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Analysis of ceRNA network identifies prognostic circRNA biomarkers in bladder cancer

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Bladder cancer remains a very challenging disease to treat with the high rates of recurrence and progression associated with current therapies. Although the association between bladder cancer pathology and circRNAs remains undetermined, circRNAs signatures may be useful as prognostic and predictive factors and clinical tools for assessing disease state and outcome. This study investigates if these circRNAs can be used as biomarkers for bladder cancer diagnosis.

Differentially expressed RNAs in bladder cancer and normal bladder tissues were screened from GEO databases (GSE37815, GSE39093, GSE97239, and GSE92675) using bioinformatics method. The related volcanic maps and the interaction network maps of differentially expressed RNAs were drawn, and the mRNA-miRNA and miRNA-circRNA interactions were predicted to establish mRNA-miRNA-circRNA competitive endogenous RNA (ceRNA) network. The differential circRNAs related to prognosis of bladder cancer patients were screened based on the influence of miRNA interacting with the circRNA above on survival rate. The expression of miRNA (hsa-mir-214), circRNA (hsa_circ_0076704, hsa_circ_0081963, hsa_circ_0001361) in bladder cancer tissues, adjacent tissues, bladder cancer cells and normal bladder epithelial cells were validated by qRT-PCR. Kaplan Meier curve analysis confirmed the relationship between circRNA (hsa_circ_0076704) and overall survival and prognosis of bladder cancer patients.

Through database screening and analysis, we found 19231 differentially expressed genes, 847 differentially expressed miRNAs, 7282 differentially expressed circRNAs. The establishment of ceRNA network consisted of 28 DERNAs (differentially- expressed RNAs), 12 Demi-RNAs and 12 DEcircRNAs. Further prognostic analysis showed that circRNA interacted miRNA hsa-miR-106b, hsa-miR-145 and hsa-miR-214 were associated with overall survival in patients with bladder cancer ($p<0.05$). Among them, hsa_circ_0076704, hsa_circ_0081963 and hsa_circ_0001361 are potential circRNAs related to the overall survival (OS) in bladder cancer and expressed in bladder cancer. The expression of hsa-mir-214 was contrary. Further Kaplan Meier survival analysis showed that hsa_circ_0076704 had significant prognostic value ($p<0.05$). In conclusion, hsa_circ_0076704 is independent prognostic factor for bladder cancer.

Key words: ceRNA, bladder cancer, prognosis, hsa_circ_0076704, miR-214

Bladder cancer, one of the most common malignant tumors in the urinary system, has gradually increased incidence in recent years. The incidence of bladder cancer ranks it as ninth among the published malignant tumors [1], which poses a serious threat to human health. Bladder cancer has biological characteristics such as high malignancy and high recurrence rate, especially invasive bladder cancer, which has strong invasive ability and poor prognosis with 5-year survival rate less than 50% [2]. Therefore, it is particularly urgent to further understand the mechanism of the occurrence, development and recurrence of bladder cancer, and to find reliable biomarkers and effective therapeutic targets to enhance survival rate of patients with bladder cancer. In molecular biology, the research on the role of oncogenes and anti-oncogenes cannot well enough explain and solve the current clinical problems. With the deepening of research and the development of technology, the regulatory network of non-coding RNA has gradually attracted people's attention and become the current research hotspot and breakthrough point, providing hope for finding biomarkers and therapeutic targets for bladder cancer diagnosis and treatment.

MicroRNA (miRNA) is a kind of endogenous non-coding single-stranded RNA, which molecule is approximately 22 nt in length. A single miRNA can inhibit target genes by complementing RNA sequence of miRNA response element (MREs) [3, 4]. Circular RNA (circRNA) is another kind of

endogenous non-coding RNA known in literature after small RNA and long non-coding RNA (lncRNA). It is a covalently closed ring structure of non-coding RNA, which widely exists in various biological cells. It is produced by reverse splicing of precursor RNA, which regulates gene expression [5]. In 2011, Salmena et al.[6] proposed a competitive endogenous RNA (ceRNA) hypothesis. CeRNA is not a new RNA, but a new regulatory mechanism. It considers that lncRNA, RNAs and other RNAs act as "sponges" of natural RNA and inhibit the regulation of miRNA through common MREs. Therefore, a large and delicate regulatory network has been established among the various RNA molecules with ceRNA function, and the various RNA molecules regulate each other and are in a dynamic equilibrium. When the balance is broken, abnormal phenomena such as disease or tumorigenesis will occur [7, 8].

