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RESEARCH ARTICLE

Circulating miR-205: a promising biomarker for the detection and prognosis evaluation of bladder cancer

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Abstract MicroRNA (miRNA) expression profile analysis indicated that miR-205 was upregulated in bladder cancer tissue compared to healthy tissue. The aim of this study is to analyze value of circulating miR-205 for the detection and prognosis evaluation of bladder cancer (BC). Eighty-nine patients with BC and 56 healthy controls (HC) were enrolled in the study. miR-205 expression was determined using TaqMan quantitative real-time polymerase chain reaction assay and further correlated with patients' clinicopathological parameters and follow-up data. The results indicated that plasma miR-205 was upregulated in BC compared with HC ($P<0.001$) and in muscle invasive BC (MIBC) compared to nonmuscle invasive BC (NMIBC) ($P=0.016$). miR-205 yielded an area under the receiver-operating characteristic curve of 0.950 with 76.4 % sensitivity and 96.4 % specificity in discriminating BC from HC, and 0.668 with 57.1 % sensitivity and 77.0 % specificity in distinguishing MIBC from NMIBC. Plasma miR-205 expression was significantly associated with tumor stage ($P<0.001$) and pathological grade ($P=0.048$). The results indicated that BC patients with high miR-205 expression experienced shorter disease-free survival and disease-specific survival ($P=0.022$ and $P=0.026$; $P=0.027$ and $P=0.034$; respectively), which was not proven by multivariate Cox regression analysis (multi-Cox) ($P=0.0765$ and $P=0.279$, respectively). Log-rank test showed that NMIBC patients with high miR-205 expression experienced shorter cancer-free survival ($P=0.044$). Log-rank test and univariate and multivariate Cox regression analyses did not indicate that high miR-205 expression in NMIBC patients was associated with cancer-specific survival ($P=0.079$, $P=0.089$, and $P=0.201$, respectively). In conclusion, miR-205 may be a promising biomarker for the detection and prognosis evaluation of BC.

Keywords Humans · miR-205 · Bladder cancer · Biomarker · Survival analysis

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Introduction

Bladder cancer is the eighth most common cancer in women and the fourth most common cancer in men. It is estimated that about 73,510 new cases of bladder cancer were diagnosed in 2012 in the USA, which led to roughly 14,880 deaths [1]. Approximately 75 % of bladder cancer patients were initially diagnosed as nonmuscle invasive bladder cancer (NMIBC); the rest is diagnosed as muscle invasive bladder cancer (MIBC) [2]. The main procedures for the diagnosis and prognosis evaluation of bladder cancer still rely on cystoscopy and histopathological analysis of bladder specimens [3]. Unfortunately, it was reported that the current prognosticators, such as tumor grade, stage, and size, failed to provide accurate information for clinical prognosis assessment and reliable risk-adjusted decision [4]. Therefore, new biomarkers are in urgent need to improve the detection rate and prognosis evaluation of bladder cancer.

MicroRNAs (miRNAs) are fragments of noncoding RNAs comprising 19 to 24 nucleotides, which can modulate gene expression by degrading mRNAs or impairing their translation [5]. It has been reported that a large number of biological processes, such as cell proliferation, differentiation, and apoptosis, were under the regulation of miRNAs [5, 6], which make them very attractive for the detection and prognosis assessment of bladder cancer. miRNAs expression profile

analysis indicated that miR-205 was upregulated in bladder cancer tissue compared to healthy tissue [3, 7] and significantly differentially expressed between NMIBC and MIBC [8]. Other studies indicated that the expression of miR-205 was reduced in invasive bladder cancer cell lines and the urine sediment of patients with bladder cancer [9, 10]. Furthermore, it is also showed that miR-205 was associated with cancer-specific survival [3].

In this study, we would like to evaluate the feasibility of miR-205 as a biomarker for bladder cancer detection and the correlation between the plasma level of miR-205 and the clinical parameters of bladder cancer. The value of miR-205 in the prognosis assessment of bladder cancer is another core of our investigation.

