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ORIGINAL RESEARCH

Microarray Expression Profiles and Bioinformatics Analyses Reveal Aberrant Circular RNAs Expression in Bladder Cancer

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Background: Increasing evidence shows that circular RNAs (circRNAs) are involved in many biological processes, functioning as microRNA (miRNA) sponges. The aim of this study is to identify differentially expressed circRNAs in bladder cancer (BCa).

Methods: The transcriptome of circRNAs in BCa was assayed by microarray. Quantitative real-time PCR was performed to verify the results. Then, potential miRNA response elements (MREs) between circRNAs and miRNAs were predicted. Pathway and ontology enrichment analyses were performed to identify mechanisms related to the gene regulation of differentially expressed circRNAs.

Results: Three hundred and eighty-six up-regulated and 394 down-regulated circRNAs were identified, and their potential MREs were predicted in BCa.

Conclusion: The differentially expressed circRNAs indicate that circRNAs could play important roles in the molecular pathogenesis of BCa.

Keywords: bladder cancer, microarray, noncoding RNAs, circular RNAs, bioinformatics analysis

Introduction

Bladder cancer (BCa) is one of the most prevalent malignant tumors worldwide. The morbidity and mortality of BCa ranks eleventh and fourteenth, respectively, of all tumor types.[1] It is estimated that there were 81,400 new BCa cases and 17,980 deaths in the United States in 2020.[2] Surgery, radiation therapy and chemotherapy only provide limited treatment for advanced BCa,[3,4] which has a high recurrence rate.[5] This high recurrence rate is partly due to our poor understanding of BCa pathogenesis. Therefore, it is necessary to search for new ways to improve the diagnosis and treatment of BCa.

Circular RNAs (circRNAs) are considered to represent splicing errors. Accumulating evidence has revealed that these low abundant but highly stable closed-loop structures could play vital roles in many biological processes,[6,7] such as transcriptional regulation,[8] RNA transport[9] and proteins binding.[10] Recently, many studies have revealed that circRNAs displayed vital roles in various tumor types.[11] Although many circRNAs have been found by high-throughput sequencing, the detailed mechanisms of their functions in tumor onset and progression remain unclear.[12–14]

In this study, we detected expressed circRNA profiles in BCa and para-carcinoma normal tissues by microarray. Seven hundred and eighty circRNAs

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were significantly differentially expressed. Two circRNAs with the most significant differences were verified by quantitative real-time PCR (qRT-PCR). The results indicated that hsa_circRNA_103670 was up-regulated and hsa_circRNA_000367 was down-regulated in 30 pairs of BCa samples, consistent with our microarray assay results.

Materials and Methods

Clinical Tissues

Human BCa and para-carcinoma normal tissues were obtained from the Department of Urology, Shengjing Hospital of China Medical University (Shenyang, China) for microarray analysis and qRT-PCR. None of the patients received surgery or other oncotherapy prior to this study. The study was conducted in accordance with the Declaration of Helsinki, and all the samples were collected for experimental purposes. Informed consent forms were signed by all patients before the study. Ethics approval was authorized by the Medical Research and New Technology Ethics Committee of Shengjing Hospital of China Medical University with an approval ID No. 2016 PS449K.

RNA Extraction and Quality Control

Total RNA was isolated using Trizol reagent (Invitrogen, Grand Island, NY, USA) according to the manufacturer's instructions. The concentrations of the RNA samples were determined by OD260 using a NanoDrop ND-1000 (Thermo NanoDrop, Wilmington, DE, USA) instrument. The integrity of the RNA was assessed by electrophoresis on a denaturing agarose gel.

RNA Labeling and Hybridization

Total RNA from each sample was treated with RNase R (EpiCentre Inc., Madison, WI, USA) to enrich for circRNAs. The enriched circRNAs were then amplified and transcribed into fluorescent circRNAs utilizing random primers according to the Arraystar Super RNA Labeling protocol (Arraystar_Inc, Rockville, MD, USA). The labeled circRNAs were purified by the RNeasy Mini Kit (Qiagen, Dusseldorf, Germany). The concentration and specific activity of the labeled circRNAs (pmol Cy3/μg complementary RNA) were measured by NanoDrop ND-1000. Each labeled complementary RNA (1 μg) was fragmented by adding 5 μL 10 × Blocking Agent and 1 μL of 25 × Fragmentation Buffer, heated at 60°C for 30 min. Finally, 25 μL 2 × hybridization buffer was added to dilute the labeled circRNAs. Fifty μL of hybridization solution was dispensed into the gasket slide and assembled onto the circRNA expression microarray slide. The slides were incubated for 17 hours at 65°C in an Agilent Hybridization oven. The hybridized arrays were washed, fixed and scanned using the Agilent Scanner G2505C.

