



# Identification of a potentially functional circRNA-miRNA-mRNA ceRNA regulatory network in bladder cancer by analysis of microarray data

Lei Du, Xin Wang, Yuewei Yin, Yanping Zhang, Jianghua Jia, Baosai Lu, Wenyong Xue, Changbao Qu, Jinchun Qi

Department of Urology, The Second Hospital of Hebei Medical University, Shijiazhuang, China

**Contributions:** (I) Conception and design: C Qu, J Qi; (II) Administrative support: C Qu, J Qi; (III) Provision of study materials or patients: C Qu, J Qi; (IV) Collection and assembly of data: B Lu, W Xue; (V) Data analysis and interpretation: L Du, X Wang, Y Yin, Y Zhang, J Jia; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

**Correspondence to:** Changbao Qu; Jinchun Qi. Department of Urology, The Second Hospital of Hebei Medical University, No. 215, Heping Xi Road, Shijiazhuang, China. Email: qu9295388@163.com; qjinchun2020@hotmail.com.

**Background:** Circular RNAs (circRNAs) have received increasing attention in cancer development. However, a substantial number of circRNAs still require characterization. The purpose of this study is to uncover novel circRNAs and their molecular mechanism in bladder cancer (BCa).

**Methods:** A combinative strategy of extensive data mining and computational biology was employed to identify BCa-related circRNAs and explore their potential mechanisms of action.

**Results:** Three differentially expressed circRNAs (has\_circ\_0023642, has\_circ\_0047322, has\_circ\_0041151) were obtained from the microarray dataset (GSE92675). Four miRNAs (miR-616, miR-515-5p, miR-647, miR-1178) with potential binding sites with these three circRNAs were identified. Pathway analysis demonstrated that all four miRNAs were closely associated with some cancer-related pathways. Survival analysis indicated that these miRNAs might potentially play a role in tumor-suppressive functions in BCa. Subsequently, 181 overlapping genes were identified from 472 up-regulated genes in BCa (TCGA database), and 10,017 predicted target genes of the four miRNAs obtained. A circRNA-miRNA-mRNA network was constructed on the identified three circRNAs, four miRNAs, and 181 overlapping genes. Besides, six hub genes (*CENPA*, *HIST1H2B7*, *HIST1H2BO*, *HIST1H3H*, *HIST1H3B*, *HIST1H3F*) were identified from establishing a protein-protein interaction (PPI) network on the same overlapping genes. Furthermore, a circRNA-miRNA-hub gene sub-network was built to delineate the links among the differential circRNAs, miRNA, and hub genes.

**Conclusions:** Our study provided significant insights into the molecular mechanisms that regulate the progression of BCa from the circRNA-miRNA-mRNA network view.

**Keywords:** Bladder cancer (BCa); circRNA; ceRNA; microarray; miRNA

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## Introduction

Bladder cancer (BCa) is the most common malignant tumor of the genitourinary system, with high mortality rates worldwide (1). BCa is categorized into two subtypes, including non-muscle-invasive bladder cancer (NMIBC) and muscle-invasive bladder cancer (MIBC). Most BCa patients are primarily diagnosed with NMIBC. However, approximately 50–70% of patients with NMIBC will experience disease recurrence within 5 years. Also, 10–30% of patients will develop MIBC, which currently has a poor prognosis (2). Although several therapeutic approaches, including surgical resection, chemotherapy, and radiotherapy, have been developed, the 5-year survival rate of MIBC patients is only approximately 60% (3,4). It is of vital importance to identify novel biomarkers and effective targets for BCa.

Circular RNAs (circRNAs) are a novel class of RNAs that are generated via endogenous splicing. These covalently closed-loop structures are resistant to exonuclease cleavage due to the absence of 5'-3' polyadenylation tails. The high stability displayed by circRNAs suggests they are strong candidates, as biomarkers (5,6). There is evidence that circRNAs play critical roles in cancer biology (7-9), and more specifically, in the development of BCa. One mechanism employed by circRNAs is via competition with microRNAs (miRNA). The competing endogenous circRNAs, known as ceRNAs, serve as miRNA sponges. For instance, a highly conserved circRNA, ciRS-7 (also termed CDR1as), exerts anti-tumor functions in BCa by sponging miR-135a (10). CircHIPK3 can effectively inhibit tumor aggression and metastasis of BCa by acting as ceRNAs of miR-558 (11). CircRIP2 accelerates BCa progression by targeting the miR-1305/Tgf- $\beta$ 2/smad3 axis (12). Meanwhile, CircFAM114A2 suppresses BCa progression via regulating  $\Delta$ NP63 by sponging miR-762 (13). Circular RNA circPICALM inhibits BCa metastasis via influencing FAK phosphorylation by acting as ceRNAs of miR-1265 (14). These findings all together strongly suggest the importance of circRNAs as novel biomarkers in the regulation and development of BCa. However, many unidentified circRNAs remain to be investigated.

In this study, a circRNA microarray from the GEO database was used to screen differentially expressed circRNAs between BCa tissues and paired-matched adjacent

normal tissues. To depict whether the differentially expressed circRNAs functioned as ceRNAs in BCa, we predicted their sponged miRNAs and miRNAs-targeted mRNAs. The signaling pathways of miRNA-targeted mRNAs were then analyzed, and a circRNA-miRNA-mRNA regulatory network was constructed. A protein-protein interaction (PPI) network was subsequently established, and the hub genes were subsequently identified. Our results provide significant insights into the molecular mechanisms that regulate the progression of BCa from the circRNA-miRNA-mRNA network. We present the following article in accordance with the MDAR reporting checklist (available at <http://dx.doi.org/10.21037/tau-20-660>).

## Methods

### Data collection

The gene expression profiles of BCa were obtained from GEO (<https://www.ncbi.nlm.nih.gov/geo/>) database. The miRNA and mRNA data of BCa were downloaded from TCGA (<https://portal.gdc.cancer.gov>). The circRNA expression profile of GSE92675 was obtained from the GEO database, including 4 pairs of matched BCa (GSM2434319, GSM2434321, GSM2434323, GSM2434325) and para-cancer tissues (GSM2434320, GSM2434322, GSM2434324, GSM2434326). A total of 433 mRNA samples (414 BCa samples and 19 normal bladder samples) were obtained from the TCGA database.