CircRNAs also contain a large number of MREs, which also play a competitive role in endogenous RNA in cells. CircRNAs can affect the level of oncogenes or tumor suppressor genes targeted by miRNA by regulating the effective level of miRNA in cells, thus affecting occurrence and development of tumors [9]. CircRNAs have the characteristics of universality, conservativeness and tissue specificity, which indicate that it is a new type of tumor diagnostic marker and can be applied in tumor diagnosis and treatment [10]. Okholm et al.[11] found that the expression of circHIPK3 in bladder cancer tissues was lower than in normal tissues and the overexpression of circHIPK3 can inhibit the proliferation and metastasis of bladder cancer cells. Therefore, the low expression level of circHIPK3 is a new prognostic biological molecule in non-muscular invasive bladder cancer. Huang et al.[12] mapped the expression profiles of lncRNAs, circRNAs and mRNAs in cancer tissues and adjacent tissues by microarray. circRNA MYLK and lncRNA H19 competed to bind to miRNA-29a-3p as ceRNA, upregulated target genes DNMT3B, VEGFA and ITGB1, suggesting that circRNAs and lncRNAs were involved in bladder cancer. Also, Wu et al.[13] found that circCEP128 as the ceRNA of miRNA-145-5p promotes bladder cancer by affecting SOX11 level. Nowadays, sequencing technology continues to develop. More and more circRNAs with potential ceRNA characteristics have been found in tumors through the analysis of increasingly abundant transcriptome data. However, in many malignant tumors, such as gastric cancer, liver cancer, breast cancer and bladder cancer, the ceRNA regulatory network with lncRNA as the main line has been constructed [14–17]. However, there are few studies on the regulation network of mRNA-miRNA-circRNA ceRNA. Systematic analysis and screening of circRNAs influencing the prognosis of bladder cancer is even rarer. Therefore, this research aims to construct differential gene interaction network for bladder cancer and elevate circRNA-miRNA-mRNA network. On the same time, screening circRNAs specifically expressed, to provide a new perspective and direction for finding effective biomarkers and therapeutic targets for bladder cancer diagnosis and treatment.

Materials and methods

Differential gene expression analysis. EdgeR was applied to identify differentially expressed RNAs. The GSE37815, GSE39093, GSE97239 and GSE92675 gene expression profile and its corresponding platform annotation files were downloaded from the GEO database. A Limma-Microarray/Counts (mRNAs, miRNAs), DESeq2-Counts (circRNAs) test for differential expression of RNAs were performed with cut-off values for the false discovery rate (FDR, the adjusted p-value) <0.05; and log2 fold change (FC)| >1.

Annotation for mRNA/miRNA/circRNA interaction. The interaction network was reconstructed based on the data of DEmRNAs. The mRNA/miRNA/circRNA interaction was predicted with Arraystar's homemade miRNA target prediction software based on miRanda; the differentially expressed circRNA were annotated using mRNA/miRNA/circRNA interaction information. The competing endogenous RNA (ceRNA) network of circRNA and the analysis of the circRNA/miRNA/mRNA regulatory network were conducted according to the target genes of circRNAs targeting miRNAs.

Patients and tissues. The validated cohort consisted of 10 patients' tumor tissues and paired adjacent non-tumor tissue samples, which were obtained during 2018. All the patients were recruited from The Second Xiangya Hospital. No recruitment restriction on age, gender, tumor stage and no previous history of other cancers for all patients existed. 70 patients of clinical prognostic information were from tissue microarrays.

Survival analysis. The overall survival (OS) was defined as the time from diagnosis to death due to any cause. Kaplan-Meier survival analyses were performed to assess the difference of overall survival time.

q-PCR assays. Trizol reagent (Invitrogen, CA, USA) was used to extract total RNA from cells. The expression of circMTO1 was measured by SsoFast EvaGreen supermix (Bio-Rad Laboratories Co., Ltd. Shanghai, China) according to manufacturers' instructions. Expression of β-actin was used as an endogenous control. QPCR was performed at the condition: 95.0 °C for 3 min, and 39 circles of 95.0 °C for 10 s and 60 °C for 30 s in ABI7900HT Fast Real-Time PCR system (Applied Biosystems, CA, USA). The primers were used as following: hsa_circ_0076704-Forward, 5'-GTTGGGACTGTTTCCCTCAA-3', hsa_circ_0076704-Reverse, 5'-CCTGATGATTTCTCCAACTCG-3'; has_circ_0081963-Forward, 5'-TGCAGCCTTCTTTGAATCCT-3', has_circ_0081963-Reverse, 5'-GACCCGATCCAAGAAAGTCA-3'; hsa_circ_0001361-Forward, 5'-CCCACTTGGTGAATGGAGAT-3', hsa_circ_0001361-Reverse, 5'-GGATTTGGTCGGTCATCATC-3'. β-actin-Forward, 5'-TTGTTACAGGAAGTCCCTTGCC-3', β-actin-Reverse, 5'-ATGCTATCACCTCCCCTGTGTG-3'. The primer of miR-214 (HmiRQP0320) and U6 (HmiRQP9001) was obtained from GeneCopoeia (Guangzhou, China).

Statistics. All data were expressed as the mean ± standard deviation using SPSS 17.0 software. The statistical significance between two experimental groups was analyzed using t-test. The statistical significance between more than two experimental groups was analyzed using One-Way ANOVA comparison method. A p-value <0.05 was defined to be statistically significant. Kaplan-Meier curves and log rank test were used to analyze the OS of patients.

