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An Evaluation of the Tumor Microenvironment through CALR, IL1R1, IFNB1, and IFNG to Assess Prognosis and Immunotherapy Response in Bladder Cancer Patients

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eLife Assessment

This study presents **useful** findings that explore the prognostic and immunotherapeutic relevance of specific immune-related genes (CALR, IL1R1, IFNB1, and IFNG) in the bladder cancer tumor microenvironment. While the analysis highlights potentially meaningful associations with survival and treatment response, the strength of evidence is **incomplete**, as some claims lack sufficient experimental or mechanistic validation. Further refinement and validation of the predictive models would enhance the impact and generalizability of the conclusions.

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Abstract

Immunogenic cell death (ICD) is a type of cell death sparking adaptive immune responses, can reshape the tumor microenvironment (TME). Exploring key ICD-related genes in bladder cancer (BLCA) could enhance personalized treatment. TCGA BLCA patients were divided into two ICD subtypes: ICD-high and ICD-low. High ICD expression linked to increased immune cell infiltration and longer survival, but with potentially suppressed immune function. The high ICD group responded better to PD1-targeted therapy. A risk-scoring model with four ICD-related genes (CALR, IL1R1, IFNB1, IFNG) was validated across TCGA, GEO datasets, and tissue samples, showing higher risk-score correlated with weaker anti-tumor immune function, more tumor-promoting elements, lower immunotherapy response rates, and shorter patient survival. This study connects ICD-related genes to BLCA prognosis and immune infiltration, offering a vital tool for personalized treatment guidance.

1 Introduction

Bladder cancer (BLCA) is the tenth most common cancer globally, divided into non-muscle-invasive (NMIBC) and muscle-invasive (MIBC) types¹. NMIBC patients often have high recurrence after surgery, so postoperative treatment like bladder instillation with BCG or chemotherapy is recommended¹. MIBC is treated with radical cystectomy, often combined with platinum-based neoadjuvant chemotherapy for better results². New immunotherapy using PD-1/PD-L1 inhibitors is showing promise for BLCA. In 2017, the FDA approved atezolizumab and pembrolizumab for advanced cases intolerant to platinum-containing chemotherapy³. Response rates of 11.68% and 24.05% were seen with atezolizumab and pembrolizumab^{4,5}. Chemotherapy can affect the tumor immune environment⁶, and ongoing trials are exploring combining immune checkpoint inhibitors (ICIs) with chemotherapy (*ClinicalTrials.gov* Identifier: NCT04383743, NCT04630730)⁷. Research in BLCA immunotherapy is in early stages, highlighting the need for innovative approaches combining chemotherapy and immunotherapy.

Immunogenic cell death (ICD) is a regulated cell death that, in an active immune system, triggers an adaptive immune response by exposing antigens from dying cells⁸. Certain chemotherapy drugs, like cisplatin⁹ and gemcitabine¹⁰, can induce ICD. While studied in preclinical BLCA models, the potential benefits of ICD-based therapies in BLCA lack conclusive evidence¹¹. It is crucial to explore the impact of ICD in clinical settings for BLCA treatment, be it through chemotherapy or immunotherapy. In this study, we discovered a connection between ICD-related genes and the prognosis and immune infiltration in BLCA patients using TCGA-BLCA, GEO datasets, and tissue microarray staining. Our validated risk-scoring model effectively evaluates immune infiltration, prognosis, immunotherapy response, and drug sensitivity in BLCA, providing guidance for personalized treatment and future research.

2 Materials and Methods

2.1 Identification of Differentially Expressed ICD-related genes

ICDs were sourced from a previous article¹². We found differentially expressed genes (DEGs) in TCGA-BLCA using the “Limma” R package. For TCGA samples: 421 tumor samples and 19 normal samples. Database release date: March 29, 2022, v36 versions. Coding package version: R version 4.1.1. This involved comparing gene expression in normal and tumor samples. DEGs interacting with ICDs were identified. Enrichment scores of ICDs were assessed using ssGSEA analysis with the “GSVA” R package. Patients were then grouped as “high” or “low” based on these scores. Finally, DEGs were visualized through volcano and heatmaps using R packages “pheatmap” and “ggplot2”. All sample data are derived from the TCGA (The Cancer Genome Atlas) and TCIA (The Cancer Immunome Atlas) databases.

2.2 Functional Enrichment Analysis

We compared ICD-high and ICD-low groups by analyzing Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) using the “clusterProfiler” R package. Gene Set Enrichment Analysis (GSEA) assessed differences in expressed gene sets between the groups using the MSigDB collection.

2.3 Analysis of Somatic Mutation

BLCA patient mutation data from TCGA were visualized using “Maftools” in waterfall plots. Boxplots summarized tumor mutational burden (TMB), and the 20 most frequently mutated genes were shown in waterfall plots.

2.4 Analysis of Immune Infiltration in the TME

Used the “ESTIMATE” R package to predict the tumor microenvironment (TME) scores for BLCA samples and assess immunocyte proportions. XCELL, TIMER, QUANTISEQ, MCPcounter, EPIC, CIBERSORT and CIBERSORT-ABS were used to explore immune cell infiltration, visualizing differences with a violin diagram and analyzing with the Wilcoxon signed-rank test. Evaluated immunotherapeutic response using the IPS algorithm and compared immune checkpoint and HLA-related gene expression in different groups. Also, accessed IPS data from The Cancer Immunome Atlas (TCIA) to compare responses to ICIs across BLCA patient groups.

2.5 Construction and validation of risk-scoring model

To explore the prognostic value of ICD-related genes in BLCA. Using TCGA-BLCA data, we conducted Cox regression analysis and LASSO Cox regression to build a prognostic model. Risk-score=CALR*(0.5378) + IFN1B*(-0.6349) + IFNG*(-0.2028) + IL1R1*(0.0919). Risk-scores were calculated, and patients were divided into low-risk and high-risk groups. We validated the model using the GSE13507 data and BLCA tissue arrays (HBlau079Su01, Shanghai Outdo Biotech Co., Ltd)including a total of 63 cancer tissues and 16 cancer-adjacent normal tissues from patients with bladder cancer. Kaplan-Meier curves for overall survival (OS) in low-risk and high-risk groups were created with the R packages “Survminer” and “Survival”. Nomogram was created using “rms” package to integrate risk-scores and other clinicopathological information to assess survival probability. ROC analysis was used to evaluate the nomogram’s ability to predict survival. The R package “pRRophetic” was used to evaluate the drug sensibility. The Remmele and Stegner’s semiquantitative immunoreactive score (IRS) scale was employed to assess the expression levels of each marker, as detailed in earlier study¹³. The R umap package was utilized to conduct the UMAP analyses of the individual cells. A two-sided P < 0.05 was considered valuable. The reagents used are as follows: anti-IFNB1 (cat #: bs-0787R, BIOSS), anti-IL1R1 (cat #: bs-20697R, BIOSS), anti-IFNG (cat #: MAB48116, Bioswamp), and anti-CALR (cat #: A1066, ABclonal).The general workflow for single-cell sequencing analysis includes sample preparation, RNA extraction, library construction, high-throughput sequencing, data preprocessing (quality control, normalization), clustering, cell type annotation, differential expression analysis, and functional enrichment.

3 Results

3.1 Stratification and pathway enrichment analysis of TCGA-BLCA based on ICD-related genes

We conducted unsupervised clustering analysis on 34 ICD-related genes to identify two ICD-associated subtypes. Subsequently, we defined ICD-high and ICD-low groups based on the expression levels of these genes and compared their ICD gene expression profiles (**Figure 1A**). A clinical heatmap demonstrated differences between the two subtypes in terms of Grade (**Figure 1B**). Furthermore, survival analysis (**Figure 1C**) indicated that the ICD-high group exhibited significantly longer survival times compared to the ICD-low group (p=0.039). Subsequently, we found that patients in the ICD-high group exhibited a significantly higher TMB (p < 0.05) (**Figure S1A**). We also found that TP53, TTN, KMT2D, MUC16, and ARID1A are the most frequently mutated genes in both groups (**Figure S1B, C**).

Next, we examined the differential gene expression between the two subgroups, identifying 4,321 DEGs, consisting of 2,177 downregulated and 2,144 upregulated genes. The heatmap displayed the top 50 upregulated and 50 downregulated genes (**Figure 1D, E**). To further identify pathways associated with immune activation specific to ICD-high and ICD-low groups, GO, KEGG, and GSEA were conducted. The results indicate a close association between elevated ICD expression and the activation of the immune microenvironment (**Figure 1F-L**).

3.2 The immune characteristics within the TME in ICD-high and ICD-low group

We employed the ESTIMATE algorithm for analysis. The ICD-high group exhibited higher ESTIMATE scores, immune scores, and stromal scores compared to the ICD-low group, while the ICD-high group demonstrated lower tumor cell purity (**Figure 2A-D**). Further analysis of immune cell infiltration between the two subtypes revealed significantly higher levels of T cells CD8+, T cells CD4+ memory activated, and T cells follicular helper in the ICD-high group, while Tregs displayed the opposite trend (**Figure 2E**). Furthermore, we compared the expression of immune function-related pathways, immune checkpoint genes, and HLA-related genes between the two subtypes. The results indicate that the ICD-high group exhibited elevated expression in nearly all 13 immune pathways (**Figure 2F**). In the 8 immune checkpoint genes, PDCD1LG2, CTLA4, PDCD1, CD274, HAVCR2, LAG3, TIGIT had higher expression levels, while SIGLEC15 had lower expression (**Figure 2G**). Among the 24 HLA-related genes, the ICD-high group had higher expression levels for almost all genes (**Figure 2H**). These results suggest that ICD-high patients have a significantly higher quantity of tumor-infiltrating immune cells, but they appear to be in a state of functional exhaustion. Thus, we evaluated the effects of ICIs PD1 and CTLA4 on the ICD-high and ICD-low groups. Treated with PD1 and CTLA4 or PD1 alone, ICD-high group had better outcomes. With CTLA4 alone, both subtypes had similar results. Without PD1 or CTLA4 treatment, ICD-high group had significantly worse outcomes than ICD-low group (**Figure 2I-L**). Demonstrating that targeting PD-1 is crucial to enhance immune cell functionality and improve the prognosis of ICD-high patients.

3.3 Construction and validation of risk-scoring model

In Cox univariate analysis, we identified 4 ICD-related genes significantly associated with OS of patients: CALR ($p=0.003$), IFNB1 ($p=0.037$), IFNG ($p=0.022$), IF1R1 ($p=0.047$) (**Figure 3A**). Subsequently, these 4 ICD-related genes were tested and selected in the LASSO regression analysis for constructing the predictive model (**Figure 3B, C**). Subsequently, patients are categorized into high-risk and low-risk groups according to the median (the boundary value is 4.372) of the risk-scores. Survival analysis indicated that, in both the TCGA-BLCA dataset and the GSE13507 dataset, the high-risk group exhibits significantly shorter survival times than the low-risk group ($P<0.05$) (**Figure 3D, H**). Next, we analyzed somatic mutations and generated waterfall plots for the top 20 mutated genes (**Figure S2A, B**) and calculated the TMB for both groups. Among the ICD-high-risk group and ICD-low-risk group, TP53, TTN, KMT2D, MUC16, and ARID1A were the most frequently mutated genes, but the TMB does not differ between the ICD high-risk group and the ICD low-risk group (**Figure S2E**). Survival analysis also indicated longer survival times in the high TMB group ($P<0.001$), with the best prognosis observed in the low-risk + high-TMB group and the worst prognosis in the high-risk + low-TMB group (**Figure S2C,D**).