### Differentially expressed circRNAs and mRNAs

The Limma package was used to identify the differentially expressed circRNAs between BCa tissue and the adjacent normal tissue from the GEO database (FDR value <0.05 and  $|\log_2FC| > 2$ ). Additionally, we used the EdgeR package to screen the differentially expressed mRNAs with thresholds of  $|\log_2FC| > 2$  and P value <0.05.

### Prediction of circRNA-miRNA binding sites

Prediction of interactions between circRNAs and miRNAs was conducted using the CircInteractome database.

MiRNAs with a context score percentile >98 were eventually selected.

#### ***Screening of miRNA-targeted mRNAs and pathway analysis***

The miRNA-targeted mRNAs were predicted with the TargetScan prediction tool and miRDB software. The signaling pathways of miRNA-targeted mRNAs were analyzed by Enrichr software. The effect of miRNAs on the survival of BCa patients was analyzed by using the online Kaplan-Meier Plotter database.

#### ***Construction of a circRNA-miRNA-mRNA regulatory network***

The ceRNA regulatory network was established on the foundation of the interactions between the differentially expressed circRNAs, the predicted miRNAs, and the differentially expressed mRNAs. Cytoscape 3.7.1 software was used to visualize the established ceRNA regulatory network.

#### ***Establishment of PPI regulatory network and identification of hub genes***

A PPI network was constructed by using the STRING (V 11.5) and visualized by the CytoCope 3.7.1 software. Additionally, the highly interacted hub genes from the PPI network were screened by the CytoHubba plugin in Cytoscape.

#### ***Verification of hub gene expression with the GEPIA database***

To further analyze the expression level of hub genes mRNA in BCa. We explored the mRNA expression level of these hub genes in the web-based GEPIA database (<http://gepia.cancer-pku.cn/>) with the settings  $P \leq 0.05$  and  $|\log_2(\text{FC})| \geq 1$ .

#### ***Statistical analysis***

All statistical analyses were performed using R (v.3.6.0) software and SPSS v.24.0 software (IBM Corp.). The correlation between miRNA expression and patients'

survival was assessed by Kaplan-Meier method. The expression of hub genes in different tissues was analyzed by Student's *t*-test. The P value <0.05 was considered statistically significant.

## **Results**

### ***Identification of differentially expressed circRNAs***

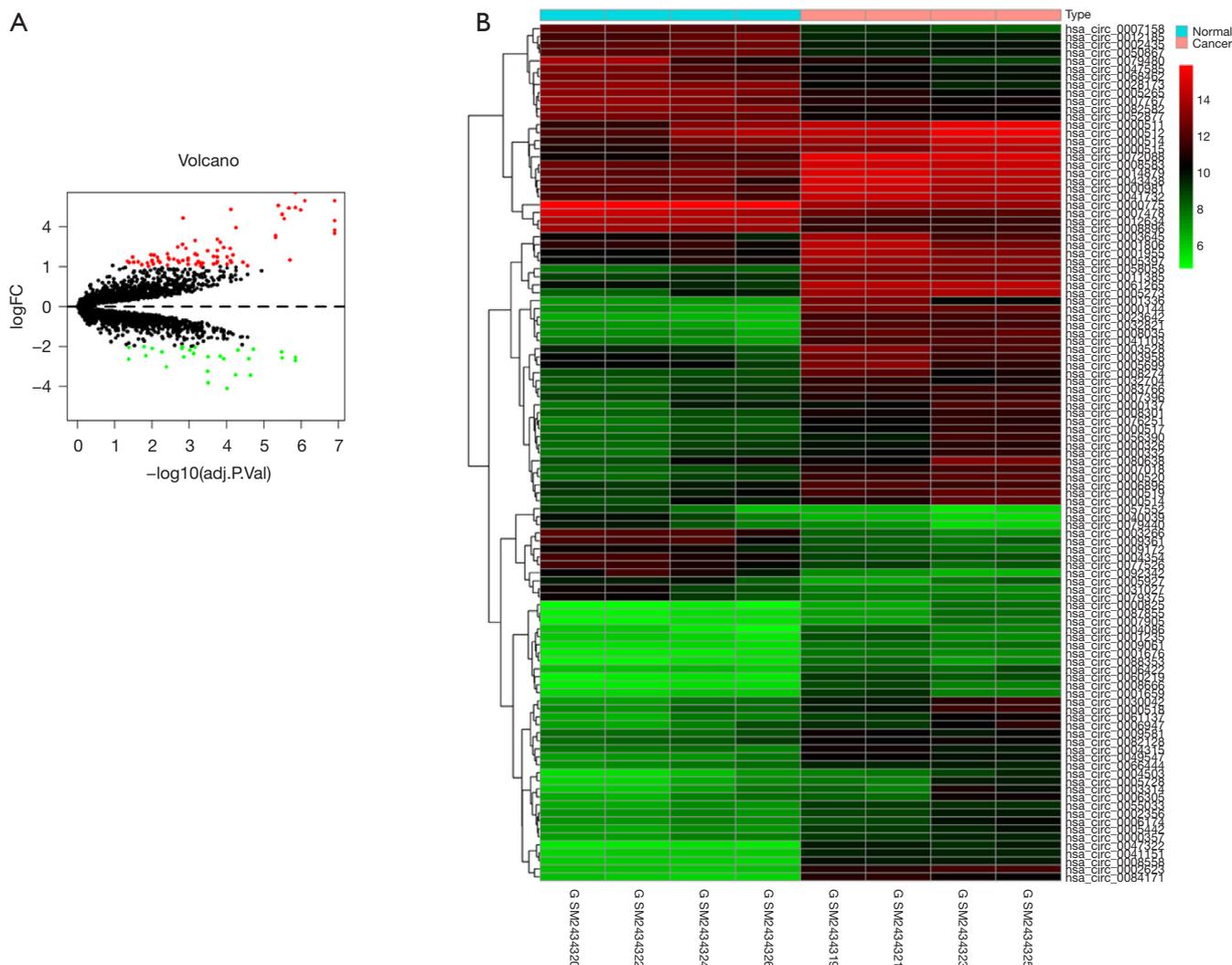
The GSE92675 dataset from GEO was analyzed by the limma package in R (P value <0.05 and  $\log_2|\text{fold change}| > 2$ ), we identified 79 up-regulated and 28 down-regulated circRNAs (*Figure 1*). Also, a total of 1,229 differential expressed mRNAs (472 up-regulated and 757 down-regulated mRNAs) were identified from the TCGA database between BCa tissues and normal bladder tissues (FDR value <0.05 and  $\log_2|\text{fold change}| > 2$ ) (*Figure 2*). Subsequently, three circRNAs were selected according to the P value. The basic structure patterns of these three circRNAs (has\_circ\_0023642, has\_circ\_0047322, has\_circ\_0041151) are exhibited in *Figure 3*.

