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Original Article

Detection of serum long non-coding RNA UCA1 and circular RNAs for the diagnosis of bladder cancer and prediction of recurrence

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Abstract: Accumulating evidence indicates that long noncoding RNAs (lncRNAs) and circular RNAs (circRNAs) may be biomarkers for the diagnosis and prediction of recurrence in tumor patients because they play an important role in tumorigenesis and progression. In this study, the expression of lncRNA urothelial carcinoma-associated 1 (UCA1) and associated circFARSA, circSHKBP1, and circBANP was investigated in serum specimens from bladder cancer (BC) patients and healthy controls using real-time PCR. When comparing patients with BC to healthy controls, the expression of lncRNA UCA1, circFARSA, and circSHKBP1 was significantly increased. The area under the curve (AUC) of the lncRNA UCA1 and circSHKBP1 signature to distinguish BC patients from controls was 0.804. The diagnostic performance of this signature was more optimal for low volume tumors (AUC = 0.870). Moreover, we determined that the expression of circFARSA, circSHKBP1, and circBANP was higher for recurrent BC than for patients without recurrence. Receiver operating characteristic (ROC) analysis revealed that a combination of circFARSA and circBANP levels was able to discriminate the patients with tumor recurrence from those without, with an area under the ROC curve of 0.737. In conclusion, our results identified an lncRNA UCA1: circSHKBP1 panel and a circFARSA: circBANP panel for BC diagnosis and prognosis, respectively.

Keywords: Bladder cancer, circRNAs, long noncoding RNA, recurrence, UCA1

Introduction

Bladder cancer (BC) is the tenth most common form of cancer worldwide, with an estimated 549,000 new cases and 200,000 deaths in 2018. The incidence and mortality rates of BC in men are approximately four times those of women [1]. Twenty-five percent of patients have muscle-invasive or metastatic disease at the time of the initial diagnosis and have a worse prognosis [2]. Moreover, the probabilities of relapse of BC at five years are up to 78% [3]. However, early diagnosis and prediction of recurrence of BC currently rely on invasive cystoscopy, and patient compliance with cystoscopy is poor [4]. Therefore, reliable noninvasive diagnostic and prognostic biomarkers for BC are needed.

Long noncoding RNAs (lncRNAs) represent a class of transcripts longer than 200 bp with no protein-coding function [5]. Accumulating evidence demonstrates that lncRNAs play critical roles in the occurrence and development of cancer and regulate gene expression at different levels [6, 7]. Circular RNAs (circRNAs) are a new type of endogenous RNAs that have received a noncanonical form of alternative splicing [8]. They can inhibit microRNAs (miRNAs) functions by serving as miRNAs sponges and are involved in the carcinogenesis of several types of cancers [9, 10]. Moreover, both lncRNAs and circRNAs are detectable and relatively stable in serum, indicating the promise of circulating lncRNAs and circRNAs for biomarker applications [11, 12].

LncRNA urothelial carcinoma-associated 1 (UCA1) is upregulated in several cancer tissues, and its aberrant expression may be associated with tumor pathogenesis [13]. Recently, it has been discovered that lncRNA UCA1 is significantly dysregulated in the serum or plasma of

Table 1. Primers used in study

Gene	Forward primer	Reverse primer
lncRNA UCA1	CTCTCCATTGGGTTCACCATTC	GCGGCAGGTCTTAAGAGATGAG
β-actin	TCCCTGGAGAAGAGCTACGA	AGCACTGTGTTGGCGTACAG
circ-FARSA	TGGAACTTTGACGCCCTCTTC	CAGCTCTGTCTCTTGCTTGGA
circ-SHKBP1	ACAACGACCTCCTTGTCAGC	TAAATGGAGCCGTTGTTGC
circ-BANP	GAGCAAGTCCAGATCACGCA	TTGGAGTCGATGCTCTGCTT

