Original Article

Integrated bioinformatics investigation of adenylyl cyclase family co-expression network in bladder cancer followed by preliminary validation of member 2 (*ADCY2*) in tumorigenesis and prognosis

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Background: The adenylyl cyclase (*ADCY*) gene family encodes enzymes responsible for the synthesis of cyclic adenosine monophosphate (cAMP) from adenosine triphosphate (ATP), which comprises nine transmembrane isoforms (*ADCYs 1–9*). Although *ADCYs* correlate with intracellular signalling and tumorigenesis in different malignancies, their roles in bladder cancer remain unclear.

Methods: Utilizing the bladder urothelial carcinoma (BLCA) dataset from The Cancer Genome Atlas (TCGA), we employed the R package 'limma' to identify differential genes. Subsequent correlation analysis with corresponding clinical data was conducted. Prognostic significance of *ADCY* family genes was assessed through survival analysis. Univariate and multivariate Cox regression determined *ADCY2* as a potential independent risk factor for BLCA. Validation was performed using immunohistochemistry results from independent cohorts. Additionally, we delved into the mechanism of genetic variations, methylation modifications, and signalling pathways of *ADCY* family genes. Evaluation of their role in the immune microenvironment was achieved through R packages single-sample gene set enrichment analysis (ssGSEA), CIBERPORT, and ESTIMATE.

Results: Cases of bladder cancer were retrieved from TCGA, and the transcriptionally differentially expressed members of *ADCY* were identified (members 2, 4, and 5). Genomic alteration, epigenomic modification, clinicopathological characteristics and clinical survival were systematically investigated. A co-expression network was established based on the intersection of correlated genes, which was centred around *ADCY2*, *ADCY4*, and *ADCY5*. Enrichment analysis revealed that correlated genes were involved in epithelial-mesenchymal transition (EMT). The *ADCY2* was selected as the most representative biomarker for prognosis in bladder cancer. Bladder tumour with higher *ADCY2* expression had higher prognostic risk and worse survival outcomes. Moreover, *ADCY2* was correlated with classic immune checkpoints, and a better responsiveness to immunotherapy was exhibited in high-expression subsets. To ameliorate universality of the conclusion, our study also included several real-world cohorts into the preliminary validation, using datasets from the Gene Expression Omnibus (GEO; GSE13507), tissue microarray (TMA) with 80 bladder cancer inclusion and clinical trial IMvigor210, which were associated with immunotherapy sensitivity, prognosis, and common biomarker presentation.

Conclusions: Our study reveals that *ADCY* family has prognostic value in patients with bladder cancer; the

ADCY2 is a prominent prognostic biomarker. The bioinformatics analyses and validation provide direction for further functional and mechanistic studies on the screened members of *ADCY* family.

Keywords: Bladder cancer; co-expression; prognosis; adenylyl cyclase (*ADCY*); tumour microenvironment (TME)

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Introduction

Bladder urothelial carcinoma (BLCA) is a common urinary malignancy, with the ninth highest annual occurrence rate worldwide (1). Nearly 90% of all bladder malignant tumours are localised in the primary region (2); however, the relapse tendency has aroused a curve of long-term disease management (3). BLCA is clinically categorised into two subtypes: non-muscular invasive bladder cancer (NMIBC) and muscular invasive bladder cancer (MIBC). Most BLCA cases (>60%) are NMIBC, of which patients have a 5-year survival rate of approximately 90%. The unfavourable course of MIBC has a 5-year survival rate of less than 50%, owing to recurrence and early occult metastatic dissemination (4).

Traditional radical resection and medications have severe limitations in treating advanced bladder cancer. Owing to the high mutational burden, immune checkpoint blockades (ICBs) are used to obtain satisfactory curative effects in a subset of patients (5). New therapeutic strategies have been approved for several indications and have revolutionised the treatment landscape of advanced bladder cancer (6,7). Nevertheless, the main dilemma is the lack of predictors of immunotherapy efficacy and identification of a population with a durable immune response to immunotherapy (8). Therefore, identifying biomarkers for disease prognosis and adjusting therapeutic options for relapsed patients can benefit individual clinical treatment. Previous work had validated this viewpoint (9).

Adenylyl cyclases (*ADCYs*) are enzymes that generate second messengers and are involved in several biological processes, including cell cycle inhibition, apoptosis, and proliferation (10). Their activities are associated with tumour cell morphology, contact inhibition restoration, and decreased growth rates (11,12). The ADCY protein increases intracellular cyclic adenosine monophosphate (cAMP) levels, which regulate cellular transport by binding to protein kinase A (PKA). In addition, activated PKA-phosphatases regulate metabolic and transcription factors, such as the cAMP response element-binding protein (CREB), consequently activating the Wnt/β-catenin pathway, which is accompanied by cell cycle arrest, apoptosis, and survival. The *ADCY*-mediated calcium signalling pathway also participates in remodelling the tumour microenvironment (TME) and regulating immune checkpoints (13). The *ADCY* family plays an essential role in dyskinesia, diabetes, cardiovascular disease, and tumorigenesis (14-16).

Since first described by Earl W. Sutherland (17), a study on *ADCY* has explored its biological function, isoforms, and protein structures. Recently, the potential of *ADCYs* as an unelucidated therapeutic target has been reviewed (18). However, to our knowledge, limited studies have focused on the potential value of *ADCYs* as a biomarker for bladder cancer prognosis.

Therefore, this study aimed to characterize the prognostic value of *ADCYs* within BLCA and estimate *ADCY*-enriched patients' response to ICBs. The *ADCY2* would be used as a potential prognostic marker for bladder cancer and applied in the stratification for disease outcome.

Highlight box

Key findings

- Adenylyl cyclase 2 (*ADCY2*), *ADCY4*, and *ADCY5* exhibit a co-expression pattern, and jointly participate in the occurrence and prognosis of bladder cancer.
- *ADCY2* can predict the efficacy of immunotherapy, and screen subgroups that may benefit from immune checkpoint blockades.

What is known and what is new?

- *ADCYs* are involved in various biological processes, involving tumour cell morphology, contact inhibition recovery, and decreased growth rate.
- *ADCYs* are first utilized in presenting pathogenesis and prognosis of bladder cancer.

What is the implication, and what should change now?

- Abundance of *ADCY2* in bladder tissue indicates tendency of therapeutic benefits for patients with bladder cancer.

We present this article in accordance with the REMARK reporting checklist (available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-1796/rc).

Methods

Data source and pre-processing

Transcriptome profiling and clinical information were retrieved from The Cancer Genome Atlas (TCGA) database on the Genomic Data Commons (GDC) data portal, including 407 bladder tumour and 19 normal samples, respectively. To enrich the diversity of the sample sources, data from nine normal bladder samples were obtained from the Genotype-Tissue Expression (GTEx) database. The Toil workflow was used to normalize the RNA-sequencing (RNA-seq) data using the transcripts per million formats. The BLCA Gene Expression Omnibus (GEO) queue was retrieved from the GEO database (https://www.ncbi.nlm.nih.gov/geo/), which contains extensive survival data. GSE13507, including 256 bladder samples, was selected based on the GPL6102 (Illumina human-6 v2.0 expression beadchip) platform to build the validation group. All included patients received conventional treatment including resection and chemotherapy. Clinical information of these included patients involving outcome and prognosis were also available in the arrays.

Moreover, a cohort of IMvigor210 queues linked with immunotherapy obtained from http://research-pub.Gene.com/imvigor210corebiologies/ was compiled. The patients in IMvigor210 cohort received treatment of immune checkpoint inhibitors.

