Original Article

Exploring the microRNA-mRNA regulatory network associated with solasonine in bladder cancer

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Background: Solasonine has been demonstrated to exert an inhibitory effect on bladder cancer (BC), but the potential mechanisms remain unclear. Therefore, the aim of this study is to explore the association between microRNAs (miRNAs)-mediated regulation and the anti-tumor activities of solasonine in BC.

Methods: MiRNA sequencing was performed to identify the differentially expressed microRNAs (DE-miRNAs) associated with solasonine in BC cells. Functional enrichment analyses of the DE-miRNAs activated and inhibited by solasonine were then conducted. The DE-miRNAs with prognostic value for BC and those differentially expressed in the BC samples were subsequently identified as the hub DE-miRNAs. After identifying the messenger RNAs (mRNAs) that were targeted by the hub DE-miRNAs and those differentially expressed in the BC samples, a protein-protein interaction analysis was performed to identify the core downstream genes, which were then used to construct a solasonine-miRNA-mRNA regulatory network.

Results: A total of 27 activated and 19 inhibited solasonine-mediated DE-miRNAs were identified that were found to be associated with several tumor-related biological functions and pathways. After integrating the results of the survival analysis and expression assessment, the following nine hub DE-miRNAs were identified: hsa-miR-127-3p, hsa-miR-450b-5p, hsa-miR-99a-5p, hsa-miR-197-3p, hsa-miR-423-3p, hsa-miR-4326, hsa-miR-625-3p, hsa-miR-625-5p, and hsa-miR-92a-3p. The DE-mRNAs targeted by the hub DE-miRNAs were predicted, and 30 core downstream genes were used to construct the solasonine-miRNA-mRNA regulatory network. miR-450b-5p was shown to be associated with the most mRNAs in this network, which suggests that it plays a crucial role in the solasonine-mediated anti-BC effect.

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Conclusions: A regulatory network, including solasonine, miRNAs, and mRNAs related to BC, was constructed. This network provides extensive insights into the molecular regulatory mechanisms that underlie the anti-cancer efficacy of solasonine in BC.

Keywords: Bladder cancer (BC); solasonine; microRNAs (miRNAs); microRNA sequencing (miRNA-seq)

Submitted Mar 29, 2024. Accepted for publication May 09, 2024. Published online May 27, 2024.
doi: 10.21037/tau-23-469
View this article at: https://dx.doi.org/10.21037/tau-23-469

Introduction

Bladder cancer (BC) is publicly recognized as the tenth most prevalent malignancy globally, with a high propensity to recurrences (1). Once muscle invasion and metastasis occur, the prognosis is unfavorable. Therefore, early detection and prompt management are very important (2). However, the exploration of early diagnosis and prevention still confronts numerous challenges. Although cystoscopy is the gold standard for diagnosis of BC, its invasive nature generally leads to the emergence of discomfort and resistance to treatment in patients (3). Despite several newer therapeutic agents such as chemotherapeutic agents, immune checkpoint inhibitors (ICIs), and targeted therapies have been already employed for patients with muscle invasion and distant metastasis, the overall survival rates remain unsatisfying (3,4). Consequently, exploring novel biomarkers and therapeutic strategies for BC diagnosis and treatment is always one of research highlights. Currently, many new techniques involving liquid biopsy and molecular beacon have been proposed with relatively good clinical potential for detecting biomarkers, such as mRNA, microRNA (miRNA) and long non-coding RNA (lncRNA), which are essential for early diagnosis and optimizing treatment outcomes (5-7). Increasing evidence has reported that the dysregulated miRNA biogenesis network is closely related to many pathological features of BC, such as tumor infiltration, recurrence, metastasis, and drug sensitivity (6,8). Given the role of miRNAs as regulators of gene expression after transcription across different tissues, and the ease of evaluation in liquid samples, making miRNA an attractive candidate biomarker (6).

With the advancement of traditional Chinese medicine, herbal medicine is gradually gaining increased attention in cancer therapy, with the advantages of multiple targets and minimal side effects. *Solanum nigrum L.* (*S. nigrum*) is one of the most commonly used anti-tumor herbs in clinical practice in China. Using contemporary extraction technologies, several components with anti-tumor properties in *S. nigrum* have been identified, including alkaloid, polysaccharide, saponin, and polyphenol (9). Among them, solasonine, a major glycoalkaloid extracted from all parts of *S. nigrum* and unripe fruits, exerts an inhibitory effect in various malignancies (10,11), including suppressing the proliferation and inducing apoptosis of BC cells (11,12). Limited studies so far have focused on the regulatory effects of solasonine on miRNA activity. For instance, solasonine has been reported to exert growth inhibitory effects in gastric cancer cells by increasing miR-486-5p expression to target phosphoinositide-3-kinase regulatory subunit-1 (PI3KR1) (13). Additionally, solasonine has shown the ability to inhibit hepatocellular carcinoma cell growth via the reciprocal regulation of miR-375-3p and lncRNA colon cancer associated transcript-1 (CCAT1) (14). Although the inhibitory effect of solasonine

Highlight box

Key findings

- We established a solasonine-micro RNA (miRNA)-messenger RNA regulatory network to provide a comprehensive understanding of the regulatory mechanisms underlying the anti-bladder cancer (BC) effect of solasonine.

What is known, and what is new?

- Solasonine has been shown to exert an inhibitory effect on BC; however, the potential mechanisms of solasonine has yet to be elucidated.
- By identifying the miRNAs that were differentially expressed in the BC cells following treatment with solasonine, we developed a regulatory network to provide a comprehensive understanding of the anti-cancer effect of solasonine.

What is the implication, and what should change now?

- The potential regulatory mechanisms underlying the anti-cancer effect of solasoine need to be elucidated. Our findings provide profound insights into the anti-cancer mechanisms of solasonine.

on BC progression has been well described (11,12), the underlying molecular mechanism of miRNA-mediated regulation has not been fully elucidated. Thus, further research is necessary comprehensively understand how solasonine modulates miRNA-mediated regulation and subsequently affect the malignant phenotype of BC.

To examine the potential relationships between miRNAs and solasonine in BC, miRNA sequencing (miRNA-seq) was performed on BC cells after treatment with solasonine. The differentially expressed microRNAs (DE-miRNAs) were then identified. The DE-miRNAs that were significantly differentially expressed in the BC samples using data sets downloaded from a public database and those associated with prognosis were identified as the hub DE-miRNAs. In addition, the differentially expressed messenger RNAs (DE-mRNAs) between the BC tissues and normal tissues were identified using a public database. Finally, the DE-mRNAs that had regulatory relationships with the hub DE-miRNAs were used to construct a solasonine-miRNA-mRNA network. Our study focused on the miRNA-mediated regulation of solasonine. By constructing a solasonine-miRNA-mRNA network using miRNA-seq information, we provided an innovative perspective on the molecular regulatory mechanisms underlying the anti-tumor activities of solasonine in BC. These findings informed the future strategies for novel biomarker in BC and clinical oncology, holding significant implications in determining personalized therapy. We present this article in accordance with the MDAR reporting checklist (available at https://tau.amegroups.com/article/view/10.21037/tau-23-469/rc).

Methods

The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

Cell culture and reagent treating

The T24 human BC cell line (RRID: CVCL_0554) was acquired from Shanghai Institutes for Biological Sciences, Shanghai, China. Solasonine, a pharmacologically active agent, was sourced from Chengdu Must Bio-Technology Co., Ltd., Chengdu, China. The T24 cell lines were cultivated in Roswell Park Memorial Institute (RPMI)-1640 medium, enriched with 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 10% fetal bovine serum. The cells were incubated in a 37 ℃ humidified atmosphere with 5% carbon dioxide in accordance with standard protocols (11,15). A concentration of 50 μM of solasonine, along with the vehicle [Dimethyl Sulfoxide (DMSO)], was used to treat the T24 cells based on prior determinations of cell viability using Cell Counting Kit-8 assays (11).