### ***Identification of three circRNA-miRNA interactions***

Accumulated evidence has demonstrated that some circRNAs derived from exons play crucial roles in tumor progression by acting as miRNAs “decoys” to sponge miRNAs. To identify whether these three circRNAs performed similar roles in BCa, we carried out a context score percentile screening in the CircInteractome database. Four miRNAs (miR-616, miR-515-5p, miR-647, miR-1178) with a context score percentile >98 were selected (*Figure 4A*). The specific binding sites between circRNAs and miRNAs were shown in *Figure 4B*. Enrichr was employed to explore the signaling pathways of these four miRNAs. As shown in *Figure 5*, all these four miRNAs are strongly associated with some cancer-related pathways.

### ***Expression of the four miRNAs with the Kaplan-Meier Plotter database***

The significance of the selected miRNA expression in BCa was further explored. We analyzed the effect of miRNA expression on the survival of BCa patients by using the online Kaplan-Meier Plotter database. As shown in *Figure 6*,



**Figure 1** The differentially expressed circRNAs between BCa and the paired-matched adjacent normal tissues. (A) The differential expressed circRNAs are displayed in the volcano plot, and (B) in a heat map. BCa, bladder cancer; circRNAs, circular RNAs.

the overall survival of BCa patients who expressed high-levels of miR-515-5p, miR-647, and miR-1178 exhibited better survival compared to patients with low miRNA expression. The median survival of BCa patients with high expression of miR-616 (45.57 months) was higher than the patients with low miR-616 expression (27.43 months). However, this difference is not statistically significant ( $P=0.18$ ). These results suggest that these miRNAs might

play potential tumor-suppressive functions in BCa.

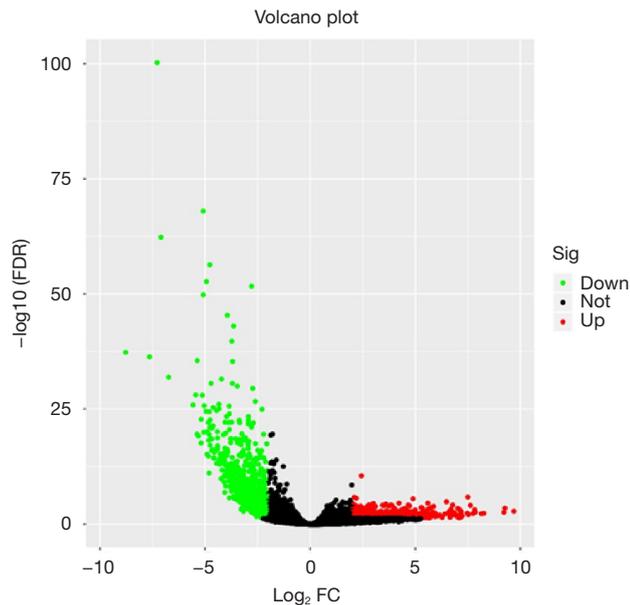
**Construction of circRNA-miRNA-mRNA regulatory networks in BCa**

Ten thousand and seventy-one target genes of the four miRNAs mentioned above were obtained from the TargetScan prediction tool. Additionally, 472 up-regulated

genes in BCa were identified from TCGA (Figure 2). These investigations led to the identification of 181 overlapping target genes that played critical roles in BCa (Figure 7A).

A circRNA-miRNA-mRNA network was constructed through integrating the circRNA-miRNA interactions and

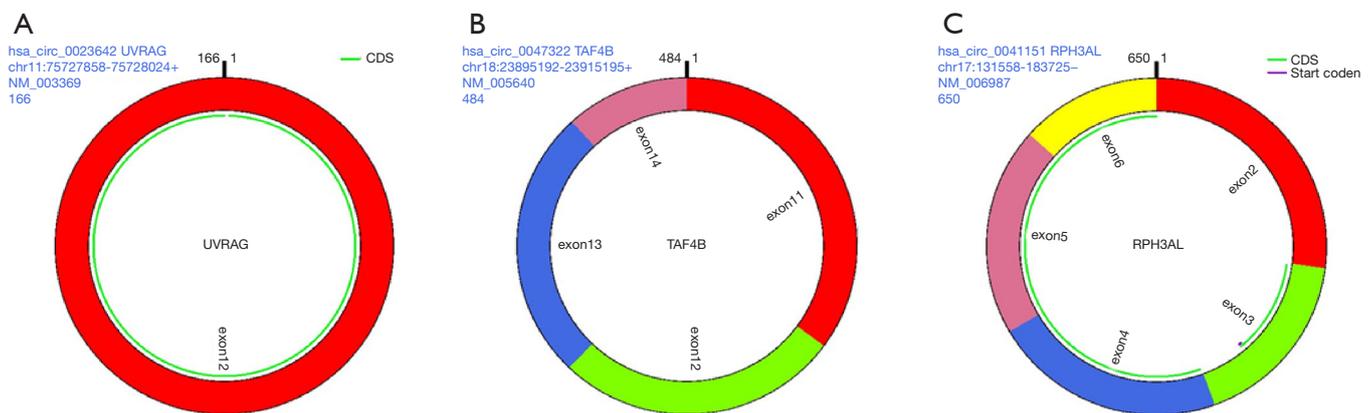
miRNA-mRNA interactions (Figure 7B). The construction of the network provided preliminary insight into the links between the differential circRNAs (has\_circ\_0023642, has\_circ\_0047322, has\_circ\_0041151), the four miRNAs (miR-616, miR-515-5p, miR-647, miR-1178) and the 181 mRNAs.