We further conducted a comprehensive analysis using the TCGA-BLCA dataset (**Figure 3E-G**) as the training set and the GSE13507 dataset (**Figure 3I-K**) as the validation set. Based on the median of the risk-scores, we categorized the datasets from TCGA and GEO into high-risk and low-risk groups. We analyzed the correlation between risk-scores and survival status, showing that higher risk-scores increased the risk of patient mortality. Additionally, we examined the

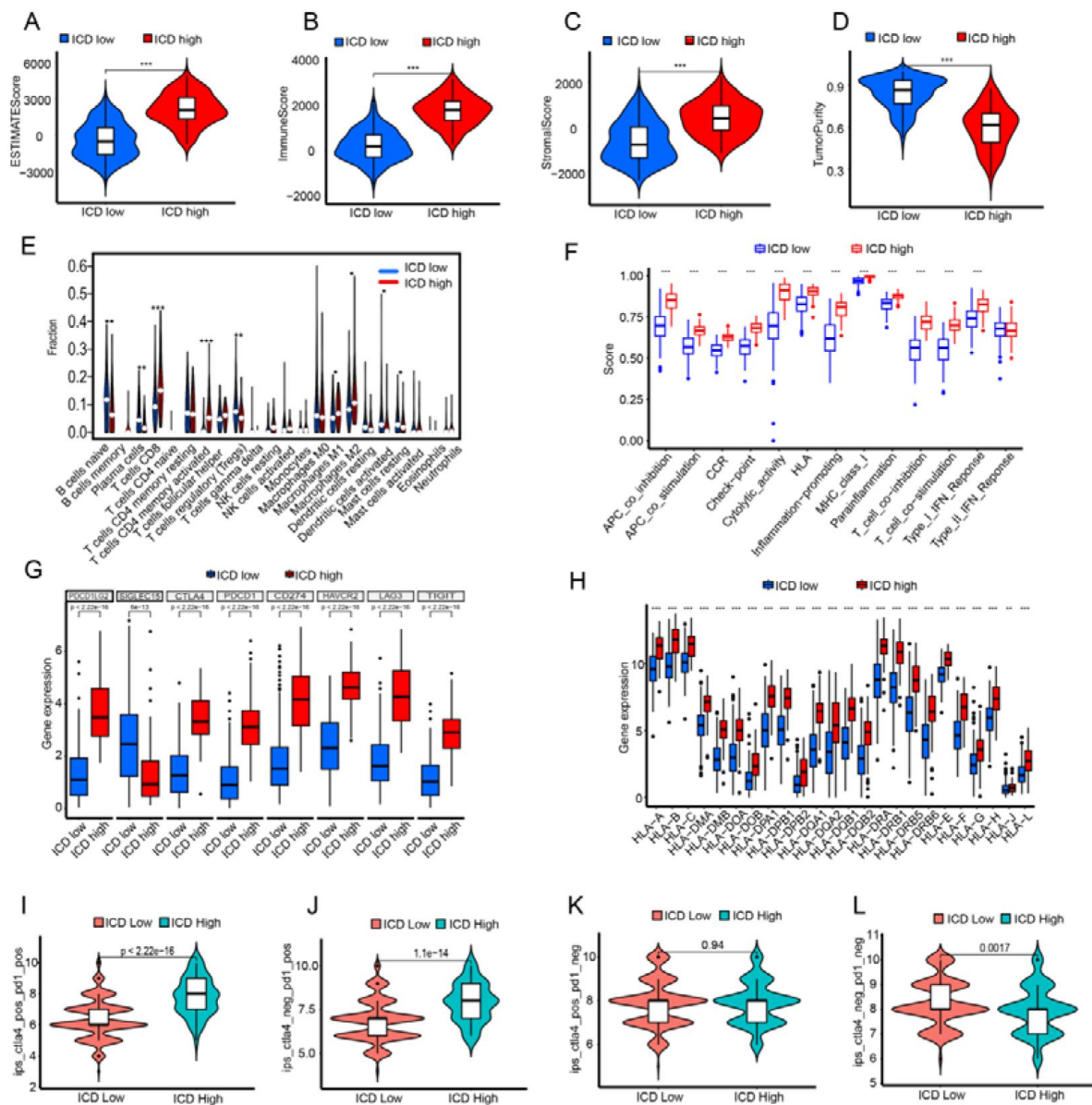


Figure 2.

The immune characteristics within the tumor microenvironment vary between ICD-high and ICD-low groups.

ESTIMATE scores (A), immune scores (B), stromal scores (C), and tumor cell purity (D) of ICD-high and ICD-low groups. E. Immune cell infiltration in the two subgroups. F-H. Expression profiles of the two subgroups in 13 immune-related pathways (F), 8 immune checkpoints (G), and HLA gene family (H). I. Efficacy of immunotherapy in the two subgroups.

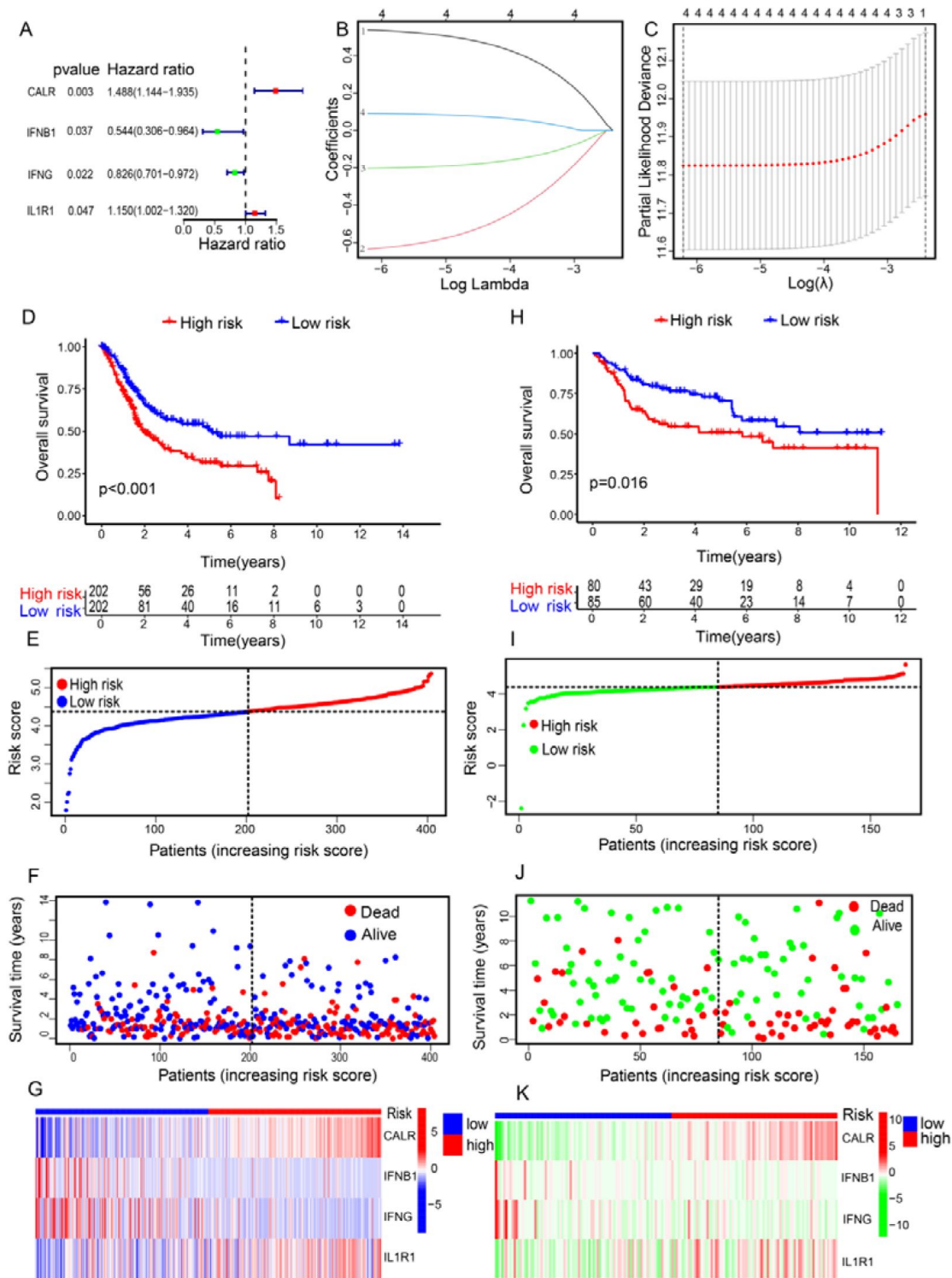


Figure 3.

Construction and validation of risk-scoring model.

A. Hazard ratio of four ICD-related genes identified by Cox regression analysis. B-C. LASSO regression analysis for constructing the prognostic model. D-E. Survival analysis of high-risk and low-risk groups in TCGA (D) and GEO (E) databases. Analyzed the correlation between risk-scores and patients, along with four key genes, in both TCGA (F-H) and GEO databases (I-K).

expression of the four genes in the risk signature, finding higher expression of CALR and IL1R1 in the high-risk group, and higher expression of IFNB1 and IFNG in the low-risk group (**Figure 3G,K**). Overall, these results suggest a less favorable prognosis for high-risk patients.

3.4 The association between risk-scores and clinical features

We analyzed the association between the four genes composing the risk-score and patient prognosis (**Figure 4A-D**). Patients with low expression of CALR, low expression of IF1R1, high expression of IFNB1, and high expression of IFNG had better prognosis, which is consistent with CALR and IF1R1 being lowly expressed and IFNB1 and IFNG being highly expressed in the low-risk group (**Figure 3G, K**). The clinical heatmap demonstrates differences between the two subtypes in terms of Stage and Grade (**Figure 4E**). In the high-risk group, there is a higher proportion of Stage III and Stage IV cases, while Stage I and Stage II cases are less common (**Figure 4F**). Additionally, the high-risk group has a higher proportion of high-grade cases and a lower proportion of low-grade cases (**Figure 4G**).

To aid clinicians in making more intuitive predictions of individual patient survival probabilities at 1, 3, and 5 years, we created a nomogram that incorporated gender, grade, age, and risk-score (**Figure 4H**). The AUC values for the 1-year, 3-year, and 5-year ROC curves are 0.630, 0.635, and 0.651, respectively (**Figure 4I**). The AUC values for risk-score, age, gender, and stage are 0.630, 0.669, 0.488, and 0.637, respectively (**Figure 4J**). Additionally, we found that the risk-score can serve as an independent prognostic factor ($p < 0.001$) (**Figure 4K, L**). This suggests that the risk-score is an effective indicator for predicting patient prognosis.

We checked risk-scores and survival in different subgroups. Higher risk-scores were seen in advanced stages (high-grade, stage III, stage IV, and M1 cases), suggesting an association with advanced disease (**Figure S3A-G**). Analyzing the relationship with patient prognosis, significant survival differences were noted between high-risk and low-risk groups in most subgroups (**Figure S3H-S**). This implies that high-risk classification is linked to shorter survival, especially in advanced stages compared to early stages. To explore the link between risk-scores and the immune microenvironment, we used the ESTIMATE algorithm. The high-risk group showed significantly lower immune scores and ESTIMATE scores than the low-risk group, while there was no notable difference in stromal scores between the two groups (**Figure 4M**). In the context of 13 immune-related pathways, the high-risk group demonstrated lower pathway activity (**Figure S4A**). Further analysis of immune cell infiltration between the two subtypes revealed that the high-risk group had significantly lower levels of T cells CD8, T cells CD4 memory activated, M1, and NK cells resting (**Figure 4N**). Correlation analysis between risk-scores and immune cells indicated a negative correlation with anti-tumor immune cells. Conversely, it showed a positive correlation with immune inhibitory cells (**Figure S5A-P**, **Figure S4B**). In addition, a significant correlation was observed between the 4 individual genes (CALR, IFN1B, IFNG and IL1R1) comprising the risk-score and immune cells, immune checkpoints or immune regulatory pathways (**Figure S6A-P**, **Figure S7A-C**). These analyses suggest that higher risk-scores are linked to weaker anti-tumor immune function and more tumor-promoting elements, partly explaining the shorter survival in the high-risk group.