Microarray Data Analysis

Scanned images were imported into Agilent Feature Extraction software for raw data extraction. Quantile normalization of raw data and subsequent data processing were performed using the R software package. When comparing two groups of profile differences (such as disease versus control), the "fold change" (ie the ratio of the group averages) between the two groups for each circRNA was computed. The statistical significance of the difference was estimated by *t*-test. circRNAs having fold changes ≥2 and *P*-values <0.05 were selected as significantly differentially expressed. A box plot was used to compare the distributions of the intensities from all the samples. A scatter plot was created to assess the circRNA expression variation between the two groups. Volcano plots and hierarchical clustering were used to show the differentially expressed circRNAs.

Quantitative Real-Time PCR Analysis

Hsa_circRNA_103670 and hsa_circRNA_000367 were analyzed with specific primers (for hsa_circRNA_103670: left primer CGGCTCTTCCAGCTACAAAC and right primer CATTGGCTACCTCCTCTGCT. For hsa_circRNA_000367: left primer ATCGAAGACTGGCGTGAAAC and right primer ATTGCACAGTGGATGGATCA). qRT-PCR was performed using the Bio-Rad CFX96 system with SYBR green to determine the expression level of each circRNA. The PCR reaction was conducted in a 15 μL volume, including 7.5 μL SYBR Mix, 6 μL cDNA, 0.75 μL forward primer (10 μM), and 0.75 μL reverse primer (10 μM). The qRT-PCR protocol was as follows: 50°C for 2 min, 95°C for 8 min 30 sec, followed by 45 cycles at 95°C for 15 sec, and 60°C for 1 min. Extension was performed at 95°C for 1 min, 55°C for 1 min and 55°C for 10 sec. GAPDH was used as a normalization control.

Statistics

Statistical analyses were performed by SPSS 17.0 (SPSS_Inc, Chicago, IL, USA). P <0.05 was considered to be statistically significant. Data differences between the two groups were analyzed by paired *t*-test. Data were expressed as mean ± SEM/SD from at least three independent experiments.

Results

Identification of Differentially Expressed circRNAs in BCa and Para-Carcinoma Normal Tissues

A microarray containing 13,617 human circRNA probes was conducted for three paired BCa and para-carcinoma normal tissues, following the Arraystar Human circRNA Array V2.0 protocol. Hierarchical clustering was performed to distinguish the circRNA expression profiles among the samples (Figure 1A). We compared the distributions of the expression intensity values from the six samples after normalization. The results showed that the normalized intensity values were similar across all six samples (Supplementary Fig. S1A). We then assessed the circRNA expression reproducibility between the two groups (Figure 1B). Volcano plots were constructed to exhibit the differentially expressed circRNAs between the two groups (Figure 1C).

Statistical significance between the two groups was determined by a fold-change ≥2.0 and P <0.05. The results showed that a total of 780 circRNAs had different expressions between the two groups, containing 386 up-regulated and 394 down-regulated circRNAs (Supplementary data 1). Fifty-three circRNAs showed a more than five-fold increase, among which 18 revealed a more than ten-fold up-regulation. Thirty-four circRNAs displayed a more than five-fold decrease, among which 7 exhibited a more than ten-fold down-regulation. Most of the above 780 circRNAs were derived from exons, namely 82.90% (320/386) in the up-regulated group and 81.73% (322/394) in the down-regulated group. The other sources were introns, sense overlapping, antisense sequences and intergenic regions (Figure 1D and E). The locations of the circRNAs on the chromosomes are shown in Figure 1F. The top ten up-regulated and down-regulated circRNAs are shown in Table 1.

Construction of circRNA-miRNA Interaction Network and miRNA Response Element (MRE) Analysis

Recent studies have demonstrated that circRNAs play important roles in fine-tuning miRNAs to regulate the expressions of genes by sequestering them.[15,16] The interactions between circRNAs and their potential targeted miRNAs were predicted according to seed matching sequences using Arraystar miRNA target prediction software based on TargetScan[17] and miRanda.[18] The networks of the top 10 up-regulated and down-regulated circRNAs and their correlated miRNAs were delineated by Cytoscape (Figure 2). We listed the top five possible MREs of the highest up-regulated and down-regulated circRNAs. The potential MREs of hsa_circRNA_103670 included miR-570-5p, miR-592, miR-542-3p, miR-628-5p and miR-148b-5p. For hsa_circRNA_000367, the potential MREs included miR-331-3p, miR-4646-5p, miR-4797-5p, miR-3919 and miR-3190-3p. The results are shown in Figure 3A and B, which displays the 2D structures of seed sequence binding, nucleotide composition with regard to AU-richness, and MRE position.

Analysis of Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO)

KEGG pathway analysis was conducted to investigate the impact of circRNAs in gene regulation. The results showed that 42 pathways were related to the up-regulated mRNAs (Supplementary data 2), while 41 pathways were associated with the down-regulated mRNAs (Supplementary data 3). The top ten KEGG pathways of the up-regulated and down-regulated mRNAs are illustrated in Figure 4A and B. Hypertrophic cardiomyopathy and the prolactin signaling pathway were the top pathways among the up-regulated and down-regulated transcripts, respectively.

