references (N = 47). (K) The subclass mapping results in three independent validation cohorts using two nivolumab-treated ccRCC cohorts as references (Checkmate, N = 172, GSE67501, N = 11).

responder group scored significantly lower than the non-responder group with a predictive power of 0.819 for anti-CTLA4/anti-PD-L1 treatment (Figure 7H). Next, we generated the hazard scores in the TCGA-KIRC, GPL570, and GPL10558 and stratified them into high and low score groups based on cohort-specific median values to further test the discrimination capability of the signature. The Tumor Immune Dysfunction and Exclusion (TIDE) algorithm was used to predict anti-CTLA4/PD-1 treatment outcome in the three external cohorts based on the immunotherapy outcomes and pretreatment expression profiles of several melanoma and non-small cell lung cancer (NSCLC) cohorts, and the results showed the responder proportions of the low-score groups were significantly higher (Figure 7I). Using a melanoma cohort (treated with CTLA-4/PD-1, N = 47) as the reference dataset to perform subclass mapping, the results...
consistently showed that the low-score groups were more likely to respond to anti-PD-1 treatment (Figure 7J). Last, we collected two nivolumab-treated ccRCC cohorts (Checkmate, N = 172; GSE67501, N = 11) as the reference cohorts for subclass mapping prediction. In both TCGA-KIRC and GPL570, the high score groups showed no response to PD-1 treatment, while the low score groups were predicted to respond to PD-1 treatment in GPL10558 (Figure 7K, p = 0.016 and 0.078). These results demonstrate that the hazard score can distinguish anti-PD1 responsiveness in ccRCC.

**DISCUSSION**

Although the presence of CSCs and stemness program activation have been hypothesized as key inducers of acquired drug resistance, their characterization in ccRCC has not been elucidated. Recently, a clinical trial report identified 9p21.3 deletion as an intrinsic factor causing immunotherapy failure in CD8+ T cell-infiltrated ccRCC.13 Subsequently, a comprehensive analysis based on pan-cancer immunotherapy cohorts concluded that 9p21.3 LOH led to the down-regulation of CDKN2A/MTAP and ultimately led to the cold immune microenvironment and ICB resistance.17 Based on the comprehensive analysis of multi-omics data, we found that the 9p21.3 deletion in ccRCC causes ICB resistance through the activation of the stemness program other than down-regulating CDKN2A/MTAP. The results of our analysis implied that the development of therapeutic strategies targeting the elimination of the CSC subpopulations may further improve ICB response rates.

The large amounts of multi-omics data generated by high-throughput sequencing now provide new opportunities for further understanding of drug treatment resistance. We noted the limitation of OCLR in describing the stemness of ccRCC,6 as the stemness index conflicted with the well-accepted assumption that tumor tissues exhibit more stemness characteristics compared with normal tissues. By collecting published stem/progenitor signatures and performing meta-analyses on multiple ccRCC datasets, we identified seven gene sets to describe the stem/progenitor features in ccRCC (Figure 1A). The identified stem/progenitor signatures showed a generally positive correlation with the CSC surface markers (Figure 1B), demonstrating that the enrichment scores reflect the existence of CSC in tumors and not just the propensity to stem cell-associated programs. The stratification strategy based on enrichment scores of the 7 gene sets was stably reproduced across multiple sequencing platforms involving 1,730 patients. The stem/progenitor subgrouping provided a new perspective on patient stratification for current frontline therapies. We found that the activated stemness program induced decreased ICB efficacy and the 9p21.3 deletion is an intrinsic driver of stemness program activation in ccRCC, as 9p21.3 loss accelerates with activated cancer stemness and is not restricted to advanced-stage ccRCC (Figures 4C and 4E). The association of recurrent 9p21 LOH with ICB resistance was recently established,13,15,17 and significant down-regulation of CDKN2A/MTAP expression because of frequent copy number loss was suggested as the underlying mechanism. However, studies in the TCGA-KIRC and Checkmate cohorts reported that the 9p21 deletion in ccRCC was predominantly heterozygous and did not result in significant down-regulation of CDKN2A/MTAP.17 We observed that in the stem/progenitor subgroups, the expression level of CDKN2A/MTAP was elevated with the increase of 9p21.3 loss events (Figure 4C). We further explored all genes encoded in the 9p21.3 region in the checkmate cohort and none of them was found to be significantly correlated with nivolumab treatment outcome (Figures S4G–S4I). Interestingly, we found the activation of tumor stemness-related signals such as EMT, WNT, and PSC signaling in both TCGA-KIRC and Checkmate (Figures 4F–4H). We also noted that mTORC1 signaling was up-regulated in the 9p21.3 loss MUT group (Figure 4H), which was reported to be associated with anti-PD-1 resistance by carrying an unfavorable PTEN mutation.23,24 We did not observe a differential distribution of PTEN mutations in the stem/progenitor subgroups. In other words, cancer stemness is an independent, unfavorable factor leading to ICB resistance in ccRCC.