3.5 The association between the risk-scores and the sensitivity to chemotherapy and immunotherapy

One of the primary factors inducing ICD is chemotherapy drugs, and targeted therapy plays a crucial role in BLCA treatment. Therefore, we further explored the correlation between the risk-scores and the sensitivity to chemotherapy drugs and targeted therapies. The high-risk group exhibited higher sensitivity (lower IC50 values) to sorafenib, epothilone B, docetaxel, elesclomol, MG132, lapatinib, and others. Conversely, the ICD-high risk group showed increased resistance (higher IC50 values) to lenalidomide, sunitinib, gefitinib, methotrexate, and camptothecin (**Figure 5A-P**). Furthermore, we investigated the prognostic characteristics of both groups in response to

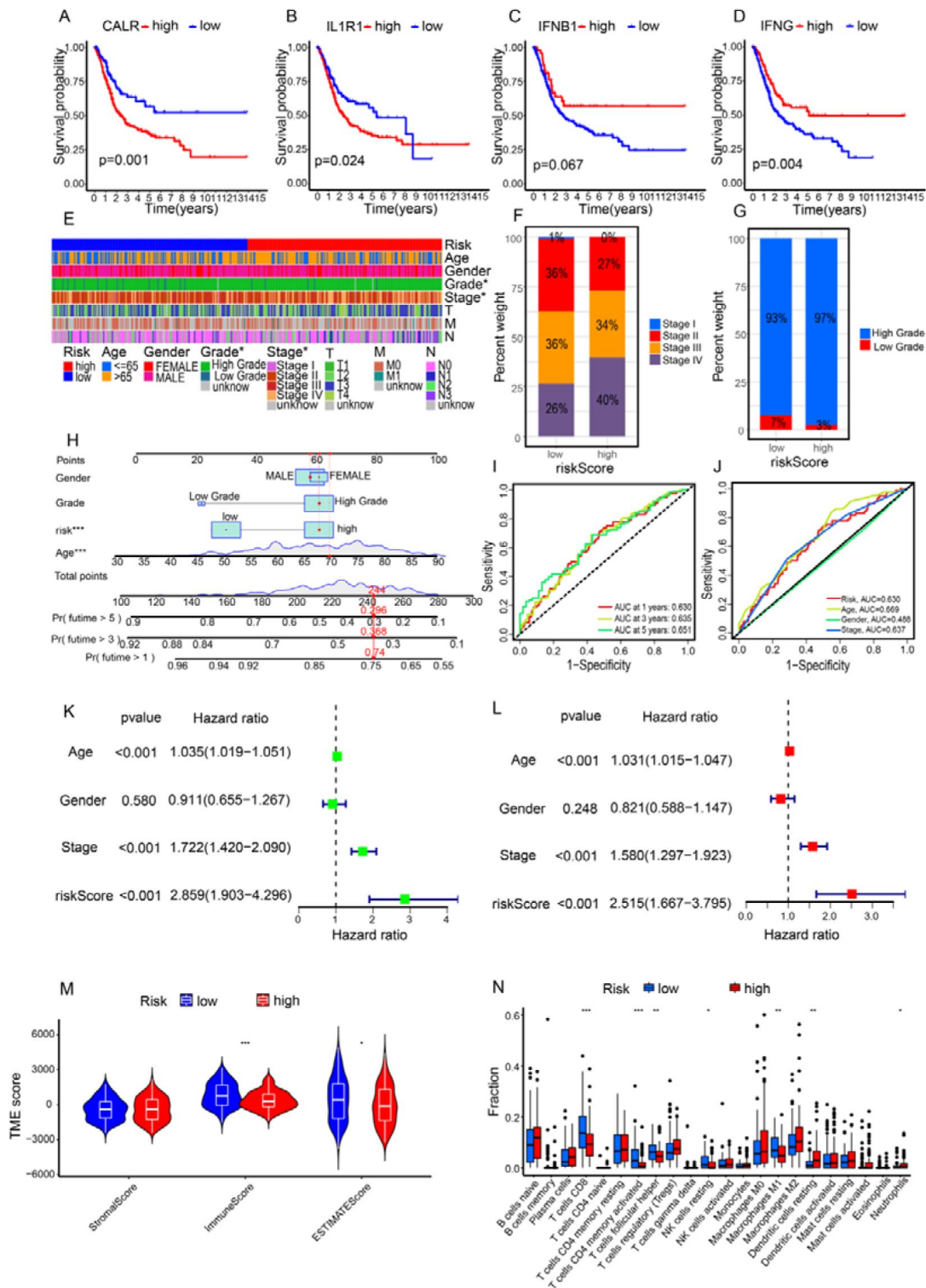


Figure 4.

The association between risk-scores and clinical features and immune characteristics.

A-D. The association between the four genes composing the risk-scores and patient prognosis. E. Clinical heatmap of the two subgroups. F-G. Differences in stage (F) and grade (G) between the two subgroups. H-J. Constructed nomogram (H) and ROC curve (I-J). K-L. Cox regression analysis. M. Stromal scores, immune scores, and ESTIMATE scores of the high-risk and low-risk groups. N. Immune infiltration in the two subgroups.

immunotherapy. The outcomes indicate that without anti-CTLA4 and anti-PD1 treatment, there was no significant difference in survival time between the groups. However, with at least one form of immunotherapy, the low-risk group had a significantly higher response rate than the high-risk group (**Figure 5Q-T**). These findings highlight the clinical potential of the risk-score in predicting sensitivity to various therapies and guiding treatment decisions for better outcomes.

3.6 Validate the risk-scoring model through BLCA tissue arrays and analyze the gene expression with single-cell sequencing

To validate the reliability of the aforementioned results, we conducted an analysis of the expression levels of CALR, IFN1B, IFNG and IL1R1 and their associations with prognosis and immune function using BLCA tissue arrays. Immunofluorescence staining revealed that CALR expression in tumor tissues was higher than in normal tissues, while the other three genes, IL1R1, IFN1B, and IFNG, showed no significant differences (**Figure 6A, B**, **Figure S8A**). This observation is consistent with the results from the TCGA-BLCA dataset exported using the GEPIA 2 tool (**Figure S8B-E**). Patients were divided into high and low expression groups based on the median expression levels (IRS scores) of CALR, IFN1B, IFNG, and IL1R1. It was found that patients in the CALR high-expression group and IL1R1 high-expression group had shorter survival times, while those in the IFN1B high-expression group and IFNG high-expression group had longer survival times (**Figure 6C**), consistent with the results of TCGA-BLCA dataset (**Figure 4A-D**). Patients' risk-scores were similarly calculated using the risk-scoring model: Risk-score = CALR-IRS* (0.5378) + IFN1B-IRS *(-0.6349) + IFNG-IRS *(-0.2028) + IL1R1-IRS *(0.0919). The high-risk group (50% vs. 50%) had significantly shorter survival times than the low-risk group (**Figure 6D**), corroborating the previous results (**Figure 3D, E**). More importantly, we found a significant positive correlation between the risk-score and CD39, a molecule we previously confirmed to be associated with immune suppression¹⁴. Additionally, the risk score showed a significant positive correlation with immune exhaustion (CD8+LAG3+), while having a negative correlation with CD8+ T cell infiltration (the IRS scores are based on our previous research¹⁴). However, there was no correlation with tumor volume and T-stage (**Figure 6E-I**). This suggests a strong link between higher risk scores and immune suppression.

Finally, using our previous single-cell sequencing data¹⁴ and the public dataset GSE135337, we analyzed the cellular expression patterns of CALR, IFN1B, IFNG, and IL1R1. CALR is expressed in nearly all cells in the TME, IL1R1 is mainly expressed in tumor-associated fibroblasts and endothelial cells, and the expression levels of IFNG and IFN1B are minimal, primarily by T lymphocytes. This suggests a crucial role of cancer-associated fibroblasts in remodeling the immune microenvironment of BLCA (**Figure 6J, K**, **Figure S8F, G**).

4 Discussion

ICD, a regulated cell death type, can be triggered by internal or external antigens, activating an adaptive immune response⁸. Some chemotherapies, such as cisplatin and gemcitabine, can induce ICD, thereby activating the immune microenvironment to achieve antitumor effects^{9,10}. ICIs are a key focus in cancer research, blocking tumors by reactivating immune cells through targeting checkpoints like PD1/PD-L1 and CTLA4¹⁵. Combining chemotherapy with ICI treatment is expected to enhance immune function and inhibit tumors, given ICD's role in activating the immune microenvironment, and some studies targeting key genes in the ICD process or combining chemotherapy with immunotherapy have also achieved encouraging results^{6,16,17}. However, the results of clinical trials indicate that the response rate of BLCA to ICIs is significantly lower than expected. The tumor immunosuppressive microenvironment is undoubtedly an important influencing factor. Considering the complexity of the TME and the

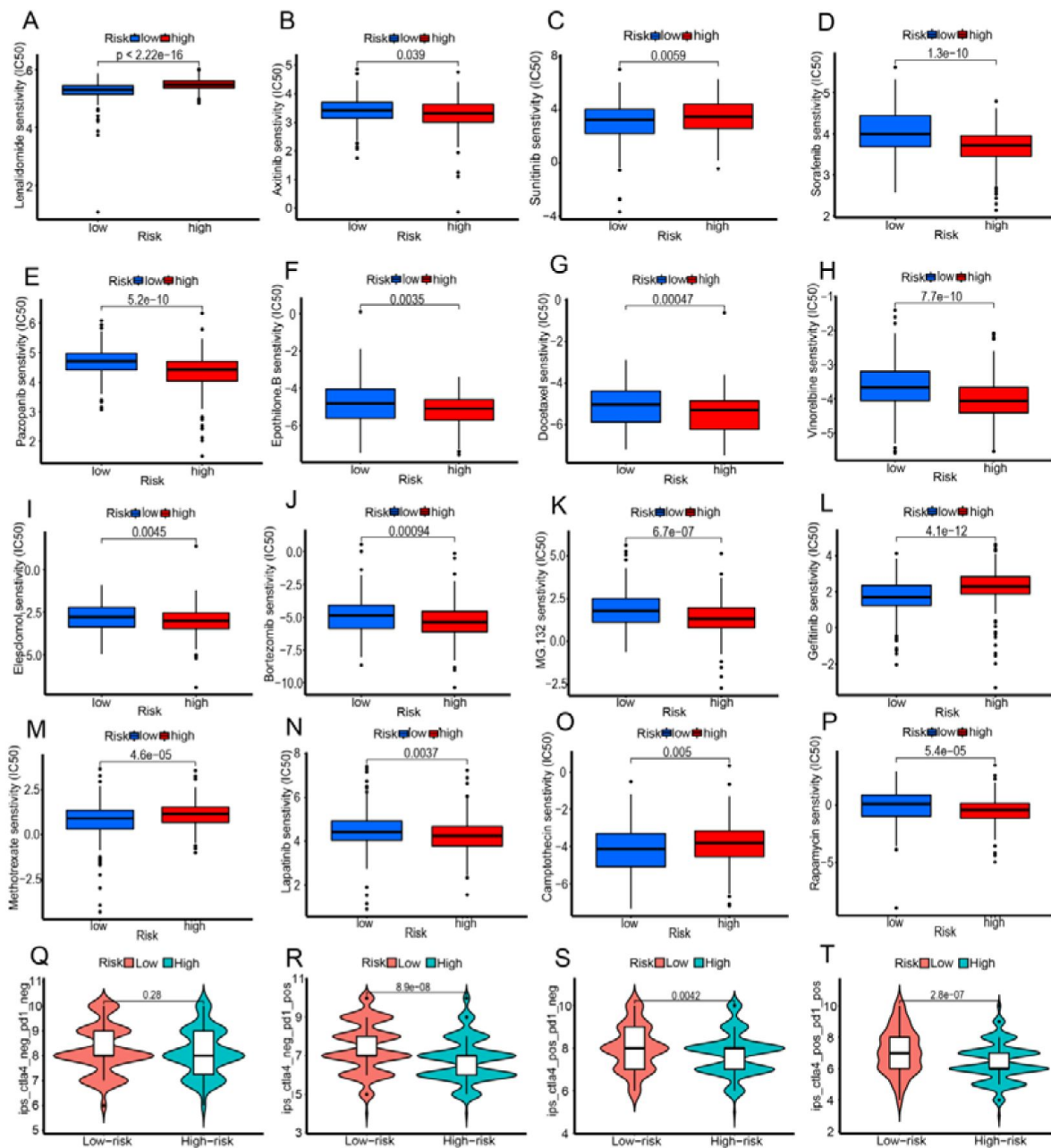


Figure 5.

