Analysis of tumor microenvironment characteristics in bladder cancer: implications for immune checkpoint inhibitor therapy

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Research

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Abstract

Background

The tumor microenvironment (TME) has a significant influence on prognosis and immunotherapy. There are no studies on the systematic analysis of bladder cancer TME and its effect on immune checkpoint inhibitor therapy.

Methods

We comprehensively evaluated the TME infiltration pattern of bladder cancer in 1,889 patients and conducted extensive immunogenomic analysis to explore the heterogeneity and prognostic significance of the TME of bladder cancer. The principal component analysis algorithm was used to calculate the immune cell (IC) score to quantify the level of IC infiltration. We used the receiver operating characteristic (ROC) curve, Tumor Immune Dysfunction and Exclusion (TIDE), and Subnetwork Mappings in Alignment of Pathways (SubMAP) algorithms to evaluate whether the IC score can predict the benefits of immune checkpoint inhibitors in bladder cancer patients.

Results

We identified three different TME phenotypes using unsupervised clustering methods. To explore the potential biological pathways that drive the formation of these microenvironmental phenotypes, we demonstrated the clinical and pathological characteristics, biological signaling pathways, cancer immune circulation, copy number, and somatic mutation differences among the different subtypes. In addition, univariate and multivariate Cox regression analyses showed that the IC score is a reliable and independent prognostic marker. The IC score can also predict immune checkpoint inhibitor responsiveness as patients with higher IC scores showed a significant therapeutic advantage in immunotherapy.

Conclusions

This study increases our understanding of the characteristics of TME infiltration in bladder cancer and provides guidance on more effective personalized immunotherapeutic strategies.

1. Background

Bladder cancer is the ninth most common cancer worldwide, is difficult to diagnose early, metastasizes rapidly, but currently has ineffective treatments [1, 2]. Immune checkpoint therapy (ICT) is an immunotherapy that targets cytotoxic lymphocyte antigen-4 (CTLA-4), programmed cell death protein 1 (PD-1), or programmed death ligand 1 (PD-L1) [3–5]. Immunotherapeutics for PD-1 or PD-L1 have greatly
improved the survival of some patients and changed the intervention measures for advanced bladder cancer. However, most patients gain little to no clinical benefits from these immunotherapeutics [6, 7]. Previous studies have found that PD-1 and PD-L1 expression, microsatellite instability status, and mutation load are not the best biomarkers for predicting immune checkpoint inhibitor responsiveness [8, 9]. Therefore, it is necessary to establish new predictive indicators for checkpoint immunotherapy.

Tumor cells grow and survive in the tumor microenvironment (TME), which not only contains cancer cells, but also stromal cells such as resident fibroblasts (cancer-associated fibroblasts), macrophages, and recruited cells such as infiltrating immune cells (bone marrow cells and lymphocytes), bone marrow-derived cells (endothelial progenitor cells and hematopoietic progenitor cells), and secreted factors (cytokines, chemokines, and growth factors) [10, 11]. These cancer cells trigger a variety of physiological changes through direct and indirect interactions with other TME components, such as inducing proliferation and angiogenesis, inhibiting apoptosis, avoiding hypoxia, and inducing immune tolerance. New evidence confirms that TME plays a key role in tumor progression, immune escape, and immunotherapeutic response [12–14]. Therefore, a comprehensive analysis of the heterogeneity and complexity of TME is a key step to improve the success rate of existing ICTs and developing new immunotherapeutic strategies. A comprehensive analysis of the heterogeneity and complexity of TME will also be beneficial to determine the different tumor immunophenotypes and improve the ability to guide and predict the responsiveness of immunotherapy.

In this study, we aimed to identify novel biomarkers of bladder cancer using ICscores of various TME subtypes. The findings of this study might provide a better understanding of the molecular mechanisms of immune microenvironmental regulation in bladder cancer, offer a more complete explanation of the response of bladder cancer to immunotherapy, and suggest novel prognostic biomarkers to guide more effective immunotherapeutic strategies for bladder cancer.

2. Materials And Methods

2.1 Bladder cancer dataset source and preprocessing

In this study, we analyzed eight cohorts with a total of 1,482 bladder cancer patients: GSE31684, GSE32548, GSE32894, GSE69795, GSE83586, GSE86411, GSE87304, and GSE120736. All gene expression data sets were subjected to log2 transformation and quantile normalization. Batch effects from non-biological technical biases were corrected using the “ComBat” algorithm of the Sva package.

