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Original Article

Prognostic features of bladder cancer based on five neddylation-related genes

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Abstract: Background: Nedylation and tumours are closely linked. The role of nedylation in bladder cancer (BCa) has rarely been reported and this study aims to explore its potential impact on the pathogenesis and progression of BCa. Methods: Leveraging gene expression data from the TCGA database, this research employs the limma software package and WGCNA for gene module identification and analysis. Subsequent steps include the construction of a PPI network, the conduct of LASSO and univariate Cox regression analyses, and utilizing GSEA and single-cell sequencing to examine the influence of hub genes in bladder cancer-related biological pathways. Results: The investigation revealed 11,361 genes with significant differential expression between normal and tumour tissues, and identified 1,500 hub genes through analysis. LASSO regression identified eight critical genes. Univariate Cox regression analysis revealed that COMMD9, GPS1, PSMB5, VHL, and WDR5 are independent prognostic factors for BCa. GSEA and single-cell sequencing highlight the potential of these genes to modulate immune responses and interactions between tumour and immune cells. Meanwhile, GSEA demonstrated that GPS1 can activate the NF-κB signalling pathway, leading to an increase in influenza virus polymerase activity. Conclusion: This study identifies COMMD9, GPS1, PSMB5, VHL, and WDR5 as significant prognostic markers in BCa, thereby underscoring their roles in immune regulation and tumour-immune cell dynamics.

Keywords: Bladder cancer, neddylation, prognostic analysis, molecular biomarkers, personalized medicine

Introduction

Bladder cancer (BC) is a common malignancy of the genitourinary system that develops primarily in the bladder mucosa. It has a global presence and is one of the genitourinary cancers with higher incidence and mortality rates, with a significantly higher incidence in men than in women [1-4]. The complex pathogenesis of BCa is closely linked to factors such as smoking, chemical exposure, chronic infections, and individual genetics [5, 6]. Clinically, BCa is characterised by different pathological types, including non-muscle invasive BCa (NMIBC) and muscle invasive BCa (MIBC), which significantly affect the choice of treatment and the patient prognosis [7, 8]. In particular, 70-80% of BCa cases are NMIBC, while muscle-invasive disease has a recurrence rate of approximately 50% [9-11]. Early-stage BCa may be asymptomatic, whereas advanced stages often present with urinary dysfunction, haematuria and back pain. Therefore, early diagnosis and accurate treatment are essential to improve survival in BCa patients.

Neddylation is central to the regulation of key cellular functions such as cell cycle, DNA damage response, apoptosis, and signalling pathways. Its dysregulation has been linked to cancer development, where abnormal neddylation triggers uncontrolled cell growth, tumour expansion and metastasis [12, 13]. As a critical player in cancer progression, neddylation represents a valuable target for cancer therapy. Specifically, the overactivity of neddylation enzymes-E1 NEDD8-activating enzyme (NAE), E2 NEDD8-conjugating enzyme, and E3 neddylation ligase [14] is prevalent in several cancers. This enzymatic overexpression often cor-

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relates with worse outcomes and reduced survival, highlighting the impact of neddylation on cancer dynamics [15]. Given neddylation's contribution to tumour development and its association with adverse clinical outcomes, it has attracted interest as a potential target for cancer therapy. Blockade of neddylation has been shown to suppress tumour growth and induce apoptosis in preclinical cancer models, which holds promise for cancer treatment strategies. This study focuses on investigating the molecular features and clinical significance of BC, with the aim of providing more precise insights into its management and prognostic assessment.

This study uses a range of analytical methods - including limma analysis, lasso regression, neddylation profiling and both univariate and multivariate Cox regression analysis - to systematically identify key gene targets and clinical prognostic markers that affect bladder cancer (BCa) patient outcomes. Through these in-depth analyses, we explored potential biomarkers and clinically relevant features of BCa. The culmination of our research is the integration of these findings into a nomogram map, providing a powerful tool for personalised medicine and improving clinical decision-making.

Materials and methods

Neddylation-related genes

We obtained 246 neddylation-related genes from the KEGG database [16]. These genes are cross-referenced with the hub genes to identify potential overlap.

Differential gene expression analysis

We conducted a differential gene expression analysis on BCa patient gene expression data from The Cancer Genome Atlas (TCGA), utilizing the [17] limma package in R [17]. Genes meeting the criteria of $P < 0.05$ and $|LogFC| > 2$ were categorized as differentially expressed. This analysis led to the creation of a volcano plot with the "ggplot" package.

WGCNA analysis

We performed Weighted Gene Co-expression Network Analysis (WGCNA) on BCa-related target genes using the R package WGCNA [18]. This analysis helped construct a weighted gene co-expression network, which identified clusters of genes associated with BCa.

Setting up a PPI network

We developed a protein-protein interaction (PPI) network using the WGCNA-identified genes via the STRING website [19]. This approach elucidated the interaction networks between the target genes at the translational level. The PPI network was visualised using Cytoscape 3.9.1 software (Cytoscape Consortium, USA). Hub genes were identified using cytoNCA [20], with criteria of degree > 2.0 and closeness > 1 to determine the most influential genes within the network.

Creation of a prediction model

LASSO-Cox regression analysis was performed using the glmnet package in R to identify optimal prognostic genes [21]. This approach applies a penalty proportional to the size of the coefficients, effectively selecting significant genes based on the shrinkage of the regression coefficients. Single-gene Cox regression was then used for further screening, followed by multiple Cox regression analysis to construct a risk scoring formula. This formula calculates the risk of poor survival probability for each sample based on the coefficient-weighted expression of prognostic genes [22].

Nomogram prediction model creation and analysis

Lasso regression analysis selected clinical information such as age, gender, and lymphatic infiltration to determine optimal prognostic factors [23]. Single-factor Cox regression and multi-factor Cox regression were then used to identify independent prognostic factors. All independent prognostic factors were then included to construct line plots to assess overall survival (OS).

Survival analysis

The Kaplan-Meier method was used to estimate the probability of OS [24]. The log-rank test was used to assess the significance of differences in OS probability between groups, with a threshold of p-value < 0.05 indicating significant differences. Relevant data were obtained from the TCGA database.

Single-cell sequencing

The analysis of single cell and bulk transcriptomic data from GSE135337 involved separate assessments. The process included quality control measures, dimensionality reduction, clustering and annotation, following the procedures outlined in the Python-based Scanpy workflow [26]. This approach allows detailed exploration of gene expression at the single cell level, providing insights into the cellular heterogeneity and molecular pathways operating in bladder cancer.

Gene Set Enrichment Analysis (GSEA)

GSEA identifies gene sets associated with specific phenotypes by comparison [25]. Independent prognostic genes, identified by Cox regression and categorised by their median values, were compared with the C2.CP.KEGG.v7.4 and C5.GO.v7.4 gene sets. The clusterProfiler package in R was used for this analysis, with the aim of elucidating the role of these genes in bladder cancer. Gene sets with $P < 0.05$ and FDR $q < 0.2$ were considered significant, indicating enriched pathways that may be critical for understanding the mechanisms of the disease.

Statistical analysis

All statistical tests were performed using R software. Kaplan-Meier curves were compared using the log-rank test and plotted using the 'survminer' R package. Forest plots were generated using the R package forest plot. $P < 0.05$ was considered statistically significant.

