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# Correlation between the immune microenvironment and bladder cancer based on a prognostic miRNA risk model

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## ABSTRACT

**Background:** Bladder cancer (BLCA), particularly invasive BLCA, has become a medical burden worldwide as it is associated with recurrence and easy metastasis. There are specific differences in the expression of various miRNAs in tumor and normal tissues. Hence, miRNAs can be used as biomarkers for tumor diagnosis and prognostic evaluation. The current study aimed to predict the downstream target genes of BLCA-related miRNAs and explore their association with immune infiltration. **Method:** Data on BLCA-related mRNA and miRNA expression levels were downloaded from The Cancer Genome Atlas. Correlation analysis and Cox regression analysis were performed to validate the miRNA risk model. The infiltration of various immune cells should be compared to determine the distinct differences between the immunological microenvironment of the two risk groups. **Results**: A predictive framework of BLCA was established using the expression levels of two miRNAs. Cox regression analysis showed that the low-risk group had a better prognosis. Then, the target genes of miRNA were predicted, and the target genes were analyzed using Gene Ontology and Kyoto Encyclopedia of Genes and Genomes. Moreover, variations in immune cells and functions between the high- and low-risk groups were assessed. **Conclusion:** The prognostic features composed of two associated miRNAs (MIR-25, MIR-548AN) may help predict the overall survival of BLCA.

## KEYWORDS

Bladder cancer; biomarker; miRNA; prognosis; GEO

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## 1. Introduction

Bladder cancer (BLCA) is a malignant tumor that occurs in the mucosa of the bladder, and > 430,000 people are diagnosed with BLCA annually [1]. Due to its prevalence in elderly men [2], it has caused a significant burden to the society in today's aging population. In the treatment of invasive BLCA, chemoradiation sensitization, radical cystectomy (RC), and the application of pelvic lymph nodes were performed at a certain extent to decrease the risk of mortality and specific diseases [3]. However, approximately 50% of the patients with disseminated transfer and ultimately disease [4] in the distance, and the adverse reaction of chemotherapy dose problems. BLCA remains a worldwide burden. Therefore, biomarkers for BLCA should be identified as they can be used for diagnosis and prognosis.

MicroRNAs are a class of evolutionarily conserved non-coding small RNAs that regulate gene expression at the translational level. Their downstream regulatory genes are also involved in different cellular function changes. miRNAs can be stably present in biological fluids [5], including urine, blood, and saliva, which facilitates the sample collection, thereby promoting the development of miRNA research into multiple fields [6]. In tumor tissues, some miRNAs have a low expression, which can inhibit tumorigenesis and are considered as tumor suppressor genes [7, 8]. Some are highly expressed in tumor tissues, and they promote tumor progression [9, 10]. These miRNAs play a role in regulating tumor progression to a certain extent. Thus, miRNAs can be diagnostic markers for tumor diagnosis [11] and prognostic evaluation [12]. There are a large number of miRNAs. However, only a few affect BLCA prognosis. Therefore, screening miRNAs that can be used as biomarkers and predicting their downstream genes are significantly important for the early diagnosis and treatment of BLCA.

Bioinformatics has played an important role in cancer research. However, in BLCA, the association between miRNAs and target genes and their prognostic value has not been evaluated. To address this problem and validate the association between BLCA-related miRNAs and downstream target genes, a risk prediction model was constructed, and the cross-target genes of miRNAs were screened out. Then, their downstream targets were successfully predicted.

## 2. Materials and Methods

### *2.1. Data acquisition*

The Cancer Genome Atlas (TCGA) (http://portal.gdc.cancer.gov/) was used to download data on BLCA expression patterns that matched the clinical data. The mRNA and MiRNA expression patterns of 431 patients were considered. To validate the outcomes of the TCGA data analysis, the datasets (training and test) were randomly grouped using the "Caret" package in version R (4.2.0).

### *2.2. Differential gene analysis*

The "limma" package was used to assess differences in mRNAs and miRNA in the R version (4.2.0). A P value of $< 0.05$ and $|\log2FC| > 0.6$ were set as the filter criteria. Finally, 1006 differentially expressed mRNAs (DEGMRNA) and 14 differentially expressed miRNAs (DEGMiRNA) were screened out.

### *2.3. Construction and assessment of the prognostic risk score model*

Univariate Cox regression screening was performed for prognostic DEGMiRNAs. The Kaplan–Meier curves and receiver operating characteristic (ROC) curves were used to assess group differences in prognosis. Finally, the correlation coefficient between prognostic risk and clinical information was calculated.

