

A multi-omics analysis and clinical relevance of regulated cell death in bladder cancer

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Background: Regulated cell death (RCD) is critical for tumor progression and treatment response, but its integrated role in bladder cancer remains unclear. This study aimed to systematically characterize the cross-talk, multi-omics alterations, and clinical relevance of major RCD pathways in bladder cancer.

Methods: Publicly available bladder cancer cohorts were analyzed to characterize the expression patterns and integrated regulation of major regulated cell death (RCD) programs. A curated set of 862 RCD-related genes was compiled from public databases and published literature, encompassing apoptosis, ferroptosis, pyroptosis, necroptosis, and autophagy-dependent cell death. Patients were stratified according to RCD-related transcriptional profiles, and associations with prognosis, tumor immune features, and therapeutic sensitivity were systematically evaluated. Selected RCD-associated hub gene was further examined through functional experiments in bladder cancer cell lines.

Results: Extensive cross-talk among RCD-related genes was observed across genomic, epigenetic, and transcriptional levels. RCD gene expression was influenced by

somatic mutation, copy number alteration, DNA methylation, and RNA modification. Bladder cancer patients (TCGA-BLCA, $n = 402$ for survival analysis) were stratified into two distinct RCD clusters demonstrating significantly different survival outcomes (log-rank $p < 0.05$), immune pathway enrichment, predicted immunotherapy responsiveness, and chemotherapy sensitivity profiles. RCD cluster 1 was predominantly associated with basal/squamous molecular subtypes, whereas RCD cluster 2 corresponded to luminal subtypes. Prognostic gene modules underlying cluster heterogeneity were identified. Among the identified hub genes, TIMP1 was functionally validated, and the silencing of TIMP1 significantly inhibited the proliferation and migration of bladder cancer cells ($p < 0.05$), supporting its association with aggressive tumor phenotypes identified from the RCD-related network.

Conclusions: RCD-based molecular stratification defines clinically distinct bladder cancer subtypes associated with prognosis and therapeutic response. These findings provide a framework for personalized treatment strategies and support the translational potential of targeting regulated cell death pathways in bladder cancer.

Key Words: regulated cell death, bladder cancer, genetic alteration, cross-talk, single-cell sequencing

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Introduction

Bladder cancer (BLCA) is one of the top 10 most common and deadly malignancies due to its high recurrence risk and treatment failures.¹ Current therapeutic options for bladder cancer include radical surgery, neo-adjuvant therapy, traditional chemotherapy, immunotherapy, and targeted therapy.^{2,3} Despite improvements in treatment modalities, a substantial proportion of patients still experience treatment failure or require alternative therapeutic strategies, such as being ineligible for cisplatin due to poor

performance status, low response rate, recurrence due to drug resistance, and severe complications.⁴⁻⁶ Therefore, it is essential to explore the mechanisms underlying drug resistance in bladder cancer cells. Improving current treatment strategies may help prevent disease progression and ultimately improve patient prognosis.⁷

Regulated cell death (RCD) is essential in removing damaged, infected, or excess cells.⁸ So far, more than 20 types of RCD have been characterized.⁹ Among these, apoptosis, ferroptosis, pyroptosis, autophagy, and necroptosis are five types of programmed cell death (PCD), and most of them participate in the breakdown of the plasma membrane.¹⁰ The dysfunction of single or mixed types of RCD contributes to human diseases, such as autoimmune diseases, infectious diseases, and cancer.¹¹ Canonical RCD programs are typically governed by distinct signaling cascades. However, increasing evidence suggests substantial cross-talk between different RCD pathways. For example, the necroptotic and apoptotic pathways are closely linked through the activity of caspase-8.¹² The pyroptosis gene GSDME has been reported to regulate the membrane permeabilization that occurs late in apoptosis recently;¹³ cells which undergoing necroptosis can trigger the pyroptotic machinery, such that their death is accompanied by the activating cleavage of IL-18 and IL-1 β .¹⁴ autophagy-dependent cell death can be inhibited by blocking the upstream Na⁺/K⁺-ATPase;¹⁵ Na⁺/K⁺-ATPase activity could lead to ferroptosis due to iron overload stimulation.¹⁶ In bladder cancer, several therapeutic agents have been reported to affect tumor cell survival through RCD-related mechanisms. For instance, sorafenib could regulate the *xCT/GPX4/ROS pathways to cause bladder cancer cells death in vitro*;¹⁷ down-regulation of miR-27a could specifically promote GSH biosynthesis and then enhance the resistance of bladder cancer cells to cisplatin;¹⁸ FGFR inhibitors exhibit significant anti-tumor activity in bladder cancer.¹⁹ However, few studies were conducted on the cross-talk, regulatory mechanisms, and clinical relevance of regulatory cell death in bladder cancer, which requires further investigation.

In this study, we systematically investigated the cross-talk, molecular characteristics, and clinical relevance of major regulated cell death-related genes (RCDGs), including apoptosis, necroptosis, pyroptosis, ferroptosis, and autophagy-dependent cell death, in bladder cancer. We aimed to characterize the multi-omics regulatory landscape of RCDGs, evaluate their associations with prognosis, immune microenvironment, and therapeutic response, and explore their

relationships with molecular subtypes and tumor heterogeneity. In addition, functional experiments were performed to validate selected hub gene associated with RCD-related networks in bladder cancer.

Methods

Identification of RCD-related genes and acquisition of public data

RCDGs were curated from publicly available databases and published literature (details in Supplementary Table S1a). Genes were included if they were annotated in curated public databases or supported by published experimental evidence indicating involvement in at least one of five major regulated cell death processes: apoptosis, ferroptosis, autophagy-dependent cell death, pyroptosis, or necroptosis. For genes appearing in more than one RCD category, overlap analysis was performed using the 'VennDiagram' R package (version 1.7.3), and these overlapping genes were defined as multifunction RCDGs. The numbers of genes in each RCD category were calculated before collapsing overlapping annotations, whereas the final integrated RCDG set was generated after duplicate gene symbols were removed. Genes lacking database annotation or literature evidence for functional involvement in regulated cell death were excluded. Gene lists obtained from different sources were merged, duplicate entries were removed, and genes involved in multiple RCD programs were retained and classified as multifunction RCDGs. Using these criteria, we ultimately identified 445 apoptosis genes, 254 ferroptosis genes, 222 autophagy genes, 40 pyroptosis genes, and 28 necroptosis genes, after removing duplicate entries for genes participating in multiple RCD programs, a total of 862 unique regulated cell death-related genes (RCDGs) were retained for subsequent analyses. Then, we downloaded the RNA-seq expression and clinical data of 33 different cancer types from the TCGA database from the UCSC Xena (Table S1b) (<https://xena.ucsc.edu/>; accessed via <https://xenabrowser.net/datapages/?hub=https://pancanatlas.xenahubs.net:443>). All the TCGA RNA-seq expression values were normalized and transformed to CPM units by utilizing the R software (version 4.3.3) and the 'edgeR' R package (version 3.42.4) to allow for subsequent analyses, and log₂(CPM + 1)-transformed values were used for downstream visualization and expression comparison analyses. CPM normalization was selected because the downstream analyses

focused primarily on relative expression differences of the same genes across samples or clinical groups after library size normalization, rather than direct comparison of absolute transcript abundance between different genes. For TCGA-BLCA, 411 samples were initially included for transcriptomic and mutation-related analyses. Because different TCGA molecular platforms and downstream analyses contain variable sample availability, analysis-specific cohorts were used according to the availability of mutation, CNV, clinical, survival, and molecular subtype annotation data. For survival analysis, samples lacking complete overall survival (OS) information were excluded, resulting in a final cohort of 402 patients for Kaplan-Meier analysis. For molecular subtype association analysis, duplicated patient entries were identified, including 10 entries from 5 patients; after removing 5 duplicated samples, 406 unique patients were retained for Fisher's exact test. Then we downloaded the GSE13507, GSE48075, GSE48276, GSE135337 normalized data for subsequent validation (<https://www.ncbi.nlm.nih.gov/gds>). The GSE135337 dataset was subsequently used for single-cell clustering, cell type annotation, and analysis of RCD-related gene distribution across bladder cancer cell subtypes. Cell-type annotation was performed using the 'SingleR' R package (version 2.0.0).

The somatic mutation, DNA methylation, and copy number variation (CNV) data of TCGA-BLCA were also downloaded from the UCSC Xena. Among the 411 TCGA-BLCA transcriptomic samples, CNV profiles were available for 408 samples and were therefore used for CNV-related analyses. We downloaded the somatic mutation data of CCLE-BLCA (<https://xenabrowser.net/hub/>) and ICGC-BLCA (<https://dcc.icgc.org/>) for validation. The copy number data of CCLE-BLCA was also obtained for validation (<https://xenabrowser.net/hub/>). The CCLE-BLCA cohort used in this study included all bladder cancer cell lines with available CNV profiles in the CCLE database at the time of analysis. Because the number of available bladder cancer cell lines was limited, CCLE-based analyses were primarily intended as supportive validation of trends observed in TCGA-BLCA rather than definitive population-level estimates.

RNA modification-related analyses included correlation analyses between RCDGs and known m⁶A/m⁵C regulators, as well as m⁵C-specific analyses based on published m⁵C modification datasets. The m⁵C modification DMGs data were obtained from previous literature.²⁰ GSE133624, GSE133621, and GSE175714 were obtained from the GEO database

(<https://www.ncbi.nlm.nih.gov/gds>), and Yang et al.'s data²¹ were used to explore the relationship between m⁵C methylation and RCDGs' expression.

A detailed summary of dataset usage across analyses is provided in the Supplementary Table S2.

Hierarchical clustering of RCDGs

The RNA-seq expression data of RCDGs from the TCGA and GEO databases were used to perform unsupervised clustering utilizing the 'factoextra' R-package (version 1.0.7). Then we computed the Euclidean distance of different samples and ward.D2 method to perform the hierarchical clustering. The GEO datasets, including GSE13507, GSE48075, and GSE48276, were not merged with the TCGA-BLCA cohort; instead, each GEO dataset was analyzed independently using the same clustering strategy as external validation.

To visualize the global expression patterns of RCDGs in tumor and normal samples, t-distributed stochastic neighbor embedding (t-SNE) analysis was performed using the 'Rtsne' R package (version 0.17). The t-SNE analysis was applied only for dimensionality reduction and visualization of transcriptomic distribution patterns and was not used to define the RCD clusters.

The number of clusters was determined based on the hierarchical clustering dendrogram together with downstream clinical relevance and biological interpretability. A two-cluster solution was selected because it produced distinct prognostic stratification in the TCGA-BLCA cohort and showed consistent clustering patterns in independent GEO validation datasets. Ultimately, we stratified the TCGA-BLCA samples into two RCD clusters.

Integrated bioinformatics analysis

Bioinformatics analyses were performed to comprehensively characterize regulated cell death-related genes (RCDGs) in bladder cancer. These analyses included somatic mutation profiling, copy number variation (CNV) analysis, DNA methylation analysis, RNA modification analysis, ceRNA and transcriptional regulatory network construction, differential expression analysis, therapeutic response prediction, molecular mechanism exploration, immune infiltration assessment, weighted gene co-expression network analysis (WGCNA), survival analysis, and single-cell sequencing analysis.

Differential expression genes (DEGs) between RCD cluster1 and cluster2 were identified and subjected to Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment

analyses using the “ClusterProfiler” R package (version 4.10.1). Gene set enrichment analysis (GSEA) and gene set variation analysis (GSVA) were further performed to explore pathway-level alterations between RCD clusters. To evaluate tumor microenvironment characteristics, immune and stromal cell activity scores were estimated using the “xCell” R package (version 1.1.0) based on transcriptomic expression profiles. ESTIMATE-derived ImmuneScore, StromalScore, and TumorPurity were calculated using normalized logCPM expression data with gene symbols as rows and TCGA-BLCA primary tumor samples as columns. Duplicate gene symbols were merged using `limma::avereps`, and ESTIMATE scores were interpreted as relative ssGSEA-derived enrichment scores rather than absolute measurements of immune or stromal cell abundance. Potential immunotherapy response was evaluated using the Tumor Immune Dysfunction and Exclusion (TIDE) web platform. The distributions of predicted responders and non-responders between RCD cluster 1 and cluster 2 were compared using the chi-square test.