Methods and materials

Patients and samples

The study was approved by the Ethic Committee of Xinqiao Hospital, and all of the patients have been given their consents for this study. Eighty-nine patients with a confirmed pathological diagnosis of bladder cancer and 56 healthy controls from Xinqiao Hospital were enrolled in the study between February 2007 and December 2007. The exclusion criteria included coagulation disorders, any other previous malignant tumors, previous radiotherapy or chemotherapy, and a platelet count less than 20.0×10^9/l. Thus, participants with other chronic but stable diseases (e.g., peptic disease, hypertension, diabetes mellitus, or heart disease) were eligible to be recruited. According to the severity of their cancer, bladder cancer patients received either transurethral resection of bladder cancer (TURBt) or radical cystectomy (RC). Eight milliliters of peripheral venous blood was collected in EDTA-containing tubes from all participants. In terms of bladder cancer patients, the blood was collected before any medical treatment. It is worthy to note that the first 2 ml of collected blood was discarded with the aim of avoiding contamination with epidermal cells. Immediately after collection, the blood was centrifuged at 4 °C and 1,000 r/min for 10 min; and then, the supernatant fluids were centrifuged at 4 °C and 15,000 r/min for 10 min in another Eppendorf tubes. At last, the supernatant fluids were stored at −80 °C until RNA extraction.

The American Joint Committee on Cancer (AJCC)/TMN staging system was used to determine the stage of bladder cancer. After surgery, NMIBC patients treated with TURBt received adjuvant therapy according to the European Association of Urology guidelines for NMIBC[11], RC-treated patients received no adjuvant or neoadjuvant treatment, and TURBt-treated patients with MIBC underwent chemotherapy or external beam radiotherapy in accordance with the European Association of Urology guidelines for MIBC [12]. For NMIBC patients, the follow-up strategy included patient assessments by urinary cytology and cystoscopy every 3 months in the first 2 years followed by every 6 months for the subsequent 3 years, and then annually thereafter [2]. MIBC patients' follow-up included physical examination, renal and liver ultrasound, and chest X-ray every 6 months for pT2 patients and every 3 months for pT3 and pT4 patients, urinary cytology every 6 months and magnetic resonance imaging or computed tomography of abdomen and pelvis every 6 months. Magnetic resonance imaging of the brain or bone scan was conducted if necessary. The recurrence of bladder cancer was defined as locoregional recurrence or distant metastasis. The clinical parameters and follow-up data were blind to the researchers who conducted the quantification of miR-205.

RNA extraction and complementary DNA (cDNA) synthesis

In accordance with the protocol from manufacturer, total RNA was extracted from 400 μl of plasma using mirVana PARIS Kit (Ambion, USA) and then eluted into 100 μl of pre-heated (95 °C) Elution Solution. RNA concentration and purity were determined using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA).

Reverse transcription was conducted by the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, USA) in 15 μl containing 6.16 μl of nuclease-free water, 5 μl of RNA extract, 1.5 μl of 10× Reverse Transcription Buffer, 1 μl of gene-specific primer, 1 μl of Multiscribe Reverse Transcriptase (50 U/μl), 0.19 μl of RNase inhibitor (20 U/μl), and 0.15 μl of 100 mM dNTPs. For synthesizing cDNA, the reaction mixtures were incubated at 16 °C for 30 min, at 42 °C for 30 min, and at 85 °C for 5 min and then held at 4 °C.

Quantitative real-time polymerase chain reaction (qRT-PCR)

miR-205 expression levels were qualified by TaqMan qRT-PCR. Each amplification reaction was performed in triplicate with a final volume of 20 μl containing 10 μl TaqMan 2× Universal PCR Master Mix with no AmpErase UNG (Applied Biosystems), 7 μl nuclease-free water, 2 μl of the cDNA (100 ng/reaction), and 1 μl gene-specific primer/probe. qRT-PCR was run on a 7300 qRT-PCR system (Applied Biosystems), and the amplification reaction initialized with a polymerase activation step at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. SDS 1.4 software was used to calculate the cycle threshold (Ct). The mirVana miRNA Reference Panel (Ambion, UK) was also amplified for each reaction and then used to construct a standard curve for the calculation of miR-205 expression.