In this report, cancer stemness was linked to a number of malignant events in RCC, including an accelerated cell cycle, stromal activation (e.g., TGF, EMT), PI3K/Akt/mTOR, and mTORC1 signaling (Figure 5A). The results also reported a unique and complex association between cancer stemness and the immune infiltration landscape in RCC (Figures 5B and 5G–5J). Specifically, increased cancer stemness was associated with abundant antitumor immune infiltration levels (M1 macrophages, activated DCs, activated and effector CD4+/CD8+ T cells), HLA-I molecule expression, and cytolytic activity. However, numerous immunosuppressive factors such as immune checkpoint molecules, CAFs, myeloid-derived suppressor cells, and T regulatory cells were also enriched in the high-stemness subgroup. The activation of cancer stemness in ccRCC is accompanied by up-regulated stromal-related signaling, such as EMT, WNT, TGF-β, hypoxia, and angiogenesis (Figures 4F–4H). It is believed that CSCs are quite plastic and they tend to form a protective microenvironment by interacting with the extracellular matrix, microvascular and surrounding endothelial cells, fibroblasts, and pericytes, and reside deeply within the microenvironment.9,28,29 Glioblastoma stem cells were first found to be enriched in the vascular niche, and this intense relevance has since been widely confirmed in a variety of cancer types.30 The vascular microenvironment maintains the initial undifferentiated dormancy of stem cells and supports self-renewal, invasion, and metastasis of CSCs, while hypoxia is an inducer of CSC plasticity.31 Chinchar et al. first reported that anti-vascular drugs may exacerbate the tumor hypoxic microenvironment and thus increase breast CSC.32 Subsequently, Mariana et al. demonstrated that sunitinib was able to induce hypoxia in areas surrounding RCC necrosis to produce resistance to its own therapeutic effects and that the number of CSCs was significantly increased in these areas.33 Therefore, although existing evidence suggests that anti-angiogenic therapy (particularly vascular endothelial growth factor inhibition) can overcome some of the immunosuppressive factors impacting response to PD-1 blockade, no survival benefit was observed in the high-stemness subgroup in the Javelin cohort.

For clinical application, we developed and demonstrated the capability of the stemness-related gene signature to distinguish
anti-PD-1 responsiveness. The robustness was validated by an independent mouse model dataset, TIDE, and subclass mapping prediction results. Of note, the stemness-related gene signature we developed based on the nivolumab-treated cohort is not applicable to predict targeted therapy responsiveness. Given the increasing use of the ICB plus targeted therapy strategy in clinical practice, the guidance value of the CSC markers in this scenario needs to be further explored in the future as more clinical trial data are disclosed. CSC cannot be specifically identified and cleared by current targeted therapies and ICB treatment strategies, so the introduction of new therapeutic modalities targeting CSC may be the future direction of adjuvant therapy for kidney cancer. Making full use of tumor-specific CSC markers to enable chimeric antigen receptor T cells to specifically identify and remove CSC may be one of the most effective ways, as Feng et al. used CAR-T anti-epidermal growth factor and anti-CD133 to target CSC.14

Conclusion
Collectively, we identified seven ccRCC-specific stemness gene sets and stratified RCC patients into three stemness subgroups with distinct prognosis and ICB treatment outcomes. Multi-omics analysis showed that cancer stemness was associated with increased genomic instability, 9p21.3 deletion, tumor-associated malignant events, immune infiltration level, and immunosuppressive factors in RCC. In addition, the stemness-related gene signature can be used to distinguish the responders to anti-PD-1 treatment.

MATERIALS AND METHODS
Multi-omics data acquisition and preprocessing
This study included multiple RCC RNA sequencing (RNA-seq) and microarray datasets (Table S1). Checkmate is a meta dataset of three phase-III clinical trial cohorts, including 311 advanced ccRCC patients treated with nivolumab or everolimus.13 Javelin is a phase III clinical trial cohort containing 726 advanced RCC patients treated with avelumab + axitinib or sunitinib.14 The TCGA-KIRC, Checkmate, and Javelin datasets contained gene mutation annotations, 450K methylation sequencing profiles, and copy number alteration (CNA) data. Raw counts or FPKM values were converted to TPM values to ensure comparability with the microarray data.35 Datasets produced on the same platforms were merged using the Combat function to remove the batch effect. The 26 published stemness signatures were obtained from the StemChecker portal (http://stemchecker.sysbiolab.eu), and the PNAS_Curated signature was obtained from Miranda et al.1 Gene aliases in different datasets were manually checked to stay consistent with the current gene symbols in Genecard (https://www.genecards.org/).

