Am J Transl Res 2022;14(7):4606-4616
www.ajtr.org /ISSN:1943-8141/AJTR0142709

Original Article

A four-miRNA signature in serum as a biomarker for bladder cancer diagnosis

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Received March 6, 2022; Accepted June 1, 2022; Epub July 15, 2022; Published July 30, 2022

Abstract: Background: Urinary bladder cancer (BCa) is globally the 10th most frequent cancer. As a novel diagnostic tool, miRNA in serum screening is non-invasive. This project aimed to determine particular serum miRNAs as novel biomarkers for diagnosing urinary BCa. Methods: We designed a three-phase study with 122 healthy controls (HCs) and 132 BCa patients. The 30 miRNAs' expressions in serum from HCs and BCa patients were detected during the screening phase. The miRNAs with the most dysregulation were tested in the training (HCs vs. BCa, 30 each) and validation (80 HCs vs. 82 BCa) phase further. The diagnostic ability of these candidate miRNAs was estimated by the receiver operating characteristic (ROC) curves as well as the area under the ROC curve (AUC). The miRNAs' target genes and their annotations to functions were predicted utilizing bioinformatic assays. Results: Six serum miRNAs (miR-124-3p, miR-182-5p, miR-1-3p, miR-196a-5p, miR-23b-3p and miR-34a-5p) had significantly different expression between BCa patients and HCs in the training and validation phase. The four-microRNA panel improved the diagnostic value, with AUC =0.985. The result of bioinformatic analysis showed that these miRNAs' target genes in the panel may be related to the MAPK signaling pathway in bladder cancer. Conclusions: Our study identified a four-miRNA panel that is a non-invasive new biomarker for diagnosing BCa.

Keywords: Bladder cancer, miRNA, biomarker, diagnosis, bioinformatics

Introduction

Bladder cancer (BCa) is globally the 10th most frequent cancer. According to the Cancer Statistics in 2018, there are estimated to be 829,620 living patients with BCa in the US. For BCa, usually, men are more likely to suffer with about a 3-fold higher incidence than women [1]. Among an estimated 80,470 new cases of 2019, men account for approximately 76.6% (61,700). Environmental or occupational exposures, especially tobacco, had largely contributed to differences in incidence rates by gender [2]. More than 17,600 deaths will be expected in 2019 [3]. BCa's five-year relative survival rate depends on the tumor stage, and the corresponding survival rate of stage 0 was 75%. From stage 1 to launch IV, the survival rate dropped from 79% to 12% [4]. Thus, to diagnose the disease early is key to enhance the prognosis of BCa. Conventionally, the measures for screening or diagnosing bladder cancer are urinary cytology, cystoscopy and Imaging. However, the advantages and disadvantages of them are worth discussing. For example, with low sensitivity (40%-60%) and high specificity (90%-100%), cytology is considered as an auxiliary examination frequently. Cystoscopy can discern cancerous lesions visually but is aggressive and risks infection [5, 6]. Therefore, diagnostic tools for bladder cancer that are effective and less invasive are urgently needed.

Recently, researchers suggested some biomarkers from body fluid could be used as screening tools, such as microRNA (miRNA) extracted from urine in stomach cancer patients [7]. The main reason, was that miRNAs by virtue of their versatile roles in biological process,

Table 1. 254 participants' demographic and clinical manifestations (BCa and HCs)

	Screening Phase (n=32)			Training Phase (n=60)			Validation Phase (n=162)		
	BCa (%)	HC (%)		BCa (%)	HC (%)		BCa (%)	HC (%)	
Total Number	20	12		30	30		82	80	
Age at diagnosis			P=0.66			P=0.42			P=0.74
≤60	8 (40.0)	5 (41.7)		8 (26.7)	11 (36.7)		31 (37.8)	36 (45.0)	
>60	12 (60.0)	7 (58.3)		22 (73.3)	19 (63.3)		51 (62.2)	44 (55.0)	
Location									
Trigonum	2 (10.0)			1 (3.3)			6 (7.3)		
Anterior Bladder wall	3 (15.0)			4 (13.3)			10 (12.2)		
Posterior Bladder wall	4 (20.0)			6 (20.0)			20 (24.4)		
Lateral Bladder wall	7 (35.0)			10 (33.4)			29 (35.4)		
Multiple locations	4 (20.0)			9 (30.0)			17 (20.7)		
Tumor stage									
pTa-pTis	8 (40.0)			11 (36.7)			37 (45.1)		
pT1	7 (35.0)			13 (43.3)			32 (39.0)		
≥ pT2	5 (25.0)			6 (20.0)			13 (15.9)		
Pathological grade									
Low grade	7 (35.0)			12 (40.0)			32 (39.0)		
High grade	13 (65.0)			18 (60.0)			50 (61.0)		

It showed insignificant difference between BCa and HCs in age among three phases. Parameters are marked as numbers (percentages). Statistical contrast was carried out via the Wilcoxon-Mann Whitney test.

such as cell growth, tissue differentiation, inflammation, and tumorigenesis, attached much attention [8]. Although without a coding function, miRNAs regulate the post-transcriptional regulation of messenger RNAs in various ways [9]. More importantly, exosomes, one kind of extracellular vesicles, enclose miRNAs and exist in body fluid [10]. Currently, many miRNAs have roles in tumors, including bladder cancer, according to their features. Therefore, it is essential to screen representative miRNAs for assisting the diagnosis of bladder cancer.

A robust 4-miRNAs panel for preliminary screening by detecting extracted miRNAs' expressions and establishing the receiver operating characteristic (ROC) curve finally was constructed in our experiment with 254 participants' serum (132 BCa patients and 122 healthy controls (HCs)) included. As a non-invasive and effective method, our 4-miRNA signature could be a diagnostic biomarker. In addition, we used bioinformatic analysis to further predict the mechanism of the 4 selected miRNAs in bladder cancer, which might also help in prognosis or therapy of BCa.

Materials and methods

Subjects' collection and disposal

Firstly, the Ethics Committee of Shenzhen Hospital, Peking University authorized all the processes of our project, and the number of the approval was 2017-007. The research was conducted based on the World Medical Association Declaration of Helsinki and each participant was informed of the serum usage. In this experiment, total 254 participants (132 BCa patients and 122 HCs) were enrolled from November 2017 to August 2019. All BCa patients were verified by pathologic diagnosis and HCs were enrolled on the premise of no underlying health problems and interfering factors. The detailed information of all participants can be found in **Table 1**.

Each serum specimen we gathered was used immediately within 2 hours. We treated them by centrifuge at 1000 g for 10 min and 15,000 g for 5 min at a temperature of 4 degrees Celsius and stored them at a temperature of -80 degrees Celsius in fresh tubes.