Statistical analysis

SPSS 18.0 and Medcalc 12.0 were employed for statistical analysis. One-way ANOVA was used to evaluate the association between miR-205 expression and tumor stage and compare ages among the three groups. Kruskal-Wallis *H* test was applied to compare gender among the three groups. Comparison of miR-205 expression between bladder cancer patients and healthy controls was calculated by *t* test, which was also applied to evaluate the association between miR-205 expression and tumor grade (WHO 2004), muscle invasion status. Receiver-operating characteristic (ROC) curves and the area under the curve (AUC) were used to evaluate the feasibility of using plasma miR-205 concentrations as a biomarker for detecting bladder cancer and differentiating MIBC from NMIBC. The association between miR-205 expression and the prognosis of bladder cancer patients was assessed by the log-rank test and Cox proportional hazards regression analysis, and tumor stage, gender, and pathological grade entered into the multivariate analysis. The levels of statistical significance were set at least at $P<0.05$ (two-sided).

Results

Patients description

Sixty-one NMIBC patients, 29 MIBC patients, and 65 healthy people were enrolled in the study; the mean age was 68.0 ± 11.2, 70.2 ± 9.8, and 68.2 ± 11.0, respectively. There was no significant difference of gender and age among the three groups ($P=0.652$ and $P=0.928$, respectively). Details of the clinical and pathological parameters are represented in Table 1.

Plasma expression of miR-205 for the detection of bladder cancer

The plasma expression of miR-205 was significantly increased in bladder cancer patients compared to healthy controls ($P<0.001$, Table 2). The ROC curve was used to explore the potential of using miR-205 as a biomarker for detecting bladder cancer, which indicated that miR-205 had AUC of 0.950 (95 % confidence interval = 0.901–0.79) (Fig. 1a). At a cut-value of 2.3 for miR-205, the sensitivity was 76.4 % and specificity was 96.4 %. Besides, the levels of miR-205 in plasma were significantly associated with tumor stage ($P<0.001$), pathological grade ($P=0.048$), and muscle-invasive status ($P=0.016$) (Table 2). Furthermore, we used the ROC curve to explore the potential of using miR-205 as a biomarker for differentiating MIBC from NMIBC, which indicated that miR-205 had AUC of 0.668 (95 % confidence interval = 0.560–0.764) (Fig. 1a). At a cut-value of 4.7 for miR-205, the sensitivity was 57.1 % and specificity was 77.0 %.

Table 1 Clinical and pathological parameters of all participants

Characteristic	NMIBC ($n=61$)	MIBC ($n=28$)	Healthy control ($n=56$)
Age (years)	68.0 ± 11.2	70.2 ± 9.8	68.2 ± 11.0
Gender			
Male	43	20	36
Female	18	8	20
pT stage			
pTa	33		
pT1	28		
pT2		19	
pT3		5	
pT4		4	
Grade (WHO 2004)			
G1	25	0	
G2	36	28	
Operation			
TURBt	56	5	
Radical cystectomy	5	23	

NMIBC nonmuscle invasive bladder cancer, *MIBC* muscle invasive bladder cancer

Table 2 miR-205 expression and tumor phenotype

Variable	No. of patients	miRNA 141 expression	*P* value
Tissue type			<0.001
Carcinoma	89	3.96 ± 1.89	
Paracarcinoma	56	1.13 ± 0.74	
Grade (WHO 2004)			0.048
G1	25	3.32 ± 1.73	
G2	64	4.20 ± 1.91	
Muscle invasive			0.016
NMIBC (Ta, T1)	61	3.63 ± 1.85	
MIBC (T2, T3, T4)	28	4.66 ± 1.82	
Stage			<0.001
pTa	33	3.00 ± 1.62	
pT1	28	4.37 ± 1.86	
pT2	19	3.97 ± 1.65	
pT3	5	5.80 ± 0.92	
pT4	4	6.55 ± 1.46	

