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Evaluation of urinary *miRNA-96* as a potential biomarker for bladder cancer diagnosis

Sanaa Eissa · Haidy Habib · Esraa Ali · Yossif Kotb

Abstract For bladder cancer, a new diagnostic marker is needed to avoid painful cystoscopy. The aim of this study was to explore the efficacy of urinary *miRNA-96* as molecular marker in bladder cancer diagnosis and its relation to bilharziasis. Urine cytology, serologic assessment of schistosomiasis and estimation of *miRNA-96* by real-time PCR were carried out for 94 bladder cancer patients, 30 benign bladder lesions and 60 healthy individuals. Expression of *miRNA-96* showed a significant difference among the three tested groups and also between benign and malignant bilharzial cases. Urinary *miRNA-96* is a good noninvasive diagnostic biomarker for bladder cancer.

Keywords Bilharziasis · Marker · Real time PCR · Urine cytology

Introduction

The standard care for bladder cancer diagnosis and follow-up is through the combination of cystoscopic examination, histology and cytology [17]. However, these methods have a significant poor sensitivity for low-grade, well-differentiated lesions and high financial cost. They are also highly subjective testes and provide little about the molecular characteristics of cancers [12].

Recently, numerous urinary markers have been under study as noninvasive tests to reduce the frequency and cost of cystoscopy. An ideal test for the bladder tumors detection should have high specificity and sensitivity; moreover, it is necessary to be objective, rapid, accurate and easy to administer [1].

In the ongoing search for new markers to improve the bladder cancer diagnosis, microRNAs (miRNAs) may thus serve as biomarkers for early detection of bladder cancer. miRNAs are small endogenous noncoding RNAs that play crucial roles in multiple biological processes through regulating translational repression or cleavage of mRNAs. Recent studies have documented that miRNAs acted as tumor suppressors or oncogenes in a variety types of cancer, such as lung, hepatic, breast and pancreatic cancer [10, 11, 13, 16, 23, 24].

Several miRNAs properties make them attractive as potential biomarkers. miRNAs can be easily detected in small amount of samples using sensitive and specific real-time quantitative PCR (qPCR). miRNAs can be detectable in bodily fluids including serum, plasma, urine, saliva and tears and are stable against degradation [2, 9]. Furthermore, expression profiles of miRNAs would be changed in the serum and/or plasma of cancer patients and miRNAs have been shown to be released from tumor cells to the circulation. So circulating miRNAs will be a novel class of noninvasive biomarkers for cancer diagnosis and prognosis [19].

miR-96 have been found to be upregulated in various human cancers such as breast, lung, liver, colon, prostate, ovary, testis cancer and lymphoma [22]. These results implied that *miR-96* is an onco-miRNA and might be a potential target of gene therapy of some human cancers. There are good results whereby expressions of miR-96 in

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urine was well correlated with tumor grade and stage; this miRNA is thus promising diagnostic tumor markers to distinguish BC patients from non-BC patients [21].

The aim of the present study was to evaluate *miRNA-96* usefulness as a urine molecular marker for bladder cancer detection. Urinary miR-96 level was measured using real-time polymerase chain reaction (qPCR) in cellular pellets from a large number of voided urine samples, especially those with bilharziasis in comparison with urine cytology.

Materials and methods

Subjects

Approval of the study was conducted by ethical committee of faculty of medicine, Ain Shams University. This study included 184 Egyptian individuals: 124 inpatients were selected from Urology Department, Ain Shams University, Faculty of Medicine, Egypt, and 60 healthy normal volunteers were enrolled as a control group after obtaining informed consent.

Patients to be enrolled in the study must presented with chronic irritative voiding symptoms or hematuria and cystoscopic evidence confirmed by biopsy consistent with proven bladder carcinoma (in situ, low grade or high grade). Participants were excluded from the study if they had a past history of occupational exposure to known bladder carcinogens, bladder cancer or another urological malignancy within the past 5 years, received chemotherapy and radiation therapy.

According to histopathological examination of cystoscopy biopsies, 124 patients included in the study were classified into malignant and benign groups. The malignant group included 94 patients (mean age 58.8 ± 11.6 years; range 28–85 years) and benign group ($n = 30$, mean age 57.1 ± 17.4 years; range 27–82 years). From the laboratory staff, 60 healthy normal volunteers (mean age 34.3 ± 14.5 years; range 11–65 years) were recruited with matched age, sex and smoking status of patients. Of this malignant group, 52 were diagnosed by histopathology as transitional cell carcinoma (TCC), 33 cases as squamous cell carcinoma (SCC) and 9 as others types (Table 1). Tumor grading and staging were determined according to World Health Organization and TNM classification [4, 18].