Experiment flow

First, searching for bladder cancer-related miRNAs, we read literature in the PubMed or referred to in the Gene Expression Omnibus database. 30 highly relevant miRNAs were confirmed through the retrieval strategy ("Urinary Bladder Neoplasms" [Mesh] OR (bladder cancer [Title/Abstract])) AND (microRNA OR "MicroRNAs" [Mesh] OR miRNA). Corresponding

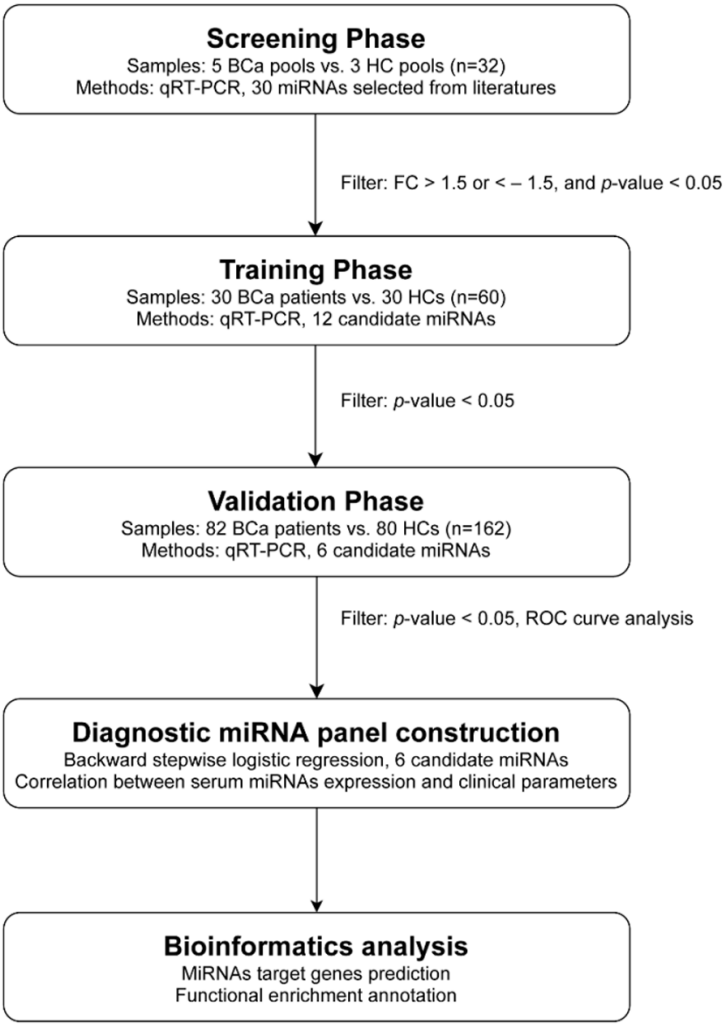

Figure 1. Study design overview. Study design overview. HCs: healthy controls; BCa: bladder cancer.

reference summary of 30 candidate miRNAs is shown in Table S1. Next, a three-phase study elaborated in **Figure 1** was conducted. In this process, high throughput qRT-PCR was employed for evaluating the miRNA profile expression level in serum specimens. 20 serum samples from bladder cancer patients and 12 healthy control serums were randomly picked up and built into 8 pools for preliminary screening. Each sample pool had 4 samples. The cut-off criterion was p<0.05, and the fold change (FC) was either more than 1.5 or less than -1.5. Then, in the training phase, other random samples including 30 BCa patients and 24 HCs were used for 12 candidate miRNAs' identification under the cut-off criterion P<0.05. Finally, we further validated the 12 candidate miRNAs with the remaining serum to assure stability and acquired 4 highly representative miRNAs. In addition, by means of backward stepwise logistic regression and ROC, the diagnostic use of 4 representative miRNAs and the 4-miRNA panel was clarified.

DAVID database (version 6.8) (http://david.abcc.ncifcrf.gov/) and MiRWalk3.0 (http://mirwalk.umm.uni-heidelberg.de/) were adopted respectively in bioinformatics analysis for predicting target genes and making functional enrichment analysis [11-13]. Gene Ontology (GO) annotation and Kyoto encyclopedia of genes and genomes (KEGG) enrichment analysis were employed. GO comprises biological process (BP), cellular component (CC) and molecular function (MF).

MiRNA extraction and qRT-PCR details

2 μl of synthesized C. elegans miR-39 (cel-miR-39) (10 nM/L, RiboBio, Guangzhou, China) was added to the prepared serum samples before miRNA extraction and purification. We used the following kits or devices after correctly complying with the manufacturer's guidelines. The miRNAs were extracted using TRIzol LS isolation kit (Thermo Fisher Scientific, Waltham, MA, USA), and then 30 μl of water free of RNase was used to dissolve miRNAs. The concentration and purity of miRNA were measured using NanoDrop 2000 spectrophotometer (NanoDrop, Wilmington, DE, USA).

Next, qRT-PCR, adding Bulge-Loop miRNA qRT-PCR Primer Set (RiboBio, Guangzhou, China) was the first important step for miRNA amplification. A sequence of steps was performed with the help of LightCycler 480 Real-Time PCR System (Roche Diagnostics, Mannheim, Germany) and SYBR Green qPCR kit (SYBR Premix Ex Taq II, TaKaRa) in 384-well plates. Step 1, 30 s was at 95 degrees Celsius; step 2, 30-time repetitions, done for 10 s at 95 degrees Celsius, 20 s at 60 degrees Celsius, and 10 s at

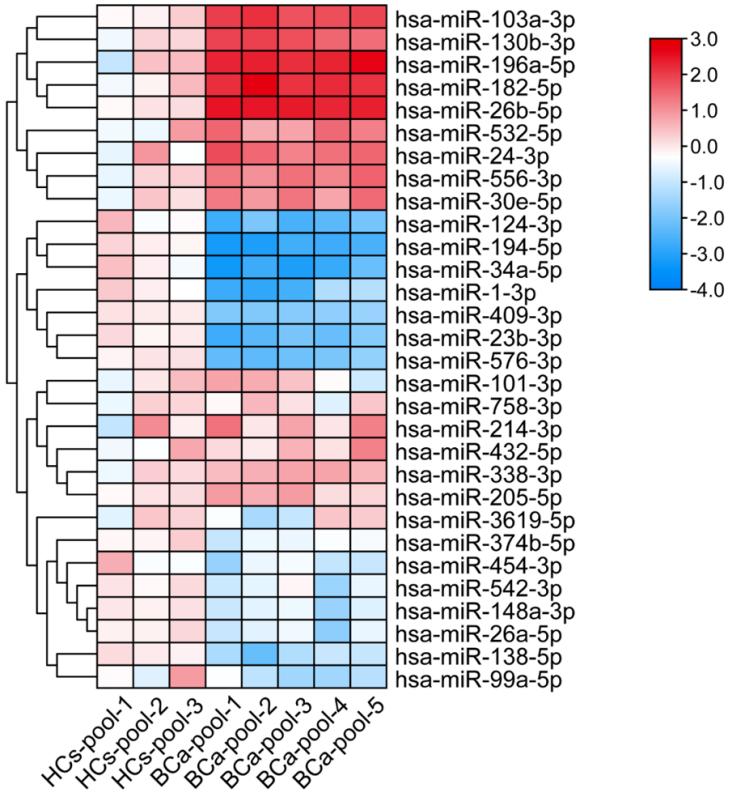

Figure 2. Heatmap of the 30 miRNAs during the screening phase. During the screening phase, 30 miRNAs' expressions are shown in the heatmap. 12 candidate miRNAs were chosen for further study with 1.5 FC and P<0.05. The red means up-regulated and the blue means down-regulated.