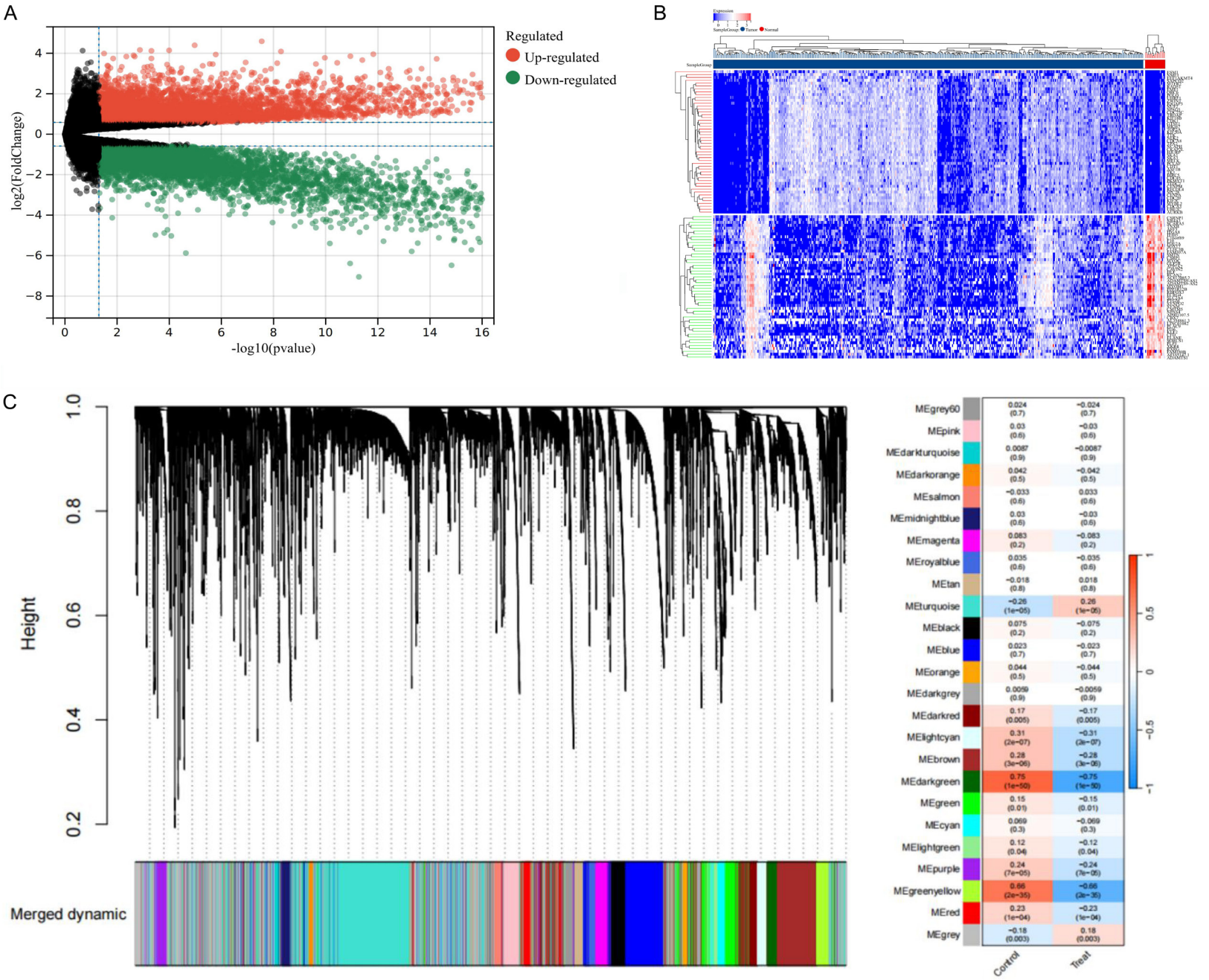
Results

Screening for bladder cancer differentiation genes

As shown in **Figure 1A**, we identified differential genes between normal and bladder cancer tissues using a heat map. Our analysis identified 11,361 genes with significant expression differences and flagged them as potential BCa-related targets. Subsequently, the expression patterns of these differential genes in the two groups were then plotted on a heat map (**Figure 1B**). In this heatmap, each row corresponds to a gene, and each column to a sample, with the colour intensity indicating gene expression changes-dark colours for downregulation and light colours for upregulation. This visualisation revealed significant changes in the expression patterns of certain genes, highlighting their potentially critical role in the onset and progression of BCa. As shown in **Figure 1C**, the WGCNA analysis classified 2,001 genes associated with prognosis in BCa patients into 25 different modules. A thorough examination of the relationships between these modules and BCa traits led to the identification of 1,578 genes in the turquoise module and 423 genes in the grey module. We then identified a total of 1,500 hub genes by protein-protein interaction (PPI) analysis (**Figure 1D**, focusing on selecting hub genes with degree scores > 2 and proximity scores > 1). Finally, we extracted endonuclease-related genes from the KEGG database and crossed them with the 1,500 previously identified hub genes, resulting in the identification of 32 BCa-endonuclease-related hub genes (**Figure 1E**).