The gene expression data of 407 patients with bladder cancer were obtained from Genome Data Commons (https://portal.gdc.cancer.gov) as a validation set(Supplementary Table 1). Then, fragments per kilobase of exon model per million reads mapped values were transformed into transcripts per kilobase million values. IMvigor 210 cohort(NCT02108652) is a multicenter, single-arm phase II clinical study for evaluating the safety and efficacy of Tecentriq, a PD-L1 inhibitor, in patients with advanced urothelial carcinoma. We obtained the corresponding clinical data and somatic mutation and copy
number data based on the Creative Commons 3.0 License. The complete expression data, detailed
clinical annotations(Supplementary Table 2), and somatic mutation data were obtained from IMvigor 210
Core Biologies (http://research-pub.gene.com/IMvigor210CoreBiologies), a complete documentation
software and data package for the R statistical computing environment.

2.2 Estimation of TME cell infiltration

Yi Xiao [17] found that a gene set that marks each TME infiltrating IC type contains 24 human IC
subtypes. We used the single-sample gene set enrichment analysis (ssGSEA) algorithm to analyze the
TME(Supplementary Table 3). The relative abundance of each cell infiltration was quantified. In addition,
we also used the ESTIMATE algorithm [18] to estimate the ratio of the immune stromal components in
the TME in each sample to further explore the differences in the TME scores among the different
immunotypes, including ICs and stromal cells using ImmuneScore and StromalScore(Supplementary
Table 4). A higher ImmuneScore or StromalScore indicates more immune or matrix components in the
TME.

2.3 Construction of molecular types based on the infiltration
level of 24 ICs in the TME

We used 407 samples from eight GEO datasets of TCGA-BLCA as the training set. Simultaneously, we
included a total of 1,482 bladder cancer patients into the meta-cohort as the validation set. By analyzing
the infiltration levels of 24 ICs and using the consensus clustering algorithm, we determined that the
optimal clustering number (k value) of the two cohorts was 3. We used unsupervised cluster analysis (K-
Means based on Euclidean distance) [19] to identify three different types. We used the
ConsensusClusterPlus R package to perform the above steps, and performed 1000 repetitions (50
iterations with a resampling rate of 80%) to ensure the stability of the classification [20].

To further interpret the different characteristics of the three different immune subtypes, we compared
them with the published classifications of several common bladder cancer subtypes in detail. The Baylor
subtype established by Mo et al. [21] divided muscle-invasive bladder cancer (MIBC) and non-muscle-
invasive bladder cancer into two subgroups each: basal and differentiated. Damrauer et al. performed a
consensus cluster analysis on the dataset, identified basal and luminal subtypes, and further identified 47
genes as subtype predictors in UNC. To perform unsupervised cluster analysis on 73 MIBC specimens,
MDA subtype [23] with 2,252 genes (2,697 probes) was used. These genes produced three subtypes,
namely, p53-like, luminal, and basal. Lund et al. [24] presented a six-class system based on global mRNA
expression: urothelial-like, genomically unstable, epithelial infiltrated, squamous carcinoma (SCO)-
like/mesenchymal (Mes)-like, SCC-like/urobasal B, and small-cell/neuroendocrine-like. CIT [25] used
2,707 genes to perform unsupervised cluster analysis on 129 MIBC patient samples and divided the
patients into four subtypes (I, II, III, and IV) [26]. These classifiers were combined into an R package
(BLCAsubtyping; https://github.com/cit-bioinfo/BLCAsubtyping) and applied independently to the GEO
and TCGA-BLCA datasets and the IMvigor 210 cohort.
2.4 Identification of differentially expressed genes (DEGs) among immunophenotypes and signal pathway enrichment analysis

The Bayesian method of the limma R package [27] was used to analyze the difference between the two groups. The DEGs were analyzed using gene ontology and Kyoto Encyclopedia of Genes and Genomes (KEGG) with the corrected $P$ value < 0.05 and the absolute value of log fold change > 1 as the criteria to determine the significance of DEGs. GSEA was used to evaluate the skewness of the two distributions of the selected gene sets in the gene list sorted by a specific phenotype. The analyzed gene set was obtained from the Hallmark gene sets from the Molecular Signatures Database (MSigDB) (h.all.v7.1.entrez.gmt) using the clusterProfiler R package [28]. We used the GSVA R package to perform Gene Set Variation Analysis (GSVA) for the assessment of gene enrichment to study the differences in biological processes among the three immune subtypes. GSVA is a non-parametric, unsupervised method used to estimate changes in pathway and biological processes in expression data set samples. We obtained the gene set "c5.all.v6.2.symbols.gmt" and 50 common biological pathway gene sets for GSVA analysis from the MSigDB database. We used the ssGSEA method to generate enrichment scores of these gene sets for each pathway in each sample, and compared the three immunotypes using the ssGSEA score of the pathways.

