Original Article

Role of a *Schistosoma haematobium*-specific microRNA-71a and its target gene MAPK3 as a tumor biomarker for early diagnosis and prognosis of bilharzial bladder cancer in Egypt

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ABSTRACT

Background: *Schistosoma* miRNAs and their target genes are utilized in diagnosis and/or prognosis of schistosomiasis.
Objective: To evaluate the diagnostic and prognostic role of *Sh*-miRNA-71a and its target gene MAPK3 in bilharzial bladder cancer (BC) in Egypt.
Subjects and Methods: This case-control study recruited 40 patients with *S. haematobium* aged 17-70, of both sexes, and 20 apparently healthy volunteers of similar age range, and gender. Based on clinical examination, and pathological characteristics, patients were divided into two equal groups; BC, and chronic bilharzial cystitis. From each patient with BC, a histopathological report for diagnosis and grading was obtained. Urine samples were collected from all participants, and miRNA, and MAPK3 RNA were extracted, followed by cDNA synthesis, amplification, and *Sh*-miRNA-71a and MAPK3 quantification using real time PCR (RT-PCR) assay. The obtained results were correlated with BC type and grade.
Results: Tested *Sh*-miRNA-71a was significantly higher in the bilharzial BC group than the chronic cystitis (P=0.005). The MAPK3 was significantly higher in patients with bilharzial BC than those with chronic cystitis, and controls. It was also significantly (P<0.05) higher in chronic cystitis, than the controls. In addition, *Sh*-miRNA-71a and MAPK3 were significantly (P<0.001) higher in patients with high-grade bilharzial BC than in patients with low-grade bilharzial BC. There was a positive correlation between MAPK3 and *Sh*-miRNA-71a (r=0.313, P=0.049) indicating that both biomarkers can significantly predict malignancy, and differentiate between high- and low-grade malignancy.
Conclusion: Urinary *Sh*-miRNA-71a and its target gene MAPK3 can be used as non-invasive prognostic markers to predict bilharzial BC and differentiate between high- and low-grade cases with positive significant correlation.

Keywords: bladder cancer; Egypt, MAPK3; tumor marker; *Sh*-miRNA-71a, *S. haematobium*.

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INTRODUCTION

In Egypt, BC is the second most common malignancy, where men are more affected than women[1]. The trematode *S. haematobium* causes urinary bilharziasis. The immunological inflammatory response to the deposited bilharzial ova is responsible for the clinical manifestations. Ova cause irritation of the urogenital system and predispose to BC. Suppressor gene inactivation, oncogene activation, and somatic chromosomal mutations are responsible for initiating the malignancy[2].

The small non-coding RNAs (miRNAs), ~22 nucleotides, had a role in regulation of tumor growth[3]. They were used as biomarkers to detect bilharzial BC using urine samples. These miRNAs are involved in the carcinogenesis process, prediction, and prognosis of bilharzial BC[4]. Their presence in different body fluids of patients, allows their use as biomarkers in diagnosis and follow-up[5]. *Schistosoma* specific miRNAs have a role in the pathogenesis of schistosomiasis and parasite growth, also there is a possibility for using them in diagnosis[6]. These miRNAs proved their importance in initiation and progression of BC disease because of their genetic and epigenetic dysregulations, and these biomarkers were approved by The USA Food and Drug Administration (FDA)[7]. These schistosomal miRNAs are found in the extracellular vesicles of host biological fluids as serum, so they can be used as a diagnostic tool to diagnose schistosomiasis *haematobium*[8]. The changes in the expression patterns of the miRNAs might regulate and affect the sexual differentiation of schistosomes[9]. Of note, related target genes detection has an importance in elucidating the biological functions of miRNAs. Variable trials were attempted to predict the target

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genes of different miRNAs using the available target prediction tools, such as miRanda, PicTar, Target ScanS, PITA, and RNAhybrid[10].

Five well determined miRNAs belonging to *S. haematobium* include: *Sh*-miRNA-71a, *Sh*-miRNA-1, *Sh*-miRNA-125b, *Sh*-miRNA-7a, and *Sh*-miRNA-let7[11]. On the other hand, specific *Sh*-miRNA-71a can bind to 30-UTR parts of 53 genes of the host suggesting its role in the regulatory functions of the host[12]. Expression of *Sh*-miRNA-71a in urine samples of patients who had BC than in those with chronic bilharziasis or in patients with benign bladder cancer was reported[13]. In addition, its expression was more in urine samples of patients who had bilharzial BC than in non-bilharzial BC. Bilharzial BC showed different values of expression of *Sh*-miRNA-71a, which adds to the importance of this miRNA in diagnosis, prediction, and follow-up of different types of bilharzial BC[13].