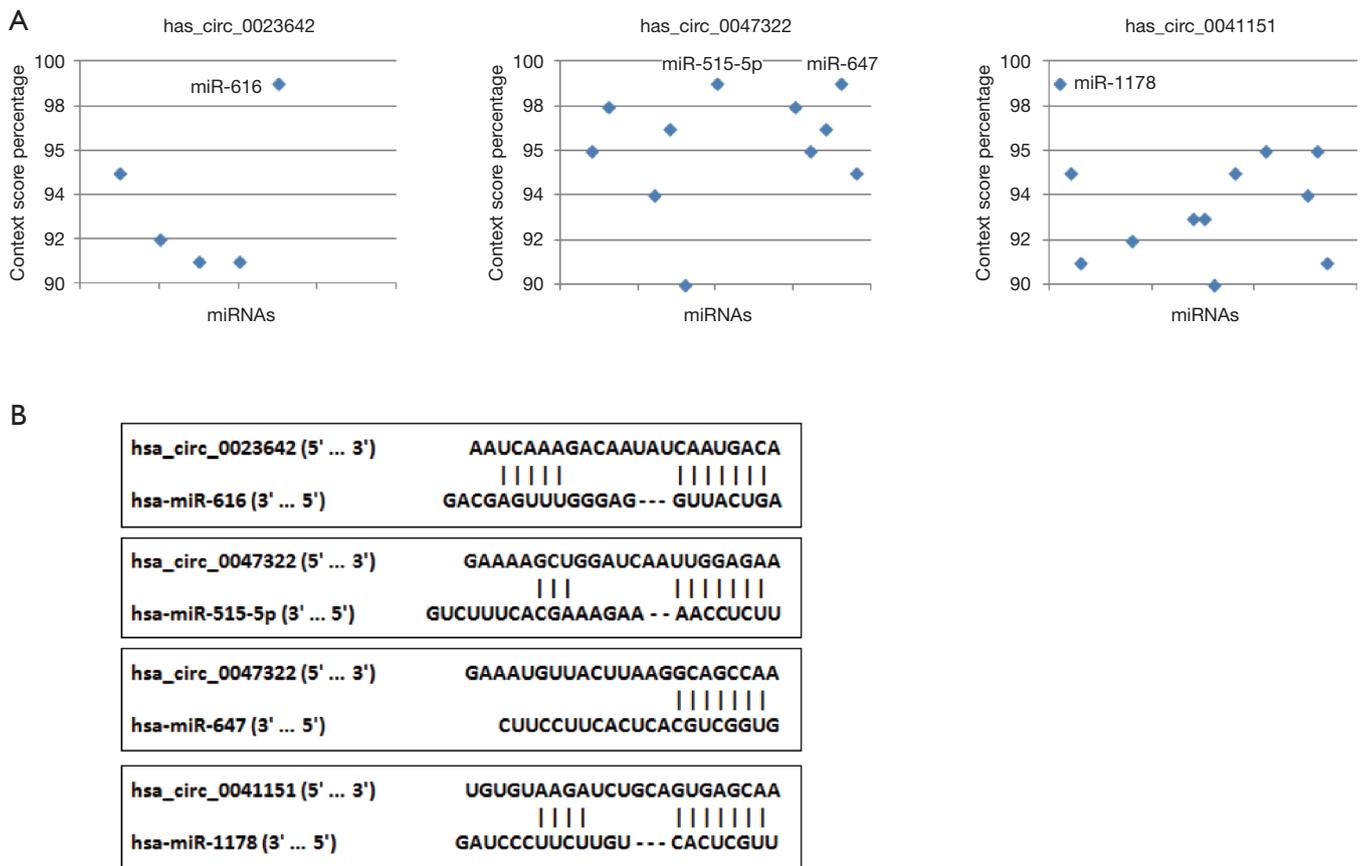
**Figure 2** Volcano plot of differentially expressed genes between BCa and normal bladder tissues in the TCGA database. BCa, bladder cancer.

### Construction of PPI network

After removing unconnected nodes, a PPI network consisting of 106 nodes and 160 edges were constructed to view the interactions among the 181 mRNAs (Figure 8A). To explore the key circRNA-miRNA-mRNA regulatory modules in the progression of BCa, we used the MCODE software to screen hub genes from the PPI network. The significant module containing 6 nodes and 15 edges was identified (Figure 8B). These hub genes were *CENPA*, *HIST1H2Bf*, *HIST1H2BO*, *HIST1H3H*, *HIST1H3B*, *HIST1H3F* (Figure 8B). The expression levels of these six genes in BCa were shown in Figure 9. A circRNA-miRNA-hub genes sub-network was then built to delineate the links among the differential circRNAs, miRNA and hub genes (Figure 10), including has\_circ\_0047322-hsa-miR-515-5p-HIST1H3B regulatory axis, has\_circ\_0047322-hsa-miR-515-5p-HIST1H3F regulatory axis, has\_circ\_0047322-hsa-miR-515-5p-HIST1H2BO regulatory axis, has\_circ\_0047322-hsa-miR-515-5p-HIST1H3H regulatory axis, has\_circ\_0047322-hsa-miR-515-5p-CENPA regulatory



**Figure 3** The schematic presentation of (A) hsa\_circRNA\_0023642, (B) hsa\_circRNA\_0047322; (C) hsa\_circRNA\_0041151.



**Figure 4** Potential interaction between circRNAs and miRNAs. (A) Screening of miRNAs that interact with circRNAs by using the CircInteractome database. (B) The potential binding sites between circRNAs and miRNAs.