RNA isolation

To obtain the RNA samples, the T24 cells in the six-well plates were collected 48 hours after the solasonine or vehicle treatment. The total RNA was extracted from each sample using Trizol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, Massachusetts, USA) in accordance with the standard protocols outlined in the TRIzol® kit's instruction manual. The RNA extraction experiments were conducted using a completely randomized design and replicated at least three times.

RNA quantification and qualification

RNA quality was evaluated by monitoring RNA degradation and contamination on 1% agarose gels. Subsequently, RNA purity was scrutinized using the NanoPhotometer® spectrophotometer (IMPLEN, CA, USA). RNA concentration was measured using the Qubit® RNA Assay Kit with the Qubit® 2.0 Fluorometer (Life Technologies, CA, USA). RNA integrity was assessed using the RNA Nano 6000 Assay Kit on the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA).

Library preparation and sequencing

Each sample used 3 μg of total RNA as input for the small RNA library preparation. The NEBNext® Multiplex Small RNA Library Prep Set for Illumina® (NEB, USA) was employed to generate sequencing libraries with index codes added for sample attribution. The total RNA served as the initial template for the complementary DNA (cDNA) synthesis through reverse transcription. After the polymerase chain reaction amplification, the target DNA fragments underwent separation via PAGE gel electrophoresis to construct a comprehensive cDNA library. Before the cluster generation, Qubit 2.0 and Agilent 2100 were used to conduct quality checks. The index-coded samples were then subjected to cluster generation using the TruSeq SR Cluster Kit v3-cBot-HS (Illumina). The final sequencing of the library preparations was performed using

the Illumina Hiseq 2500/2000 platform.

Data analysis and miRNA prediction

The raw data in the fastq format underwent initial processing via custom Perl and Python scripts for quality control. The small RNA tags were subsequently aligned to the reference sequence using Bowtie (16) without any mismatches, which facilitated the analysis of their expression and distribution in relation to the reference genome. The miRBase20.0 was used as a reference, and modified software miRDeep2 (https://github.com/rajewsky-lab/mirdeep2) (17) and UEA SRNA workbench (https://github.com/sRNAworkbenchuea/UEA_sRNA_Workbench/) were used to identify the known miRNAs. Additionally, the novel miRNA prediction was performed using miRDeep2 (17) and miREvo (https://github.com/akahanaton/miREvo) (18) by exploring the secondary structure, the Dicer cleavage site, and the minimum free energy of the small RNA tags unannotated in the former steps.

Differential expression analysis of miRNAs

The miRNA expression levels were quantified using the transcript per million (TPM) method whereby the normalized expression was calculated using the following formula: normalized expression = the mapped read count/by the total reads × 1,000,000. The DESeq R package (version 1.8.3) (https://bioconductor.org) (19) was used to identify the DE-miRNAs; a q value <0.01 and a $|\log_2(\text{fold change})|$ >1 was set as the default threshold for significant differential expression.

Prediction of the potential target genes of the DE-miRNAs

The potential target genes of the DE-miRNAs were predicted using the following four databases: Starbase (version 2.0; https://starbase.sysu.edu.cn) (20), miRDB (http://mirdb.org/miRDB/) (21), TargetScan (version 8.0; https://www.targetscan.org) (22), and TarBase (version 9; https://dianalab.e-ce.uth.gr/tarbasev9) (23). Only the target genes predicted by all four databases were included in the further analysis.

Function and pathway enrichment analyses

To investigate the function of the predicted targets of the DE-miRNAs, we used the DOSE (Disease Ontology Semantic and Enrichment analysis) (http://www.bioconductor.org/packages/release/bioc/html/DOSE.html) (24) and clusterProfiler (http://bioconductor.org/packages/release/bioc/html/clusterProfiler.html) (25) packages in the R statistical software (version 4.1.0). This facilitated Gene Ontology (GO) classification and the identification of enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (26). We generated high-quality graphs using the ggplot2 (https://ggplot2.tidyverse.org/) and pROC (https://CRAN.R-project.org/package=pROC) packages. Three GO categories (biological process, cellular component, and molecular function) were examined. The GO terms and KEGG pathways with an adjusted P value <0.05 were considered significantly enriched.

The Cancer Genome Atlas (TCGA) data processing

To investigate the expression patterns of the identified DE-miRNAs in the BC tissues, we validated their expression using TCGA data sets (https://portal.gdc.cancer.gov/). We obtained RNA-sequencing raw data comprising 19 normal samples and 418 tumor samples of the bladder from TCGA database. Subsequently, the raw data underwent standardization through log2 transformation and normalization procedures using the "limma" package (https://bioconductor.org/packages/limma/) (27) in the R environment. The "normalizeBetweenArray" function was employed for the normalization process.

Survival analysis

Univariate Cox regression models were employed to compute the hazard ratios and 95% confidence intervals based on the expression levels of the DE-miRNAs of the BC patients in TCGA database. A Cox analysis was conducted using the "survival" package (accessible at CRAN; https://cran.r-project.org/web/packages/survival/index.html). To visualize the survival data, Kaplan-Meier (K-M) curves were generated using the "survminer" package (accessible via GitHub; https://github.com/kassambara/survminer). Subsequently, all the BC patients were stratified into low and high expression groups based on the optimal cut-off values for the miRNA expression levels determined by the smallest P value.

Protein-protein interaction (PPI) network construction and module analysis

Using the Search Tool for the Retrieval of Interacting

Genes (STRING) database (version 11.0; http://string-db.org), a preliminary PPI network was established (28). To ensure the robustness of the network, a minimum threshold of 0.7 was set, and any proteins that did not form connections were excluded. The network underwent clustering via the Molecular Complex Detection (MCODE) algorithm (version 1.4.2), an extension of Cytoscape software (version 3.7.1), to delineate regions with high connectivity (29). The following filtering parameters were applied to the data: a MCODE score >5, a maximum depth of 100, a node score threshold of 0.2, a minimum degree of 2, and a k-score of 2.

Construction of the solasonine-miRNA-mRNA network

To further elucidate the potential regulatory mechanism of solasonine in BC, we constructed a solasonine-miRNA-mRNA network. The miRNAs and the expression data of the corresponding target genes were obtained from TCGA database. The target genes that were significantly differentially expressed in TCGA-BLCA (bladder urothelial carcinoma) dataset and inversely correlated with the expression of the miRNA candidates were included in this network.

Statistical analysis

For the statistical analysis, the Student's *t*-test was used for comparisons between two distinct groups, and a one-way analysis of variance followed by the Newman-Keuls post-hoc test was used for comparisons of more than two groups. In the context of the differential expression analysis, a gene expression variation was deemed significant at an absolute log2 fold change of 1 or greater, coupled with a P value <0.05. For the survival analysis, a Cox proportional hazards model P value <0.05 was established as the threshold for statistical significance.

Results

Identification of the DE-miRNAs regulated by solasonine in the BC cells

A differential analysis of the miRNA-seq data was performed to identify the potential miRNAs involved in the process in which solasonine suppressed the malignant progression of BC cells. The volcano plot (*Figure 1A*) and heatmap (*Figure 1B*) clearly showed the number and hierarchical clustering of the DE-miRNAs in the solasonine-treated and control (untreated) groups. A total of 46 DE-miRNAs were identified, including 27 activated miRNAs, and 19 inhibited miRNAs, of which two were novel miRNAs.