2.5 Correlation analysis with core biological pathways of bladder cancer

We referred to a gene set related to certain biological processes provided by Mariathasan et al. [15] that included (1) immune checkpoint (2) antigen-processing machinery, (3) CD8 T-effector signature, (4) epithelial-mesenchymal transition (EMT) markers including EMT1, EMT2, and EMT3, (5) angiogenesis signature, (7) pan-fibroblast transforming growth factor (TGF)-β response signature, (8) Wnt targets, (9) DNA damage repair, (10) mismatch repair, (11) nucleotide excision repair, (12) DNA replication, and (13) antigen processing and presentation. Simultaneously, we obtained the Hallmark gene set from the MSigDB database and quantified the scores of 50 signal pathways using the ssGSEA algorithm to further reveal the differences in biological pathways related to different immunophenotypes.

2.6 Correlation with cancer-immunity cycle

The cancer-immunity cycle is an important framework for tumor immunotherapy research. It describes a cyclical process involving the immune system to eradicate cancer, which mainly includes seven steps: (1) cancer antigen release, (2) cancer antigen presentation, (3) initiation and activation, (4) T-cell transport to the tumor, (5) T cells penetration into the tumor, (6) T-cell recognition of cancer cells, and (7) T cell killing of cancer cells [29]. The genetic information from each step were obtained from Tracking Tumor ImmunePhenotype (http://biocc.hrbmu.edu.cn/TIP/index.jsp). We quantified the scores of the seven steps using the ssGSEA algorithm, and compared the differences in the three scores to the seven steps in immunophenotyping.
2.7 Analysis of mutations and copy number differences

The waterfall chart of the maftools software package was used to show the top 20 genes with high mutation frequency in the TCGA-BLCA cohort. For copy number analysis, GISTIC2.0 was used to identify significantly amplified or missing genomes [30]. The burden of copy number loss or gain was calculated as the total number of genes with copy number changes at the focal and arm levels.

2.8 The construction and evaluation of ICscore

By analyzing the level of 24 IC infiltration in each sample, we used the PCA method to construct a scoring system to evaluate the level of IC infiltration in a single patient with bladder cancer, and termed it ICscore. We tested the distribution differences of immune scores in different immune subtypes. We further explored and evaluated its predictive effects on the prognosis of patients with bladder cancer and their response to immunotherapy.

2.9 Correlation analysis with ICIS response

To explore the predictive effect of our immunophenotyping on anti-PD-L1 immunotherapy, we used the IMvigor 210 cohort. In this study, we classified all or a portion of the responders as responders, and classified patients with stable disease or progressive disease as non-responders. We used the TIDE algorithm to evaluate the potential response to immune checkpoint blockade (ICB) treatment. The TIDE algorithm is a calculation method that uses gene expression profiles to predict the ICB response. It evaluates two different tumor-immune escape mechanisms, including the dysfunction of tumor-infiltrating cytotoxic T lymphocytes (CTLs) and the rejection of CTLs by immunosuppressive factors. The TIDE score can be a good assessment of the efficacy of anti-PD-1 and anti-CTLA4 treatments [31]. Patients with a higher TIDE score have a higher chance of anti-tumor-immune escape and thus show a lower ICB treatment response rate. SubMAP [32] is used to compare the similarity of expression profiles and reflects the response to treatment. We used the SubMAP algorithm to predict the possibility of high and low ICscore of anti-PD1 and anti-CTLA4 response immunotherapy. The related annotation data were obtained from the research supplementary materials of Lu et al. [33].

2.10 Statistical analysis

The comparison between the two groups of normally distributed variables was performed using unpaired t- and Mann-Whitney U tests (also called Wilcoxon rank sum test) for non-normally distributed variables. The Kruskal-Wallis test and one-way analysis of variance were used as non-parametric and parametric analytical methods for comparison between the two groups, respectively. The differences between the groups of categorical variables were evaluated using the chi-squared test. The correlation coefficient between the two variables was calculated using Spearman and distance correlation analyses. The survminer R package was used to determine the demarcation point of each data set of each subgroup. The surv-cutpoint function was used to dichotomize the ICscore, and then the patients were divided into high and low subgroups according to the largest selected log-rank statistic to reduce the calculated batch effect. The Kaplan-Meier method was used to draw the survival curve of prognosis analysis, and the log-
rank test was used to determine the significance of the difference. Univariate Cox regression model was used to calculate the hazard ratio (HR) of the ICscore, and a multivariate Cox regression model was used to determine the independent prognosis factor. Statistical analysis was performed on R software, with a P value < 0.05 (two-tailed) indicating significant significance.