The association between the risk-score and the sensitivity to chemotherapy (A-P) and immunotherapy (Q-T).

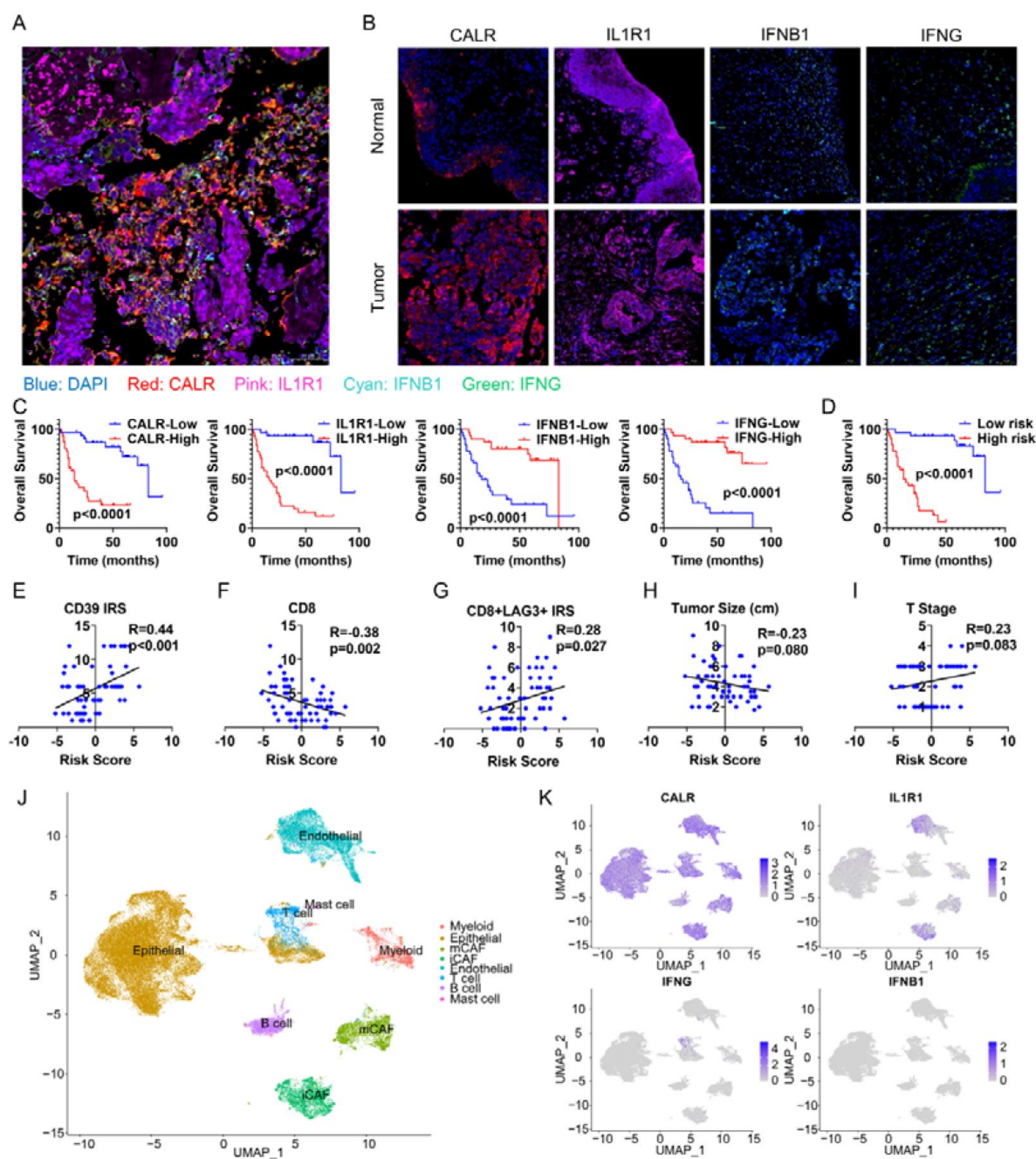


Figure 6.

Validate the risk-scoring model through tissue arrays and single-cell sequencing.

A-B. Analyzing the expression of CALR, IFN1B, IFNG, and IL1R1 using immunofluorescence staining. C-D. Survival analysis of the four genes (C) and risk-scores (D) in patients. E-I. The relationship between risk-scores and CD39, CD8+, CD8+LAG3+, Tumor Size, and T Stage. J-K. Expression analysis of CALR, IFN1B, IFNG, and IL1R1 in single-cell sequencing data.

impact of ICD, we investigate and confirm the clinical utility of ICD-related genes using BLCA public databases, BLCA tissue microarrays, and single-cell sequencing data. This offers theoretical guidance for applying ICD in clinical settings.

Through unsupervised clustering analysis, we categorized TCGA-BLCA patients into two groups: ICD-high and ICD-low. Pathway enrichment analysis revealed that the ICD-high group predominantly clustered in pathways related to the immune system, consistent with previous literature reports^{18,19}. In the analysis of immune infiltration, we found that the ICD-high group showed higher immune cell infiltration, coupled with lower tumor purity, indicating a lower invasive ability of the tumor and an activated immune state²⁰. We checked if ICD boosts ICI effectiveness by studying the connection between ICD-related gene expression and immunotherapy response rates. Turns out, the ICD-high group showed a much better response compared to the ICD-low group. This suggests that ICI treatment could work better in people with high ICD. Thus, we created a risk-scoring model using four ICD-related genes (CALR, IL1R1, IFNB1, and IFNG) identified through Cox regression and LASSO regression analyses. This model helps classify BLCA and predict immune infiltration, prognosis, and response to immunotherapy. Of them, IFNB1 and IFNG, both belonging to the interferon family, play crucial roles in anti-tumor immunity^{21,22}. IL1R1 is a receptor for IL1 α and IL1 β , a member of the interleukin family, and plays an immunosuppressive role in immune regulation²³. In ICD, the CALR calcium-binding protein moves to the cell membrane to aid antigen presentation, a widely accepted process²⁴. Yet, the general increase in CALR levels does not necessarily have a positive regulatory effect. Studies indicate that elevated CALR expression in breast cancer can enhance tumor cell metastasis and resistance to chemotherapy²⁵. Similarly, our study found that high CALR expression in BLCA has an inhibitory effect. Survival analysis showed that high CALR and IL1R1 expression was detrimental to patient survival, while high IFNB1 and IFNG expression was beneficial.

To verify our risk-scoring model, we used univariate and multivariate Cox regression analyses. The results indicate that the ICD scoring system can independently predict the prognosis of BLCA patients. Subgroup analysis based on clinical characteristics showed no difference between high-risk and low-risk groups in the early stages of the disease. However, in later stages, the high-risk group had shorter survival times. This may be due to changes in the TME at different disease stages, with a decrease in immune-killing molecules and an increase in inhibitory molecules as the tumor advances²⁶. Additional analysis of the correlation between the risk-scores and immune cell infiltration confirmed this: as the risk-score increased, the infiltration of cells with anti-tumor effects decreased, while the infiltration of M2-type macrophages and cancer-associated fibroblasts increased. These findings confirm the efficacy of our risk-scoring model in assessing patients' immune infiltration, prognosis, and tumor progression.

In treating BLCA, chemotherapy, targeted therapy, and immunotherapy are effective but have limitations in sensitivity and specificity²⁷. Our risk signature predicts drug sensitivity. High-risk group responds better to chemotherapy, and low-risk group shows higher response in immunotherapy (PD1 or CTLA4). This matches the higher immune infiltration in the low-risk patient group, emphasizing that immune infiltration level is crucial for successful immunotherapy. However, we find here that the quantity of immune cells in the tumor does not directly decide the patient's prognosis. This is because many infiltrating immune cells are functionally exhausted, and only immunotherapy can activate the immune response within the tumor, improving prognosis. This is confirmed in BLCA tissue microarray stains. Specifically, the risk-score shows a negative correlation with CD8⁺ T cell infiltration but a positive correlation with T cell exhaustion (CD8⁺LAG3⁺ or CD39). This shows our risk scoring model can categorize patients based on immune infiltration, specifically identifying a high-risk group (lower immune infiltration) and a low-risk group (higher immune infiltration). Personalized treatment is crucial for patients in this context. Additionally, in BLCA single-cell sequencing, we found IL1R1 is abundantly expressed in

cancer-associated fibroblasts, linked to tumor promotion and immunosuppression²⁸[\[link\]](#). This suggests a crucial role for these fibroblasts in shaping the immune microenvironment of BLCA, guiding our next research.

5 Conclusions

ICD, a type of cell death triggered in an immunogenic environment, influences immune response. Our study links ICD-related genes to BLCA prognosis and tumor immune infiltration. We created a validated risk-scoring model with four key genes (CALR, IL1R1, IFNB1, and IFNG) using TCGA-BLCA, GEO datasets, and tissue microarray staining. This model effectively assesses immune infiltration, prognosis, immunotherapy response, and drug sensitivity in BLCA, guiding personalized treatment and future research.

Supplementary Materials

Figure S1.

Somatic mutations in ICD-high and ICD-low groups.

A. Tumor mutation burden in the two subgroups. Most common mutated genes in the ICD-high (B) and ICD-low (C) groups.

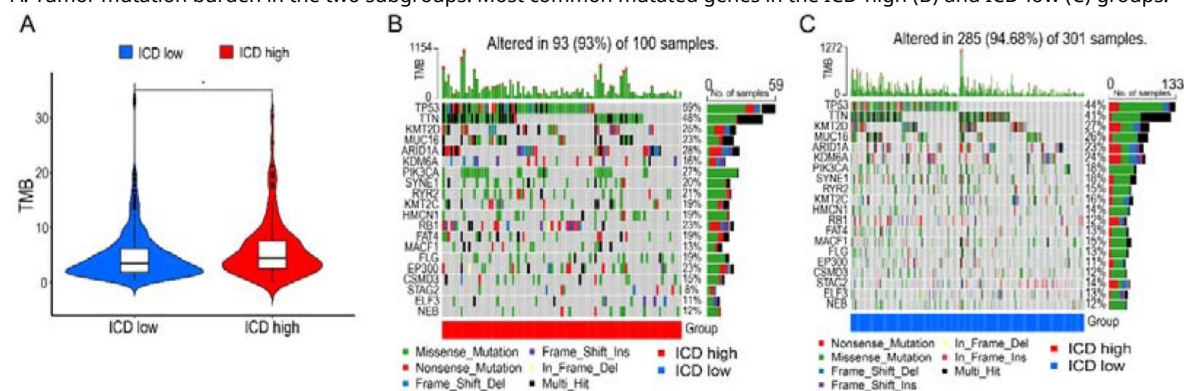
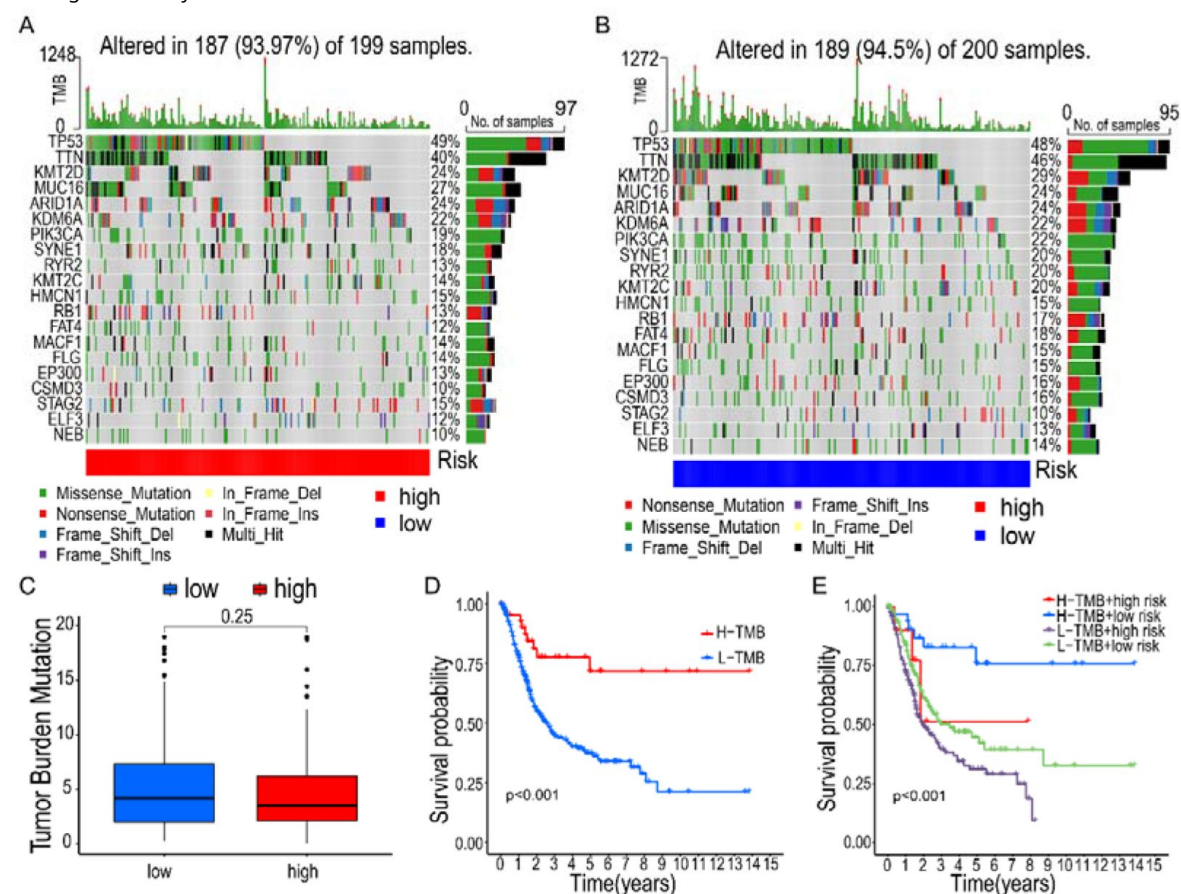


