Three Prognostic Biomarkers Correlate With Immunotherapy Response in Bladder Urothelial Carcinoma

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Abstract

**Background:** There are currently no satisfactory biomarkers to predict prognosis and evaluate the benefit of immunotherapy in bladder urothelial carcinoma (BLCA) patients. This study aimed to develop a predictive signature that could accurately predict prognosis and evaluate the response to immunotherapy in BLCA.

**Methods:** Differentially expressed genes (DEGs) were identified using the GEPIA and Oncomine databases, and the common genes between the two database were selected using a Venn diagram. In addition, gene ontology enrichment and protein–protein interaction (PPI) network analyses were performed. We further identified the prognosis-related hub genes using the survival R package and confirmed in three online databases (PROGgenesV2, PrognoScan, and OSblca). Moreover, the correlation between prognosis-related hub genes and clinical characteristics was analyzed. Finally, comprehensive bioinformatics analysis was carried out to investigate the association between the three genes and immunity.

**Results:** A total of 750 and 1881 DEGs were identified from GEPIA and Oncomine, respectively, and 69 common DEGs were selected. The most significantly enriched term among the 69 common DEGs was “mitotic cell cycle”, and 11 hub genes were detected by PPI analysis. Moreover, three prognosis-related hub genes, AURKA, BIRC5, and CKS1B, were identified, and which were associated with clinical characteristics, in particular, histological subtypes and TP53 mutation status. Furthermore, our results showed that the expression levels of the three genes were positively correlated with CD8+ T cells and tumor mutation burden (TMB), and with PD-L1, which had higher expression in responders to immunotherapy and in the C2 (IFN-gamma dominant) subtype. Drug–gene interaction network analysis demonstrated that these genes and related drugs could be used to help develop new targets for BLCA immunotherapy.

**Conclusions:** Our study suggested that three key genes in BLCA were correlated with poor prognosis and immune cell infiltration, especially that of CD8+ T cells. The responses of these prognosis-related genes to immunotherapy in BLCA may be associated with CD8+ T cells, TMB, and PD-L1 expression. These key genes and their related drugs may help to develop new targets for BLCA immunotherapy.

Background

Bladder cancer is the ninth most common malignant tumor worldwide (1), and bladder urothelial carcinoma (BLCA) is its most frequent pathological type. Despite the development of diagnostic and treatment techniques, the 5-year survival rate of patients with BLCA varies from 5% to 70%, and their prognosis remains unfavorable (2). Detection of biomarkers can indicate a particular disease state and can be used in screening for differential prognosis, evaluation of treatment response, and monitoring of disease progression (3). Increasing numbers of prognosis-related biomarkers in BLCA have been identified using publicly available bioinformatics databases and tools. Unfortunately, there are still no
reliable and repeatable biomarkers to assess the prognosis of BLCA patients (4–7). Therefore, there is an urgent need to develop reliable molecular markers for predicting prognosis in BLCA.

In recent years, immunotherapy has attracted considerable attention for its influence on the treatment of locally advanced and metastatic BLCA (8). However, only a proportion of patients respond to treatment with immune-checkpoint inhibitors (9, 10). Therefore, identification of biomarkers to effectively predict prognosis and evaluate the benefit of immunotherapy is of great significance. Previous studies have reported that highly infiltrating lymphocytes are related to prognosis of BLCA (11). The evidence indicates that CD8+ T cells are involved in tumor adaptive immunity, and their infiltration is associated with prognosis (12, 13). Programmed death ligand 1 (PD-L1), also known as B7-H1 or CD274, is involved in inhibiting T cell-mediated antitumor immunity through interaction with PD-1 (14–15). Previous studies have reported that high expression of PD-L1 is associated with worse cancer outcomes in various malignancies (16). CD8+ T cell infiltration and tumor mutation burden (TMB) have been reported to be correlated with response to atezolizumab (anti-PD-L1) in metastatic urothelial cancer (mUC) (17–19). Therefore, identification of biomarkers related to CD8+ T cell infiltration, PD-L1 expression, and TMB will help to predict prognosis and immunotherapy response in patients with BLCA.

In this study, we first identified DEGs from the GEPIA (Gene Expression Profiling Interactive Analysis) and Oncomine databases, and then identified the overlapping DEGs between them using a Venn diagram. We further performed gene enrichment and protein–protein interaction (PPI) analyses to select hub genes. Moreover, prognosis-related hub genes were identified using the survival R package and confirmed in three online databases. We then explored the expression of the key prognosis-related genes with different clinical factors using the UALCAN database. Finally, we evaluated whether three key genes related to CD8+ T cell infiltration, TMB, and PD-L1 expression could be used to predict prognosis and monitor immunotherapy response in BLCA based on comprehensive bioinformatics analysis.