3. Results

3.1 Identification of three different immune subtypes in bladder cancer

We included 24 IC subtypes that are divided into three categories: adaptive and activated innate immune cells, activated innate immune cells, and other stromal cells. We drew the correlation network diagram of 24 ICs in bladder cancer (Fig. 1A), and observed IC interaction and its significance in the prognosis of patients with bladder cancer. We found that most cells had a significantly positive correlation with the level of infiltration, while a few had a negative correlation, including activated memory CD4 T cells and resting T cells, memory CD4 T cells, resting mast and endothelial cells, and activated mast cells and resting mast cells. To select the optimal number of clusters, we analyzed 407 sample data from TCGA-BLCA to evaluate the stability of the clusters using the ConsensusClusterPlus package, and determined the optimal number of clusters to be 3 (Fig. 1B-C). Then, we performed unsupervised clustering of the aforementioned bladder cancer samples and divided them into three clusters: A, B, and C. We found that most ICs were highly infiltrated in cluster C with the lowest infiltration in cluster A (Fig. 1D, Supplementary Fig. 1). The clustering results are in line with the immunological principles reported in a previous article: clusters A and B were similar to cold tumors, but they had different microenvironment composition phenotypes, and cluster C was a similar hot tumor. Eight meta-cohorts (GSE57303, GSE34942, GSE84437, ACRG/GSE62254, GSE15459, GSE29272, and TCGA-stomach adenocarcinoma) were used to verify repeatability (Supplementary Fig. 2A-B), and we obtained almost the same clustering results.

3.2 Correlation analysis of immunophenotyping with clinical features and common molecular typing of bladder cancer

Based on the OS data from TCGA, we performed survival analysis on the TME phenotype and found that cluster B had the worst prognosis (log-rank test, \(P = 0.0076; \) Fig. 2A) while clusters A and C showed better survival, and the relapse-free survival (RFS) results were similar to the OS results (log-rank test, \(P = 0.027; \) Fig. 2B).

Then we compared the clinical characteristics of different immunotypes and displayed them through heat maps, and found that there was little difference in gender and age between the three groups (\(\chi^2\) test, \(p > 0.05\)), but there are significant differences in race, smoking time, TNM and tumor grade status (\(\chi^2\) test, \(p < 0.05\)) (Fig. 2C). We compared and analyzed the immunophenotyping of several published subtypes of common bladder cancers. Compared with the Baylor subtype, cluster A had lesser basal subtypes with the differentiated subtype being predominant, while cluster C was the opposite. Compared with the UNC
subtype, luminal subtype was predominant in cluster A, while basal subtype was predominant in cluster C. Compared with the CIT subtype, MC1 subtype was predominant in cluster A, while MC7 subtype was predominant in cluster C. Compared with the Lund subtype, urothelial-like A (UroA-Prog) was predominant in cluster A, while basal/squamous (Ba/Sq)-Inf, and Mes-like were the majority in cluster C. Compared with the MDA subtype, luminal subtype was predominant in cluster A, while the basal subtype was predominant in cluster C. Compared with the TCGA subtype, luminal papillary accounted for the vast majority in cluster A, and Ba/Sq was predominant in cluster C ($\chi^2$ test, all $P$ values < 2.2e-16; Fig. 2D).

3.3 Analysis of the differences among the three immunophenotyping signal pathways

We first compared the differences in the scores of important biological gene sets of bladder cancer among the three immunotypes. We found that CD8 T effector, immune checkpoint, EMT, and antigen processing machinery were significantly higher in cluster A and lowest in cluster C, while nucleotide excision repair, DNA damage response, DNA replication, and base excision repair were the lowest in cluster B (Fig. 3A, Supplementary Table 5). In addition, we used the ESTIMATE algorithm to obtain the ImmuneScore and StromalScore, and compared the differences in immune and matrix components among the different immunotype TMEs. We found that ImmuneScore and StromalScore were the highest in cluster C, followed by cluster B, with cluster A having the lowest ImmuneScore and StromalScore (Fig. 3B, C). We obtained 50 common biological pathway gene sets (Hallmark gene set) from the MSigDB database, and quantified the score of each signal pathway using the ssGSEA algorithm to further reveal the differences in biological pathways related to various immunophenotyping. We found that there were significant differences in the scores of most biological pathways among the three different immunotypes (Supplementary Fig. 3A).