Tissue microarrays (TMAs)

Formalin-fixed paraffin-embedded (FFPE) samples were obtained from patients with BLCA receiving conventional first-line therapeutic resection. The TMAs were constructed using FFPE samples by Shanghai Outdo Biotech Co., Ltd. (https://www.superchip.com.cn/). Details of the TMA cohort are provided in Figure S1. The hole-making instrument was applied to the recipient array blocks and used to acquire tissue cores from the donor blocks. A stereotactic microscope was used to identify the areas of interest, with an additional bright light source under each block. After block construction, 8 μm sections of the resulting tumour TMA block were cut using a microtome knife, cutting underneath a tape piece placed over the block surface. The thin tissue section was rolled upon an adhesive-coated microscope slide. The malignant tumour tissues retained their histological pattern throughout the entire 1.5-mm-deep block. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Medical Ethics Committee of Second Military Medical University (No. CHEC2019-134). Individual consent for this retrospective analysis was waived due to the retrospective nature.

Immunohistochemical (IHC) staining evaluation

IHC staining was graded by two skilled pathologists for blinded rechecking. The staining index (SI) was used to integrate the intensity and percentage of positive cells for semiquantitative assessment of protein marker expression.

The staining intensity was graded as follows: 0, no staining; 1, pale yellow; 2, yellow; and 3, light brown. The percentage of cells stained was graded as follows: 0, <5%; 1, 5–25%; 2, 26–50%; 3, 51–75%; and 4, >75% in tumour cells. The SI was calculated by multiplying the two scores and was classified into the following grades: 0, negative (–); 1–4, weak (+); 5–8, moderate (++); and 9–12, strong (+++). Specimens with SIs ≥5 and ≤4 were defined as high and low *ADCY2* expression, respectively. A third pathologist was consulted in case of a disagreement between the two observers. Low-magnification images and examples of high- and low-expression strains are shown in Figures S1,S2.

Enrichment analysis and annotation of differentially expressed genes (DEGs)

DEGs were analysed using the limma package (19). Gene Ontology (GO) enrichment and Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway analyses were performed with hub genes using the R package "clusterprofiler". GO terms and KEGG pathways were visualised using the package "ggplot2". The "clusterprofiler" package was used to conduct gene set enrichment analysis (GSEA) for signature genes, and the "gene set variation analysis (GSVA)" package was used to identify significantly correlated pathways. The gene sets "c2.cp.kegg.v6.2.symbols.gmt" and "h.all.v7.2.symbols.gmt" were selected as reference gene sets.

Genome alteration and DNA methylation

CBioPortal was used to retrieve the genomic data to

query the genome alterations in *ADCY* gene family. The CBioPortal Cancer Genomics is an open-access resource (https://www.cbioportal.org) for interactive exploration of multiple cancer genomics data sets. Cytosine-phosphate-guanine (CpG) site information in the promoter region of *ADCY2*, *ADCY4*, and *ADCY5* was extracted using Illumina Human Methylation 450 data, examining the Pearson correlation between the methylation level (beta value) and gene expression.

Immune cell infiltration analysis

Single-sample GSEA (ssGSEA) (20) was proposed for a single sample that could not be utilised for GSEA. The R package GSVA can be used to implement it. The extent of tumour immune cell infiltration was calculated using GSVA. We retrieved the expression properties of each immune cell using a deconvolution technique. The method of CIBERPORT (21) was used to determine immune cell infiltration. To estimate the abundance of immune cells, it uses linear support vector regression to deconvolute the expression matrix of immune cell subtypes.

TME analysis

We used ESTIMATE (22) to evaluate the tumour immune microenvironment scores of samples, and comparisons of their differential distribution in different subtypes was performed. Based on expression profiles, ESTIMATE provided us with tumour purity scores and level of stromal cells and immune cell infiltration in tumour tissues. Linear regression analysis was used to determine the link between the components and the expression of each immune cell and the final combination.

Statistical analysis

Differences in clinicopathological features between the two *ADCY* groups were investigated using the chi-square test. Differences between the high- and low-*ADCY2* groups were investigated using a Student's *t*-test. Correlation analysis was performed using the Pearson correlation coefficient. Statistical analysis was conducted in R (version 4.0.2). Significance was set as $P<0.05$. The cut-off for DEGs was set as $|\log_2(\text{fold change})| > 1$ and false discovery rate (FDR) <0.05.

Results

Expression of ADCY gene family in bladder specimens

The BLCA datasets from TCGA and GTEx were integrated to analyse the messenger RNA (mRNA) levels of *ADCYs* in bladder tissues. The expression of *ADCY2*, *ADCY4*, *ADCY5*, and *ADCY9* was significantly downregulated in tumour tissues ($P<0.001$); however, no statistically significant difference was observed in the other *ADCY* genes (*Figure 1A*). To further elucidate the potential function of *ADCY2*, *ADCY4*, *ADCY5*, and *ADCY9* in tumour malignancy, the relationship between *ADCY* expression levels and clinicopathological characteristics, including pathological stage, T stage, histologic grade, lymphovascular invasions, and pathological subtypes, were analysed (*Figure 1B*, Figure S3A). In contrast to the differential expression levels between normal and tumour tissues, *ADCY* expression levels, especially *ADCY2*, *ADCY4*, and *ADCY5*, were positively associated with clinical features indicating tumour progression. *ADCY2* levels were significantly higher in patients with metastatic risk than in those with focal lesions. The non-papillary subtypes showed significantly higher *ADCY2* and *ADCY9* expression than the papillary subtypes. *ADCY* expression levels were also associated with earlier T stage and histological grade. Moreover, the mRNA expression levels in 21 bladder cancer cell lines were compared with those in telomerase reverse transcriptase-normal human urothelial cells (TERT-NHUCs) bladder epithelial cells, and the results confirmed the above conclusion (*Figure 1C*).

Elevated ADCYs expression is associated with poor clinical outcomes in BLCA patients

To further elucidate the relationship between survival promotion and transcriptional levels, a prognostic analysis was performed by retrieving TCGA clinical data (*Figure 2*, Figure S3B). Kaplan-Meier (KM) curves showed that multiple *ADCY* genes were correlated with improved prognosis. Patients with higher *ADCY2*, *ADCY4*, *ADCY5*, and *ADCY9* expressions had significantly prolonged overall survival (OS) and disease-specific survival (DSS) time.

TMA cohort validated the relation between ADCY2 expression level and clinical outcomes