Non-synonymous mutations were extracted from the mutation annotation files according to the definition of Braun et al.13 For methylation data, probes with missing samples more than 30% were removed, and the missing values were compensated for using the ‘impute’ function. Data filtering and normalization were completed using the ‘ChAMP’ package.16 For CNA data, the Masked Copy Number Segment file and reference markers were downloaded from the TCGA portal. Copy number variation analysis was completed using the GISTIC_2.0 module provided on the GenePattern portal (https://cloud.genepattern.org/gp/pages/index.jsf). The Hg38 reference genome was set as the reference genome, and the threshold for amplification/deletion was ±0.3 (q < 0.05), the confidence level was 0.95.

Identification of stemness signatures and consensus clustering
The cancer stemness of bulk tissue transcriptome was measured by the ‘ssgsea’ algorithm provided in the ‘GSVA’ package as described by Miranda et al.1 In addition, the OCLR algorithm was used to regenerate the stemness index according to the description of Malta et al.6 The comparison of the stemness scores between normal and tumor samples was completed using the ‘limma’ package. The correlation between stemness scores and CSC markers was evaluated using Pearson’s coefficient. The meta-analysis was performed using the ‘meta’ package. Consensus clustering was performed using the ‘ConsensusClusterPlus’ package, which was repeated 1,000 times to ensure the stability of the classification results. The clustering algorithm was set as “km” and the sample similarity was determined by the Euclidean distance. According to the description of the ‘ConsensusClusterPlus’ algorithm, the most appropriate K value of the classification number occurred at the node with a smooth CONSENSUS CDF curve and a significant change in the Delta area curve.37

Transcriptional factors and histone modifier analysis
We downloaded 1,317 proofread TF-target pairs from Garcia-Alonso et al.38 Considering the tissue specificity of TF target pairs, we inferred the ccRCC-specific TF regulatory network by performing a Java platform-based pipeline called ARACNe.59,60 In detail, ARACNe established a regulatory network in which non-directly regulatory pairs were removed by running data processing inequality (DPI) with a mutual information threshold of 1e-08 using a list of 1,317 TFs and the expression profile as input files. After running 100 reproducible bootstraps, ARACNe used the Poisson distribution to test the significance of the regulatory pairs in multi-bootstrap mode and generated a TF target pair list. The assessment of TF activity using target gene expression is becoming a widely accepted method to depict the functional status of transcriptional regulatory circuits.38 In other words, TF activity is a comprehensive reflection of TF expression level, chromatin accessibility, and methylation of the TF binding site (TFBS). Here, we used the ‘viper’ package to calculate the regulon activity based on the TF-target pairs.41 Sample replacement and t-tests were executed between the subgroups to generate a null model, and then the ‘mviper’ function was run to obtain the normalized enrichment score for each TF.

The 19 H3K27me3, H3K4me3, and H3K27ac enzymes were obtained from the HISTome2 portal (http://www.actrec.gov.in/histome2/index.php). The DNA methylation writers and erasers were collected from Meng et al.42 The ‘RTN’ package was used to build potential regulatory networks of the histone and DNA methylation modifiers, and then ‘mi.bootstrap’ and ‘ini.dpi.filter’ functions were run sequentially to remove unstable interactions.43 Finally, the regulatory activity of the epigenetic modifiers was assessed by two-sided GSEA.
Assessment of the promoter region methylation probes in regulation of the stemness subtypes

Because TF binding to DNA can be influenced by (or influences) DNA methylation levels nearby, target gene expression levels result from the synergistic effects of both TF and DNA methylation. In other words, DNA methylation can directly regulate gene expression or indirectly affect gene level by impacting TF activity. Here, we introduced a CpG-TF-target regulation model provided by ‘MethReg’ to evaluate the regulatory potential of CpGs. The methylation probes were mapped into the 2-kb region upstream or downstream of the promoter region using the TFBS information from the JASPAR database. Then, the effects of methylation probes on TF regulation of target genes were measured using a robust linear model:

\[ \text{Target gene expression} \sim \text{TF activity} + \text{DNA methylation} + \text{TF} \]

The regulatory role of TFs or probes was confirmed when the Bonferroni corrected p value was less than 0.001.