To further explore the differences in biological behavior among the three different immunophenotypes, we first performed GSVA enrichment analysis. Cluster A was significantly enriched in immunosuppressive signaling pathways such as extracellular structure organization, inflammatory response, collagen trimer, response to interferon $\gamma$, extracellular matrix, regulation of leukocyte migration, multicellular organismal macromolecule metabolic process, granulocyte migration, positive regulation of leukocyte migration, and proteinaceous extracellular matrix. Cluster B was enriched in mRNA metabolic process, ribonucleoprotein complex subunit organization, ribonucleoprotein complex biogenesis, RNA splicing via transesterification reactions, translational initiation, RNA splicing, mRNA processing, spliceosomal complex, fatty acid beta oxidation, and microbody part. However, cluster C was significantly related to biological processes related to immune activation, such as adaptive immune response, positive regulation of cell activation, innate immune response, positive regulation of immune response, regulation of cell activation, cytokine-mediated signaling pathway, cell chemotaxis, and chemokine-mediated signaling pathway (Fig. 3D). We used the limma package to determine DEGs related to immunophenotyping (Fig. 3E, Supplementary Table 6) and performed KEGG signaling pathway enrichment analysis (Fig. 3F, Supplementary Table 7). We found that cluster A were mainly enriched in Citrate cycle (TCA cycle), Glutathione metabolism, Natural killer cell mediated cytotoxicity, Cytokine – cytokine receptor interaction, and Th1 and Th2 cell
differentiation compared with the other two groups. However, cluster C were mainly related to B cell receptor signaling pathway, PD − L1 expression and PD − 1 checkpoint pathway in cancer, Fatty acid biosynthesis, Butanoate metabolism. Cluster C were mainly related to Spliceosome, Toll − like receptor signaling pathway, Primary immunodeficiency, and IL-17 signaling pathway compared with the other two groups. Finally, we verified the differences in pathway enrichment among the three immunotypes using GSEA. We found that most of the immune signaling pathways were activated in clusters B and C, while immune activation-related biological pathways in cluster A were in an inhibited state (Fig. 3G).

3.4 The differences in expression of immune cycle activation, tumor immunogenicity, and immune checkpoint molecules in the three immunotypes

An anti-tumor-immune response must initiate a series of step-by-step events to effectively kill cancer cells. These steps are crucial to the cancer-immunity cycle. The dead tumor cells release antigens (step 1). The antigen and the major histocompatibility complex (MHC) complex on the surface of the antigen-presenting cells (APC) such as dendritic cells (a type of professional APC) form an antigen peptide-MHC complex (step 2). The T-cell receptor recognizes the binding between the antigen peptide-MHC complex on the surface of APC, the B7 molecule on the surface of APC and the dimer molecule CD28 on the surface of T cells, and the dual signal starts to activate T cells (the dual signal system regulation can review the immune response and tumor immunotherapy) (Step 3). Among them, CTLs are transported to the tumor tissue through blood circulation (step 4). CTLs enter the tumor tissue (step 5) and recognize tumor cells; (step 6) CTLs kill tumor cells(step 7), and release additional tumor-associated antigens (step 1 again). We obtained the important regulatory genes of each step and calculated the score of each step using the ssGSEA algorithm. We then explored the differences in the scores of the seven steps among the three immunotypes, and the results showed that the seven steps in cluster C had the highest score, followed by cluster B with cluster A having the lowest score (all $P<0.05$; Fig. 4A). We further studied the potential intrinsic immune escape mechanism of bladder cancer. Innate immune escape indicates that tumor cells directly mediate their own immune escape. The inherent immune escape has at least two aspects: the immunogenicity of the tumor and the expression of immune checkpoint molecules. Both subtypes had lower expression of MHC I-related antigen-presenting molecules than cluster C (all $P<0.001$; Supplementary Fig. 3A, top), and their immunogenicity was low. Overall, the tumor immunogenicity of cluster C was relatively high, while that of clusters A and B was relatively low. We demonstrated that cluster C had higher expression of costimulatory molecules (most $P<0.05$) and immune checkpoint molecules compared with other clusters (most $P<0.05$; Supplementary Fig. 3A, bottom). We further demonstrated that immune infiltration is positively correlated with immunogenicity and the expression of most checkpoint molecules. We obtained the gene set “c5.all.v6.2.symbols.gmt” and 50 common biological pathway gene sets for GSVA analysis from the MSigDB database. We used the ssGSEA method to generate enrichment scores of these gene sets for each pathway in each sample, and compared the three immunotypes using the ssGSEA score of the pathways (Supplementary Fig. 3B).
3.5 The characteristics of the tumor somatic mutation and immune subtype copy number

We explored the underlying mechanisms leading to the formation of these microenvironmental phenotypes, aiming to identify potential targets to reverse the formation of low immune infiltration. We used the maftools package to analyze the distribution of somatic mutations and the difference in tumor mutational burden (TMB) among the three subtypes (Supplementary Fig. 3C). Clinical trials and preclinical studies have showed that when ICB therapy is used, higher somatic TMB is associated with enhanced response, long-term survival, and lasting clinical benefits in patients. A single altered gene can mediate resistance or sensitivity to immunotherapy. In addition, we also analyzed the copy number characteristics of the different immune subtypes (Supplementary Fig. 3D) and found three different immunotype composite copy number profiles, including GISTIC score and percentage/frequency (Fig. 4B). Our analysis shows that there are certain genomic changes in different immune subtypes, which may be the cause of the difference in immunotyping.