Furthermore, GO analysis was also performed to parse the linear transcripts of the differentially expressed circRNAs. The results revealed that negative regulation of biological process, intracellular part, and protein binding were the top gene and gene product attributes in the biological process, cellular component, and molecular function, respectively, for the up-regulated transcripts (Figure 4C-E). For the down-regulated transcripts, the top GO terms were regulation of transcription from RNA polymerase II promoter, and nucleus and protein binding (Figure 4F-H). The above results revealed that these pathways may take part in the onset and progression of BCa.

Confirmation of Differentially Expressed circRNAs in Clinical Samples and Their Correlations with Clinicopathological Features

We chose hsa_circRNA_103670 and hsa_circRNA_000367 for further validation because they had the highest up- and

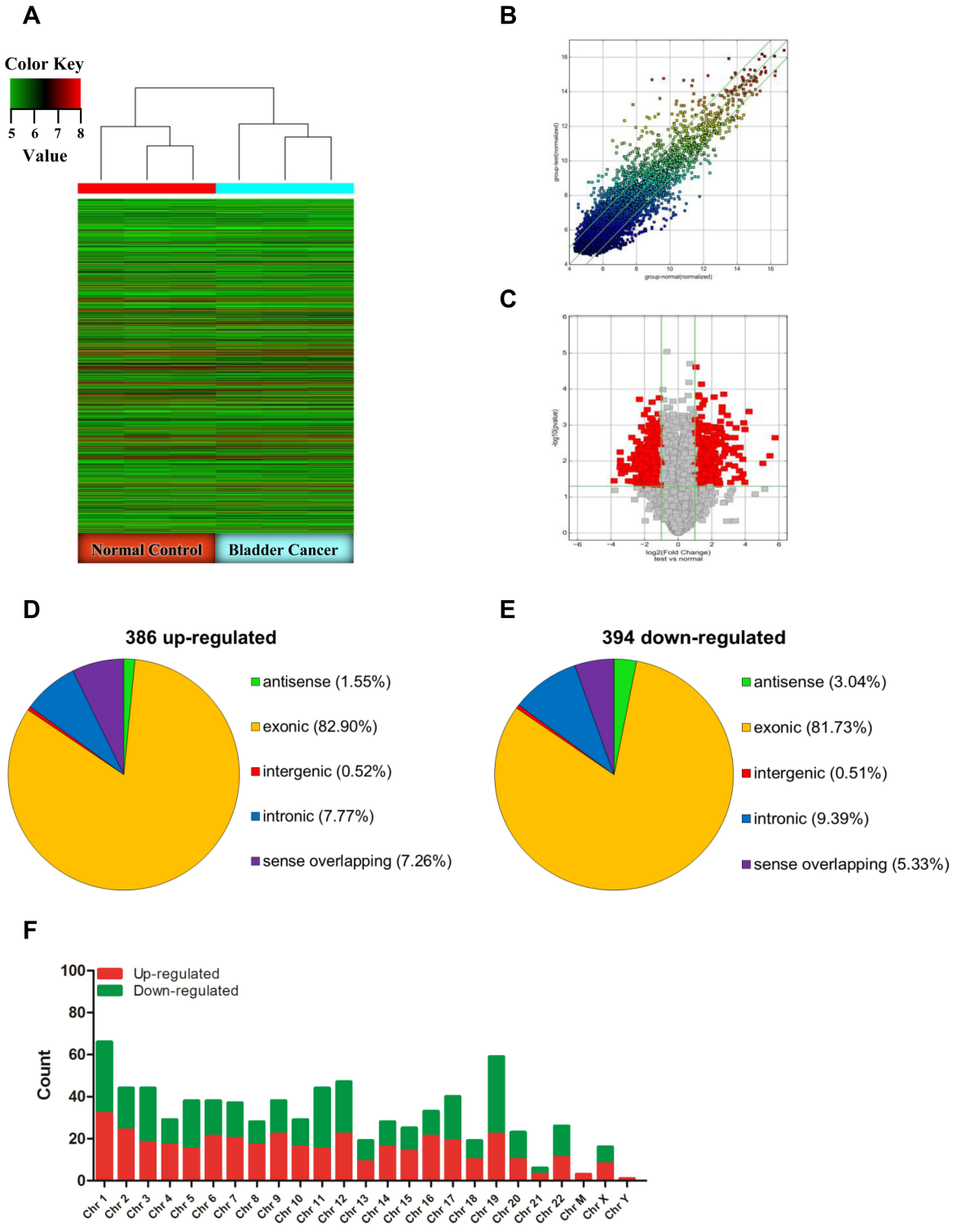

Figure 1 Differentially expressed circRNAs in BCa. (**A**) Hierarchical clustering shows distinguishable circRNA expression profiles among the samples. Each column represents a sample, including the normal control (first three columns) and BCa (last three columns). Each row represents a circRNA. Red band means relative high expression, while green one means relative low expression. (**B**) Scatter plot of circRNAs for assessing the expression reproducibility between the paired BCa and para-carcinoma normal tissues. The values plotted on X and Y axes are averaged normalized signal values of BCa and normal control (log2 scaled). Two green slashes represent 2.0 fold changes while the middle slash means no difference. CircRNAs above the upper green slash or below the lower green slash indicate a more than 2.0 fold change. (**C**) Volcano plots of circRNAs for exhibiting the differentially expressed circRNAs between the two groups. The vertical lines correspond to 2.0 fold up and down, respectively, and the horizontal line represents a *P*-value of 0.05. The red points in the plot represent the differentially expressed circRNAs with statistical significance. (**D-E**) The two pie charts display the sources of circRNAs. Most of the differentially expressed circRNAs derive from exons in up-regulated group (**D**) and down-regulated group (**E**). The other sources are intronic, sense overlapping, antisense and intergenic. (**F**) The bar diagram shows the locations of the circRNAs on the chromosomes.