Collection of samples

Voided urine (30–60 mL) and sera (1 mL) samples were obtained from all individuals before any surgical or other therapeutic intervention. Urine samples were collected using the urine collection cup that measures volume, sealed immediately and placed on ice and then centrifuged at 2,500–4,000×g for 15–20 min. The urinary pellets were

Table 1 The clinicopathological factors among the different groups of study

	Malignant group (%)	Benign group (%)	Healthy control (%)
Overall (184)	94 (51.1 %)	30 (16.3 %)	60 (32.6 %)
Age			
Range	28–85	27–82	11–65
Mean ± SD	58.8 ± 11.6	57.1 ± 17.4	34.3 ± 14.5
<52	25 (26.6 %)	10 (33.3 %)	48 (80 %)
≥52	69 (73.4 %)	20 (66.7 %)	12 (20 %)
P	0.000*		
Sex			
Male	74 (78.7 %)	20 (66.7 %)	40 (66.7 %)
Female	20 (21.3 %)	10 (33.3 %)	20 (33.3 %)
P	0.185		
Smoking			
Positive	56 (59.6 %)	6 (20 %)	4 (6.7 %)
Negative	38 (40.4 %)	24 (80 %)	56 (93.3 %)
P	0.000*		
Bilharziasis			
Positive	35 (37.2 %)	12 (40 %)	0 (0 %)
Negative	59 (62.8 %)	18 (60 %)	60 (100 %)
P	0.000*		
Urine cytology			
Positive	32 (34 %)	2 (6.7 %)	0 (0 %)
Negative	62 (66 %)	28 (93.3 %)	60 (100 %)
P	0.000*		
Clinical stage			
Early (0 + 1)	66 (70.2 %)		
Late (2 + 3)	28 (29.8 %)		
Histological grade			
Low (1 + 2)	69 (73.4 %)		
High (3)	25 (26.6 %)		
Pathological type			
TCC	52 (55.3 %)		
SCC	33 (35.1 %)		
Others	9 (9.6 %)		

* Significant correlation between investigated groups is detected at $P < 0.001$ using Chi-square test

washed twice with phosphate-buffered saline (PBS) at pH 7.0. A portion of the pellet was used for microscopic and cytological examination [15], and the other portion was treated with 0.15 ml of RNA later, RNA Stabilization Reagent (Qiagen, Valencia, CA, USA), and stored at −80 °C for further processing to extract miRNAs.

Schistosomiasis antibodies detection in serum

Assessment of bilharzial infestation was done by schistosomiasis antibodies detection in sera via indirect

Table 2 Real-time qPCR of *miRNA-96* level using the RQ values and positivity rates of urine cytology and bilharziasis among the different groups of the study

Groups (no)	miRNA-96 Quantitative real-time PCR			Positive cytology[a]	Positive bilharziasis[a]
	Median	Mean rank[b]	>cutoff value[a]		
Control (n = 60)	0.10	55.02	2 (3.3 %)	0 (0 %)	0 (0 %)
Benign (n = 30)	0.34	76.63	8 (26.7 %)	2 (6.7 %)	12 (40 %)
Malignant (n = 94)	5.26	121.49	68 (72.3 %)	32 (34 %)	35 (37.2 %)

* Significant difference between investigated groups is detected at $P < 0.001$ using Chi-square test[a] and nonparametric test[b]

haemagglutination test, using the Cellognost Schistosomiasis H kit (Dade Behring Marburg GmbH, Marburg, Germany) [5].

Extraction of miRNA from urine pellet samples

Total miRNAs were extracted from all urine sediments using miRNeasy Mini Kit (Qiagen, Germany, Cat. no. 217004). The miRNeasy Mini Kit combines phenol-/guanidine-based lysis of samples with silica-membrane-based purification of total miRNAs according to the manufacturer's instructions. Then, total miRNAs were treated with RNase inhibitors and kept at -80 °C until its use in the reverse transcription quantitative polymerase chain reaction (RT-qPCR) for detection of *miRNA-96* expression. The concentrations of RNA were determined spectrophotometrically.