Methods

Identification of DEGs

GEPIA (http://gepia.cancer-pku.cn/) is an online network tool based on data from The Cancer Genome Atlas (TCGA) and GTEx, which can be used to study interactions between DEGs, as well as for survival analysis, profile plotting, and detection of similar genes (20). Oncomine is an online resource containing microarray data (https://www.Oncomine.org) (21). In this study, we first used the GEPIA and Oncomine databases to identify DEGs by comparison of tumor samples with normal samples. In the GEPIA database, mRNAs with q<0.01 and |log2 fold change (FC)|≥2 were chosen as DEGs. In the Oncomine database, the selection criteria for DEGs are P<0.01, |log2FC|≥2, and gene rank ≤10%. Then, we identified the overlapping DEGs between them using a Venn diagram (http://bioinformatics.psb.ugent.be/webtools/Venn/) (22).

Functional Analysis and Pathway Enrichment Analysis
Metascape (http://metascape.org/) is an online resource for gene annotation and analysis (23). In the present study, Metascape was used to perform gene ontology (GO) and pathway analyses of 69 common hub genes. Pathway and process enrichment analyses were conducted based on several sources, including GO biological processes, The Kyoto Encyclopedia of Genes and Genomes pathways, reactome gene sets, and CORUM. Terms with a P-value less than 0.01, a minimum count of 3, and an enrichment factor greater than 1.5 were considered to represent significant processes or pathways.

Construction of PPI Network and Identification of Hub Genes

We evaluated PPI information of common genes using the STRING online database (https://string-db.org/cgi/input.pl) and then visualized the resulting interaction network using Cytoscape software (http://www.cytoscape.org/) (24, 25). A confidence score greater than 0.4 was defined as significant. The Molecular Complex Detection (MCODE) plugin in Cytoscape was used to further screen key genes in the PPI network with degree cutoff = 5, K-score = 2, and node score cutoff = 0.2.

Prognostic Value of Hub Genes

We downloaded gene expression profile and clinical data from TCGA (https://portal.gdc.cancer.gov/). The prognostic value of 11 identified hub DEGs that were analyzed using the R survival package (26). PROGgenesV2 (http://genomics.jefferson.edu/proggene/filter.php) is a web resource that allows researchers to study the correlations between genes and overall survival (OS) in multiple cancers based on TCGA and GEO data (27). PrognoScan (http://www.prognoscan.org/) was used to evaluate the associations between gene expression and patient prognosis, according to measures including OS and disease-free survival (DFS), across a large collection of publicly available cancer microarray datasets (28). The OSblca database (http://bioinfo.henu.edu.cn/BLCA/BLCAList.jsp) provides a useful tool to assess novel prognostic biomarkers in bladder cancer, based on data from 1,075 bladder cancer patients, including OS, disease-specific survival (DSS), disease-free interval, and progression-free interval (29). In this study, we further confirmed the prognostic value of key genes based on the abovementioned three databases. The hub genes identified in this way were considered to be key prognosis-related genes.

Association between Prognosis-related Key Genes and Clinical Characteristics

The UALCAN database (http://ualcan.path.uab.edu/) is a comprehensive web resource for analyzing cancer OMICS data (TCGA and MET500) (30, 31). A previous study reported that the expression and prognostic value of DEGs were associated with clinical characteristics, including TNM stage, smoking history, lymph invasion, and histological type (32). Therefore, we explored the relationship between the three key prognosis-related genes and clinical characteristics using UALCAN.

Immune Cell Infiltration and TMB analysis

TIMER is a user-friendly web portal for the systematic analysis of immune infiltrates across different types of cancer (https://cistrome.shinyapps.io/timer/) (33). In this study, we used TIMER to analyze the associations between the three key genes and immune cell infiltration. P<0.05 was considered to be
statistically significant. In addition, we downloaded 33 cancer expression profiles with associated mutation data from TCGA, and assessed the association between the prognosis-related hub genes and TMB (26, 34).