### *2.4. Target gene network construction*

The miRNA target genes were screened using the miRDB, TarBase, and Targetscan databases, and the intersection target genes in the three databases were obtained using the R software Veen package. Finally, Cytoscape was used to construct the target gene regulatory network.

### *2.5. Comparison of the target gene for gene enrichment*

A group of R-packets different expressions of target gene were analyzed via profiler's gene ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses to identify which biological pathways might be involved.

### *2.6. Correlation between high and low prognostic risk and immune cell infiltration*

To assess the amount of tumor-infiltrating immune cells in HCC tumor samples, the CIBERSORT R-packet was utilized. Finally, the morphological and functional differences of tumor immune cells were examined using the R software packages "reshape2," "GSVA," and "GSEABase" (4.2.0).

### *2.7. Separation of RNA and Real-Time PCR Quantification (qRT-PCR)*

The total RNA of the cells was isolated using the RNA Isolation Kit (Vazyme, Nanjing, China). Reverse transcription was performed using the HiScript II Q RT SuperMix for qPCR (Vazyme, Nanjing, China). The mRNA concentration was determined using the ChamQ Universal SYBR qPCR Master Mix based on the manufacturer's instructions (Vazyme, Nanjing, China). Supplemental Material 1 shows the primer fragments. The mRNA expression levels was analyzed using the 2Ct method after normalization to ACTB.

### *2.8. Statistical analysis*

All statistical analyses were performed using GraphPad Prism 8.0. (GraphPad Software, San Diego, CA, the USA). To determine if there was a statistically significant difference between the means of multiple groups, one-way analysis of variance was conducted. The mean and standard deviation were displayed for all statistical data (SD). A P value of $< 0.05$ was considered statistically significant.

## 3. Results

### *3.1. Differentially expressed mRNA and miRNA were obtained*

The TCGA-BLCA dataset was differentially analyzed using the "limma" package in R language. Then, 1006 mRNA differentially expressed genes and 14 miRNA differentially expressed genes were obtained. Figure 1A, 1B, 1C, and 1D present the volcano plot and heatmap of the DEGmRNAs and DEGMiRNAs.

**Prognostic risk signature construction of DEGMiRNAs**

Based on the univariate Cox regression analysis, 14 miRNAs were identified as prognostic factors. MIR25 and MIR548AN were the risk factors of the prognosis model. The following equation was used for the risk score of each patient with BLCA: risk score = (-0.26 × MIR25 expression) + (-0.31 × MIR548AN expression). In the end, the median risk score was used to classify patients with TCGA-BLCA into the high- and low-risk categories.