Figure S2.

Prognostic analysis of somatic mutations in high-risk and low-risk groups.

A-B. Most common mutated genes in high-risk (A) and low-risk (B) groups. C. Tumor mutation burden in the two subgroups. D. Prognostic analysis of different risk burdens with risk scores.



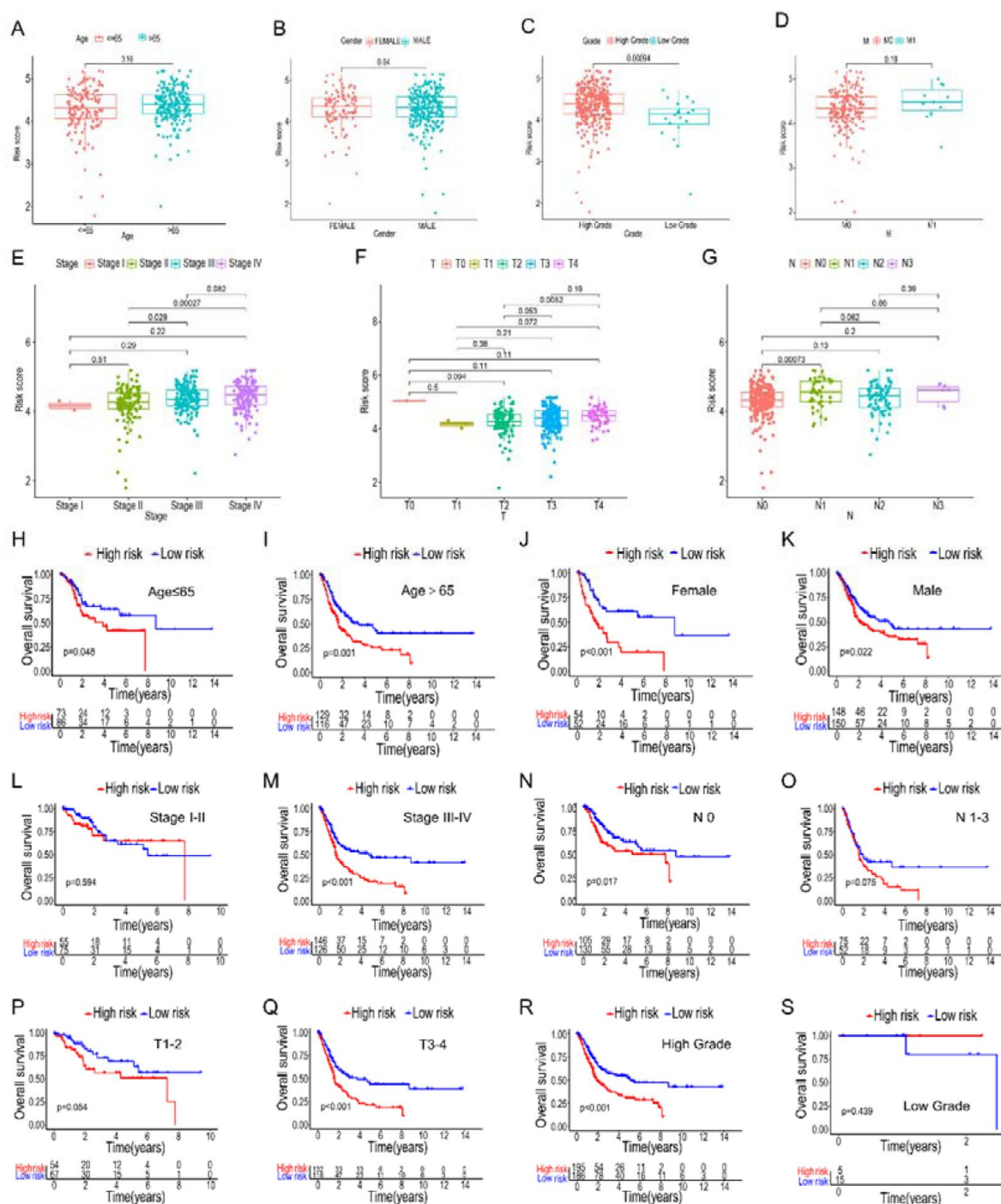


Figure S3.

Relationship between risk scores and different clinical features.

A-G. Relationship between Age, Gender, Grade, M, Stage, T, N, and risk scores. H-S. Survival analysis of subgroups based on Age, Gender, Grade, M, Stage, T, N, and risk scores.

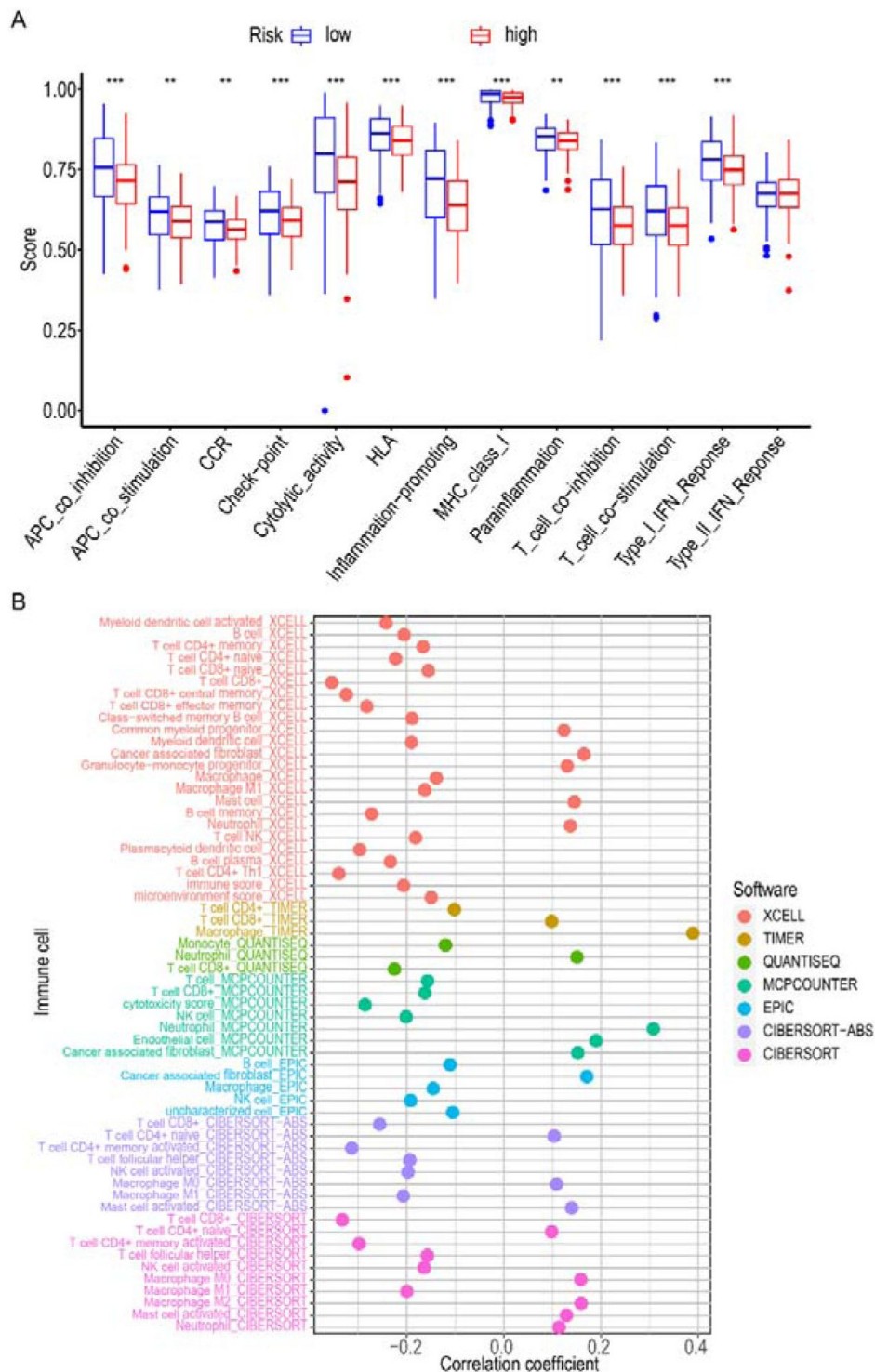


Figure S4.

The relationship between risk scores and immune-related pathways and immune infiltration.

A. The relationship between risk scores and immune-related pathways. B. The relationship between risk scores and immune infiltration.