3.6 ICscore is a reliable and independent prognostic biomarker for evaluating bladder cancer patients

Considering the individual heterogeneity and complexity of the tumor-immune microenvironment, we constructed a scoring system to quantify the level of immune cell infiltration based on these immune cells in a single bladder cancer patient using the ICscore. We first analyzed the differences in ICscores among the three immunotypes, and found that the ICscore of cluster C was the highest, followed by cluster B, with cluster A having the lowest ICscore (Fig. 5A). This result is in line with our previous trend of results. We further determined the value of ICscore in predicting patient prognosis. Patients were divided into low or high ICscore groups according to the survminer software package. The Kaplan-Meier curve showed that ICB treatment may be more beneficial in patients with higher ICscores, regardless of OS (Fig. 5B; HR = 0.65, 95 % confidence interval (CI): 0.55–0.77) or RFS (Fig. 5C; HR = 0.8, 95 % CI: 0.65–0.99). Simultaneously, we tested whether the ICscore could be used as an independent prognostic factor for bladder cancer. We used univariate and multivariate Cox regression models to analyze factors including patient age, sex, tumor, node, metastasis, smoking, and stage statuses, and confirmed that the ICscore is a reliable and independent prognostic biomarker for assessing patient prognosis (Fig. 5D,E). We further explored the correlation between the ICscore and scores of important biological pathways, and found that the ICscore was negatively correlated with DNA replication, mismatch repair, homologous recombination, base excision repair, cell cycle, and nucleotide excision repair (Fig. 5F).

3.7 ICscore is a reliable prognostic biomarker and predictor of immune checkpoint inhibitor response

Immunotherapy using anti-PD-1/PD-L1 and anti-CTLA4 has become a breakthrough in cancer treatment. We systematically studied whether our immune classification and ICscore can predict the patient response to ICB therapy.
First, we verified the three immunotypes in bladder cancer (Supplementary Fig. 4A-B) based on the IMvigor 210 cohort. Simultaneously, we confirmed that there were fewest patients with complete response (CR) in cluster A, and The levels of PD-L1 expression on ICs and tumor cells were significantly lower than those of the other two groups (Fig. 6A, Supplementary Fig. 4C-F). We used a composite heat map to show the differences in TMB and important mutation driver genes of bladder cancer among the three immunotypes (Fig. 6A). We found that there was a significant increase in mutations in the fibroblast growth factor receptor 3 (FGFR3) gene in cluster A. In addition, gene sets were significantly upregulated in cluster A, such as FGFR3 gene signature, MKI67, cell cycle genes, DNA replication-dependent histones, and DNA damage repair genes, while genes such as CD8 T-effector signature, antigen-processing machinery, immune checkpoint signature were inhibited in Cluster A (Fig. 6A, Supplementary Fig. 4G), which have been demonstrated to have an important effect on immunotherapy.

Based on the OS data from IMvigor 210 cohort, we performed survival analysis on the TME phenotype and found that cluster C showed better survival(Supplementary Fig. 5A),The predictive effect of ICscore on anti-PD-L1 immunotherapy response was also systematically evaluated. In patients treated with anti-PD-L1, the Kaplan-Meier curve showed that a high ICscore had better prognosis for anti-PD-L1 therapy (HR = 0.85, 95% CI: 0.73–0.98; Fig. 6B), and the ICscore of the CR/partial response (PR) group was higher than that of the stable disease/progressive disease group (Wilcoxon test, p = 0.0029; Fig. 6C). We found that the ICscore was positively correlated with PD-L1 expression on ICs and tumor cells. It was confirmed that high ICscores are closely related to immune checkpoint expression (Supplementary Fig. 5B, C).

Comparison of the differences in the ICscore in immune subtypes revealed that ICscore was the lowest in desert tumors, moderate in excluded tumors, and highest in inflamed tumors (Supplementary Fig. 5D). These results suggest that our immunophenotyping method and ICscore had a prominent predictive effect on anti-PD-L1 therapy.