Results

Differential expression of RNA in bladder cancer and normal samples. The differentially expressed RNAs between bladder cancer and normal bladder tissues were analyzed by bioinformatics tools through GEO databases (GSE37815, GSE39093, GSE97239, GSE92675). We found 19231 differentially expressed RNAs, 847 differentially expressed miRNAs and 7282 differentially expressed circRNAs. Based on the differential analyses, we inferred 231 upregulated and 551 downregulated mRNAs, 10 upregulated and 5 downregulated miRNAs, 5 upregulated and 13 downregulated circRNAs (Figures 1A, 1B, 1C). The gene interaction network was established by the significant upregulated and downregulated DEGs, respectively (Figure 2).

Construction of the circRNA/miRNA/mRNA network. The interactions of mRNA-miRNA and miRNA-circRNA were predicted according to the differential expression. We focused on differential expression of miRNAs and constructed a ceRNA network (Figure 3). We selected a few top differential circRNAs in ceRNA network and plotted their expression heat-maps in Figure 4. Specifically, there were 12 circRNAs related to miRNAs, we focused on the node degree of the ceRNA network (Table 1). Among the miRNAs, hsa-mir-214 had higher degree, which had been demonstrated as a diagnostic and prognostic biomarker in bladder cancer (BC) [18, 19]. The expression of predicted target genes of hsa-miR-214 is shown in Figure 5. It might indicate that the lower expression of the hsa-mir-214 meant the poor OS in patients with BC.

Detection of RNAs expression in cell and tissue samples. To determine which circRNA played the most important role in BC, real-time qPCR was used to detect 3 selected

Table 1. The Kcore and Degree of ceRNA (circRNA, miRNA, mRNA).

AccID	KCore	Degree	Category	AccID	KCore	Degree	Category
GSK3B	1	1	mRNA	hsa-miR-125b	2	8	miRNA
hsa-miR-106a	4	12	miRNA	C1S	1	1	mRNA
ITGAL	3	4	mRNA	hsa_circ_0001361	2	2	circRNA
hsa-miR-182	1	2	miRNA	LPAR2	1	1	mRNA
CCND2	1	1	mRNA	hsa_circ_0042103	4	4	circRNA
FGF9	1	1	mRNA	HSD17B6	1	1	mRNA
hsa_circ_0076704	2	3	circRNA	FUT3	1	1	mRNA
hsa_circ_0051401	1	1	circRNA	hsa_circ_0005406	4	4	circRNA
hsa-miR-92a	4	8	miRNA	hsa_circ_0004119	3	4	circRNA
SH3GL2	1	1	mRNA	RELN	1	1	mRNA
HLA-DPA1	1	1	mRNA	hsa-miR-200a	2	3	miRNA
hsa_circ_0006041	4	8	circRNA	E2F2	2	2	mRNA
PDGFRB	1	1	mRNA	SFN	2	2	mRNA
PDGFRA	2	2	mRNA	MYH11	1	1	mRNA
SLC38A1	1	1	mRNA	ST3GAL6	1	1	mRNA
FCER1G	1	1	mRNA	FLNC	1	1	mRNA
hsa-miR-20a	4	12	miRNA	hsa-miR-18a	2	4	miRNA
hsa_circ_0081963	2	3	circRNA	SH2B3	2	2	mRNA
INMT	1	1	mRNA	JAM3	1	1	mRNA
SFRP1	2	2	mRNA	hsa-miR-106b	4	10	miRNA
CYP2U1	2	2	mRNA	hsa_circ_0017636	4	5	circRNA
hsa-miR-138	3	11	miRNA	SLC37A1	1	1	mRNA
DPYD	1	1	mRNA	EHD2	1	1	mRNA
ITGA8	2	2	mRNA	hsa_circ_0004689	3	3	circRNA
EPHA2	1	1	mRNA	hsa_circ_0002387	3	4	circRNA
hsa_circ_0018064	4	5	circRNA	hsa-miR-210	3	7	miRNA
hsa-miR-145	2	4	miRNA	IL7R	2	2	mRNA
TSTA3	1	1	mRNA	hsa-miR-214	2	9	miRNA
PFKFB4	1	1	mRNA				