Detailed computational workflows, datasets, parameter settings, and corresponding references are provided in the Supplementary Methods.

Cell culture and reagents

The human bladder cancer cell lines T24 and 5637 were obtained from the Cell Resource Center at the Shanghai Institute of Biological Sciences, Chinese Academy of Sciences (Shanghai, China). Cell line authentication was performed by the provider using short tandem repeat (STR) profiling, with no evidence of cross-contamination. The cell lines were provided as mycoplasma-free by the supplier. Cells were cultured in RPMI-1640 medium (Gibco, Grand Island, NY, USA, Cat#11875093) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin antibiotics (Gibco, USA Cat#15140122). All cells were maintained in a humidified atmosphere containing 5% CO₂. To achieve TIMP1 knockdown, three candidate siRNAs and a non-specific negative control were synthesized (Genepharma, China). The sequence for the most effective siRNA was sense, 5'-GCAAUCCGACCUCGUCAUTT-3' and antisense, 5'-AUGACGAGGUCGGAUUGCTT-3'. T24 and 5637 cells were transfected using Lipo3000 (Beyotime Biotechnology, Shanghai, China, Cat# R33136) according to the manufacturer's instructions. Cells were harvested or subjected to functional assays 48 h after transfection unless otherwise specified. The sequences of all tested siRNAs are provided in the Supplementary Methods.

Western blot

Total protein was extracted from T24 and 5637 cells using RIPA lysis buffer (Beyotime Biotechnology, China, Cat#P0013C) supplemented with protease inhibitors. Protein concentrations were determined using a BCA Protein Assay Kit (Beyotime Biotechnology, China, Cat#P0009). Samples were loaded at 20 µg per lane, and separated by 10% SDS-PAGE, then transferred onto PVDF membranes (Beyotime Biotechnology, China, Cat#FFP103-1roll) at 300 mA constant current for 90 min. Membranes were blocked with 5% non-fat milk in TBST for 1 h at room temperature and then incubated overnight at 4°C with primary antibodies against TIMP1 (Proteintech, Wuhan, China, Cat#16644-1-AP, 1:1000) and β-actin (Proteintech, China, Cat#60008-1-Ig, 1:10000). After washing, membranes were incubated with HRP-conjugated secondary antibodies (Proteintech, China, Cat#SA00001-2, 1:10000) for 1 h at room temperature. Protein bands were visualized using an enhanced chemiluminescence (Beyotime Biotechnology, China, Cat#P0018S) and quantified using ImageJ software (version 1.54, National Institutes of Health, Bethesda, MD, USA). Band intensities were normalized to β-actin expression. Western blot was performed with three independent biological replicates (n = 3).

Cell counting kit-8 (CCK8) assay

Cell viability was assessed using the Cell Counting Kit-8. Transfected T24 and 5637 cells were seeded into 96-well plates at a density of 5×10^3 per well and incubated for 0, 24, 48, and 72 h. Subsequently, 10 µL of CCK-8 reagent (Beyotime Biotechnology, China, Cat#C0038) was added to each well and incubated for 2 h at 37°C. The absorbance was measured at 450 nm using a microplate reader (Molecular Devices, San Jose, CA, USA, SpectraMax i3x). CCK-8 assay was performed with three independent biological replicates (n = 3).

Transwell assay

Cell migration was assessed using transwell chambers. Transfected T24 and 5637 cells in serum-free medium were seeded into the upper chamber, while medium containing 10% FBS was added to the lower chamber as a chemoattractant. After 24 h, non-migrated cells were removed, and migrated cells were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet for observation. The Transwell assay was performed with three independent biological replicates. For quantification, three random non-overlapping visual fields per well of migrated cells were counted using ImageJ software. Each replicate was measured in triplicate technical replicates.

Wound healing assay

Cell migration was evaluated using a wound healing assay. T24 and 5637 cells were initially seeded into 6-well plates at a density of 5×10^5 cells per well, and cultured until reaching 100% confluence, respectively, and a sterile 200 μ L pipette tip was used to create a vertical wound. After washing with PBS (0.01 mol/L, pH 7.4), cells were cultured in low-serum RPMI-1640 medium containing 1% FBS for 24 h. Images were captured at 0, 12, and 24 h to observe the wound closure rate. Each group included three independent biological replicates.

Statistical analyses

The unpaired *t*-test was utilized to compare the difference between the two groups. The chi-square test was used to detect the constituent ratio differences in different groups. The log-rank test was used to estimate the relationship between the variant and the prognosis of patients. Meanwhile, Pearson and Spearman were common methods to assess the correlation between the two variants. For all correlation analyses, $|r| > 0.3$ and adjusted. *p*-value (FDR) < 0.05 was significant. For differential expression analysis, genes with $|\log FC| > 0.5$ and adjusted *p*-value < 0.05 were regarded as DEGs. This moderate fold-change threshold was selected because the study focused on pathway-level and network-level alterations of curated RCD-related genes, where coordinated changes of moderate magnitude may be biologically meaningful. For differential methylation analysis, methylation differences were represented as $\Delta\beta$ values, calculated as the difference in mean β values between bladder cancer and normal tissues. Sites with $|\Delta\beta| > 0.2$ and adjusted *p*-value < 0.05 were regarded as differentially methylated sites. Genes associated with these sites were defined as differentially methylated genes (DMGs). This threshold was selected because moderate DNA methylation differences may still contribute to transcriptional regulation. These thresholds were used for exploratory integrative analyses and were interpreted together with correlation, pathway enrichment, WGCNA, survival, and validation analyses rather than as standalone evidence.

Results

Introduction of main RCD types and the workflow in this study

As shown in [Figure 1A](#), the main morphological characteristics of apoptosis are cell shrinkage, nuclear

condensation, karyorrhexis, and apoptotic body formation.²² Unlike apoptosis, pyroptosis could be triggered by activation of CASP1 or CASP11, and the Gasdermin D (GSDMD) formed a GSDMD-C and GSDMD-N fragment. The GSDMD-N binds to cell membrane phospholipids and induces the formation of cleavage pores, which leads to membrane lysis.^{22,23} Ferroptosis is driven by iron accumulation-induced ROS production and lipid peroxidation, which lead to dysmorphic small mitochondria with decreased crista, and condensed, ruptured outer membranes.²² In the process of autophagy, the main steps include the fusion of the autophagosome with the lysosome and autolysosome, and membrane rupture.²⁴ Necroptosis is mainly regulated by activation of threonine kinase 3 (RIPK3), activation of mixed lineage kinase domain-like pseudokinase (MLKL), and membrane rupture.²⁵ These RCDs mentioned above were the primary manner of programmed cell death, most of which are related to the rupture of the plasma membrane.¹⁰ To explore the cross-talk, molecular mechanisms, transcriptional regulation, and clinical relevance of RCDs in bladder cancer, we generated a 5 step analysis pipeline: i, Checking databases and relevant literature to determine these five types of RCD-related genes, then reclassified these genes, identifying the complex relationship is exist in mRNA correlation and PPI network analysis; ii, Analyzing the cross-talk and regulatory relationship of RCDGs in the aspect of somatic mutation, CNVs, DNA methylation, RNA modification, ceRNA network, and regulator network; iii, based on the gene Dependency Score (gDS), IC₅₀ of chemotherapeutic drug, aneuploidy score, and radiosensitivity index, investigating the importance of RCDGs in bladder cancer; iv, RCD cluster construction, molecular mechanisms, immune infiltration, and therapy; v, exploring the relationship between RCD clusters and consensus cluster, and the distribution of RCDGs in BLCA single-cell subtypes ([Figure 1B](#)). Referring to the public database and high-quality literature, we ultimately identified 445 apoptosis genes, 254 ferroptosis genes, 222 autophagy genes, 40 pyroptosis genes, and 28 necroptosis genes ([Figure 1C](#), [Table S1a](#)). The Venn diagram showed that several genes functioned in different RCD types ([Figure 1D](#), [Table 1](#)). To facilitate subsequent studies, we named these genes multifunction genes because they do not fall into any single category of RCD.

Multi-omics landscape reveals extensive regulation of RCDGs in bladder cancer

To characterize the global regulatory landscape of RCDGs in bladder cancer, we performed

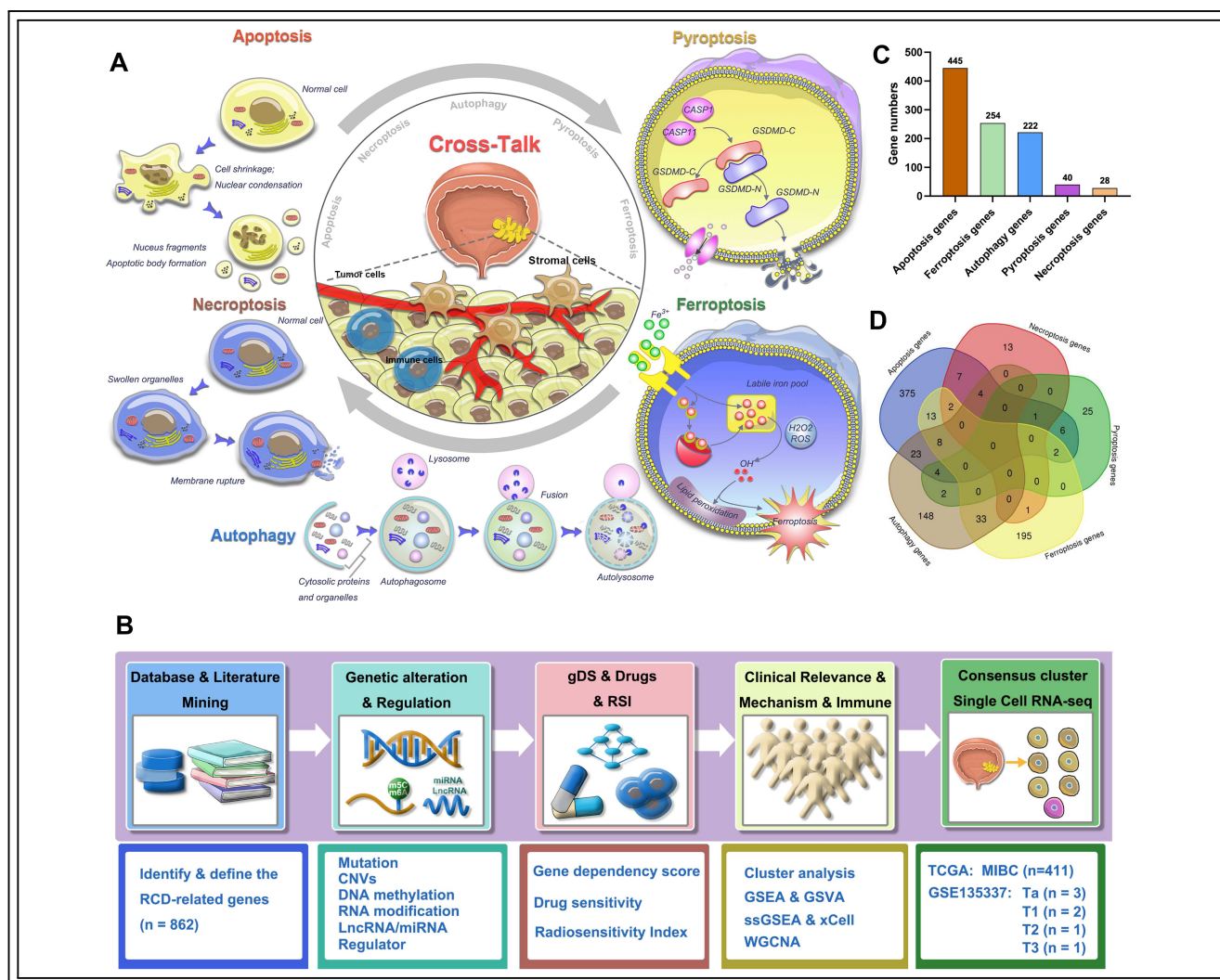


FIGURE 1. Introduction of main RCD types and the workflow. (A) The progression of apoptosis, necroptosis, autophagy, pyroptosis, and ferroptosis. (B) Main workflow in this study. The TCGA-BLCA cohort initially included 411 transcriptomic samples prior to analysis-specific filtering. Different downstream analyses used analysis-specific cohorts according to the availability of molecular platform data and clinical annotations. The label “RNA modification” in the workflow summarizes the exploratory RNA modification regulator analysis and the subsequent m5C-focused analysis. (C) The number of apoptosis genes, ferroptosis genes, autophagy genes, pyroptosis genes, and necroptosis genes. (D) The Venn diagram shows the overlapping genes between five types of RCDGs

comprehensive multi-omics analyses encompassing transcriptional interaction, somatic mutation, copy number variation (CNV), DNA methylation, RNA modification, and transcriptional regulatory networks.