The TIDE score integrates T-cell dysfunction and removal characteristics, and simulates tumor-immune escape at the level of tumor-infiltrating CTLs. Compared with other biomarkers, TIDE has an advantage in predicting the efficacy of anti-PD1 and anti-CTLA4 treatments. We further explored the correlation between the IC and TIDE scores. The ICscore was negatively correlated with TIDE (r = -0.35, P = 2.9e-13; Fig. 6D). This again suggests that patients with high ICscores may show a better response to immunotherapy, and the combination of IC and TIDE scores can improve the prediction of patient prognosis (Fig. 6E). We found that ICscore is negatively correlated with IFNG and CD274 (r = -0.69, P = 4.6e-58; r = -0.70, P = 6.6e-62; Supplementary Fig. 5E, F). Using the receiver operating characteristic (ROC) algorithm, we found that the ICscore(AUC 0.716, 95% CI: 0.647–0.787) can predict the responsiveness of anti-PD-L1 therapy well (area under the curve = 0.716), and compared with TMB(AUC 0. 0.723, 95% CI: 0.643–0.793),tumor neoantigen burden(TNB, AUC 0.763, 95% CI: 0.688–0.829), CD274 (AUC 0.723, 95% CI: 0.643–0.793) and IFNG (AUC 0.661, 95% CI: 0.574–0.735),the ICscore can equally predict the responsiveness of immunotherapy (P > 0.05; Supplementary Fig. 5G,H). We used the SubMAP algorithm to predict the possibility of response to anti-PD1 and anti-CTLA4 immunotherapy in the high and low ICscore groups. We also confirmed that the group with a high ICscore may respond better to treatment (Bonferroni-corrected P = 0.01; Fig. 6F).
4. Discussion

We analyzed 1,889 bladder cancer samples using a series of unsupervised learning methods, explored the existence of three different microenvironmental phenotypes in bladder cancer, and verified its reproducibility. Our clustering results are in line with the immunological principles described in previous studies [34, 35]. We found that cluster C had hot tumors with relatively high innate and adaptive IC infiltration while cluster A had immune desert type tumors (cold tumors), which was characterized by relatively low microenvironmental cell infiltration. In addition, our data clearly show that there were large differences in clinical characteristics among the different immune subtypes. Compared with other clusters, cluster B had poor prognosis in both OS and RFS, but there was not much of a difference in prognosis between clusters C and A. The difference in survival was consistent with previous studies.

In many solid tumors, although CTLs infiltrate the tumors to a greater extent, T-cell dysfunction is also strong, which may weaken the ability of CTLs to kill cancer cells and may promote the growth and progression of tumors, resulting in invasion and metastasis [12, 31, 36]. We found that the poor prognosis of cluster C may be due to higher immunosuppression and lower immunoreactivity in the TME. Though cluster C had the highest IC infiltration, it exhibited a matrix-activated state with highly expressed EMT, TGF-β pathway, and angiogenesis, which are T-cell inhibitory [17, 37, 38]. In addition, our results confirmed that there was a significantly high expression of the immune checkpoint molecules in cluster C. These results emphasize that though cluster C had more immune cells infiltrated in the tumors, its IC dysfunction and immune escape were also stronger, which may weaken the ability of ICs to kill cancer cells. This result is consistent with previous research reports, and is due to higher immunosuppression and lower immunoreactivity in the TME. Such patients are often more suitable for immune checkpoint inhibitor treatment [39, 40]. Our research may also help promote research on the regulation mechanism of immune infiltration in bladder cancer. The transition from cold to hot tumors is currently being researched [41], and specific biological pathways may drive the formation of these microenvironmental phenotypes.

In this study, differences in genes among different subtypes were shown to be significantly related to immune-related biological pathways. The differential genes obtained by comparing cluster A with the other two groups revealed that the characteristic genes related to cluster A were mainly enriched in signaling pathways such as cytokine-cytokine receptor interaction, Wnt signaling pathway, and Toll-like receptor signaling pathway. In addition, our GSEA and GSVA analyses revealed that many biological pathways related to immune activation of cluster A were significantly inhibited, while the signal pathways such as inflammatory response, response to interferon γ, and extracellular matrix were significantly activated. We confirmed that one of the most prominent biological characteristics was the significant change in FGFR3, including mutations and overexpression, and a significant increase in FGFR3 gene mutations in cluster A. Previous studies have confirmed that FGFR oncogenic mutations occur in a fifth of bladder cancers [42, 43]. The protein encoded by FGFR3 is a member of the FGFR family and is related to Ras protein kinase/mitogen-activated protein kinase activation, angiogenesis inhibition, fibroblast activation, and EMT [44]. We speculate that the low chemotaxis of innate ICs induced by FGFRs may be the cause of the poor immune permeability in cluster A. FGFR3 is a promising therapeutic target in
multiple preclinical trials, but further studies are needed to verify the benefits of FGFR3 inhibitors in these subgroups. In addition, we have shown the copy number and mutation differences in different immune subtypes, providing evidence for exploring the reasons for the formation of immune subtypes at the genomic level. These results further reverse the poor infiltration characteristics of cells in the TME, and provide new ideas and targets for transforming cold tumors into hot tumors, which will help the development of new combinations of drugs or new immunotherapy drugs.