To validate the hypothesis that *ADCY2*, *ADCY4*, *ADCY5*,

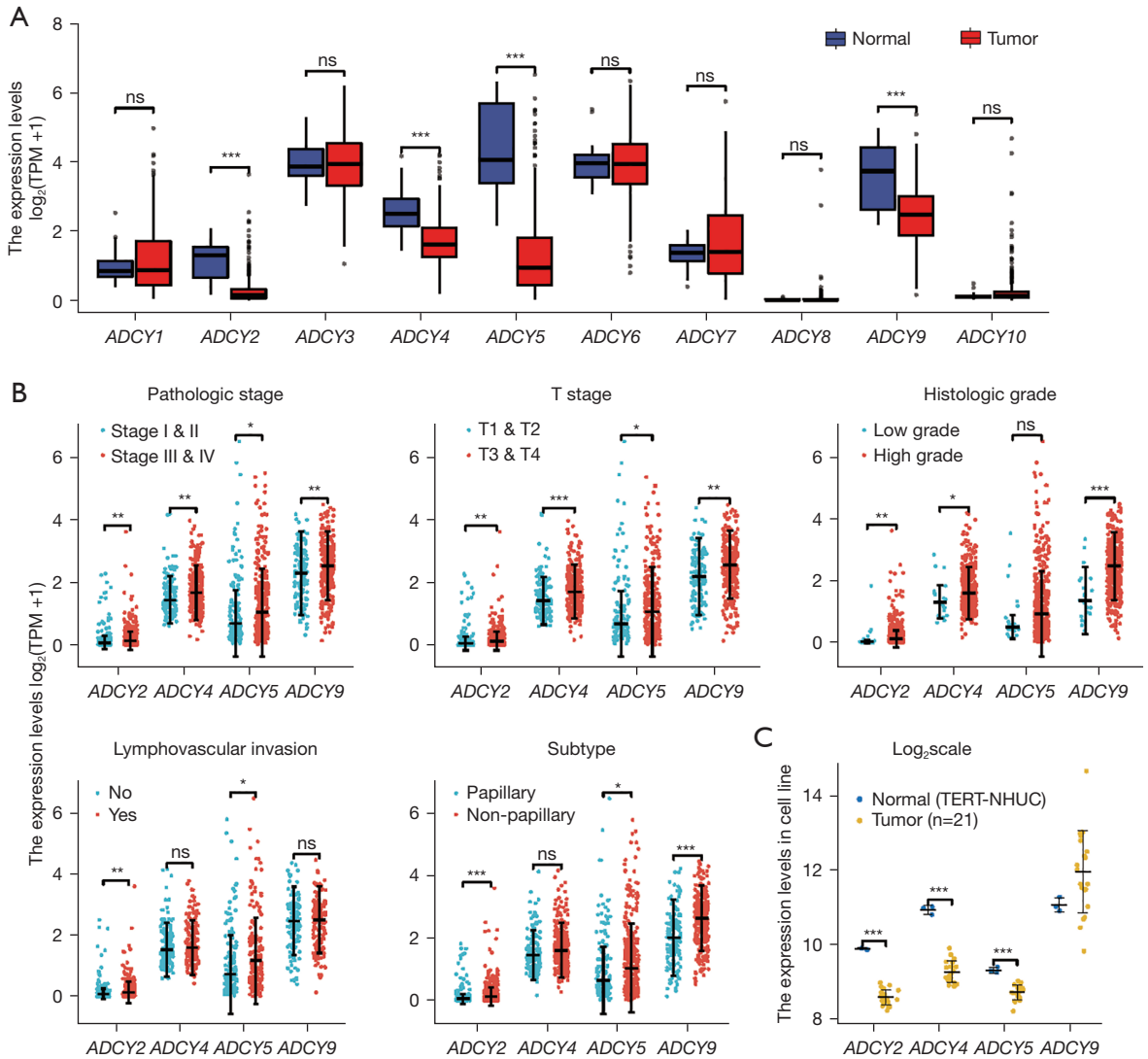


Figure 1 Expression of *ADCY* genes in bladder cancer tissues and cell lines. (A) Expression levels of *ADCYs* in bladder cancer. (B) Clinical features and *ADCY* expression were relevant examined as determined by Wilcoxon test; that patients with later tumour stage and worse prognosis might have higher of *ADCYs* mRNA expression. (C) Expression level of *ADCY* genes in normal bladder epithelial cell line (TERT-NHUC; n=3) and tumour cell line (n=21) from the Genevestigator dataset. ***, P<0.001; **, P<0.01; *, P<0.05; ns, P>0.05. *ADCY*, adenylyl cyclase; TPM, transcript per million; TERT-NHUC, telomerase reverse transcriptase-normal human urothelial cell; mRNA, messenger RNA.

and *ADCY9* are prognostic indicators, pathological tissues of 80 in-patients in urological department of Changhai Hospital were included into TMA. *ADCY2* was selected as a candidate for IHC staining, and IHC scores were quantitatively evaluated (Figure S1A). The staining standard was classified into three levels (*Figure 3A*). The clinical outcome of the TMA cohort was illustrated using the KM curve, showing a significant difference between the high- and low-*ADCY2* expression groups (*Figure 3B*). The KM curve of disease-free survival (DFS) further demonstrated that *ADCY2* expression had no statistical relationship with tumour recurrence (*Figure 3C*), consistent with the KM curve of disease-free interval (DFI) in TCGA cohort (Figure S1B). In the high-*ADCY2* expression group, Ki67, a proliferative indicator of cancer cells, was significantly increased (*Figure 3D*).

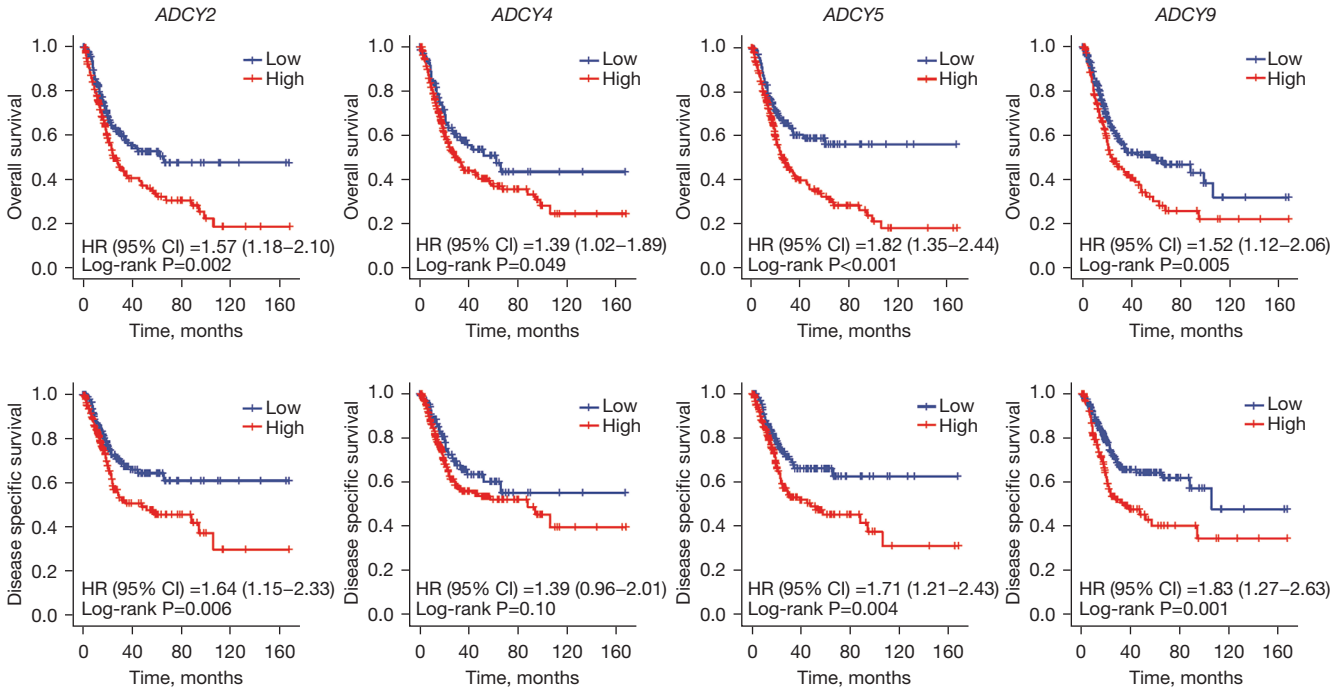


Figure 2 Relationship between *ADCY* expression and survival prognosis of cancers. KM curves for OS and DSS of *ADCY* expression in BLCA patients in TCGA cohort. KM analysis was conducted to determine the prognosis difference between the groups divided using the minimal P value approach, and the P value was examined using the log-rank test. *ADCY*, adenylyl cyclase; HR, hazard ratio; CI, confidence interval; KM, Kaplan-Meier; OS, overall survival; DSS, disease-specific survival; BLCA, bladder urothelial carcinoma; TCGA, The Cancer Genome Atlas.

To verify *ADCY2* expression in other datasets, the GSE13507 dataset (23) was downloaded from the GEO database. In addition to observing significant differences in *ADCY2* expression between tumour and normal tissues, we counted and combined the expression in tumour occurrence sites in GSE13507; the difference between the recurrent and primary sites was not statistically significant (P>0.05, Figure S1C,S1D).