70 degrees Celsius. Ultimately, we standardized expression level of control cel-miR-39 and identified the target miRNAs' relative expression levels utilizing the $2^{-\Delta\Delta Cq}$ method [14].

Statistical analysis

The software used in our study included SPSS software (SPSS 20.0 Inc, Chicago, IL), Medcalc (Version 19), and GraphPad Prism 8 (GraphPadSoftware Inc, La Jolla, CA). The parameters regarding clinical features of participants were expressed according to digital traits, as countable numbers with percentages and continuous ones with mean ± standard deviation. Different data were analyzed by optimal approaches. The Kruskal-Wallis rank test, a method of nonparametric analysis, was applied for the multiple independent sample comparisons. Moreover, we utilized Mann-Whitney test or Student's t-test for the difference between BCa patients and HCs samples regarding each miRNA's expression level, and then the profile of candidate miRNAs was depicted by multiple logistic regression analysis. Then, to assess the diagnostic ability of representative miRNAs and the miRNA panel, we used ROC curves and the area under the ROC curve (AUC).

Results

Characteristics of subjects

254 participants were enrolled in this project, including 122 HCs and 132 BCa patients. Complying with the protocols of the WHO criteria and the TNM staging system, the histologic classification and the staging were confirmed. The demographics, clinical and pathologic characteristics of these 240 subjects are shown in **Table 1**. BCa patients were not significantly different from HCs in gender or age distribution (P> 0.05).

MiRNA screening

During the screening phase, 30 miRNAs' expression levels in five BCa pools and three HCs pools were screened out to determine candidate miRNAs, as shown in **Figure 2**. Based on the criterion of FC being either more than 1.5 or less than -1.5 and P being less than 0.05, the candidate miRNAs were selected. Then, 12 serum miRNAs that were compared between BCa patients and HCs, including five up-regulated ones and seven down-regulated ones, were most differentially expressed (Table S2). Thus, we conducted further validation with these 12 candidate miRNAs during the training phase.

Evaluation of candidate miRNAs during the training phase

In the training phase, the 12 candidate miRNAs selected were verified by qRT-PCR analysis in BCa patients and HCs (30 each). Among these candidate serum miRNAs, between BCa patients and HCs, 6 miRNAs were still differentially expressed, including miR-124-3p, miR-182-5p, miR-1-3p, miR-196a-5p, miR-23b-3p,

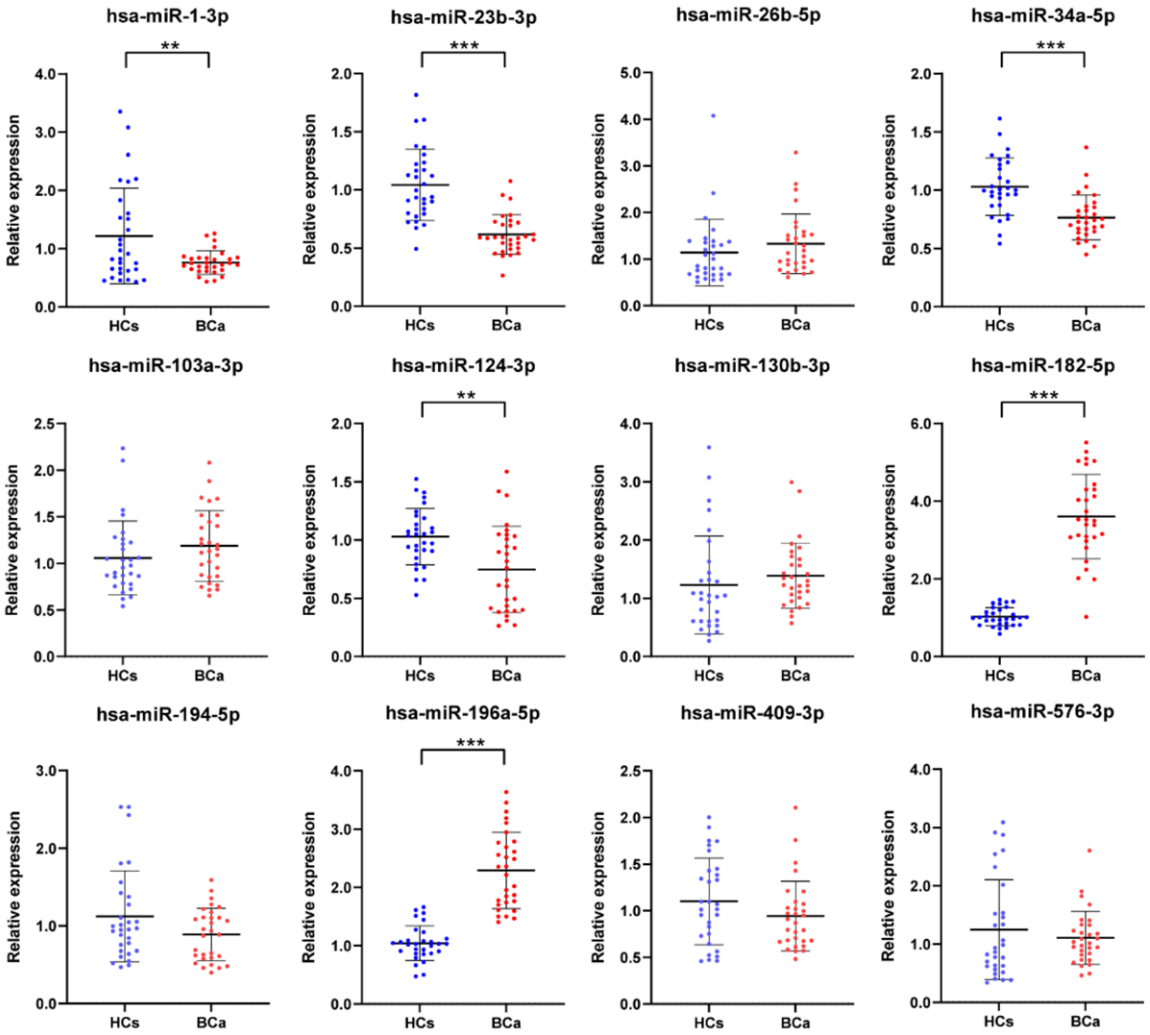

Figure 3. The 12 candidate miRNAs' expression during the training phase. The 12 candidate miRNAs' expression during the training phase with 30 BCa patients and 30 HCs. *represents P<0.05, **represents P<0.01, ***represents P<0.001.

and miR-34a-5p, as shown in **Figure 3**. Thus, these 6 candidate miRNAs were chosen for more investigation.

Diagnostic ability of candidate miRNAs during the validation phase

In the validation phase, we performed thevalidation of the expression of these 6 miRNAs in a larger population with 82 BCa patients and 80 HCs, to verify a potential role as serum biomarkers in bladder cancer diagnosis. Consistent with the results in the first two phases, as shown in **Figure 4**, miR-182-5p and miR-196a-5p had increased serum expressions in BCa patients and the other 4 miRNAs (miR-124-3p, miR-1-3p, miR-23b-3p and miR-34a-5p) were downregulated.