GO and KEGG pathway analyses of the target genes for the DE-miRNAs inhibited by solasonine

The potential targets of the 17 known mature inhibited DE-miRNAs were identified and numbered in a Venn plot (*Figure 2A*). The duplicate DE-miRNAs were eliminated. Information about these identified targets available at https://cdn.amegroups.cn/static/public/tau-23-469-1.pdf. We identified 2,728 unique genes in the merged union for further GO term and KEGG pathway analyses (table available at https://cdn.amegroups.cn/static/public/tau-23-469-2.pdf). The top five terms in each GO category and the 15 tumor-related enriched KEGG pathways, including the proteoglycans in cancer, estimated glomerular filtration rate (EGFR) tyrosine kinase inhibitor resistance, pathways regulating the pluripotency of stem cells, mitogen-activated protein kinase (MAPK) pathway, and phosphatidylinositol-3-kinase (PI3K)-Akt pathway, are presented in the bar graphs (*Figure 2B,2C*).

GO and KEGG pathway analyses of the target genes for the DE-miRNAs activated by solasonine

The target genes corresponding to the 27 DE-miRNAs up-regulated by solasonine were identified using four distinct databases (table available at https://cdn.amegroups.cn/static/public/tau-23-469-3.pdf). Following the post-removal of redundant entries, a convergence of 3,626 unique overlapping genes was established (*Figure 3A*). A subsequent GO terms and KEGG pathways enrichment analyses were then conducted (table available at https://cdn.amegroups.cn/static/public/tau-23-469-4.pdf). The predominant five GO terms across each category, along with the 15 cancer-related KEGG pathways exhibiting significant enrichment, were then visualized in bar graphs (*Figure 3B,3C*). These pathways encompassed a range of biological processes, notably including the proteoglycans in cancer, focal adhesion, the Erb-B2 Receptor Tyrosine Kinase-2 (ErbB) signaling pathway, resistance to EGFR tyrosine kinase inhibitors, and the MAPK signaling pathway.

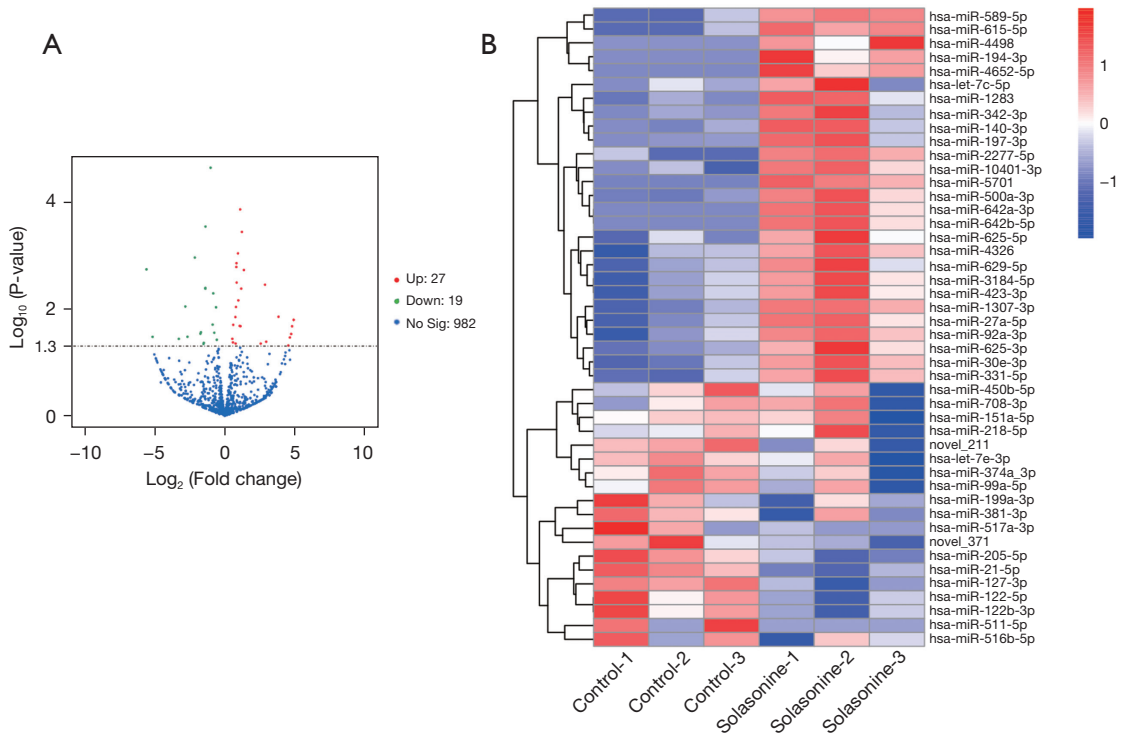

Figure 1 Identification of the DE-miRNAs regulated by solasonine in BC cells. (A) Volcano plot visualizing the DE-miRNAs in the T24 cells between solasonine-treated and control (untreated) groups. The red dots on the right side represent 27 significant activated miRNAs, and the green dots on the left side represents 17 known and 2 unknown novel repressed miRNAs (log2FC >1 and P<0.05). (B) Heatmap visualizing the hierarchical clustering of the DE-miRNAs in the two groups. The color indicates expression intensity from high (red) to low (blue). Each column represents one sample, and each row indicates a transcript. no Sig, no significant difference, P>0.05. DE-miRNAs, differentially expressed microRNAs; BC, bladder cancer; miRNA, microRNA; FC, fold change.

Survival analysis of the DE-miRNAs regulated by solasonine

To explore the effect of the DE-miRNAs regulated by solasonine on the prognosis of BC patients, a K-M analysis of disease-specific survival (DSS) was performed. The significant results are presented in *Figure 4A*,*4B*, which show that a total of 15 solasonine-mediated DE-miRNAs (seven inhibited and eight activated) were significantly associated with DSS in BC. In the inhibition group, the low expression of four DE-miRNAs (i.e., hsa-miR-127-3p, hsa-miR-199a-3p, hsa-miR-450b-5p, and hsa-miR-99a-5p) was associated with favorable outcomes. In the activation group, the high expression of seven DE-miRNAs (i.e., hsa-miR-140-3p, hsa-miR-197-3p, hsa-miR-423-3p, hsa-miR-4326, hsa-miR-625-3p, hsa-miR-625-5p, and hsa-miR-92a-3p) was associated with a better prognosis. Thus, these 11 DE-miRNAs were selected as candidate hub DE-miRNAs for the next analysis to identify the functional regulators in the solasonine-mediated anti-BC effect.

The expression of the candidate DE-miRNAs in the BC samples

To investigate the expression of the candidate hub DE-miRNAs in the BC samples, we compared the DE-miRNAs expression levels between the 418 BC samples and 19 adjacent normal samples in TCGA database. As *Figure 5* shows, only hsa-miR-199a-3p and hsa-miR-140-3p showed no significant difference in gene expression. Integrating the results of the survival analysis and expression assessment, the remaining nine DE-miRNAs were considered the hub regulators with a major functional contribution for solasonine in BC. The nine hub DE-miRNAs were hsa-miR-127-3p, hsa-miR-450b-5p, hsa-miR-99a-5p, hsa-miR-197-3p, hsa-miR-423-3p, hsa-miR-4326, hsa-miR-625-3p,