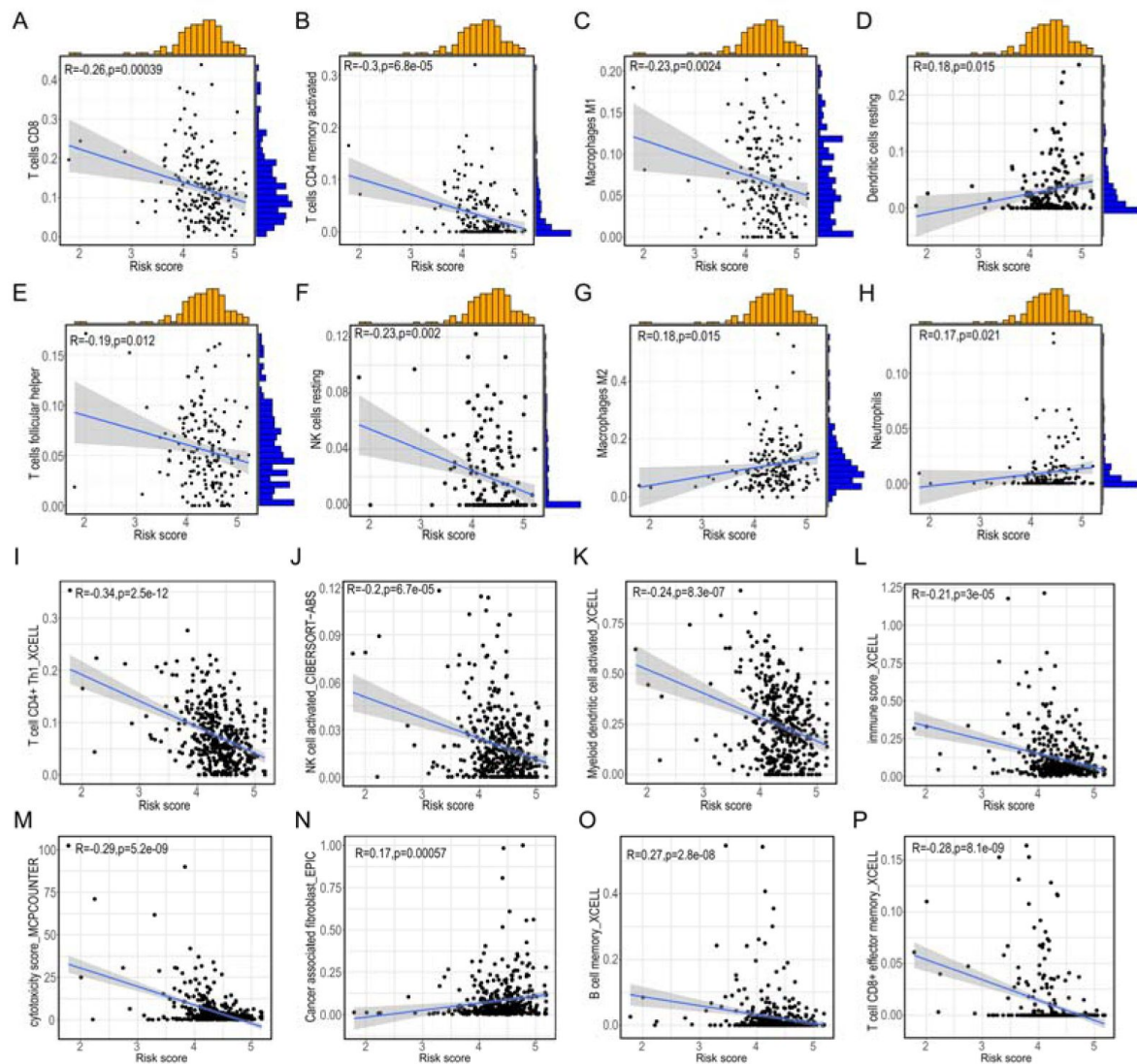


Figure S5.

The association between risk-scores and immune cells.

A-H. Correlation analysis of risk scores with immune cells. I-P. Analysis of the correlation between risk scores and immune cells using various algorithms.

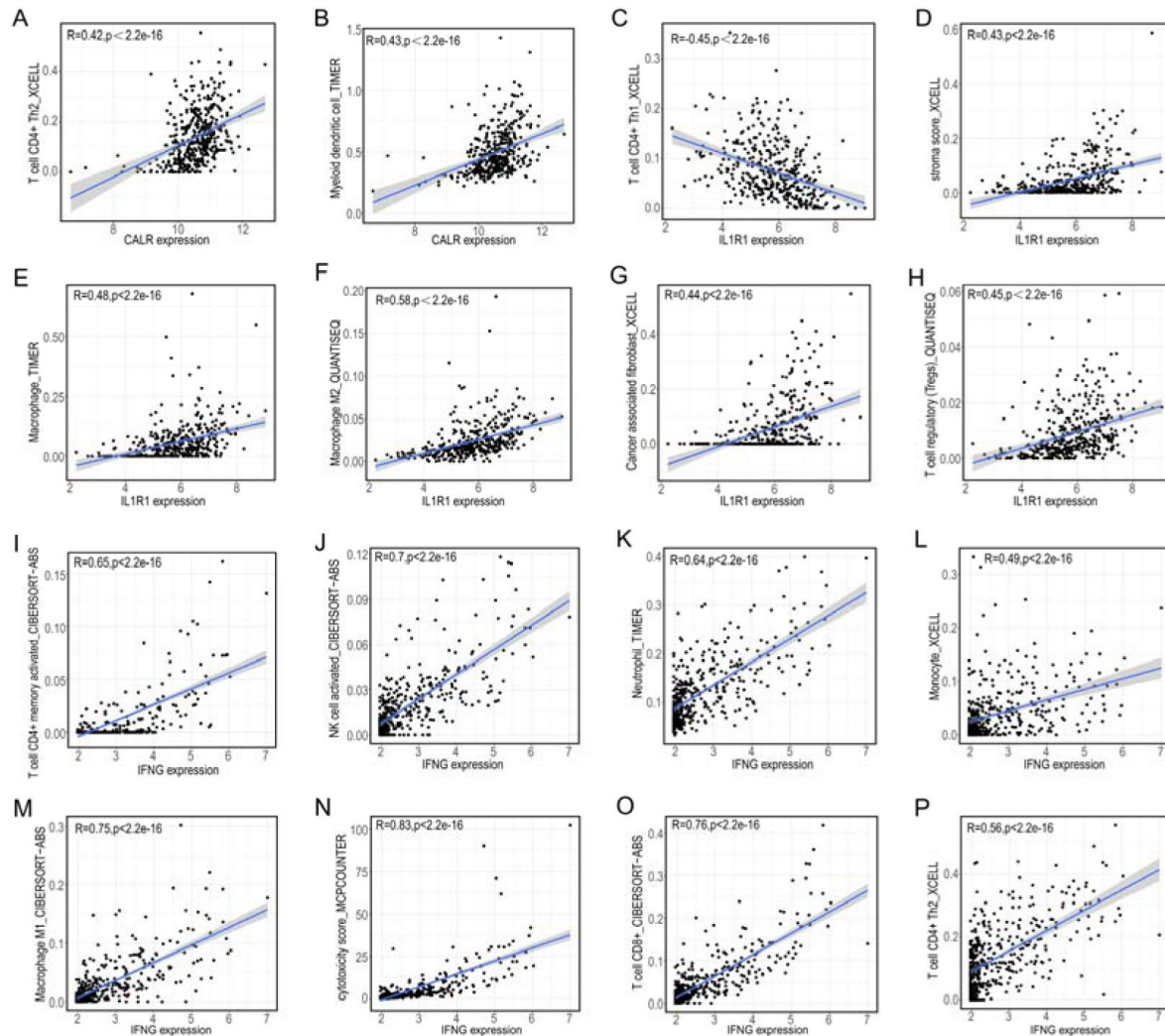
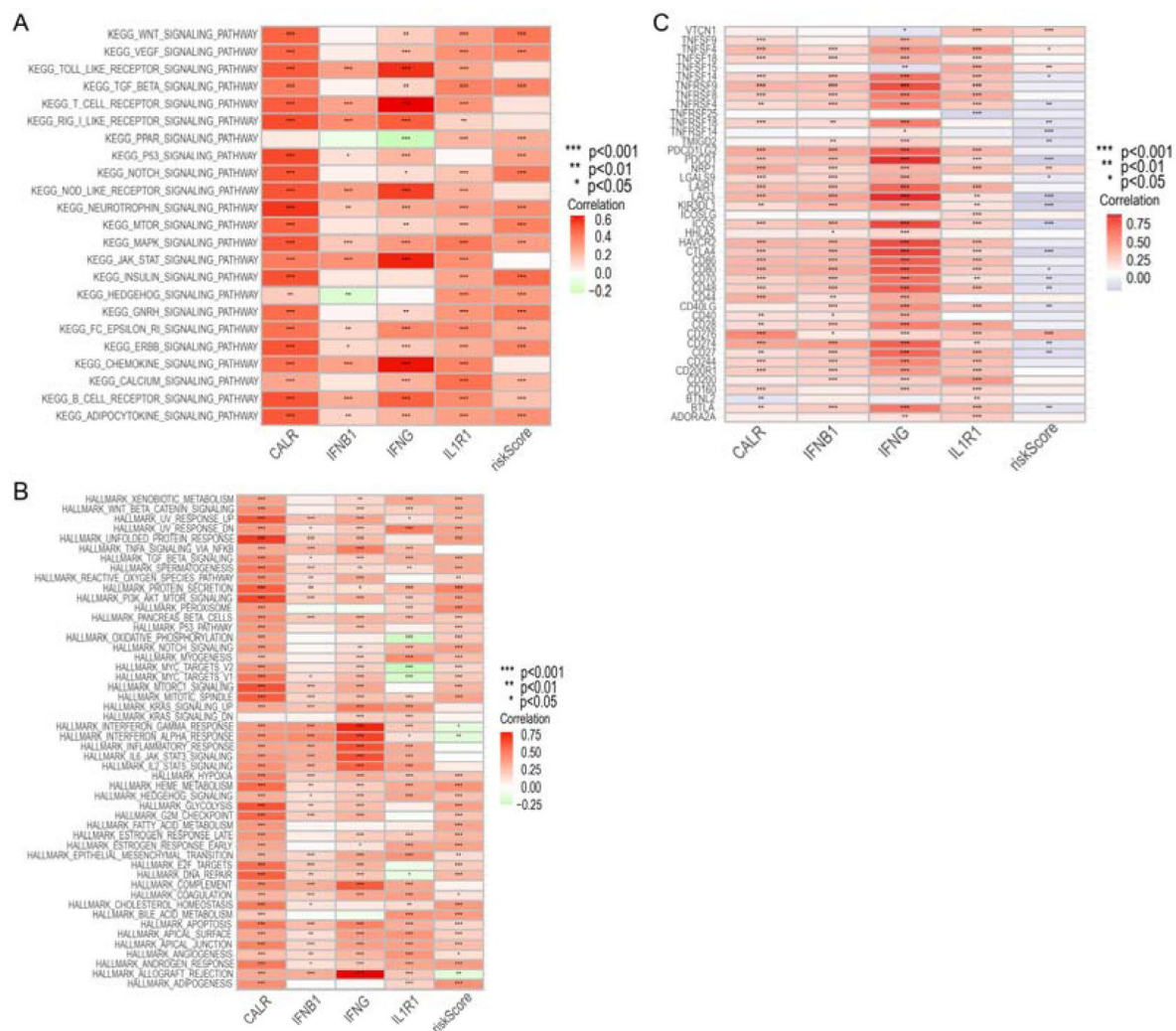


Figure S6.

The correlation of CALR, IFNG, and IL1R1 with immune cell infiltration.

A-B. The correlation of CALR with immune cell infiltration. C-H. The correlation of IL1R1 with immune cell infiltration. I-P. The correlation of IFNG with immune cell infiltration.



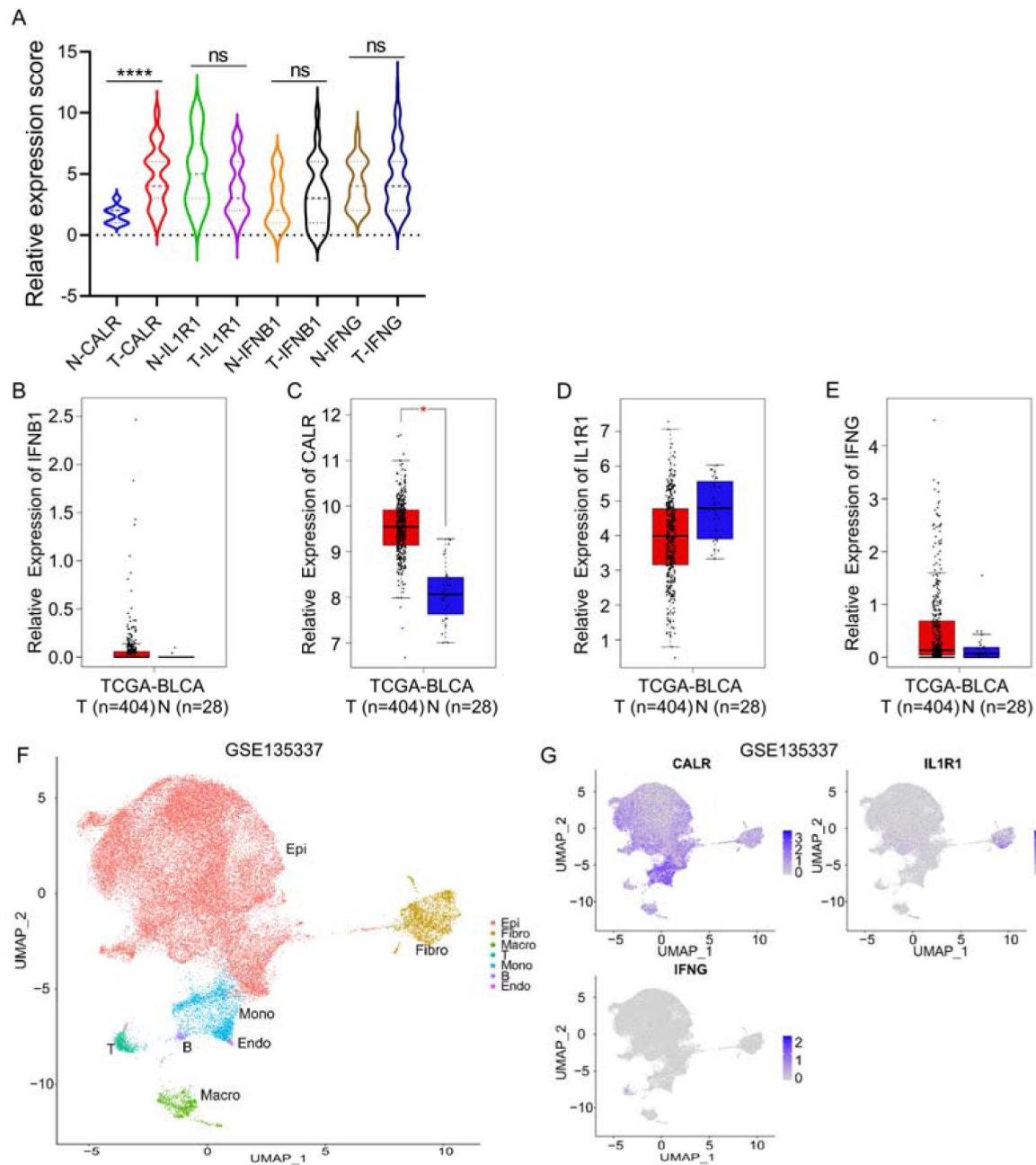


Figure S8.