Univariate and multivariate Cox regression analysis

Subsequent lasso regression analysis on these 32 genes narrowed down the list to 8 critical genes that are likely to have a significant impact on the prognosis of BCa patients (**Figure 2A** and **2B**). Univariate and multivariate Cox regression analyses were performed to assess whether the risk scores derived from the 8 identified genes could serve as independent prognostic predictors of BCa beyond traditional clinical features. Univariate Cox regression analysis highlighted five genes - COMMD9, GPS1, PSMB5, VHL, and WDR5 - as independent prognostic factors for BCa (**Figure 2C**, **2D**). The results of the analysis were as follows: COMMD9 (HR=1.018, 95% confidence interval (CI) 1.004-1.032, P=0.01), GPS1 (HR=1.007, 95% CI 1.001-1.012, P=0.015), PSMB5 (HR=1.007, 95% CI 1.001-1.003, P=0.001), VHL (HR=0.991, 95% CI 0.984-0.998, P=0.007), and WDR5 (HR=1.006, 95% CI 1-1.011, P=0.042), all with *P*-values less than 0.05, signifying their statistical significance as individual predictors of BCa prognosis. Further multivariate Cox regression analyses confirmed that COMMD9, GPS1, PSMB5, VHL and WDR5 could be used as independent indicators of bladder cancer prognosis (**Figure 3A-D**). To further substantiate the findings from the multivariate Cox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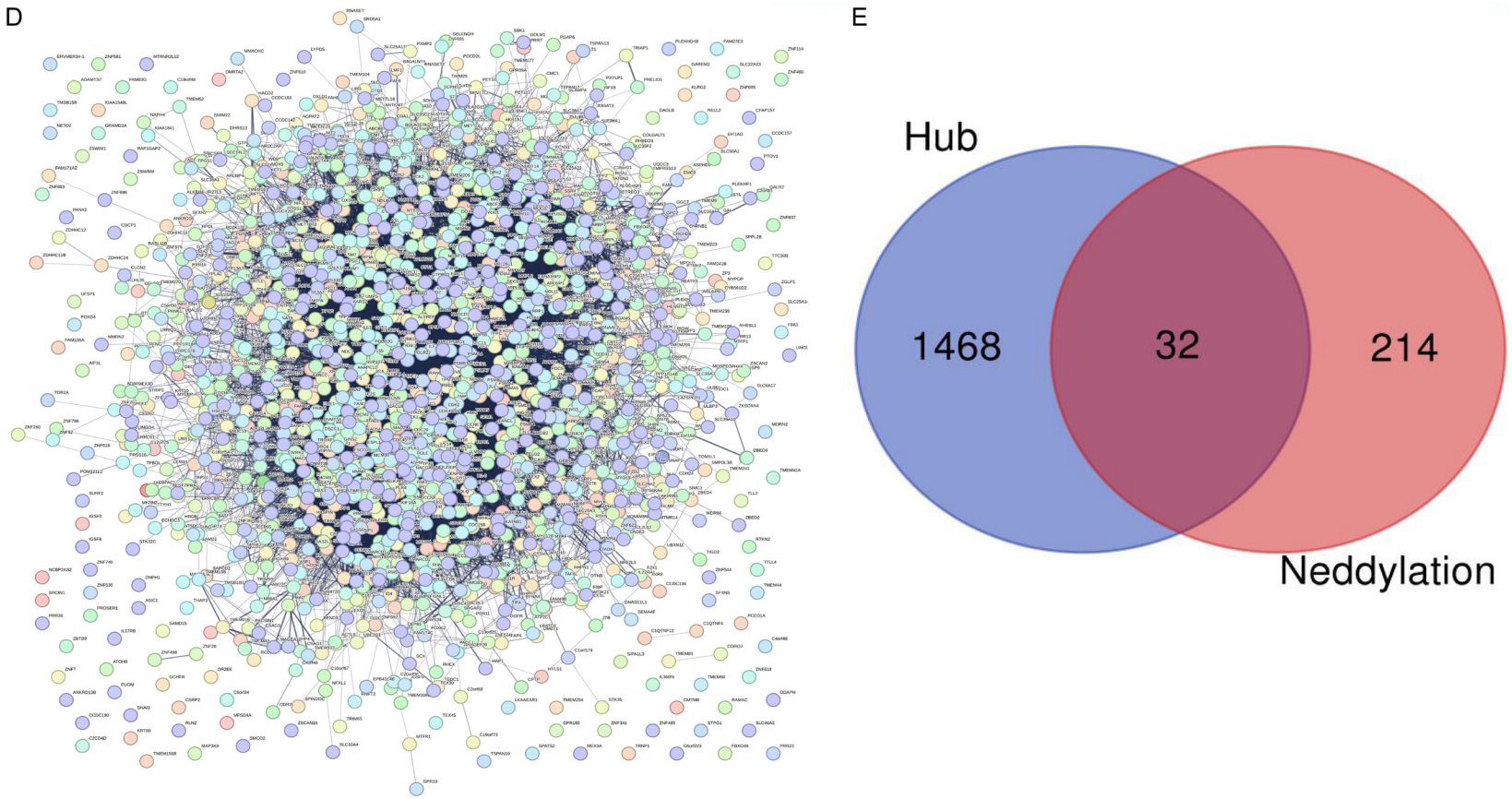


Figure 1. Differentially expressed genes between BCa tissues and normal tissues. A. Volcano plot. B. Identification of BCa gene modules in the TCGA dataset using WGCNA. C. Heatmap of differentially expressed genes between normal and tumour groups in BCa. Blue represents down-regulated genes, red represents up-regulated genes. D. Construction of protein-protein interaction (PPI) network of different proteins, and connections between proteins represent interactions. E. Intersection results of hub genes between BCa and neddylation.

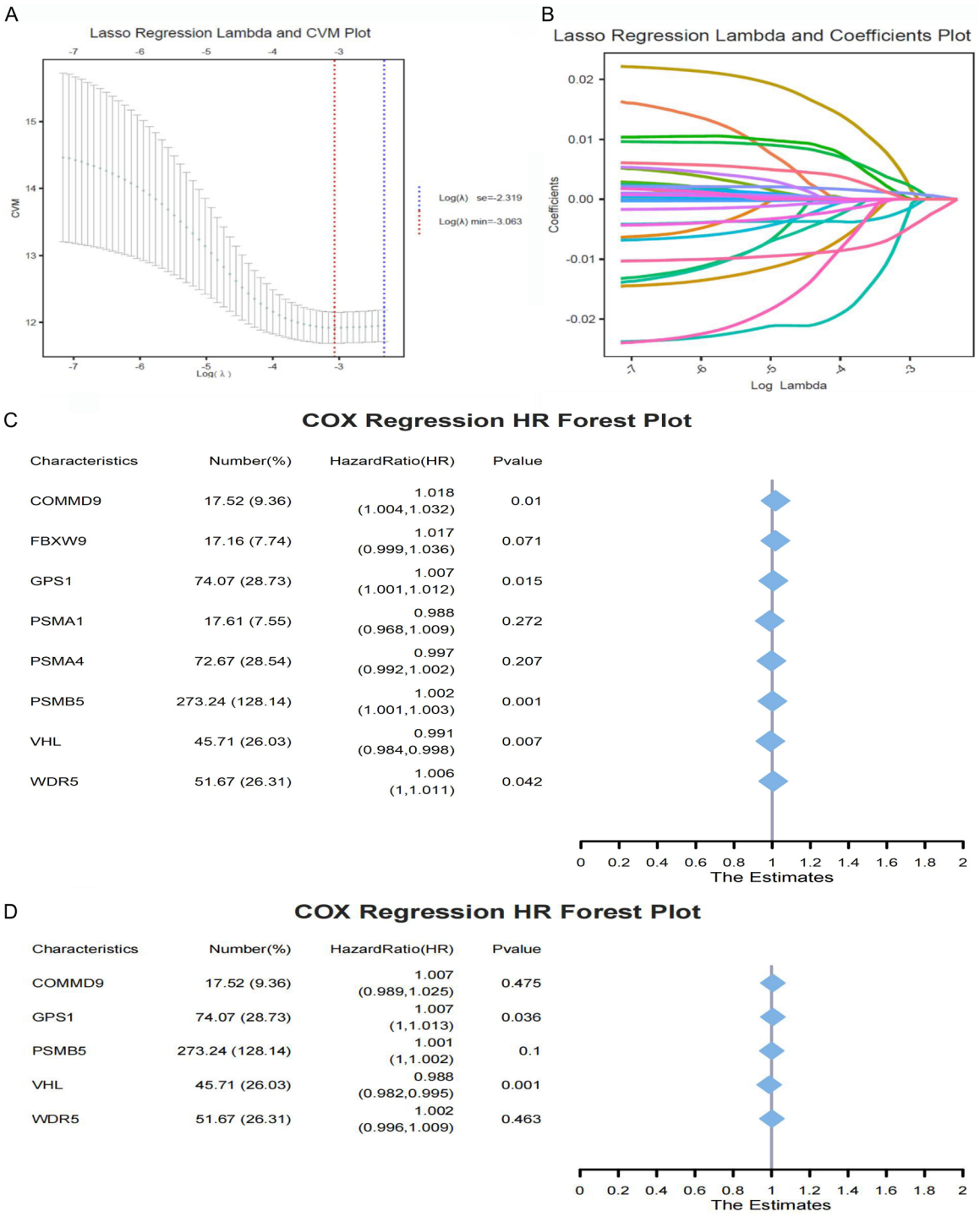


Figure 2. Univariate and multivariate Cox regression analyses. A and B. LASSO regression analysis of the timing of the minimised model error. The optimal value for log(λ) was -3.036, and eight genes were selected for further survival analysis. C. Univariate Cox Regression Analysis. D. Multivariate Cox Regression Analysis.

regression analysis, a hazard survival analysis was performed (**Figure 3E**). This analysis showed that overall survival in the high-risk group was significantly lower than overall survival (OS) in the low-risk group.

