RESEARCH PAPER

Transcriptome analysis reveals PER2 and CRY2 as biomarkers in bladder cancer and circadian rhythm pathways

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Abstract

Bladder cancer RNA-Seq data analysis highlighted significant insights into the molecular mechanisms and biological processes underlying tumorigenesis. Utilizing R packages such as edgeR, limma, and DESeq2, we preprocessed and normalized the data to identify differentially expressed genes (DEGs). Unsupervised learning methods, including PCA, facilitated the classification of these DEGs, revealing critical pathways associated with DNA replication, DNA repair, and cell cycle control. Gene ontology enrichment analysis further delineated the affected biological processes, cellular components, and molecular functions. Notably, our findings identified potential biomarkers, particularly the genes PER2 and CRY2, which are integral to the circadian rhythm pathway. Additionally, we observed that protein complexes CLOCK/BMAL1, CLOCK/BMAL2, and BMAL1/NPAS2 regulate the expression of several clock genes via E-boxes. The study also constructed a differential gene network in bladder cancer, providing insights into the regulatory functions of key genes involved in tumor progression. These findings not only enhance our understanding of bladder cancer biology but also pave the way for potential therapeutic targets and biomarkers.

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Introduction

Bladder cancer is a very malignant tumor of urinary bladder (Dobruch and Oszczudłowski, 2021). It is more frequent in men compare to women. It is generally diagnosed in mid age people over 56 (Saginala *et al.*, 2020). Some of the major risk causes reported for bladder cancer are smoking, rubber manufacturing chemicals, oil paints and textile chemicals (Pramod *et al.*, 2020).

There are various genetic mutations that increase the risk of bladder cancer (Zhang and Zhang, 2015). Epigenetic changes such as DNA methylation and acetylation of histone are also increase the risk of bladder cancer (Bošković *et al.*, 2022). Bladder cancer can be developed in bladder neck, dome of bladder, anterior wall of bladder, bladder nos, lateral wall of bladder, trigone of bladder, posterior wall of bladder and ureteric orifice. Malignant bladder tumor can invade nearby tissues and may infect to rest of the body (Okbah *et al.*, 2022). Genomics of bladder cancer helps to understand the main molecular mechanisms. Bladder cancer genetic makeup and clinical behavior could be easily understood by genomic profiling (Green and Milowsky, 2016).

There are many ways of treatment such as chemotherapy, radiation therapy, immunotherapy and surgery (DeGeorge *et al.*, 2017). Gene expression data can be used to understand genes regulation in normal and disease process. Transcriptome profiling is used to decipher the underlying molecular mechanisms of normal and disease processes in cancer (Cieślik and Chinnaiyan, 2018). RNA sequencing (RNA-Seq) is a very important technique used tool for transcriptome profiling that enables extensive analysis of gene expression at the transcript level (Maeda *et al.*, 2018). RNA-Seq analysis has been applied to bladder cancer to get important insights of the molecular mechanism and biological process of tumorigenesis (Hwang *et al.*, 2018). Several studies have examined RNA-Seq data of bladder cancer, identifying numerous genes and pathways linked to tumorigenesis. Genes involved in DNA replication, repair, and cell regulation, such as cyclins, CDKs,

and CHKs, have been extensively researched (Zhao *et al.*, 2017).

Upregulation of these genes has been linked to increased bladder cancer metastasis and proliferation rate. Earlier study has reported down regulation of genes such as E-cadherin and integrins in bladder cancer cells (Zhu *et al.*, 2012). Furthermore, many genes are involved in immune response, inflammation, and tumor progression in bladder cancer (Dyck and Mills, 2017).

RNA-Seq can be used to develop gene expression signatures that can predict the prognosis and response to therapy of bladder cancer patients (Li *et al.*, 2022). Differentially expressed genes of bladder cancer help to understand the gene expression level between different samples or conditions. Recent studies have identified several key pathways such as the PI3K/AKT/mTOR pathway (Li *et al.*, 2022), the MAPK pathway (Dangle *et al.*, 2009), and the Wnt/β-catenin pathway (Garg and Maurya, 2019) that are dysregulated in bladder cancer. These pathways are involved in cell proliferation, survival, and differentiation, and their dysregulation is thought to contribute to the development and progression of bladder cancer.