NMIBC nonmuscle invasive bladder cancer, *MIBC* muscle invasive bladder cancer

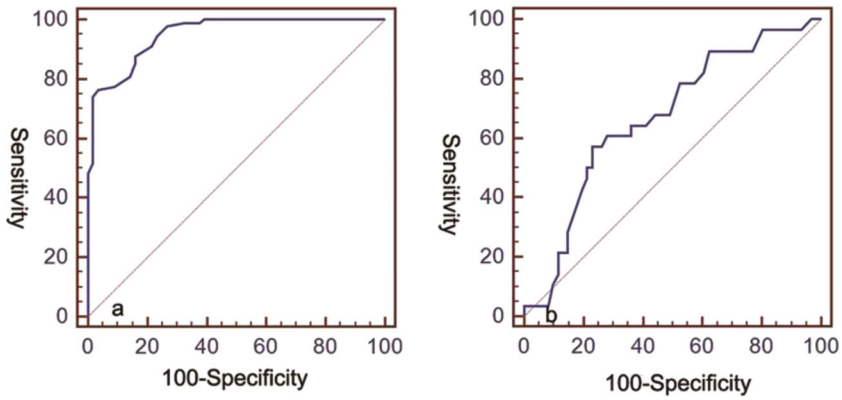

Fig. 1 Plasma expression of miR-205 for the detection of bladder cancer and muscle invasive bladder cancer. **a** Receiver-operating characteristics curve analysis using plasma miR-205 for the detection of bladder cancer. Area under the curve is 0.950. **b** Receiver-operating characteristics curve analysis using plasma miR-205 for differentiating muscle invasive bladder cancer from nonmuscle invasive bladder cancer. Area under the curve is 0.668

Plasma expression of miR-205 in relation to prognosis

Five MIBC patients treated with TURBt were not included in the survival analysis; 17 (20.2 %) patients were lost to follow-up; the number of patients with cancer recurrence was 37 (44.0 %); and the dead patients accounted to 30 (35.7 %) during the follow-up. The median postoperative follow-up time was 48.2 ± 10.6 months. The samples with miR-205 expression at the level less than 3.94 were assigned to the low expression group ($n = 51$, 60.7 %), and these samples with expression above the median value were assigned to the high expression group ($n = 33$, 39.3 %). Both the log-rank test and univariate Cox proportional hazards regression analysis indicated that bladder cancer patients with higher miR-205 expression experienced shorter disease-free survival ($P = 0.022$ and $P = 0.026$, respectively) (Fig. 2a), which was not confirmed by multivariate Cox proportional hazards regression analysis ($P = 0.076$) (Table 3). A statistically significant reduction in disease-specific survival for bladder cancer patients with higher miR-205 expression was highlighted by both the log-rank test ($P = 0.027$, Fig. 2b) and univariate Cox proportional hazards regression analysis ($P = 0.034$). Unfortunately, the abovementioned results were not confirmed by multivariate Cox proportional hazards regression analysis ($P = 0.279$) (Table 3).

It has also demonstrated that the log-rank test showed that NMIBC patients with higher miR-205 expression experienced shorter cancer-free survival ($P = 0.044$, Fig. 2c), which was not confirmed by univariate and multivariate Cox proportional hazards regression analysis ($P = 0.051$ and $P = 0.243$, respectively). There was no evidence indicating that higher miR-205 expression in NMIBC patients was associated with cancer-specific survival by log-rank test and univariate and multivariate Cox proportional hazards regression analyses ($P = 0.079$, $P = 0.089$, and $P = 0.201$, respectively, Fig. 2d, Table 3).

Discussion

Bladder cancer is the most common malignant tumor of urinary system in both men and women, which has diverse functional and biological characteristics. At present, the most reliable method for the detection of bladder cancer is cystectomy, which is expensive, invasive, time-consuming, and cannot be utilized for mass screen, especially for developing countries. Compared to NMIBC, MIBC has the characters of frequent recurrences, and the clinical outcome of which is poor [13]. Even though CT and MRI are commonly used for the staging of MIBC, they fail to detect T2 and T3a bladder cancer [12]. Transurethral resection of bladder cancer and histopathological staging is still the main method for the diagnosis of MIBC, especially for MIBC in early stage. Less costly and noninvasive biomarkers are urgently needed to be developed.