Differential and prognostic analysis

A detailed examination of differential gene expression between the high-risk and low-risk groups was performed, focusing on the expres-

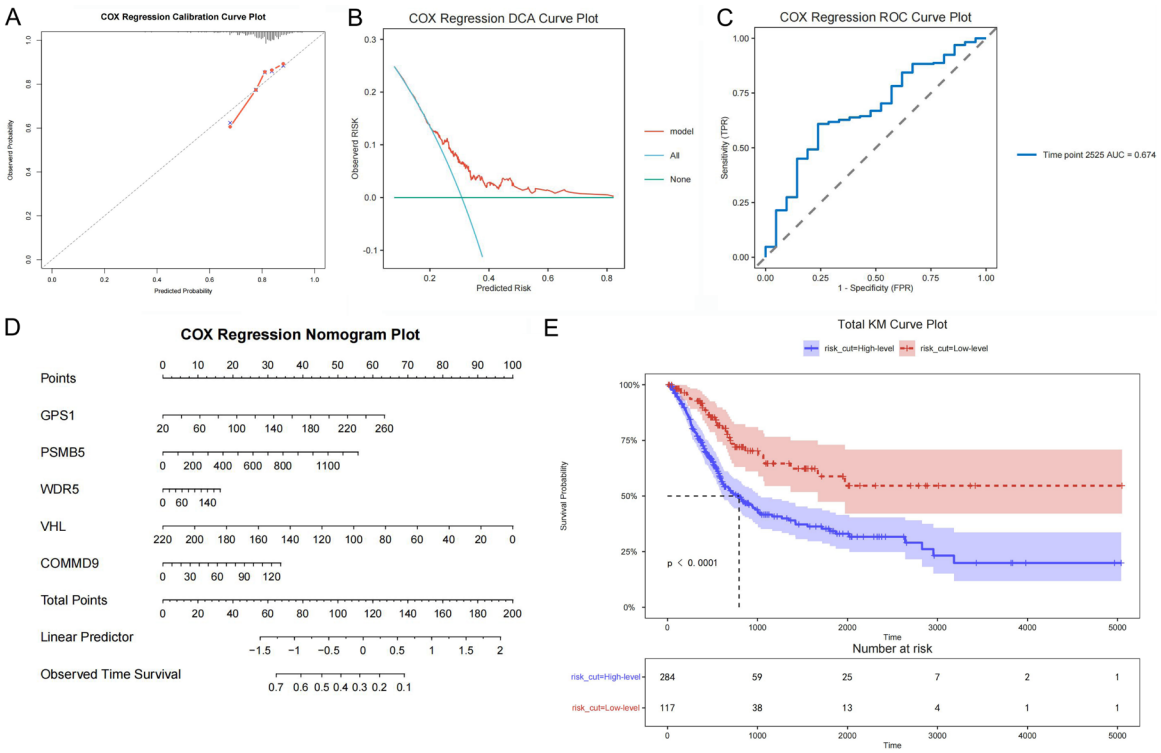


Figure 3. Receiver Operating Characteristic (ROC) and survival analysis. A. Calibration curve of the multivariate Cox regression analysis. B. Decision curve analysis (DCA) curve of the multivariate Cox regression analysis. C. Receiver operating characteristic (ROC) curve of the multivariate Cox regression analysis. D. Nomogram chart using risk scores from GPS1, PSMB5, WDR5, VHL, COMMD9. E. Multivariate Cox regression risk survival analysis.

sion levels of the identified prognostic genes. The analysis revealed that in the high-risk group, genes such as COMMD9, GPS1, PSMB5 and WDR5 showed significantly higher expression levels (**Figure 4**). Conversely, VHL was the only gene that showed a significantly lower expression level compared to the low-risk group. This pattern of expression underscores the distinct molecular characteristics that differentiate the prognostic outcomes between the two groups, highlighting the potential of these genes as markers of bladder cancer aggressiveness and patient stratification. Prognostic survival analysis was performed for the above five genes (**Figure 5**). The results indicate that, with the exception of VHL, the high expression groups of the other genes have significantly lower survival rates compared to their low expression counterparts.

Nomogram prediction model creation and analysis

Building on the knowledge gained from univariate and multivariate Cox regression analyses focusing on genetic markers, attention was expanded to include key clinical factors such as age to provide a more rounded understanding of patient survival. A lasso regression analysis was conducted to sift through various factors for those most predictive of prognosis, resulting in the identification of three significant clinical factors: age, metastasis (M), and nodal (N) status (**Figure 6**). We then performed univariate Cox regression analysis on these factors, and the results indicated that these three factors were clinically independent predictors of BCa (**Figure 7A**). After establishing models for the 3 prognosis-related factors from the univariate Cox regression analysis, we performed multivariate Cox regression using the survival package in R to assess the independence of risk scores in prognosis estimation (**Figure 7B-E**). The results indicated that age, metastasis (M) and nodal status (N) could serve as independent indicators for predicting the clinical prognosis of BCa patients.

Single-cell RNA sequencing analysis

To further verify the above results, we performed single cell RNA sequencing. We

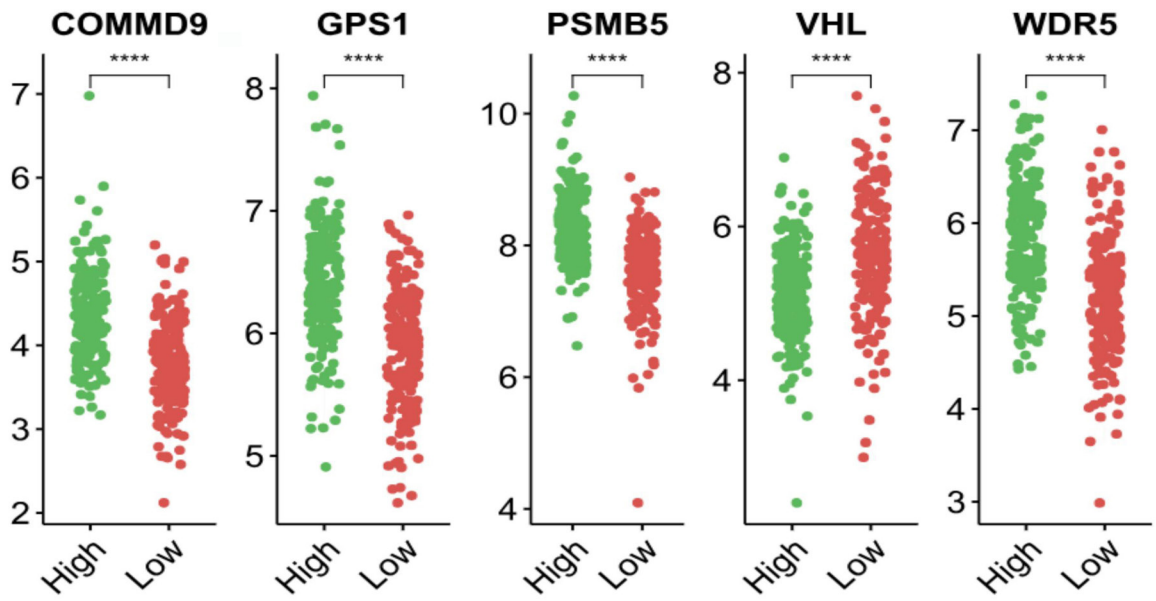


Figure 4. Differential gene expression in high and low risk groups.

