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Original Article

MiR-20a in cell-free urine as a potential diagnostic biomarker for non-muscle invasive bladder cancer: a Chinese population-based study

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Abstract: The purpose of this study was to examine urinary microRNA-20a (miR-20a) levels in non-muscle-invasive bladder cancer (NMIBC) and its diagnostic value as a noninvasive biomarker. Eighty patients with NMIBC and 86 healthy individuals were enrolled in the present study. Urinary miR-20a expression was detected by qRT-PCR analysis, and correlated with tumor tissue miR-20a expression. The receiver operating characteristic (ROC) curve was analyzed to obtain the AUC (area under the curve), sensitivity and specificity of urinary miR-20a for NMIBC diagnosis. Overall survival (OS) and disease-free survival (DFS) in the patient groups after surgery were determined by Kaplan-Meier survival curve and Log-rank test. Multivariate Cox proportional hazards regression was performed to verify whether urinary miR-20a expression could be considered as a risk factor for OS of NMIBC patients. We found that urinary levels of miR-20a were significantly higher in NMIBC patients than in healthy controls ($P<0.001$). High expression of urinary miR-20a was obviously associated with larger tumor size and advanced tumor grade (all $P<0.05$). Also, Pearson correlation analysis indicated that miR-20a levels in tumor tissues were positively correlated with urinary miR-20a levels. The AUC of urinary miR-20a was 0.804 when the cut-off value was set at 5.28, and the optimal sensitivity and specificity were 72.1% and 87.5%, respectively. Additionally, miR-20a in urine supernatant could function as an independent prognostic biomarker and risk factor for predicting OS of patients with NMIBC. Moreover, urinary miR-20a expression was found to be significantly reduced after transurethral resection (TUR). Our results suggested that urinary miR-20a levels are markedly elevated in patients with NMIBC and can be used as potential biomarkers in the diagnosis of NMIBC at early stage.

Keywords: Non-muscle-invasive bladder cancer, urine, microRNA-20a, diagnosis, prognosis

Introduction

Bladder cancer (BCa) is one of the most prevailing urological malignancies with an annual incidence rate of over 350,000 cases being documented in the world [1]. Multiple genetic factors, such as chromosomal anomalies, genetic polymorphisms, genetic and epigenetic alterations, have been found to be associated with the tumorigenesis and progression of BCa. There are two clinical phenotypes of BCa, including non-muscle-invasive bladder cancer (NMIBC) and muscle-invasive bladder cancer (MIBC) [2]. Emerging evidence showed that NMIBC frequently recur at approximate rate of 50~70% and develop to MIBC at rate of 1~2% and ~45% in low-grade and high-grade tumors, respectively [3]. Although great advances have been made in clinical diagnosis and treatment strategies, many urologists still face serious challenges of how to improve diagnostic accuracy for BCa through identification of promising biomarkers that can uncover malignancies at the early stages. As a gold standard for the initial diagnosis of BCa, cystoscopy is an invasive and relatively expensive tool for patients, and its disadvantages largely limit its clinical application [4].

Many molecules engaged in the genetic alterations might serve as diagnostic markers of tumor growth and disease progression. Asa diverse class of endogenous small non-coding RNAs measuring 17-24 nucleotides in length, microRNAs (miRNAs, miRs) play important roles in a wide variety of biological processes, including cell proliferation, differentiation, apoptosis and tumorigenesis, through direct binding to

Table 1. The demographic features of case group and control group

Characteristics	Case group (n=80)	Control group (n=86)	*P* value
Age (year)			0.313
<60	49	46	
≥60	31	40	
Gender			0.661
Male	41	47	
Female	39	39	
History of smoking			0.314
Yes	46	56	
No	34	30	
History of drinking			0.881
Yes	40	44	
No	40	42	

the 3'-untranslated region (UTR) of their target mRNAs at post-transcriptional level [5, 6]. Recent studies have reported that miRNAs could function as oncogenes or tumor suppressors in various types of cancers [7-9]. Furthermore, miRNAs have been demonstrated to be released from tumor cells to the body fluids, including serum, plasma, saliva, urine and tears, and thus circulating miRNA could be easily exploited as promising diagnostic and prognostic biomarkers for cancers [10, 11].