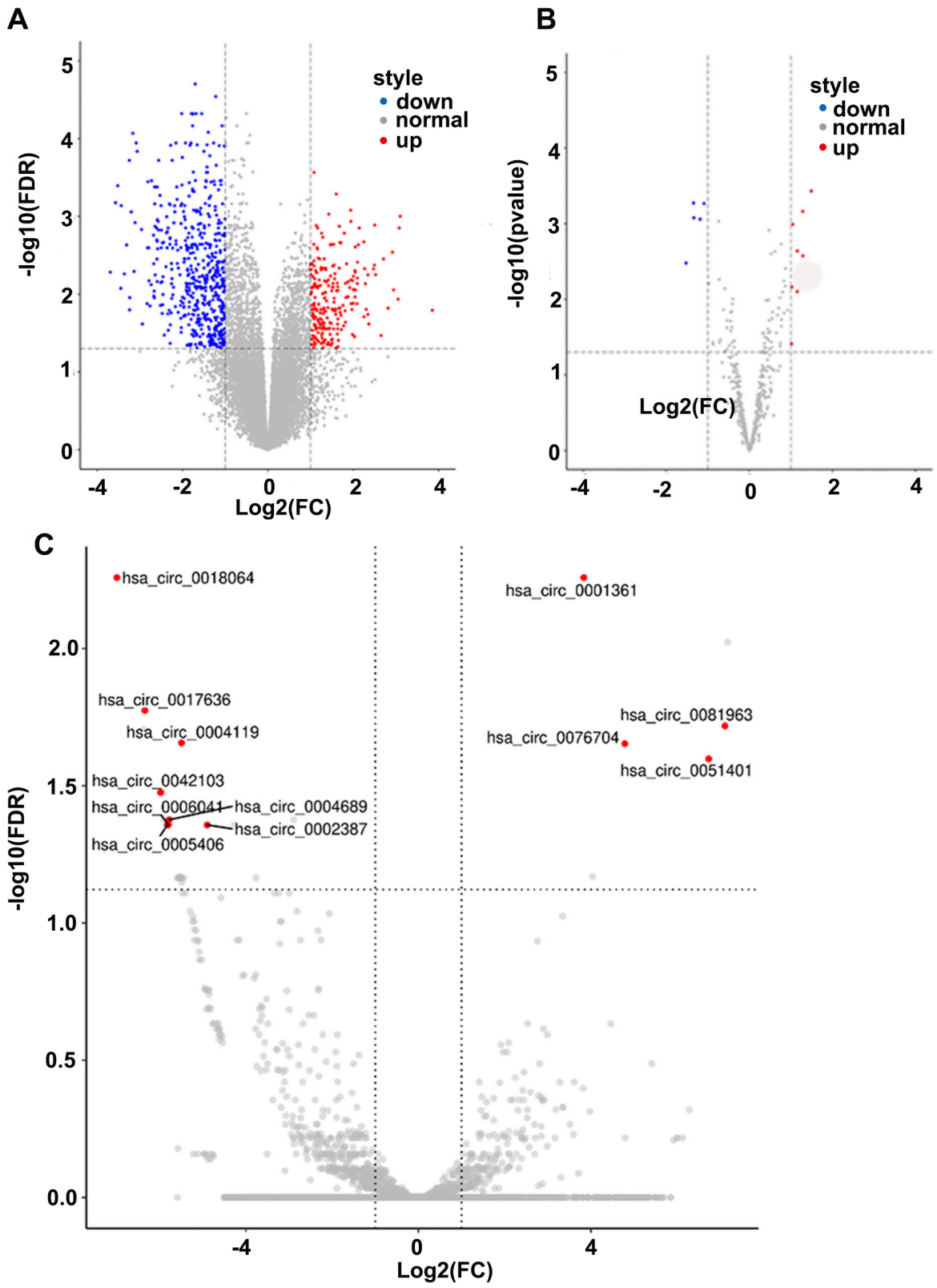

Figure 1. Differential expression of RNAs in bladder cancer and normal samples. A) Volcanic maps of differentially expressed mRNAs, B) Volcanic maps of differentially expressed miRNAs, C) Volcanic maps of differentially expressed circRNAs.