obtained 1,996 cells from the GEO database and sorted them into clusters to obtain 9 cell clusters (**Figure 8A**). The expression levels of COMMD9, GPS1, PSMB5, VHL and WDR5 genes were then analysed in these 9 cell clusters (**Figure 8B** and **8C**). The results showed that COMMD9, GPS1, PSMB5, VHL and WDR5 were expressed in the above 9 cell clusters.

Analysis of key pathways associated with bladder cancer by GPS1 and VHL

Using Gene Set Enrichment Analysis (GSEA) with "C5-GO" and "C2-KEGG" as reference gene sets, our study focused on GPS1 and VHL, which were identified as independent prognostic factors for BCa by multivariate Cox regression analysis. For GPS1, the initial GSEA using the C5-GO dataset (**Figure 9A**) highlighted the enrichment of its genes in pathways integral to skin structure and function, including epidermal cell differentiation, keratinisation and keratinocyte differentiation, as well as structural components such as intermediate filament and cytoskeleton. Subsequent analysis using the C2 KEGG dataset (**Figure 9B**) further revealed the involvement of GPS1 genes in fundamental cellular processes, in particular base excision repair, DNA replication, olfactory transduction, proteasome function and spliceosome operations. Similarly, analysis of VHL using the C5-GO dataset (**Figure 9C**) highlighted the enrichment of its genes in pathways critical for skin integrity and development, such as cornification, epidermal cell differentiation, keratinisation, keratinocyte differentiation and skin development. Extending this to the C2 KEGG dataset (**Figure 9D**), VHL genes were found to be enriched in pathways critical for immune response and cell lineage determination, including allograft rejection, antigen processing and presentation, cytokine-cytokine receptor interaction, graft-versus-host disease and haematopoietic cell lineage. These GSEA results highlight the important roles that GPS1 and VHL may play in orchestrating a variety of key biological processes associated with BCa, suggesting their potential as targets for therapeutic intervention and as markers of disease progression and prognosis.

Discussion

Bladder cancer (BCa) is a major public health challenge worldwide, characterised, characterised by its high morbidity and mortality. The complexity of its aetiology, involving a variety of pathogenic mechanisms and clinical manifestations, poses significant barriers to effective diagnosis and treatment [3, 6, 27]. This study examines the role of neddylation in BCa, a post-translational protein modification process comparable to ubiquitination, to uncover its implications in the disease. Neddylation, a key post-

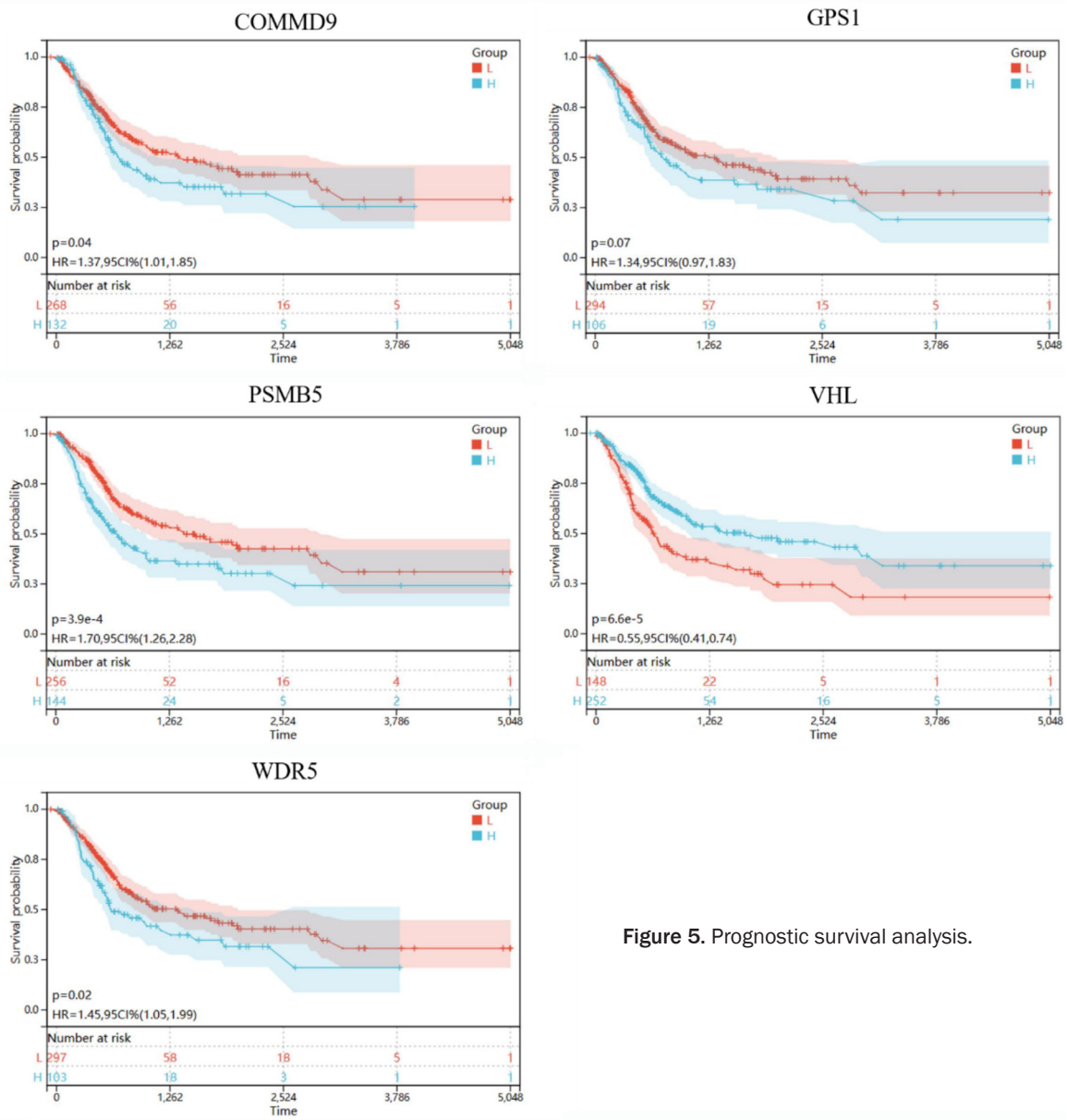


Figure 5. Prognostic survival analysis.

translational modification similar to ubiquitination, plays an essential role in cellular regulation. It involves the covalent attachment of the NEDD8 protein to lysine residues on target proteins, affecting their activity, stability and localisation [28-31]. This modification is central to the control of several cellular processes, including cell cycle progression, DNA repair, apoptosis, and signalling pathways. Dysregulation of neddylation has been implicated in several diseases, particularly cancer, where abnormal neddylation activity can drive tumour growth, spread and resistance to therapy [12, 14, 15, 32]. Therefore, a deeper understanding of the mechanisms of neddylation and its effects on cell biology is essential for unravelling disease mechanisms and developing targeted treatments.