Our research may also guide more effective patient-specific immunotherapy strategies such as PD-1/PD-L1 therapy [15, 16]. However, its efficacy is high only in a small number of patients. Several studies have found the TMB are not effective biomarkers for predicting the benefits of ICB [36]. Therefore, the establishment of predictive biomarkers for checkpoint immunotherapy is essential to maximize the benefits of treatment [5, 45, 46].

Considering the heterogeneity and complexity of the individual TME, we established a scoring system to evaluate the level of IC infiltration in the TME. Univariate and multivariate Cox regression analyses showed that ICscore is a reliable and independent prognostic biomarker. We demonstrated the predictive value of the ICscore for the use of anti-PD-L1 drugs (atezolizumab) using the IMvigor 210 cohort. There was a significant difference in ICscore between non-responders and responders.

The TIDE score integrates T-cell dysfunction and removal characteristics, and simulates tumor-immune escape with different levels of tumor-infiltrating CTLs. Compared with other biomarkers, its advantages are very prominent [31, 47]. A higher tumor TIDE score is related to a poorer efficacy of immune checkpoint suppression therapy. Our research confirmed that ICscore is negatively correlated with TIDE, which is consistent with our previous inference. In addition, the ICscore is negatively correlated with interferon γ and CD274, and these factors are T-cell inhibitory [36, 45]. We could predict the possibility of anti-PD1 and anti-CTLA4 immunotherapy response in high and low ICscore groups using the SubMAP algorithm. This also confirmed that the high ICscore group may respond better to treatment.

Although important results were obtained in this study, there were some limitations. First, although our study included a large sample size of the bladder cancer cohort, sampling deviations caused by using different platforms may cause some subjectivity in gene expression values. Second, our research provides new insights into the stromal microenvironment of bladder cancer and related treatment strategies. However, this study is limited by being prospective. Therefore, our findings should be further confirmed in clinical studies. In addition, immunogenomic analysis cannot reflect causality. The potential driving factors in our research, such as the FGFR3 pathway require further functional verification. This part of our study is currently undergoing experimental research and verification.

5. Conclusion

In conclusion, this study defined three heterogeneous bladder cancer microenvironmental phenotypes and illustrated their clinical significance.
We conducted a comprehensive analysis of the characteristics and differences of the three immunophenotypes. This will help elucidate the response of bladder tumors to immunotherapy and provide new strategies for cancer treatment. In addition, we identified a TME-based score that can effectively predict the survival outcome of bladder cancer patients. The TME score is a potential and powerful biomarker for the prognosis and clinical response evaluation of immunotherapy. Our findings provide novel ideas for improving the clinical response of bladder cancer patients to immunotherapy, identifying different tumor immunophenotypes, and promoting personalized cancer immunotherapy in the future.

List Of Abbreviations

TCGA
The Cancer Genome Atlas
MIBC
Muscle-Invasive Bladder Cancer
TIDE
Tumor Immune Dysfunction And Exclusion
SubMAP
Subnetwork Mappings In Alignment Of Pathways
ICs
Immune Cells
ROC
Receiver Operating Characteristic
GEO
Gene-Expression Omnibus
KEGG
Kyoto Encyclopedia Of Gene And Genome
GO
Gene Ontology
GSEA
Gene Set Enrichment Analysis
SsGSEA
The single-sample gene set enrichment analysis
TME
Tumor Microenvironment
TMB
Tumor Mutation Burden
ICT
The Immune Checkpoint Therapy
ICB
Immune Checkpoint Blockade

CTLA-4
Cytotoxic Lymphocyte Antigen-4

CTLs
Cytotoxic T Lymphocytes

PD-1
Programmed Cell Death Protein 1

PD-L1
Programmed-Death Ligand 1

DCs
Dendritic Cells

DEGs
Differentially Expressed Genes

EMT
Epithelial-Mesenchymal Transition

ICB
Immunological Checkpoint Blockade

TGFb
Transforming Growth Factor Beta

CR
Complete Response

PR
Partial Response

Declarations

Ethical Approval and Consent to participate

Not applicable.

Consent for publication

All authors have agreed on the contents of the manuscript.

Availability of supporting data

All data used in this work can be acquired from the GDC portal (https://portal.gdc.cancer.gov/) and the IMvigor210CoreBiologies http://research-pub.gene.com/IMvigor210CoreBiologies}
Competing interests

The authors have declared that no conflict of interest exists.

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Authors' contributions

XYC and KC designed the study, XYC, HTC, KC and DH analyzed and interpreted the data, XYC,YXZ, and YXC wrote this manuscript. MQX,HL and ZWW edited and revised the manuscript. All authors have seen and approved the final version of the manuscript.

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