Table 1 The Top 10 Up- and Down-Regulated circRNAs Ranked by Fold Changes in Microarray Data

circRNA ID	Alias	GeneSymbol	Regulation	Fold Change	circRNA_type	Chrom	P-value	FDR
hsa_circRNA_103670	hsa_circ_0006168	CNOT6L	up	54.7093218	exonic	chr4	0.0022321	0.1196595
hsa_circRNA_104327	hsa_circ_0006169	FAM126A	up	44.1459689	exonic	chr7	0.0071093	0.1378173
hsa_circRNA_104328	hsa_circ_0006170	FAM126A	up	32.977412	exonic	chr7	0.0115224	0.150833
hsa_circRNA_089761	hsa_circ_0006171	JA760602	up	18.6163907	exonic	chrM	0.0004185	0.1101169
hsa_circRNA_406483		AASDH	up	15.7366936	exonic	chr4	0.0013187	0.1101169
hsa_circRNA_101833	hsa_circ_0039908	DUS2	up	15.6961356	exonic	chr16	0.0386546	0.1965778
hsa_circRNA_404458		C1orf63	up	15.0162568	intronic	chr1	0.0008632	0.1101169
hsa_circRNA_001937	hsa_circ_0000700	CHD9	up	14.5273013	intronic	chr16	0.0226398	0.1704275
hsa_circRNA_104640	hsa_circ_0001806	CSPP1	up	14.4051901	exonic	chr8	0.008879	0.1427345
hsa_circRNA_103809	hsa_circ_0072088	ZFR	up	13.5925795	exonic	chr5	0.0291555	0.1803554
hsa_circRNA_000367	hsa_circ_0000367	SIAE	down	14.1671638	exonic	chr11	0.035155	0.1908276
hsa_circRNA_405571		TADA2A	down	11.0026207	exonic	chr17	0.0149866	0.1583509
hsa_circRNA_103224	hsa_circ_0063329	DDX17	down	11.0011827	exonic	chr22	0.0056186	0.1339006
hsa_circRNA_406587		TRIO	down	10.8661301	intronic	chr5	0.0109593	0.1496619
hsa_circRNA_405283		LINC00618	down	10.8657857	sense overlapping	chr14	0.009501	0.1457163
hsa_circRNA_102049	hsa_circ_0043278	TADA2A	down	10.4337054	exonic	chr17	0.0223921	0.1704275
hsa_circRNA_401977		NEDD4L	down	10.1938804	exonic	chr18	0.016923	0.1605343
hsa_circRNA_404686		GPR137B	down	9.2662399	exonic	chr1	0.0160127	0.1600703
hsa_circRNA_102504	hsa_circ_0050386	ANKRD27	down	9.1541462	exonic	chr19	0.0202362	0.1664709
hsa_circRNA_001226	hsa_circ_0001226	MYH9	down	8.4160761	antisense	chr22	0.0134785	0.1545473

down-regulated fold changes in the microarray assay. A qRT-PCR assay was performed in 30 paired of BCa and para-carcinoma normal tissues. The results were consistent with our microarray data and showed that the expression of hsa_circRNA_103670 was higher in the BCa group than the para-carcinoma normal tissues, while hsa_circRNA_000367 expression was lower in the BCa group than the para-carcinoma normal tissues (Figure 5A and B). The results of a clinicopathological analysis showed that the high expression of hsa_circRNA_103670 was related to the high histological grade, poor T stage. Early T stage had a positive correlation with the expression of hsa_circRNA_000367 (Table 2).

Discussion

CircRNAs were found in the 1970s as an abnormal splicing product by Sanger et al.[19] Due to technological limitations, circRNAs were not highly studied in the following years.[20–22] However, studies investigating circRNAs have become more common since two sequencing analyses were done by Jeck et al,[23] and Memczak et al.[24] With the improvement of high-throughput sequencing, circRNAs were found to exist in many tissues abundantly and stably.[25] More and more studies have demonstrated that circRNAs have tissue or cell specificity, are relatively conserved, and have dynamic expression, which reflects their possible functions in eukaryotes.[26–28] Many studies have shown that circRNAs function as miRNA sponges in various tumors.[29,30] Our study shows evidence that some circRNAs are differentially expressed in BCa compared with para-carcinoma normal tissues.