Dysregulation of neddylation, particularly the overexpression of neddylation enzymes, has been observed in a number of cancers, including BCa. This overactivity suggests a critical role in cancer development, but the specific contributions of neddylation to BCa pathology remain underexplored. Our investigation aims to elucidate the influence of neddylation on BCa, potentially opening up new avenues for

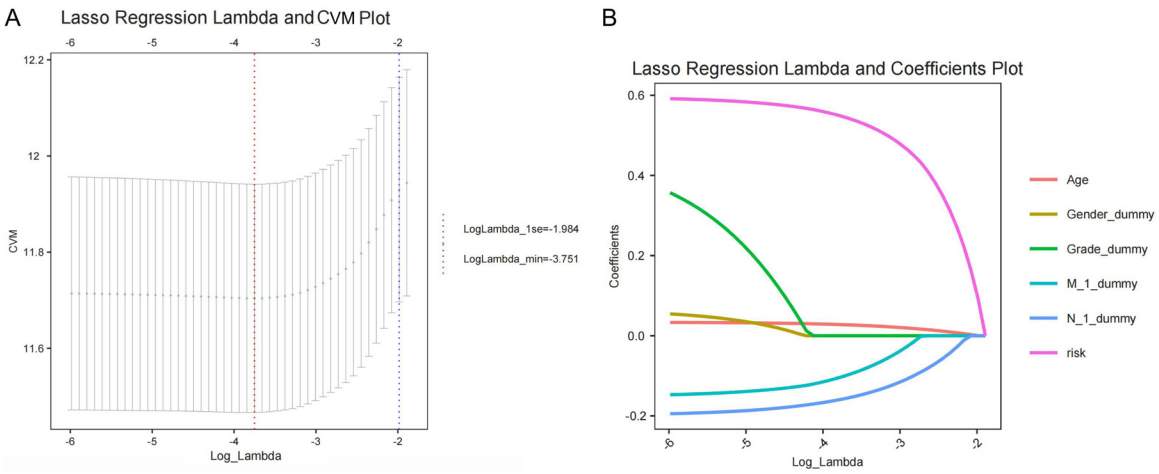


Figure 6. LASSO regression analysis (A and B) shows that when the model error is minimized, log(λ) =-3.751, and 3 prognosis-related factors are selected for further survival analysis.

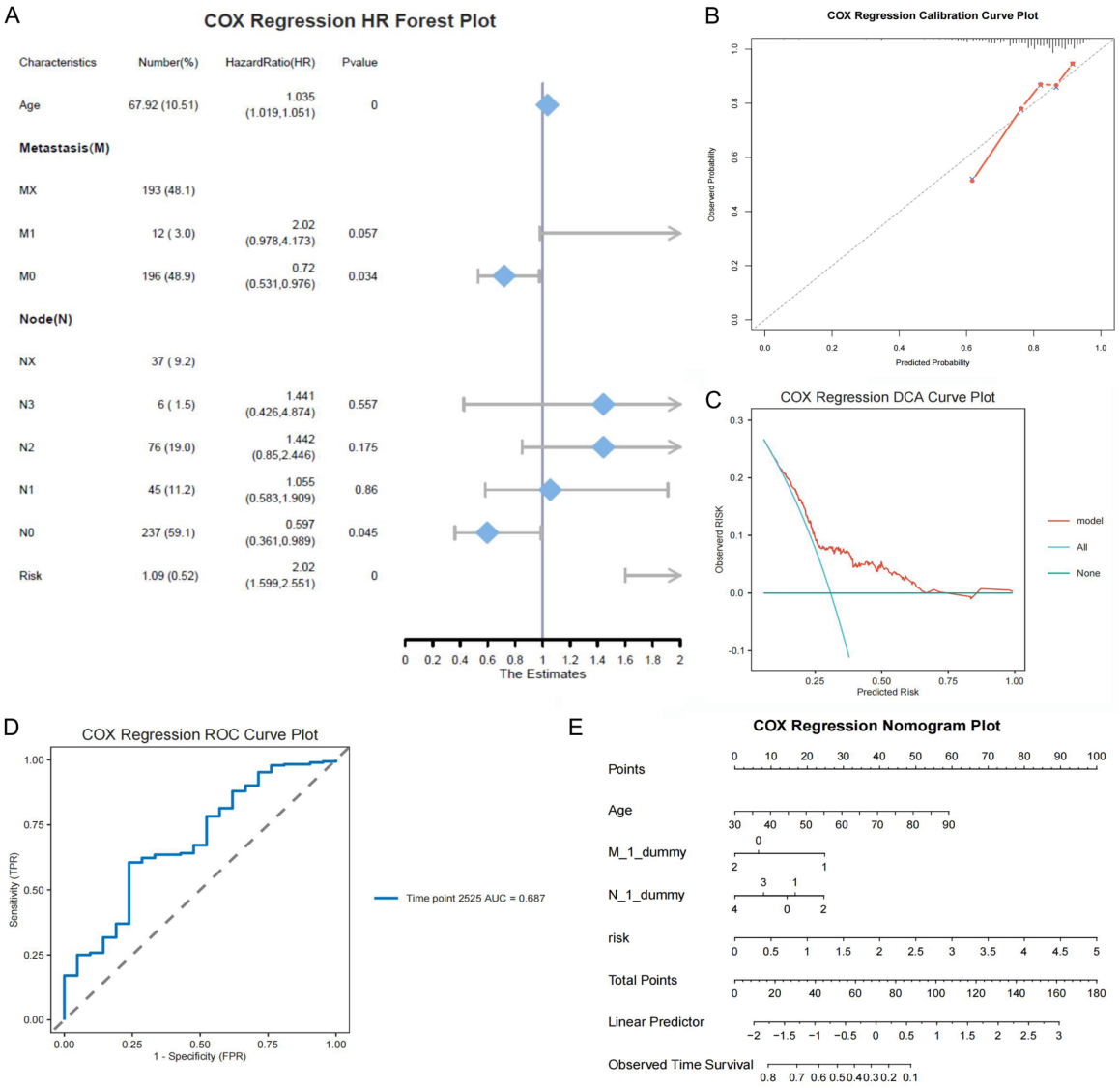


Figure 7. Nomogram prediction model creation and analysis. A. Multivariate Cox Regression Analysis. B. Calibration curve of the Multivariate Cox Regression Analysis. C. Decision Curve Analysis (DCA) curve of the Multivariate Cox Regression Analysis. D. Receiver Operating Characteristic (ROC) curve of the Multivariate Cox Regression Analysis. E. Nomogram chart using risk scores from GPS1, PSMB5, WDR5, VHL, COMMD9.