With the development of microarray technology, a large number of biomarkers have been developed for cancer classification and identification of tumor subclass [14], which definitely accelerate the speed for the exploration of novel biomarkers for bladder cancer. Microarray-based miRNA analysis showed that miR-205 was upregulated in bladder cancer tissue compared to healthy tissue [3, 7] and significantly differentially expressed between NMIBC and MIBC [8]. Other studies indicated that the expression of miR-205 was reduced in invasive bladder cancer cell lines and the urine sediment of patients with bladder cancer [9, 10]. In our study, it was found that the plasma expression of miR-205 in bladder cancer patients was significantly higher than in healthy controls, which yielded an AUC of 0.950. At a cut-value of 2.3 for

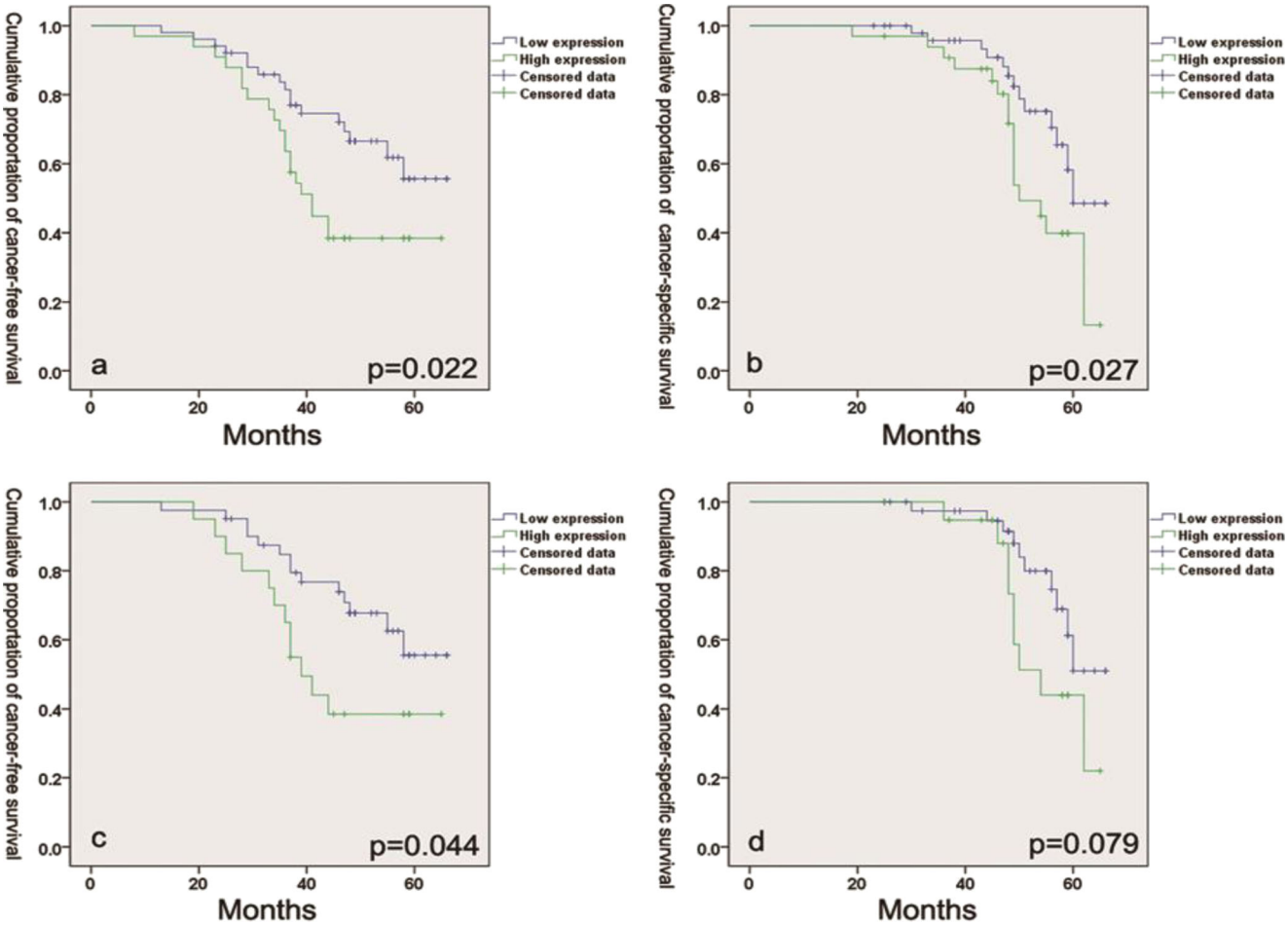

Fig. 2 Kaplan-Meier survival curves show the correlation of plasma miR-205 with the prognosis of bladder cancer. **a** Kaplan-Meier survival curves for the disease-free survival of bladder cancer patients. **b** Kaplan-Meier survival curves for the disease-specific survival of bladder cancer patients. **c** Kaplan-Meier survival curves for the disease-free survival of patients with nonmuscle invasive bladder cancer. **d** Kaplan-Meier survival curves for the disease-specific survival of the patients with nonmuscle invasive bladder cancer