Important drug targets, such as the HDAC inhibitor vorinostat, DNMT inhibitor decitabine, and proteasome inhibitor ixazomib target cancer pathways that are dysregulated in bladder cancer and may improve therapeutic outcomes (Sato *et al.*, 2017). Different types of bladder cancers have distinct molecular profiles that could be used to develop targeted therapies (Sanguedolce *et al.*, 2022). In a recent study, several genes were linked to chemotherapy resistance in bladder cancer patients who received neoadjuvant chemotherapy. Patients with highly expressed genes had a poorer prognosis than those with low expression. These findings could assist clinicians in identifying patients at risk of treatment failure and developing more effective treatment approaches (Vlachostergios and Faltas, 2019).

According to a study, bladder cancer can lead to the dysregulation of vital biological processes such as cell migration, angiogenesis, and cell adhesion. Since these processes play a crucial role in the development and progression of bladder cancer, targeting their dysregulation could open up new avenues for therapeutic intervention. In our article, we delved into the transcriptomics profile of bladder cancer by analyzing RNA-Seq data. Our research findings revealed differentially expressed genes, ontology and pathway enrichment analysis, and network analysis, all of which hold significant importance for the diagnosis and treatment of bladder cancer.

Materials and methods

Data collection

Transcriptomic profiling data for bladder cancer was collected from the Cancer Genome Atlas (TCGA) (https://www.cancer.gov/ccg/research/genome-sequencing/tcga) and the Genomic Data Commons (GDC) (https://gdc.cancer.gov/access-data/gdc-data-portal) . We have downloaded the TCGA data by using TCGAbiolinks (Colaprico *et al.*, 2016) an R package directly from Genomic Data Commons (GDC). We have downloaded 431 count cases out of which 412 were primary tumor and 19 were normal count cases. Gender wise bladder cancer was observed in 117 female and 314 male. All the material and methods were performed in R v4.2.3 environment (https://www.R-project.org/) (Schwarzer, 2007) and Bioconductor (Ihaka and Gentleman, 1996) package.

Data preprocessing and normalization

Preprocessing can help to detect true biological signal and correct the biases in the data to make it comparable across various samples. Preprocessing makes data smooth and easy analysis of differential expressed genes (DEGs). For preprocessing of data we have used edgeR (Robinson *et al.*, 2009) and voom (Law *et al.*, 2014) R packages. We have also used limma (Ritchie *et al.*, 2015) and DESeq2 (Love *et al.*, 2014) an R package for DEGs analysis. After preprocessing RNA-Seq data, normalization was performed for that we used limma R package (Ritchie *et al.*, 2015). Our normalization process has used the trimmed mean of M-values (TMM) method (Robinson and Oshlack, 2010).

Clustering of data

Normalized RNA-Seq data was processed through the classification to get the important insights into the subtypes of cancer. We used unsupervised learning method to identify patterns and structures in our data. To reduce the dimensionality of data we used principal component analysis (PCA) method (Maćkiewicz and Ratajczak, 1993). For non-linear reduction of data UMAP method (Ghojogh *et al.*, 2023) was used, and clustering was performed on UMAP data. For classification we used DESeq2 (Love *et al.*, 2014), edgeR (Robinson *et al.*, 2009), and limma (Ritchie *et al.*, 2015) R packages. An R packages caret was used to train and testing machine learning method for evaluating the performance of the models. R package glmnet (Friedman *et al.*, 2023) was used to fitting generalized linear models for analyzing high-dimensional gene expression data.

Differentially expressed genes and survival analysis

For analysis of differentially expressed genes (DEGs) we have used limma (Ritchie *et al.*, 2015), edgeR (Robinson *et al.*, 2009), DESeq2 (Love *et al.*, 2014), and voom (Law *et al.*, 2014) R packages. DEGs in bladder cancer RNA-Seq data were analyzed using language R (v4.2.3) and Bioconductor. Cutoff values a log2|fold change|>1 and a false discovery rate of <0.05 were set for the DEGs identification. SummarizedExperiment (Morgan *et al.*, 2021) is an R package was used to integrate gene expression and clinical information, and genomic annotations. We have used the method "survival" R package (Therneau, 2023) for cancer data survival analysis and visualization. The Cox analysis (Abd Elhafeez *et al.*, 2021) was deployed to identify relationship between gene expression and patient survival we used coxph function. Survival analysis hazard ratio (HR) and P value for bladder cancer data were calculated, and the difference we got was considered significant at $P < .05$.