Detection of *miRNA-96* by reverse transcription quantitative polymerase chain reaction (RT-qPCR)

First, the cDNA was synthesized from the total miRNAs of the urine pellets using Miscript II RT kit in accordance with the manufacturer's recommended protocol (Qiagen, Germany). The resultant cDNA was subjected to real-time quantitative polymerase chain reaction (qPCR) using miScript SYBR® Green PCR Kit with miScript Primer assays (Qiagen, Germany). This kit includes QuantiTect SYBR Green PCR Master Mix and the miScript Universal Primer (reverse primer that use to detect miRNAs) in combination with a miScript Primer Assay (Cat. no. MS00003360) that specifically recognizes the targeted miRNA. The real-time qPCR was performed on a Step One Plus™ System (Applied Biosystems Inc, Foster, CA). The PCR conditions were as follows: 95 °C for 15 min, then 94 °C for 15 s, 55 °C for 30 s and 70 °C for 30 s for 40 cycles. The data were normalized using the endogenous RNU U6 as reference control. The threshold cycle (Ct) value of each sample was calculated with Step One Plus™ software v2.2.2 (Applied Biosystems), and the $2^{-\Delta\Delta CT}$ method was used in the analysis of PCR data for relative quantification of *miRNA-96*.

Statistical methods

Data analyses were performed using Chi-square and nonparametric tests, and the level of significance was determined to be less than 0.05. The threshold value for optimal sensitivity and specificity of *miRNA-96* was determined by receiver operating characteristics (ROC) curve. All analyses were performed using Statistical Package for the Social Sciences software (SPSS Inc, Chicago, IL, USA).

Results

The present study included 184 subjects. Ninety-four bladder cancer: 35 of them were bilharzial and 59 were non-bilharzial. Thirty patients with benign urological diseases: 12 of them with bilharzial lesions and 18 cases without. All 60 normal volunteers were without bilharziasis (Table 1).

Urinary *miRNA-96* level in investigated groups

In urine sediment cells, *miRNA-96* expression was measured using real-time qPCR and normalized to *RNU 6* as reference control. As shown in Table 2, the expression level of *miR-96* was significantly upregulated in the bladder cancer group (mean rank = 121.49), in comparison with the normal and benign groups (mean rank = 55.02 and 76.63, respectively, $P < 0.001$) using the RQ values. The best cutoff point for *miRNA-96* using the ROC curve was 1.63 (Fig. 1). Using this cutoff value, 68 out of 94 (72.3 %) malignant patients, 8 out of 30 (26.7 %) benign patients and 2 out of 60 (3.3 %) normal individuals were *miRNA-96* positive ($P < 0.001$), as shown in Table 2.

Positive urine cytology results were reported in 6.7 % of benign cases and in 34 % of malignant cases (Table 2). Bilharziasis was found in 40 % of benign cases and 37.2 % of malignant cases (Table 2). There was a significant difference in urinary *miRNA-96* expression between benign and malignant bilharzial cases (Table 3).
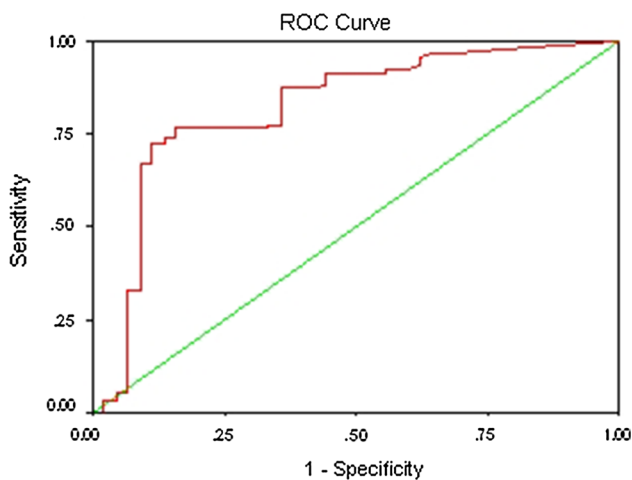
Fig. 1 ROC curve analysis for *miRNA-96* to calculate the best cutoff point that discriminates between malignant and non-malignant groups. Area under the curve = 0.822 and standard error = 0.033. The best cutoff point of *miRNA-96* was 1.63, 95 % confidence limits range = 0.758–0.887, sensitivity = 72.3 % and specificity = 88.9 % and $P < 0.001$

Table 3 Expression of urinary *miRNA–96* in benign and malignant bilharzial bladder cancer

Investigated marker	Benign group Positive bilharziasis (n = 12)	Malignant group Positive bilharziasis (n = 35)
Positive urine miRNA-96 (n = 31)	4 (33.3 %)	27 (77.1 %)
Negative urine miRNA-96 (n = 16)	8 (66.7 %)	8 (22.9 %)
P	0.006*	

* Significant difference is detected between bilharzial benign groups and bilharzial malignant groups at $P < 0.05$ using Chi-square test

Relation between urinary *miRNA-96* and different clinicopathological factors in the malignant group

No significant difference was detected between *miRNA-96* expression and any of the studied clinicopathological factors in the malignant group ($P > 0.05$) as shown in Table 4.