ADCYs co-expression analysis

To explore the interconnection among *ADCY* isoforms in BLCA regulation, Spearman's rank correlation analysis was used to calculate the correlation scores among the *ADCY* gene family members at the transcriptional level. The heatmap (*Figure 4A*) displayed two clustered gene groups with significant correlations (*ADCY2*, *ADCY4*, and *ADCY5*; and *ADCY3*, *ADCY7*, and *ADCY9*), indicating a potential biological connection among these clustered genes. Further analysis of this phenomenon by overlapping the 500 most correlated genes of each *ADCY* family revealed a probable co-expression relationship among *ADCY2*, *ADCY4*, and *ADCY5*, containing 165 candidate genes at the intersection, ranking the highest in the clustered groups (*Figure 4B*).

The mechanism of co-expression of *ADCY2*, *ADCY4*, and *ADCY5* was preliminarily explored from genetics and epigenetics perspectives, respectively; no apparent coherence in copy number variation (CNV) was observed among the genes (*Figure 4C*), and each gene had a unique chromosomal distribution (Figure S2A). The methylation levels of CpG sites in the *ADCY2*, *ADCY4*, and *ADCY5* promoter regions were assessed using bladder cancer samples (n=440) from TCGA database. A significant inverse correlation was observed between methylation level and mRNA expression in almost all CpG sites, indicating that *ADCY2*, *ADCY4*, and *ADCY5* are regulated by DNA methylation (*Figure 4D*). However, direct evidence that this co-expression phenomenon is regulated by epigenetics requires further exploration.

To better identify the transcriptional co-expression patterns in *ADCY* genes, co-expression network was constructed using correlated genes (*Figure 4E*); the

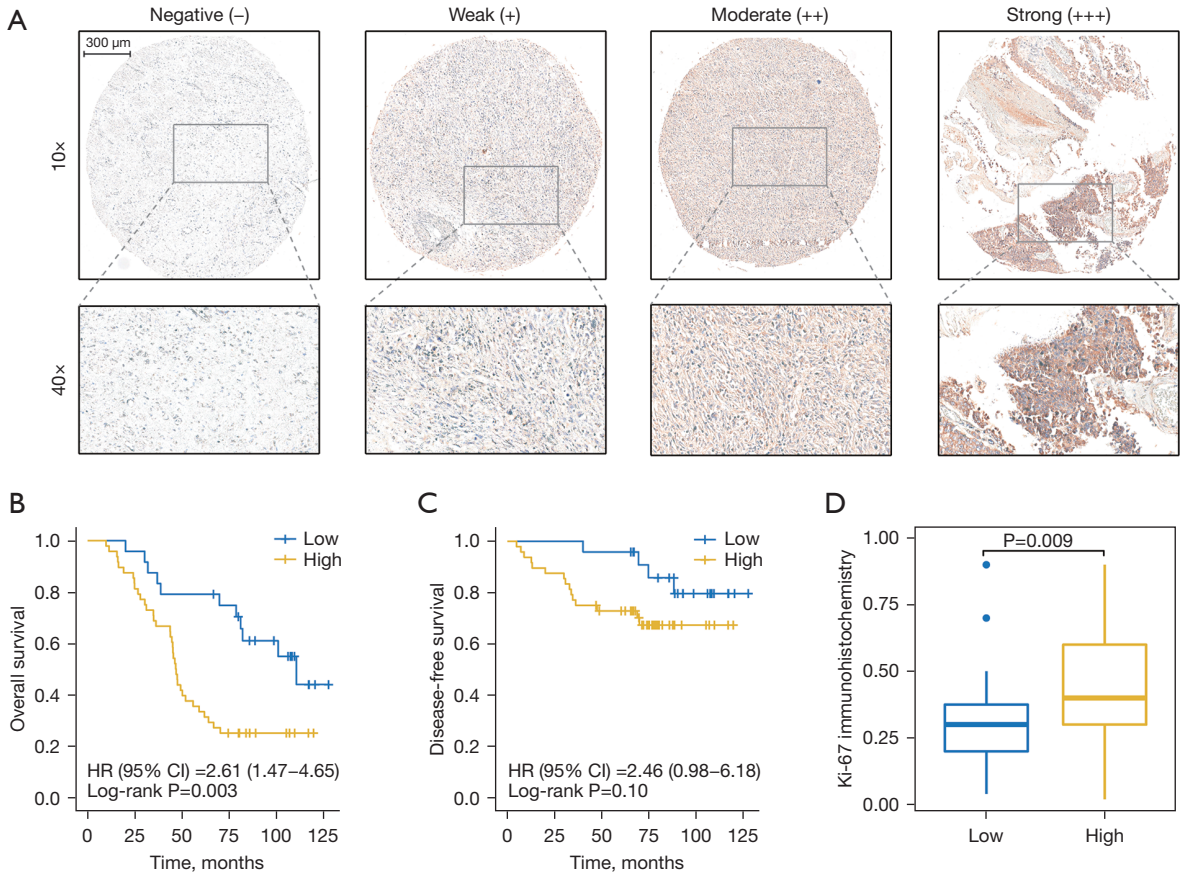


Figure 3 Validation expression and prognosis of *ADCY2* in bladder cancer clinical specimens. (A) IHC staining of TMAs. Four groups based on the expression level of *ADCY2* were identified (negative, weak, moderate, and strong) according to the IHC. (B) KM curve for OS of *ADCY2* expression in TMA cohort. The figure confirms that individuals with elevated expression of *ADCY2* experience poorer OS rates. (C) KM curve for DFS of *ADCY2* expression in TMA cohort. (D) Relationship of *ADCY2* gene expression with cancer proliferative activity in TMA cohort. Proliferation was measured by IHC expression of Ki67, and P was determined by two-sided Mann-Whitney *U* test. HR, hazard ratio; CI, confidence interval; *ADCY*, adenylyl cyclase; IHC, immunohistochemical; TMA, tissue microarray; KM, Kaplan-Meier; OS, overall survival; DFS, disease-free survival.

network containing *ADCY2*, *ADCY4*, and *ADCY5* were clearly separated from the other *ADCYs*. Overall, the transcriptional regulatory network validated the close correlation among *ADCYs* in BLCA, and co-expression was potentially regulated by epigenetics.

Differential expression gene enrichment analysis

To evaluate the clinical values and understand the biological functions of *ADCY2*, *ADCY4*, and *ADCY5* in BLCA, DEGs were extracted based on the median expression values, and the pathways enriched using DEGs were investigated. The receiver operating characteristic (ROC) curve revealed that *ADCY2*, *ADCY4*, and *ADCY5* were favourable diagnostic biomarkers for bladder cancer, and the area under the curve (AUC) were 0.96, 0.92, and 0.88 for *ADCY5*, *ADCY2*, and *ADCY4*, respectively (*Figure 5A*). Moreover, 2,017, 2,904, and 2,496 DEGs of *ADCY2*, *ADCY4*, and *ADCY5*, respectively, displayed by volcano plots to separate the up- and down-regulated groups, were identified (Figure S2B). DEGs were intersected using a Venn diagram to observe the overlap between these three groups (*Figure 5B*). The common genes were used for subsequent enrichment analysis. The significantly correlated genes and DEGs of the three groups were intersected, and five common genes *ADRA2A*, *SNED1*, *RASL12*, *LIMS2*, and *JAM2* were