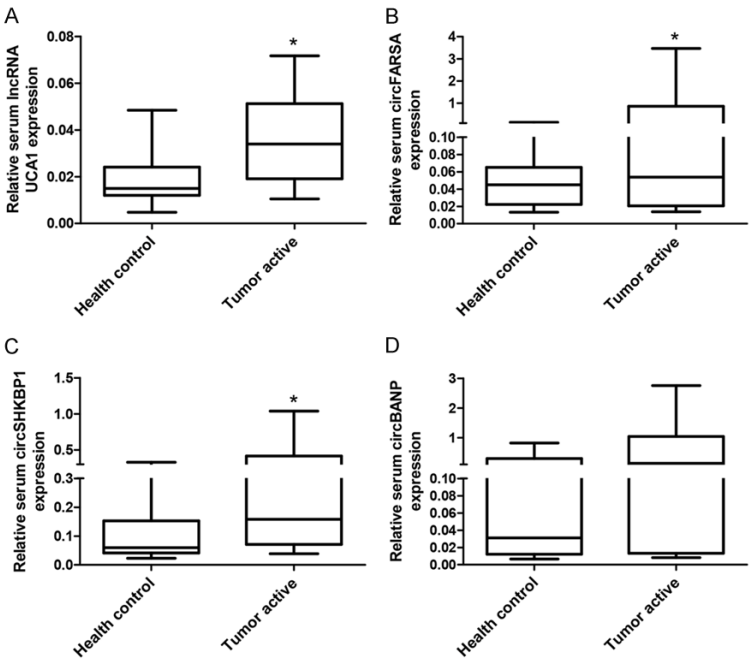

Figure 1. The serum expression of lncRNA UCA1 (A), circFARSA (B), circSHKBP1 (C), and circBANP (D) are quantitatively analyzed in patients with bladder cancer and healthy controls. *$P < 0.05$ bladder cancer vs. healthy controls.

patients with different types of cancer and may be used to discriminate between patients with cancer and healthy controls [14-16]. However, the potential significance of serum lncRNA UCA1 in BC remains elusive.

Differential expression of circFARSA (hsa_circ_0000896), circSHKBP1 (hsa_circ_0000936), and circBANP (hsa_circ_0040823) in BC were collected from the GEO database and predicted to be endogenous sponges for miR-382-5p, miR-635 and miR-516b-5p, respectively [17]. These miRNAs also have binding sequences with lncRNA UCA1. In addition, circFARSA can significantly promote migration and invasion of non-small cell lung cancer (NSCLC) cells, making plasma circFARSA as a potential noninvasive biomarker for NSCLC [18]. CircSHKBP1 may regulate the angiogenesis of glioma-exposed endothelial cells through the miR-544a/FOXP1 and miR-379/FOXP2 pathways [19]. It has been reported that dysregulated circBANP appears to have an important role in colorectal cancer (CRC) cells and could serve as a prognostic and therapeutic marker for CRC [20]. Nevertheless, the role of these circRNAs in BC is not clear.

Although there are several BC-associated lncRNAs and circRNAs, there are few reports on their co-expression levels, which may be useful as markers for clarifying BC diagnosis and prognosis. This prompted us to conduct research regarding the serum levels of lncRNA UCA1 and related circRNAs (including circFARSA, circSHKBP1 and circBANP) in patients with BC and control subjects. This study was designed to investigate whether the combined detection of circRNAs and lncRNA UCA1 in patient serum can be used to distinguish healthy controls from BC patients and predict relapse.

Materials and methods

Patients and sample collection

This study was approved by the Ethical Review Committee of the First Affiliated Hospital of Zhengzhou University, Zhengzhou, China. Written informed consent was provided by all patients. Between 18 June 2018 and 30 November 2018, a total of 70 patients at the first affiliated hospital of Zhengzhou University were enrolled in the current study. Patients were divided into four distinct groups: (1) no previous history of BC (healthy control group, n = 16); (2) first-time bladder cancer detected by cystoscopy (tumor active group, n = 14); (3) with a previous history of BC and exhibiting recurrence (recurrent group, n = 15) or (4) no recurrence (non-recurrent group, n = 25) by cystoscopy.