Overwhelming articles on the application of urinary miRNAs for the diagnosis of BCa with high detection rate and high sensitivity have been published previously [12]. Available in large quantities obtained through noninvasive methods, urinary diagnosis enjoys several advantages, such as repeated measurements and continuous surveillance [13]. Notably, the article of Zhang *et al.* have indicated that the sensitivity and specificity of urinary miR-99a for BCa diagnosis were 78.0% and 85.7%, while the corresponding data were 84.8% and 76.2% as for miR-125b in urine [14]. miR-20a, a member of the miR-17-92 cluster, which is a common oncogene in diverse cancer subtypes [15], was proved to be significantly up-regulated in BCa patients through screening 723 miRNAs by microarray in malignant bladder tissue samples compared to healthy tissue [16]. However, the extracellular circulating expression pattern of miR-20a in BCa patients at the early stages and its diagnostic values remain largely obscure.

Accordingly, in this study, we aimed to discuss whether the expression of miR-20a in cell-free urine is significantly altered in patients with NMIBC compared with cancer-free individuals, and to evaluate whether urinary miR-20a expression was associated with the clinicopathologic features and prognostic predictions of NMIBC. The results might provide a promising circulating biomarker in BCa diagnosis at the early stages.

Material and methods

Study subjects

A total of 80 patients with a verified histopathological diagnosis of NMIBC in accordance to the American Joint Committee on Cancer (AJCC) TMN staging system, as well as 86 healthy donors were recruited for collection of urine samples in Luodian Hospital of Baoshan District from January 2010 to December 2010. No patients had received chemotherapy or radiotherapy prior to sample collection. De mographic characteristics of all participants were summarized in **Table 1**. There was no obvious difference of age (P=0.313), gender (P=0.661), history of smoking (P=0.314) and history of drinking (P=0.881) between the two groups. Voided urine samples obtained from patients before cystoscopy and healthy donors in the morning. After surgery, all NMIBC patients should undergo urinary cytology and cystoscopy every 3 months in the first 2 years followed by every 6 months for the subsequent 3 years [17].

The study protocol was performed following the principles of the Declaration of Helsinki and approved by the Medical Ethics Committee of Luodian Hospital of Baoshan District. Written informed consent was obtained from all the subjects before enrollment.

RNA extraction and qRT-PCR analysis

The urine supernatant aliquots were obtained through centrifugation at 3,000 g for 10 min at 4°C followed by another centrifugation at 16,000 g for 10 min at 4°C to wipe off any residual cells, and then stored at -80°C until further application. Total RNA was extracted from urine supernatants and tissues using the mirVana PARIS kit (Ambion, Austin, TX, USA) and TRizol reagent (Thermo, Massachusetts,

Table 2. The sequences of primers used for qRT-PCR in this study

Gene name	Primer sequences	Primer length (bp)
miR-20a		
Forward	5'-GCGGCGGTAAAGTGCTTATAGTG-3'	23
Reverse	5'-TGCAGGGTCCGAGGTAT-3'	18
U6		
Forward	5'-CTCGCTTCGGCAGCACA-3'	17
Reverse	5'-AACGCTTCACGAATTTGCGT-3'	20

USA), respetively. Total RNA was quantified and purity was investigated through using the Nanodrop® 1000 (Thermo Scientific). cDNA was synthesized from the total RNA using a universal cDNA synthesis kit (Exiqon, Vedbaek, Denmark). Quantitative Real-time PCR (qRT-PCR) was performed using SYBR® Prime ScriptTMmiRNA RT-PCR kit (Takara, Japan) and microRNA LNA PCR primer (Exiqon) that specifically recognized the targeted miRNA (**Table 2**) in an StepOne Real Time PCR System (Applied Biosystem, Grand Island, USA). Human U6 small nuclear RNA (snRNA) was used as the reference control. All of the samples were tested in duplicate. The relative level of microRNA was determined using the $2^{-\Delta Ct}$ method.