Expression of CALR, IFN1B, IFNG, IL1R1 in tissues.

A. Expression of the four genes in cancer and adjacent tissues in tissue microarrays. B-E. Expression of CALR, IFN1B, IFNG, IL1R1 in cancer and adjacent tissues in TCGA. F-G. Expression of CALR, IFNG, IL1R1 in public database single-cell sequencing data (GSE135337).

Data Availability Statement

We obtained TCGA data from <https://portal.gdc.cancer.gov/> and GEO data from <https://www.ncbi.nlm.nih.gov/geo/>. Access our single-cell sequencing data¹⁴ at <https://ngdc.cnbc.ac.cn/gsa-human/browse/HRA000212> or <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA662018/>.

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None.

Additional information

CRedit authorship contribution statement

Lilong Liu: Funding acquisition; Data curation; Formal analysis; Writing – original draft; and Writing - review & editing. Zhenghao Liu, Lei Fan and Zhipeng Yao: Data curation; Formal analysis; Writing - original draft; and Writing - review & editing. Junyi Hu, Yaxin Hou, Yang Li, Yingchun Kuang, and Yuhong Ding: Data curation and Writing - review & editing. Yi Hao played crucial roles in Funding acquisition, conceptualization and Writing - review & editing. Zheng Liu, and Ke Chen played crucial roles in conceptualization and Writing - review & editing. All authors approved the final manuscript for publication.

Ethics approval and consent to participate

Patients provided informed consent for this work. All experimental procedures were approved by the Institutional Review Board of Tongji Medical College, Huazhong University of Science and Technology (IRB: S116).

Consent for publication

Not applicable.

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Reviewer #1 (Public review):

The authors aimed to explore the prognostic and therapeutic relevance of immunogenic cell death (ICD)-related genes in bladder cancer, focusing on a risk-scoring model involving CALR, IL1R1, IFNB1, and IFNG. The research indicates that higher expression of certain ICD-related genes is associated with enhanced immune infiltration, prolonged survival, and improved responsiveness to PD1-targeted therapy in bladder cancer patients.

Major strengths:

- The establishment of an ICD-related gene risk model based on publicly available datasets (TCGA and GEO) and further validated through tissue arrays and preliminary single-cell RNA sequencing data provides potential but weak clinical guidance.
- The integration of multi-dimensional data (gene expression, mutation burden, immune infiltration, and treatment responses) strengthens the clinical applicability of the model.

Key limitations and concerns:

(1) Gene Selection and Novelty:

The selection of genes predominantly reflects known regulators of immune responses, somewhat limiting the novelty. Exploring less-characterized ICD markers or extending validation beyond bladder cancer could improve the model's innovative aspect and wider clinical relevance.

(2) Reliance on RNA-Seq for Immune Infiltration:

Immune infiltration analyses based primarily on bulk RNA-Seq data have inherent methodological limitations, such as inability to distinguish cell subsets accurately. Incorporation of robust single-cell sequencing would significantly enhance the reliability of these findings. Although the authors recognize this limitation, future studies should directly address it.

(3) Drug Sensitivity and Immunotherapy Response Data:

While the authors clarify that the drug sensitivity analysis was performed using established databases (TCGA via pRRophetic), the unexpected correlations between ICD-related genes and various targeted therapies need further mechanistic validation. The observed relationships may reflect indirect associations rather than direct biological relevance, which warrants cautious interpretation.

(4) Presentation and Clarity Issues:

Initially noted formatting inconsistencies across figures compromised professional presentation; these have been corrected by the authors. Additionally, the authors have now provided essential methodological details, including clear sample sizes and database versions, enhancing reproducibility.

(5) Immunotherapy Response Evidence:

Conclusions regarding differences in immunotherapy response rates between patient subgroups, although intriguing, remain based on retrospective database analyses with relatively limited demographic and clinical detail. Future prospective studies or more detailed patient characterization would be required to robustly confirm these associations.

(6) Interpretation of ICD Gene Signatures:

The ICD-related gene set includes many genes broadly associated with immune activation rather than specifically ICD. Although this was addressed by the authors, clearly distinguishing ICD-specific versus general immune-response genes in future studies would help clarify biological implications.

Summary and Recommendations for Readers:

Overall, this study presents an interesting and clinically relevant risk-scoring approach to stratify bladder cancer patients based on ICD-related gene expression profiles. It provides useful information about prognosis, immune infiltration, and potential immunotherapy

responsiveness. However, readers should interpret the results within the context of its limitations, notably the need for broader validation and careful consideration of the biological significance underlying the observed associations. This work lays a valuable foundation for further investigation into the integration of ICD and immune response signatures in personalized cancer therapy.

<https://doi.org/10.7554/eLife.95326.2.sa1>

Author response:

The following is the authors' response to the original reviews.

Reviewer #1 (Recommendations for the authors):

Thank you for your thorough review of our manuscript and your valuable suggestions. Here are our responses to each point you raised:

(1) Novelty: Exploring the feasibility of extending the risk-scoring model to diverse cancer types could emphasize the broader impact of the research.

Thank you so much for your thoughtful and insightful feedback. Your suggestion to explore extending the risk-scoring model to diverse cancer types is truly valuable and demonstrates your broad vision in this field. We deeply appreciate your interest in our research and the effort you put into providing such constructive input.

After careful consideration, we have decided to focus our current study on the specific cancer type(s) we initially set out to explore. This decision was made to ensure that we can thoroughly address the research questions at hand, given our current resources, time constraints, and the complexity of the topic. By maintaining this focused approach, we aim to achieve more in-depth and reliable results that can contribute meaningfully to the understanding of this particular area.

However, we fully recognize the potential significance of your proposed direction and firmly believe that it could be an excellent avenue for future research. We will definitely keep your suggestion in mind and may explore it in subsequent studies as our research progresses and evolves.

(2) Improvement in Figure Presentation: The inconsistency in font formatting across figures, particularly in Figure 2 (A-D, E, F-H, I), Figure 3 (A-C, D-J, H, K), and the distinct style change in Figure 5, raises concerns about the professionalism of the visual presentation. It is recommended to standardize font sizes and styles for a more cohesive and visually appealing layout. This ensures that readers can easily follow and comprehend the graphical data presented in the article.

The text in the picture has been revised as requested.

(3) Enhancing Reliability of Immune Cell Infiltration Data: Address the potential limitations associated with relying solely on RNASeq data for immune cell infiltration analysis between ICD and ICD high groups in Figure 2. It is advisable to discuss the inherent challenges and potential biases in this methodology. To strengthen the evidence, consider incorporating bladder cancer single-cell sequencing data, which could provide a more comprehensive and reliable understanding of immune cell dynamics within the tumor microenvironment.

Thank you very much for your meticulous review and the highly constructive suggestions. Your insight regarding the limitations of relying on RNASeq data for immune cell infiltration analysis and the proposal to incorporate bladder cancer single-cell sequencing data truly reflect your profound understanding of the field. We deeply appreciate your efforts in guiding our research and the valuable perspectives you've offered.

After careful deliberation, given our current research scope, timeline, and available resources, we've decided to focus on further discussing and addressing the challenges and biases inherent in RNASeq-based immune cell infiltration analysis. By delving deeper into the methodological limitations and conducting more in-depth statistical validations, we aim to provide a comprehensive and reliable interpretation of the data within our study framework. This focused approach allows us to maintain the integrity of our original research design and deliver robust findings on the relationship between immune cell infiltration and ICD in the current context.

However, we fully acknowledge the significant value of your proposed single-cell sequencing approach. It is indeed a powerful method that could offer more detailed insights into immune cell dynamics, and we believe it holds great promise for future research in this area. We will keep your suggestion in mind as an important direction for potential future studies, especially when we plan to expand and deepen our exploration of the tumor microenvironment.

(4) Clarity in Data Sources and Interpretation of Figure 5: In the results section, provide a detailed and transparent explanation of the sources of data used in Figure 5. This includes specifying the databases or platforms from which the chemotherapy, targeted therapy, and immunotherapy data were obtained. Additionally, elucidate the rationale behind the chosen data sources and how they contribute to the overall interpretation of the study's findings. And, strangely, these immune-related genes are associated with cancer sensitivities to different targeted therapies.

Thank you very much for your detailed and valuable feedback on Figure 5. We sincerely appreciate your careful review and insightful suggestions, which have provided us with important directions for improvement.

Regarding the data sources in Figure 5, we used the pRRophetic algorithm to conduct a drug sensitivity analysis on the TCGA database. The reason for choosing these data sources is multi-faceted. Firstly, these databases and platforms are well-established and widely recognized in the field. They have strict data collection and verification processes, ensuring the accuracy and reliability of the data. For example, TCGA has a large-scale, long-term-accumulated chemotherapy case database, which can comprehensively reflect the clinical application and treatment effects of various chemotherapeutic drugs.

Secondly, these data sources cover a wide range of cancer types and patient information, which can meet the requirements of our study's diverse sample size and variety. This comprehensiveness enables us to conduct a more in-depth and representative analysis of the relationships between different therapies and immune-related genes.

In terms of the overall interpretation of the study's findings, the use of these data sources provides a solid foundation. The accurate chemotherapy, targeted therapy, and immunotherapy data help us clearly demonstrate the associations between immune-related genes and cancer sensitivities to different treatments. This allows us to draw more reliable conclusions and provides a scientific basis for understanding the complex mechanisms of cancer treatment from the perspective of immune-gene-therapy interactions.

As for the unexpected association between immune - related genes and cancer sensitivities to different targeted therapies, this is indeed a fascinating discovery. In our analysis, we hypothesized that immune - related genes may affect the tumor microenvironment, thereby influencing the response of cancer cells to targeted therapies. Although this finding is currently beyond our initial expectations, it has opened up a new research direction for us. We will further explore and verify the underlying mechanisms in future research.

Once again, thank you for your guidance. We will make corresponding revisions and improvements according to your suggestions to make our research more rigorous and complete.

(5) Legends and Methods: Address the brevity and lack of crucial details in the figure legends and methods section. Expand the figure legends to include essential information, such as the number of samples represented in each figure. In the methods section, provide comprehensive details, including the release dates of databases used, versions of coding packages, and any other pertinent information that is crucial for the reproducibility and reliability of the study.

We would like to express our sincere gratitude for your valuable feedback on the figure legends and methods section of our study. We highly appreciate your sharp observation of the issues regarding the brevity and lack of key details, which are crucial for further improving our research.