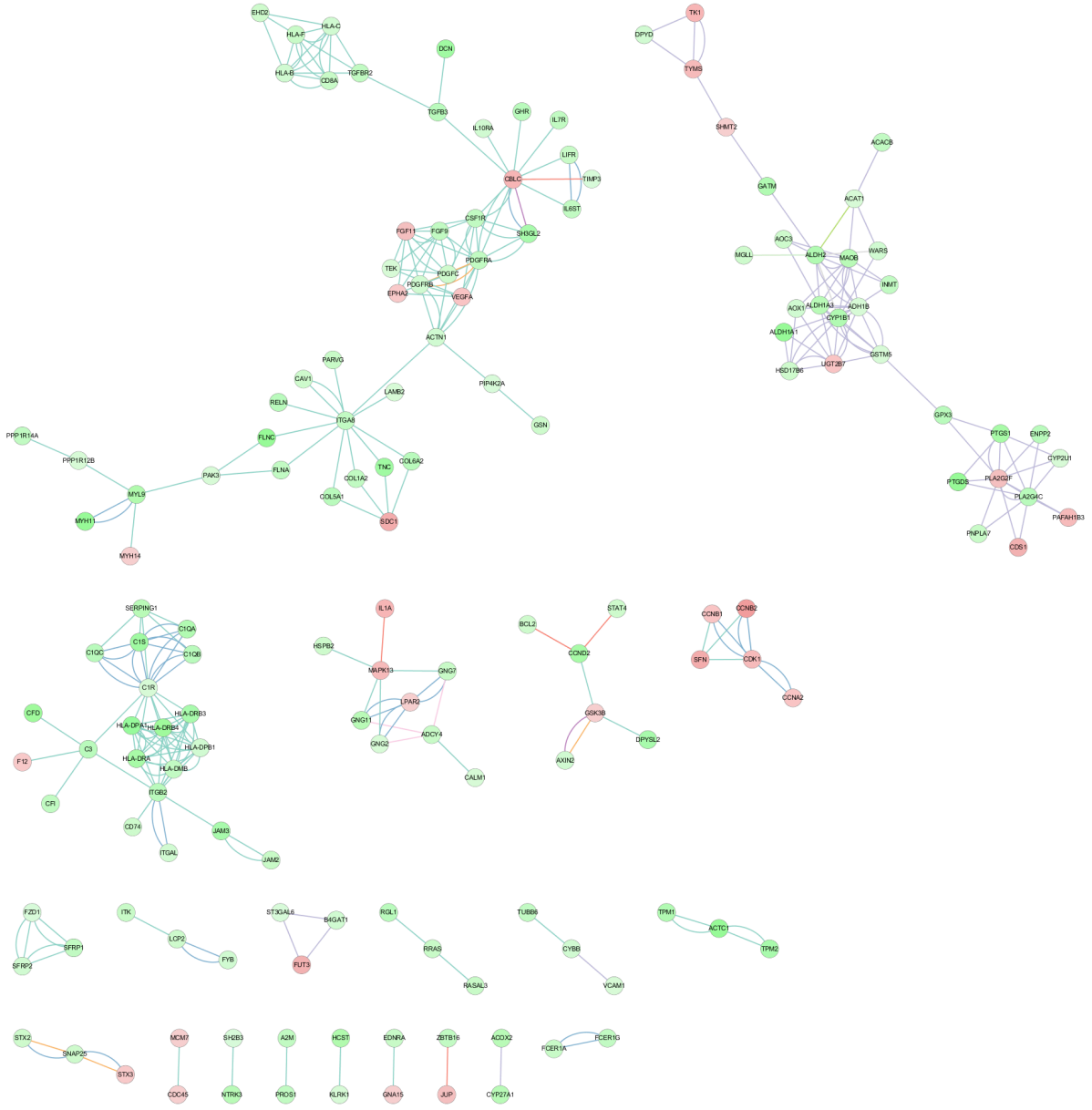

Figure 2. Differentially expressed mRNAs interaction network map.

differentially expressed circRNAs (hsa_circ_0076704, hsa_circ_0081963 and hsa_circ_0001361) and hsa-miR-214 expression noted above in 10 pairs of fresh frozen tissue samples. Levels of hsa_circ_0076704, hsa_circ_0081963 and hsa_circ_0001361 were upregulated (Figures 6B–D) and hsa-miR-214 was downregulated in tissue samples (Figure 6A). Compare to the SV-Huc-1 cell lines, the expression level of hsa_circ_0076704 was upregulated in bladder cancer cells (5637/T24) (Figure 6E). In brief, above data further improved the specificity of our assay, verified that miR-214 expression might play a tumor-suppressive role in bladder cancer. On the other hand, verified the hsa_circ_0076704, hsa_circ_0081963 and hsa_circ_0001361 upregulation in cancer progression.

Survival analysis of hsa_circ_0076704 in validated cohort. We studied the association of hsa_circ_0076704 with patients' survival, which can be used to evaluate their prognostic potential. The Kaplan-Meier overall survival curves for hsa_circ_0076704 was plotted in Figure 6F. As the result shown, hsa_circ_0076704 is significantly associated with survival, indicating that it might be a prognostic maker and drug target for bladder cancer.