**TISIDB Analysis**

TISIDB ([http://cis.hku.hk/TISIDB](http://cis.hku.hk/TISIDB)) is a publicly available resource that allows the user to explore the function of a gene and its role in tumor-immune features (35). TISIDB consists of 10 modules: function, literature, screening, immunotherapy, lymphocyte, immunomodulator, chemokine, subtype, clinical, and drug. In this study, the lymphocyte module was used to evaluate the relationships between the expression of selected genes and tumor-infiltrating lymphocytes. The immunomodulator module was used to examine the associations between PDL1 and selected genes. The subtype module was used to assess the distribution of selected genes’ expression across immune and molecular subtypes. We used the screening module to explore whether the expression of prognosis-related genes showed significant differences between responders and non-responders to immunotherapy. Using the drug module, we analyzed the drugs in the DrugBank database targeting these three genes.

**Results**

**Identification of Hub Genes**

A total of 750 DEGs were identified from the GEPIA database, and 1,881 DEGs were identified from Oncomine. Sixty-nine common genes were screened out using the Venn diagram (Fig. 1 A). We then performed GO and pathway enrichment analyses for the common genes using Metascape. The results showed that these common genes were involved in 20 main GO terms and pathways, of which the top five were mitotic cell cycle, cGMP-PKG signaling pathway, extracellular matrix organization, muscle contraction, and response to hydrogen peroxide (Fig. 1 B). Finally, a PPI network of DEGs was constructed using the STRING and Cytoscape software, containing 69 nodes and 83 edges (Fig. 1 C). One significant module was identified using MCODE. This module contained 11 genes, Aurora-A kinase (AURKA), BIRC5, CENPA, CKS1B, ECT2, MYBL2, NUF2, RRM2, TK1, TPX2, and UBE2C, which were considered to be hub genes (Fig. 1 D).

**Prognosis-related Hub Genes**

The association between the expression of 11 hub genes and overall survival was evaluated using the R survival package. High expression of three genes (AURKA, BIRC5, CKS1B) was related to an unfavorable prognosis (Fig. 2 A–K). We further validated the prognostic value of the three genes in BLCA using the PROGgenesV2, PrognoScan, and OSblca databases. Our results showed that BLCA patients with higher expression levels of the three hub genes exhibited poorer OS, DFS, and DSS, indicating that the three hub genes may be associated with unfavorable prognosis (Table 1). In summary, our data suggested that the three key genes could serve as biomarkers of poor prognosis.
Association between Three Key Genes and Clinical Parameters in BLCA

A previous study reported that the OS of BLCA patients was significantly associated with clinical characteristics, including TNM stage, smoking history, lymph invasion, and histological type (29). The three hub genes identified here were associated with various clinical characteristics including age, histological subtype, molecular subtype, nodal metastasis status, sample type, smoking, cancer stage, and TP53 mutation status (Table 2). Most importantly, overexpression of the three hub genes was positively correlated with histological subtypes (Fig. 3 A–C). The expression of the three hub genes was higher in the basal squamous and neuronal subtypes than in the luminal subtype (Fig. 3 D–F). BLCA patients with TP53 mutations also showed high expression of the three hub genes (Fig. 3 G–I). Taken together, these results suggest that increased expression of the three key genes might predict poor prognosis in patients with BLCA.

CD8+ T Cell Infiltration Predicts Poor Prognosis in BLCA

TheTIMER and TISIDB databases were used to explore the relationship between the three prognosis-related genes and tumor-infiltrating immune cells. The three genes were positively associated with levels of infiltrating CD8+ T cells, neutrophils, and dendritic cells. Expression of BIRC5 was negatively correlated with infiltration of B cells (Table 3, Fig. 4 A–F). High levels of infiltration of CD8+ T cells were associated with poor prognosis (Fig. 4 G). These results suggest that these genes may affect prognosis via regulation of CD8+ T cells.

Three Key Genes Correlated with Immunotherapy Response

Previous studies have suggested that TMB and PD-L1 expression are correlated with response to atezolizumab in mUC (17). Our results indicated that the three key genes were associated with TMB in multiple cancers, including adrenocortical carcinoma, BLCA, and breast invasive carcinoma (Fig. 5 A–C). We further discovered that the expression levels of the three hub genes were positively correlated with infiltration of PD-L1 (CD274) expression (Fig. 5 D–F). Finally, our results showed that the three hub genes exhibited a significant difference in expression between responders and non-responders to atezolizumab in urothelial cancer via TISIDB analysis (Table 4). Taken together, these results suggest that the impact of the three hub genes on response to immunotherapy in BLCA may be associated with TMB and PD-L1 expression. The three key genes may thus represent a promising immunotherapeutic target. Finally, we found that the three prognosis-related genes had the highest expression levels in the C2 (IFN-gamma dominant) subtype and the lowest in the C3 (inflammatory) subtype (Fig. 5 G–I).