First, correlation analysis demonstrated extensive co-expression relationships among RCDGs, and unsupervised clustering revealed both positive and negative transcriptional associations across different RCD categories, indicating substantial cross-talk

among distinct cell death programs (Figure S1A,B; Tables S3 and S4). Protein-protein interaction analysis further supported the existence of a highly interconnected RCD regulatory network (Figure S1C). The dense structure of the PPI network reflects the widespread interactions among multiple RCD pathways and was intended to illustrate the overall complexity of RCD cross-talk.

At the genomic level, somatic mutation analysis across TCGA-BLCA, ICGC-BLCA, and CCLE-BLCA

TABLE 1. The genes that were correlated with multiple RCD types

RCD types	Number	Genes
Apoptosis/Necroptosis/Pyroptosis	1	TNF
Apoptosis/Ferroptosis/Necroptosis	2	RIPK1, CAV1
Apoptosis/Autophagy/Necroptosis	4	FADD, FAS, SIRT2, CFLAR
Apoptosis/Ferroptosis/Pyroptosis	2	GPX4, IL6
Apoptosis/Autophagy/Pyroptosis	4	CASP8, CASP1, CASP4, CASP3
Apoptosis/Autophagy/Ferroptosis	8	BID, SQSTM1, DDIT3, MAPK8, RELA, TP53, CDKN1A, CDKN2A
Apoptosis/Necroptosis	7	FASLG, RIPK3, CYLD, DNM1L, BIRC2, BIRC3, CD14
Apoptosis/Pyroptosis	6	CASP6, GZMB, CASP9, PRKACA, IL1B, IL-18
Apoptosis/Ferroptosis	13	CD44, HSPB1, HMOX1, ATF3, PIK3CA, AURKA, ATM, GCH1, JUN, TXNIP, VDAC2, SAT1, HMGB1
Apoptosis/Autophagy	23	BAK1, PEA15, IKBKB, CAPN2, BNIP1, ERBB2, CDKN1B, TNFSF10, BCL2, BCL2L1, CAPN1, HSPA8, PRKCD, PRKCQ, PRKAR1A, BNIP3L, CAPN10, DLC1, NFKB1, DAPK2, GAPDH, BAX, DAPK1
Ferroptosis/Necroptosis	1	TLR4
Autophagy/Pyroptosis	2	NLRC4, FOXO3
Autophagy/Ferroptosis	33	MAPK1, EGFR, IFNG, HSPA5, WIPI1, ULK1, MAPK3, HIF1A, RB1, MAPK9, NFE2L2, MTOR, BNIP3, MAP1LC3A, LAMP2, ULK2, ATG5, EIF2S1, ATG7, WIPI2, SIRT1, ATG4D, GABARAPL1, BECN1, ATG13, VEGFA, ATG16L1, GABARAPL2, SESN2, ATF4, ATG3, TP63, KLHL24

cohorts showed that RCDGs exhibited higher mutation frequencies compared with randomly selected genes (Table S5). Missense mutations and C > T substitutions were the predominant alteration patterns (Figure S2A–C). Multifunction RCDGs were significantly enriched among highly mutated genes, and mutation co-occurrence and mutually exclusive patterns suggested coordinated genomic alterations across different RCD pathways (Figure S2D–G; Table S6). The network-style visualization in Figure S2G was designed to illustrate the overall interaction architecture and systems-level cross-talk among highly mutated RCDGs rather than individual pairwise statistical comparisons alone. The above mutational characteristics were further examined in independent CCLE-BLCA and ICGC-BLCA cohorts (Figure S3A–G). However, the CCLE-BLCA cohort contained a relatively limited number of bladder cancer cell lines (n = 24), which may not fully represent the molecular heterogeneity of bladder cancer. Therefore, mutation frequency estimates and related patterns derived from the CCLE-BLCA dataset should be interpreted cautiously and were

primarily used as supportive validation evidence rather than definitive population-level conclusions.

CNV analysis revealed widespread genomic instability affecting RCDGs, particularly with distinct amplification and deletion tendencies across different RCD categories, consistent CNV patterns in multiple genes across independent datasets, and significant correlations between linear copy number levels and gene expression that implied CNV-driven transcriptional dysregulation of RCDGs (Figure S4A–K, Table S7). Chromosomal distribution of CNV alterations prominently involved chromosomes 8, 9, 17, and 20 (Figure S5). However, the CCLE-BLCA cohort included a relatively limited number of bladder cancer cell lines with available CNV data, which may reduce the robustness of frequency-based analyses such as high-CNV-rate gene identification. Therefore, the CCLE-based CNV results were interpreted primarily as supportive validation of trends observed in TCGA-BLCA rather than definitive estimates of CNV frequency in bladder cancer.

Epigenetic analyses further demonstrated that DNA methylation widely participated in RCDG regulation. Differential methylation sites were

broadly distributed across genomic regions, with both hypermethylation and hypomethylation events distinguishing tumor from normal tissues (Figure S6; Table S8). All differential methylation analyses were performed as tumor versus normal; therefore, hypermethylated probes indicate increased methylation in tumor samples relative to normal tissues, whereas hypomethylated probes indicate decreased methylation in tumor samples. The volcano plot reflects the magnitude and significance of differential methylation, while the regional barplots summarize the proportional distribution of hypermethylated and hypomethylated probes across CpG island-related or genomic functional elements. Correlation analyses identified multiple genes whose expression levels were strongly associated with methylation status, suggesting methylation-driven transcriptional regulation. Notably, although promoter-associated DNA methylation is classically linked to transcriptional repression, a substantial proportion of positively correlated methylation-expression relationships were observed in gene body regions. Previous studies have suggested that gene body methylation may be associated with active transcriptional elongation, alternative splicing regulation, or maintenance of transcriptional stability, indicating that DNA methylation may exert context-dependent regulatory effects on RCDGs in bladder cancer.

At the post-transcriptional level, RNA modification analyses indicated extensive associations between RCDGs and RNA modification regulators, demonstrating widespread post-transcriptional regulation (Table S9). Known m6A and m5C regulators were summarized in Table 2, and m5C methylation-related changes in RCDGs were first examined using bladder cancer-specific m5C datasets (Figure S7). We then performed exploratory correlation analyses between RCDGs and known m6A and m5C regulators (Figures S8 and S9; Table S9). The m6A regulator analysis was included as a contextual comparison because m6A is another major mRNA modification involved in post-transcriptional regulation, whereas the mechanistic focus of this section remained on m5C due to the availability of bladder cancer-specific m5C methylation and perturbation datasets. Because bladder cancer-specific m5C methylation and perturbation datasets were available, the subsequent mechanistic analyses focused primarily on m5C-associated regulation (Figure S10). Differential m5C methylation sites overlapped with numerous RCDGs, and integrative analyses combining RIP-seq and RNA-seq datasets suggested that several RCDGs may be regulated

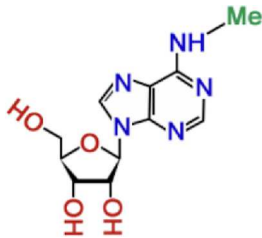
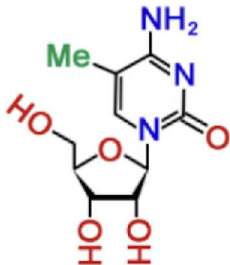
in an m5C-dependent manner (Table S10). However, the ALYREF-PKM/PKM2 correlation shown in Figure S7G was not statistically significant in the GSE133624 dataset, indicating that this specific relationship was not validated at the mRNA correlation level. Therefore, this example was interpreted only as an illustrative case suggesting that m5C reader-target relationships may not always be reflected by simple transcript-level correlation, and it should not be considered a key validated interaction in this study. We re-verified the direction of differential expression analysis in GSE133621 and confirmed that the comparison was performed as T24-siNSUN2 versus siNC. NSUN2 knockdown was associated with 223 differentially expressed RCDGs, including 158 upregulated and 65 downregulated genes (Supplementary Table S11). Because NSUN2-mediated m5C can promote mRNA stability for selected transcripts, the increased expression of some RCDGs after NSUN2 depletion may reflect indirect transcriptional responses, compensatory pathway activation, or loss of m5C-dependent regulation of upstream repressors rather than direct stabilization by NSUN2. Therefore, these genes were interpreted as NSUN2-perturbation-associated RCDGs rather than direct m5C-stabilized targets. Similarly, analysis of the GSE175714 dataset (sgYBX1 vs. sgLuci) identified 52 upregulated and 73 downregulated RCDGs following YBX1 perturbation, indicating that m5C-associated RNA-binding proteins may contribute to the transcriptional regulation of RCDGs (Table S12). We re-checked the genes with high logFC values in this dataset and found that these values were retained after normalization and DEG filtering, but several large fold-change estimates may be influenced by low baseline expression in the sgLuci control group; therefore, these results were interpreted as YBX1-perturbation-associated expression changes rather than direct evidence of m5C-dependent regulation. Consistently, transcriptional regulatory network analysis revealed complex lncRNA-miRNA-mRNA interactions and strong correlations between RCDGs and known bladder cancer regulators (Figure S11, Tables S13 and S14).

Collectively, these results demonstrate that RCDGs in bladder cancer are extensively shaped by coordinated genomic, epigenomic, and transcriptional regulatory mechanisms, supporting a systems-level cross-talk among multiple regulated cell death programs.