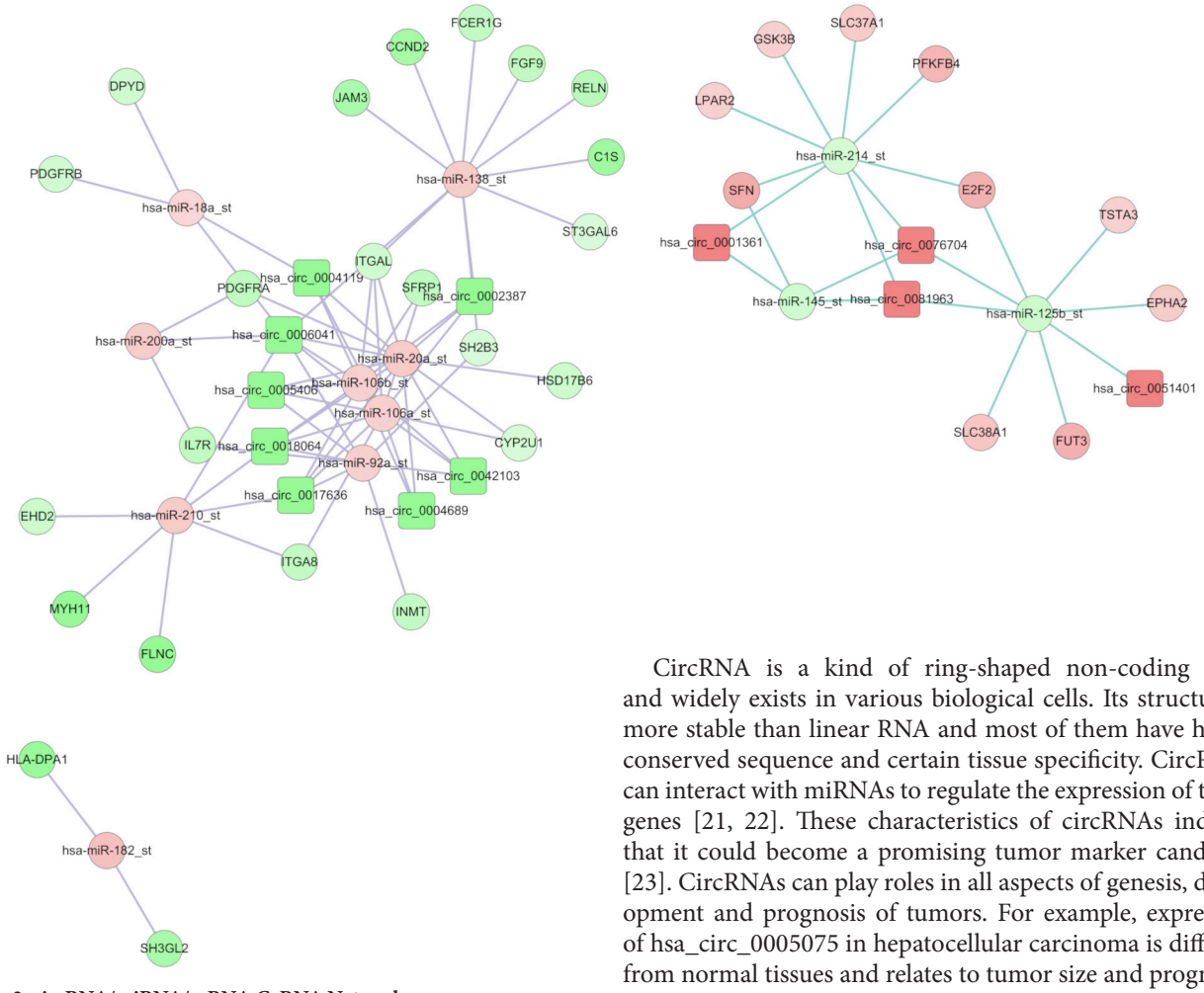

Figure 3. circRNA/miRNA/mRNA CeRNA Network.

Discussion

Bladder cancer, with high malignancy, easy recurrence and poor prognosis is an urgent problem to be solved in clinic [20]. In order to find reliable biomarkers for survival rate of patients with bladder cancer, we constructed differential gene interaction network of bladder cancer. Then ceRNA network of related node genes were analyzed and constructed. Differential circRNAs related to prognosis of bladder cancer were screened out and confirmed by quantitative real-time PCR (qRT-PCR). The expressions of circRNAs (hsa_circ_0076704, hsa_circ_0081963, hsa_circ_0001361) were upregulated in tumors and expression of hsa-mir-214 was downregulated in tumors. The results of Kaplan-Meier curve analysis showed that hsa_circ_0076704 was closely related to the overall survival prognosis of bladder cancer patients. The experimental results indicated that hsa_circ_0076704 had a potential to become a molecular marker for the overall survival prognosis of bladder cancer patients.

CircRNA is a kind of ring-shaped non-coding RNA and widely exists in various biological cells. Its structure is more stable than linear RNA and most of them have highly conserved sequence and certain tissue specificity. CircRNAs can interact with miRNAs to regulate the expression of target genes [21, 22]. These characteristics of circRNAs indicate that it could become a promising tumor marker candidate [23]. CircRNAs can play roles in all aspects of genesis, development and prognosis of tumors. For example, expression of hsa_circ_0005075 in hepatocellular carcinoma is different from normal tissues and relates to tumor size and prognosis. Gene interaction analysis shows that hsa_circ_0005075 can interact with miRNA-23b-5p, miRNA-93-3p, miRNA-581 and miRNA-23a-5p and then further affect hepatocellular carcinoma [24]. Xuan et al.[25] found that the most significant upregulated and downregulated circ-RNAs in laryngeal squamous cell carcinoma were hsa_circ_100855 and hsa_circ_104912, respectively. Further studies shown that hsa_circ_104912 was closely related to cervical lymph node metastasis, clinical stage and prognosis of laryngeal cancer patients. CircRNA circHIPK3 can be used as a prognostic marker in glioma and promote the occurrence and development of cancer through the miRNA-654/IGF2BP3 pathway [26]. There are few studies on circRNAs influencing the prognosis of bladder cancer. Zeng et al.[27] found that circVANGL1 affects the progression and prognosis of bladder cancer by regulating the miRNA-605-3p/VANGL1 pathway. Chen et al.found that CircPRMT5 could promote epithelial-mesenchymal transition and/or aggressiveness of urothelial carcinoma of the bladder and is a prognostic biomarker of this disease [28]. However, the results of these studies have not been widely recognized and the related functions need to be further validated.