We plotted ROC curves to analyze the diagnostic ability of the candidate miRNAs. AUCs for miR-1-3p, miR-23b-3p, miR-34a-5p, miR-124-3p, miR-182-5p, and miR-196a-5p were 0.682 (95% confidence interval (CI): 0.604 to 0.753; **Figure 4B**), 0.748 (95% CI: 0.674 to 0.813; **Figure 4D**), 0.804 (95% CI: 0.734 to 0.862; **Figure 4F**), 0.688 (95% CI: 0.611 to 0.758; **Figure 4H**), 0.867 (95% CI: 0.805 to 0.915; **Figure 4J**) and 0.731 (95% CI: 0.656 to 0.757; **Figure 4L**), respectively.

Construction of a diagnostic panel

In order to precisely diagnose bladder cancer, combining several miRNAs might enhance accuracy in distinguishing BCa patients from HCs better than individual miRNAs. A stepwise

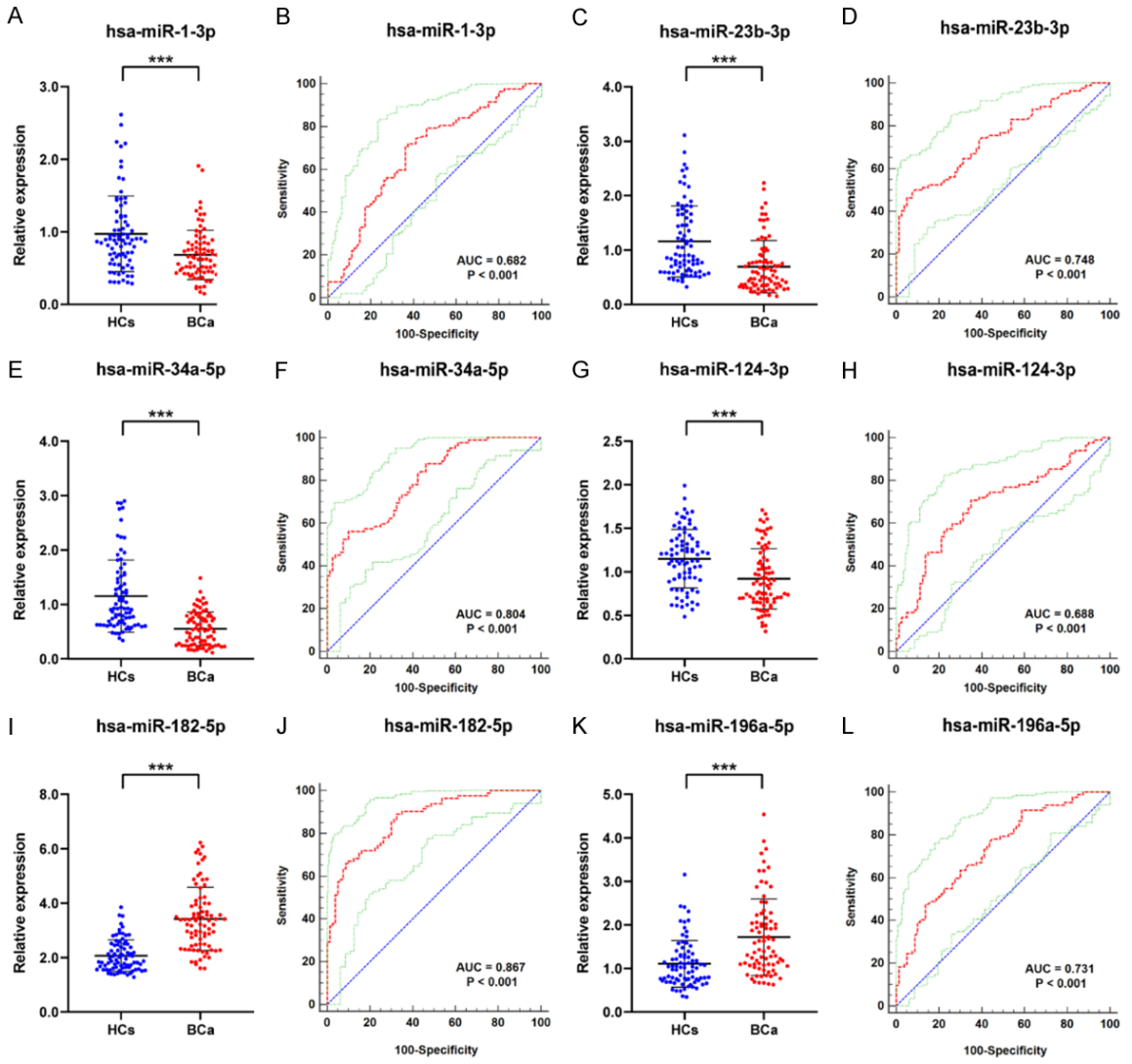

Figure 4. The expression and ROC curve analyses of six candidate miRNAs in the validation phase. In this phase, 82 BCa patients' and 80 HCs' serum were involved. The relative expression level of (A) miR-1-3p, (C) miR-23b-3p, (E) miR-34a-5p and (G) miR-124-3p were remarkably downregulated in BCa patients' serum. (I) miR-182-5p and (K) miR-196a-5p were significantly upregulated. The AUC were (B) 0.682 for miR-1-3p with 95% CI: 0.604 to 0.753, (D) 0.748 for miR-23b-3p with 95% CI: 0.674 to 0.813, (F) 0.804 for miR-34a-5p with 95% CI: 0.734 to 0.862, (H) 0.688 for miR-124-3p with 95% CI: 0.611 to 0.758, (J) 0.867 for miR-182-5p 95% CI: 0.805 to 0.915 and (L) 0.731 for miR-196a-5p with 95% CI: 0.656 to 0.757. *represents P<0.05, **represents P<0.01, ***represents P<0.001.

logistic regression model was utilized to build diagnostic panels. As a result, a four-miRNA panel was produced to diagnose bladder cancer best, and below is the equation the model followed: Logit(P) = -3.576 + 3.304 × miR-182-5p + 3.361 × miR-196a-5p - 2.508 × miR-124-3p - 7.579 × miR-34a-5p. The four-miRNA panel's AUC was 0.985 (95% CI: 0.952 to 0.998; sensitivity =98.78%, specificity =93.75%; **Figure 5**) by ROC analysis.

Correlation of expression of serum miRNAs with clinical manifestations

We explored the correlation of expression of serum miRNAs with clinical manifestations to explore their clinical application, as shown in **Table 2**. All the training phase and validation phase data were included for analysis. Our results showed that candidate serum miRNAs expression levels did not correlate with location, tumor stage. or pathological grade.