Enrichment analysis, data visualization and plotting

We have performed gene enrichment analysis over differentially expressed genes. Bladder cancer DEGs were analyzed by enrichR (Chen *et al.*, 2013) server to identify the genes associated with biological process, cellular components and molecular functions. Further we have analyzed the cell types by using PanglaoDB (Franzén *et al.*, 2019). Enrichment for Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway (Kanehisa and Goto, 2000) and WikiPathway (Martens *et al.*, 2021) was also performed. To creates all the plots for bladder cancer data analysis we have use gplots (Warnes *et al.*, 2019), ggplot2 (Gómez-Rubio, 2017), cowplots (Wilke, 2019). For the interactive visualization of gene expression data we have used Glimma R package (Su *et al.*, 2017). RColorBrewer (Neuwirth, 2014) is an R package that provides a collection of color palettes for creating visually appealing plots.

Results

Bladder cancer affects many people across the world. The bladder cancer RNA-Seq dataset is provided by The Cancer Genome Atlas (TCGA) project. We have performed data normalization before conducting any downstream analysis. Our study used Mean-Variance Trend (MVT) to analyze the bladder cancer RNA-Seq from different tissue types. Our resulting plots for bladder cancer showed the mean-variance trend for two different tissue types: primary tumor, and normal tissue (Fig. 1A). Results show a clear separation between primary tumor and normal tissue samples. Both tissues have shown significantly different expression levels. We have also studied the gender-specific differences in cancer RNA-Seq data using this approach. The data plot showed different patterns for female and male bladder cancer samples. MVT for males was more variable compared to females. This trend suggests a higher degree of variance due to male-specific factors in bladder cancer data. MVT for females shows a more consistent pattern along the expression level across samples (Fig. 1D). MVT for bladder cancer types and based on race are shown in Fig. 1B and 1C, the variance was consistent with the expression pattern of bladder cancer samples.
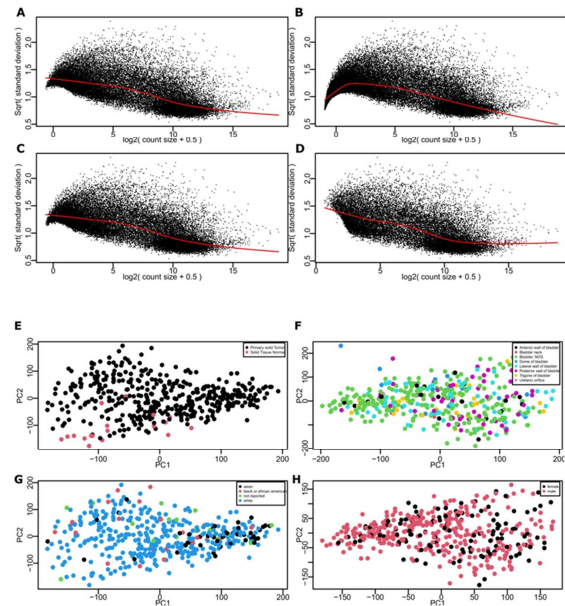

Fig. 1. Voom normalized data of bladder cancer. Mean variance trend (MVT) plots are shown for bladder cancer condition variable (A) MVT based on definition group primary tumor and normal tissue samples, (B) MVT for bladder cancer types, (C) MVT based on race, and (D) MVT for gender based male and females are shown. PCA plots are shown for the first two principal components (E) PCA plot for two sample groups, tumor tissue and healthy tissue, (F) PCA plot based on bladder cancer type, (G) PCA plot of bladder cancer based on race, (H) PCA plot for gender, male and female.

Dimensionality reduction and PCA analysis

Principal Component Analysis (PCA) was performed over bladder cancer RNA-Seq data. We have analyzed condition variable definition that have the primary solid tumor and solid tissue normal samples and visualized as PCA plots of PC1 and PC2 (Fig. 1E). Our result shows that both sample groups (tumor and normal) have well-separated RNA expression profiles. PCA capture the largest amount of variance and showed a clear separation between primary solid tumor and solid tissue normal samples along PC1 and PC2. There was some degree of heterogeneity revealed within tumor samples. This indicates that not all bladder tumors are the same, and there may be subtypes of bladder cancer having different expression profile (Fig. 1F). In addition to our analysis of bladder