Microarray assays have been previously used to detect circRNAs, lncRNAs, miRNAs, or mRNAs in various tumors,[31–33] including in BCa samples.[34–37] Li et al identified 16,535 circRNAs and found that 524 circRNAs were significantly down-regulated, while 47 circRNAs were up-regulated in BCa compared to para-carcinoma normal tissues.[35] Yang et al also performed next-generation sequencing in 5 BCa patients and revealed that 42 circRNAs were down-regulated and 14 circRNAs were up-regulated in BCa compared to non-cancerous tissues.[36] The results of Zhong et al showed that 469 circRNAs were significantly altered based on microarray data, including 184 down-regulated circRNAs and 285 up-regulated circRNAs.[37] In our study, we determined the profiles of circRNAs in three paired BCa and para-carcinoma normal tissues by microarray sequencing. The results showed that 386 circRNAs were significantly up-regulated and 394 circRNAs were down-regulated between the two groups. Moreover, 18 up-regulated and 7 down-regulated circRNAs exhibited a more than 10-fold change, which implied the possibility of these circRNAs functioning as oncogenes or tumor suppressors in BCa. Comparing the top 20 up- and down-regulated circRNAs of Zhong et al's microarray data, we find that only

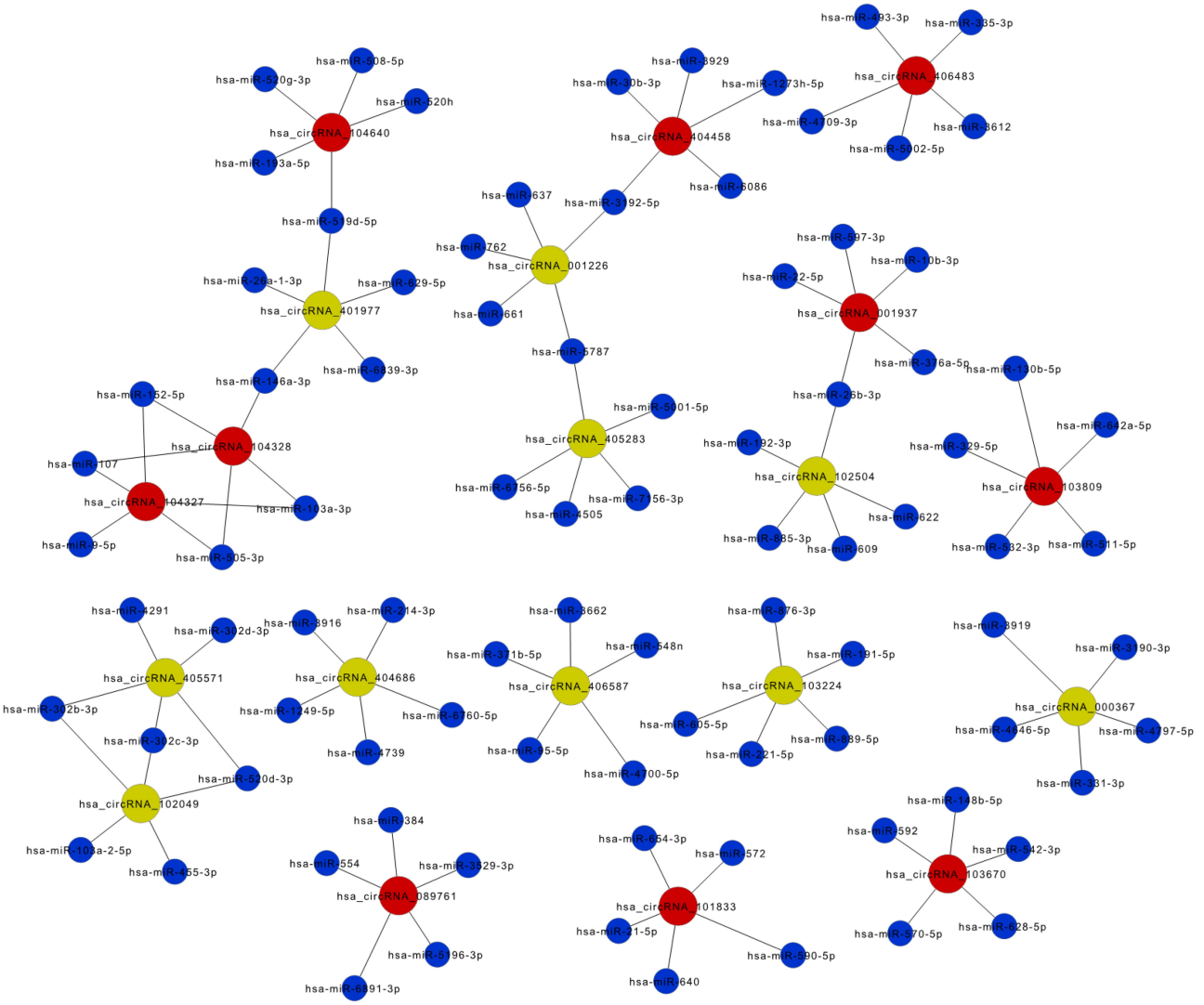

Figure 2 The circRNA-miRNA network analysis, which comprises the top 10 up- and down-regulated circRNAs and their targeted miRNAs.