Therapeutic relevance of RCDGs in bladder cancer

After characterizing the multi-omics regulatory landscape of RCDGs, we next investigated whether these

TABLE 2. The main mRNA regulators of m⁶A and m⁵C modification

Modification	Chemical structure	Writers	Readers	Erasers
m ⁶ A		METTL3	YTHDF1 YTHDF2 YTHDF3 YTHDC1 YTHDC2	FTO ALKBH5
m ⁵ C		NSUN2	ALYREF, YBX1	TET2

alterations translate into functional importance and therapeutic vulnerability in bladder cancer. To understand the importance of these RCDGs, we first utilized a gene dependency score (gDS) to estimate the “Essentiality” of RCDGs in bladder cancer cells²⁶ (Table S15a). The genes with gDS < −1 indicated their deletion severely affects cell viability; gDS between −1 and 0 indicated these genes influence cell viability to some degree but are not lethal; gDS > 0 of genes indicated whose deletion does not affect cell viability. Among these, 214 genes with gDS < 0 in at least one of the three bladder cancer cells, we called these genes potential essential genes (Figure 2A, Table S15b). Ninety-three genes with gDS > 0 in all three bladder cancer cells, we called these non-essential RCDGs (Figure S12A, Table S15c). Several RCD-promoting genes (e.g., CASP2, RIPK3, TFRC, GLS2, and ACSL4) were classified as non-essential based on gDS, indicating their deletion does not impair cell viability. These genes represent potential therapeutic targets whose activation might trigger RCD and suppress tumor cell survival. In contrast, the deletion of several RCD-inhibited genes showed to be essential for cell viability, such as NFE2L2, AURKA, BCL2L2, and BCL2L10, indicating that up-regulating these genes could play a cancer-promotion role by inhibiting their corresponding RCD types. Aneuploidy, indicating the copy number alteration of whole chromosome arms or chromosomes, has been revealed to be a general and remarkable feature of malignancy genomes.²⁷ For the RCDGs, we found several genes (including

TOP2A, MSH2, and E2F1) showed a positive correlation with the aneuploidy score, whereas NLRP1 and GNB2L1 showed a negative correlation with the aneuploidy score, indicating these genes could drive the aneuploidy in BLCA (Figure S12B, Table S16). For advanced bladder cancer, neo-adjuvant therapy and traditional chemotherapy were mainly therapeutic options. Current chemotherapy therapy drugs, including gemcitabine,²⁸ cisplatin,²⁸ methotrexate,²⁹ vinblastine,²⁹ paclitaxel.²⁸ Drug sensitivity influenced the therapeutic effect. To determine drug sensitivity-related genes, we calculated the estimated IC₅₀ of main drugs in CCLE BLCA cells and TCGA-BLCA tissues; the results were consistent in these two datasets (Figure S12C,D, Table S17a,b). Then we calculated the PCC between RCDGs’ expression and IC₅₀ of the drugs, and the correlation network with |PCC| > 0.3 and adjusted *p*-value < 0.05 was identified (Figures 2B and S12E, Table S17c,d). The positive correlation indicated that the genes might increase the IC₅₀ of the drug, and the negative correlation indicated that the genes might decrease the IC₅₀ of the drug. Though the CCLE samples are small, multiple correlations did not reach the threshold, but we also observed some consistent results. For example, TOP2A could decrease the IC₅₀ of the drugs, revealing it may play a potentially important role in chemotherapeutic sensitization. To further validate the consistency of our findings across independent cohorts, we compared the significant RCDG-drug correlations between the TCGA-BLCA

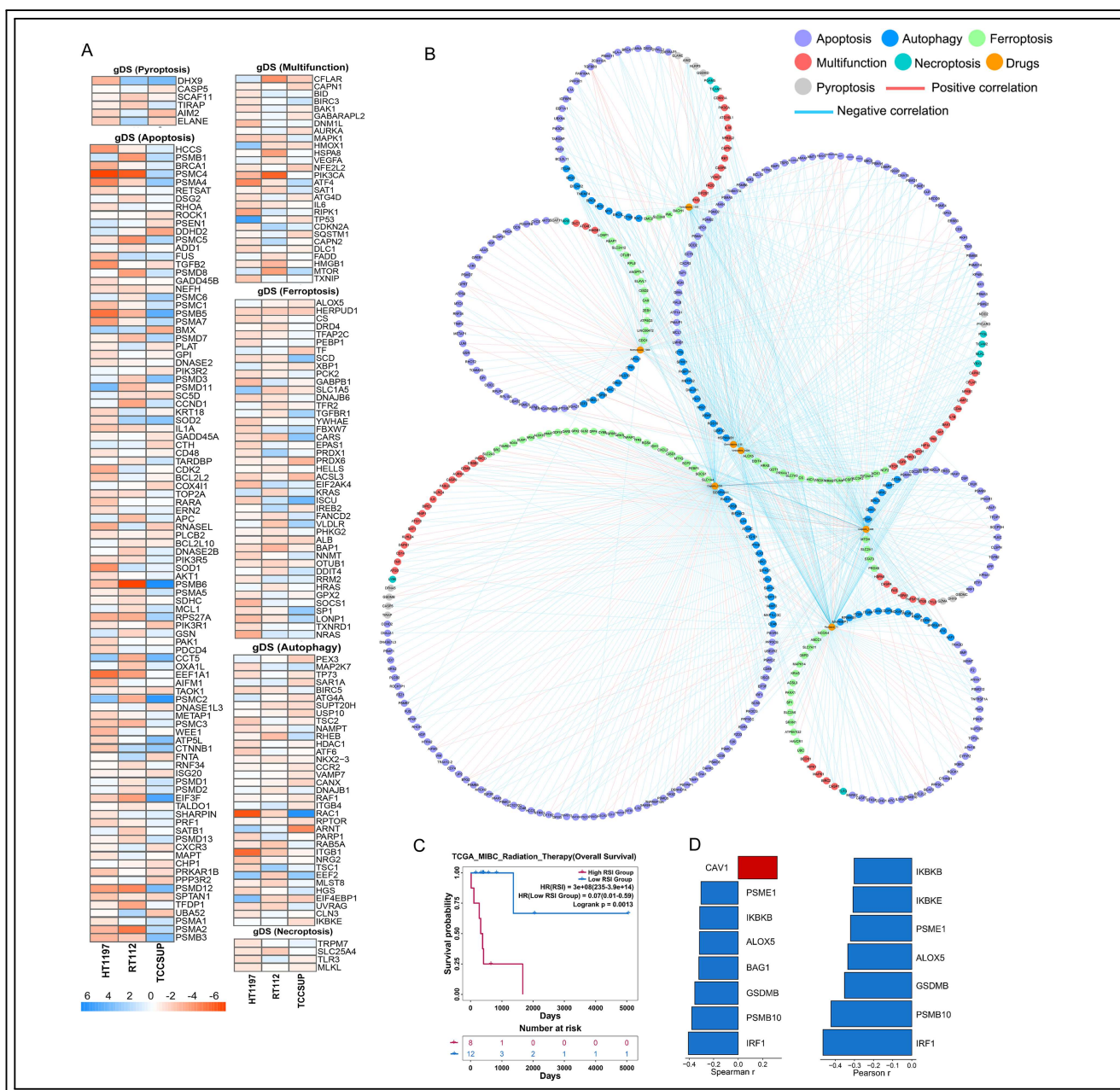


FIGURE 2. Importance of RCDGs in bladder cancer. (A) gDS of RCDGs in three bladder cancer cells (HT1197, RT112, and TCCSUP). (B) The network showed the correlation between IC_{50} of chemotherapeutic drugs and the mRNA expression of RCDGs. (C) The Kaplan-Meier survival curves of the high-/low-RSI-group in the subset of TCGA-BLCA patients who received the radiotherapy. (D) Barplot shows the correlation between RSI and mRNA expression of RCDGs

and CCLE-BLCA cohorts. A total of 15 overlapping RCDGs met the significance criteria ($|PCC| > 0.3$, $p < 0.05$) in both cohorts, indicating consistent chemotherapeutic response correlations of these RCDGs across independent bladder cancer datasets.

In addition to cystectomy and chemotherapy, radiotherapy (RT) for bladder cancer is an effective alternative.³⁰ To understand the effect of RCDGs' alteration for radiotherapy, we used a radiosensitivity index (RSI) constructed by a previous study.³¹ High RSI indicated radioresistance, and Low RSI

indicated no radioresistance.³² In the TCGA-BLCA cohort, we found that the prognosis of the patients in the high RSI group is poor than that in the low RSI group (Figure S12F). Although RSI was associated with OS in Cox regression analysis, the very high HR estimate indicated potential model instability, likely due to sparse event distribution or separation after dichotomizing patients into high- and low-RSI groups (Figure S12G). Therefore, this Cox regression result should be interpreted as exploratory rather than definitive evidence of RSI as an independent prognostic factor. In addition, in the subset of TCGA-BLCA patients who received radiotherapy, the prognosis of the high RSI group was worse than that of low-RSI group (Figure 2C), whereas Figure S12F presents the RSI-associated survival analysis in the overall TCGA-BLCA cohort. However, due to the limited sample size (high RSI: $n = 8$; low RSI: $n = 12$), the Cox regression model yielded an unstable hazard ratio estimate. Therefore, these findings should be interpreted as exploratory, and larger cohorts are needed to validate the potential role of RSI in predicting radiotherapy response in bladder cancer. These results revealed that RSI could predict the prognosis and radiotherapy effect of BLCA patients. Further analysis revealed that several RCDGs (including PSME1, IKBKB, ALOX5, BAG1, GSDMB, PSMB10, IRF1, and IKBKE) were negatively correlated with RSI, representing that these genes could enhance the radiosensitivity (Figure 2D, Table S18a,b).

Clinical relevance and molecular mechanism of RCD clusters

Given the extensive multi-omics alterations and therapeutic relevance of RCDGs, we next investigated whether RCD expression patterns could define biologically and clinically distinct subgroups of bladder cancer. To visualize the global transcriptomic distribution patterns of RCDGs between tumor and normal samples, we first performed t-SNE analysis (Figure 3A). The RCD clusters were not defined by t-SNE; instead, tumor samples were stratified by hierarchical clustering based on RCDG expression profiles, using Euclidean distance and the ward. D2 method as described in the Methods section. On a two-dimensional axis, the normal sample straddles the tumor sample, indicating that the expression of the RCD gene alters in two different directions. Interestingly, the BLCA patients were divided into two clusters based on the expression of RCDGs, and the prognosis of the patients in cluster 1/cluster 2 was significantly discriminative (Figures 3B,C). These findings indicate that RCD transcriptional programs stratify BLCA patients into clinically distinct

subtypes with prognostic significance. We conducted the same analysis in the other three MIBC datasets (including GSE13507, GSE48075, and GSE48276). The results in these external GEO cohorts were used as supportive validation rather than definitive prognostic evidence. In particular, the GSE13507 survival analysis should be interpreted cautiously because Cluster 2 contained only 6 patients, resulting in a highly unbalanced comparison and potentially unstable survival estimation. The results also showed that the patients could be separated into two different OS rate groups based on the RCDGs (Figure S13A–I). To further explore whether RCDG expression patterns also distinguish tumor and normal transcriptional states beyond BLCA, we performed a supportive pan-cancer t-SNE analysis using representative TCGA cancer types with sufficient normal samples. This analysis was intended as contextual evidence for the broad relevance of RCDG expression alterations, whereas all subsequent mechanistic and clinical analyses remained focused on BLCA. Intriguingly, when we divided the patients based on the differential expression of RCDGs and prognosis-related RCDGs, the patients in clusters showed no significant difference in overall survival, revealing that all RCDGs caused the patients' prognosis in different clusters. In other cancer types, we conducted the same analysis (Figures S14 and S15). Exploratory pan-cancer analyses suggested that RCD-based clustering was associated with survival differences in several cancer types; however, these results should be interpreted cautiously because some cohorts showed small subgroup sizes or overlapping survival curves despite statistical significance. RCD clusters of multiple cancer types showed different prognoses, revealing that the various expression characterization of RCDGs in cancers could cause different clinical outcomes. Mutation analysis further showed distinct genomic alteration patterns between the two RCD clusters (Figure S16A–D). Cluster 1 showed a higher proportion of C > G mutations than cluster 2, with nine variants per sample in cluster 1 and eight variants per sample in cluster 2. In addition, several recurrently mutated genes, including TP53 and RB1, showed significantly higher mutation frequencies in cluster 1 than in cluster 2 (Fisher's exact test, TP53: $p = 0.022$; RB1: $p < 0.001$), whereas PIK3CA showed a numerically higher but non-significant mutation frequency. These findings suggest that patients in cluster 1 exhibited a relatively higher mutational burden and genomic instability. We further explored RCD clusters' clinical relevance, mechanism, and immunoreactivity. We observed that the T stage, M stage, histological

grade, and pathological stage showed a significantly different distribution in cluster1/cluster2 (Figures 3D–G). Then, the differential expression genes (DEGs) in cluster1 versus cluster2 were calculated and presented to the ‘Clusterprofile’ R package to estimate the potential mechanisms (Table S19a). Multiple terms of gene ontology enriched in cluster 1, such as antigen binding, extracellular matrix, immune receptor activity, inflammatory response, and adaptive immune response (Figures 3H–J, Table S19b). Multiple immune-related KEGG pathways, including phagosome, systemic lupus erythematosus, complement, and coagulation cascades, were enriched in cluster 1 (Figure 3K, Table S19c). In contrast, pathways with negative NES values represented biological processes relatively enriched in cluster 2, including transcriptional regulation, lipid metabolic processes, ion homeostasis, and urothelial differentiation-related programs. Through the GSEA software analysis, we also found that some hallmark pathways, including inflammatory response, apoptosis, complement, and interferon-gamma response, were highly enriched in cluster1 (Figure 3L, Table S19d). GSVA also revealed that the activity of these Hallmark pathways was higher in cluster 1 than in cluster 2 (Figure S17A, Table S19e). We further analyzed several BLCA-hallmark pathways—urothelial differentiation pathways were enriched in cluster2, whereas the myofibroblasts pathways were enriched in cluster1 (Figure 3M, Table S19f).