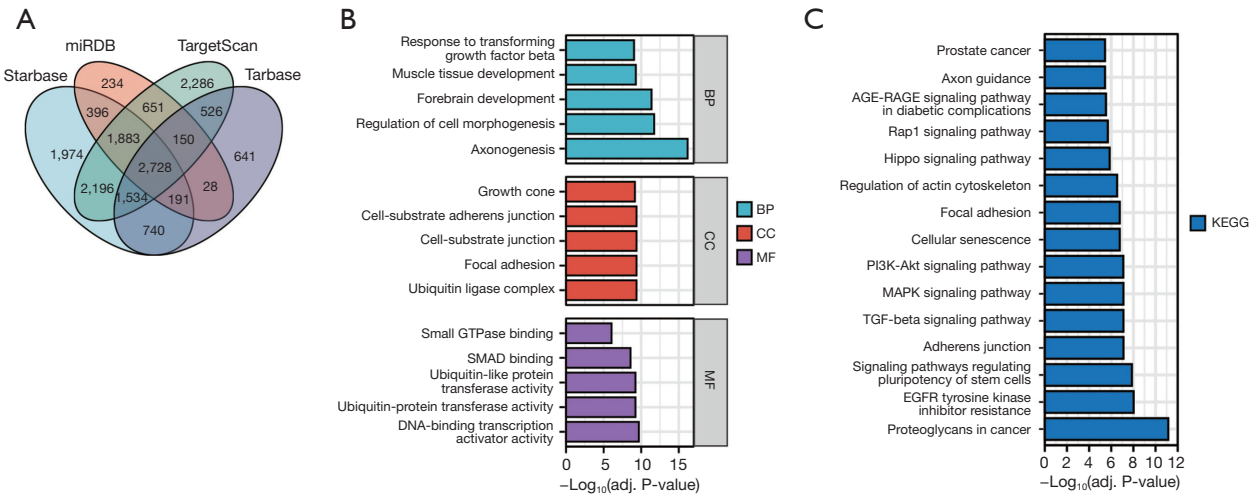

Figure 2 GO and KEGG pathway analyses of the target genes for the DE-miRNAs inhibited by solasonine. (A) Venn plot visualizing the potential targets for the 17 known mature inhibited DE-miRNAs identified from four databases (Starbase, miRDB, TargetScan, and Tarbase). A total of 2,728 unique genes, common to all four databases, were identified for the functional enrichment analysis. (B) Bar graph showing the top five significantly enriched BP/CC/MF-GO terms, sorted by adjusted P values in increasing order. (C) Bar graph showing the 15 tumor-related enriched KEGG pathways. BP, biological process; CC, cellular component; MF, molecular function; KEGG, Kyoto Encyclopedia of Genes and Genomes; GO, Gene Ontology; DE-miRNAs, differentially expressed microRNAs.

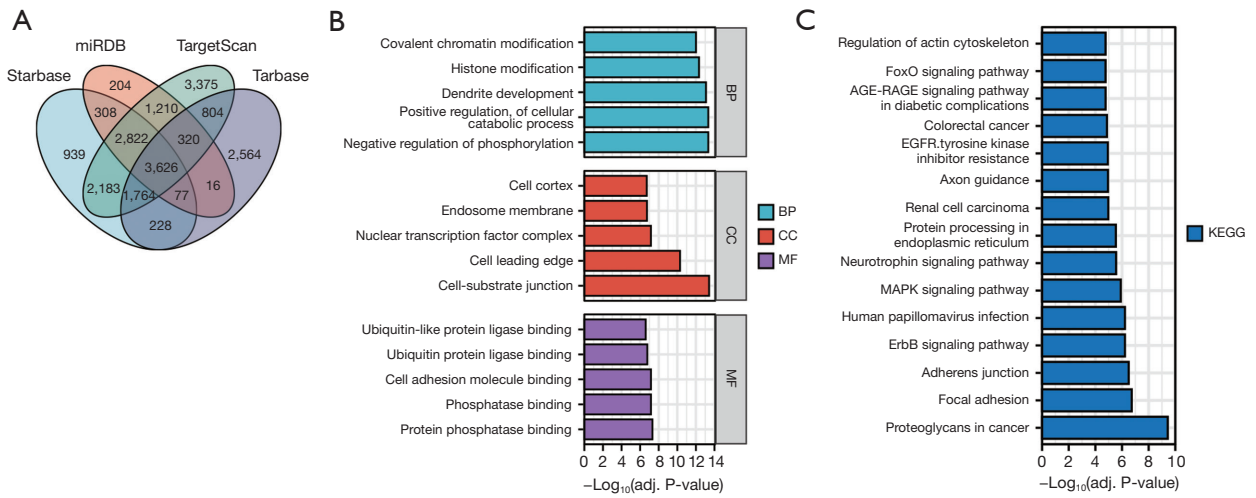

Figure 3 GO and KEGG pathway analyses of the target genes for the DE-miRNAs activated by solasonine. (A) Venn plot visualizing the target genes for the 27 activated DE-miRNAs identified from the four databases. A total of 3,626 unique genes, common to all four databases, were identified for the functional enrichment analysis. (B) Bar graph showing the top five significantly enriched BP/CC/MF-GO terms, sorted by adjusted P values in increasing order. (C) Bar graph showing the 15 tumor-related enriched KEGG pathways. BP, biological process; CC, cellular component; MF, molecular function; KEGG, Kyoto Encyclopedia of Genes and Genomes; GO, Gene Ontology; DE-miRNAs, differentially expressed microRNAs.