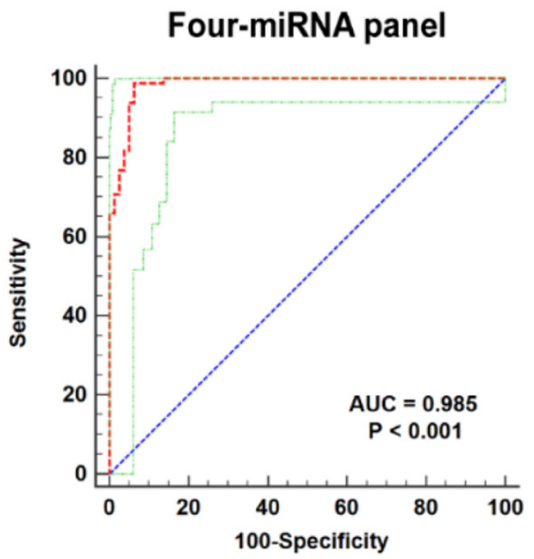

Figure 5. Four-miRNA panel's ROC curve analyses. The four-miRNA panel's AUC (miR-182-5p, miR-196a-5p, miR-124-3p and miR-34a-5p) was 0.985 (95% CI: 0.952 to 0.998; sensitivity =98.78%, specificity =93.75%).

Bioinformati analysis

The miRWalk 3.0 was adopted for predicting candidate miRNAs' possible target genes. Target genes were selected if they were predicted by two or more miRNAs. The 1840 genes were picked as target genes for the next analysis. Then, they were mapped into the DAVID database for KEGG pathway analysis and GO functional annotation. We showed the top 5 of the BP, CC, MF, and KEGG pathways in **Figure 6**, including GO:0007399~neurogenesis, GO:0043065~positive regulation of the cell death process and GO:0006397~mRNA processing in the BP category; GO:0005829~cytosol, GO:0005794~Golgi apparatus and GO:0005737~cytoplasm in the CC category; GO:0005515~binding of protein, GO:0004672~kinase activity of protein and GO:0004702~serine/threonine kinase activity of receptor signaling protein in the MF category; hsa04010:MAPK signaling pathway, hsa04012:ErbB signaling pathway; and hsa05205:Proteoglycans in KEGG pathway analysis-enriched cancer.

Discussion

On the basis of the prediction from the American Cancer Society in 2020, for males bladder cancer is the fourth most common malignant tumor in US [15]. Especially with its non-invasive nature, screening serum miRNA is a widely applicable and novel diagnostic tool. Numerous studies have concentrated on the serum miRNAs' diagnostic ability in BCa [6, 16, 17]. In our project, we devised a three-phase study to determine serum miRNAs' detection performance. First, 30 miRNAs' differential expression profiles were analyzed in 5 BCa pools and 3 HCs pools. Afterwards, 12 miRNAs were most dysregulated between BCa patients and HCs, and were chosen as candidate miRNAs. As a result, 6 serum miRNAs (miR-1-3p, miR-23b-3p, miR-34a-5p, miR-124-3p, miR-182-5p and miR-196a-5p) were significantly dysregulated in bladder cancer patients compared to HCs. Lastly, a four-miRNA panel was constructed and may play a serum marker role in bladder cancer early diagnosis (AUC =0.985 (95% CI: 0.952 to 0.998; sensitivity =98.78%, specificity =93.75%)).

Among the 4 miRNAs in the panel, miR-182-5p might help distinguish BCa patients from HCs with AUC =0.867; 95% CI: 0.805 to 0.915. Evidence has shown that miR-182-5p might have an oncogene role, activating the Wnt-beta-catenin signaling pathway through the knockdown of RECK and Smad4 in BCa [18]. Through targeting miR-182-5p, the long-chain noncoding RNA (lncRNA) ADAMTS9-AS2, which is a gene suppressing tumors, regulates proliferation and migration of BCa cells, and induces cell death [19]. Moreover, Fei Xie et al. revealed that circular RNA BCRC-3, through the miR-182-5p/p27 axis, could suppress bladder cancer cell proliferation functioning as a tumor inhibitor [20]. UBAC2 (the ubiquitin-associated domain-containing protein 2) gene could inhibit the expression of p27 and promote proliferation of BCa by the BCRC-3/miRNA-182-5p/p27 axis [21]. All these findings show that miR-182-5p is important in urinary bladder cancer.

Herein, the miR-34a-5p was a suitable biomarker for BCa diagnosis with (AUC =0.804; 95% CI: 0.734 to 0.862). Cavallari et al. maintained that high urine levels of miR-34a-5p in BCa patients were related to a poor prognosis ($P<0.05$ and hazard ratios >3.1) by univariate Cox proportional hazards regression analyses [22]. In urothelial cancer, SLIT3 was reported as a prognostic biomarker of survival, and miR-34a-5p may function in the changed tumor

Table 2. Correlation of the serum miRNAs' relative expression levels and clinical parameters (training and validation phases)

	hsa-miR-124-3p		hsa-miR-182-5p		hsa-miR-196a-5p		hsa-miR-34a-5p	
Location		P=0.40		P=0.87		P=0.16		P=0.98
Single location	0.90±0.34		3.50±1.29		1.76±0.83		0.54±0.30	
Multiple locations	0.98±0.36		3.40±1.14		1.56±1.06		0.55±0.31	
Tumor stage		P=0.29		P=0.28		P=0.33		P=0.76
< pT2	0.83±0.41		3.84±1.39		1.87±1.01		0.53±0.33	
≥ pT2	0.93±0.39		3.57±1.33		1.74±0.97		0.58±0.29	
Pathological grade		P=0.75		P=0.42		P=0.08		P=0.89
Low grade	0.95±0.35		3.39±1.44		1.82±0.91		0.52±0.29	
High grade	0.91±0.32		3.66±1.35		1.61±0.98		0.55±0.32	

The parameter value is displayed as mean ± SD. Statistical analysis was made via the Wilcoxon-Mann Whitney test.