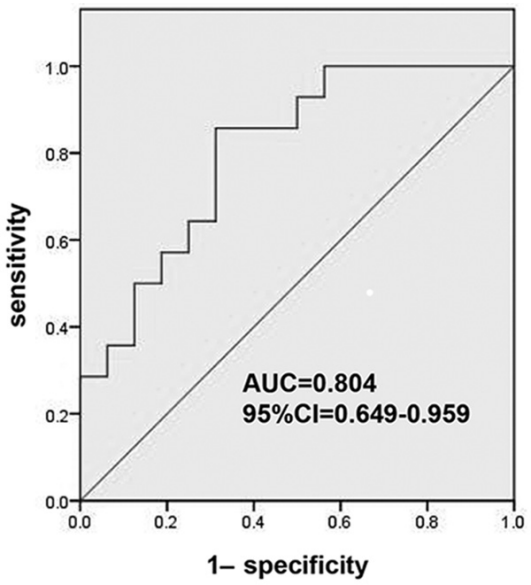

Figure 2. ROC curve using lncRNA UCA1 and circSHKBP1 to separate patients with bladder cancer from healthy controls.

Patients had first-time or recurrent bladder cancer confirmed by a clinician with cystoscopy results. Urothelial carcinoma of the bladder cancer and tumor grade were determined by pathology. Tumors larger than 3 cm were referred to large volume tumors. Serum specimens from patients were kept at -80°C until RNA extraction.

RNA extraction

Total RNA was extracted from 200 µl of serum using a Blood Total RNA Isolation Kit (DP443, Tiangen Biotech Co., Ltd., Beijing, China) and eluted in 25 µl of RNase-free ddH_2O according to the manufacturer's instructions. Briefly, 900 µl of lysate was added to the 200 µl serum specimen and vortexed for 30 seconds. Then 200 µl of chloroform was added and the mixture was vortexed for 15 seconds, maintained at room temperature for 5 minutes, and then centrifuged at 12,000 g for 15 minutes. The upper aqueous phase was transferred to a new EP tube and 100% ethanol at twice the volume of this aqueous phase was added. A total of 700 µl of this solution was placed in a filter collection tube and centrifuged at 12,000 g for 30 seconds. The flow-through was discarded and the procedure was repeated until all the sample had been filtered. Then, 700 µl of RW1H buffer and 500 µl of the washing solution were sequentially added to the collection tube, followed by centrifugation for 30 seconds. The filter cartridge was transferred to a labeled tube. Then 25 µl of RNase-Free ddH_2O was added to the filter and centrifuged at 12,000 g for 2 minutes. RNA elutions were stored at -80°C.

Quantification of serum circRNAs and lncRNA UCA1

Reverse transcription (RT) and real-time PCR kits were used to evaluate the expression levels of lncRNA UCA1 and the related circRNAs. RT reactions were performed in a volume of 20 µl using a PrimeScript® RT reagent kit (Roche, USA) with incubation for 10 minutes at 25°C, 30 minutes at 55°C and 5 seconds at 85°C, followed by storage at 4°C. For real-time PCR, 2 µl of RT product was mixed with 10 µl of 2×SYBR® Premix Ex TaqTM (Roche, USA), 0.6 µl of gene-specific forward and reverse primers (10 µM), and 6.8 µl of nuclease-free water for a final volume of 20 µl according to the manufacturer's instructions. The primers used in this study are listed in **Table 1**. All reactions were performed using the following conditions: 95°C for 10 seconds, followed by 40 cycles of 95°C for 5 seconds and 60°C for 60 seconds. Samples were analyzed in triplicate. Amplification of the appropriate product was confirmed by melting curve analysis following amplification. The relative expression of each circRNA and lncRNA UCA1 was calculated using the comparative cycle threshold (CT) ($2^{-\Delta\Delta CT}$) method with β-actin as the endogenous control for data normalization. The CT was defined as the number of cycles required for the SYBR signal to cross the threshold. Samples with a CT of greater than 40 were considered negative.