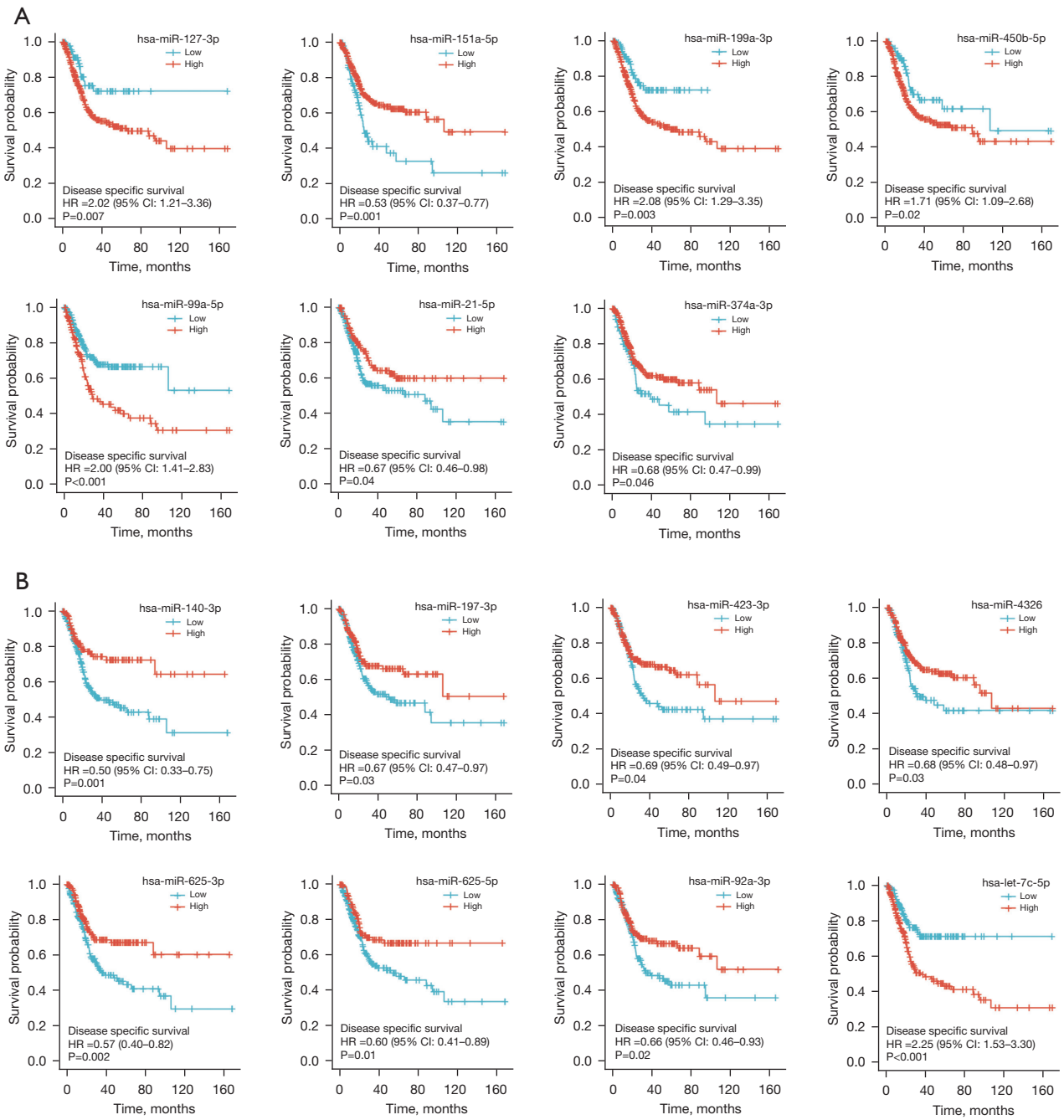

Figure 4 Kaplan-Meier plots showing the DSS of patients with BC in relation to the solasonine-mediated DE-miRNAs. (A) Kaplan-Meier survival curve showing the DSS of the DE-miRNAs inhibited by solasonine and (B) the DE-miRNAs promoted by solasonine in BC. Only the significant DE-miRNAs are presented in this figure. HR, hazard ratio; DSS, disease-specific survival; BC, bladder cancer; DE-miRNAs, differentially expressed microRNAs; CI, confidence interval.

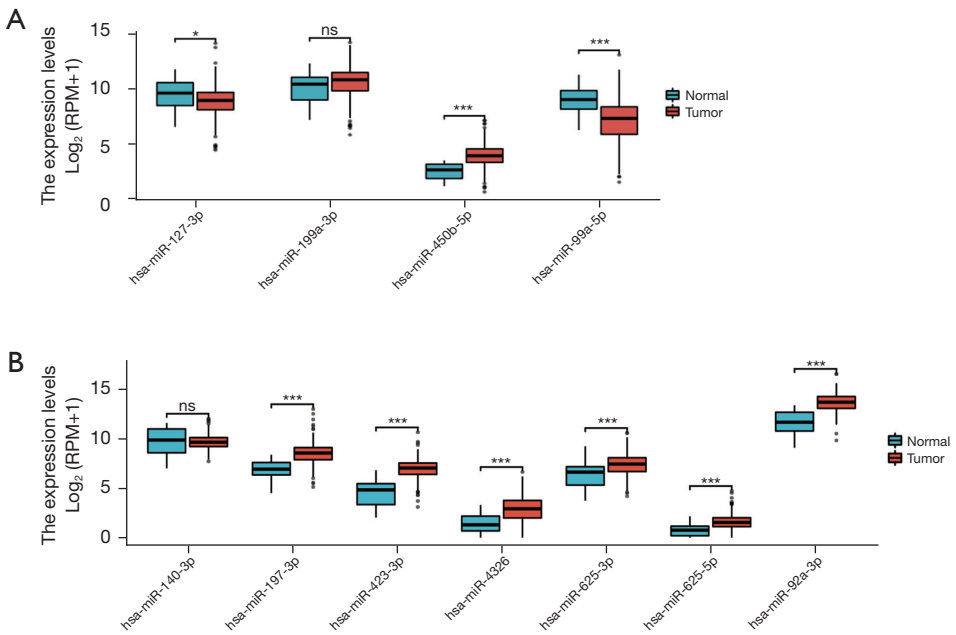

Figure 5 The expression analysis of the candidate DE-miRNAs in the BC samples. (A) The difference in the expression of the four candidate DE-miRNAs inhibited by solasonine, and (B) the seven candidate DE-miRNAs promoted by solasonine, between the 418 BC samples and 19 adjacent normal samples in TCGA database. ns, P≥0.05; *, P<0.05; ***, P<0.001. RPM, reads per million; DE-miRNAs, differentially expressed microRNAs; BC, bladder cancer; TCGA, The Cancer Genome Atlas.

hsa-miR-625-5p, and hsa-miR-92a-3p.

Identification of the candidate mRNAs

To identify the candidate mRNAs that were differentially expressed in BC and regulated by the hub DE-miRNAs, we first identified the overlapping DE-mRNAs between the BC samples and adjacent normal samples in TCGA database. As *Figure 6A* and the table (available at https://cdn.amegroups.cn/static/public/tau-23-469-5.pdf) show, a total of 4,052 down-regulated DE-mRNAs and 8,202 up-regulated DE-mRNAs were identified. Based on the criteria that the target genes should display an inverse relationship with the miRNAs (30), we then identified 151 highly expressed candidate DE-mRNAs for the down-regulated hub DE-miRNAs, and 1,708 lowly expressed DE-mRNAs for the up-regulated hub DE-miRNAs in the BC tissues as compared to the normal tissues (*Figure 6B,6C*).

GO and KEGG pathway analyses of the candidate mRNAs

The results of the functional enrichment analyses of the 151 highly expressed candidate mRNAs and 1,708 down-regulated candidate mRNAs are presented in Table S1 and the table (available at https://cdn.amegroups.cn/static/public/tau-23-469-6.pdf), respectively. The top terms in each GO category and the related KEGG pathways are presented in bar graph (*Figure 7A,7B*). The constructed network, which integrated the gene pathways and GO terms, showed the interplay between the selected candidate mRNAs and the most significantly enriched pathways/GO terms (*Figure 7C,7D*).

Construction of the PPI network and module analysis

A PPI network comprising the candidate mRNAs was established, consisting of 1,076 nodes and 3,485 edges (*Figure 8A*). Based on their degree values, the top 30 mRNAs in this network were subsequently ranked (*Figure 8B*). A module analysis of the PPI network led to the identification of three predominant modules (*Figure 8C-8E*), each exhibiting enrichment in various KEGG pathways (see Table S2). Specifically, Module 1 comprised 13 genes predominantly involved in transforming growth factor-beta (TGF-β) signaling, BC, and the p53 signaling pathway. Module 2 comprised 12 genes significantly associated

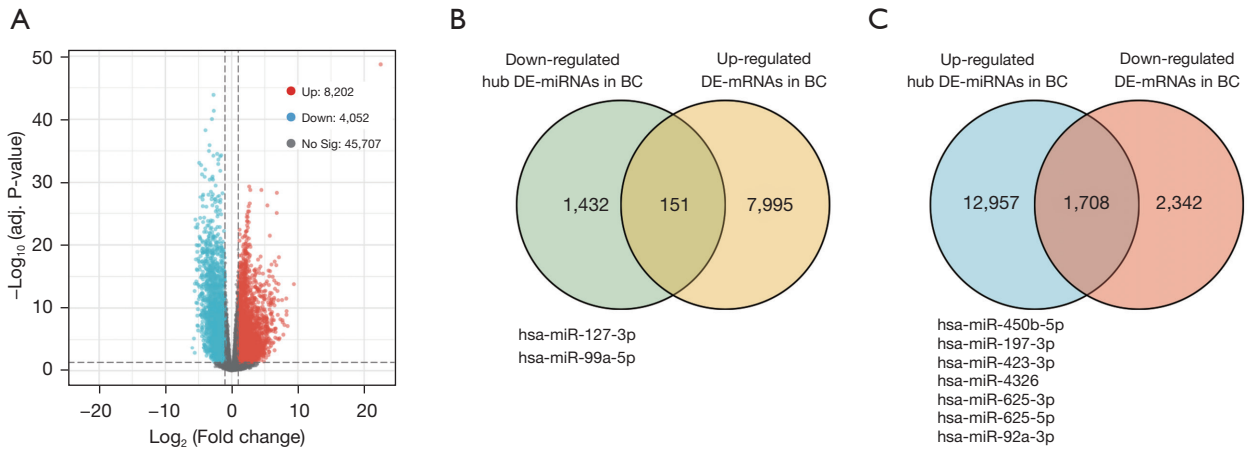

Figure 6 Identification of the candidate DE-mRNAs targeted by the hub DE-miRNAs. (A) Volcano plot visualizing the DE-mRNAs between the BC samples and normal samples from TCGA database. The red dots on the right side represent significant up-regulated mRNAs, and the blue dots on the left side represent the down-regulated mRNAs (log2FC >1 and adjusted P value <0.05). (B) Venn plot visualizing 151 candidate mRNAs for the hub DE-miRNAs that were down-regulated in the BC samples. (C) Venn plot visualizing 1,708 candidate mRNAs for the hub DE-miRNAs that were up-regulated in the BC samples. no Sig, no significant difference, P>0.05. DE-miRNA, differentially expressed microRNA; BC, bladder cancer; mRNA, messenger RNA; TCGA, The Cancer Genome Atlas; FC, fold change.