Immuno-/chemo-/target-therapy prediction of RCD clusters

Given that RCD clusters demonstrated distinct biological and prognostic characteristics, we next evaluated their potential therapeutic implications. We first investigated immunotherapy-related features of the RCD clusters. The expression levels of immune checkpoint molecules PD1, PDL1, and CTLA4 were significantly higher in cluster1 compared with cluster2 (Figure 4A). Consistently, ESTIMATE-derived relative ImmuneScore and StromalScore were higher, whereas estimated tumor purity was lower, in cluster 1 than in cluster 2 (Figure 4B), suggesting a more immune-active tumor microenvironment. Because ESTIMATE scores are relative enrichment scores, negative values reflect lower relative signature enrichment rather than negative immune or stromal abundance. In addition, the activity of multiple immune cells was higher in cluster1, such as Treg, aDC, and macrophage cells (Figure 4C, Table S20a). We further used the ‘xCell’ R package to calculate the activity score of the tumor micro-environment, and the activity of

the immune cell is consistent with Figure 4C (Figure S17B, Table S20b). For the stromal cell, the activity of adipocytes, smooth muscle, pericytes, chondrocytes, and fibroblasts was higher in cluster 1 than in cluster 2 (Figure 4D, Table S20c). Through the Tumor Immune Dysfunction and Exclusion (TIDE) analysis, we found the TIDE score in cluster 1 was lower than in cluster 2, and the number of responders was higher than in cluster 2 (p -value < 0.05, Chi-squared test) (Figures 4E,F, Table S20d). These predicted responder proportions were derived using the default TIDE classification framework and should be interpreted as relative transcriptomic indicators of potential immunotherapy-associated sensitivity rather than actual clinical response rates. The higher predicted responder proportion in cluster 1 may reflect its immune-inflamed tumor microenvironment characterized by increased immune checkpoint expression and inflammatory pathway enrichment. Therefore, patients in cluster 1 may demonstrate greater relative sensitivity to immune checkpoint blockade therapy.

Furthermore, the estimated IC_{50} of vinblastine, cisplatin, and paclitaxel in cluster 1 was lower than in cluster 2, revealing that patients in cluster 1 exhibit predicted sensitivity associations with these chemotherapeutic agents. However, substantial overlap between the distributions suggests that these predicted transcriptome-derived sensitivity differences should be interpreted cautiously and may represent modest relative therapeutic associations rather than clinically validated response differences (Figure 4G). We further provided the DEGs between cluster1/cluster2 on the design website. A connectivity score closer to 1 indicates that the drug has the best efficacy, and closer to -1 indicates that the drug has the minimal efficacy.³³ The results showed that doxorubicin (targeting TOP2A) or cytarabine (targeting POLA1, POLB, POLD1, and POLE) were sensitive to the patients in cluster 1 (Figure 4H). Collectively, these findings suggest that RCD-based stratification may inform personalized immunotherapy and chemotherapy selection in bladder cancer.

Identification of RCD cluster prognosis-related genes

To explore the biological basis underlying the prognostic differences between RCD clusters, we performed weighted gene co-expression network analysis (WGCNA). We achieve a scale-free co-expression network by using a power of $\beta = 5$ in RCD cluster1 and cluster2 cohorts, respectively (Figure S18A). The k and $p(k)$ reached or exceeded 0.85, indicating that an optimal scale-free topology

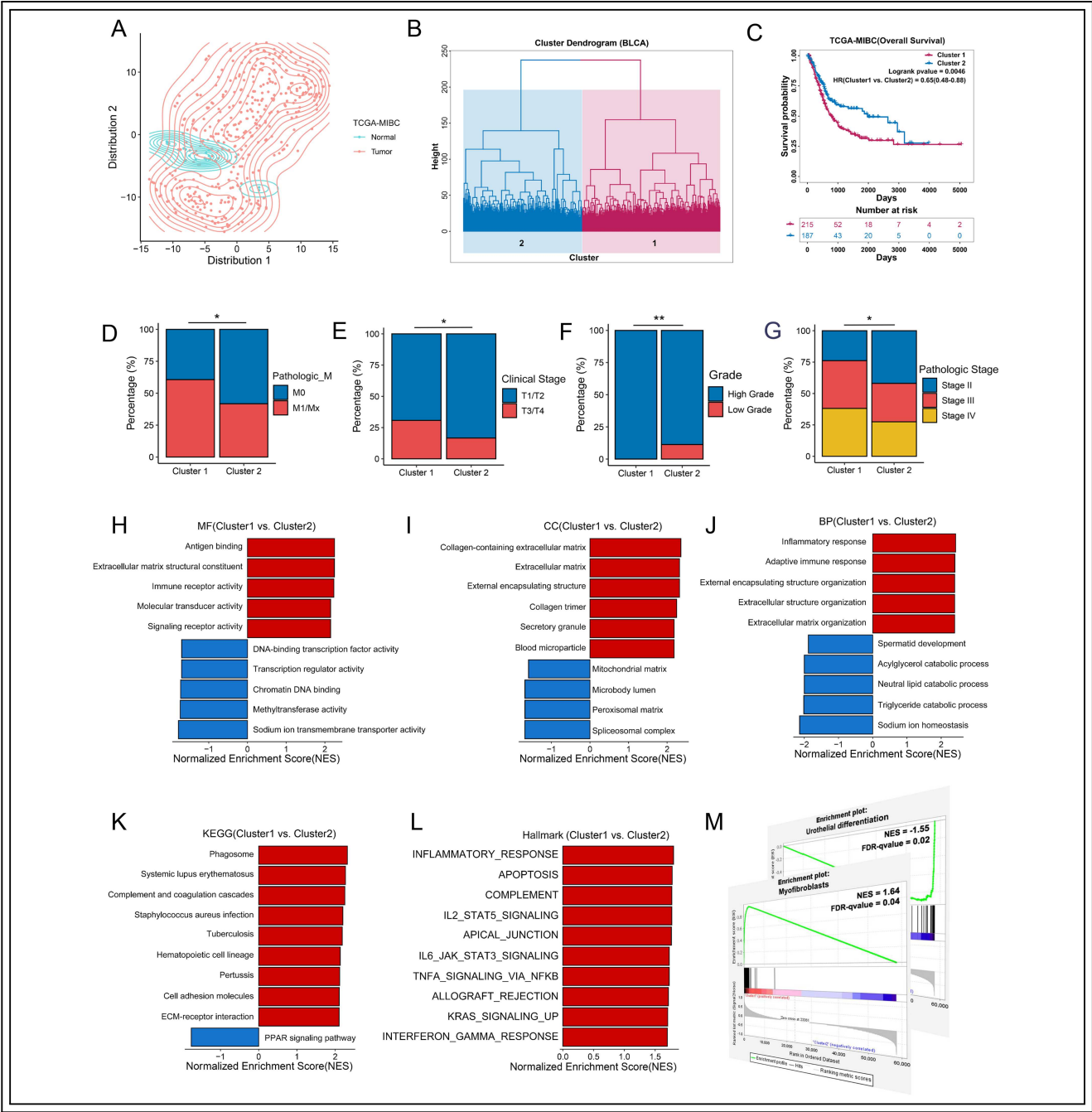


FIGURE 3. Construction and mechanisms of RCD clusters. (A) The t-SNE visualization of RCDG expression pattern in TCGA-MIBC tumor and normal groups. (B) Hierarchical clustering dendrogram used to define the RCD cluster. (C) The Kaplan-Meier survival curves of RCD cluster 1 and RCD cluster 2 based on 402 TCGA-BLCA patients with complete OS information. (D–G) Distribution of pathologic_M, clinical stage, histological grade, and pathological stage in RCD cluster1/cluster2. The chi-square test was used to compare the distribution of clinical characteristics between RCD cluster 1 and cluster 2 in [Figures 3D–G](#). (H–M) Differential enrichment GO terms (molecular function, cell component, and biological progression), KEGG pathways, Hallmark-related cancer pathways, and BLCA-specific pathways in cluster1/cluster2. Positive NES values indicate pathways relatively enriched in RCD cluster 1, whereas negative NES values indicate pathways relatively enriched in RCD cluster 2. *p-value < 0.05, **p-value < 0.01