We have supplemented the methods section with data including the number of samples, the release dates of the databases used, and the versions of the coding packages, etc. For TCGA samples: 421 tumor samples and 19 normal samples. Database release date: March 29, 2022, v36 versions. Coding package version: R version 4.1.1. We will immediately proceed to supplement these key details, making the research process and methods transparent. This will allow other researchers to reproduce our study more accurately and enhance the persuasiveness of our research conclusions.

(6) Evidence Supporting Immunotherapy Response Rates: The importance of providing a robust foundation for the conclusion regarding lower immunotherapy response rates. Strengthen this section by offering a more detailed description of sample parameters, specifying patient demographics, and presenting any statistical measures that validate the observed trends in Figure 5Q-T. More survival data are required to conclude. Avoid overinterpretation of the results and emphasize the need for further investigation to solidify this aspect of the study.

Thank you very much for your professional and meticulous feedback on the content related to immunotherapy response rates in our study! Your suggestions, such as providing a solid foundation for the conclusions and supplementing key information, are of great value in enhancing the quality of our research, and we sincerely appreciate them.

The data in Figures 5Q to T are from the TCGA database, which has already been provided. The statistical measure used for Figures 5Q to T is the P-value, which has been marked in the figures. The survival data have been provided in Figure 3D.

Reviewer #2 (Recommendations for the authors):

Thank you for your thorough review of our manuscript and your valuable suggestions. Here are our responses to each point you raised:

(1) There is no information on the samples studied. Are all TCGA bladder cancer samples studied? Are these samples all treatment naïve? Were any excluded? Even simply, how many samples were studied?

Thank you so much for pointing out the lack of sample - related information. Your attention to these details has been extremely helpful in identifying areas for improvement in our study.

All the samples in our study were sourced from the TCGA (The Cancer Genome Atlas) and TCIA (The Cancer Immunome Atlas) databases. It should be noted that the patient data in the TCIA database are originally from the TCGA database. Regarding whether the patients received prior treatment, this information was not specifically mentioned in our current report. Instead, we mainly relied on the scores of the prediction model for evaluation. Since all samples were obtained from publicly available databases, we understand the importance of clarifying their origin and characteristics.

We sincerely apologize for the omission of the sample size and other relevant details. We will promptly supplement this crucial information in the revised version, including a detailed description of the sample sources and any relevant characteristics. This will ensure greater transparency and help readers better understand the basis of our research.

For TCGA samples: 421 tumor samples and 19 normal samples. Database release date: March 29, 2022, v36 versions. Coding package version: R version 4.1.1.

(2) What clustering method was used to divide patients into ICD high/low? The authors selected two clusters from their "unsupervised" clustering of samples with respect to the 34 gene signatures. A Delta area curve showing the relative change in area under the cumulative distribution function (CDF) for k clusters is omitted, but looking at the heatmap one could argue there are more than k=2 groups in that data. Why was k=2 chosen? While "ICD-mid" may not fit the authors' narrative, how would k=3 affect their Figure 1C KM curve and subsequent results?

Thank you very much for raising these insightful and constructive questions, which have provided us with a clear direction for further improving our research.

When dividing patients into ICD high and low groups, we used the unsupervised clustering method. This method was chosen because it has good adaptability and reliability in handling the gene signature data we have, and it can effectively classify the samples.

Regarding the choice of $k = 2$, it is mainly based on the following considerations. Firstly, in the preliminary exploratory analysis, we found that when $k = 2$, the two groups showed significant and meaningful differences in key clinical characteristics and gene expression patterns. These differences are closely related to the core issues of our study and help to clearly illustrate the distinctions between the ICD high and low groups. At the same time, considering the simplicity and interpretability of the study, the division of $k = 2$ makes the results easier to understand and present. Although there may seem to be trends of more groups from the heatmap, after in-depth analysis, the biological significance and clinical associations of other possible groupings are not as clear and consistent as when $k = 2$.

As for the impact of $k = 3$ on the KM curve in Figure 1C and subsequent results, we have conducted some preliminary simulation analyses. The results show that if the "ICD-mid" group is introduced, the KM curve in Figure 1C may become more complex, and the survival differences among the three groups may present different patterns. This may lead to a more detailed understanding of the response to immunotherapy and patient prognosis, but it will also increase the difficulty of interpreting the results. Since the biological characteristics and clinical significance of the "ICD-mid" group are relatively ambiguous, it may interfere with

the presentation of our main conclusions to a certain extent. Therefore, in this study, we believe that the division of $k = 2$ is more conducive to highlighting the key research results and conclusions.

Thank you again for your valuable comments. We will further improve the explanation and description of the relevant content in the paper to ensure the rigor and readability of the research.

(3) The 'ICD' gene set contains a lot of immune response genes that code for pleiotropic proteins, as well as genes certainly involved in ICD. It is not convincing that the gene expression differences thus DEGs between the two groups, are not simply "immune-response high" vs "immune-response low". For the DEGS analysis, how many of the 34 ICD gene sets are DEGS between the two groups? Of those, which markers of ICD are DEGs vs. those that are related to immune activation?

a. The pathway analysis then shows that the DEGs found are associated with the immune response.

b. Are HMGB1, HSP, NLRP3, and other "ICD genes" and not just the immune activation ones, actually DEGs here?

c. Figures D, I-J are not legible in the manus.

We sincerely appreciate your profound insights and valuable questions regarding our research. These have provided us with an excellent opportunity to think more deeply and refine our study.

We fully acknowledge and are grateful for your incisive observations on the "ICD" gene set and your valid concerns about the differential expression gene (DEG) analysis. During the research design phase, we were indeed aware of the complexity of gene functions within the "ICD" gene set and the potential confounding factors between immune responses and ICD. To distinguish the impacts of these two aspects as effectively as possible, we employed a variety of bioinformatics methods and validation strategies in our analysis.

Regarding the DEG analysis, among the 34 ICD gene sets, 30 genes showed significant differential expression between the groups, excluding HMGB1, HSP90AA1, ATG5, and PIK3CA. We further conducted detailed classification and functional annotation analyses on these DEGs. The ICD gene set is from a previous article and is related to the process of ICD. Relevant literature is in the materials section. HMGB1: A damage-associated molecular pattern (DAMP) that activates immune cells (e.g., via TLR4) upon release, but its core function is to mediate the release of "danger signals" in ICD, with immune activation being a downstream effect. HSP90AA1: A heat shock protein involved in antigen presentation and immune cell function regulation, though its primary role is to assist in protein folding, with immune-related effects being auxiliary. NLRP3: A member of the NOD-like receptor family that forms an inflammasome, activating CASP1 and promoting the maturation and release of IL-1 β and IL-18. Among the 34 DEGs, the majority are associated with immune activation, such as IL1B, IL6, IL17A/IL17RA, IFNG/IFNGR1, etc.

(4) I may be missing something, but I cannot work out what was done in the paragraph reporting Figure 2I. Where is the ICB data from? How has this been analysed? What is the cohort? Where are the methods?

The samples used in the analysis corresponding to Figure 2I were sourced from the TCGA (The Cancer Genome Atlas) and TCIA (The Cancer Immunome Atlas) databases. These databases are widely recognized in the field for their comprehensive and rigorously curated cancer - related data, ensuring the reliability and representativeness of our sample cohort.

Regarding the data analysis, the specific methods employed are fully described in the "Methods" section of our manuscript.

(5) How were the four genes for your risk model selected? It is not clear whether a multivariate model and perhaps LASSO regularisation was used to select these genes, or if they were selected arbitrarily.

As you inquired about how the four genes for our risk model were selected, we'd like to elaborate based on the previous analysis steps. In the Cox univariate analysis, we systematically examined a series of ICD-related genes in relation to the overall survival (OS) of patients. Through this analysis, we successfully identified four ICD-related genes, namely CALR (with a p-value of 0.003), IFNB1 (p = 0.037), IFNG (p = 0.022), and IF1R1 (p = 0.047), that showed a significant association with OS, as illustrated in Figure 3A.

Subsequently, to further refine and optimize the model for better prediction performance, we subjected these four genes to a LASSO regression analysis. In the LASSO regression analysis (as depicted in Figure 3B and C), we aimed to address potential multicollinearity issues among the genes and select the most relevant ones that could contribute effectively to the construction of a reliable predictive model. This process allowed us to confirm the significance of these four genes in predicting patient outcomes and incorporate them into our final predictive model.

(6) How related are the high-risk and ICD-high groups? It is not clear. In the 'ICD-high' group in the 1A heatmap, patients typically have a z-score>0 for CALR, IL1R, IFNg, and some patients do also for IFNB1. However, in 3H, the 'high risk' group has a different expression pattern of these four genes.

Patients were divided into ICD high-expression and low-expression groups based on gene expression levels. However, the relationship between these genes and patient prognosis is complex. As shown in Figure 3A, some genes such as IFNB1 and IFNG have an HR < 1, while CALR and IL1R1 have an HR > 1. Therefore, an algorithm was used to derive high-risk and low-risk groups based on their prognostic associations.

(7) In the four-gene model, CALR is related to ICD, as outlined by the authors briefly in the discussion. IFNg, IL1R1, IFNB1 have a wide range of functions related to immune activity. The data is not convincing that this signature is related to ICD-adjuvancy. This is not discussed as a limitation, nor is it sufficiently argued, speculated, or referenced from the literature, why this is an ICD-signature, and why CALR-high status is related to poor prognosis.

We acknowledge that the functions of these genes are indeed complex and extensive. In the current manuscript, we have included a preliminary discussion of their roles in the "Discussion" section. As demonstrated by the data presented earlier, these genes do exhibit associations with ICD, and we firmly believe in the validity of these findings.

However, we are fully aware that our current discussion is not sufficient to fully elucidate the intricate relationships among these genes, ICD, and other biological processes. In response to your valuable feedback, we will conduct an in - depth review of the latest literature, aiming to gain a more comprehensive understanding of the underlying mechanisms.

(8) Score is spelt incorrectly in Figures 3F-J.

Figures 3F-J have been revised as requested.

(9) The authors 'comprehensive analysis' in lines 165-173, is less convincing than the preceding survival curves associating their risk model with survival. Their 'correlations' have no statistics.

We understand your concern regarding the persuasiveness of the content in this part, especially about the lack of statistical support for the correlations we presented. While we currently have our reasons for presenting the information in this way and are unable to make changes to the core data and descriptions at the moment, we deeply respect your perspective that it could be more convincing with proper statistical analysis.

(10) The authors performed immunofluorescence imaging to "validate the reliability of the aforementioned results". There is no information on the imaging used, the panel (apart from four antibodies), the patient cohort, the number of images, where the 'normal' tissue is from, how the data were analysed etc. This data is not interpretable without this information.

a. Is CD39 in the panel? CD8, LAG3? It's not clear what this analysis is.

The color of each antibody has been marked in Fig 2B. The cohort information and its source have been supplemented. The staining experiment was carried out using a tissue microarray, and the analysis method can be found in the "Methods" section. Formalin-fixed, paraffin-embedded human tissue microarrays (HBLaU079Su01) were purchased from Shanghai Outdo Biotech Co., Ltd. (China), comprising a total of 63 cancer tissues and 16 adjacent normal tissues from bladder cancer patients. Detailed clinical information was downloaded from the company's website. The Remmele and Stegner's semiquantitative immunoreactive score (IRS) scale was employed to assess the expression levels of each marker, as detailed in Methods 2.5. CD39, CD8, and LAG3 were also stained, but the results were not presented.

(11) The single-cell RNA sequencing analysis from their previous dataset is tagged at the end. CALR expression in most identified cells is interesting. Not clear what this adds to the work beyond 'we did scRNA-seq'. How were these data analysed? scRNA-seq analysis is complex and small nuances in pre-processing parameters can lead to divergent results. The details of such analysis are required!

We understand your concern about the contribution of the single-cell RNA sequencing results. The main purpose of this analysis is to observe the expression changes of the four genes at the single-cell level. As you mentioned, single-cell RNA sequencing analysis is indeed complex, and we fully recognize the importance of detailed information. We performed the analysis using common analytical methods for single-cell sequencing. It has been supplemented in the Methods section.

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