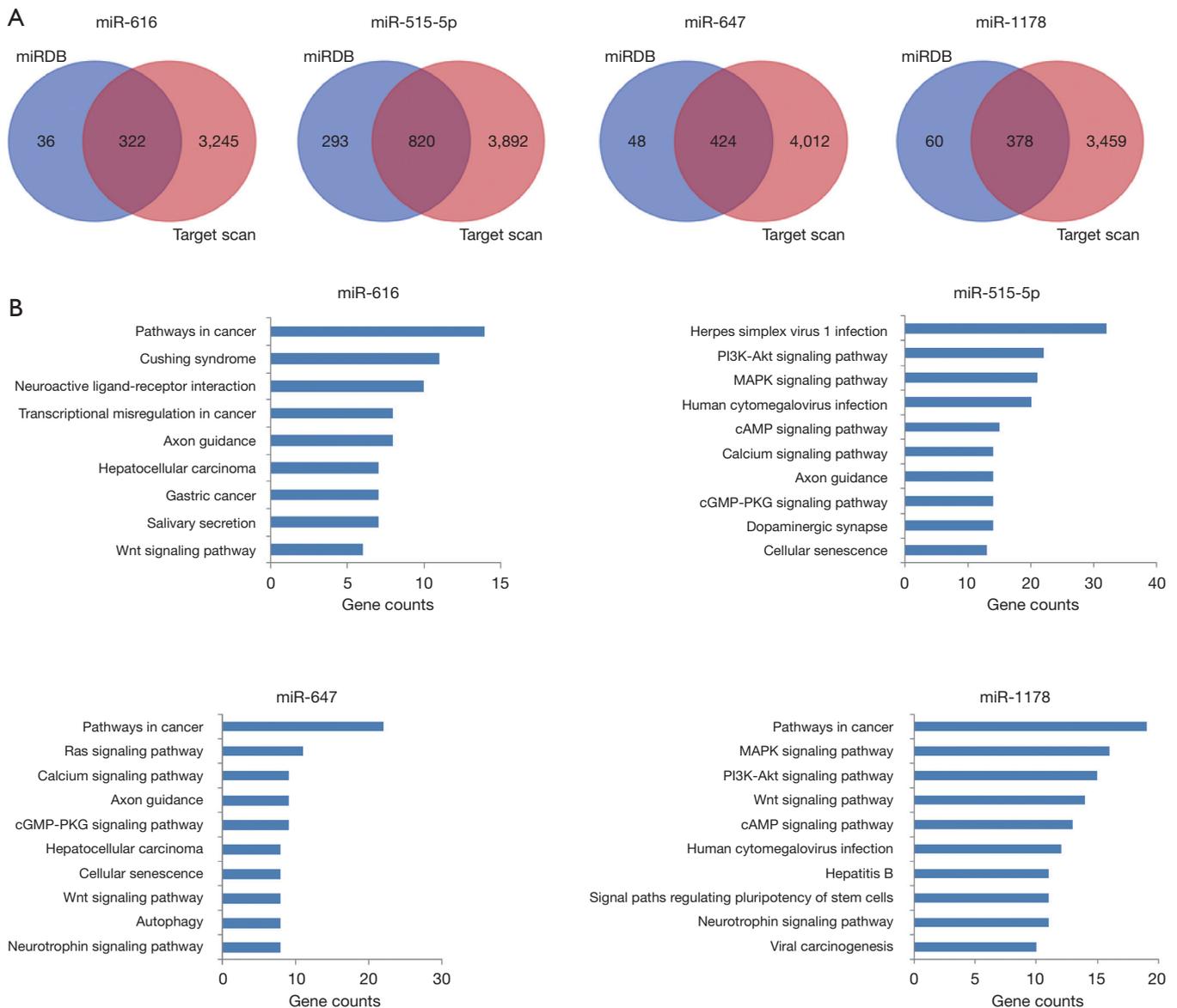
axis, has\_circ\_0023642-hsa-miR-616-3p-HIST1H3H regulatory axis, has\_circ\_0023642-hsa-miR-616-3p-HIST1H2BJ regulatory axis, has\_circ\_0023642-hsa-miR-616-3p-CENPA regulatory axis, has\_circ\_0041151-hsa-miR-1178-3p-CENPA regulatory axis, were found from the network.

### Discussion

Accumulating evidence suggests that circRNAs are abundant and stable in eukaryotic cells, demonstrating high conservation and disease specificity (15-17). It has been reported that circRNAs that are abnormally expressed play crucial roles in various cancers (18-22). Their resistance

to exonuclease cleavage due to their covalently closed structure has resulted in their routine use as prognostic and diagnostic biomarkers. In BCa, various circRNAs, including Cdr1as (10), circHIPK3 (11), circRIP2 (12), circFAM114A2 (13), circPICALM (14), circZKSCAN1 (23), circACVR2A (24), circDOCK1 (25), have been reported to exert crucial roles in regulating the disease progression. CircRNAs have also been used to aid in the clinical diagnosis of BCa and subsequent courses of therapy. However, many circRNAs remain unidentified.

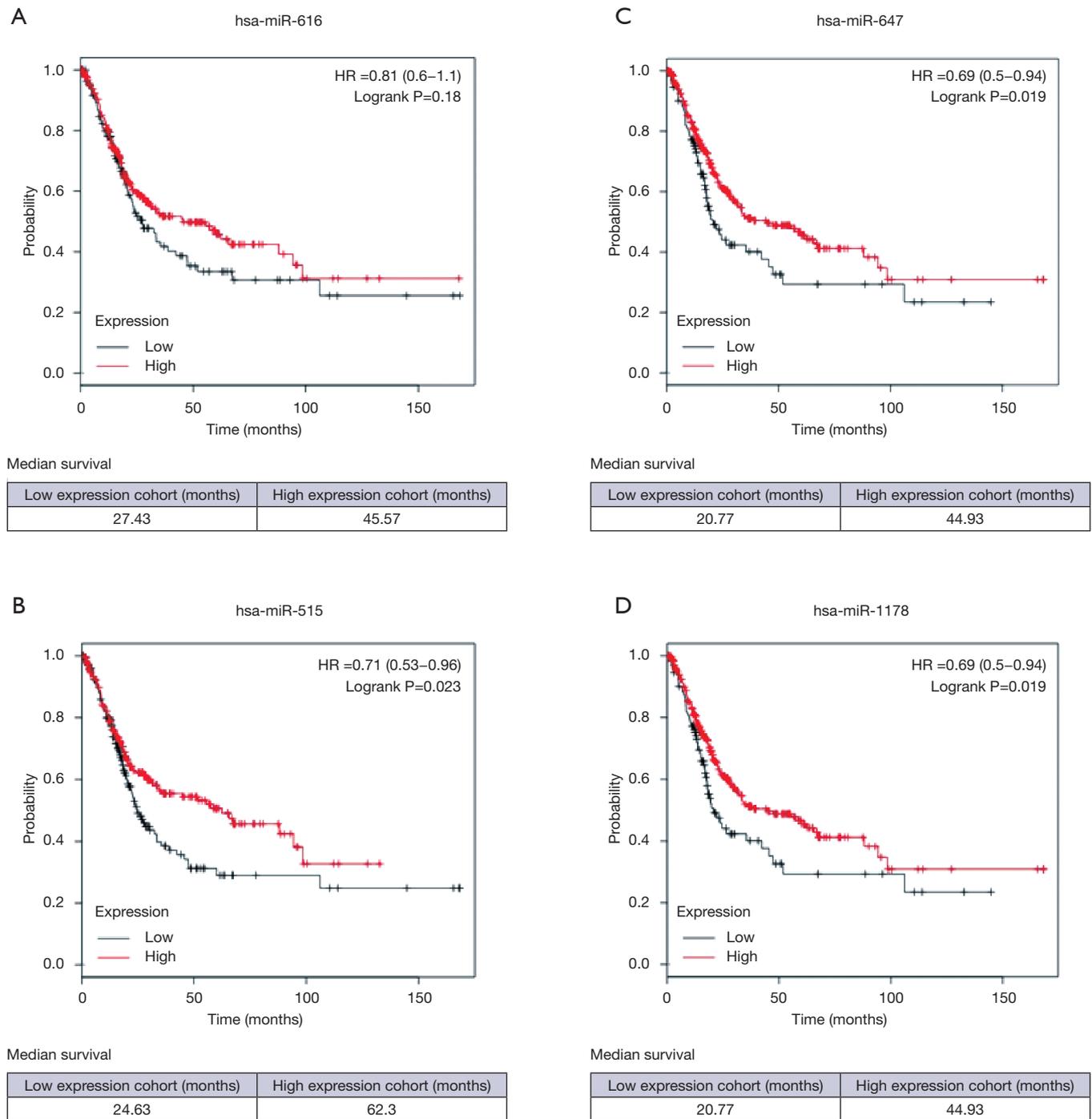
In this study, microarray data were collected from the GEO database. Three up-regulated circRNAs (has\_circ\_0023642, has\_circ\_0047322, has\_circ\_0041151) were subsequently screened in BCa for further analysis. To