cancer based on gender, we also investigated the differences in RNA expression based on race. Our PCA plot revealed that RNA expression profiles were well-separated based on race (Fig. 1G). Furthermore, we applied PCA to RNA-Seq data from male and female bladder cancer patients to explore gender-specific patterns in RNA expression. Our visualization of PC1 vs PC2 showed significant differences in RNA expression between males and females (Fig. 1H). Specifically, we found that genes related to inflammation and immune response were more strongly expressed in female samples, while genes involved in DNA repair and cell proliferation were more strongly expressed in male samples.
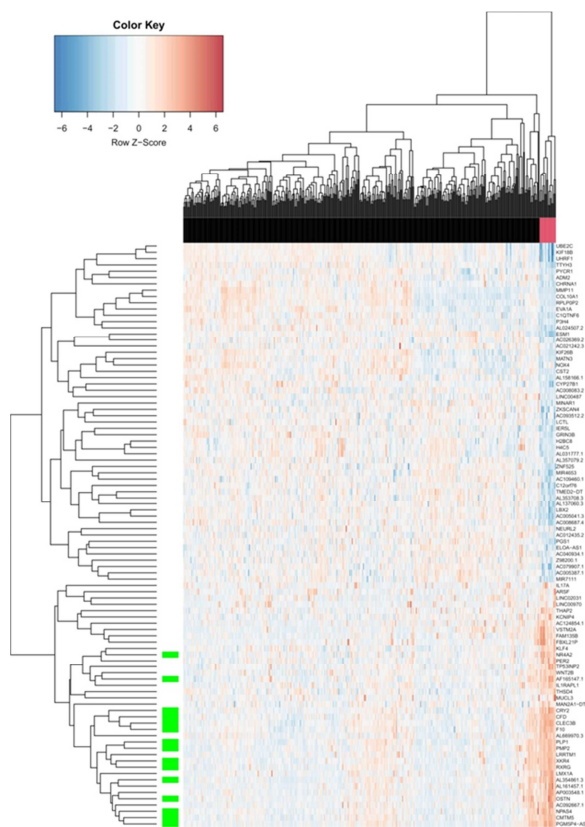

Fig. 2. Gene Heatmap Dendogram. Bladder cancer samples cluster together by hierarchical clustering. The bladder cancer samples in red are solid tissue normal, and the samples highlighted in black are primary solid tumor. The genes in green colors are earlier predicted in differential expression analysis of bladder cancer. Based on z-score upregulated genes are in maroon color and down regulated are shown in blue color.

Hierarchical clustering and gene heatmap

The data was normalized and split into a train and test set. An elastic net model was trained, which combines LASSO and Ridge Regression to select genes that predict each condition. The selected genes were used to build a classification model, which showed great accuracy with sensitivity, specificity, and precision all equal to 1. Relevant genes with non-zero values were selected. After normalization, we run a hierarchical clustering algorithm to cluster our samples genes. We have visualized the gene expression pattern of gene clusters in a heatmap format using the limma gene heatmap function (Fig. 2). This study has analyzed RNA-Seq data from bladder cancer patients and healthy controls over 28088 differentially expressed genes. DEGs were further differentiated between up and down regulated genes based on expression levels. Our study shows that DNA replication, DNA repair, and cell cycle control were among the significant pathways enriched in the differentially expressed genes. Because these pathways are linked with the development and spread of cancer, their identification in bladder cancer advances our knowledge of the disease.

Differentially expressed genes and survival analysis

Differentially expressed genes in human bladder cancer were visualized using a volcano plot and an enhanced volcano plot (Fig. 3). The volcano plot shows the log2 fold change of gene expression on the x-axis and the negative log10 of the p-value on the y-axis (Fig. 3A).