Drug–Gene Interaction Network

Drugs targeting three key genes were collected from the DrugBank database. AURKA and 18 other targets were correlated with 15 drugs. BIRC5 and two targets were related to 15 drugs. CKS1B and another three targets interacted with three drugs (Fig. 6 A–C). Our results may contribute to the development of new targets for BLCA immunotherapy.
Discussion

BLCA is one of the most common urinary cancers in the world, with high recurrence and mortality rates that limit the efficacy of treatment (36). It is therefore essential to understand the molecular mechanism of BLCA. With the development of bioinformatics technology, increasing numbers of studies are using bioinformatics analysis to develop biomarkers and explore the molecular mechanism of BLCA (3, 7). However, there are still no reliable biomarkers associated with prognosis of BLCA patients. In recent years, immunotherapy has attracted considerable attention owing to its influence on the treatment of locally advanced and metastatic BLCA (8), but limited information is available about biomarkers related to immunotherapy response. Overall, there are no satisfactory signatures to effectively predict prognosis and evaluate the benefit of immunotherapy in BLCA patients.

AURKA is a member of the serine/threonine kinase family and has a role in regulation of the cell cycle (37). Accumulating evidence indicates that AURKA is overexpressed in various cancers, including breast cancer, head and neck cancer, esophagus cancer, hematological malignancies, colorectal cancer, stomach cancer, pancreatic cancer, and ovarian and prostate cancers (38-40). Pathological overexpression of AURKA is correlated with shorter survival of cancer patients. According to previous reports, high expression of Aurora-A in tumor cells is closely related to poor prognosis (37, 40, 41). BIRC5 is a member of the inhibitor of apoptosis gene family, which has dual roles in promoting cell proliferation and preventing apoptosis (42). Overexpression of BIRC5 has been reported in several malignancies, and higher BIRC5 expression was also found to be associated with decreased survival (43). CKS1B is an oncogene that has been reported to show increased expression in various tumors (44). In accordance with previous studies, our results demonstrated that high expression of the three hub genes was associated with poor prognosis (Fig. 2 L–V). Further analysis revealed that these three key genes were overexpressed in non-papillary tumors, the basal squamous subtype, and TP53 mutation patients (Fig. 3 A–I). Moreover, our results indicated that the three hub genes were highly correlated with TMB and PD-L1 expression (Fig. 5 A–F). Expression of the three unfavorable-prognosis-related genes was highest in the C2 (IFN-gamma dominant) subtype and lowest in the C3 (inflammatory) subtype (Fig. 5 G–I). Previous studies have reported that TP53 mutation is associated with poor prognosis (45). TMB has prognostic roles in various cancer types, including BLCA (46). A previous study reported that the C2 subtype had the highest levels of CD8+ T cells, as well as having less favorable outcomes, whereas the C3 (inflammatory) subtype had the best prognosis (47). Taken together, these results indicate that the expression of these three key genes may represent a marker of poor prognosis in BLCA, as well as in non-papillary tumors, the basal squamous subtype, TP53 mutation patients, tumor with high TMB, and the C2 subtype.

Many types of human tumors are affected by tumor-infiltrating immune cells (TIICs) (48). A considerable number of studies have indicated that TIICs affect the prognosis of patients and alter response to immunotherapy (49) (50). Higher infiltration levels of CD8+ T cells are associated with poor prognosis in gastric cancer (51). In line with previous studies, our results demonstrated that high infiltration of CD8+ T cells was associated with poor prognosis (Fig. 4 G). Moreover, we found that the three prognosis-related genes were positively related to CD8+ T cells (Fig. 4 A–F). These data suggest that CD8+ T cell infiltration
has prognostic value in BLCA, and that the three genes comprising our prognosis-related signature may affect prognosis via CD8+ T cell infiltration. Increased CD8+ T cell infiltration has been reported to be correlated with better immunotherapeutic effect (49). A previous study showed that biomarkers related to CD8+ T cell infiltration could facilitate the monitoring of immunotherapy response and the exploration of the immune infiltration mechanism in clear cell renal cell carcinoma (19). AURKA is overexpressed in cancer cells but not in normal tissues, making it a potential target for immunotherapy. AURKA-specific CD8+ T cells can selectively lyse leukemia cells (52). BIRC5 is correlated with T cell survival and proliferation, which can increase the accumulation and persistence of CD8(+) T cells following an encounter with Ag (53). A miR-197/CKS1B/STAT3-mediated network that promotes tumor PD-L1 expression and miR-197 replacement therapy may be a potential treatment for chemotherapy-resistant non-small-cell lung cancer (54). In this study, a positive association between the expression of three key genes and immune cells, particularly CD8+ T cells, was examined using the TIMER/TISIDB databases. Previous studies have reported that high expression of PD-L1 is associated with worse cancer outcomes (16). TMB has been investigated in various malignancies and found to be correlated with response to atezolizumab in mUC (17-19). We found that the expression levels of the three hub genes were positively correlated with PD-L1 expression and TMB (Fig. 5 A–F). Patients have previously been classified into immunotherapeutic responders and non-responders based on number and distribution of TIICs (55). We found that the three prognosis-related genes were overexpressed in responders to atezolizumab among patients with urothelial cancer (Table 4). Finally, our results demonstrated that AURKA and another 18 targets were correlated with 15 drugs. BIRC5 and two targets were related to 15 drugs. CKS1B and another three targets interacted with three drugs (Fig. 6 A–C). Collectively, these data suggest that the response of the three hub genes to PD-L1 in BLCA may be associated with CD8+ T cells, TMB, and PD-L1 expression. The three key genes may represent a promising immunotherapeutic target. Drug–gene interaction network analysis indicated that these genes and their related drugs could be used in the development of new targets for BLCA immunotherapy.