The results of this study certified that hsa_circ_0076704, hsa_circ_0081963 and hsa_circ_0001361 were expressed higher in cancer tissues than adjacent tissues. Further, hsa_circ_0076704 was confirmed relative to bladder cancer prognosis. The role of hsa_circ_0076704, hsa_circ_0081963 and hsa_circ_0001361 in bladder cancer has not been reported yet. The role of hsa_circ_0076704 in bladder cancer was revealed first time and suggests that circ_0076704 is promising to serve as a prognostic marker of bladder cancer. However, the further in-depth mechanism studies are indispensable. Also, hsa-miR-214 was found downregulated in bladder cancer tissues. MiR-214 could interact with multiple target genes to regulate cell growth, differentiation and apoptosis [29, 30]. In different tumors, it could function as oncogene or anti-oncogene [31, 32]. Early study discovered that the expression of miRNA-214 was downregulated in myeloma cell. The overexpression of miRNA-214 could promote myeloma cell apoptosis and inhibit proliferation, which severed as an anti-oncogene [33]. In bladder cancer, Wang et al.showed that downregulated miR-214 was related to tumor stage, grade, multifocality, lymph node status, history of non-muscle-invasive bladder cancer and is an independent factor of recurrence-free survival and overall survival for muscle-invasive bladder cancer [19]. Other studies revealed that the upregulation of miRNA-214 expression was related to poor prognosis [19, 34]. In this study, expression of miRNA-214 was reduced in bladder cancer

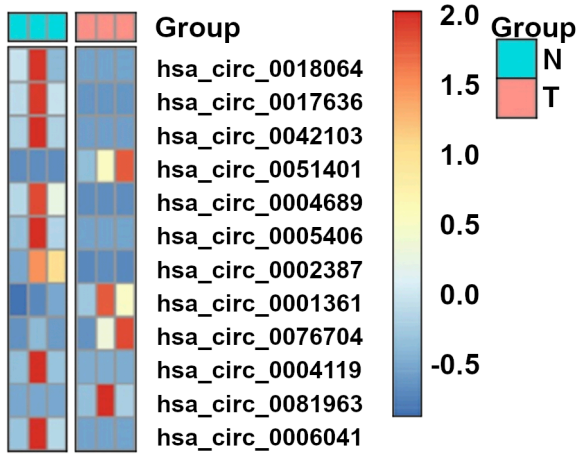

Figure 4. Differentially expressed circRNAs thermogram in bladder cancer and normal bladder tissues.