Differences in gene expression between two conditions are represented by log2 fold change. The p-value on the y-axis represents the statistical importance of the difference in gene expression of bladder cancer. The smaller p-value is the indicator of the more significant difference among expression levels. Each point in the enhanced volcano plot represents the gene. The color of each point or gene in the plot indicates the level of significance. Genes with a high log2 fold change and a low p-value are colored in red, while blue-colored points have a low log2 fold change and a

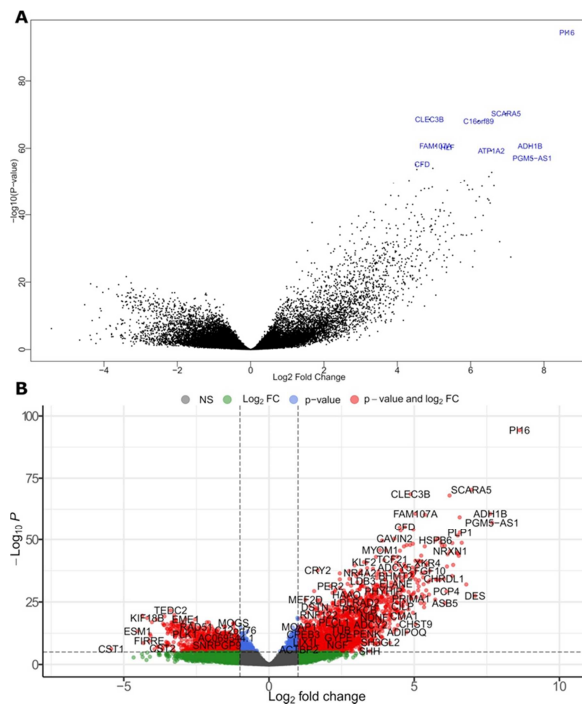high p-value. Genes with intermediate values are colored in gray or green (Fig. 3B).

Fig. 3. Differentially expressed genes are plotted as; (A) Volcano plot, top ten differentially expressed genes are labeled in blue color, and (B) Enhanced volcano plot, The top genes by P value are labeled, and genes that meet the FDR and Log Fold Change thresholds are colored (red for upregulation and blue for down regulation).

Expression of genes in bladder cancer was analyzed through Kaplan-Meier (KM) plots. The relationship between time and survival probability for differentially expressed genes of bladder cancer has visualized through KM plots (Fig. 4). Our result in KM plot shows that there is very similar sloppy trend until almost the 2800 day mark, where the survival probability for females seems to have worse compare to the males. Despite we have tested event curves through logrank test that is a repeated test of independence. Plot shows that gender alone does not importantly shift prognosis in this bladder cancer dataset (Fig. 4A). We have also visualized the KM plot where the number of patients dying or being censored as time increases (Fig. 4B). KM plot shows that most of the bladder cancer patients die or are censored before the 3000-day mark.
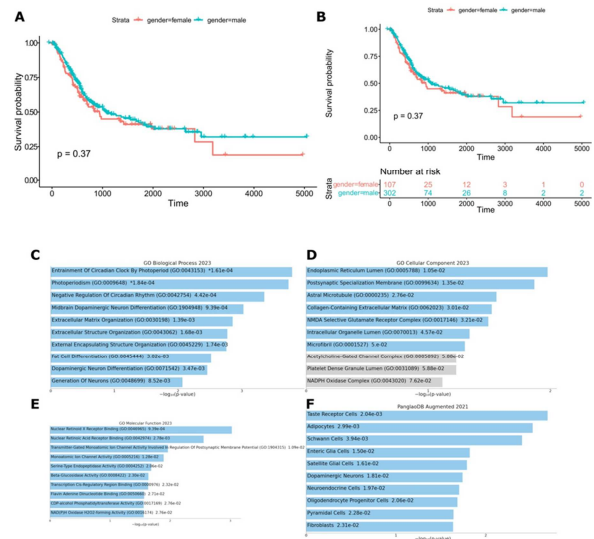

Fig. 4. KM plots; (A) Kaplan-Meier plot displays two very similar trajectories, up until roughly 2800 days, when females appear to have a lower chance of survival. The logrank test (p value) also determines that both "event curves" have not very much differ from one another. (B) KM plot with "risk table". Bar chart of top enriched terms from the (C) GO_Biological_Process_2023, (D) GO_Cellular_Component_2023, (E) GO_Molecular_Function_2023, and (F) PanglaoDB_Augmented_2021 gene set library.

Gene ontology and pathway enrichment analysis
Gene ontology enrichment shows that significant biological processes (Fig. 4C), cellular component (Fig. 4D) and molecular functions (Fig. 4E) were affected by bladder cancer.