Conclusions

In summary, three key genes in BLCA were found to be correlated with poor prognosis and immune cell infiltration, especially that of CD8+ T cells. The responses of these prognosis-related genes to immunotherapy in BLCA may be associated with CD8+ T cells, TMB, and PD-L1 expression. These three key genes and their related drugs may help to develop new targets for BLCA immunotherapy. Further investigations and experiments using methods such as quantitative real-time PCR and western blotting, and clinical data analyses are required to validate the results of the present study. We will perform such further experiments to support our results.

Abbreviations

BLCA, bladder urothelial carcinoma; DFS, disease-free survival; DSS, disease-specific survival; GEPIA, Gene Expression Profiling Interactive Analysis; GO, gene ontology; mUC, metastatic urothelial cancer; OS,
overall survival; PPI, protein–protein interaction, TIIC, tumor-infiltrating immune cell; TMB, tumor mutation burden.

Declarations

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Authors’ contributions

Y.G. wrote the main text of the article and designed the experiments. Y.B.Z. reviewed and edited the article. Y.L., W.H.S., and S.P.P. prepared Figures 1–4. S.H. analyzed part of the data. K.X. and W.H.K. prepared Figures 5 and 6. All authors reviewed the manuscript.

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Availability of data and materials

The data that support the findings of this study are openly available from GEPIA (http://gepia.cancer-pku.cn/) and Oncomine (https://www.Oncomine.org).

Ethics approval and consent to participate

This research was supported by the Independent Ethics Committee (IEC) of the Second Affiliated Hospital of Xi’an JiaoTong University.

Competing interests

The authors declare that there are no competing interests associated with this manuscript.

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Tables
Table 1 Confirmation of the associations of three hub genes with prognosis in three different databases

PROGgeneV2, PrognoScan, and OSblca databases were used to confirm the prognostic value of three hub genes in BLCA. HR, hazard ratio.