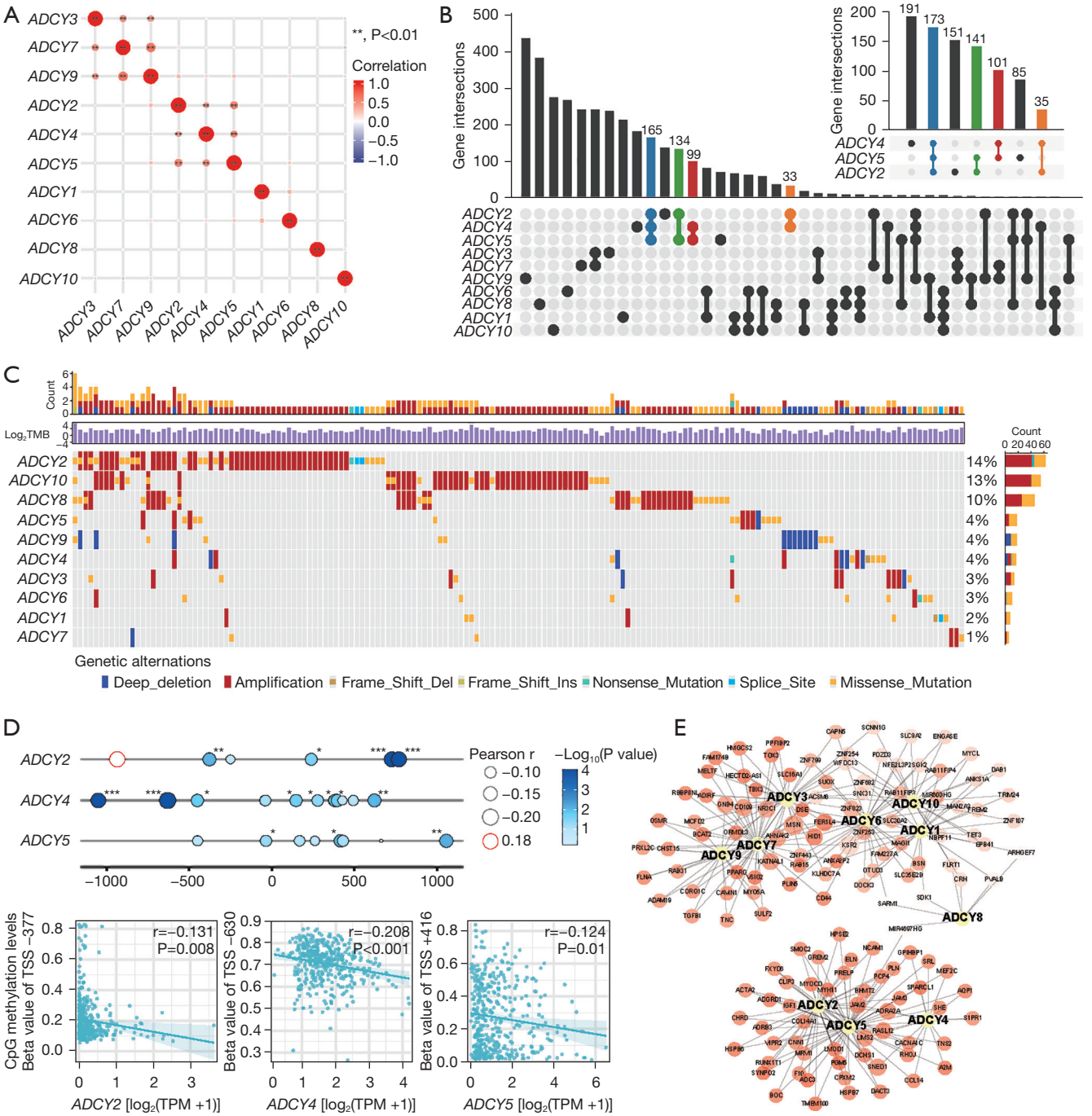


Figure 4 Co-expression analysis of *ADCYs* in bladder cancer. (A) Correlation between 10 *ADCYs* at the transcriptional level in BLCA patients of TCGA cohort. The coefficient and P value are assessed by Spearman's rank correlation. (B) An UpSet diagram shows the overlap of the top 500 correlated genes for *ADCYs*, suggesting that *ADCY2*, *ADCY4*, and *ADCY5* have the highest number of intersecting genes. (C) Genomic variation landscape suggests that co-expression between *ADCYs* in bladder cancer cannot be attributed to DNA copy number alterations. Data are publicly available from the *cbioportal* website (https://www.cbioportal.org/), and we selected the BLCA (TCGA, Pan-Cancer Atlas) cohort. (D) Association between promoter methylation and expression of *ADCY2*, *ADCY4*, and *ADCY5*. The top panel shows the Pearson correlation coefficient and P value assessed using the beta value of each CpG site and gene expression. The promoter region is defined as −1 kb/+1 kb of TSS. The bottom panel shows the correlation at representative CpG sites (*ADCY2*, TSS −377 bp; *ADCY4*, TSS −630 bp; *ADCY5*, TSS +416 bp). (E) Co-expression network constructed by the top 100 correlated genes of *ADCY* in bladder cancer. In the matrix representation of each network, a darker colour indicates a stronger correlation. ***, P<0.001; **, P<0.01; *, P<0.05. *ADCY*, adenylyl cyclase; TMB, tumour mutation burden; TSS, transcriptional start site; CpG, cytosine-phosphate-guanine; TPM, transcript per million; BLCA, bladder urothelial carcinoma; TCGA, The Cancer Genome Atlas.