We have also figured out cell types (Fig. 4F) which are high-flown in these processes. Main pathways that are affected by bladder cancer were also explored. We have found most significant KEGG Pathway from human which are highly disturbed by bladder cancer. Wiki pathways was also identified which are important for bladder cancer therapeutics. According to our research, bladder cancer appears to involve three pathway networks: Entrainment of circadian clock (GO-0009649) implicated in various physiological processes, including metabolism, sleep, and cancer. Entrainment of circadian clock by photoperiod (GO-0043153) involved in the regulation

of seasonal behaviors, such as migration and hibernation. Photoperiodism (GO-0009648) implicated in various biological processes, including flowering in plants and seasonal changes in animal behavior. Our research has identified two pathway networks that may play a role in human biology: Glial cell differentiation (WP2276) process is critical for the proper functioning of the nervous system and enriched in bladder cancer disorders (Fig. 5A).
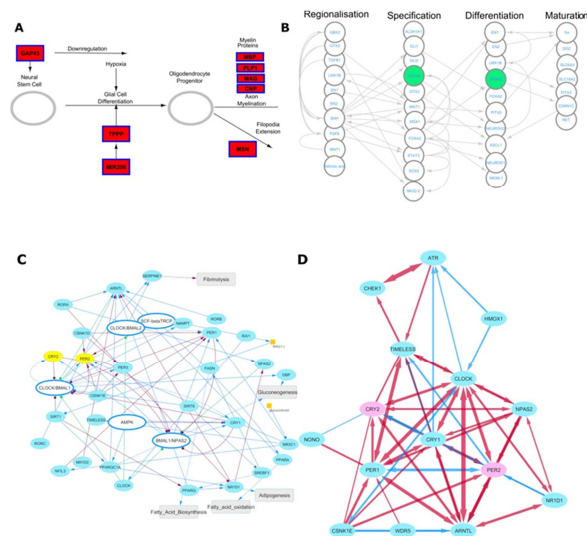

Fig. 5. Pathway enrichment (A) WP2276-Glial cell differentiation-Homo sapiens, (B) WP2855 - Dopaminergic neurogenesis - Homo sapiens, and DEGs predicted genes LMX1A and NR4A2 are shown in green color actively takes part in cell differentiation process. Gene network pathway; (C) Circadian clock, DEGs PER2 and Cry2 are represented by yellow color, major protein complexes are represented in large blue circle eclipses, (D) Circadian rhythm pathway, DEGs PER2 and CRY2 are shown in magenta color.

Dopaminergic neurogenesis (WP2855) involved in various neurological and psychiatric disorders, but has shown enrichment in bladder cancer (Fig. 5B). Further research is needed to fully understand the mechanisms underlying these pathways and their potential therapeutic targets. We have identified two related pathway networks that may play important roles in various biological processes: Circadian clock and Circadian rhythm pathway. Disruptions in circadian clock function have been implicated in various diseases; including cancer, metabolic

disorders, and sleep disorders. Circadian rhythm pathway refers to the various molecular and cellular mechanisms that regulate the circadian clock and its outputs. This pathway involves a complex network of genes and proteins that interact with each other to maintain the proper timing of various physiological processes, such as sleep/wake cycles, hormone secretion, and metabolism. In this study both pathways has shown enrichment in bladder cancer (Fig. 5C and 5D).

Discussion

Our study has analyzed RNA-Seq data from bladder cancer patients, revealing gender-specific differences and distinct variations between primary tumor and normal tissue samples. The study used PCA plots, hierarchical clustering, and gene heatmap to group the data and identify patterns in RNA expression linked to inflammation and immune response specific to particular genders. The study used volcano and improved volcano plots to find the genes that were differently expressed and to visualize them. The study also found important pathways involved in the initiation and spread of cancer, such as cell cycle regulation, DNA replication, and DNA repair. Furthermore, gene enrichment analysis and Kaplan-Meier plots were used to examine the expression of genes in bladder cancer and identify potential biomarkers. The study discovered that prognosis was not significantly influenced by gender in this dataset. EZH2 was identified as a potential treatment strategy based on its expression, while CDK1 may be a helpful predictive indicator. The findings provide important insights into the prognosis of patients with bladder cancer and identify potential targets for future research.

In this study we found that bladder cancer involves various biological processes, including gene regulation and environmental factors. One such process is the regulation of the circadian clock, which controls metabolism, cell proliferation, and sleep-wake cycles.