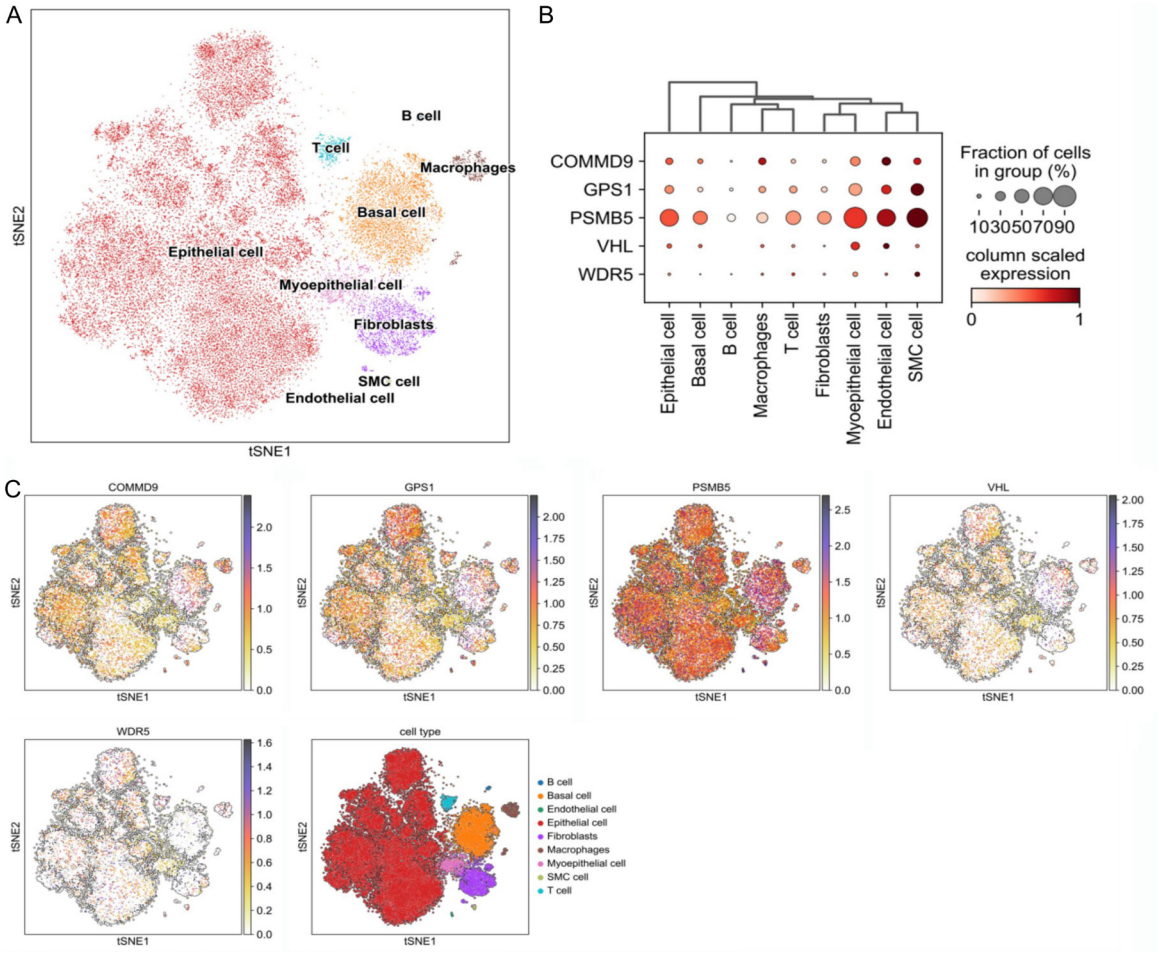
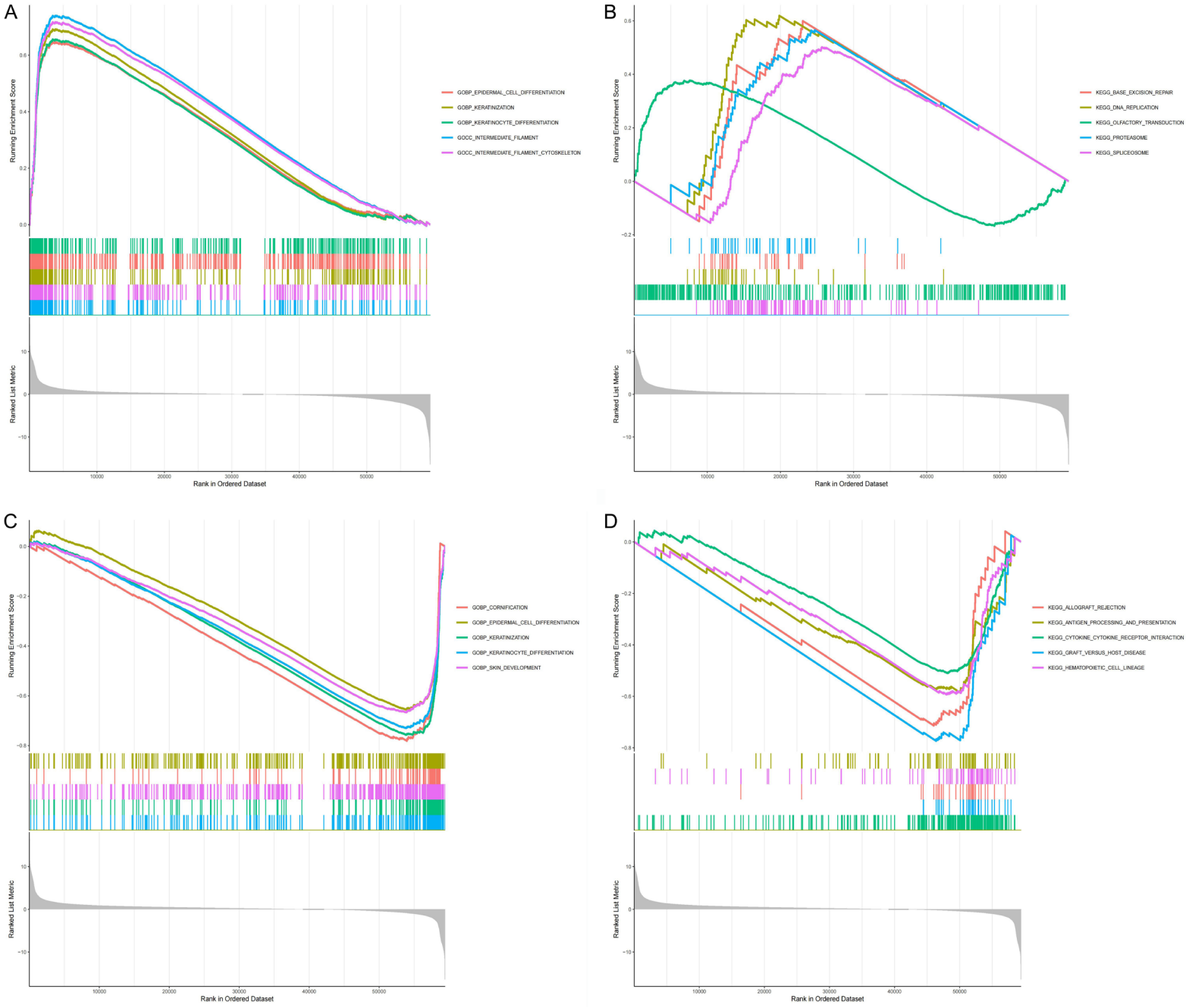