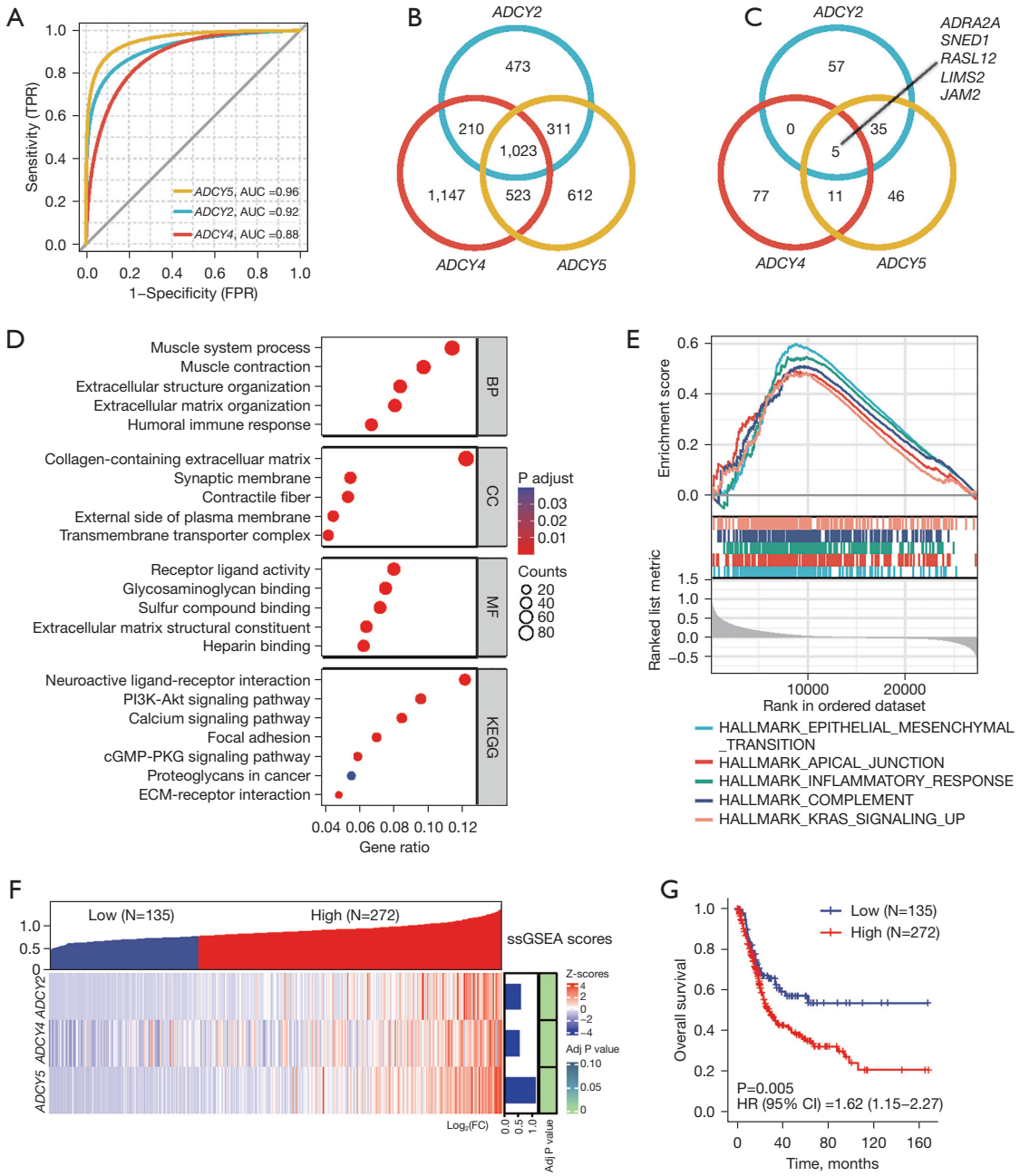


Figure 5 Differential expression gene enrichment analysis for *ADCY2*, *ADCY4*, and *ADCY5*. (A) ROC curve of patients with bladder cancer in TCGA cohort. (B) Venn diagram of the differential expression of *ADCY2*, *ADCY4*, and *ADCY5*. Statistically significant DEGs were defined as those with $\log_2$(FC) >1 and adjusted P<0.05. (C) The diagram of the top 100 DEGs according to the expression correlation of *ADCY2*, *ADCY4*, and *ADCY5* and there are five genes in the intersection of DEGs of *ADCY2*, *ADCY4*, and *ADCY5*, namely *ADRA2A*, *SNED1*, *RASL12*, *LIMS2*, *JAM2*. (D) Pathway enrichment analysis shows the top 5 GO terms and top 7 enriched KEGG pathways based on 1,023 DEGs overlapping between *ADCY2*, *ADCY4*, and *ADCY5*. (E) Enrichment plots from GSEA. Pathway analysis was enriched by 1,023 DEGs, and plots show the representative pathways. (F) Heatmap of 407 bladder cancer samples in TCGA using the Z-scores from *ADCY2*, *ADCY4*, and *ADCY5* expressions. The ssGSEA was used to divide the samples into groups with high and low expression of *ADCY2*, *ADCY4*, and *ADCY5* according to the minimal P value approach. (G) KM curve for OS of two groups based on the ssGSEA scores. The plot shows that patients with higher scores exhibited worse OS than those with lower scores (P=0.005). *ADCY*, adenylyl cyclase; AUC, area under the curve; TPR, true positive rate; FPR, false positive rate; BP, biological progress; CC, cellular component; MF, molecular function; KEGG, Kyoto Encyclopaedia of Genes and Genomes; ECM, extracellular matrix; ssGSEA, single-sample gene set enrichment analysis; FC, fold change; HR, hazard ratio; CI, confidence interval; ROC, receiver operating characteristic; TCGA, The Cancer Genome Atlas; DEG, differentially expressed gene; GO, Gene Ontology; GSEA, gene set enrichment analysis; KM, Kaplan-Meier; OS, overall survival.

obtained (*Figure 5C*).

KEGG analysis revealed that the *ADCY2*, *ADCY4*, and *ADCY5* DEGs were enriched in the calcium and PI3K-Akt signalling pathways (*Figure 5D*). GO analysis revealed phenotypic features, including collagen-containing extracellular matrix, extracellular structure organization, and a series of components focusing on epithelial-mesenchymal transition (EMT) and tumour metastasis phenotypes (*Figure 5D*). Considering the difference between the absolute expression trends in specific pathways and general expression alterations, GSEA was performed. The GSEA results further validated that the *ADCY2*, *ADCY4*, and *ADCY5*-related gene sets were associated with metastasis and tumour immunosuppressive-related process, such as hallmarks of EMT and the interleukin 2-signal transducer and activator of transcription 5 (IL2-STAT5) pathway (*Figure 5E*). ssGSEA clustered samples with relatively high or low *ADCY2*, *ADCY4*, and *ADCY5* expressions (*Figure 5F*). KM plots revealed a significant survival difference between the upregulated and downregulated groups (*Figure 5G*).

Boosting immune infiltration status and TME formation

Investigating the immunological role of *ADCY2*, *ADCY4*, and *ADCY5* could elucidate their potentially critical role in treating BLCA, especially in immunotherapy. Thus, the difference in immune cell enrichment between the two groups (*Figure 5F*) was evaluated; the upregulated group was characterised by increased immune infiltration by memory T cells, natural killer (NK) cells, macrophages, and other innate immune cells (*Figure 6A*). GSEA illustrated the pathways involved in the recruitment of innate immune cells, focusing on effector $CD8^+$ T, memory $CD4^+$ T cells, and macrophages (*Figure 6B*).

Next, *ADCY2*, *ADCY4*, and *ADCY5* enhancements in the formation of the TME were explored. Regression analysis showed that the mRNA levels of *ADCY2*, *ADCY4*, and *ADCY5* were positively correlated with the number of stromal cells present and infiltration of immune cells (*Figure 6C*). Markers of immune cells were also significantly correlated with *ADCY2*, *ADCY4*, and *ADCY5* expression levels, especially in $CD4^+$/$CD8^+$ T, memory B, and mast cells (Figure S4A). Survival analyses were performed; patients with higher infiltration of $CD4^+$/$CD8^+$ T, memory B, and mast cells had a relatively short OS, suggesting that the upregulation of *ADCY2*, *ADCY4*, and *ADCY5* co-expression affected the immune-mediated process and influenced survival time (Figure S4B). To further elucidate the role of *ADCY* in immunotherapy (particularly ICBs) (24), an immunotherapy cohort of bladder cancer patients was used to further explore the associations between *ADCYs* and several typical immune checkpoint genes. In TCGA-BLCA cohort, *ADCY* expression was positively correlated with programmed death ligand 1 (*PD-L1*), cytotoxic T lymphocyte-associated antigen-4 (*CTLA-4*), indoleamine 2,3-dioxygenase-1 (*IDO1*), B and T lymphocyte attenuator (*BTLA*), hepatitis A virus cellular receptor 2 (*HAVCR2*), and T cell immunoreceptor with Ig and ITIM domains (*TIGIT*) expression, and strong agreement was observed in the IMvigor210 cohort (*Figure 6D*). The population with higher *ADCY* expression in this cohort had a better prognosis and higher responsiveness to immunotherapy (*Figure 6E*, Figure S4C). It is noteworthy that the correlation between *ADCYs* and immunomodulators, chemokines, and chemokine receptors in both the TCGA and IMvigor datasets is consistent (Figure S5).

In order to explore whether *ADCY2* has the ability to independently predict the prognosis of BLCA and its role in the immune microenvironment, we first analysed the correlation between the clinical indicators of *ADCY2* and BLCA, and found that its expression levels were significantly different in lymph node metastasis of bladder cancer at different levels (*Figure 7A*). Subsequently, we conducted an in-depth analysis of the patterns and prognostic roles of tumour mutation burden (TMB) in bladder cancer (Figure S6A-S6C). The plot illustrated that TMB was significantly leading to a bad outcome, especially in bladder cancer. Our findings revealed a significant correlation between *ADCY2* and tumour purity (R=0.37, P<0.001, as illustrated in *Figure 7B*). Then, we assessed the infiltration levels of diverse immune cells in both high and low *ADCY2* expression groups. In particular, the infiltration levels of T cells, B cells, dendritic cell (DC), Eosinophils, immature DC (iDC), macrophages, mast cells, neutrophils, NK cells, plasmacytoid DC (pDC), effector memory T cell (Tem), T follicular helper (TFH), T helper type 1 (Th1) cells, and regulatory T cell (Treg) were all found to be correlated with the expression of *ADCY2*, and this phenomenon is universal in bladder cancer compared to the normal tissues (*Figure 7C*, Figure S6D,S6E). *Figure 7C* summarized the results, incorporating tumour-node-metastasis (TNM) stage, pathological grade, age, and categorization based on high and low *ADCY2* expression levels. Remarkably, *ADCY2* stands as an independent risk factor for BLCA. These findings collectively underscore the pivotal role of *ADCY2* in the immune microenvironment and