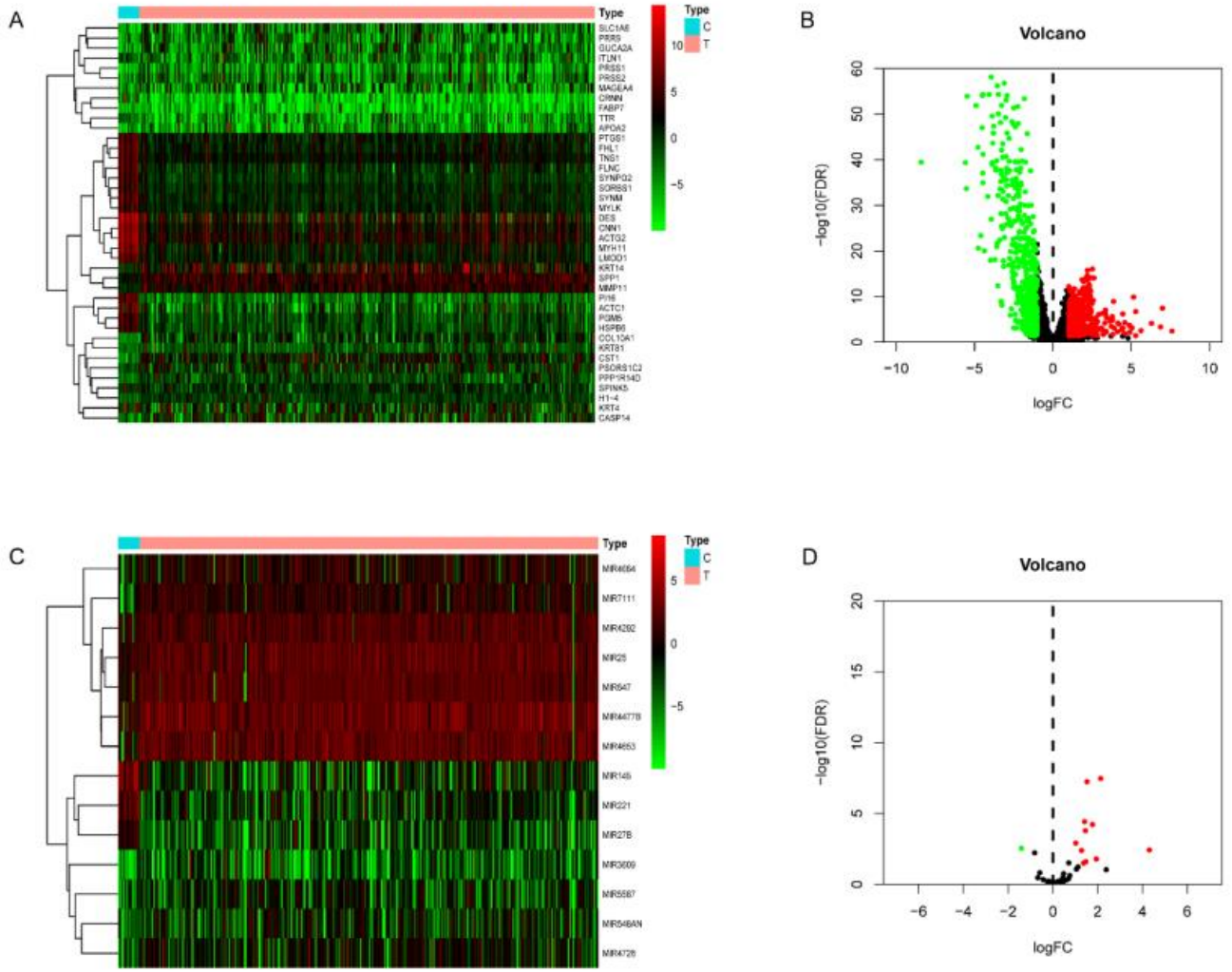


**Figure 1.** Differentially expressed mRNA and miRNA. (A, B) Heatmaps and volcano plots of differentially expressed mRNAs. (C, D) Heatmaps and volcano plots of differentially expressed miRNAs.

### *3.2. Assessment of the predictive performance of the ARGLnExps signature*

According to the median risk score, patients with TCGA-BLCA were divided into the low- and high-risk groups. The low-risk group had a better prognosis and longer survival time (Fig. 2A). In addition, the external validation training and testing datasets were used to validate the accuracy of the prognostic risk. Results showed that the low-risk group also had a better prognosis than the high-risk group in the training and testing groups (Figures 2B, C). The scatter plot and validation set in Figure 3D confirmed that patients with high-risk BLCA had a higher mortality than those with low-risk BLCA (Figure 2E, 2F). The model was validated using the ROC curve, and the AUC values were 0.607, 0.574 and 0.635, respectively (Fig. 2G–I). Cox regression analysis revealed that the DEGMiRNA prognostic risk model was an independent predictor of BLCA prognosis (Figure 3A, 3B).