Single cell RNA-seq analysis

We downloaded single cell transcriptome data from seven ccRCC samples from GSE159115 and used the ‘Seurat’ package to quality control the data according to the original report. We included 17,665 cells for analysis and selected highly variable 3,000 genes for clustering after normalizing the transcriptome data. The cells were manually annotated using the published ccRCC cell marker. Cell markers were determined by the ‘FindAllMarkers’ function with a logFC of greater than 0.25 and an adjusted p value of less than 0.05. Cytolytic activity was calculated using the ‘Cytolytic Activity’ package. We used CytoTRACE to measure the degree of dedifferentiation of ccRCC subpopulations and to assist us in identifying CSC subpopulations.

Bioinformatic analysis

A DEG analysis was performed using the “limma” package. The fraction of genome alteration was calculated using the ‘MOViCS’ package developed by Lu et al. GO enrichment and GSEA were performed using the “ClusterProfiler” package. Cytolytic activity was calculated as the geometric mean of GZMA and PRF1 expression (TPM value) as described before. Reference gene sets of 28 immune cells were described by Charoentong et al. and the relative infiltration abundance of 28 immune cells was estimated by the “ssgsea” algorithm. In addition, the R package ‘IOBR’, which integrates published tumor microenvironment (TME) signatures and several TME deconvolution methods, was adopted to deconstruct the immune features of RCC samples. CB, ICB, and NCB were set as numerical variables, and the Pearson’s coefficients were calculated to assess the correlation of DEGs with ICB treatment clinical benefit. The hazard score was calculated as the Z score of expression values of the identified stemness-related genes. To validate the predictive value of the hazard score, we performed TIDE and subclass mapping analysis to predict the responsiveness of ccRCC samples to immunotherapy. The TIDE algorithm was developed to predict anti-PD-1/CTLA4 therapy responses based on melanoma and NSCLC cohorts.

We collected a melanoma cohort treated with anti-PD-1/CTLA-4 (N = 47), and two ccRCC cohorts treated with nivolumab (Checkmate, N = 172; GSE67501, N = 11) as reference cohorts. In addition, 770 immune genes closely correlated with ICB response were collected from the nCounter PanCancer Immune Profiling Panel portal for subclass mapping.

Statistical analyses

All statistical analyses were conducted in R 4.1.1. Numerical variates were expressed as mean ± standard deviation, and the Wilcoxon test and the Kruskal-Wallis test were used to assess the difference between two-grouped or multi-grouped variates. Fisher’s exact test was used to compare the distribution differences of categorical variates. The Kaplan-Meier curves were used to visualize the censoring status and the Log rank test was used to detect OS differences. Univariate and multivariate Cox regression were used to assess the prognosis value, and receiving operating characteristic curves were used to evaluate the predictive efficacy. Restricted mean survival time ratios were estimated using the ‘survRM2’ package. For delayed clinical benefit, long-term survival differences after a certain time of dose initialization were inferred by a non-proportional risk approach using the ‘ComparisonSurv’ package. All statistical comparisons were bilateral, and a p value of less than 0.05 was considered statistically significant.

DATA AVAILABILITY STATEMENT

Code to reproduce the main results of this study would be available from the corresponding author upon reasonable request. Open access dataset was summarized in Table S1.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omto.2022.10.005.

ACKNOWLEDGMENTS

Supported by the National Natural Science Foundation of China, Youth Science Fund Project (Grant Number: 81777279) and the Chen Xiao-ping Foundation for The Development of Science and Technology of Hubei Province (No. 202094). The funders have no role in designing and completing this study. The data source involved in this present study was open access; therefore, ethics approval was not applicable. The corresponding author declared full access to data involved in this study and final responsibility for the decision to submit for peer review. The authors thank Dr Xiaofan Lu (State Key Laboratory of Natural Medicines, Research Center of Biostatistics and Computational Pharmacy, China Pharmaceutical University) for the methodology support of this study.

AUTHOR CONTRIBUTIONS

S.W. and J.H. proposed and designed the framework of this study; J.X. and C.L. completed the collation and pre-processing of the raw data required for subsequent analysis; P.Z., H.H., and Y.L. performed a detailed analysis the raw data; J.X. and Y.W. visualized the analytical results; P.Z. interpreted the results and drafted this manuscript;
Y.X., J.H., and S.W. reviewed and critically revisited the manuscript. The manuscript was confirmed by all authors before submitting for peer review.

DECLARATIONS OF INTEREST
The corresponding authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest on behalf of all authors.

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