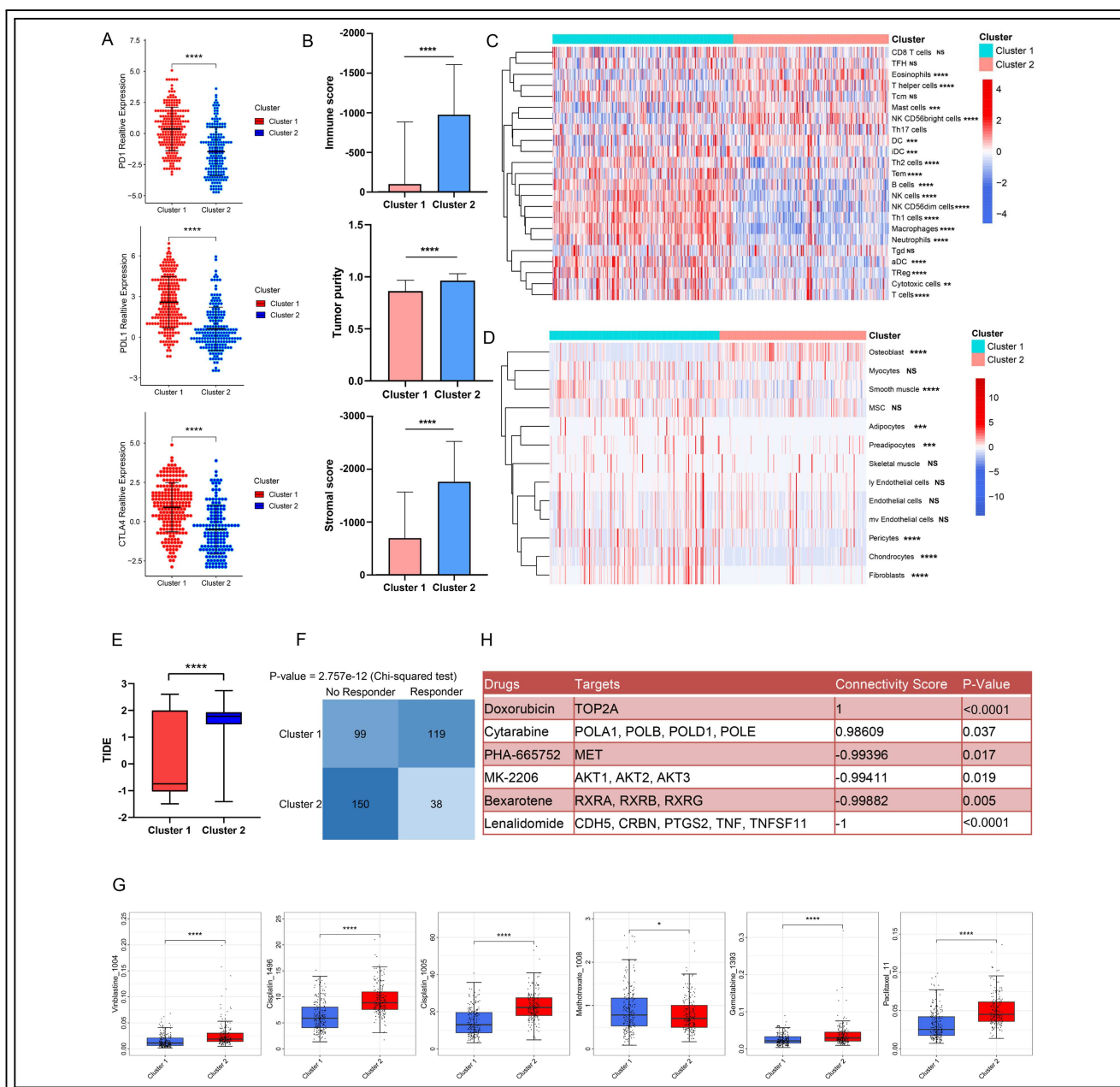


FIGURE 4. Immune infiltration and predicted therapeutic associations of RCD clusters. (A) The mRNA expression of PD1, PDL1, and CTLA4 in RCD cluster1 and cluster2. (B) ESTIMATE-derived relative ImmuneScore, TumorPurity, and StromalScore in RCD cluster1 and cluster2. Negative ImmuneScore or StromalScore values indicate lower relative enrichment of immune or stromal-related signatures and should not be interpreted as negative cell abundance. (C) Activity Score of 23 immune signatures in RCD cluster 1 and cluster 2. (D) Activity score of stromal cells in RCD cluster1 and cluster2. (E) TIDE score in cluster1 and cluster2. (F) The distribution of TIDE-predicted responder and non-responder categories in RCD cluster1 and cluster2 based on the default TIDE classification framework. These categories represent computationally predicted immunotherapy-associated sensitivity rather than clinically observed response rates. (G) IC₅₀ of current chemotherapeutic drugs in cluster1 and cluster2. (H) Potential sensitive drug prediction based on the RCD clusters. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, NS represents non-significant

network was constructed (Figure S18B). Then we merge the highly familiar modules by choosing a cut line of 0.25 and minimum module size of 30 (Figure 5A). Finally, the interest modules of RCD cluster1 (Yellow module: $r = 0.49$, $p = 3e-26$) and cluster2 (Brown module: $r = 0.62$, $p = 1e-43$; Turquoise module: $r = 0.7$, $p = 5e-60$) were identified (Figure 5B, Table S21a). Among these, 73 genes were identified as risk-associated genes, and 47 were identified as protective genes for MIBC (Figure 5C, Table S21b,c). Although some individual genes in the Brown or Turquoise modules showed $HR > 1$, WGCNA modules represent co-expression patterns at the module level rather than uniform prognostic direction for every member gene. Interestingly, among these 73 risk genes, 34 genes come from the yellow module, which is positively correlated with RCD cluster1 (p -value < 0.0001 , hypergeometric test) (Figure 5D). Among the 47 protective genes, 41 genes come from the RCD-cluster 2-related module including the Brown and Turquoise modules (p -value < 0.0001 , hypergeometric test) (Figure 5E). Notably, WGCNA modules represent co-expression patterns at the module level. Therefore, the prognostic interpretation was based on enrichment patterns of risk- and protective genes within each module, rather than assuming that all genes in a cluster-associated module shared the same HR direction. These results indicate that distinct co-expression gene modules underlie the prognostic heterogeneity between RCD cluster 1 and cluster 2.

The relationships between RCD clusters and consensus clusters in bladder cancer

Previous studies have proposed several transcriptome-based molecular classification systems for MIBC. Rebouissou et al. identified a basal-like subtype associated with EGFR activation,³⁴ whereas the Lund taxonomy further characterized urothelial carcinoma molecular subtypes using TCGA cohorts.³⁵ Choi et al. classified MIBC into basal and luminal subtypes with distinct chemotherapy sensitivities,³⁶ and Damrauer et al. demonstrated that intrinsic subtypes of bladder cancer share biological similarities with breast cancer subtypes.³⁷ In addition, TCGA-based molecular characterization studies and tumor differentiation signature analyses further refined the molecular taxonomy and prognostic stratification of bladder cancer.^{38,39} Kamoun et al. combined these methods and constructed a consensus cluster recently.⁴⁰ To understand the correlation between these molecular classifications and the RCD cluster, we construct these classifications to conduct a comparison (Figure 6, Table S22). We discovered that RCD cluster 1 is highly correlated

with basal/squamous clusters, whereas RCD cluster 2 is highly correlated with luminal clusters (Figure 6). It should be noted that several molecular subtypes contained limited sample sizes, and thus, findings related to these subgroups should be interpreted cautiously. The patient with the basal subtype usually showed a poor prognosis,⁴⁰ which further explains why the prognosis of the patients in RCD cluster 1 is poor. To explore the distribution of these RCD cluster prognosis-related genes, we used a recent single-cell RNA-sequence data published in a recent paper.⁴¹ We determined 16 clusters based on the data (Figure S19A). The cell types were annotated by the 'SingleR' R-package (Table S23a,b). Among these, most clusters were epithelial cells validated by specific markers, EPCAM, KRT8, and KRT18 (Figures S19B and S20A). In addition, the basal or luminal subtype cells were determined by GATA3, KRT5, and CD44 (Figure S20B).⁴² We found multiple yellow module risk genes (such as EMP1, ZEB1, F2R, VIM, CAV1, and TIMP2) distributed in fibroblasts (Cluster 13), tissue stem cells (Cluster 13), and endothelial cells (Cluster 16) (Figure S21). Although the Yellow module was predominantly enriched for risk-associated genes, it also contained a small number of protective genes, indicating that module-level correlation does not require all genes within the module to have identical survival effects. EMP1, ANXA1, SLC7A11, DUSP1, MAP3K5, JUN, TP63, SAR1A, and FTL showed a high expression in basal type cells (Figure S21). For the RCD cluster 2-related module genes, multiple genes showed high expression in luminal type cells, such as P4HB, QPRT, PRDX6, SCD, CISD1, SLC3A2, BAG3, SLC20A1, ERBB3, and so on (Figures S22 and S23). The single-cell analysis was used to visualize the cellular distribution patterns of module-related prognostic genes rather than to define cell-type-specific markers. Some genes in the Brown module, such as HSPA5, HSPA8, and ITGB1, were broadly expressed across epithelial clusters, suggesting that they may reflect general malignant or stress-related programs rather than cell type specific regulators. These results support the correlation between the RCD cluster and the consensus cluster. In CCLE bladder cancer cells, we also observed a similar phenomenon (Figure S24). In luminal-type cells, the expression of some RCDGs (such as AIFM3, SRC, ERBB3, ALOX5, ACSF2, PRKCD, BLCAP, and TP63) was higher. In basal-type cells, expression of some RCDGs (such as MLKL, SPHK1, F2R, AKT3, TXNRD1, VIM, and ZEB1) was higher (Figure S19C, Table S23c).

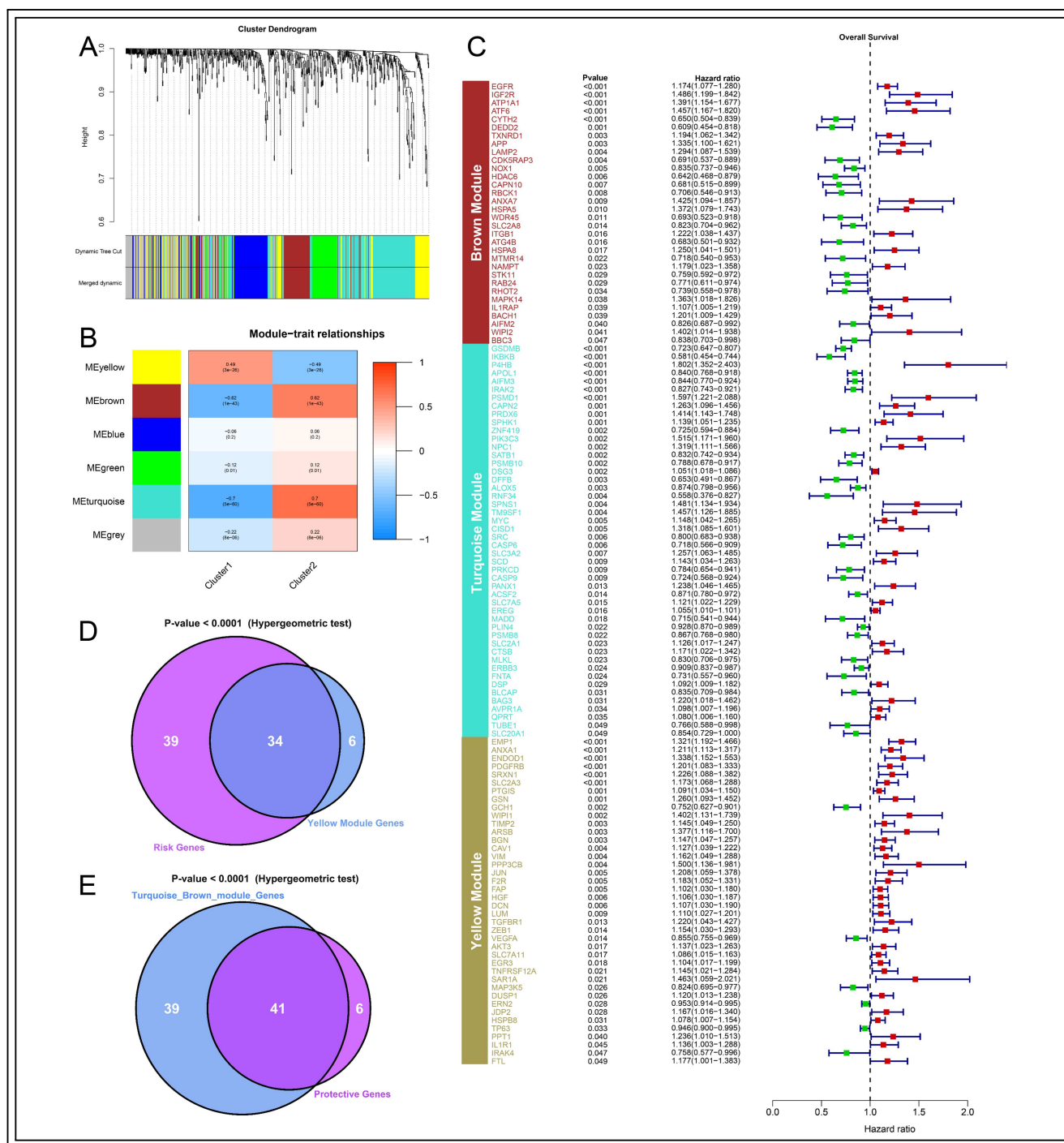


FIGURE 5. Identification of RCD cluster-related prognosis genes. (A) Dendrogram of the differentially expressed RCDGs between RCD cluster 1/cluster 2 based on a dissimilarity measure (1-TOM). (B) The heatmaps were depicted to show the correlation between module Eigengenes and clinical status (including RCD cluster 1 and cluster 2). Correlation coefficients and *p*-values were shown in every module. (C) The Forest plot shows the distribution of RCD cluster-related prognostic gene hazard ratios (HR) in TCGA-BLCA. The red point indicates the high HR, and the green point indicates the low HR. (D) Venn diagrams show the overlapping risk genes and yellow module genes. (E) Venn diagrams show the overlapping genes, protective genes and turquoise-brown module genes