hsa_circRNA_0072088, hsa_circRNA_0000520, hsa_circRNA_0003528, hsa_circRNA_0028173, hsa_circRNA_0082582, and hsa_circRNA_0005265 are consistent with our microarray results. The two results do not fit very well due to some reasons. One possible reason may be that the samples of each microarray are limited and insufficient to assess the differential expression of circRNAs, for which the heterogeneity among the patients is usually noticeable. Another reason may be that the clinical samples are derived from different regions and population ancestries. Research in large samples and different regions and population are needed to reduce the bias.

Consistent with our microarray data, the results of the qRT-PCR assay confirmed that hsa_circRNA_103670 and hsa_circRNA_000367 were up-regulated and down-regulated, respectively, in 30 paired BCa and para-carcinoma normal tissues. Hsa_circRNA_103670 was spliced from the CCR4-NOT transcription complex subunit 6 like (CNOT6L or CCR4b) gene. Zhou et al found that miR-146a-Cnot6L affected the stability of Zeb1 mRNA and induced epithelial-mesenchymal transformation.[38] Hsa_circRNA_000367 was generated from the sialic acid acetylesterase (SIAE) gene and had been shown to participate in immune system disease.[39,40] Due to the inseparable relationships between tumor formation and the immune system, we believe that SIAE may be correlated with tumorigenesis.

Patients with high histological grade and poor T stage had a high expression of hsa_circRNA_103670. Conversely, patients with poor T stage had a low expression of hsa_circRNA_000367. These data showed that circRNAs might play vital roles in carcinogenesis and the progression of BCa.