miR-205, the sensitivity was 0.764 and specificity was 0.964. These encouraging results indicated that miR-205 could serve as a reliable marker for the detection of bladder cancer. On the other hand, there was significant difference in plasma expression of miR-205 between MIBC and NMIBC; the AUC of miR-205 for distinguishing MIBC from NMIBC was 0.668, and the sensitivity and specificity of which were 0.571 and 0.770 separately. Even though the results were not appealing, it showed us that miR-205 had the potential to differentiate MIBC from MIBC, which needs to be further studied. It is worth to note that in a study on bladder cancer, it was demonstrated that a miR21/miR-205 expression ratio could distinguish invasive bladder tumors from noninvasive phenotype with 100 % sensitivity and 78 % specificity [15].

It was also found that the aberrant expression of miR-205 in plasma was significantly associated with tumor stage, pathological grade, and muscle invasive status, which presented an indication that miR-205 played an important role in the development of bladder cancer from the clinical aspect. It has been demonstrated that miR-205 appeared to act as a devil or angel in malignant tumors determined by its target genes or the specific cancer context. Acting as a devil, recent studies indicated that miR-205 could promote tumor initiation, progression, and resistance to anti-tumor therapy [16, 17]. Results from research studies on endometrial tumors demonstrated that miR-205 promoted cancer cell proliferation through targeting PTEN [18]. As an angel, accumulating studies indicated that miR-205 plays essential roles in epithelial biogenesis and maintenance, hold the ability of counteracting epithelial to mesenchymal transition (EMT), and inhibit invasion and proliferation in various cancer, such as breast cancer, melanoma, and prostate cancer [19]. In a recent research on bladder cancer, ΔNp63α was found to promote miR-205 transcription and then suppress EMT [20]. Wiklund et al. found that miR-205 and miR-200 family are often concurrently silenced and gain DNA hypermethylation

Table 3 Cox proportional regression analysis for assessing the correlation of plasma miR-205 with the prognosis of bladder cancer

Covariate	Univariate analysis			Multivariate analysis		
	HR	95 % CI	*P* value	HR	95 % CI	*P* value
Patients with bladder cancer						
Cancer-free survival						
miR-205						
Low	1.000			1.000		
High	2.091	1.093–4.002	0.026	2.217	0.919–5.350	0.076
Tumor stage						
Ta + T1	1.000			1.000		
T2 + T3 + T4	1.427	0.702–2.899	0.326	0.863	0.382–1.950	0.724
Grade						
G1	1.000			1.000		
G2	2.035	0.928–4.463	0.076	2.092	0.790–5.540	0.138
Gender						
Female	1.000			1.000		
Male	1.764	0.913–3.405	0.091	0.804	0.310–2.090	0.655
Cancer-specific survival						
miR-205						
Low	1.000			1.000		
High	2.184	1.059–4.502	0.034	1.708	0.648–4.500	0.279
Tumor stage						
Ta + T1	1.000			1.000		
T2 + T3 + T4	2.572	1.212–5.460	0.014	1.466	0.580–3.702	0.419
Grade						
G1	1.000			1.000		
G2	3.269	1.242–8.604	0.016	2.623	0.839–8.196	0.097
Gender						
Female	1.000			1.000		
Male	1.752	0.841–3.652	0.134	0.877	0.324–2.373	0.796
Patients with nonmuscle invasive bladder cancer						
Cancer-free survival						
miR-205						
Low	1.000			1.000		
High	2.166	0.997–4.707	0.051	1.861	0.655–5.286	0.243
Tumor stage						
Ta	1.000			1.000		
T1	2.572	1.212–5.460	0.014	0.598	0.134–2.677	0.502
Grade						
G1	1.000			1.000		
G2	1.989	0.863–4.581	0.106	2.194	0.618–7.784	0.224
Gender						
Female	1.000			1.000		
Male	2.358	1.079–5.151	0.031	1.578	0.341–7.297	0.559
Cancer-specific survival						
miR-205						
Low	1.000			1.000		
High	2.186	0.887–5.390	0.089	2.395	0.628–9.138	0.201
Tumor stage						
Ta	1.000			1.000		