Statistical analysis

Statistical analyses were performed by SPSS 17.0 (Chicago, USA) and Graph PAD prism 6.0 (GraphPad Software, Inc., US). Categorical variables were compared by Chi-square test, and Student's *t*-test was used for comparison of continuous variables. A Pearson correlation test was performed to correlate urinary miR-20a levels with tissue miR-20a levels. Receiver operating characteristic (ROC) curve was plotted to determine the potential predictive value of urinary miR-20a to discriminate between NMIBC and healthy individuals. The area under the curve (AUC) value and 95% confidence intervals (CI) were calculated, and the optimal cutoff value was set according to the Youden index (sensitivity + specificity - 1). Survival durations in the case group after surgery were calculated with Kaplan-Meier survival curve and Log-rank test. Overall survival (OS) was calculated as the time from cancer diagnosis to death or date of last follow-up, and disease-free survival (DFS) was calculated as the time from complete remission to treatment failure such as relapse, death, or date at last follow-up. The joint effect of covariates was investigated with multivariate Cox proportional hazards regression to determine whether urinary miR-20a expression is an independent prognostic factor for NMIBC patients. A *P* value of < 0.05 was considered statistically significant.

Results

Expression level of urinary miR-20a was elevated in NMIBC

The levels of miR-20a expression in the urine samples from 80 NMIBC patients and 86 healthy individuals were investigated by qRT-PCR analysis. As exhibited in **Figure 1A**, the case group had significantly higher miR-20a expression in urine than that in control group (*P*<0.001), indicating that urinary miR-20a expression was elevated in NMIBC.

Next, we examined the expression of miR-20a in 37NMIBC tumor tissues and their corresponding normal tissues. As shown in **Figure 1B**, the average level of miR-20a in the NMIBC tumor tissues was remarkably higher compared to the normal counterparts (*P*<0.001). Next, we analyzed the relevance between urinary miR-20a and tissue miR-20a in 37NMIBC patients. Pearson correlation analysis showed a positive correlation between urinary miR-20a and tissue miR-20a expression (r^2=0.229, *P*=0.003; **Figure 1C**).

Correlations between urinary miR-20a level and clinicopathologic variables in NMIBC

Next, 80 NMIBC patients were allocated to low level group (n=58) and high level group (n=22) according to their urinary miR-20a expression. The associations between clinicopathological variables and urinary miR-20a expression were presented in **Table 3**. The results of Chi-square test indicated that down-regulated expression of urinary miR-20a was closely associated with tumor size (*P*=0.042) and tumor grade (*P*=0.035), whereas no significantly correlated with age, gender, history of smoking, history of drinking, tumor number and T stage (all *P*>0.05).