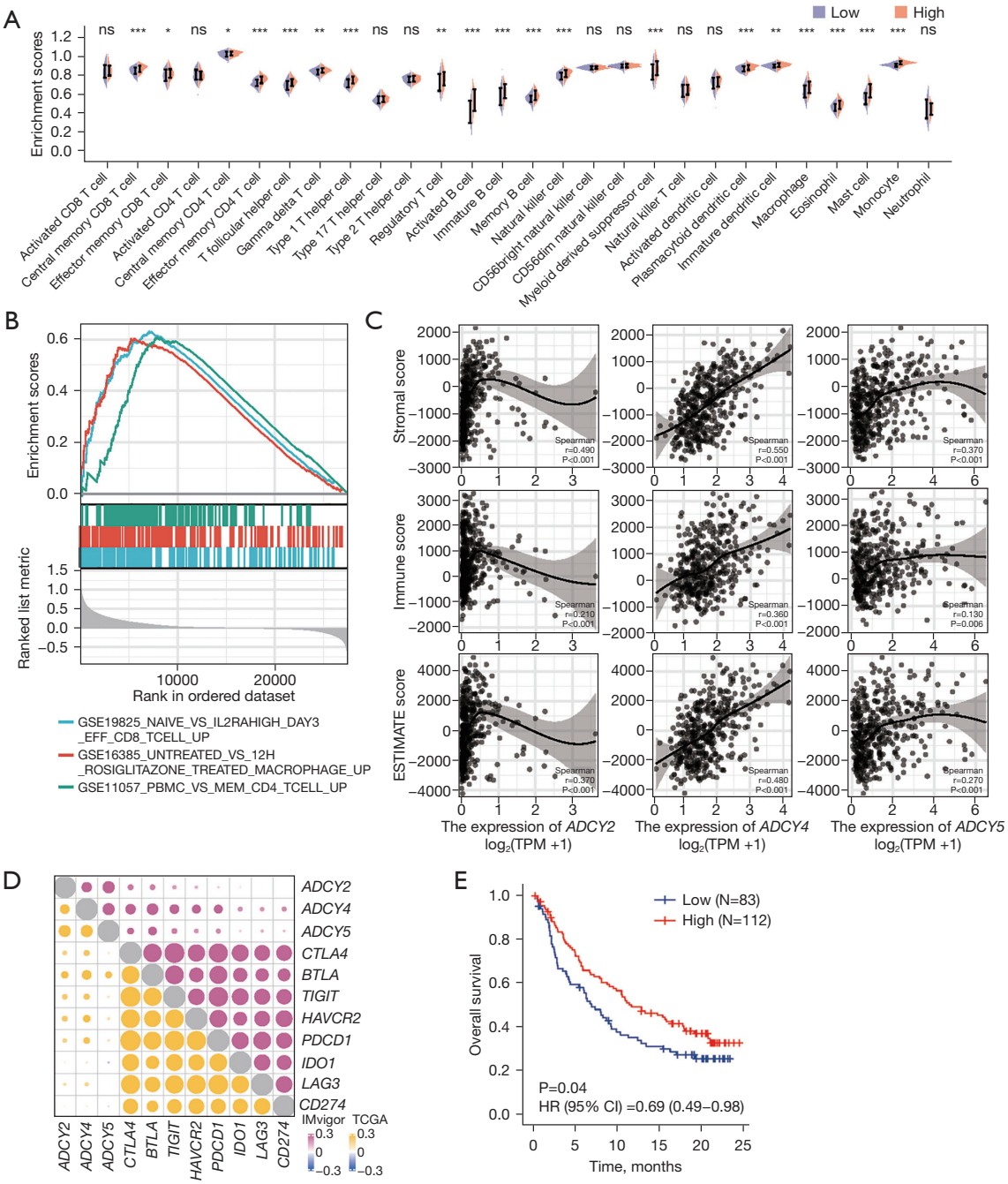
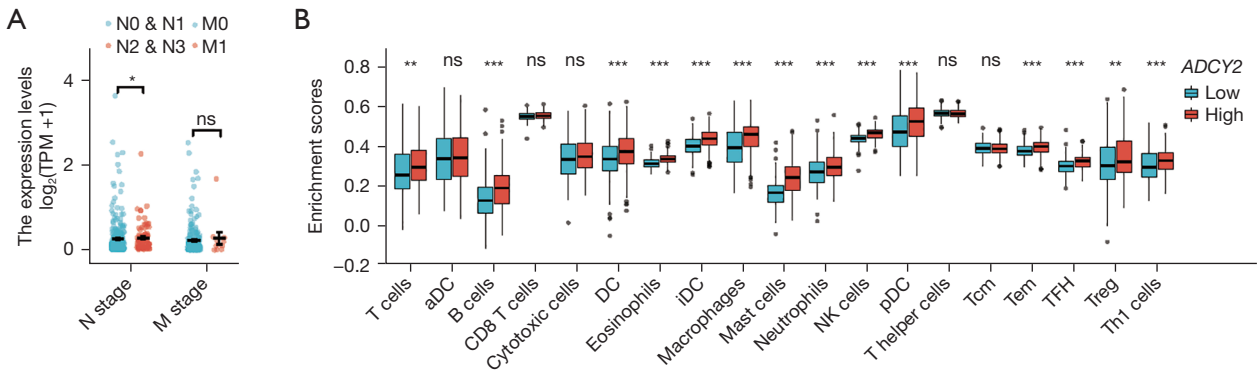


Figure 6 Relationship of *ADCY2*, *ADCY4*, and *ADCY5* gene expression with the immune infiltration status. (A) Clinical cases with high expression showed tumour immune infiltration. The grouping of the samples (high and low) is consistent with *Figure 5F*. (B) GSEA analysis indicated that four immune/inflammation-related pathways were enriched in the *ADCY2*, *ADCY4*, and *ADCY5* high expression group in the BLCA cohort. The top of the figure represents the enrichment score of the pathways; the bottom of the figure represents the ranked differential expression gene list between the high and low *ADCY2*, *ADCY4*, and *ADCY5* expression groups. (C) Stromal, and immune, and ESTIMATE scores in TCGA of BLCA patients using ESTIMATE algorithm analysis. The figure indicates that high expression is related to the phenotypes with increasing tumour immune infiltration. (D) Positive correlation between *ADCY2*, *ADCY4*, and *ADCY5* expression and levels of immune checkpoint molecules in TCGA and IMvigor210 cohorts. (E) KM curve for OS of two groups based on the ssGSEA scores in the IMvigor210 cohort. This figure is related to Figure S4C. ***, P<0.001; **, P<0.01; *, P<0.05; ns, P>0.05. *ADCY*, adenylyl cyclase; TPM, transcript per million; TCGA, The Cancer Genome Atlas; HR, hazard ratio; CI, confidence interval; GSEA, gene set enrichment analysis; BLCA, bladder urothelial carcinoma; KM, Kaplan-Meier; OS, overall survival; ssGSEA, single-sample gene set enrichment analysis.