Table 3 (continued)

Covariate	Univariate analysis			Multivariate analysis		
	HR	95 % CI	*P* value	HR	95 % CI	*P* value
T1	1.712	0.692–4.235	0.245	0.415	0.075–2.296	0.314
Grade						
G1	1.000			1.000		
G2	2.642	0.944–7.393	0.064	4.235	0.994–18.041	0.051
Gender						
Female	1.000			1.000		
Male	2.231	0.892–5.579	0.086	1.177	0.166–8.324	0.871

HR hazards ratio, *CI* confidence interval

in poorly differentiated bladder cell lines and muscle invasive bladder tumors [21]. Moreover, the regression of miR-200/miR-205 is accompanied by a gain of H3K27me3 and a loss of H3K9Ac in T24 and HU609 cells, which demonstrated regional epigenetic remolding encompassing miR-205 and miR-200 family [21, 22].

The present prognosis evaluation systems, such as tumor stage, tumor grade, and lymph node, failed to accurately detect the whole spectrum of bladder cancer in routine clinical practice [23]. Given the limited value of these established prognostic biomarkers, we further analyzed the correlation of plasma expression of miR-205 with the prognosis of bladder cancer. The univariate analysis using both log-rank test and Cox proportional hazards regression analysis indicated that bladder cancer patients with high miR-205 expression experienced shorter disease-free survival and disease-specific survival period, which was not confirmed by multivariate Cox proportional hazards regression analysis after considering tumor stage, tumor grade, and gender as covariates. Furthermore, the log-rank test showed that NMIBC patients with low miR-205 expression experienced longer disease-free survival period, which was not supported by both the univariate and multivariate Cox proportional hazards regression analyses. In our study, there was no evidence demonstrating that plasma miR-205 expression status was associated with disease-specific survival in NMIBC patients. Of note, the *P* value for the association between plasma miR-205 expression and disease-free survival in NMIBC patients calculated by univariate Cox proportional hazards regression analysis was 0.051. So, if the sample size increased, more encouraging results will be gotten. Tran et al. also found that both bladder cancer patients and MIBC patients with high miR-205 expression experienced shorter disease-free survival and overall survival [20]. In their opinion, even though miR-205 was associated with a lethal bladder cancer phenotype, miR-205 might be not the reason for the lethal biology. Instead, miR-205 was found to be associated with poor prognosis because it was a marker of ΔNp63α [20]. On the contrary, Ratert et al. found that bladder cancer patients with high miR-205 expression in tissue experienced longer overall survival calculated by Kaplan-Meier analysis. No significant difference in overall survival between different expression patterns of miR-205 was detected in NMIBC and MIBC patients [3]. Besides, miR-205 was also found to be a positive prognostic marker for certain types of cancers, such as esophageal squamous cell carcinoma and head and neck squamous cancer [24–26].

Even though compelling, the study has several limitations. First, the recruited participants were from a single institution, and it is unclear whether the sample can represent the general patient population with bladder cancer. Second, the methods of postoperative treatments and patients' compliance with the postoperational management was not considered in the multivariate Cox proportional hazards regression analyses, which may bring some bias into the results. Third, the results of the study may have been more persuasive if the sample size had been increased. Fourth, we did not consider the expression status of miR-205 in other types of cancer, so the ability of miR-205 in distinguishing bladder cancer from other types of cancer was not evaluated.

In summary, the study showed that miR-205 may be a promising biomarker for the detection of bladder cancer and its upregulation may be potentially associated with unfavorable prognosis of bladder cancer, suggesting that miR-205 might serve as a potential biological marker for further risk stratification of bladder cancer.

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Conflict of interest None

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