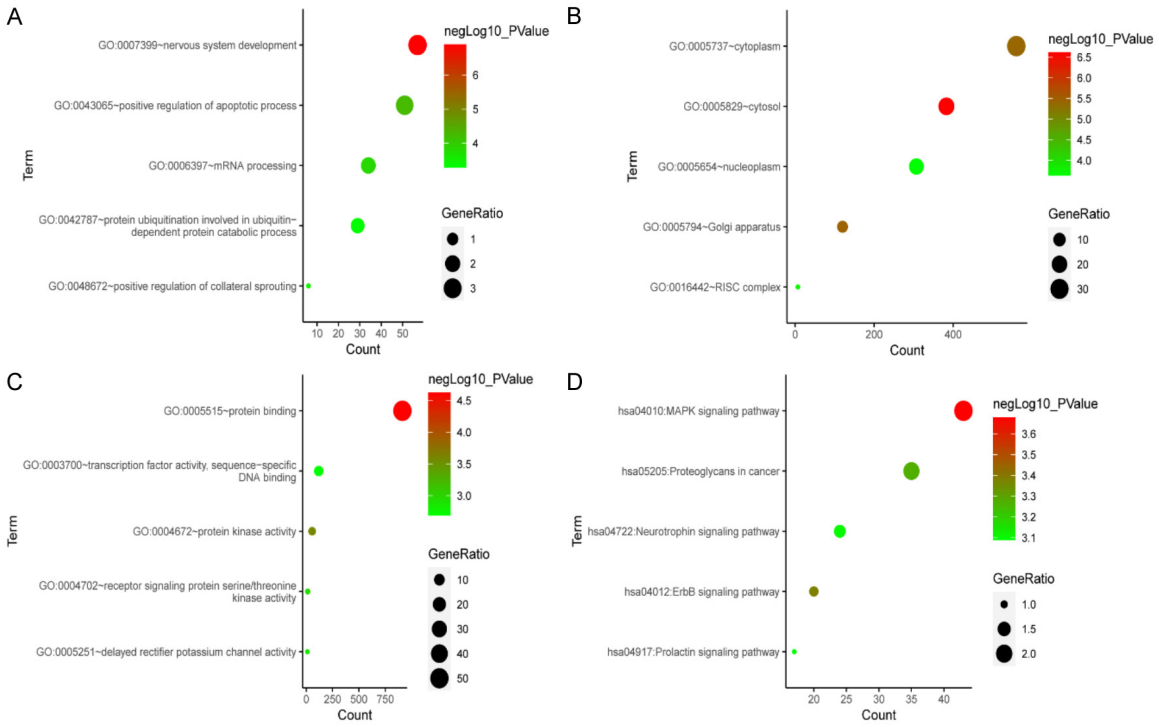

Figure 6. KEGG pathway enrichment analysis and GO annotation of the target genes of miR-182-5p, miR-196a-5p, miR-124-3p and miR-34a-5p. A. Biological process analysis; B. Cellular component analysis; C. Molecular function analysis; D. KEGG analysis.

microscale environment in upper tract urothelial carcinoma by regulating SLIT3 [23]. In the field of cancer, immune checkpoint inhibitors have been an important therapeutic advancement. Through regulating the miR-34a-5p/PNUTS regulation axis, the exosomes produced by macrophages treated with PD-1 inhibitor can promote aging [24].

Many studies have focused on miR-196a-5p's role in cancer. In colorectal cancer, miR-196a-5p, by targeting the IκBα, may affect invasion, metastasis, and epithelial-mesenchymal transition (EMT) [25]. By targeting Smad4, miR-196a-5p has an important role in invasion and EMT in gastric cancer stem cells, and miR-196a-5p may function as a possible target for treating gastric cancer [26]. Xu et al. reported that a novel LOC134466/hsa-miR-196a-5p/TAC1 regulatory axis by activating TACR3, activated neuroactive ligand-receptor interaction in endometrial adenocarcinoma [27]. In BCa,

through modulating CREB-upregulated miR-196a-5p targeting p27Kip1, lncRNA UCA1 enhances cisplatin/gemcitabine resistance [28]. Further research on miR-196a-5p in urinary BCa is needed.

Studies show has-miR-124-3p is crucial in suppressing BCa progression.Xu et al. said that miR-124-3p by regulating ROCK1, could suppress BCa cells' invasion and migration [29]. Also, research showed that miR-124-3p suppressed BCa cell invasion and migration by targeting integrin α3 (ITGA3) and downstream FAK/Src and FAK/PI3K/AKT signaling pathways [30]. Through targeting DNA methyltransferase 3B (DNMT3B), miR-124-3p can restrain the invasion, migration, and proliferation, and boost cell death of BCa cells [31]. By suppressing the expression of endothelin receptor type B (EDNRB), miR-124-3p can regulate BCa cell proliferation and induce cell death [32]. By targeting Aurora A kinase (AURKA), miR-124-3p influences migration, apoptosis, and proliferation of BCa cells [33]. The aforementioned studies show that miR-124-3p can suppress tumor and regulate tumorigenesis and progression in urinary BCa.

The MAPK signaling pathway, ErbB signaling pathway, and proteoglycans in cancer were enriched by KEGG pathway analysis. Previous studies have reported that the MAPK signaling pathway is related to urinary bladder cancer. For example, through regulating MiR-145-5p and Myd88, circular RNA CEP128 can promote BCa progression by the MAPK signaling pathway [34]. Maslinic acid induced BCa cell death by inhibiting p38 MAPK pathway activation [35]. By regulating the MAPK signaling pathway, IL-6 could accelerate proliferation and improve the immune suppressive ability of myeloid-derived suppressor cells in bladder cancer [36]. As for the proteoglycans in cancer pathway, Papadaki et al. reported that two proteoglycans, osteomodulin and proline/arginine-rich end leucine repeat protein, activators of urothelial cell-cell adhesion, suppressed initiation and progression in bladder cancer [37].

Though the results of our project are meaningful, there are some limitations in our project. First, our project had a small sample size, and it could be more reliable if we added an external validation set to the experiment. Second, in the initial phases, we explored only 30 miRNAs to avoid possibly neglecting quantitative experiments and data processing. We should continue to investigate the potential possibilities of other miRNAs in diagnosing BCa, verifying their value as clinical biomarkers and studying their biologic effects. There have been some published studies on the signature of miRNA prognosis related to bladder cancer [16, 38-40]; we could further explore the prognostic value of this panel in the future. Our study concentrated on the diagnostic ability of serum miRNAs in bladder cancer and there were some studies paying attention to miRNA in urine to detect bladder cancer [17, 41-44]. We might enhance the diagnostic value by applying this panel in serum and urine.

In summary, we showed 6 serum miRNAsdysregulated between BCa patients and HCs. We identified a four-miRNA panel (miR-182-5p, miR-196a-5p, miR-124-3p and miR-34a-5p) in serum for bladder cancer diagnosis, with prominent diagnostic ability and AUC =0.985. The four-miRNA panel could become a new biomarker for diagnosing BCa, which is non-invasive.

Acknowledgements

This study received support from Clinical Research Project of Peking University Shenzhen Hospital (LCYJ2017001), Basic Research Project of Peking University Shenzhen Hospital (JCYJ2017001, JCYJ2017004, JCYJ2017005, JCYJ2017006, JCYJ2017007, JCYJ2017012), Clinical Research Project of Shenzhen Health Commission (No. SZLY2018023), Science and Technology Development Fund Project of Shenzhen (No. JCYJ20180507183102747) and Shenzhen High-level Hospital Construction Fund.

Disclosure of conflict of interest

None.

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Table S1. Corresponding reference summary of 30 candidate miRNAs