| Database      | Dataset                        | Gene  | Endpoint | P-Value | HR [95% CI low - CI upp] |
|---------------|--------------------------------|-------|----------|---------|-------------------------|
| PROGgeneV2    | GSE13507                       | AURKA | OS       | 0.00135 | 1.39 [1.14-1.7]         |
|               | GSE13507                       | BIRC5 | OS       | 0.01299 | 1.94 [1.15-3.26]        |
|               | GSE13507                       | CKS1B | OS       | 0.03256 | 1.24 [1.02-1.5]         |
|               | GSE19915                       | BIRC5 | OS       | 0.00009 | 2.62 [1.62-4.24]        |
| PrognoScan    | GSE13507_ILMN_1680955          | AURKA | OS       | 0.00128 | 1.39 [1.14-1.70]        |
|               | GSE13507_ILMN_1680955          | AURKA | DFS      | 0.00011 | 1.91 [1.40-2.62]        |
|               | GSE5287_210334_x_at            | BIRC5 | OS       | 0.00560 | 7.68 [2.55-23.14]       |
|               | GSE5287_202095_s_at            | BIRC5 | OS       | 0.00183 | 2.43 [1.35-4.38]        |
|               | GSE13507_ILMN_1710082          | BIRC5 | OS       | 0.00179 | 1.94 [1.15-3.26]        |
|               | GSE13507_ILMN_1710082          | BIRC5 | DFS      | 0.00077 | 3.22 [1.65-6.31]        |
|               | GSE13507_ILMN_1719256          | CKS1B | DFS      | 0.04721 | 1.56 [1.17-2.08]        |
| OSblca        | GSE13507_ILMN_1710082          | BIRC5 | OS       | 0.01880 | 1.837 [1.1059-3.0515]   |
|               | GSE13507_ILMN_1680955          | AURKA | OS       | 0.00400 | 2.1412 [1.2742-3.5983]  |
|               | GSE19915                       | BIRC5 | DSS      | 0.00030 | 4.4378 [1.9813-9.9398]  |
|               | GSE32548_ILMN_2349459          | BIRC5 | OS       | 0.04900 | 2.2344 [1.0036-4.9749]  |
|               | GSE48507_ILMN_2349459          | BIRC5 | OS       | 0.00400 | 2.5862 [1.3551-4.936]   |
|               | GSE48075_ILMN_1803124          | BIRC5 | OS       | 0.00900 | 2.3966 [1.2444-4.6154]  |
|               | GSE32548_ILMN_1719256          | CKS1B | OS       | 0.03500 | 2.3667 [1.026-5.2711]   |
|               | GSE32548_ILMN_2041046          | CKS1B | OS       | 0.00790 | 2.9205 [1.3245-6.4394]  |
Table 2 Relationships of three prognostic genes with clinical characteristics

Clinical characteristics included age, histological subtype (papillary or non-papillary tumor), molecular subtype (luminal papillary, luminal infiltrated, luminal, basal squamous, neuronal), nodal metastasis status (N0, no regional lymph node metastasis; N1, metastases in 1–3 axillary lymph nodes; N2, metastases in 4–9 axillary lymph nodes; N3, metastases in 10 or more axillary lymph nodes), sample type, smoking, cancer stage, and TP53 mutation status.
| Gene Symbol | Clinical Characteristic | Comparison                          | P-value    |
|-------------|-------------------------|-------------------------------------|------------|
| AURKA       | Age                     | Normal-vs-Age(41-60 Y)              | 4.00E-15   |
|             |                         | Normal-vs-Age(61-80 Y)              | <1E-12     |
|             |                         | Normal-vs-Age(81-100Y)              | 7.10E-10   |
| BIRC5       | Age                     | Normal-vs-Age(41-60 Y)              | 1.82E-10   |
|             |                         | Normal-vs-Age(61-80 Y)              | 3.99991E-12|
|             |                         | Normal-vs-Age(81-100 Y)             | 2.40E-09   |
| CKS1B       | Age                     | Normal-vs-Age(41-60 Y)              | 8.88E-16   |
|             |                         | Normal-vs-Age(61-80 Y)              | 1.62E-12   |
|             |                         | Normal-vs-Age(81-100 Y)             | 2.617E-07  |
| AURKA       | Histological Subtypes   | Normal-vs-Papillary tumors          | 6.55E-15   |
|             |                         | Normal-vs-NonPapillary tumors       | <1E-12     |
|             |                         | Papillary tumors-vs-NonPapillary tumors | 3.00E-03 |
| BIRC5       | Histological Subtypes   | Normal-vs-Papillary tumors          | 6.40E-07   |
|             |                         | Normal-vs-NonPapillary tumors       | 8.40E-10   |
|             |                         | Papillary tumors-vs-NonPapillary tumors | 1.10E-03 |
| CKS1B       | Histological Subtypes   | Normal-vs-Papillary tumors          | 1.77E-04   |
|             |                         | Normal-vs-NonPapillary tumors       | 3.27E-08   |
|             |                         | Papillary tumors-vs-NonPapillary tumors | 2.38E-04 |
| AURKA       | Molecular Subtypes      | Normal-vs-Neuronal                  | 1.54E-06   |
|             |                         | Normal-vs-Basal squamous            | <1E-12     |
|             |                         | Normal-vs-Luminal                   | 2.26E-10   |
|             |                         | Normal-vs-Luminal_Infiltrated       | 1.49E-11   |
|             |                         | Normal-vs-Luminal_Papillary         | 1.67E-12   |
|             |                         | Neuronal-vs-Luminal                 | 1.05E-02   |
|             |                         | Neuronal-vs-Luminal_Infiltrated     | 2.48E-03   |
|             |                         | Neuronal-vs-Luminal_Papillary       | 6.00E-03   |
| Molecular Subtypes          | Normal-vs-Neuronal | Normal-vs-Basal squamous | Normal-vs-Luminal | Normal-vs-Luminal_Infiltrated | Normal-vs-Luminal_Papillary | Neuronal-vs-Basal squamous | Neuronal-vs-Luminal | Neuronal-vs-Luminal_Infiltrated | Neuronal-vs-Luminal_Papillary | Basal squamous-vs-Luminal | Basal squamous-vs-Luminal_Infiltrated | Basal squamous-vs-Luminal_Papillary |
|----------------------------|--------------------|--------------------------|-------------------|-------------------------------|-----------------------------|-----------------------------|-------------------------|-------------------------------|-------------------------------|--------------------------|-------------------------------|-------------------------------|
| BIRC5                      | 3.20E-07           | 1.65E-12                 | 2.93E-07          | 4.66E-07                       | 1.15E-08                    | 3.40E-03                    | 9.81E-05                | 4.18E-05                       | 9.99E-05                       | 3.10E-06                 | 1.03E-11                       | 1.36E-08                      |
| CKS1B                      | 9.74E-07           | 1.62E-12                 | 2.40E-08          | 5.97E-11                       | 4.44E-15                    | 4.57E-02                    | 1.74E-03                | 1.46E-03                       | 9.79E-04                       | 9.60E-04                 | 9.92E-05                       | 2.26E-07                      |
| AURKA                      | Normal-vs-N0       |                          |                   |                               |                             |                            |                         |                               |                               |                         |                               | 1.62E-12                      |
|                | Nodal Metastasis Status | Normal-vs-N0       |
|----------------|-------------------------|--------------------|
| BIRC5          | Normal-vs-N0            | 1.34E-11           |
|                | Normal-vs-N1            | 1.69E-10           |
|                | Normal-vs-N2            | 8.57E-09           |
|                | Normal-vs-N3            | 7.04E-03           |
|                | N1-vs-N2                | 2.50E-02           |
| CKS1B          | Normal-vs-N0            | 1.62E-12           |
|                | Normal-vs-N1            | 1.04E-09           |
|                | Normal-vs-N2            | 1.63E-12           |
|                | Normal-vs-N3            | 8.60E-04           |
|                | N0-vs-N1                | 4.35E-02           |
|                | N1-vs-N2                | 1.70E-02           |
|                | N2-vs-N3                | 2.42E-02           |