It was estimated that miRNAs aberrant expression was used as novel biomarker detected in the urine of bilharzial BC and non-bilharzial BC patients[14]. Moreover, the role of some small RNAs in carcinogenesis and refining prognosis in cell lines has been elucidated[15]. The miRNAs can also be used in screening of some cancers in their early stages[16]. It was reported that some miRNAs can predict treatment merit and prognosis[12]. The MAPK3 is the gene target because its dysregulated pathway is involved in bladder cancer[17]. Therefore, the diagnostic and prognostic role of *S. haematobium*-specific miRNA, *Sh*-miRNA-71a in bilharzial bladder cancer in Egypt should be evaluated.

SUBJECTS AND METHODS

This case-control study was conducted from November, 2022 to July, 2025 in Urology and Oncology Polyclinics, Sohag University Hospital, Molecular Biology Research and Studies Institute, Assiut University, Egypt.

Study design: Based on cystoscopy and histopathology examination, cases were divided into two groups; group I: patients with bilharziasis who developed BC, group II: patients with chronic bilharzial cystitis. Group III cases were with no urinary bladder conditions (control group). Complete history was taken from all participants. Reports of clinical examination, full laboratory tests, histopathological characteristics were obtained. Urine samples were collected for determination of *Sh*-miRNA-71a and its target gene MAPK3.

Study participants and samples collection: The study recruited 40 outpatients attending Sohag Urology Clinic, aged from 17- 70 y of both sexes. Cases with urinary symptoms (urinary retention, hematuria, dysuria, *etc*.) who were suspected to have bilharzial BC or bilharzial cystitis were selected, and scheduled for cystoscopy. Exclusion criteria included patients who had received chemotherapy or were previously diagnosed having any malignancy within the past 5 y. For the control group, additional 20 apparently healthy volunteers of both sexes were selected from apparently healthy employees of Sohag university hospital. For all participants, full history was recorded, clinical examination and full laboratory tests including complete blood count, liver and kidney function tests, were performed. Urine samples were collected and stored at -80°C for estimated expression of *Sh*-miRNA-71a and its target gene MAPK3.

Urine expression of *Sh*-miRNA-71a and host MAPK3

1. **RNA extraction[13]** using ABT total RNA mini extraction kit (Applied biotechnology, Eygpt, Catalogue number: ABT002) following the manufacturer's instructions.
2. **cDNA synthesis, amplification, and detection (reverse transcription)[13]:** This was performed following the manufacturer's protocol using the ABT H-minus cDNA synthesis kit (Applied biotechnology, Eygpt, Catalogue number: ABT009).
3. **RT-PCR assay for miRNA quantification[13]:** Following the manufacturer's protocol using the HERA PLUS SYBR® Green qPCR Kit (Applied biotechnology, Eygpt. WF1030800X), 5 µl of cDNA was employed to run the experiment on the Stepone™ real-time PCR apparatus. The U6 primer was used as an internal control for *Sh*-miRNA-71a and ACTB for MAPK3 to normalize the expression. At the end of the reaction the Ct value data was analyzed for gene expression (Table 1).

Statistical analysis: The SPSS v27 (IBM©, Chicago, IL, USA) was used in data processing. Shapiro-Wilk, ANOVA (*F*) with post hoc test (Tukey), Mann-Whitney, Kruskal-Wallis, and Chi-square tests were used in data analysis. Fisher's exact test was used when dealing with small sample sizes or low frequencies. In receiver operating characteristic curve (ROC-curve) analysis, the area under the curve (AUC) evaluates the overall test performance, where the area under the curve >50% denotes acceptable performance, and area ~100% is the best performance for the test. Variables for diagnostic performance were calculated as follow: Sensitivity= [(TP)/(TP+FN)] ×100, Specificity= [(TN)/(TN+FP)] ×100, Positive predictive value (PPV)= [(TP)/(TP+FP)] ×100, Negative predictive value (NPV)= [(TN)/(TN+FN)] ×100, where TP: true positive, FN: false negative, TN: true negative, FP: false positive. On the other hand, diagnostic accuracy= (sensitivity * prevalence) + (specificity * (1 - prevalence), where prevalence means the proportion of the population that actually has the condition. A two tailed *P* value < 0.05 was considered statistically significant.

Table 1. The primers used for qPCR amplification.

Primer	Description	Annealing temp (Ta) °C
U6		
Forward (5'-3')	CTC GCT TCG GCA GCA CAT	59
Reverse (5'-3')	TTT GCG TGT CAT CCT TGC G	
***Sh*-miRNA-71A**		
Forward (5'-3')	GCA GTG AAA GAC GAT GGT AGT	50
Reverse (5'-3')	GGT CCA GTT TTT TTT TTT TTT TCT C	
ACTB		
Forward (5'-3')	AGC ACA GAG CCT CGC CTT	60
Reverse (5'-3')	CAT CAT CCA TGG TGA GCT GC	
MAPK3		
Forward (5'-3')	GGC AAG CAC TAA CCT GGA TCA G	65
Reverse (5'-3')	GCA GAG ACT GTA GGT AGT ATT CGG	

U6: Small nuclear RNA used as endogenous control for miRNA; ***Sh*-miRNA-71A:** *S. haematobium* miRNA-71A; **ACTB:** Beta-actin used as endogenous control for MAPK3; **MAPK:** Mitogen-activated protein kinase.