Overall sensitivity, specificity, PPV, NPV and accuracy of urine *miRNA-96*

When *miRNA-96* was tested independently using real-time qPCR, it showed the highest sensitivity and specificity (72.3, 88.9 %) even in low-grade, early-stage or bilharzial bladder cancer (Table 5). Moreover, the sensitivity of urine cytology (34 %) when combined with *miRNA-96* was improved to 79.8 %.

Discussion

For bladder cancer, a new diagnostic marker has been under study in order to reduce the cost and the frequency of cystoscopy or replace them by noninvasive tests during the initial diagnosis and follow-up period. In recent years, the aberrant expression of miRNAs in bladder cancer has been studied. Some miRNAs have been reported to be upregulated in tissues of bladder cancer. For example, *miR-129* was the most commonly upregulated and its upregulation was associated with poor outcome [3].

In the ongoing search for new markers to improve the bladder cancer diagnosis, the efficacy of urinary *miRNA-96* in diagnosis of bladder cancer and its relation to bilharziasis was performed in this study.

In the current study, real-time qPCR was used to detect the expression level of *miRNA-96* in 184 voided urine samples collected from patients with different types of bladder cancer (n = 94), benign bladder lesions (n = 30) and normal volunteers (n = 60). The expression level of *miR-96* was significantly upregulated in bladder cancer group (mean rank = 121.49), in comparison with the normal and benign groups (mean rank = 55.02 and 76.63, respectively, $P < 0.001$) using the RQ values. Upregulation of *miR-96* in TCC tumorigenesis is one of the mechanisms of repression of transcription factors *(FOXO)* of Forkhead Box O subfamily, which is a tumor-suppressor gene-causing G1 cell cycle arrest and cell death ([6, 14]). Also, *hsa-miR-96* by upregulating *MAP4K1* and *insulin receptor substrate 1 (IRS1)* levels may affect the growth of bladder cancer cells [20].

Yamada et al. [21] and Han et al. [7] identified a great number of miRNAs that were significantly upregulated in bladder cancer group using miRNA qRT-PCR and microarray profiling. Yamada et al. [21] observed that *miR-96* was significantly higher expressed in urine of 100 bladder cancer group than in healthy controls (miR-96, $P = 0.0059$) and significantly correlated with tumor grade and stage. Han et al. [7] also revealed that *miR-96* was the most significantly upregulated miRNA in bladder cancer group. This miRNA, therefore, can be regarded as a promising diagnostic marker in bladder cancer. In addition, expression of this miRNA decreased significantly after radical surgery, suggesting that it can be used also as a prognostic molecular marker of cancer recurrence [21].

Yan et al. [22] and Wang et al. [20] observed that *miR-96* expression was higher in bladder carcinoma compared with normal bladder tissues using northern blot analysis and quantitative real-time PCR (qRT-PCR). Also, *miR-96* expression in the superficial bladder tumors was lower than in invasive tumors and significantly related to the clinical stages of bladder carcinoma and the pathological types. These results revealed that *miR-96* maybe plays a role in

Table 4 Real-time qPCR of *miRNA-96* levels using the RQ values and positivity rate of urine cytology and bilharziasis in relation to different clinicopathological factors of the bladder cancer group