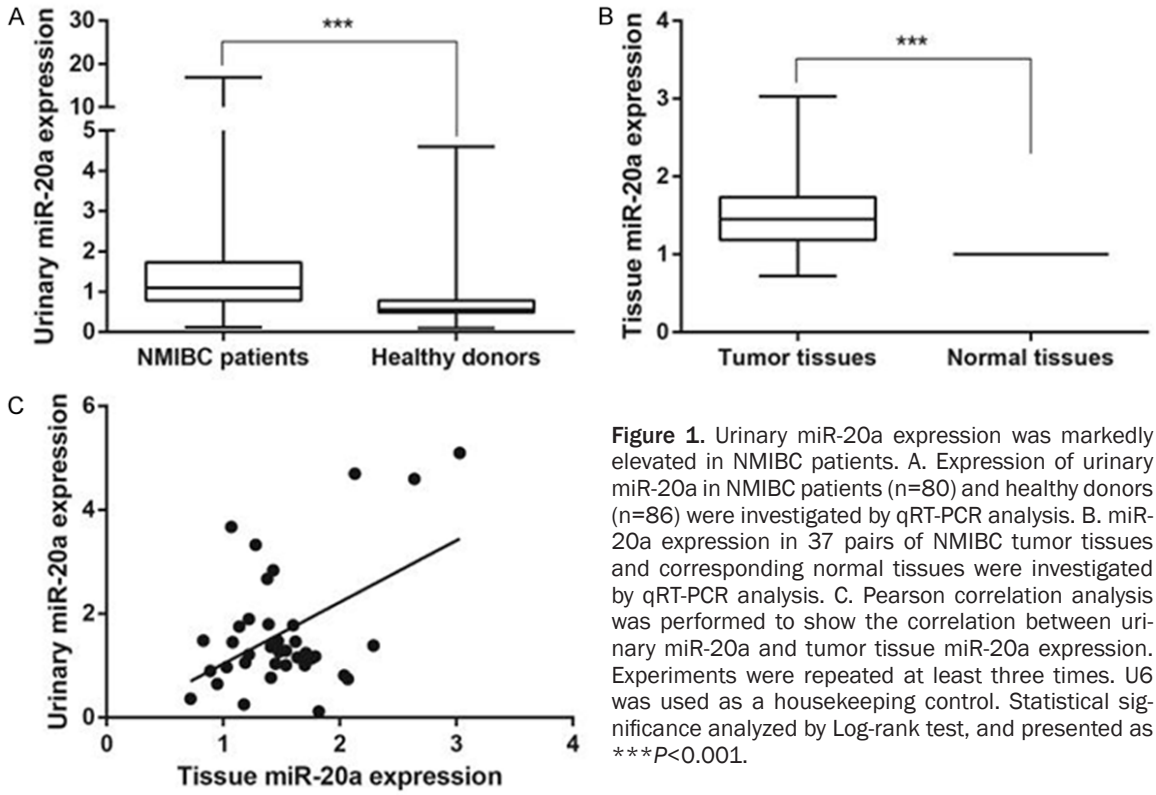

Figure 1. Urinary miR-20a expression was markedly elevated in NMIBC patients. A. Expression of urinary miR-20a in NMIBC patients (n=80) and healthy donors (n=86) were investigated by qRT-PCR analysis. B. miR-20a expression in 37 pairs of NMIBC tumor tissues and corresponding normal tissues were investigated by qRT-PCR analysis. C. Pearson correlation analysis was performed to show the correlation between urinary miR-20a and tumor tissue miR-20a expression. Experiments were repeated at least three times. U6 was used as a housekeeping control. Statistical significance analyzed by Log-rank test, and presented as ***P<0.001.

Diagnostic value of urinary miR-20a level for NMIBC

ROC curve analysis was subsequently performed to assess the diagnostic value of urinary miR-20a to differentiate NMIBC patients from healthy controls. As illustrated in **Figure 2**, the AUC of ROC curve of urinary miR-20a was 0.804 (95% confidence interval (CI): 0.733-0.875), with optimal specificity and sensitivity of 87.5% and 72.1%, respectively at a diagnostic threshold of 5.28, indicating a relatively clear separation between the NMIBC patients and the healthy controls.

High expression of miR-20a in urine was associated with lower 5-year survival rates in the patients with NMIBC

As showed in **Figure 3A**, Kaplan-Meier survival curves manifested that NMIBC patients with higher urinary miR-20a levels had more unfavorable OS (OS: 37.7 months versus 61.3 months, P=0.039) compared with those with lower urinary miR-20a levels. However, intriguingly, compared with those with lower urinary miR-20a levels, NMIBC patients with higher urinary miR-20a levels had shorter DFS, but the difference was not statistically significant (DFS: 32.3 months versus 54.3 months, P=0.056) (**Figure 3B**). Therefore, these results demonstrated that urinary miR-20a could be considered as a promising prognostic indicator for OS of NMIBC patients.

Multivariate Cox's regression analysis of the prognostic value of parameters in NMIBC patients

To date, it still remains elusive whether the independent parameters of urinary miR-20a level in prognosis of NMIBC were significantly correlated to OS. In this study, to identify whether urinary miR-20a expression is a risk factor for unfavorable prognosis of NMIBC patients, the relevant clinicopathologic variables, including urinary miR-20a expression, tumor grade, tumor size and T stage, were subjected to multivariate Cox's proportional hazard regression analysis. The results indicated that urinary miR-20a expression (P=0.022) and tumor grade (P=0.006) could be regarded as risk factors for OS in NMIBC (**Table 4**).