|                | Sample Types            | Normal-vs-Primary  |
|----------------|-------------------------|--------------------|
| AURKA          | Normal-vs-Primary       | 1.62E-12           |
| BIRC5          | Normal-vs-Primary       | 5.35E-11           |
| CKS1B          | Normal-vs-Primary       | 1.62E-12           |

|                | Smoking Habit            | Normal-vs-Non smoker| Normal-vs-Smoker | Normal-vs-Reformed smoker1 | Normal-vs-Reformed smoker2 | Non smoker-vs-Reformed smoker1 | Non smoker-vs-Reformed smoker2 |
|----------------|--------------------------|---------------------|----------------|---------------------------|---------------------------|-------------------------------|-------------------------------|
| AURKA          | Normal-vs-Non smoker     | 1.24E-14            | 1.63E-12       | <1E-12                   | 1.33E-15                   | 8.60E-03                      | 9.46E-03                      |
| BIRC5          | Normal-vs-Non smoker     | 1.82E-09            | 2.35E-10       | 3.83E-12                 |                           |                               |                               |
| Gene       | Phenotype                        | Comparison                           | p-value     |
|------------|----------------------------------|--------------------------------------|-------------|
| CKS1B      | Smoking Habit                    | Normal-vs-Non smoker                 | 1.63E-12    |
|            |                                  | Normal-vs-Smoker                     | 5.55E-16    |
|            |                                  | Normal-vs-Reformed smoker1           | <1E-12      |
|            |                                  | Normal-vs-Reformed smoker2           | 1.62E-12    |
| AURKA      | Cancer Stage                      | Normal-vs-Stage2                     | 1.62E-12    |
|            |                                  | Normal-vs-Stage3                     | 1.62E-12    |
|            |                                  | Normal-vs-Stage4                     | 1.62E-12    |
| BIRC5      | Cancer Stage                      | Normal-vs-Stage2                     | 1.53E-10    |
|            |                                  | Normal-vs-Stage3                     | 3.81E-13    |
|            |                                  | Normal-vs-Stage4                     | 7.86E-12    |
| CKS1B      | Cancer Stage                      | Normal-vs-Stage2                     | <1E-12      |
|            |                                  | Normal-vs-Stage3                     | <1E-12      |
|            |                                  | Normal-vs-Stage4                     | 1.62E-12    |
| AURKA      | TP53 Mutation Status             | Normal-vs-TP53-Mutant                | 1.62E-12    |
|            |                                  | Normal-vs-TP53-NonMutant             | 3.63E-13    |
|            |                                  | TP53-Mutant-vs-TP53-NonMutant        | 3.80E-12    |
| BIRC5      | TP53 Mutation Status             | Normal-vs-TP53-Mutant                | 1.63E-12    |
|            |                                  | Normal-vs-TP53-NonMutant             | 8.02E-09    |
|            |                                  | TP53-Mutant-vs-TP53-NonMutant        | 7.83E-08    |
| CKS1B      | TP53 Mutation Status             | Normal-vs-TP53-Mutant                | 1.62E-12    |
|            |                                  | Normal-vs-TP53-NonMutant             | 8.44E-15    |
|            |                                  | TP53-Mutant-vs-TP53-NonMutant        | 2.20E-12    |