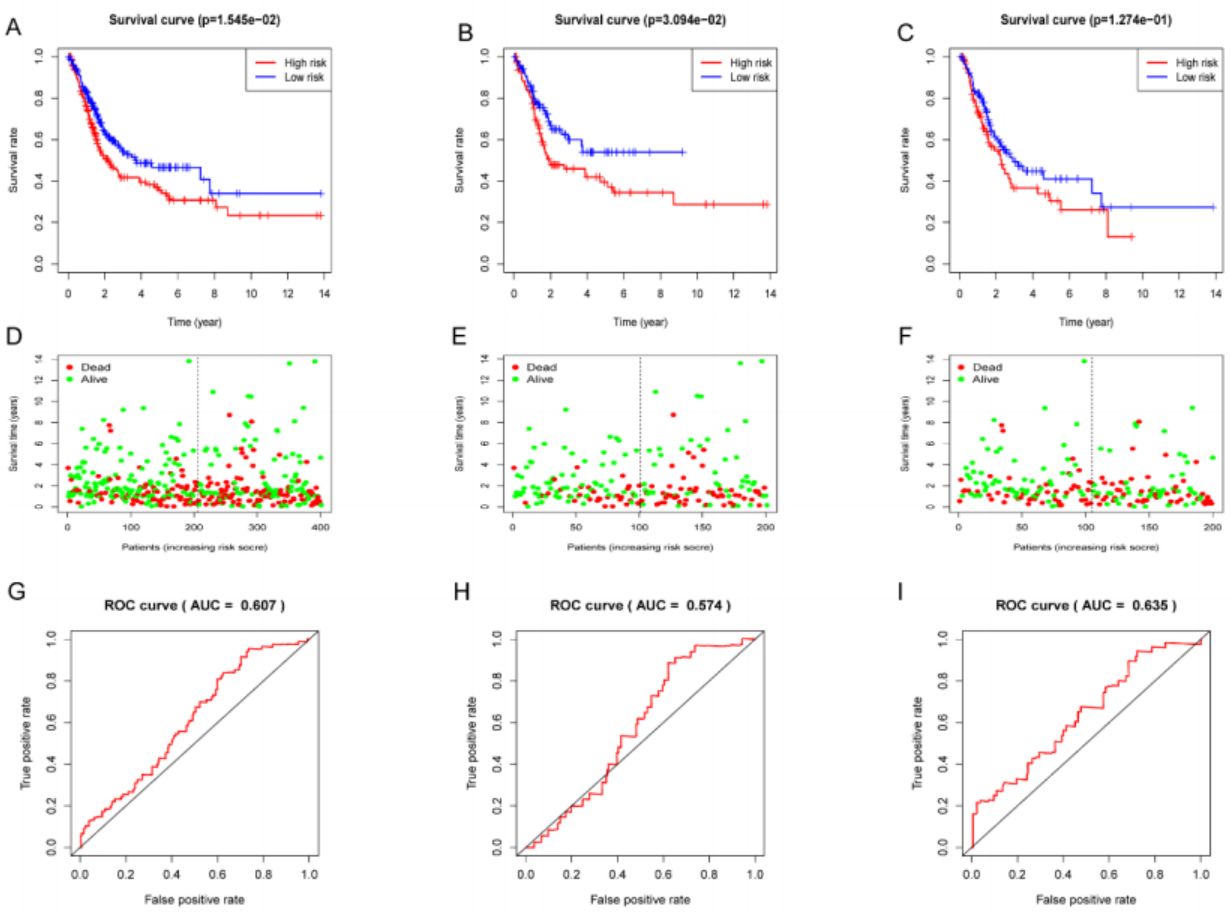


**Figure 2.** Construction of a prognostic model in TCGA-BLCA. (A-F) Kaplan-Meier (KM) curve and survival status of patients; (J-I) ROC curve of the model.

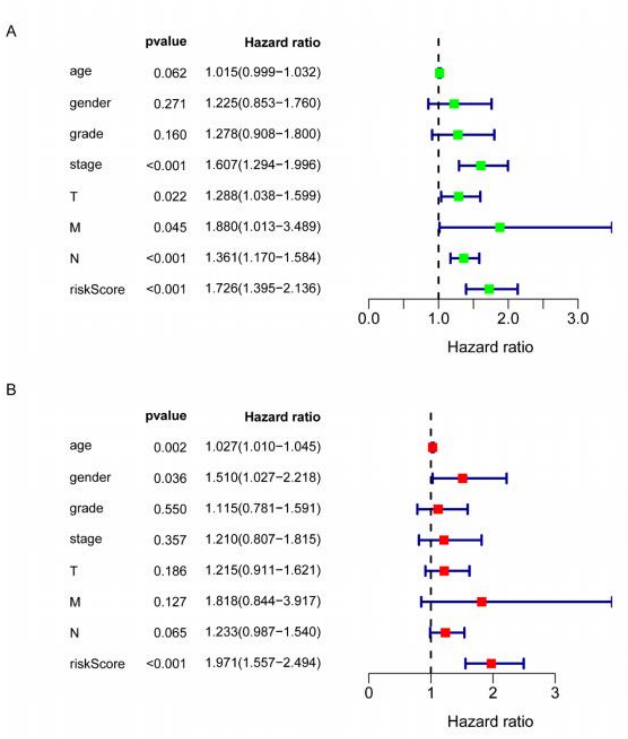


**Figure 3.** Evaluation of the prognostic signature in TCGA-LIHC. (A, B) Cox univariate and multivariate analysis.

### *3.3. Construction of the miRNA target gene regulatory network*

The target genes of MIR25 and MIR548AN were predicted in miRDB, Tarbase, and Targetscan. Subsequently, the intersection target genes in the three gene pools were screened using the Veen package in R language (Figure 4A, 4B). We obtained 166 predicted target genes of MIR-25 and 54 target genes of MIR-548AN. Since all the miRNAs screened were upregulated, the related downregulated target genes were screened. Finally, visualization was performed using Cytoscape (Figure 4C).