Disruptions to this clock, caused by non-functionality or environmental factors, can increase the risk of

bladder cancer (Fekry and Eckel-Mahan, 2022). Photoperiodism, a biological process regulated by external cues such as light, also plays a role in bladder cancer growth. The PER2, CRY2, and FBXL21P genes are interconnected and involved in regulating the circadian rhythm. Disruptions to these genes can lead to misaligned circadian rhythms and bladder cancer development. Additionally, the biological processes of midbrain dopaminergic neuron differentiation, extracellular matrix organization, and fat cell differentiation have been linked to bladder cancer growth. Further studies are needed to fully understand the role of these genes and processes in bladder cancer development.

The Endoplasmic Reticulum (ER) plays a crucial role in protein synthesis, folding, and transport. The activation of the ER stress response pathway is linked to bladder cancer cells' survival and proliferation, making it a promising therapeutic strategy for bladder cancer. The Astral Microtubule is essential for cell division, and overexpression of microtubule-associated proteins is associated with bladder cancer proliferation. Dysregulated collagen deposition and organization in cells lead to bladder cancer development. The NMDA selective glutamate receptor complex plays a role in synaptic plasticity, learning, and memory, and targeting it may be a potential therapeutic strategy for bladder cancer. Alterations in organelle functions lead to changes in cellular components that may cause bladder cancer growth. Microfibril organization is also a potential therapeutic target for bladder cancer. These findings offer insight into potential targets for therapeutic interventions in bladder cancer treatment (Yang *et al.*, 2019).

Our study identified unique molecular functions such as nuclear retinoic acid receptor binding and nuclear retinoid X receptor binding, which are involved in gene expression.

Dysregulation of these receptors may lead to bladder cancer growth. Similarly, dysregulation of beta-glucosidase and serine-type endopeptidase activities,

which break down proteins and carbohydrates, respectively, can also lead to bladder cancer growth and invasion. Transcription cis-regulatory region binding controls the regulation of gene expression, and dysregulation of this function can cause the development of cancer (Champion *et al.*, 2020). Flavin adenine dinucleotide binding and CDP-alcohol phosphatidyl transferase activity are involved in energy and lipid metabolism, respectively, and their dysregulation may also cause bladder cancer development. Furthermore, NAD (P) H oxidase H_2O_2-forming activity is linked to the production of reactive oxygen species, which can damage DNA and other cellular components and lead to cancer development. Our enrichment analysis shows a link between various cell types such as taste receptor cell, adipocytes, Schwann cells, glial cells, progenitor cells and fibroblast and bladder cancer progression.

The study's findings have identified key pathways involved in the molecular functions of bladder cancer, with circadian rhythms playing a direct role in the development and progression of the disease. Disrupting the circadian rhythm can alter gene expression and impair the immune system's ability to recognize and remove cancer cells. Targeting potential therapeutics for bladder cancer requires a thorough understanding of the circadian rhythm. The PER2 and CRY2-regulated (Chan and Lamia, 2020) circadian clock pathways manage the sleep-wake cycle and other physiological processes, while CLOCK/BMAL1 and CLOCK/BMAL2 transcription factors (Mazzoccoli *et al.*, 2012) bind to DNA to drive the expression of target genes. Parathyroid hormone (PTH) synthesis, secretion, and action are also crucial for regulating calcium and phosphate metabolism in the body. PTH promotes angiogenesis, which is crucial for tumor development and metastasis. Targeting PTH pathways and inhibiting PTH secretion using drugs can effectively slow down bladder cancer growth and metastasis (Gislefoss *et al.*, 2018). Some of the most well-known WikiPathways in human bladder cancer have been anticipated by our study. Bladder cancer is a complex disease that involves dysregulation of multiple

pathways. Our analysis has identified several key pathways that play a crucial role in the development and progression of bladder cancer. These pathways include gastric cancer network 1, dopaminergic neurogenesis, melatonin metabolism and effects, nuclear receptors, let-7 inhibition of ES cell programming, SRF and miRs in smooth muscle differentiation and proliferation, extracellular structure organization, complement and coagulation cascade, glial cell differentiation, and vitamin D metabolism. Dysregulation of these pathways results in the activation of various signaling pathways that promote tumor growth and invasiveness.