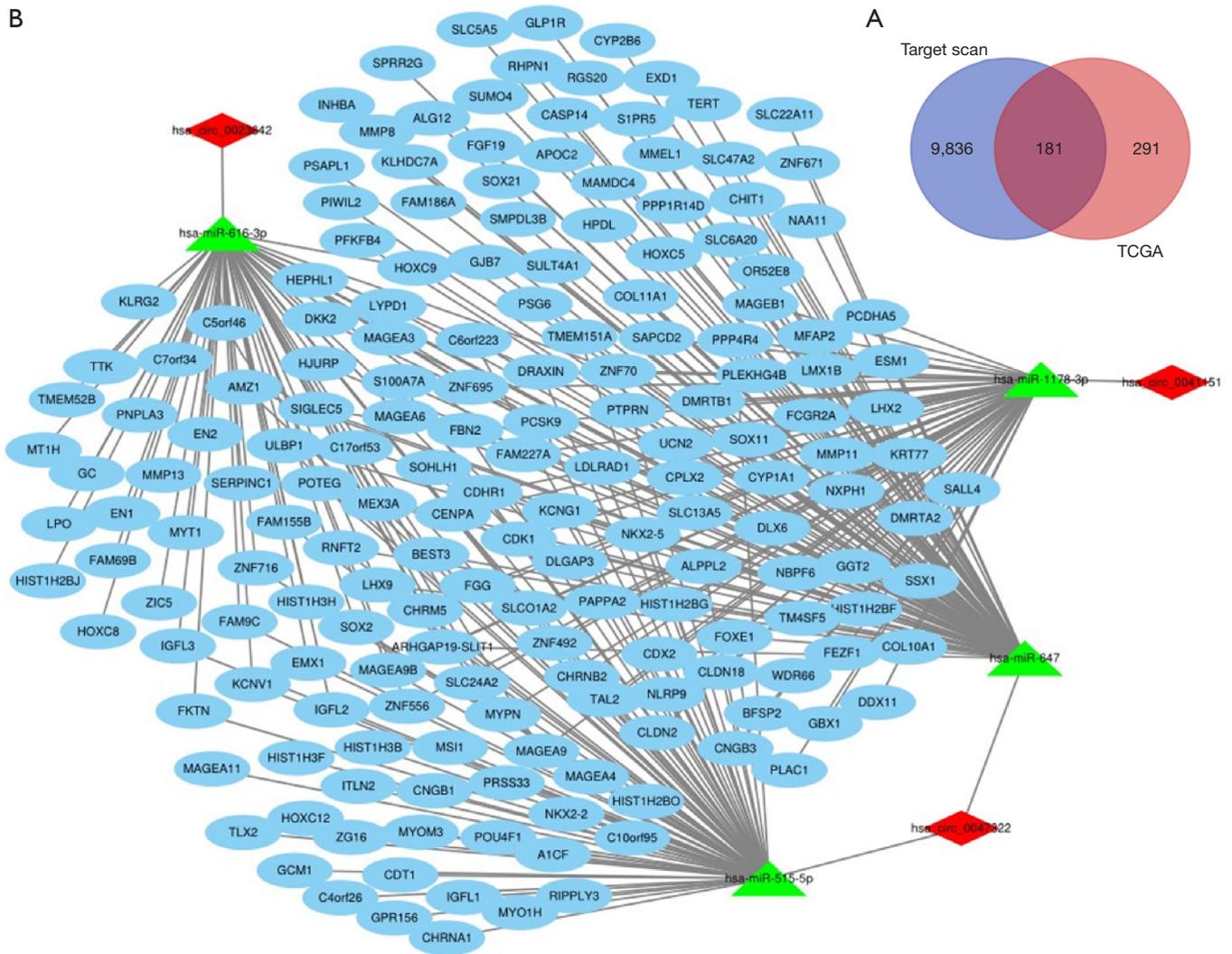
**Figure 5** The potential signaling pathways of miRNAs. (A) Venn diagram of overlapping targeted mRNAs from TargetScan and miRDB. (B) Signaling pathway analysis of predicted miRNAs.

ascertain whether those above three circRNAs function as miRNA sponges in BCa, the CircInteractome online tool was utilized to predict miRNAs with binding potential to circRNAs. Some circRNAs harbor abundant miRNA binding sites, thereby acting as “decoys” or “sponges” to regulate gene expression. Four miRNAs (miR-616, miR-

515-5p, miR-647, miR-1178) were determined to have high binding potentials with these three circRNAs. We then analyzed the effect of the miRNAs on the survival of BCa patients via the online Kaplan-Meier Plotter database. According to the results, three miRNAs, including miR-515-5p, miR-647, and miR-1178, showed a