with the immune checkpoint pathway in cancer, MAPK signaling, and various immune cell-mediated pathways. While Module 3 comprised nine genes that were significantly enriched in the cell-cycle process.

Construction of the solasonine-miRNA-mRNA regulatory network

To elucidate the regulatory mechanisms governing the effect of solasonine on BC, this study then established a solasonine-miRNA-mRNA regulatory network. This network incorporated nine critical solasonine-responsive DE-miRNAs and the top 30 candidate mRNAs exhibiting the highest values of degree in the PPI network. Using the predicted miRNA-mRNA regulatory pairs identified in the BC samples from TCGA database, a solasonine-miRNA-mRNA regulatory network associated with anti-tumor effect in BC was established (*Figure 9*).

Discussion

BC remains a leading cause of cancer-associated mortality worldwide, negatively affecting quality of life and causes a significant burden on the healthcare system (1,4). BC exhibits high clinical morphological and biological heterogeneity, and is typically pathologically categorized into non-muscle invasive BC (NMIBC) and muscle invasive BC (MIBC). Alterations common in NMIBC include deletions of chromosome 9 and FGFR3 point mutation. However, MIBC requires inactivation of one or more tumor suppressor genes, such as TP53, RB1, and PTEN, and is considered to originate from flat urothelial dysplasia and carcinoma *in situ* (2). Although advancements in understanding the genomic landscape and tumor microenvironment have motivated the clinical implementation of targeted treatments, new ICIs and antibody-drug conjugates, the resulting clinical benefits have only been observed in partial patients (31). In the field of oncological therapy, herbal medicine has shown unique advantages characterized by multiple targets, multiple biological processes, and multiple pathways. Similar to the comprehensive regulation of herbal medicine, the post-transcriptional regulation of miRNA is also complex, as a single miRNA typically targets multiple mRNAs, thereby affecting the expression of both oncogenes and tumor suppressor genes in a tumor-specific fashion (6,32). Meanwhile, a single mRNA transcript can also be regulated by several miRNAs. Furthermore, due to their small size and binding properties, miRNAs exhibit relatively greater stability compared to other RNA-based molecules, making them promising candidates for cancer detection and therapy (33). Therefore, investigating the molecular

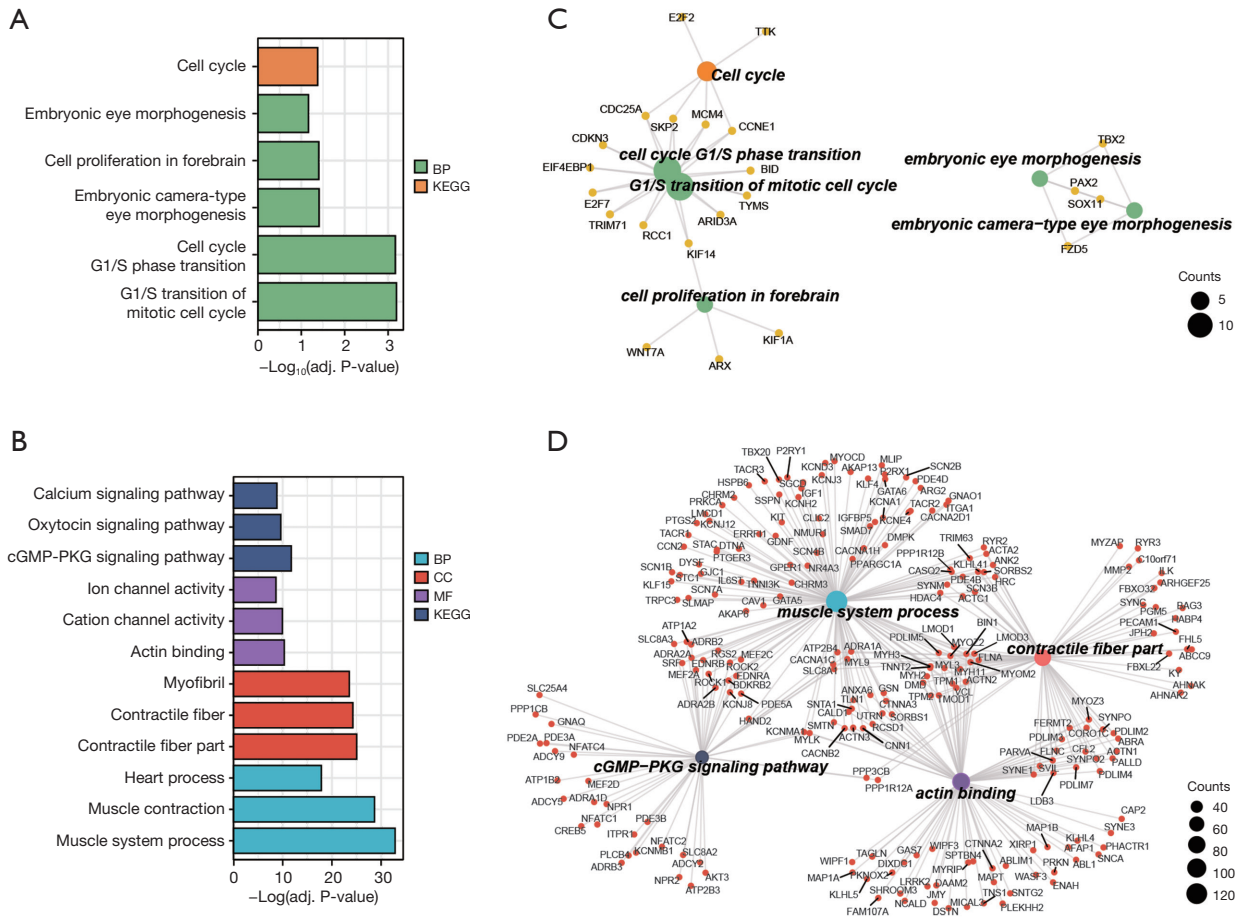

Figure 7 GO and KEGG pathway analyses of the candidate mRNAs. (A) Bar graph showing the top three significantly enriched BP/CC/MF-GO terms and KEGG pathways for the up-regulated candidate mRNAs, and (B) the down-regulated candidate mRNAs, sorted by adjusted P values in increasing order. (C) Network of the gene pathways/GO terms showing the interactions among the candidate down-regulated mRNAs and the top significantly enriched pathways/GO terms, and (D) the interactions among the candidate up-regulated mRNAs and the significantly enriched pathways/GO terms. BP, biological process; KEGG, Kyoto Encyclopedia of Genes and Genomes; CC, cellular component; MF, molecular function; GO, Gene Ontology; mRNA, messenger RNA.

mechanisms underlying the effects of solasonine based on the miRNA regulatory network holds certain guiding significance for its clinical application in patients with BC.