Glial cell differentiation refers to the process by which glial cells differentiate and mature into their various subtypes, providing support and protection for neurons in the nervous system. Disruptions in this pathway have been linked to various neurological disorders. The dopaminergic neurogenesis pathway plays a crucial role in brain function by generating dopaminergic neurons, which are involved in the regulation of movement, motivation, and reward. This pathway involves different stages, including regionalization, specification, differentiation, and maturation, as well as regulation of various molecular networks.

Our findings suggest that further research is needed to fully understand the mechanisms underlying these pathways and their potential therapeutic targets. Specifically, the DEGs LMX1A and NR4A2 have been implicated in the specification, growth, and differentiation of dopaminergic neurons. Further research is needed to fully understand the mechanisms of these pathways and their potential as drug targets for the treatment of bladder cancer. Our findings suggest that targeting these pathways could lead to the development of new therapies for this disease. In our study, we conducted a GWAS enrichment analysis to identify key terms associated with bladder cancer. Our study revealed several significant molecular functions linked to bladder cancer. The predicted GWAS terms that stood out in our analysis include periodontitis, response to

methotrexate and acetaminophen, vestibular neuritis, leisure time exercise behavior, bipolar disorder, personality traits in bipolar disorder, diffusing capacity of the lung for carbon monoxide, and economic and political preferences. These findings shed light on the potential genetic factors that may contribute to the development of bladder cancer. Our study provides valuable insights that could aid in the identification of new therapeutic targets for this disease.

In this study, we explore the gene network pathways and enrichment analysis networks associated with bladder cancer. The circadian clock and rhythm pathways are represented by differentially expressed genes (DEGs) PER2 and CRY2. We also compared miRNA and RNA expression levels using the TCGA-BLCA dataset and identified the best predicted miRNAs hsa-mir-199b, hsa-mir-199a-1, hsa-mir-199a-2, hsa-mir-214, and hsa-mir-1245. The gene network of DEGs from bladder cancer was predicted using GeneMANIA, and the annotated gene network showed interactions with various genes regulating different pathways. Our predicted DEGs play differential roles in gene networks, including cell proliferation, differentiation, DNA repair processes, and ECM protein synthesis (Lin *et al.*, 2020). We predicted a gene network associated with gastric cancer in Homo sapiens (human), which includes DEGs UBE2C and ESM1 showing interaction with genes indicating stronger evidence in bladder cancer. The network includes several well-known cancer-related genes involved in DNA replication, angiogenesis, and regulation of inflammation and immune response. This network provides a valuable resource for investigating the molecular mechanisms underlying gastric cancer and identifying potential targets for therapeutic intervention in bladder cancer.

Our study depicts a gene network of clustered genes NPAS4, ESM1, MMP11, TRIM, GYPA, PAX9, and TTYH3 that have not been studied together in bladder cancer. These genes may be functionally related, and their differential expression may be associated with a particular biological process or disease state.

Overexpressed gene ESM1 is involved in angiogenesis and cancer progression. Matrix metalloproteinase 11 encoding gene MMP11 is overexpressed in bladder cancer and degrades extracellular matrix protein (Chen *et al.*, 2020). The gene PAX9 expresses a transcription factor that controls the modulation of cell differentiation in bladder cancer (Chen *et al.*, 2022). Innate immune response, antiviral defense, and cell cycle regulation in bladder cancer have been modulated by the gene TRIM (TRM). Glycophorin A encoding gene GYPA and TTYH3 gene are over-expressed in bladder cancer and involved in tumor grade, stage, and prognosis (Biswas *et al.*, 2022). Our study highlights the intricate gene networks and pathways associated with bladder cancer, revealing potential therapeutic targets through the differential expression of key genes.

The identified miRNAs and DEGs provide valuable insights into the molecular mechanisms driving the disease, paving the way for future research and targeted interventions.

Conclusion

This study has identified important pathways linked to DNA replication, DNA repair, and cell cycle control. Gene ontology enrichment analysis showed that bladder cancer impacts key biological processes, cellular components, and molecular functions. We identified potential biomarkers, including FBXL21P, PER2, and CRY2, which play roles in the circadian rhythm pathway. Additionally, our gene network analysis helped us predict the functions of specific genes involved in regulatory networks. Overall, RNA-Seq analysis has given us valuable insights into the molecular mechanisms and biological processes of bladder cancer. In summary, our study offers a detailed look at the gene network pathways related to bladder cancer and their interactions with other biological systems. Further research is necessary to validate our findings and explore how these genes contribute to bladder cancer progression.

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