C

Characteristics	Total (N)	HR (95% CI) Univariate analysis	P value	HR (95% CI) Multivariate analysis	P value
Pathologic T stage	173				
T1 & T2	57	Reference		Reference	
T3 & T4	116	2.282 (1.226–4.248)	0.009	3.066 (0.804–11.693)	0.10
Pathologic N stage	173				
N0	129	Reference		Reference	
N1	21	1.811 (0.946–3.467)	0.07	0.882 (0.110–7.053)	0.91
N2 & N3	32	2.584 (1.526–4.375)	<0.001	0.958 (0.124–7.427)	0.97
Pathologic M stage	173				
M0	166	Reference			
M1	7	2.301 (0.921–5.745)	0.07		
Pathologic stage	173				
Stage I & II	52	Reference		Reference	
Stage III	66	1.533 (0.756–3.109)	0.24	0.416 (0.097–1.778)	0.24
Stage IV	55	2.947 (1.495–5.807)	0.002	0.923 (0.083–10.275)	0.95
Age (years)	173				
≤70	105	Reference			
>70	68	1.203 (0.755–1.917)	0.44		
ADCY2	173				
Low	95	Reference		Reference	
High	78	2.907 (1.768–4.781)	<0.001	2.609 (1.551–4.386)	<0.001

Figure 7 Relationship between clinical indicators and *ADCY2*. (A) Correlation of clinical characteristics and *ADCY2* expression, including metastasis and lymph node invasion. (B) Immune infiltration in *ADCY2* high- and low-expressed groups. (C) Cox regression analysis illustrated the expression level of *ADCY2* significantly reflect the common clinical indicators in prognosis and outcome (P<0.05). ***, P<0.001; **, P<0.01; *, P<0.05; ns, P>0.05. TPM, transcript per million; *ADCY*, adenylyl cyclase; aDC, activated DC; DC, dendritic cell; iDC, immature DC; NK, natural killer; pDC, plasmacytoid DC; Tcm, central memory T cell; Tem, effector memory T cell; TFH, T follicular helper; Treg, regulatory T cell; Th1, T helper type 1; HR, hazard ratio; CI, confidence interval.

its potential as a prognostic indicator for BLCA.

Discussion

This study aimed to evaluate the potential value of *ADCYs* as diagnostic biomarkers or therapeutic targets for bladder cancer. Four of the 10 members of the *ADCY* gene family exhibited transcriptional differences between tumour and normal specimens. Among them, *ADCY2*, *ADCY4*, and *ADCY5* exhibited closer interactions than other members in the corresponding expression network, indicating a joint interaction mechanism or similar functions. Thus, these members can be used as prognostic biomarkers to indicate OS/DSS time in BLCA patients; the clinical data of the TMA cohort validated *ADCY2* efficacy in disease prognosis. Moreover, *ADCYs* can predict the efficacy of immunotherapy and screen for the subpopulation that may benefit from ICBs.

Abnormal methylation of *ADCY2* can affect clinical outcomes in colorectal cancer (25). SNP(rs4702484) in *ADCY2* region was identified significantly associated with capecitabine/5-fluorouracil (5-FU) susceptibility, which is

directly influencing the chemotherapy outcome of bladder cancer (26). *ADCY4* mediated the activation of intracellular Ca^{2+}, which might influence carcinogenesis and adverse invasion of lung adenocarcinoma cells (27). Some studies have reported that *ADCY5* plays a role in the genesis and development of neurobiological diseases, including dyskinesia, early onset autosomal dominant chorea, and dystonia (28,29).

This study explored the correlation between features of *ADCY2*, *ADCY4*, and *ADCY5* gene and BLCA prognosis. The effect of *ADCY2* on survival was validated using real-world clinical data and verified using TMA experiments. In the TMA cohort, the correlation between included clinical characteristics and *ADCY2* expression was investigated; *ADCY2* levels were not associated with distant metastasis but were positively correlated with lymph node invasion.

A previous study has demonstrated that *ADCYs* are not homologous regarding degrees of amino acid sequence and can be divided into distinct isoform subgroups with similar amino acid sequences and biochemical properties (30). Therefore, we explored co-expression from a genetic and epigenetic perspective. Amplification and missense mutation were the major DNA alteration in *ADCY2*, while deep deletion was the main genomic alteration in *ADCY4*. Although the mutation distribution could account for the downregulation in BLCA samples compared to normal tissues, the DNA alteration types in *ADCY2*, *ADCY4*, and *ADCY5* were not coincident. Given that the transcription of these different *ADCY* genes is regulated by distinct promoter/enhancer elements (30,31), we sought clues from a DNA methylation perspective. We found that methylation of the transcriptional start site was negatively correlated with *ADCY* gene expression, similarly to the results of previous studies (32,33). Owing to the consistent trend in *ADCY2*, *ADCY4*, and *ADCY5*, methylated modifications might function in constructing the co-expression network and work as a synchronous system. These results are consistent with those of previous studies (31,34), suggesting that the expansion of the *ADCY* gene family is not due to gene duplication. Subsequent studies should explore the regulatory mechanism of epigenetic modifiers on the *ADCY* co-expression network to evaluate the potential therapeutic value of targeting *ADCY* genes.

Pathway enrichment in neuroactive ligand-receptor interaction, EMT, PI3K-AKT signalling, inflammation response, and IL2-STAT5 signalling might account for the worsened prognosis in the *ADCY*-upregulated groups. These pathways modulate tumour cells to transfer and escape immune surveillance (35,36). The intersection of DEGs also revealed five correlated genes (*ADRA2A*, *SNED1*, *RASL12*, *LIMS2*, and *JAM2*). These screened genes have been reported as intermediate elements in tumour biological processes and are involved in tumour metastasis and drug resistance (37-39). Potential regulatory mechanisms exist among these five correlated genes in the interaction networks, in which *ADCY2* might also affect tumour malignancies.

The activities of anticancer immunity comprehensively reflect the ability of tumours to escape immune surveillance and develop drug resistance, which is closely related to the complicated immunomodulatory interactions in the TME. *ADCY2* was associated with several steps in tumour evolution from innate immunity. Consequently, the infiltration levels of effector immune cells, such as $CD8^+$ T cells, NK cells, mast cells, and macrophages, were also significantly increased in the high-*ADCY2* enrichment population. These patients were classified as better responders to the immune-killing effect. Thus, this subset of patients with advanced bladder cancer may benefit from immunotherapy and targeted therapy (40). The effect of *ADCY* on the regulation of immune checkpoint activity was tentatively analysed; mRNA expression of *ADCY2*, *ADCY4*, and *ADCY5* indicated a significant correlation with well-known immune checkpoint markers, and the survival data conformed to the immune status evaluated. Thus, our results improve the selection of populations that may achieve better clinical outcomes and durable responses to immunotherapy.

TME was demonstrated to largely participate into the process of tumour recurrence. The relationship between *ADCY2* level and TME formation has been statistically disclosed in the research. We expected that the succedent studies could further validated the role of *ADCY2* in formation of TME and promoting long-term recurrence. *ADCY2* could be identified as an indirect bridge to measure long-term tumour recurrence from the perspective of TME formation.

Nevertheless, there are still some limitations in our study. Firstly, owing to the difficulty in retrieving datasets containing both transcriptomic and clinical survival data from public databases, the sample size of TCGA raw data was small and thus unpersuasive. Some differences were observed between the cohorts. Then, the clinical cohort of TMA mainly comprised patients with invasive advanced bladder cancer, whereas TCGA cohort had a higher proportion of patients at earlier stages. Finally, the majority

of our research findings were based on bioinformatics analysis, without experimental validation of the associated conclusions. We will conduct laboratory studies in the future to further investigate and validate the phenomenon of co-expression and molecular mechanisms of *ADCYs* in EMT.

Conclusions

In this research, the role of *ADCY2* in predicting prognosis and immunotherapy responsiveness in bladder cancer was investigated. *ADCY2* could be used as a prognostic marker for BLCA. We will further explore the potential molecular mechanism of *ADCY2* in modulating and alternating the biologic features of bladder cancer cells.

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Footnote

Reporting Checklist: The authors have completed the REMARK reporting checklist. Available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-1796/rc

Data Sharing Statement: Available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-1796/dss

Peer Review File: Available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-1796/prf

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-1796/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Medical Ethics Committee of Second Military Medical University (No. CHEC2019-134). Individual consent for this retrospective analysis was waived due to the retrospective nature.

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