RCD Clusters	Baylor.subtype								P-value
	Basal (N=161)		Differentiated (N=245)						
	Cluster 1 (N = 218)	145(90.1%)	73(29.8%)						
Cluster 2 (N = 188)	16(9.9%)		172(70.2%)					< 2.2e-16	

RCD Clusters	UNC.subtype				P-value
	Basal (N=197)		Luminal (N=209)		
	Cluster 1 (N = 218)	177(89.8%)	41(19.6%)		
Cluster 2 (N = 188)	20(10.2%)		168(80.4%)		< 2.2e-16

RCD Clusters	CIT.subtype							P-value
	MC1 (N=165)	MC2 (N=5)	MC3 (N=19)	MC4 (N=74)	MC5 (N=1)	MC6 (N=3)	MC7 (N=139)	
	Cluster 1 (N = 218)	20(12.1%)	3(60.0%)	0(0.0%)	66(89.2%)	0(0%)	0(0%)	
Cluster 2 (N = 188)	145(87.9%)	2(40%)	19(100%)	8(10.8%)	1(100%)	3(100%)	10(7.2%)	< 2.2e-16

RCD Clusters	Lund.subtype										P-value
	Ba/Sq (N=54)	Ba/Sq-Inf (N=45)	GU (N=30)	GU-Inf (N=26)	Mes-like (N=35)	Sc/NE-like (N=16)	Uro-Inf (N=29)	UroA-Prog (N=96)	UroB (N=22)	UroC (N=53)	
	Cluster 1 (N = 218)	53(98.1%)	45(100%)	2(6.7%)	24(92.3%)	31(88.6%)	4(25.0%)	20(69.0%)	9(9.4%)	19(86.4%)	
Cluster 2 (N = 188)	1(1.9%)	0(0%)	28(93.3%)	2(7.7%)	4(11.4%)	12(75.0%)	9(31.0%)	87(90.6%)	3(13.6%)	42(79.2%)	< 2.2e-16

RCD Clusters	MDA.subtype			P-value
	basal (N=137)	luminal (N=155)	p53-like (N=114)	
	Cluster 1 (N = 218)	126 (92.0%)	22 (14.2%)	
Cluster 2 (N = 188)	11 (8.0%)	133 (85.8%)	44 (38.6%)	< 2.2e-16

RCD Clusters	TCGA.subtype					P-value
	Basal_squamous (N=133)	Luminal (N=46)	Luminal_infiltrated (N=75)	Luminal_papillary (N=133)	Neuronal (N=19)	
	Cluster 1 (N = 218)	129(97.0%)	4(8.7%)	64(85.3%)	17(12.8%)	
Cluster 2 (N = 188)	4(3.0%)	42(91.3%)	11(14.7%)	116(87.2%)	15(78.9%)	< 2.2e-16

RCD Clusters	Consensus.subtype						P-value
	Ba/Sq (N=153)	LumNS (N=21)	LumP (N=127)	LumU (N=53)	NE-like (N=6)	Stroma-rich (N=45)	
	Cluster 1 (N = 218)	144(94.1%)	9(42.9%)	20(15.7%)	7(13.2%)	0(0%)	
Cluster 2 (N = 188)	9(5.9%)	12(57.1%)	107(84.3%)	46(86.8%)	6(100%)	7(15.6%)	< 2.2e-16

FIGURE 6. Relationship between RCD cluster and current clusters of bladder cancer based on 406 unique TCGA-BLCA patients after removal of duplicated patient entries. Fisher’s exact test was used for subtype association analysis

Biological validation of the RCD-associated hub gene TIMP1 in BLCA cells

Among the hub genes identified in the RCD co-expression network, TIMP1 was prioritized for functional validation. This selection was based on TIMP1’s classification as a yellow module gene, which was significantly enriched in the risk module RCD cluster 1 and associated with poor prognosis. In addition, TIMP1 expression was elevated in high-risk RCD clusters and correlated with advanced pathological stages. Consistent with our subtype analysis,

RCD cluster 1 is associated with the basal/squamous subtype, which exhibits more aggressive clinical behavior. Single-cell RNA sequencing data further showed that TIMP1 was expressed across multiple tumor-associated cellular clusters (Figure S21). Based on these observations, TIMP1 was selected as a representative gene for loss-of-function validation in bladder cancer cells. T24 was selected as a representative basal-type bladder cancer cell line because it was classified as basal in our CCLE-based analysis (Figure S24), consistent with the basal

features of RCD cluster 1. Consistent with these predictions, we performed siRNA-mediated loss-of-function experiments in two human bladder cancer cell lines, T24 and 5637, to validate the tumor-associated functional relevance of TIMP1 suggested by our bioinformatic analyses. Western blot analysis confirmed the expression of TIMP1 protein was successfully reduced in cells transfected with siTIMP1 compared to the control group (siNC) (Figure 7A). The CCK-8 assay demonstrated that TIMP1 knockdown suppressed the proliferation of both T24 and 5637 cells (Figure 7B). Transwell assay revealed the reduction in the number of migrated cells following TIMP1 knockdown (Figure 7C). In wound healing assay, TIMP1 knockdown delayed the wound closure of both T24 and 5637 cells in 12 and 24 h compared to the control (Figure 7D).

Discussion

In this study, we systematically characterized the multi-omics landscape and clinical relevance of regulated cell death (RCD) programs in bladder cancer and identified two biologically and clinically distinct RCD subtypes associated with prognosis and therapeutic response. Although previous studies have reported the expression characteristics and clinical associations of RCD pathways in pan-cancer or bladder cancer contexts, and some have mentioned inter-pathway interactions,⁴³ which focus on the independent functions of a single RCD pathway. This study provides a comprehensive integrative analysis of cross-talk among five core RCD pathways, including apoptosis, ferroptosis, autophagy, pyroptosis, and necroptosis in bladder cancer, offering insights into their coordinated regulatory patterns and partially addressing the current gap in understanding network-level regulatory mechanisms. Importantly, our findings extend current understanding by linking integrated RCD programs with clinically actionable features, including patient prognosis, immune microenvironment status, and therapeutic responsiveness in bladder cancer. Regulated cell death was an essential biological process in diverse physiological settings, including maintenance of tissue homeostasis and host defense.⁴⁴ Multiple regulated cell death pathways share interconnected molecular signaling mechanisms, providing biological support for the extensive cross-talk among RCD-related genes observed in our integrated multi-omics analyses. Therefore, developing a standard panel of biomarkers and functional tests, such as genetic and pharmacological inhibition studies, is necessary to discriminate

different forms of RCD that might occur in a single or mixed form.²² In-depth exploration of these complex mechanisms, exploiting the chemical modulators currently available for blocking or triggering these cell death methods is essential to treat cancer, infectious diseases, and autoinflammatory by promoting the development of precise therapeutic targets.⁴⁵ Collectively, these established interactions among RCD pathways provide a mechanistic framework supporting our observation of extensive cross-talk among RCD-related genes in bladder cancer.

Some RCD types have been found to correlate to multiple chemotherapeutic drugs in treating bladder cancer.^{46,47} However, few mechanism studies were performed on RCD types in bladder cancer.⁴⁸ Our integrated genomic analyses indicate that dysregulation of RCD programs in bladder cancer is driven by coordinated somatic mutations and copy number alterations. These findings suggest that genomic instability contributes to the simultaneous activation or suppression of multiple RCD pathways, potentially influencing tumor evolution and therapeutic responsiveness. Beyond genomic alterations, our analyses demonstrate that epigenetic and post-transcriptional mechanisms further shape RCDG expression patterns. DNA methylation and RNA modification collectively contribute to multilayer regulation of RCD programs, supporting the concept that RCD dysregulation in bladder cancer arises from coordinated genomic and epigenomic control.

As mentioned above, it has been found that various manners regulate RCDGs. Next, we explore RCDGs' clinical therapy relevance in BLCA. Currently, the research on RCD in cancers mainly focuses on novel RCD-based strategies for tumor elimination and drug sensitivity.⁴⁹ Triggering RCD shows the potential for cancer therapy in several malignancies resistant to traditional therapies. Our research analyzed some RCDGs that may be correlated with the IC₅₀ of traditional chemotherapeutic drugs or RSI. For example, NLRP3, a pyroptosis-related gene, showed a negative correlation with cisplatin, suggesting that upregulated NLRP3 could increase the sensitivity of cisplatin in bladder cancer. IKBKB was negatively correlated with RSI, suggesting that IKBKB overexpression could increase the radiosensitivity in bladder cancer. All in all, these results provide a bioinformatic basis for predicted optimization of conventional therapeutic efficacy based on the RCD gene intervention.

We finally determined two RCD clusters in bladder cancer based on the expression of RCDGs. We found that the prognosis of these two clusters was significantly different. RCD cluster 1 is predicted to

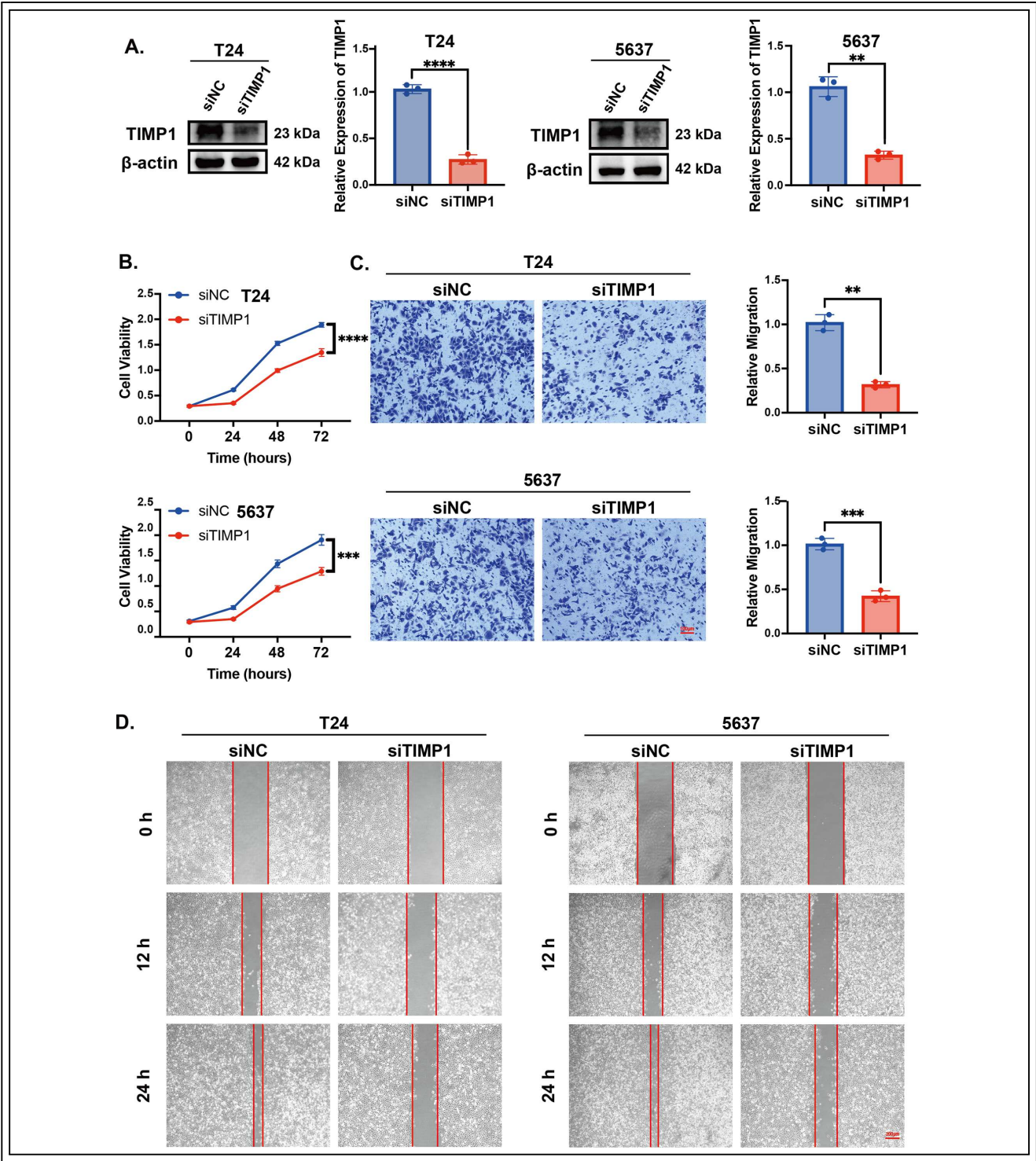


FIGURE 7. Validation of the hub gene TIMP1 in BLCA cells. (A) Western blot and its quantitative analysis confirming the knockdown of TIMP1 in T24 and 5637 cells (n = 3). (B) CCK-8 assay results showing the suppressed proliferation of both T24 and 5637 cells after TIMP1 knockdown. (n = 3) (C) Representative images and quantitative analysis of Transwell migration assays in T24 and 5637 cells following TIMP1 knockdown (n = 3). Scale bar: 100 μ m (D) Representative images of the wound healing assay at 0, 12, and 24 h (n = 3). Scale bar: 200 μ m. Data are presented as mean $\pm$ SEM. ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001, ns represents non-significant