Figure 8. Single-cell RNA sequencing analysis. A. Nine major immune cell clusters. B. The amount of hub gene expression in nine major immune cell clusters, with dot size representing the proportion of cells in the cell type expressing a given gene and colour intensity representing the amount of expression in the expressing cells based on the file count. C. Cell annotation and the expression of model genes in each cell, the color depth is proportional to the expression amount of genes.

therapeutic intervention [12, 14, 15, 32]. In the present study, we identified 11,361 genes that were significantly differentially expressed in normal and tumour tissues and highlighted their potential role in the development and progression of BCa. Subsequent weighted gene co-expression network analysis (WGCNA) of the differentially expressed genes isolated 25 modules and identified 2,001 genes significantly associated with BCa prognosis, and further screened 1,500 key genes. By cross-tabulation with neddylation-related genes in the KEGG database, we identified 32 BCa neddylation-related hub genes. Finally, we identified five genes, COMMD9, GPS1, PSMB5, VHL and WDR5, as independent prognostic factors for BCa by LASSO regression, univariate and multivariate Cox regression analyses. In clinical practice, age, metastasis and lymph node status are important prognostic factors in BCa [1-4]. Using a holistic approach integrating molecular and clinical prognostic factors, we found that five neddylation-related genes (COMMD9, GPS1, PSMB5, VHL and WDR5),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GOBP_KERATINIZATION
GOBP_KERATINOCYTE_DIFFERENTIATION
GOCC_INTERMEDIATE_FILAMENT
GOCC_INTERMEDIATE_FILAMENT_CYTOSKELETON
Ranked List Metric
Rank in Ordered Dataset
B
Running Enrichment Score
KEGG_BASE_EXCISION_REPAIR
KEGG_DNA_REPLICATION
KEGG_OLFACTORY_TRANSDUCTION
KEGG_PROTEASOME
KEGG_SPLICEOSOME
Ranked List Metric
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KEGG_CYTOKINE_CYTOKINE_RECEPTOR_INTERACTION
KEGG_GRAFT_VERSUS_HOST_DISEASE
KEGG_HEMATOPOIETIC_CELL_LINEAGE
Ranked List Metric
Rank in Ordered Dataset

Figure 9. Gene Set Enrichment Analysis (GSEA).

age, metastasis (M) and nodal status (N) served as independent predictors of clinical prognosis in BCa patients.

Gene Set Enrichment Analysis (GSEA) was employed to delve into the roles of GPS1 and VHL-related genes in specific biological pathways relevant to bladder cancer, shedding light on the intricate mechanisms underlying disease progression. For GPS1, the analysis highlighted its enrichment in pathways critical for epidermal cell differentiation, keratinisation, DNA replication, and olfactory transduction, highlighting its diverse functional implications. Notably, research by Tomoko Kuwahara et al. [33] highlighted the ability of GPS1 to activate the NF-κB signalling pathway, thereby enhancing influenza virus polymerase activity and influencing the transcription and replication of viral genomic RNA. Wei et al. further explored GPS1's biological functions in breast cancer, revealing significantly elevated GPS1 expression in breast cancer tissues compared to adjacent non-cancerous tissues [34]. Their findings associate high GPS1 expression with adverse prognosis in breast cancer patients, suggesting that GPS1 influences cancer development and prognosis through multiple pathways. Specifically, GPS1 has been implicated in the regulation of ribonucleoprotein complex production, modulation of RNA expression and increased responsiveness of the Wnt signalling pathway. In addition, GPS1 silencing was shown to inhibit the proliferation, invasion and migration of MCF7 and MDA-MB-231 breast cancer cell lines in vitro. The pathways identified by GSEA, which are associated with cellular proliferation, differentiation and sensory perception, are critical in the context of cancer progression. These findings highlight the complex role of GPS1 in modulating cellular processes that contribute to cancer initiation and progression, and provide insights into potential therapeutic targets and prognostic markers in bladder cancer and beyond.

In studying VHL and its association with bladder cancer, Gene Set Enrichment Analysis (GSEA) revealed its involvement in key pathways such as keratinisation, antigen processing and presentation, cytokine-cytokine receptor interaction and haematopoietic cell lineage. These pathways are integral to immune response regulation, cell adhesion, and tissue development, suggesting a role for VHL in influencing the immune response and tumour microenvironment in bladder cancer. Previous studies have highlighted the importance of VHL in carcinogenesis; for example, Fen et al. [35] demonstrated that VHL gene expression correlates with Jada and significantly impacts the clinical prognosis of renal cell carcinoma patients. Gong et al. [36] found that VHL gene expression could significantly inhibit proliferation and induce apoptosis in the 786-0 renal cell carcinoma cell line, suggesting its therapeutic potential in renal cancer. The enrichment of these pathways by GSEA highlights the intricate biological mechanisms affected by VHL and GPS1 in the progression of bladder cancer, highlighting promising avenues for therapeutic intervention and further research into their roles.

To further elucidate the prognostic relevance of neddylation-related genes such as COMMD9, GPS1, PSMB5, VHL, and WDR5 in BCa, scRNA-seq was used. The results confirmed the expression of these genes across various immune cell clusters, aligning with previously reported findings. For instance, COMMD9's expression in non-small cell lung cancer, WDR5's presence in pancreatic cancer cells, and PSMB5's role as a cancer therapy target [37, 38] have been documented. This widespread expression pattern across different cancers underscores the potential role of the genes in immune modulation and tumour-immune cell interactions, and reinforces their importance in the immune regulation of the tumour microenvironment. In conclusion, our investigation sheds light on the molecular intricacies and clinical significance of BCa and highlights the importance of neddylation-related genes as prognostic markers. By demonstrating the complex relationship between molecular alterations and clinical manifestations, this study lays the foundation for improved, tailored therapeutic strategies in BCa management. However, further research and validation efforts are essential to fully unravel the contributions of neddylation to BCa development and to translate these findings into

actionable clinical applications. With continued research and understanding, the potential for more effective BCa treatments and management strategies is increasingly within reach, promising improved outcomes for patients facing this challenging disease.

Conclusion

After a thorough screening process, our study identified five genes - GPS1, PSMB5, WDR5, VHL and COMMD9 - as potential independent indicators of survival in patients with bladder cancer (BCa). In addition to these genetic markers, age, metastasis (M) and lymph node (LN) status emerged as significant clinical predictors of patient survival. These discoveries herald a step forward in identifying potential biomarkers and clinical factors that could guide personalised treatment strategies and clinical decision-making for individuals battling BCa. However, it's important to treat these findings with caution; their validity needs to be confirmed by validation in broader independent cohorts and through detailed mechanistic studies. Such studies are essential to improve our understanding of the precise contribution of these genes to the initiation and progression of BCa. By substantiating these initial findings, we can move closer to integrating these biomarkers and clinical factors into a more refined, effective approach to BCa treatment and patient care.

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Disclosure of conflict of interest

None.

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