**Figure 6** The survival analysis of the predicted miRNAs with the Kaplan-Meier Plotter database.

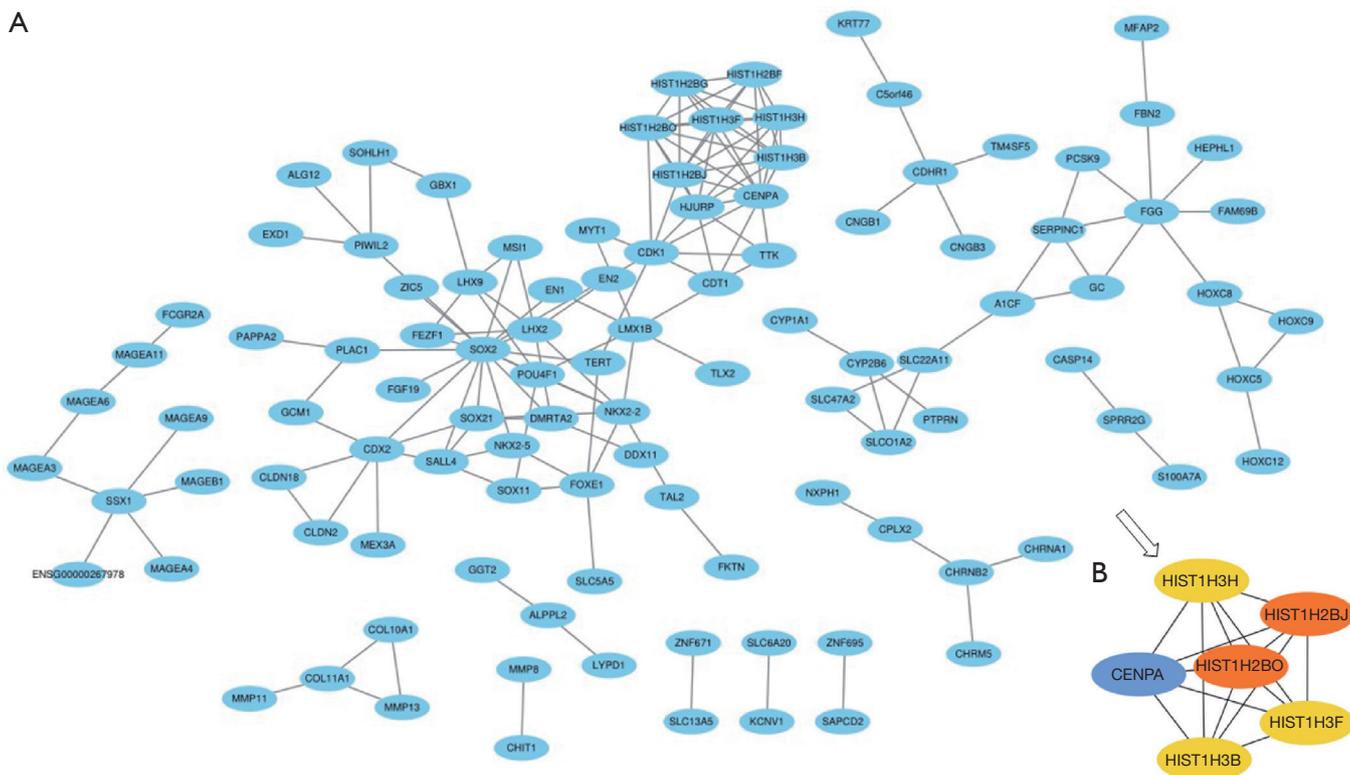


**Figure 7** Construction of ceRNA network in bladder cancer. (A) Venn diagram of 181 overlapping, differentially expressed genes, identified from TCGA and Target gene prediction software (TargetScan). (B) The ceRNA regulatory network of circRNA-miRNA-mRNA in bladder cancer. Diamonds indicate circRNAs, triangles represent miRNAs, and ovals represent mRNAs.

positive association with the better survival of BCa, which suggested their potential tumor-suppressive functions in BCa.

Following the collection of the 181 overlapping genes between the up-regulated genes from TCGA and the target genes of the four miRNAs in BCa, we constructed

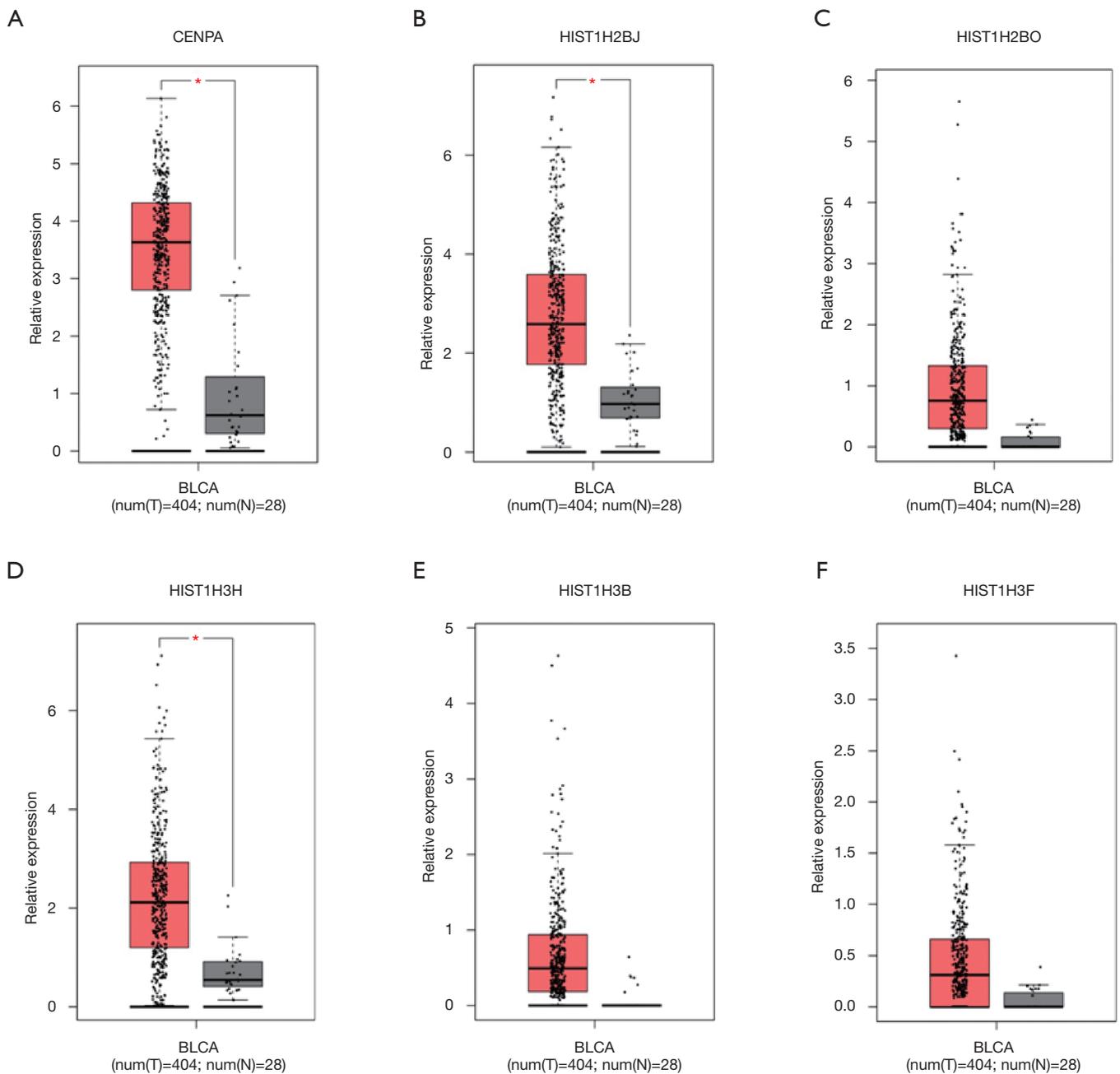
a circRNA-miRNA-mRNA regulatory network. We found that has\_circ\_0023642, has\_circ\_0047322, has\_circ\_0041151 may act as miRNA sponges to capture miR-616, miR-515-5p, miR-647, miR-1178, and subsequently regulate the mRNA expression of the 181 overlapping genes. Our results provide clinical evidence of the