Table 3. Relationships between urinary miR-20a levels and clinicopathological characteristics in 80 NMIBC patients

Characteristics	Total number	Urinary miR-20a expression		*P* value
		Low (n=58)	High (n=22)	
Age, years				0.802
<60	49	36	13	
≥60	31	22	9	
Gender				0.291
Male	47	32	15	
Female	33	26	7	
History of smoking				0.09
Yes	46	30	16	
No	34	28	6	
History of drinking				0.617
Yes	40	28	12	
No	40	30	10	
Tumor size, cm				0.042
<3	57	45	12	
≥3	23	13	10	
Tumor number				0.674
Simple	52	38	14	
Multiple	28	20	8	
Tumor grade				0.035
G1	41	32	9	
G2	27	21	6	
G3	12	5	7	
T stage				0.467
Ta	42	29	13	
T1	38	29	9	

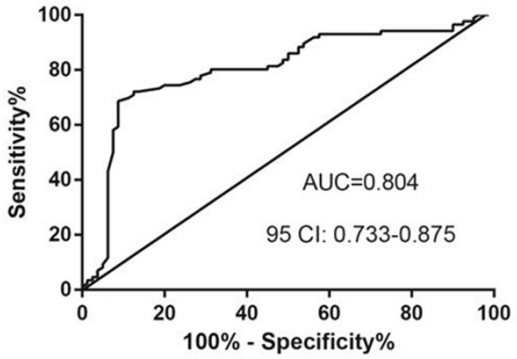

Figure 2. Receiver operating characteristics (ROC) curve analysis for evaluating the predictive value of urinary miR-20a in NMIBC. AUC, area under the curve; CI, confidence interval.

Urinary miR-20a level was decreased after transurethral resection (TUR)

Postoperative voided urine was obtained from 26 patients at three weeks after transurethral resection (TUR). As shown in **Figure 4**, urinary miR-20a levels of 19 patients decreased markedly after TUR, indicating that the urinary miR-20a levels of the patients with elevated miR-20a levels before TUR decreased to the normal range after TUR.

Discussion

BC are mains one of the most prevailing malignancies worldwide [18], and currently, the common diagnostic tool for BC-aisurethrocystoscopy, which is costly, invasive and uncomfortable for patients [19]. It is extensively accepted that early detection and early treatment are the effective methods to improve the prognosis of cancer patients [20]. Therefore, from a clinical perspective, there is a pressing need to find low-cost sensitive and specific screening methods for BCa diagnosis at early stage.

Quantitative changes of miRNAs in urine, blood, and tissues have gradually become the primary focus in the search for new biomarkers for BCa [21]. Stability is a main consideration when evaluating the efficacy of anypotential diagnostic tool. Harsh environment, such as extremes of pH, multiple freeze-thaw cycles and RNase treatment could not exert obvious effect on the levels of miRNAs mainly because of their small size that makes them less likely to fragment than large RNAs [22]. Thus, miRNAs could be easily investigated by qRT-PCR in various body fluids, including serum, plasma, gastric liquids, or urine [23]. Motawi TK *et al.* revealed that plasma miR-92a, miR-100 and miR-143 could be promising novel circulating biomarkers in clinical diagnosis of BCa [24]. However, urine collection is non-invasive, simple, low-cost and comfortable compared with other invasive methods, such as blood collection. Thus, urine could be considered as an ideal body fluid for disease detection in clinical applications.

To date, it might be the first time to describe a non-invasive diagnostic method using cell-free urinary miR-20a biomarker for detecting BCa.

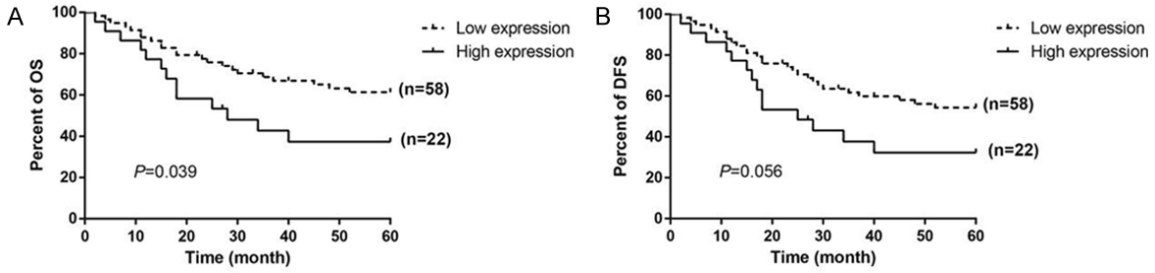

Figure 3. Increased expression of urinary miR-20a was closely correlated with unfavorable 5-year survival rates of NMIBC patients. A. The association between urinary miR-20a expression and OS of NMIBC patients. B. The relationship between urinary miR-20a expression and DFS of NMIBC patients. Statistical significance analyzed by Log-rank test.