Statistical analysis

Student's *t*-test was used to evaluate differences in the expression of lncRNA UCA1 and the chosen circRNAs in serum from the BC patients and controls. The values were expressed as the means ± SD and $P < 0.05$ was considered significant. A receiver operating characteristic (ROC) curve was constructed and the area under the curve (AUC) was calculated to assess the specificity and sensitivity of the predicted BC. Data analysis was performed using IBM SPSS 17.0 software (SPSS, Inc., NY, USA).

Table 2. Clinical and pathologic characteristics of patients in the study

	Healthy control (n = 16)	Bladder cancer active (n = 14)	Recurrent (n = 15)	Non-recurrent (n = 25)
Age (years)				
Median	52.5	66.5	65	61
Range	50-71	38-93	49-89	27-87
Gender				
Male	14	13	15	25
Female	2	1	0	0
Tumor size				
≥ 3 cm		9	5	
< 3 cm		5	10	
Tumor number				
> 2		5	6	
≤ 2		9	9	
Tumor grade				
High		8	10	
Low		6	5	

Results

Expression of serum lncRNA UCA1, circFARSA, and circSHKBP1 in patients with BC and healthy controls

In order to determine the feasibility of the selected circRNAs and lncRNA UCA1 as biomarkers for the detection of BC, we first detected and analyzed the relative expression of serum lncRNA UCA1 and circRNAs in each sample using real-time PCR. It was demonstrated that lncRNA UCA1, circFARSA, and circSHKBP1 levels in patients with BC were significantly higher than those in healthy controls ($P < 0.05$), while there was no significant difference between the patients with BC and healthy controls with respect to serum circBANP expression (**Figure 1**). Then, we performed ROC curve analyses to evaluate whether serum lncRNA UCA1, circFARSA, and circSHKBP1 could be used as potential diagnostic biomarkers for BC. It was found that combined lncRNA UCA1 and circSHKBP1 provided the ability to predict tumor presence with an AUC of 0.804 (95% confidence interval, 0.649-0.959) (see **Figure 2** and Table S1).

Diagnostic value of lncRNA UCA1 combined with circSHKBP1 in patients with BC

We also tested the ability of combined lncRNA UCA1 and circSHKBP1 to predict the presence of tumors in different subgroups dependent on tumor size, tumor grade, and number of tumors. Clinical and pathologic characteristics of the patients with BC are shown in **Table 2**. It was observed that the best performance of combined detection occurred with low volume tumors, with an AUC of 0.870 (95% confidence interval, 0.662-1.000) (see **Figure 3** and Table S2).

Validation of serum circFARSA, circSHKBP1, and circBANP expression in patients with tumor recurrence and no recurrence

To further explore the viability of the selected circRNAs and lncRNA UCA1 as biomarkers for predicting relapse among patients with BC, we compared the expression of each gene in the two groups, those with recurrence and those without. It was found that the expressions of circFARSA, circSHKBP1, and circBANP in the recurrence group were significantly higher than that in the non-recurrence group except lncRNA UCA1 (**Figure 4**), which suggested that these circRNAs could be used as biomarkers to differentiate the two groups. It was demonstrated that combined serum circFARSA with circBANP could distinguish patients with tumor recurrence from those without, with an area under the ROC curve of 0.737 (95% confidence interval, 0.532-0.942) (see **Figure 5** and Table S3).

Prognostic value of circFARSA combined with circBANP for BC recurrence

To further explore whether serum circFARSA and circBANP levels act as prognostic factors for BC patients with recurrence, we studied the predictive performance of combined circFARSA and circBANP in different tumor subgroups based on number of tumors, tumor size, and grade using ROC curves (**Table 2**). It was found that the best predictive value was for low grade tumors with an AUC of 0.839 (95% confidence interval, 0.690-0.989), while there were no significant differences among the remaining clinicopathologic characteristics (Table S4).