In this study, the investigation into the biological roles of the DE-miRNAs modulated by solasonine revealed that the target genes of these down-regulated DE-miRNAs are significantly involved in critical cellular pathways, including those associated with the proteoglycans in cancer, and the TGF-β, MAPK, and PI3K-Akt signaling pathways. Intracellular proteoglycans are highly expressed in breast, lung, prostate, colon, and hepatocellular carcinoma, and contribute to the aggressiveness and metastasis of tumors (34). The TGF-β, MAPK, and PI3K/Akt signaling pathways are well known and have served as therapeutic targets in cancer treatments (35,36). Additionally, the activated DE-miRNAs are mainly associated with the proteoglycans in cancer, focal adhesion, Ras protein signal transduction, and the ErbB signaling pathway. Ras proteins are commonly activated in many types of tumors, including BC (37). Research has shown that the aberrant expression of Ras-association domain family 1 (RASSF1A) is related to a greater likelihood of BC recurrence and muscle infiltration (38). Aberrant ErbB activation has been reported in various cancers, can increase transcriptional expression, and is frequently associated with drug resistance and a poor prognosis (39).

After integrating the results of the survival analysis

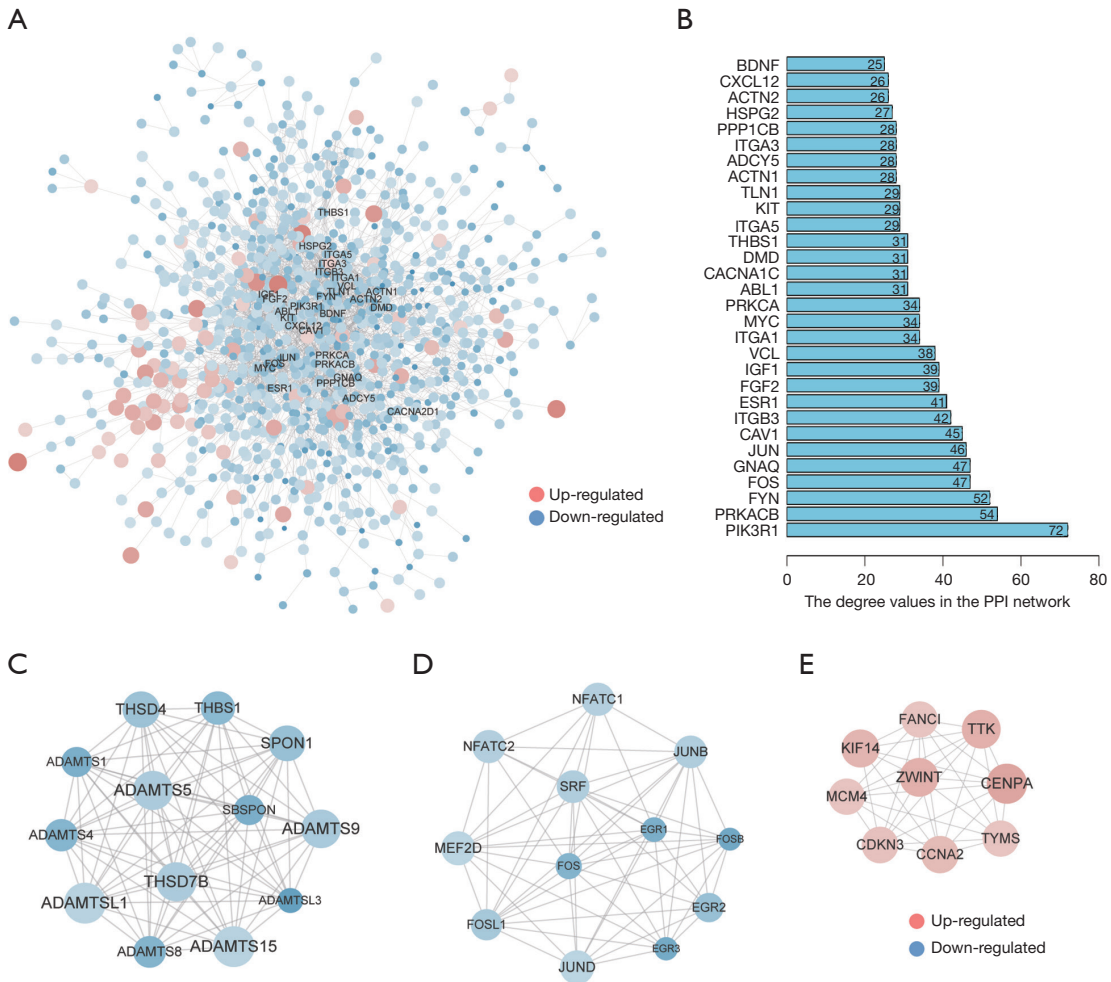

Figure 8 PPI network construction and module analysis. (A) A PPI network was constructed, and genes with ≤3 nodes and ≤3 edges were removed from the network. The up-regulated genes are marked in red, and the down-regulated genes are marked in blue. The genes with the top 30 degrees in the PPI network were labeled. The larger size or deeper color of a node indicates that the corresponding gene had a greater log2FC. (B) Bar plot of the nodes with the top 30 degrees in the PPI network. Higher numbers and longer bar lengths represent greater interactions among the genes. (C) The most significant module in the PPI network with 13 nodes and 78 edges. (D) The second most significant module in the PPI network with 12 nodes and 57 edges. (E) The third most significant module in the PPI network with 9 nodes and 34 edges. PPI, protein-protein interaction; FC, fold change.

and expression assessment, nine DE-miRNAs ultimately remained, which were considered the hub regulators with a major functional contribution for solasonine in BC. Among them, miR-127-3p, miR-99a-5p, and miR-450b-5p were inhibited by solasonine. MiR-127-3p has been shown to be aberrantly expressed in several types of tumors and is associated with malignant clinicopathological features (40). The elevated expression of miR-450b-5p has been observed to enhance cell proliferation and impede apoptosis in colorectal cancer cells (41). Evidence also indicates that miR-450b-5p could be targeted for treating glioblastoma. Its downregulation can promote the expression level of gelsolin, inhibiting cell proliferation and invasion (42). In addition, studies indicate that the up-regulation of miR-99a-5p is correlated with an adverse prognosis in gastric cancer patients (43). Serum miR-99a-5p is significantly elevated in patients with ovarian cancer, suggesting its potential as a target for inhibiting the progression of the disease (44).

Additionally, miR-197-3p, miR-423-3p, miR-4326, miR-625-3p, miR-625-5p, and miR-92a-3p are shown to be