candidate miRNA	Corresponding reference title
hsa-miR-26b-5p	1. miRNA-26a-5p and miR-26b-5p inhibit the proliferation of bladder cancer cells by regulating PDCD10
	2. Tumor-suppressive miRNA-26a-5p and miR-26b-5p inhibit cell aggressiveness by regulating PLOD2 in bladder cancer
	3. MicroRNA dysregulation and non-muscle-Invasive bladder cancer prognosis
hsa-miR-196a-5p	1. Long non-coding RNA UCA1 promotes cisplatin/gemcitabine resistance through CREB modulating miR-196a-5p in bladder cancer cells
	2. Gene expression profiling of microRNAs associated with UCA1 in bladder cancer cells
hsa-miR-182-5p	1. miRNA-182-5p promotes human bladder cancer proliferation and migration through the FOXF2/SHH axis
	2. LncRNA UCA1/miR-182-5p/MGMT axis modulates glioma cell sensitivity to temozolomide through MGMT-related DNA damage pathways
	3. Long-chain noncoding RNA ADAMTS9-AS2 regulates proliferation, migration, and apoptosis in bladder cancer cells through regulating miR-182-5p
	4. UBAC2 promotes bladder cancer proliferation through BCRC-3/miRNA-182-5p/p27 axis
hsa-miR-103a-3p	1. Screening differential circular RNA expression profiles reveals the regulatory role of circTCF25-miR-103a-3p/miR-107-CDK6 pathway in bladder carcinoma
	2. Serum microRNA expression signatures as novel noninvasive biomarkers for prediction and prognosis of muscle-invasive bladder cancer
hsa-miR-130b-3p	1. Genome-Wide Screen of miRNAs and Targeting mRNAs Reveals the Negatively Regulatory Effect of miR-130b-3p on PTEN by PI3K and Integrin β1 Signaling Pathways in Bladder Carcinoma
	2. Pharmacological Inhibition of miR-130 Family Suppresses Bladder Tumor Growth by Targeting Various Oncogenic Pathways via PTPN1
	3. miR-130b promotes bladder cancer cell proliferation, migration and invasion by targeting VGLL4
hsa-miR-24-3p	1. miR-24-3p regulates bladder cancer cell proliferation, migration, invasion and autophagy by targeting DEDD
	2. MicroRNA-24 upregulation inhibits proliferation, metastasis and induces apoptosis in bladder cancer cells by targeting CARMA3
hsa-miR-556-3p	1. MicroRNA Dysregulation and Non-Muscle-Invasive Bladder Cancer Prognosis
hsa-miR-30e-5p	1. miR-30e-5p suppresses cell proliferation and migration in bladder cancer through regulating metadherin
	2. Up-regulation of microRNA in bladder tumor tissue is not common
	3. A novel messenger RNA and long noncoding RNA signature associated with the progression of nonmuscle invasive bladder cancer
hsa-miR-532-5p	1. Downregulation of microRNA-532-5p promotes the proliferation and invasion of bladder cancer cells through promotion of HMGB3/Wnt/β-catenin signaling
	2. Prognostic Stratification of Bladder Cancer Patients with a MicroRNA-based Approach
hsa-miR-214-3p	1. MiR-214-3p regulates the viability, invasion, migration and EMT of TNBC cells by targeting ST6GAL1
	2. Non-coding RNA NEAT1/miR-214-3p contribute to doxorubicin resistance of urothelial bladder cancer preliminary through the Wnt/β-catenin pathway
	3. miR-199a-3p and miR-214-3p improve the overall survival prediction of muscle-invasive bladder cancer patients after radical cystectomy
hsa-miR-338-3p	1. Circular RNA_0000326 promotes bladder cancer progression via microRNA-338-3p/ETS Proto-Oncogene 1/phosphoinositide-3 kinase/Akt pathway
	2. MicroRNA-338-3p inhibits the progression of bladder cancer through regulating ETS1 expression
	3. Identification of circRNA-miRNA-mRNA Regulatory Network in Bladder Cancer by Integrated Analysis
hsa-miR-205-5p	1. Circ0001429 regulates progression of bladder cancer through binding miR-205-5p and promoting VEGFA expression
	2. miR-21-5p, miR-141-3p, and miR-205-5p levels in urine-promising biomarkers for the identification of prostate and bladder cancer
	3. MicroRNA Profiling in Patients with Upper Tract Urothelial Carcinoma Associated with Balkan Endemic Nephropathy
	4. Prognostic Stratification of Bladder Cancer Patients with a MicroRNA-based Approach
hsa-miR-432-5p	1. Down-regulated RBM5 inhibits bladder cancer cell apoptosis by initiating an miR-432-5p/β-catenin feedback loop
	2. Negative Correlation Between Circular RNA SMARC5 and MicroRNA 432, and Their Clinical Implications in Bladder Cancer Patients
hsa-miR-101-3p	1. MicroRNA-101-3p advances cisplatin sensitivity in bladder urothelial carcinoma through targeted silencing EZH2
	2. 1,25D 3 differentially suppresses bladder cancer cell migration and invasion through the induction of miR-101-3p
	3. LncRNA SPRY4-IT1 sponges miR-101-3p to promote proliferation and metastasis of bladder cancer cells through up-regulating EZH2
	4. LncRNA-MALAT1 mediates cisplatin resistance via miR-101-3p/VEGF-C pathway in bladder cancer

4-miRNA panel for bladder cancer diagnosis

hsa-miR-758-3p	1. lncRNA CASC9 sponges miR-758-3p to promote proliferation and EMT in bladder cancer by upregulating TGF-β2
	2. miR-758-3p suppresses human bladder cancer cell proliferation, migration and invasion by targeting NOTCH2
hsa-miR-3619-5p	1. MicroRNA-3619-5p suppresses bladder carcinoma progression by directly targeting β-catenin and CDK2 and activating p21
hsa-miR-374b-5p	1. Bladder cancer-associated transcript 1 promotes melanoma cell proliferation and invasion via the miR-374b-5p/U2-associated factor homology motif kinase 1 axis
	2. MiR-454-3p and miR-374b-5p suppress migration and invasion of bladder cancer cells through targetting ZEB2
hsa-miR-542-3p	1. Circular RNA circ_0000515 adsorbs miR-542-3p to accelerate bladder cancer progression via up-regulating ILK expression
	2. MicroRNA-542-3p suppresses cellular proliferation of bladder cancer cells through post-transcriptionally regulating survivin
hsa-miR-148a-3p	1. miR-148a-3p inhibits the proliferation and migration of bladder cancer via regulating the expression of ROCK-1
	2. lncRNA CCAT1 promotes bladder cancer cell proliferation, migration and invasion
	3. Expression of miR-490-5p, miR-148a-3p and miR-608 in bladder cancer and their effects on the biological characteristics of bladder cancer cells
	4. miR-148a-3p represses proliferation and EMT by establishing regulatory circuits between ERBB3/AKT2/c-myc and DNMT1 in bladder cancer
hsa-miR-26a-5p	1. miRNA-26a-5p and miR-26b-5p inhibit the proliferation of bladder cancer cells by regulating PDCD10
	2. Identification of circulating microRNA signatures for upper tract urothelial carcinoma detection
	3. Enhanced plasma miR-26a-5p promotes the progression of bladder cancer via targeting PTEN
	4. Investigation of key miRNAs and target genes in bladder cancer using miRNA profiling and bioinformatic tools
hsa-miR-454-3p	1. Low Expression of hsa_circ_0018069 in Human Bladder Cancer and Its Clinical Significance
	2. MiR-454-3p and miR-374b-5p suppress migration and invasion of bladder cancer cells through targeting ZEB2
hsa-miR-99a-5p	1. microRNA-99a-5p induces cellular senescence in gemcitabine-resistant bladder cancer by targeting SMARCD1
	2. Benzyl isothiocyanate suppresses IGF1R, FGFR3 and mTOR expression by upregulation of miR-99a-5p in human bladder cancer cells
	3. MiR-99a-5p inhibits bladder cancer cell proliferation by directly targeting mammalian target of rapamycin and predicts patient survival
	4. miR-99a-5p acts as tumor suppressor via targeting to mTOR and enhances RAD001-induced apoptosis in human urinary bladder urothelial carcinoma cells
hsa-miR-138-5p	1. miR-138-5p contributes to cell proliferation and invasion by targeting Survivin in bladder cancer cells
	2. MicroRNAs in tumor samples and urinary extracellular vesicles as a putative diagnostic tool for muscle-invasive bladder cancer
	3. Evaluating adipose-derived stem cell exosomes as miRNA drug delivery systems for the treatment of bladder cancer
hsa-miR-409-3p	1. MicroRNA-409-3p inhibits migration and invasion of bladder cancer cells via targeting c-Met
	2. c-Met and CREB1 are involved in miR-433-mediated inhibition of the epithelial-mesenchymal transition in bladder cancer by regulating Akt/GSK-3β/Snail signaling
	3. Serum microRNAs as predictors of risk for non-muscle invasive bladder cancer
hsa-miR-576-3p	1. MiR-576-3p is a novel marker correlated with poor clinical outcome in bladder cancer
	2. MicroRNA-576-3p inhibits proliferation in bladder cancer cells by targeting cyclin D1
hsa-miR-1-3p	1. MiR-1/GOLPH3/Foxo1 Signaling Pathway Regulates Proliferation of Bladder Cancer
	2. MiR-1-3p inhibits cell proliferation and invasion by regulating BDNF-TrkB signaling pathway in bladder cancer
	3. RNA-binding protein HuR promotes bladder cancer progression by competitively binding to the long noncoding HOTAIR with miR-1
	4. miR-1-3p suppresses proliferation of hepatocellular carcinoma through targeting SOX9
	5. MiR-1-3p inhibits the proliferation and invasion of bladder cancer cells by suppressing CCL2 expression
hsa-miR-23b-3p	1. Deregulation of seven CpG island-harboring miRNAs in bladder cancer: miR-155 and miR-23b as the most promising oncomiRs
	2. MicroRNA-23b functions as a tumor suppressor by regulating Zeb1 in bladder cancer
	3. Investigation of key miRNAs and target genes in bladder cancer using miRNA profiling and bioinformatic tools
hsa-miR-124-3p	1. The effect of miR-124-3p on cell proliferation and apoptosis in bladder cancer by targeting EDNRB
	2. MicroRNA-124-3p suppresses cell migration and invasion by targeting ITGA3 signaling in bladder cancer
	3. MiR-124-3p suppresses bladder cancer by targeting DNA methyltransferase 3B
	4. MicroRNA-124-3p affects proliferation, migration and apoptosis of bladder cancer cells through targeting AURKA
	5. MicroRNA-124-3p inhibits cell migration and invasion in bladder cancer cells by targeting ROCK1