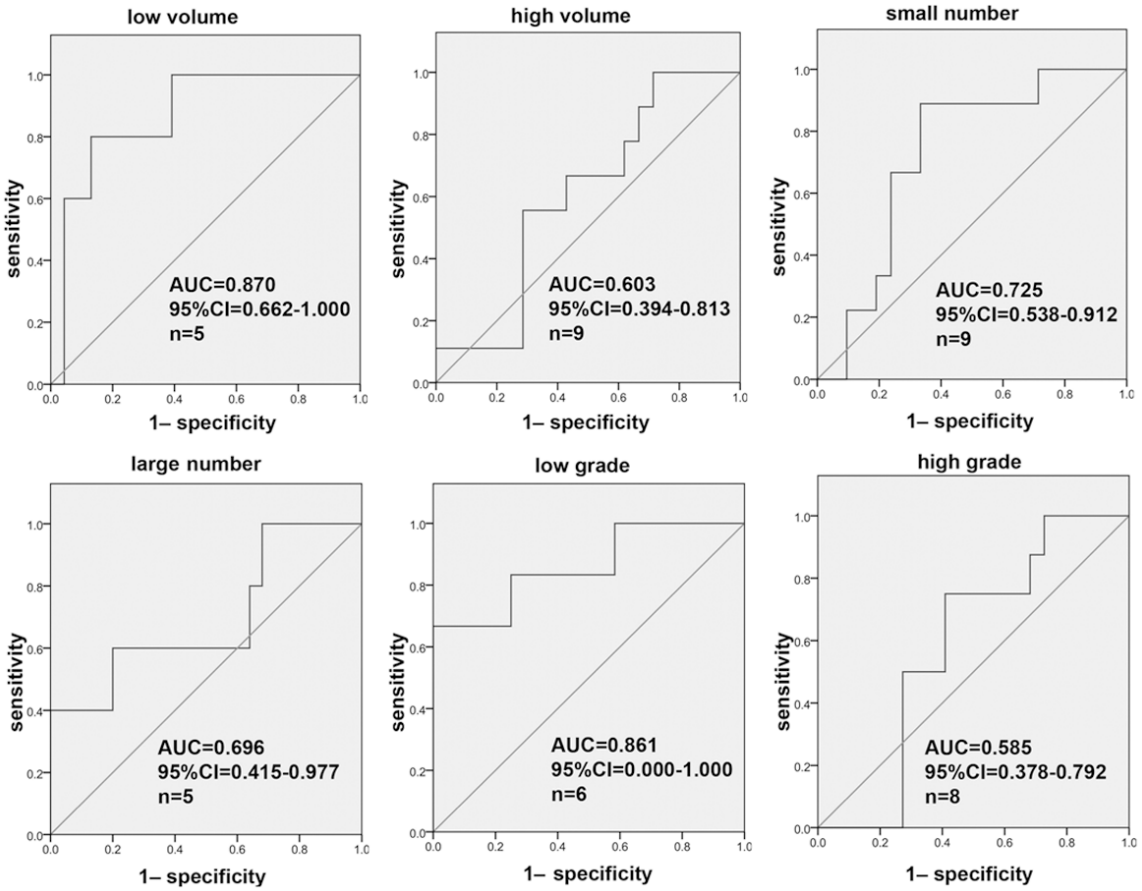

Figure 3. ROC curve using lncRNA UCA1 and circSHKBP1 to distinguish clinical and pathologic characteristics of the patients with bladder cancer.

Discussion

Recent studies have revealed that lncRNA UCA1 and circRNAs expression levels may be associated with the progression and prognosis of human malignancies. However, it is still unknown whether combining different types of non-coding RNAs may improve their diagnostic and prognostic value for BC. Blood-based testing is ideal for evaluating biomarkers in cancer care because it is easy and less invasive than other methods. Moreover, lncRNA UCA1 and circRNAs in serum are quite stable and readily detected by real-time PCR [14], since lncRNA UCA1 may be protected by exosomes [21] and covalent closed loops of circRNAs [18]. Therefore, the expression levels of lncRNA UCA1 and related circRNAs, including circFARSA, circSHKBP1, and circBANP were determined in the serum specimens from BC patients and control subjects in this study. Because it exhibited greater stability and abundance, β-actin was selected as a suitable reference housekeeping gene [15].