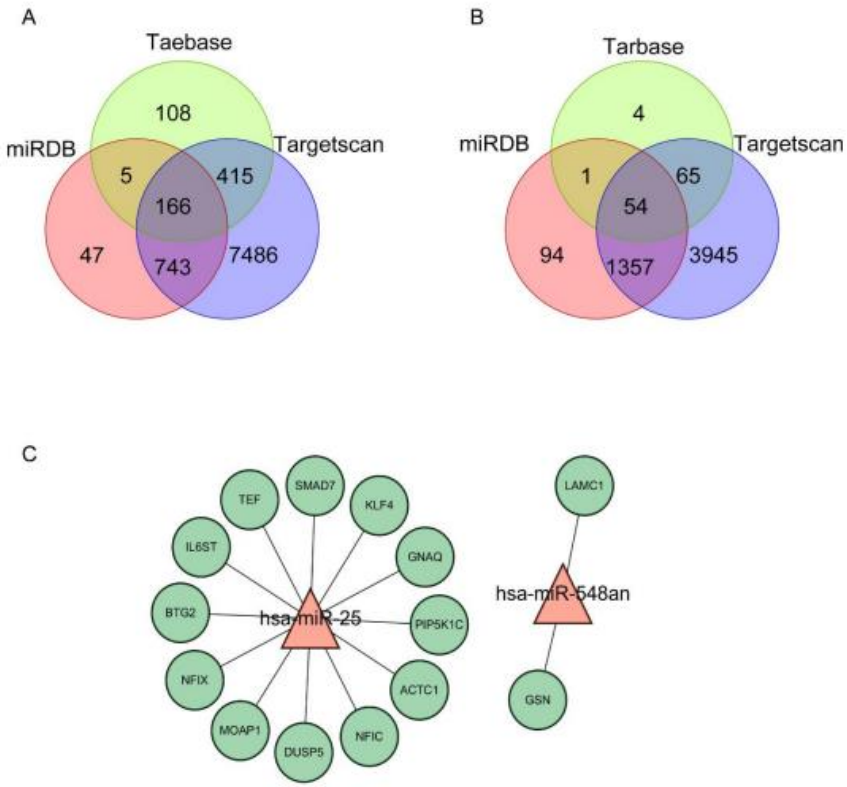


**Figure 4.** Target gene network construction. (A) Venn diagram of MIR-25 target gene prediction; (B) Venn diagram of MIR-548AN target gene prediction; (C) target gene regulatory network.

### *3.4. Gene enrichment analysis*

To further explore the association between mRNA and miRNA, we performed GO and KEGG enrichment analysis on the target gene. In total, 9 KEGG signaling pathways and 35 significantly enriched biological processes were obtained. GO enrichment analysis revealed the enrichment of adherens junction assembly and response to laminar fluid shear stress and fluid shear stress in biological processes. In addition, we showed enrichment in the cellular component of cell leading edge, focal adhesion, cell–substrate junction. Furthermore, the molecular functions included enrichment of myosin binding, beta-catenin binding, DNA-binding transcription activator activity, RNA polymerase II-specific, and DNA-binding transcription activator activity (Fig. 5A). Importantly, KEGG enrichment analysis revealed the main enrichment pathways of the target gene, including Fc gamma R-mediated phagocytosis, Amoebiasis, Yersinia infection, signaling pathways regulating the pluripotency of stem cells, adrenergic signaling in cardiomyocytes, focal adhesion, and viral carcinogenesis (Fig. 5B).