Ethical consideration: The study received agreement from the Ethical Committee of the Faculty of Medicine, Sohag University, Sohag, Egypt, with IBR Registration number (Soh-med-22-11-12). Informed written consent was obtained from all participants.

RESULTS

The age parameter was significantly (P=0.048) higher in the chronic bilharziasis group (II) than in the bilharzial BC group (I), and was insignificantly different from the control group. Sex was insignificantly higher in males in the three groups. Residence was rural in all patients of the three groups. High-grade transitional cell carcinoma (TCC) was the predominant type in 14 (70%) of BC cases, followed by low-grade TCC (3; 15%), then high-grade squamous cell (SCC) (2; 10%), and lastly low-grade squamous cell carcinoma (1; 5%) (Table 2, Fig. 1).

Our results revealed that *Sh*-miRNA-71a was significantly (P<0.005) higher in the bilharzial BC group than the chronic bilharziasis group. In addition, MAPK3 was significantly (P<0.05) higher in the bilharzial BC group than the chronic bilharziasis group, and control group. It was also significantly (P<0.05) higher in the chronic bilharziasis group than the control group (Table 3).

Table 2. Demographic data and histopathological results of the studied groups.

Variable	Groups			Statistical analysis
	GI (No. =20)	GII (No. =20)	GIII (No. =20)	
Age (mean ± SD)	49.7±17.76	61.1±9.01	53.7±10.95	***P*1**=0.048*, ***P*2**=0.494, ***P*3**=0.408, ***P*4**= <0.001*
Gender (No., %)				
Male	16 (80%)	17 (85%)	16 (80%)	***P*** = 0.895
Female	4 (20%)	3 (15%)	4 (20%)	
Grading (No., %)		---	---	
1A	14 (70%)			
2A	2 (10%)			***P*** < 0.001*
1B	3 (15%)			
2B	1 (5%)			

GI: Bladder cancer; **GII:** Chronic cystitis; **GIII:** Control group; ***P*1:** *P* value between chronic bilharziasis group and bilharzial BC group, ***P*2:** *P* value between chronic bilharziasis group and control group, ***P*3:** *P* value between bilharzial BC group and control group, ***P*4:** *P* value between the three groups; **1A:** High-grade TCC, **1B:** High-grade SCC, **2A:** Low-grade TCC, **2B:** Low-grade SCC, ***:** Significant (P<0.05).

Table 3. Expression of *Sh*-miRNA-71a and host MAPK3 in the studied groups.

Variable	Groups			Statistical analysis
	GI (No. =20)	GII (No. =20)	GIII (No. =20)	
***Sh*-miRNA-71a**	28.13 ± 4.39	22.94 ± 6.35	--	P < 0.005*
Host MAPK3	28.16 ± 4.50	23.47 ± 6.38	3.87 ± 1.12	P < 0.05*

GI: Bladder cancer; **GII:** Chronic cystitis; **GIII:** Control group; ***:** Significant (P<0.05).

Expression of both biomarkers was significantly (P<0.001) higher in patients with high grade than in patients with low grades. Their expressions were also higher in patients with TCC than in patients with SCC with insignificant differences (Table 4). Figure (2) shows that both expressions in urine samples were positively correlated (r=0.313, P=0.049).

Both biomarkers were significantly higher in patients with high-grade bilharzial BC than in patients with low-grade bilharzial BC (P<0.001). There was a positive correlation between MAPK3 and *Sh*-miRNA-71a (r=0.313, P=0.049). The *Sh*-miRNA-71a and MAPK3 can significantly predict malignancy (at cut-off =25.75 and 26.67 respectively), with 85% and 85% sensitivity, 75% and 80% specificity, 78% and 81.5% accuracy and P<0.001, AUC = 0.848 and 0.832, respectively (Fig. 3). Additionally, *Sh*-miRNA-71a and MAPK3 can significantly differentiate between high- and low-grade malignancy at cut-off 27.24 and 27.68 with 87.5% and 93.75% sensitivity, 75.0% and 75.0% specificity, and 78.75% and 80.6% accuracy, and P<0.001, AUC = 0.969 and 0.984, respectively (Fig. 3).