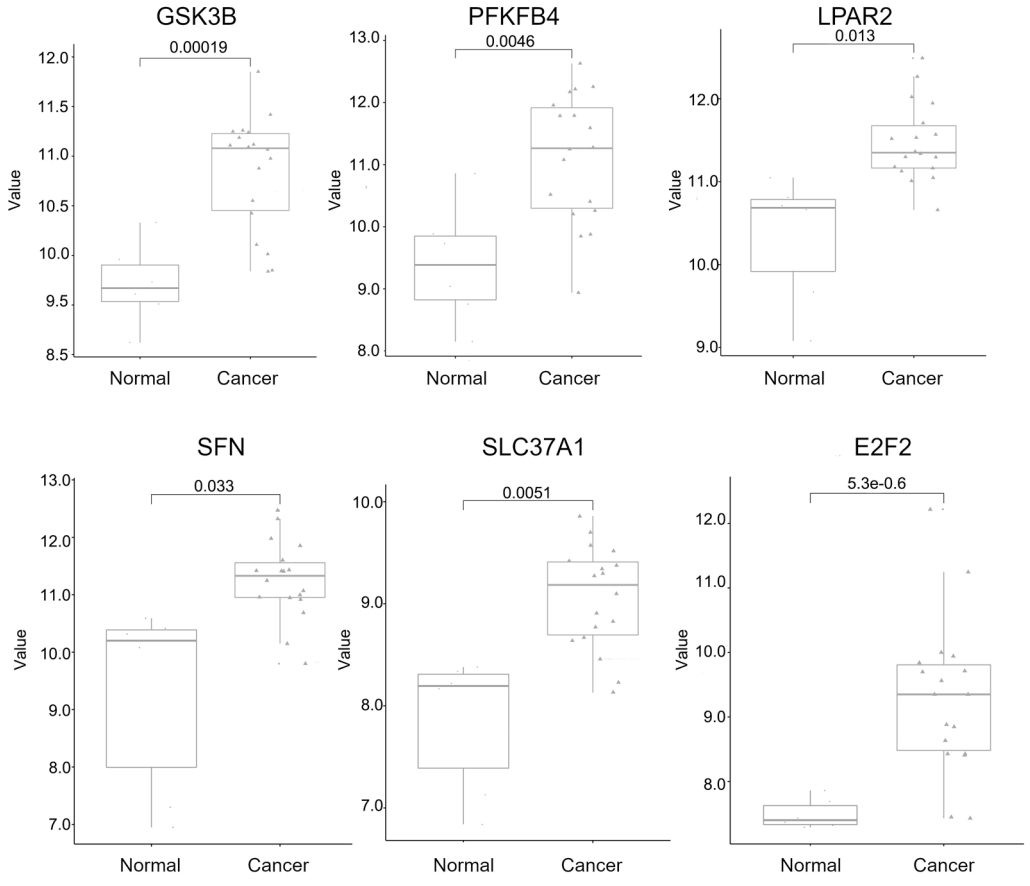

Figure 5. hsa-mir-214 predicts differential expression box map of target genes.

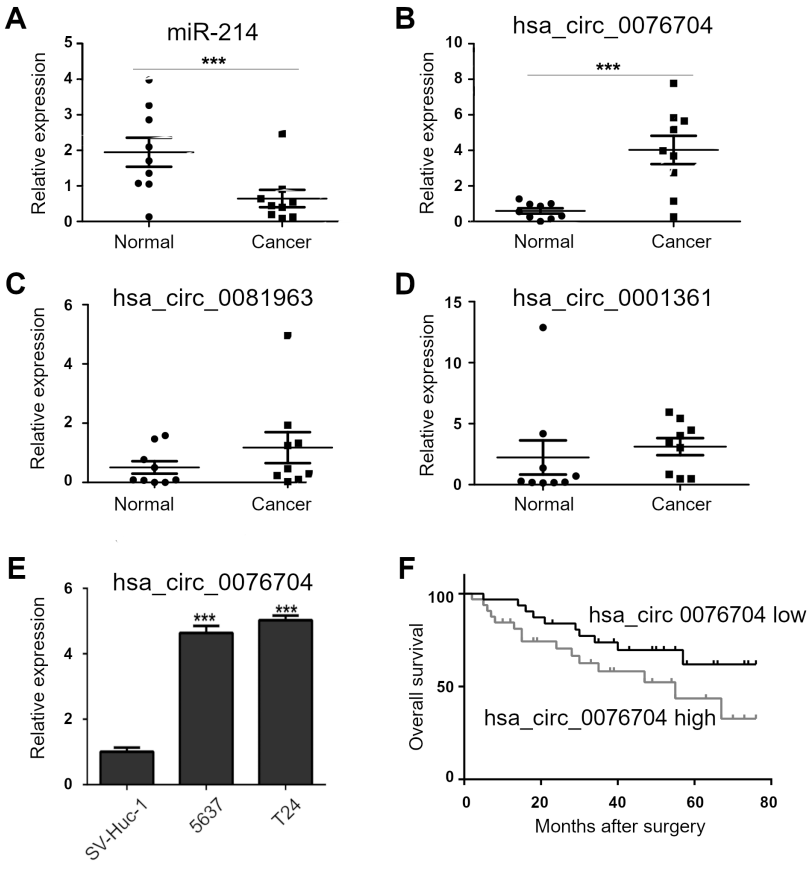

Figure 6 Differential expressions of RNAs in cells and tissues. Expression of hsa-mir-214 (A), hsa_circ_0076704 (B), hsa_circ_0081963 (C), and hsa_circ_0001361 (D) in bladder cancer and adjacent tissues. E) Expression of hsa_circ_0076704 in bladder cells (5637 and T24) and normal bladder epithelial cells (SV-Huc-1) and F) Kaplan-Meier analysis of overall survival for hsa_circ_0076704 expression.

tissues. The results were consistent with the research reports, which further confirmed the role of miRNA in bladder cancer.

In conclusion, the circRNA-miRNA-mRNA network of differentially expressed genes in bladder cancer was constructed. Three high expression circRNAs (hsa_circ_0076704, hsa_circ_0081963 and hsa_circ_0001361) in cancer tissues were screened. The level of hsa_circ_0076704 was found to have significant prognostic value by Kaplan-Meier survival analysis, suggesting that hsa_circ_0076704 can become prognostic maker and drug target for bladder cancer. Furthermore, the expression of miR-214, which predicted can bind with circ_0076704, was lower in cancer tissues than normal ones. The same, many predicted target genes of miR-214 such as GSK3B, PFKFB4 were all upregulated in bladder cancer. All these results demonstrated that circ_0076704/ miR-214/target gene could construct a ceRNA net and open up new avenues for treatment or prognostic of bladder cancer. On the other hand, hsa_circ_0076704 has significant prognostic value and may be biomarkers of survival rate of bladder cancer patients.

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