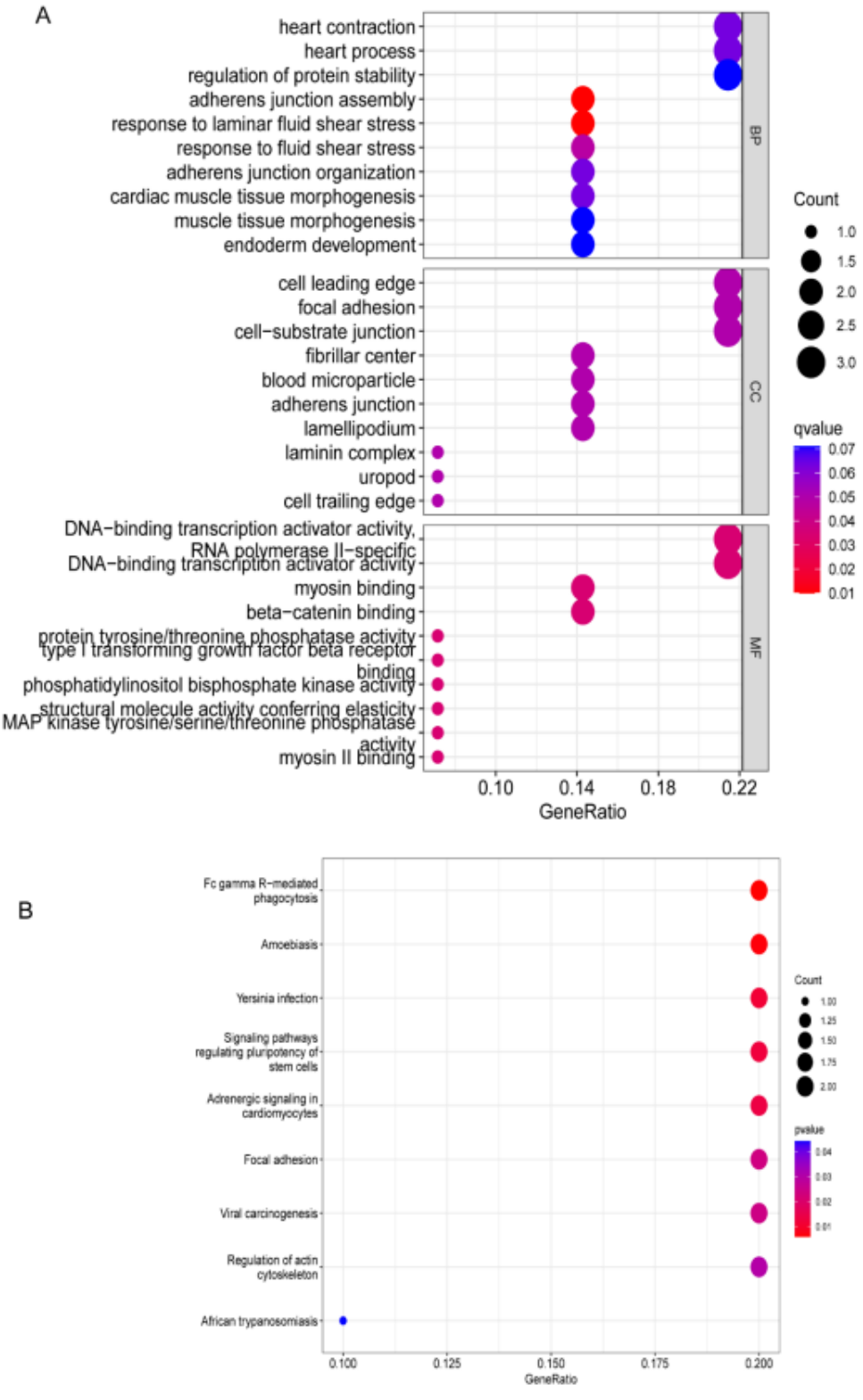


**Figure 5.** Enrichment analysis. (A) GO enrichment analysis; (B) KEGG enrichment analysis.

### *3.5. Association between tumor immune cell invasion and threat score*

The CIBERSORT algorithm was used to determine the score of immune cells in the tumor group, and the

difference in immune cells between the high- and low-risk groups was calculated (Fig. 6A). As depicted in the image, neutrophils were more evident in the high-risk groups. However, the expression of T cells regulatory significantly increased in the low-risk group. Immune cell function analysis (Figure 6B) revealed that the rest, except type II IFN response, was more significant in the high-risk group.