hsa-miR-34a-5p	1. Combined miRNA and SERS urine liquid biopsy for the point-of-care diagnosis and molecular stratification of bladder cancer
	2. Knockdown of long non-coding RNA metastasis associated lung adenocarcinoma transcript 1 inhibits the proliferation and migration of bladder cancer cells by modulating the microRNA-34a/cyclin D1 axis
	3. miR34a/GOLPH3 Axis abrogates Urothelial Bladder Cancer Chemoresistance via Reduced Cancer Stemness
hsa-miR-194-5p	1. lncRNA TUG1 Promotes Cisplatin Resistance by Regulating CCND2 via Epigenetically Silencing miR-194-5p in Bladder Cancer
	2. MiR-194-5p inhibits cell migration and invasion in bladder cancer by targeting E2F3
	3. LncRNA PVT1 accelerates malignant phenotypes of bladder cancer cells by modulating miR-194-5p/BCLAF1 axis as a ceRNA

Table S2. The statistical difference of 30 miRNAs between 5 BCa pools and 3 HCs pools at the screening phase

ID	logFC	AveExpr	t	*P*-value	adj.P.Val	B
hsa-miR-26b-5p	2.422666667	1.514166667	12.61739715	8.43E-08	1.48E-06	8.581782264
hsa-miR-196a-5p	2.384844445	1.490527778	7.08496765	2.25E-05	6.13E-05	2.796793211
hsa-miR-182-5p	2.298	1.43625	8.663604858	3.46E-06	1.30E-05	4.752748643
hsa-miR-103a-3p	1.942888889	1.214305556	9.231766822	1.88E-06	9.52E-06	5.388317563
hsa-miR-130b-3p	1.751333333	1.094583334	7.257811189	1.81E-05	5.42E-05	3.025706043
hsa-miR-24-3p	1.470666666	0.9525	4.443442425	0.001035908	0.002061791	-1.207752092
hsa-miR-556-3p	1.302222223	0.813888889	5.225278036	0.000301333	0.000753332	0.077868746
hsa-miR-30e-5p	1.176	0.735	4.406960788	0.001099622	0.002061791	-1.26963538
hsa-miR-532-5p	1.160777778	0.725486111	3.31145727	0.007108107	0.012543717	-3.184628509
hsa-miR-214-3p	0.701111111	0.438194444	1.467304841	0.170817629	0.197097264	-6.224476838
hsa-miR-338-3p	0.682444444	0.426527778	2.950571088	0.013445306	0.020049741	-3.825656981
hsa-miR-205-5p	0.566222222	0.353888889	2.25817615	0.045670835	0.054805002	-5.022681909
hsa-miR-432-5p	0.409777778	0.256111111	1.145016679	0.276957094	0.307730105	-6.618894038
hsa-miR-101-3p	0.168666667	0.105416667	0.418590323	0.683723407	0.707300077	-7.197109416
hsa-miR-758-3p	0.036666667	0.022916667	0.116013518	0.909772111	0.909772111	-7.284316041
hsa-miR-3619-5p	-0.387777778	-0.242361111	-0.894471969	0.390579262	0.418477781	-6.871957645
hsa-miR-374b-5p	-0.541111111	-0.338194444	-2.340751658	0.039532743	0.049415929	-4.884377801
hsa-miR-542-3p	-0.749777778	-0.468611111	-2.518356592	0.028922284	0.037724719	-4.581798714
hsa-miR-148a-3p	-0.861111111	-0.538194444	-3.273225939	0.007602242	0.012670403	-3.252625562
hsa-miR-26a-5p	-0.866222222	-0.541388889	-2.937184787	0.013768246	0.020049741	-3.849344529
hsa-miR-454-3p	-0.898666667	-0.561666667	-2.688621006	0.021397602	0.029178549	-4.286538689
hsa-miR-99a-5p	-1.098	-0.68625	-2.926370814	0.014034819	0.020049741	-3.868472053
hsa-miR-138-5p	-1.346888889	-0.841805556	-4.638096616	0.000755676	0.001619305	-0.880269787
hsa-miR-409-3p	-1.753333333	-1.095833333	-9.207385084	1.93E-06	9.52E-06	5.361721366
hsa-miR-576-3p	-2.052444445	-1.282777778	-9.343601827	1.67E-06	9.52E-06	5.5095536
hsa-miR-1-3p	-2.140777778	-1.337986111	-5.052503274	0.000392946	0.000906798	-0.199234267
hsa-miR-23b-3p	-2.212888889	-1.383055556	-9.074050874	2.22E-06	9.52E-06	5.215220702
hsa-miR-124-3p	-2.317888889	-1.448680556	-8.202084451	5.82E-06	1.94E-05	4.211076294
hsa-miR-34a-5p	-2.827555556	-1.767222223	-9.399554376	1.58E-06	9.52E-06	5.569747419
hsa-miR-194-5p	-2.862444444	-1.789027778	-12.42236602	9.88E-08	1.48E-06	8.421281137