Table 3 Associations of three prognosis-related genes with immune cell infiltration by TIMER
### Table 4 Differences in expression of three genes between responders and non-responders

| Gene Symbol | Description                                      | Drug                                  | AveExpr | P       |
|-------------|--------------------------------------------------|---------------------------------------|---------|---------|
| AURKA       | aurora kinase A                                  | Anti-PD-L1 (atezolizumab)             | 5.632   | 0.0007978 |
| BIRC5       | baculoviral IAP repeat containing 5              | Anti-PD-L1 (atezolizumab)             | 2.755   | 0.0001359 |
| CKS1B       | CDC28 protein kinase regulatory subunit 1B       | Anti-PD-L1 (atezolizumab)             | 1.739   | 0.001768  |

### Figures
Figure 1

Identification of 11 hub genes. (A) Identification of common genes between GEPIA and Oncomine by Venn diagram. (B) Enriched terms of common genes by Metascape. Network of enriched terms colored by cluster ID. (C) PPI network of DEGs constructed with STRING software: nodes represent proteins; continuous lines represent direct interactions (physical), while indirect ones (functional) are represented by interrupted lines; line thickness indicates the strength of data support. (D) Identification of hub genes using MCODE. Upregulated genes are marked with red nodes, while downregulated genes are denoted by green nodes. The size of the nodes is positively correlated with P-value. The line color is determined by the combined score provided by STRING.
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Figure 2

Overexpression of three genes is correlated with poor prognosis. (A–K) The associations between the expression of 11 hub genes and OS was evaluated using the R survival package. (A) AURKA, (B) BIRC5, (C) CENPA, (D) CKS1B, (E) ECT2, (F) MYBL2, (G) NUF2, (H) RRM2, (I) TK1, (J) TPX2, (K) UBE2C. Only three key genes were associated with prognosis in BLCA.
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Figure 3

Evaluation of association between three prognosis-related key genes and clinical factors using UACLAN. Expression of three key prognosis-related genes based on different sample types, according to (A–C) histological subtypes, (D–F) molecular subtypes, and (G–I) TP53 mutation status.
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Figure 4

Association between three hub genes and immune infiltration. (A, B) Correlations of (A, B) AURKA, (C, D) BIRC5, and (E, F) CKS1B expression with immune infiltration level in BLCA. (G) Kaplan-Meier survival curves for different immune cells. Levels are divided into low and high by a defined slider. P-value of log-rank test for comparing survival curves of the two groups is shown in each plot.
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Figure 5

TISIDB analysis of correlations between three key genes and immune system. (A–C) TMB is positively related to three hub genes in pan-cancer, including BLCA: (A) AURKA, (B) BIRC5, (C) CKS1B. (D–F) Correlations between three hub genes and PD-L1 mRNA expression: (D) AURKA, (E) BIRC5, (F) CKS1B. (G–I) Associations between the three prognosis-related genes’ expression and immune subtypes across BLCA: (G) AURKA, (H) BIRC5, (I) CKS1B. Act_CD8, activated CD8 T cell, Tcm_CD8, central memory CD8 T
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Figure 6

Drugs targeting three prognosis-related genes. (A–C) Drugs targeting three key genes were collected from DrugBank database. (A) AURKA, (B) BIRC5, (C) CKS1B. Red rectangle represents the current gene, blue rectangle represents a drug, green rectangle indicates other targets.
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