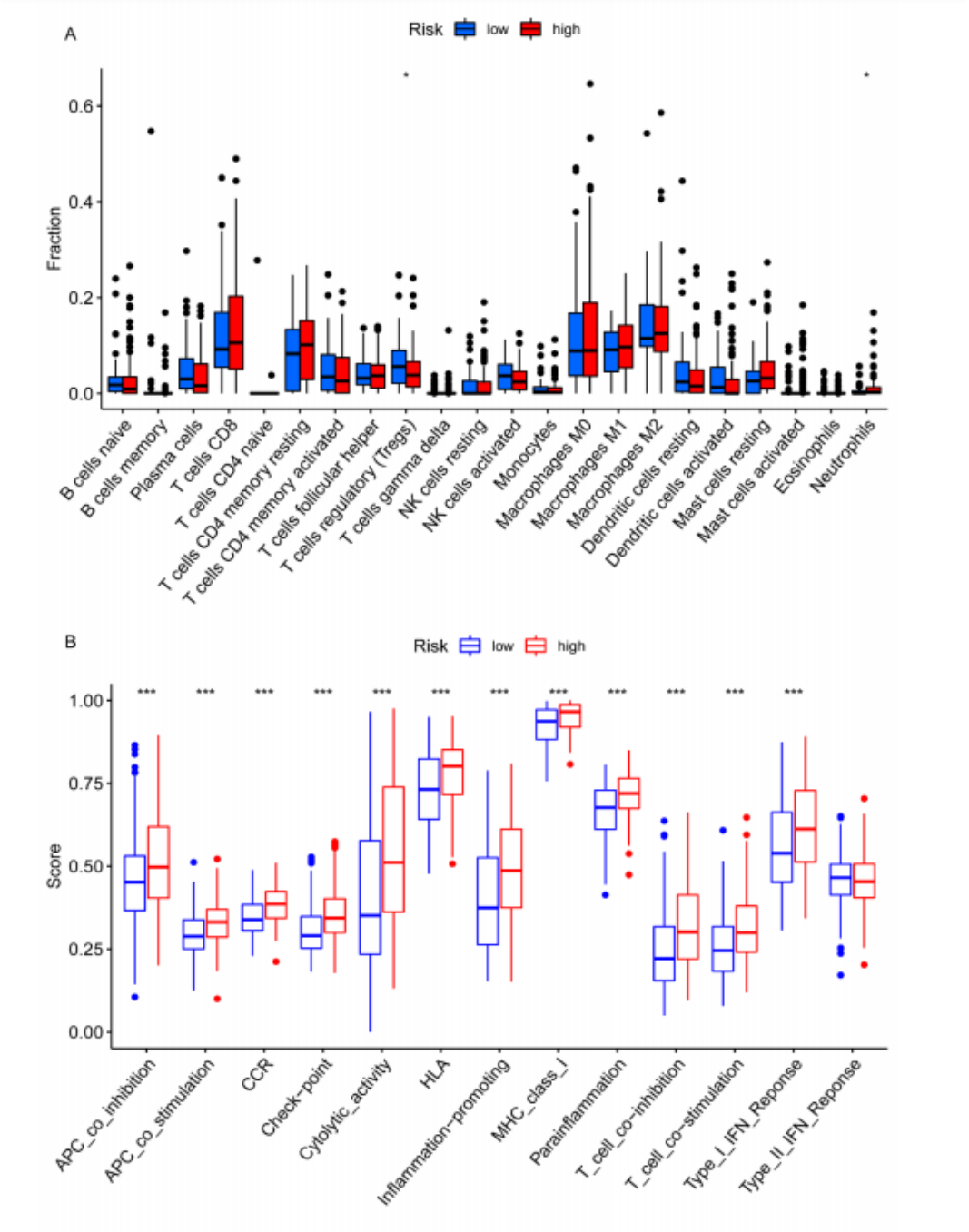


**Figure 6.** Immunoinfiltration analysis. (A) Correlation between risk score model and tumor infiltrating immune cells; (B) Correlation between risk score model and tumor infiltrating immune cell function. * P < 0.05; ** P < 0.01; *** P < 0.001.

### *3.6. miRNA expression in the BLCA cells*

To verify the validity of the TCGA dataset, the expression levels of two miRNAs in the cells was measured using

quantitative reverse transcription polymerase chain reaction (qRT-PCR). MIR-25 and MIR-548AN have a high expression in T24 cells (Figure 7).