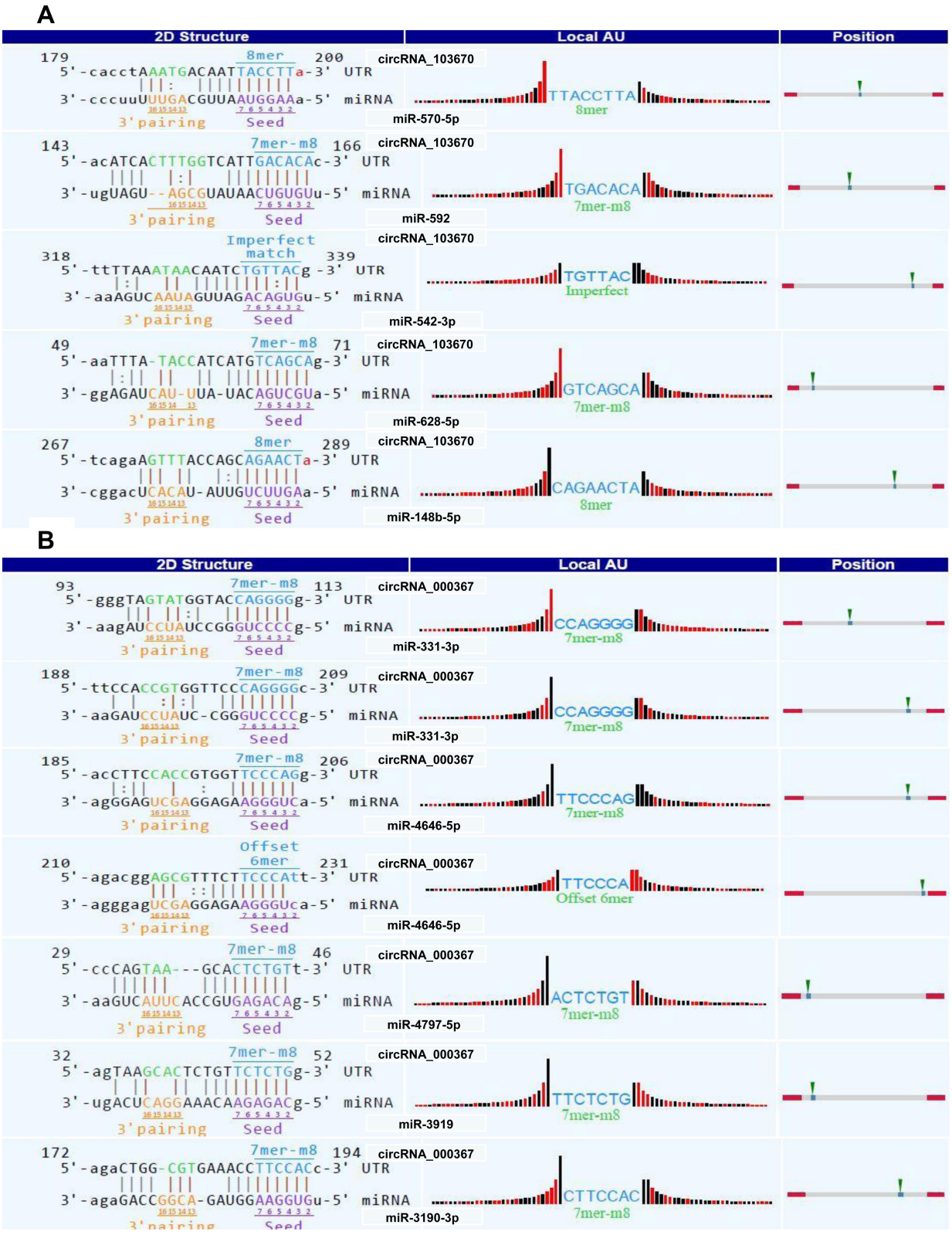

Figure 3 The detailed annotation for circRNA-miRNA interactions. (**A-B**) The top five MREs of hsa_circRNA_103670 (**A**) and hsa_circRNA_000367 (**B**) are predicted.

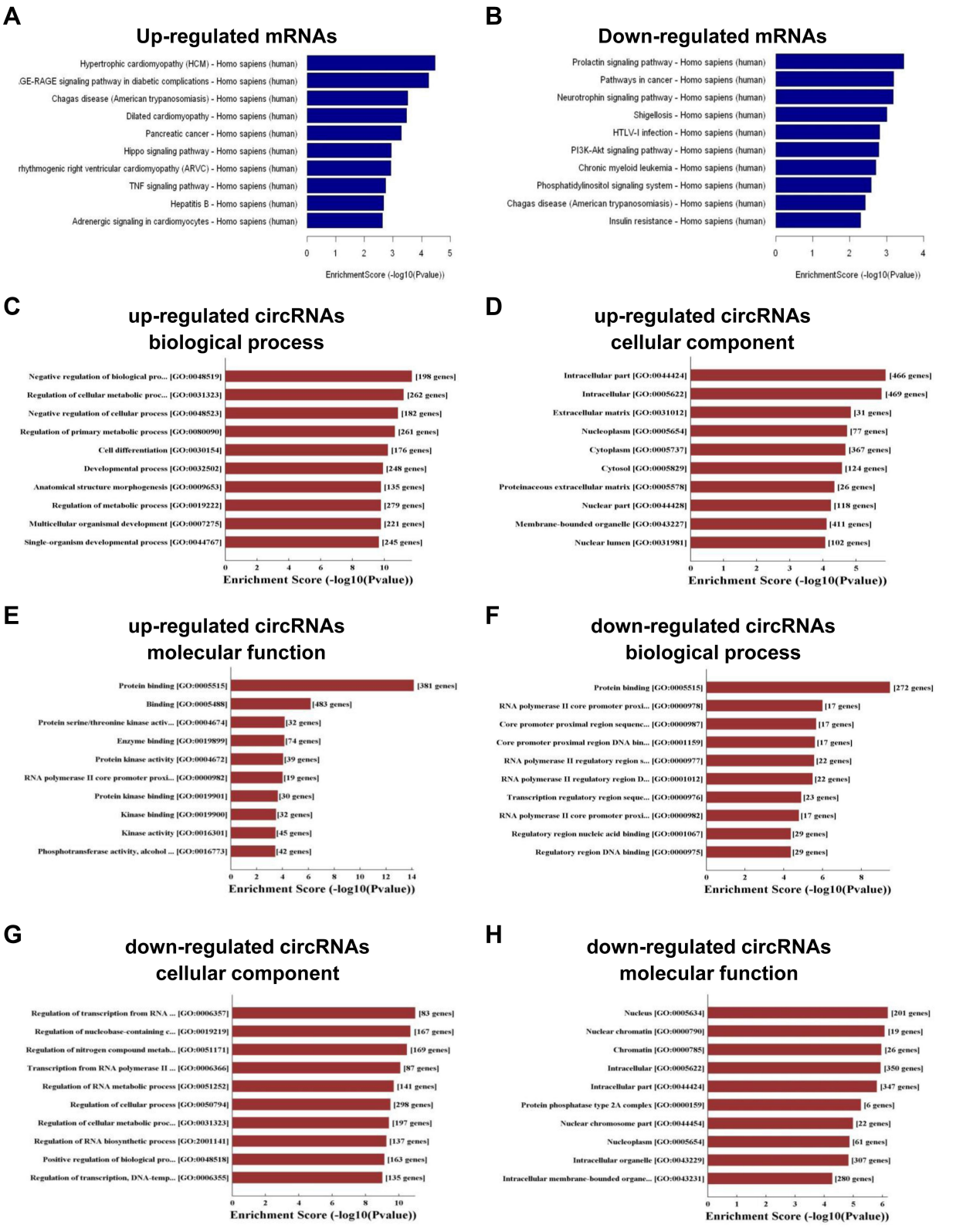

Figure 4 KEGG and GO analyses of the genes that produce up-regulated and down-regulated circRNAs. (**A-B**) KEGG pathway enrichment analyses of up-regulated mRNAs (**A**) and down-regulated mRNAs (**B**). (**C-H**) GO enrichment analyses of up-regulated circRNAs in biological process (**C**), cellular component (**D**) and molecular function (**E**) and down-regulated circRNAs in biological process (**F**), cellular component (**G**) and molecular function (**H**).