have a higher TMB than that in RCD cluster 2. Interestingly, we observed that the proportion of C > G mutations was higher in RCD cluster 1 compared to cluster 2. C > G transversions have been associated with oxidative DNA damage and distinct mutational processes in cancer.^{50,51} Given that RCD cluster1 also exhibited higher tumor mutational burden and more aggressive clinical characteristics, the enrichment of such mutation types may contribute to genomic instability and potentially affect the regulation of RCD-related genes. However, the direct functional impact of specific mutation types on RCDG activity remains to be further elucidated. Further analysis found that the expression of the immune checkpoint, such as PD1, PDL1, and CTLA4, was higher in cluster 1. TIDE analysis showed that RCD cluster1 TIDE scores were lower than RCD cluster2. Importantly, the responder proportions generated by TIDE represent transcriptomic prediction categories derived from immune dysfunction and exclusion signatures rather than clinically validated objective response rates. Therefore, the relatively high predicted responder proportion observed in cluster 1 should be interpreted as reflecting a comparatively immune-inflamed tumor state rather than a direct estimate of real-world immunotherapy efficacy. Although TIDE analysis suggested differential immunotherapy responsiveness between RCD clusters, direct survival validation in immunotherapy-treated bladder cancer cohorts was not feasible due to limited availability of annotated clinical datasets; thus, further validation in prospective immunotherapy cohorts is needed. RCD cluster1 was correlated with multiple immune-related pathways. These results indicated that the patients in cluster 1 might benefit more from immune therapy than those in cluster 2. Consistent with the importance of metabolic-immune interactions in bladder cancer, a recent single-center multi-omics study reported that elevated uric acid was associated with poorer prognosis, reduced CD8+ and CD4+ T cell infiltration, and worse response to PD-1 blockade in bladder cancer.⁵² This supports the concept that metabolic and cell-death-associated tumor states may jointly shape the immune microenvironment and influence immunotherapy responsiveness. Besides, RCD can be activated in response to various mutations.⁵³ Therefore, the patients of RCD cluster1 with a higher mutation burden may be sensitive to the RCD-based therapeutic methods. These findings suggest that RCD transcriptional programs may serve as a biologically meaningful framework for molecular stratification and therapeutic decision-making in bladder cancer. The immune

checkpoint expression was assessed at the transcriptomic level in this study. Therefore, future investigations incorporating protein level validation, such as immunohistochemistry, in clinical samples will be necessary to confirm the translational relevance of RCD-associated immune phenotypes.

Due to the different prognoses of RCD clusters, we finally identified the RCD clusters' related genes. Weighted correlation network analysis (WGCNA) can be used for finding clusters (modules) of highly correlated genes.⁵⁴ Interestingly, most RCD cluster1-related yellow module genes are risk genes. Most protective genes are from the RCD cluster2-related turquoise/brown module, revealing the potential reason which caused the prognostic difference. In addition, we analyzed the relationship between RCD clusters and consensus clusters and observed the distribution of these RCD clusters-related genes in the subtype of the urothelial cancer cell. We observed that several RCDGs were expressed in the different subtypes of the urothelial cancer cell. It is reported that different clusters of bladder cancer showed different sensitivity to therapy drugs, and molecular subtypes could have a role in the selective administration of such chemotherapy.⁵⁵ Therefore, we speculate that these different expressions of RCDGs maybe play a potential role in drug sensitivity based on the subtypes.

Mechanistically, the extensive cross-talk among RCD programs observed in this study is likely to have significant effects on the tumor immune microenvironment and therapeutic responsiveness in bladder cancer. Multiple RCD modalities, including apoptosis, ferroptosis, pyroptosis, necroptosis, and autophagy-dependent cell death, share overlapping molecular regulators and can be co-activated within the same tumor context, resulting in composite cell death phenotypes rather than isolated pathways.⁴³ Such coordinated activation may influence the release of damage-associated molecular patterns (DAMPs), inflammatory cytokines, and tumor antigens, thereby reshaping immune cell recruitment and activation within the tumor microenvironment. Notably, RCD cluster 1 exhibited both enhanced immune infiltration and enrichment of inflammatory and interferon-related pathways, suggesting that heightened RCD cross-talk may promote an immunologically active tumor state. Pyroptotic and necroptotic signaling, for example, can amplify inflammatory cascades, while dysregulated apoptosis and ferroptosis may increase tumor antigen availability, collectively facilitating immune checkpoint engagement and sensitivity to immune checkpoint blockade. This concept is also supported by recent therapeutic studies showing

that ferroptosis induction can reinforce antitumor immune responses. For example, a metal-phenolic nanoplateform was reported to amplify a ferroptosis-immunotherapy cascade loop by reducing GSH, promoting GPX4 inhibition, and enhancing CD8+ T cell-mediated antitumor immunity in colorectal cancer.⁵⁶ Conversely, RCD cluster 2, characterized by more limited immune activation and luminal differentiation, may reflect a tumor state in which RCD programs are preferentially wired towards cell survival or immunologically silent death. In addition to immunotherapy responsiveness, RCD cross-talk may also condition sensitivity to chemotherapy and radiotherapy. The convergence of multiple RCD pathways could lower the threshold for treatment-induced lethal signaling, whereas selective suppression of specific RCD branches may contribute to resistance. Together, these findings suggest that integrated RCD network activity, rather than individual cell death pathways alone, acts as a central determinant of tumor-immune interactions and treatment efficacy in bladder cancer.

In order to validate our previous findings, we performed functional experiments on one of our top-ranked hub gene, TIMP1. By performing loss-of-function studies in T24 and 5637 cell lines, we experimentally confirmed that silencing TIMP1 impairs the proliferative capacity and migratory potential of BLCA cells. These results support the biological relevance of TIMP1 as a hub gene identified from the RCD-associated transcriptional network. However, these functional assays mainly demonstrate the contribution of TIMP1 to malignant phenotypes, including proliferation and migration, rather than directly proving that TIMP1 regulates a specific RCD pathway. Therefore, TIMP1 should be interpreted as an RCD-associated prognostic hub gene with tumor-promoting functional relevance, while its direct mechanistic role in individual RCD programs requires further validation.

Several limitations of this study should be acknowledged. First, the analyses were primarily based on public transcriptomic and multi-omics datasets, and *in vivo* animal models were not employed to directly validate the functional role of RCD cross-talk in tumor progression. Second, key findings, such as immune checkpoint activation and RCD, associated hub gene functions, were assessed mainly at the mRNA level, and validation using clinical tissue samples or protein-level assays such as immunohistochemistry, was not performed. The gene dependency scores were derived from a limited number of bladder cancer cell lines due to data availability; therefore, further validation in additional

in vitro and *in vivo* models will be necessary to fully characterize RCDG dependencies. Future studies incorporating animal models and well-annotated clinical cohorts will be necessary to further validate the mechanistic and translational relevance of the RCD-based stratification framework proposed in this study. In addition, although functional validation demonstrated that TIMP1 influences malignant phenotypes, the effects of TIMP1 and other hub genes on specific regulated cell death pathways were not directly investigated. Dedicated mechanistic experiments, including pathway-specific assays and rescue studies, will be required to determine whether TIMP1 directly modulates apoptosis, ferroptosis, pyroptosis, necroptosis, or other RCD programs. Future studies will be required to systematically elucidate how these genes modulate distinct RCD signaling mechanisms.

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Author Contributions

The authors confirm contribution to the paper as follows: Conceptualization, Zhihong Zhang, Changwen Zhang, Shiyong Qi and Yue Chen; methodology, Jingxian Li, Yan Zhao and Haiwen Zhou; software, Jingxian Li and Yuanjiong Qi; validation, Haiwen Zhou, Yan Zhao and Yang Liu; formal analysis, Haiwen Zhou and Yan Zhao; investigation, Jingxian Li; resources, Zhihong Zhang; data curation, Haiwen Zhou and Jingxian Li; writing—original draft preparation, Jingxian Li, Yan Zhao and Haiwen Zhou; writing—review and editing, Shiyong Qi and Zhihong Zhang; visualization, Jingxian Li, Haiwen Zhou and Yan Zhao; supervision, Shiyong Qi and Zhihong Zhang; project administration, Shiyong Qi and Zhihong Zhang; funding acquisition, not applicable. All authors reviewed and approved the final version of the manuscript.

Availability of Data and Materials

Data supporting the findings of this study are available within the paper and its Supplementary Information files. All publicly available data referenced herein can be retrieved from TCGA (<https://portal.gdc.cancer.gov/>), GEO (<https://www.ncbi.nlm.nih.gov/gds/>), Genecode database (<https://www.genecodegenes.org/human/>), UCSC Xena (<https://xena.ucsc.edu/>), CCLE (<https://xenabrowser.net/hub/>), ICGC-BLCA (<https://dcc.icgc.org/>), GSEA (<https://www.gsea-msigdb.org/gsea/index.jsp>), miRcode (<http://www.mircode.org/>). Scripts and datasets used in this study are available at the following link: <https://www.jianguoyun.com/c/sd/1c1c96e/4ea7b42daae9a0c0>.

Ethics Approval

Not applicable.

Conflicts of Interest

The authors declare no conflicts of interest.

Supplementary Materials

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Abbreviations

The following abbreviations are used in this manuscript

RCD	Regulated cell death
BLCA	Bladder cancer
RCDGs	Regulated cell death genes
WGCNA	Weighted gene co-expression network analysis
PCD	Programmed cell death
GSEA	Gene set enrichment analysis
GSVA	Gene set variant analysis
GSDMD	Gasdermin D
RIPK3	Threonine kinase 3
MLKL	Mixed lineage kinase domain-like pseudokinase

Gds	Gene Dependency Score; PCC: Pearson Correlation Coefficient
CNVs	Copy number variants
DMSs	Differential methylated sites
ceRNAs	Competitive endogenous RNAs
RT	Radiotherapy
RSI	Radiosensitivity index
DEGs	Differential expression genes
TIDE	Tumor Immune Dysfunction and Exclusion
DMGs	Different methylation genes
FDR	False discovery rate
ssGSEA	Single-sample gene set enrichment
AM	Adjacency matrix
TOM	Topological overlap matrix
ME	Module Eigengenes
TMB	Tumor mutational burden
ICI	Immune checkpoint inhibitor
DPP4	Dipeptidyl peptidase-4

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