Table 4. Multivariate Cox proportional hazards regression analysis of independent predictors on OS

Characteristics	Categories	*P* value	OR	95.0% CI for Exp (B)	
				Lower	Upper
Urinary miR-20a expression	High/Low	0.022	11.081	1.421	86.392
Tumor grade	G2+G3/G1	0.006	13.179	2.058	84.399
Tumor size, cm	≥3/<3	0.187	3.212	0.568	18.172
T stage	T1/Ta	0.281	0.214	0.433	17.887

OS, overall survival; CI, confidence interval.

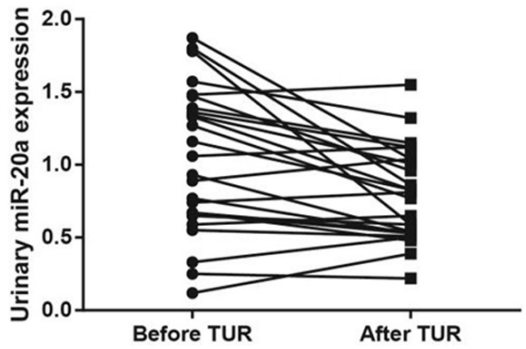

Figure 4. Change of urinary miR-20a levels in 26 NMIBC patients before and after TUR. Experiments were repeated at least three times. U6 was used as a housekeeping control.

miR-20a, a member of the miR-17 miR precursor family [25], has been previously reported to be participated in multiple tumorigenesis, including gastric cancer [26], non-small cell cancer [27] and cervical cancer [28], making it ideal as therapeutic targets as well as diagnostic biomarkers. Cheng *et al.* reported that miR-20a facilitates the invasion and metastasis capacities through directly inhibiting Smad4 expression in colorectal cancer [29]. Moreover, miR-20a was found to be significantly elevated in BCa tissues and urine of BCa patients [30]. Accordingly, we speculated that urinary miR-20a might be mainly derived from BCa cells. Aberrant circulating miR-20a expression has been also reported in other solid cancers, including esophageal squamous cell carcinoma [31], cervical cancer [32], and nasopharyngeal carcinoma [33]. However, to date, amore direct correlation between extracellular (biofluid based) and cellular (BCa tumor tissue based) miRNA has yet not been clearly verified. In our research, we observed a positive correlation between urinary miR-20a and tumor tissue miR-20a expression.

Currently, staging systems based on pathological grade and TNM stage are insufficient to predict clinical outcome. Different outcome for individuals with same pathological grade and TNM stage calls for novel prognostic biomarkers and therapeutic targets [34]. Several studies demonstrated that miRNA expression in BCa tissues significantly correlated with tumor aggressiveness and patient survival [35-37]. However, prognostic values of circulating miRNAs for BCa have not been fully explored. Herein, we found that urinary miR-20a expression was greatly associated to tumor size, tumor grade. Further analyses also revealed a strong relationship between elevated urinary miR-20a expression and unfavorable prognosis of NMIBC patients. Although it did not have forceful value as a single diagnostic biomarker, we believe that combining with conventional assessments such as urine cytology might

improve its diagnostic accuracy for the detection of NMIBC.

We are aware of several limitations exist in this study. First, microarray was not performed to obtain miRNA profiles of urine samples. So other potential urinary miRNA biomarkers may also exist. Second, the cohort of samples was quite small, and all patients were Chinese. Multicentre clinical trials in a larger cohort of samples are required to further validate our findings in the future. Third, the biological functions of miR-20a were largely based on previous reports. In-depth analysis of their biological functions is in urgent needed.

Urinary miRNAs derive from organs of urinary system making them ideal for global biomarker discovery [38-40]. To our knowledge, this is the first comprehensively study provide the evidences that dysregulated expression of cell-free urinary miR-20a might be closely associated to NMIBC, and it could be considered as a novel non-invasive biomarker for NMIBC with good sensitivity and specificity. This study provides a new method and prospect for the early detection of BCa through using a noninvasive screening method.

Disclosure of conflict of interest

None.

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