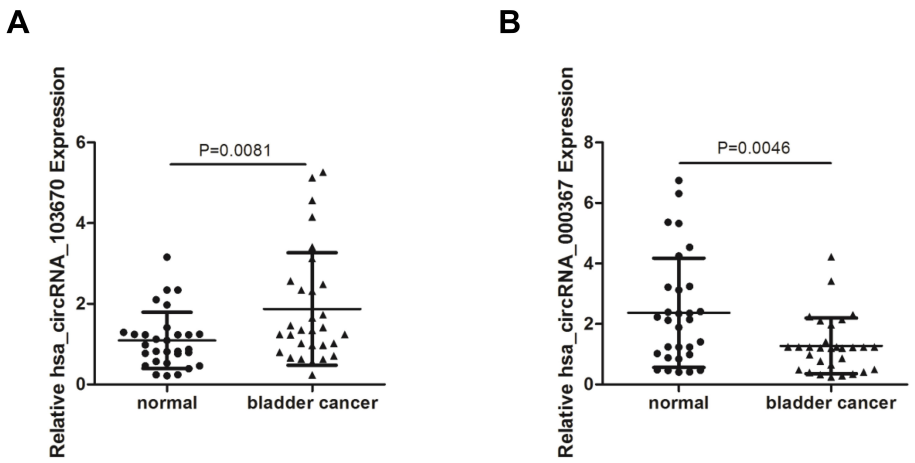

Figure 5 The verification of two differentially expressed circRNAs by qRT-PCR in 30 pairs BCa samples. (**A** and **B**) Hsa_circRNA_103670 (**A**) and hsa_circRNA_000367 (**B**) expression in 30 paired BCa samples. Each sample is run in triplicate and in multiple experiments for mean ± SEM.

CircRNAs are known to act as miRNA sponges to regulate protein expression by sequestering miRNAs. Zhong et al found that the circRNA MYLK could promote BCa progression as a competing endogenous RNA through modulating VEGFA/VEGF2 signals.[37] Yang et al showed that the circRNA ITCH could inhibit BCa progression through sponging miR-17/miR-224 and regulating p21, and PTEN expression.[41] Our study predicted circRNA-miRNA interactions, which could provide information for further research. We also constructed a network of the top 10 up- and down-regulated circRNAs and their correlated miRNAs.

Furthermore, we detected the top five possible MREs of the highest up- and down-regulated circRNAs in BCa. The potential MREs of hsa_circRNA_103670 included miR-570-5p, miR-592, miR-542-3p, miR-628-5p, and

Table 2 Relationships Between hsa_circRNA_103670, hsa_circRNA_000367 Expression and Clinicopathologic Features of BCa

Parameter	Case	hsa_circRNA_103670 Expression	*p* value	hsa_circRNA_000367 Expression	*p* value
Gender			0.0895		0.2810
Female	12	1.344±0.925		1.504±1.204	
Male	18	2.227±1.560		1.128±0.670	
Age(years)			0.7163		0.0777
≤55	20	1.807±1.318		1.488±1.043	
>55	10	2.008±1.603		0.860±0.378	
Histological grade			0.0432		0.523
Low	12	1.250±1.184		1.413±1.273	
High	18	2.290±1.398		1.189±0.615	
T stage			0.0159		0.0123
T_{a-1}	16	1.314±0.697		1.661±1.034	
T_{2-4}	14	2.514±1.718		0.841±0.522	
Lymph node metastasis			0.0526		0.1062
No	24	1.629±1.141		1.415±0.966	
Yes	6	2.854±1.962		0.734±0.427	
Distant metastasis			0.0871		0.2466
No	25	1.679±1.269		1.367±0.975	
Yes	5	2.848±1.738		0.836±0.390	

miR-148b-5p. For hsa_circRNA_000367, the potential MREs included miR-331-3p, miR-4646-5p, miR-4797-5p, miR-3919, and miR-3190-3p. The results exhibited the 2D structure of seed sequence binding, nucleotide composition with regard to AU-richness, and MRE position.

Although we identified hundreds of differentially expressed circRNAs by microarray assay and predicted the potential interactions between circRNAs and their target miRNAs or mRNAs, additional in vitro cellular functional assays are needed to verify the effects of these circRNAs.

In summary, we showed that circRNAs may be dysregulated in BCa compared with para-carcinoma normal tissues. Confirmed by 30 paired clinical samples, the expression of hsa_circRNA_103670 was up-regulated and hsa_circRNA_000367 was down-regulated in BCa. Our study provides new data on circRNAs in the tumorigenesis of BCa.

Abbreviations

circRNAs, circular RNAs; BCa, bladder cancer; MREs, miRNA response elements; KEGG, Kyoto Encyclopedia of Genes and Genomes; GO, Gene Ontology; SIAE, sialic acid acetylesterase.

Ethical Conduct of Research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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Disclosure

The authors declare that they have no competing interests.

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