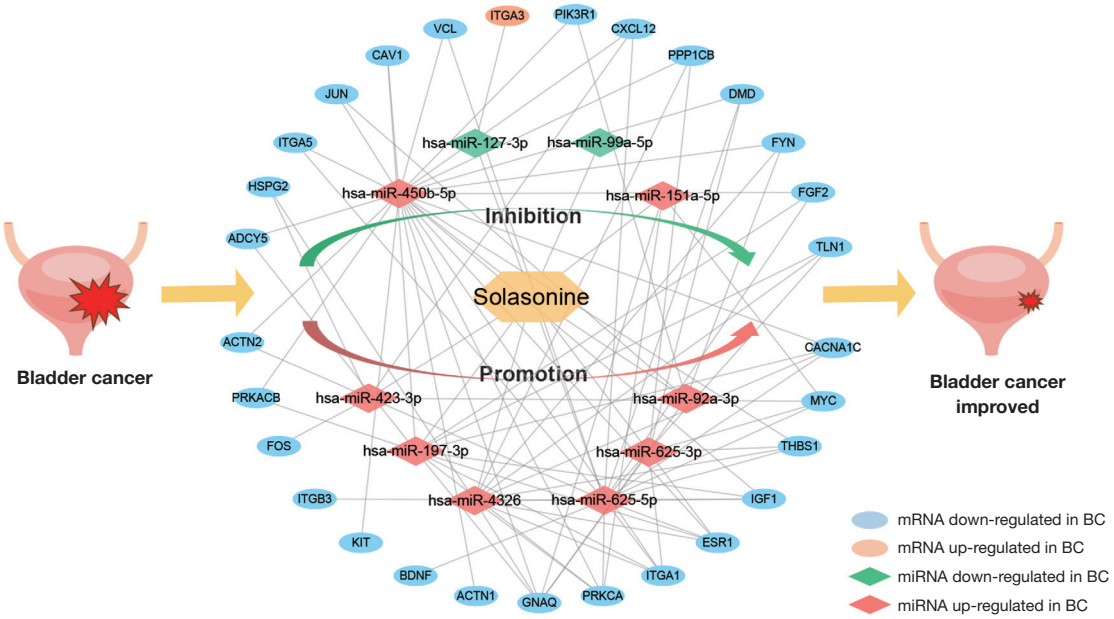

Figure 9 The solasonine-miRNA-mRNA regulatory network in BC. mRNA, messenger RNA; BC, bladder cancer; miRNA, microRNA.

promoted by solasonine in our study. The elevated expression of miR-197 has been shown to decrease both proliferation and epithelial-mesenchymal transition in ovarian cancer cells (45). Further, miR-625-3p has been validated as an inhibitor of gastric cancer metastasis, primarily through the modulation of enhancer of zeste 2 polycomb repressive complex 2 subunit (EZH2) expression (46). Conversely, the dysregulated expression of miR-625-5p has been implicated in the enhancement of tumor angiogenesis and modulation of tumor immunity in BC (47). MiR-92a-3p has been identified as a suppressor of cell proliferation, migration, and invasion in Wilms tumors, and acts by targeting FRS2 (48). Additionally, exosomal miR-423-3p plays a role in attenuating the malignant progression of cervical cancer (49). Despite miR-4326 being up-regulated and linked to cell proliferation in lung cancer (50), its role in other cancer types, particularly BC, necessitates further exploration.

In accordance with the established inverse relationship between miRNA and mRNA expression (30), the target genes of the pivotal DE-miRNAs in BC tissues were determined. GO and KEGG enrichment analyses of these downstream genes were then conducted. The results revealed that the up-regulated genes were significantly enriched in processes related to the G1/S transition of the mitotic cell cycle and the G1/S phase transition. Additionally, the KEGG pathway analysis highlighted a pronounced enrichment in the cell-cycle pathway. Conversely, the down-regulated genes were predominantly enriched in biological processes, such as muscle system processes, muscle contraction, actin binding, cation channel activity, and ion channel activity. Ion channels mediate a range of cellular processes and are dysregulated in all cancers, which have been described as a cancer-related channelopathy (51). The KEGG pathway analysis revealed that the examined genes predominantly participate in pathways such as focal adhesion, cyclic guanosine monophosphate-dependent protein kinase (GMP-PKG) signaling, oxytocin signaling, calcium signaling, and MAPK signaling. These pathways have been implicated in the processes of oncogenesis and tumor progression (52).

To more accurately delineate the regulatory mechanisms underpinning the effects of solasonine in BC, a solasonine-miRNA-mRNA regulatory network was established. This network comprised nine central solasonine-responsive DE-miRNAs and the top 30 essential downstream mRNAs, which were selected based on the degree of their interactions in the PPI network. The most connected mRNAs were estrogen receptor-1 (ESR1), Integrin Subunit Alpha-1 (ITGA1), and G protein subunit alpha q (GNAQ), with five connections per mRNA. Followed by calcium voltage-gated channel subunit alpha1 c (CACNA1C), protein kinase c alpha (PRKCA), Thrombospondin-1 (THBS1), and insulin like growth factor-1 (IGF1), each of them had a

connection number of 4. The most connected miRNA was miR-450b-5p with a connection number of 22, suggesting a central role in the solasonine-related network. In addition, miR-625-5p and miR-197-3p ranked the second and third, with a connection number of 16 and 15, respectively. The anti-cancer effects by suppressing miR-450b-5p has been confirmed in colorectal cancer and glioblastoma (41,42). Collectively, it is reasonable to consider that miR-450b-5p may exert an oncogenic function and serve an important role in BC development. Further experimental validation is required for this hypothesis.

This study had some limitations. First, only one BC cell line was used for the miRNA-seq in this study, which might limit the precision of the data. Second, while the DE-mRNAs were obtained by screening TCGA database, this method lacks specificity compared with sequencing experiments and might have resulted in the exclusion of vital genes associated with solasonine. Third, this study did not involve the construction of silenced or overexpressed miRNAs as a method to substantiate our findings *in vivo* and *in vitro* experiments. This omission may limit the strength of the evidence supporting the regulatory mechanisms of the effect of solasonine in BC. Future experiments should seek to address these limitations.

Conclusions

This study identified the miRNAs associated with solasonine in BC using miRNA-seq. After evaluating the expression and prognostic values in the BC samples using a public database, the hub DE-miRNAs were identified and a solasonine-miRNA-mRNA regulatory network was established. MiR-450b-5p was shown to play a central role and had the most connections to the mRNAs in this network. Further experiments should be conducted to validate these results. We believe that this study extends understandings of the regulatory mechanisms underlying the anti-tumor activities of solasonine in BC.

Acknowledgments

Funding: This work was supported by funding from the National Natural Science Foundation of China (No. 82204866 to Y.D., and No. 12271467 to C.H.H.), the Xuzhou Youth Medical Science and Technology Innovation Project (No. XWKYHT20210545, to Y.D.), the Pengcheng Talent—Medical Young Reserve Talent Training Project (No. XWRCHT20220012, to Y.D.), the Natural Science Foundation of Jiangsu Province (No. BK20231163, to Y.D.), and Postgraduate Research & Practice Innovation Program of Jiangsu Province (No. SJCX23_1399, to Y.A.C.).

Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://tau.amegroups.com/article/view/10.21037/tau-23-469/rc

Data Sharing Statement: Available at https://tau.amegroups.com/article/view/10.21037/tau-23-469/dss

Peer Review File: Available at https://tau.amegroups.com/article/view/10.21037/tau-23-469/prf

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tau.amegroups.com/article/view/10.21037/tau-23-469/coif). Y.A.C. reports receiving funding from Postgraduate Research & Practice Innovation Program of Jiangsu Province (No. SJCX23_1399, to Y.A.C.). Y.D. reports receiving funding from the National Natural Science Foundation of China (No. 82204866, to Y.D.); the Xuzhou Youth Medical Science and Technology Innovation Project (No. XWKYHT20210545, to Y.D.); the Pengcheng Talent—Medical Young Reserve Talent Training Project (No. XWRCHT20220012, to Y.D.); and the Natural Science Foundation of Jiangsu Province (No. BK20231163, to Y.D.). C.H.H. reports receiving funding from the National Natural Science Foundation of China (No. 12271467, to C.H.H.). The other authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work, including ensuring that any questions related to the accuracy or integrity of any part of the work have been appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

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Cite this article as: Fang K, Fang DL, Yu H, Chen YA, Yu PZ, Wang ZF, Zhang RB, Yang W, Tao L, Fukushima H, Dong Y, Han CH. Exploring the microRNA-mRNA regulatory network associated with solasonine in bladder cancer. Transl Androl Urol 2024;13(5):812-827. doi: 10.21037/tau-23-469