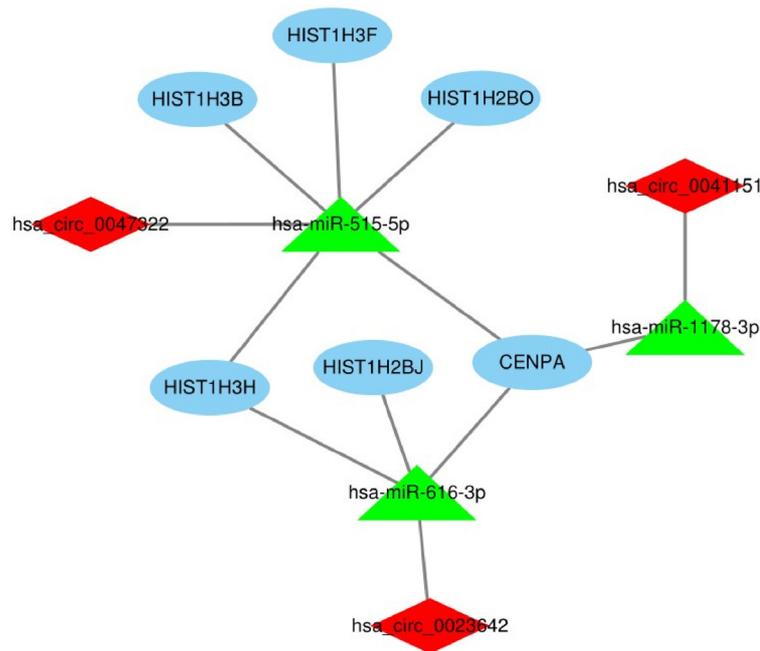
**Figure 8** PPI network construction and hub gene selection. (A) PPI network was constructed by using the string database and visualizing it with Cytoscape software. (B) Top six hub genes selected from the PPI network by the CytoHubba plugin in Cytoscape. PPI, protein-protein interaction.

ceRNA regulatory mechanism of *has\_circ\_0023642*, *has\_circ\_0047322*, and *has\_circ\_0041151* in BCa. To further demonstrate the action of the ceRNA network, we subsequently constructed a PPI network, screening six hub genes, including *CENPA*, *HIST1H2B7*, *HIST1H2BO*, *HIST1H3H*, *HIST1H3B*, *HIST1H3F*. The important roles of these six genes in cancer have also been demonstrated previously (26-30). In our study, we identified nine circRNA-miRNA-mRNA axes (*has\_circ\_0047322*-*hsa-miR-515-5p*-*HIST1H3B* regulatory axis, *has\_circ\_0047322*-*hsa-miR-515-5p*-*HIST1H3F* regulatory axis, *has\_circ\_0047322*-*hsa-miR-515-5p*-*HIST1H2BO* regulatory

axis, *has\_circ\_0047322*-*hsa-miR-515-5p*-*HIST1H3H* regulatory axis, *has\_circ\_0047322*-*hsa-miR-515-5p*-*CENPA* regulatory axis, *has\_circ\_0023642*-*hsa-miR-616-3p*-*HIST1H3H* regulatory axis, *has\_circ\_0023642*-*hsa-miR-616-3p*-*HIST1H2BJ* regulatory axis, *has\_circ\_0023642*-*hsa-miR-616-3p*-*CENPA* regulatory axis, *has\_circ\_0041151*-*hsa-miR-1178-3p*-*CENPA* regulatory axis), indicating competitive regulatory relationships of *has\_circ\_0023642*, *has\_circ\_0047322* and *has\_circ\_0041151* with the six hub genes in BCa. However, further in-depth studies are needed to verify the possible roles of these nine axes in BCa.



**Figure 9** Relative expression of six hub genes in BCa and normal bladder tissues in the GEPIA database. (A) *CENPA*; (B) *HIST1H2BJ*; (C) *HIST1H2BO*; (D) *HIST1H3H*; (E) *HIST1H3B*; (F) *HIST1H3F*.



**Figure 10** CircRNA-miRNA-hub genes network. The network consists of three circRNAs, three miRNAs, and six hub genes. Diamonds indicate circRNAs, triangles represent miRNAs, and ovals represent mRNAs.

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## Footnote

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*Conflicts of Interest:* All authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/tau-20-660>). The authors have no conflicts of interest to declare.

*Ethical Statement:* The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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