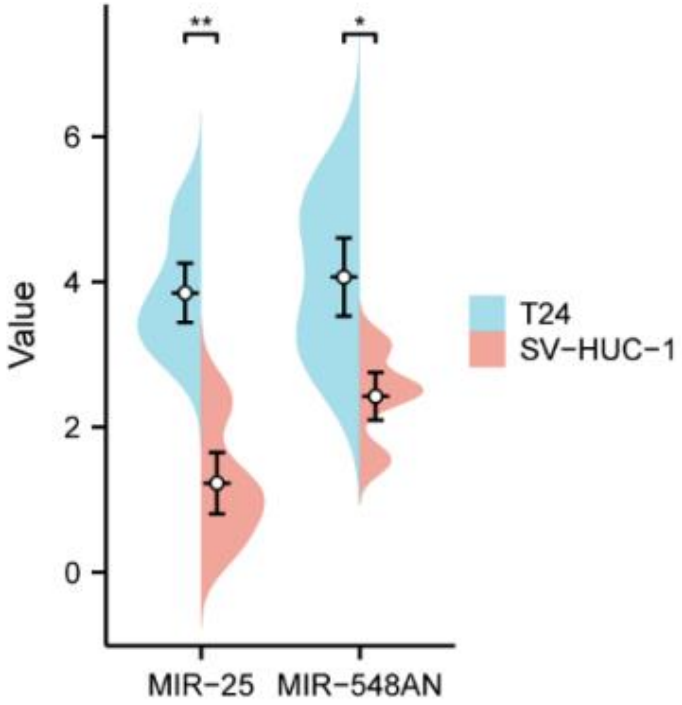


**Figure 7.** Validation of the prognostic miRNA.

## 4. Discussion

BLCA is a common tumor of the urinary system, among which invasive BLCA has a poor prognosis and is associated with distant metastasis. Current studies have revealed that miRNA plays an important role in different cancers, including BLCA [13, 14]. The dysregulation of miRNA is closely related to the prognosis of tumor metastasis. Vishnu Suresh Babu et al. revealed that miR-181a promoted epithelial–mesenchymal transition and chemotherapy resistance in advanced-stage retinoblastoma [15]. Elena Andreucci et al. [16] elaborated the role of miR-214 in tumor metastasis caused by immune cell abnormalities, and these novel functions have attracted significant attention. Some miRNAs play an essential role in the diagnosis and prognosis of cancer. However, the association between them and the target has not been validated due to disease complexity and diversity. Bioinformatics analysis plays a key role in the study of disease mechanism. Therefore, it is a powerful tool for evaluating the association between miRNAs and target genes.

Our study performed differential analysis on the TCGA-BLCA dataset. Then, patients with TCGA-BLCA were classified into the high- and low-risk groups using the median risk score. The miRNA cross-target genes were screened using data from the miRDB, TarBase, and Targetscan databases. Subsequently, their involvement in key tumor-related pathways were validated. Results showed that Fc gamma R-mediated phagocytosis, amoebiasis, Yersinia infection, the signaling pathways regulating pluripotency of stem cells, adrenergic signaling in cardiomyocytes, focal adhesion, and the viral carcinogenesis pathways were statistically significant. Moreover, the downstream target genes of miR-25 and miR-548an associated with BLCA were successfully predicted. The downstream genes of miR-25 included SMAD7, TEF, IL6ST, BTG2, NFIX, MOAP1, DUSP5, NFIC, ACTC1, PIPI5K1C,

GNAQ, and KIF4. The target genes of miR-548an were LAMC1 and GSN. Previous studies have found that miR-25 is highly differentially expressed in several cancers, including BLCA [17-19].Thus, this is not the first study showing the role of miR-25 in BLCA. Jin-Zhuo Ning [20] et al. revealed that miR-25 promotes BLCA metastasis and proliferation and that miR-25 can be used as a biomarker for predicting BC progression. However, there is no clear correspondence for the downstream targets. Thus, we screened out the target genes to match them, thereby laying the foundation for the mechanism research. However, for miR-548an with few target genes, some studies have found that HIF1α/HDAC1 can be downregulated by transcription to inhibit the tumorigenesis of pancreatic cancer [21]. Therefore, it is important to evaluate the downstream targets of miR-548an in the treatment of pancreatic cancer. In addition, some studies have shown that the tumor microenvironment plays an important role in BLCA [22]. We assessed the distribution of immune cells in the high- and low-risk groups. In our study, neutrophils were more evident in the high-risk group. However, the low-risk group had a significantly increased expression of T cells regulatory. The exact mechanism by which these miRNAs interact with immune cells is unknown, and more research should be performed to investigate the role of BLCA.

Our study revealed a few key miRNAs and related target genes. Nevertheless, they may be in the growth, development and metastasis of BLCA plays an important role, but how specific target genes regulating the disease process is not careful, not sure these target genes involved in cancer metastasis or through regulating immune cells of the tumor microenvironment. These notions should be further explored. Undeniably, for the diagnosis and prognosis of BLCA, it is significantly important to validate the association between BLCA-related MiRNA and corresponding target genes and the expression of immune cells.

## 5. Conclusion

A prognosis model for aging based on two miRNAs with a high predictive value was developed. This study presented a novel approach for individualized therapy in patients with BLCA.

## Data Availability Statement

The corresponding author can provide data that were utilized to support the study's conclusions upon request.

## Funding statement

This research received no external funding.

## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Author contributions

All authors made a significant contribution in the conception, study design, execution, data acquisition, analysis, and interpretation, or in all these areas; took part in drafting, revising, or critically reviewing the article; provided final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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