Groups (n = 94)	miRNA-96 Real-time qPCR			Positive cytology[a] (n = 32)	Positive bilharziasis[a] (n = 35)
	Median	Mean rank[b]	>cutoff value[a]		
Age					
<52 (n = 25)	4.00	40.34	15 (22.1 %)	7 (21.9 %)	7 (20 %)
≥52 (n = 69)	5.43	50.09	53 (77.9 %)	25 (78.1 %)	28 (80 %)
P		0.126	0.107	0. 457	0.265
Sex					
Male (n = 74)	5.02	47.80	54 (79.4 %)	27 (84.4 %)	28 (80 %)
Female (n = 20)	6.70	46.40	14 (20.6 %)	5 (15.6 %)	7 (20 %)
P		0.839	0.792	0.336	0.810
Smoking					
Positive (n = 56)	6.10	51.10	42 (61.8 %)	23 (71.9 %)	20 (57.1 %)
Negative (n = 38)	4.40	42.20	26 (38.2 %)	9 (28.1 %)	15 (42.9 %)
P		0.121	0.484	0.081	0.711
Bilharziasis					
Positive (n = 35)	5.70	47.54	27 (39.7 %)	11 (34.4 %)	–
Negative (n = 59)	5.02	47.47	41 (60.3 %)	21 (65.6 %)	–
P		0.991	0.423	0.680	–
Urine cytology					
Positive (n = 32)	5.96	49.92	25 (36.8 %)	–	11 (31.4 %)
Negative (n = 62)	5.05	46.25	43 (63.2 %)	–	24 (68.6 %)
P		0.536	0.368	–	0.680
Clinical stage					
Early (0 + 1) (n = 66)	5.02	45.86	48 (70.6 %)	26 (81.3 %)	24 (68.6 %)
Late (2 + 3) (n = 28)	7.08	51.38	20 (29.4 %)	6 (28.7 %)	11 (31.4 %)
P		0.370	0.898	0.093	0.789
Histological grade					
Low (1 + 2) (n = 69)	5.43	47.50	51 (75 %)	27 (84.4 %)	26 (74.3 %)
High (3) (n = 25)	5.02	47.50	17 (25 %)	5 (15.6 %)	9 (25.7 %)
P		1.00	0.571	0.084	0.882
Pathological type					
TCC (n = 52)	6.64	50.46	40 (58.8 %)	20 (62.5 %)	13 (37.1 %)
SCC (n = 33)	5.09	46.14	22 (32.4 %)	10 (31.3 %)	19 (54.3 %)
Others (n = 9)	3.66	35.39	6 (8.8 %)	2 (6.3 %)	3 (8.6 %)
P		0.291	0.543	0.544	0.10
Cutoff of miRNA-96					
<cutoff (n = 28)	0.41	13.50	0 (0 %)	7 (21.9 %)	8 (22.9 %)
≥cutoff (n = 68)	9.37	60.50	68 (100 %)	25 (78.1 %)	27 (77.1 %)
P		0.000*	0.000*	0. 368	0.423

* Significant difference is detected between investigated groups at $P < 0.001$ using Chi-square test[a] and nonparametric test[b]

the process of development, occurrence and infiltration of bladder carcinomas [20].

This is the first study to investigate *miRNA-96* expression in bilharzial bladder cancer. Interestingly, 27 out of 35 bilharzial malignant group showed positive *miRNA-96* with no statistical significance between them ($P > 0.05$). While in bilharzial benign group, 4 out of 12 showed positive *miRNA-96* with also no statistical significance between them ($P > 0.05$). This study is among the first to investigate urinary *miRNA-96* in bilharzial bladder cancer. There was significant difference between malignant and benign bilharzial cases regarding urinary *miRNA-96*, indicating a

Table 5 Overall sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy of urinary *miRNA-96* and cytology in detecting bladder carcinoma, superficial bladder cancer, low-grade bladder cancer and bilharzial bladder cancer when tested independently or in combinations

Parameters	Bladder cancer					Superficial bladder cancer					Low-grade bladder cancer					Bilharzial bladder cancer				
	Sen.	Spec	PPV	NPV	Acc	Sen.	Spec.	PPV	NPV	Acc	Sen.	Spec.	PPV	NPV	Acc	Sen.	Spec.	PPV	NPV	Acc
RT-qPCR of miRNA-96	72.3	88.9	87.2	75.5	80.4	72.7	88.9	82.8	81.6	82	73.9	88.9	83.6	81.6	82.4	77.1	88.9	73	90.9	85.6
Cytology	34	97.8	94.1	58.7	65.2	39.4	97.8	92.9	68.7	73	39.1	97.8	93.1	73.3	72.3	31.4	97.8	84.6	78.6	79.2
RT-qPCR of miRNA-96+cytology	79.8	86.7	86.2	80.4	83.1	81.8	86.7	81.8	86.7	84.6	81.2	86.7	82.3	85.7	84.3	80	86.7	70	91.8	64.8

potential role for bilharziasis in the aberrant expression of urinary *miRNA-96* underlying carcinogenesis.

An ideal urine biomarker for bladder cancer diagnosis should have high positive predictive value (PPV) and sensitivity [8]. In this study, *miRNA-96* showed high sensitivity and specificity even in low-grade, early-stage or bilharzial bladder cancer than that of cytology (Table 4). Accordingly, urinary *miRNA-96* was superior to urine cytology for bladder cancer diagnosis. Moreover, the sensitivity of urine cytology was improved when combined with *miRNA-96* detected by RT-qPCR.

In conclusion, the results of this study revealed that *miRNA-96* expression level in urine sample is a potentially useful urinary biomarker for early diagnosis of bladder cancer including bilharzial bladder cancer and it improves the sensitivity of urine cytology.

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