It has been reported that deregulation of serum or plasma lncRNA UCA1 also occurs in several other malignancies. The expression of serum lncRNA UCA1 in osteosarcoma patients is related to clinical stage and metastasis, and it can be used as a prognostic biomarker [22]. Plasma or serum lncRNA UCA1 is also upregulated in patients with hepatocellular carcinoma, gastric cancer and NSCLC, and it shows good diagnostic value [14, 23, 24]. LncRNA UCA1 in serum exosomes is downregulated in CRC. The combined ROC curve of circHIPK3: lncRNA UCA1 shows high sensitivity and specificity to CRC [16]. It is suggested that lncRNA UCA1 does not appear to be a specific marker for BC and circRNAs in combination with lncRNAs may be more suitable biomarkers for BC, which is a heterogeneous disease. It was demonstrated that the expression levels of lncRNA UCA1, circFAR-

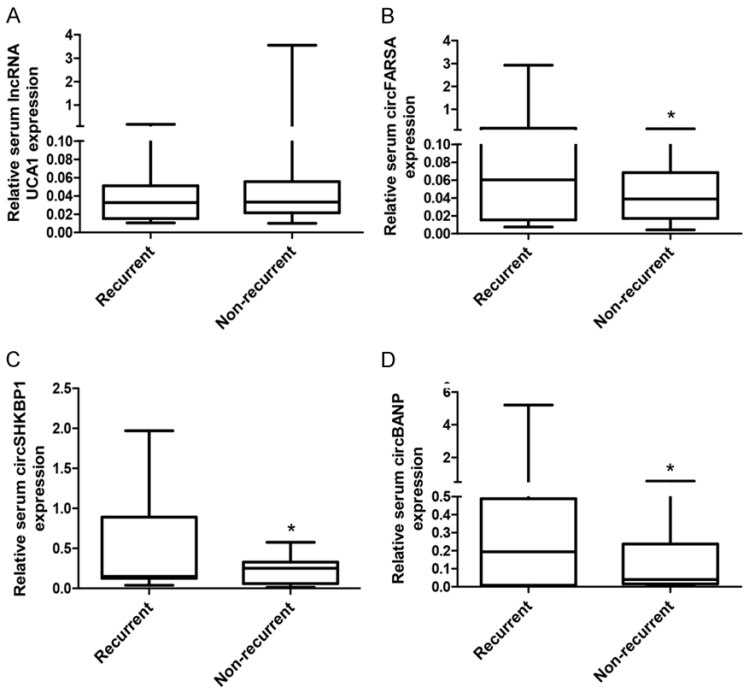

Figure 4. The serum expression of lncRNA UCA1 (A), circFARSA (B), circSHKBP1 (C), and circBANP (D) are quantitatively analyzed in patients with recurrence versus non-recurrent. *$P < 0.05$ recurrent vs. non-recurrent.

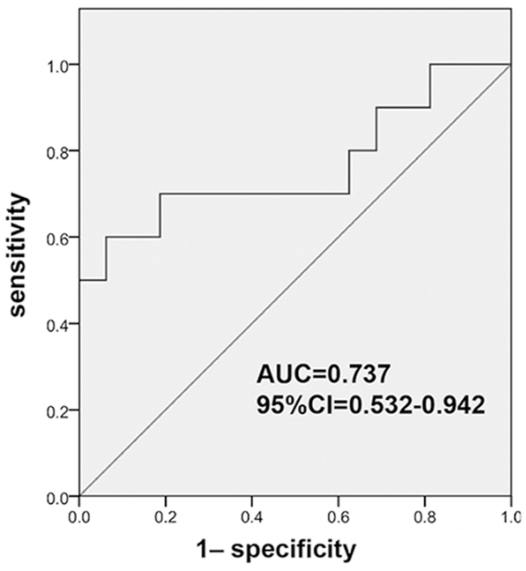

Figure 5. ROC curve using circFARSA and circBANP to distinguish patients with recurrence from non-recurrent patients.

SA, and circSHKBP1 were significantly higher in the serum of patients with BC than healthy controls in the current study. The combined ROC curve of lncRNA UCA1 and circSHKBP1 distinguishing BC patients from controls had an AUC of 0.804. The diagnostic performance of lncRNA UCA1 and circSHKBP1 was more optimal for bladder tumor with low volume, small number, and low grade. It seems to be related to the limited number of study cases and baseline clinical characteristics of participants.

Previous research has shown that circPRMT5 is upregulated in serum from patients with BC, and high expression of circPRMT5 indicates poorer disease-free survival, suggesting that circPRMT5 may provide prognostic potential for predicting relapse [25]. It has also been observed that circVANGL1 is highly expressed in BC tissues compared with adjacent normal tissues, indicating that circVANGL1 may serve as a prognostic marker for patients with BC [26]. However, none of these studies has investigated the use of these biomarkers in patients with BC recurrence.

The present study demonstrated that the expression levels of circFARSA, circSHKBP1, and circBANP were significantly higher in the serum of the recurrent group than in that of the non-recurrent group. The ROC curve of the combined circFARSA and circBANP distinguishing recurrent from non-recurrent patients had an AUC of 0.737. Our study was similar to a recent study which indicated that a 6-miRNA signature (miR-16, 21, 34a, 200c, 205, 221) provided an improved ability to predict the presence of BC [27], however, the difference depends whether or not an independent verification group was established.

This study had limitations. First, the number of study participants was small, which might have slightly overestimated the precision of diagnostic performance. Secondly, there were slight age differences between patients in the bladder cancer group and patients in the healthy control group, which might have mildly affected the sensitivity of results.

The current study revealed that the combinations of circRNAs and lncRNA in serum from BC patients might serve as diagnostic and prognostic indicators. A larger independent validation study is planned to consolidate our conclusions.

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Disclosure of conflict of interest

None.

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Table S1. Summary of the area under the ROC curve using lncRNA UCA1 and circRNAs to distinguish patients with bladder cancer from healthy controls

	AUC	*P*-value	95% CI
lncRNA UCA1	0.754	0.018	0.573-0.936
circFARSA	0.567	0.533	0.344-0.790
circSHKBP1	0.719	0.042	0.533-0.904
circBANP	0.594	0.383	0.384-0.804
lncRNA UCA1+circFARSA+circSHKBP1	0.679	0.096	0.483-0.874
lncRNA UCA1+circSHKBP1	0.804	0.005	0.649-0.959

Table S2. Summary of the area under the ROC curve lncRNA UCA1 and circSHKBP1 to separate clinicopathologic characteristics of patients with bladder cancer from healthy controls

	AUC	*P*-value	95% CI
Tumor size			
≥ 3 cm (n = 9)	0.603	0.378	0.394-0.813
< 3 cm (n = 5)	0.870	0.011	0.662-1.000
Tumor number			
> 2 (n = 5)	0.696	0.173	0.415-0.977
≤ 2 (n = 9)	0.725	0.054	0.538-0.912
Tumor grade			
High (n = 8)	0.585	0.482	0.378-0.792
Low (n = 6)	0.861	0.007	0.000-1.000

Table S3. Summary of the area under the ROC curve using lncRNA UCA1 and circRNAs to distinguish recurrent from non-recurrent

	AUC	*P*-value	95% CI
lncRNA UCA1	0.590	0.385	0.379-0.800
circFARSA	0.705	0.047	0.502-0.908
circSHKBP1	0.615	0.256	0.411-0.820
circBANP	0.699	0.045	0.506-0.891
circFARSA+circSHKBP1+circBANP	0.569	0.562	0.322-0.816
circSHKBP1+circBANP	0.737	0.025	0.532-0.942

Table S4. Summary of the area under the ROC curve using circFARSA and circBANP to distinguish clinicopathologic characteristics of recurrent from non-recurrent

	AUC	*P*-value	95% CI
Tumor size			
≥ 3 cm (n = 5)	0.642	0.333	0.327-0.977
< 3 cm (n = 10)	0.700	0.082	0.460-0.941
Tumor number			
> 2 (n = 6)	0.603	0.440	0.306-0.899
≤ 2 (n = 9)	0.743	0.053	0.498-0.987
Tumor grade			
High (n = 10)	0.599	0.390	0.333-0.865
Low (n = 5)	0.839	0.030	0.690-0.989