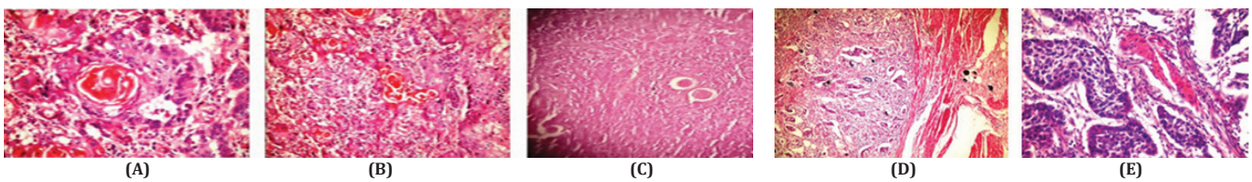

Fig. 1. (A) Well-differentiated SCC (low grade); **(B)** poorly differentiated SCC (high-grade); **(C)** Chronic cystitis; **(D)** poorly differentiated TCC (high-grade); **(E)** well-differentiated TCC (low grade).

Table 4. Relation of *Sh*-miRNA-71a and MAPK3 expressions in urine samples to histopathology and grading in the studied groups.

Markers	Histopathology		Statistical analysis
	TCC (N=17)	TCC (N=17)	
***Sh*-miRNA-71a**	18.76 - 31.96	17.77 - 28.77	P = 0.56
MAPK3	18.89 - 32.68	16.48 - 28.3	P = 0.64
	Grading		
	High grade (N=16)	Low grade (N=4)	
***Sh*-miRNA-71a**	26.66 - 31.96	17.77 - 26.07	P<0.001*
MAPK3	27.69 - 32.68	16.48 - 31.07	P<0.001*

Numbers represent range of threshold RT-PCR cycles; ***:** Significant (P<0.05).

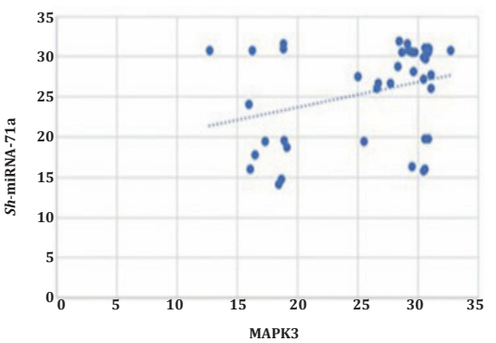

Fig. 2. Correlation between MAPK3 and *Sh*-miRNA-71A.

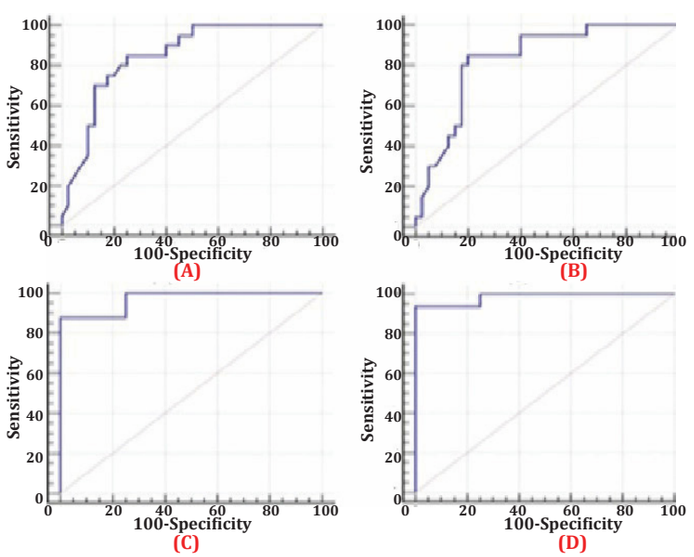

Fig. 3. ROC curve of **(A)** *Sh*-miRNA-71a, and **(B)** MAPK3 in the prediction of malignancy, **(C)** *Sh*-miRNA-71a, and **(D)** MAPK3 in differentiation between high and low-grade.

DISCUSSION

Bladder cancer is the second most common malignancy of the urogenital system, affecting mainly men[18]. In our study, age was significantly lower in the chronic bilharziasis group than BC group and was insignificant between (chronic cystitis, and BC groups) and the control group. Sex was insignificantly different among the three groups. Residence was rural in all patients of the three groups, which could be attributed to the fact that bilharzial infestation requires an agricultural environment[19]. Chronic infection by *S. haematobium* can lead to bladder cancer, and this can explain why age was significantly lower in chronic bilharziasis than bilharzial BC[20]. This study agreed with Shih *et al*.[21] who showed that age was higher in patients with BC than patients with chronic cystitis.

In our study, in the chronic bilharziasis group, biopsy results showed chronic bilharziasis in all 20 (100%) patients. In the bilharzial BC group, the biopsy was 1A (high-grade transitional cell carcinoma) in 14 (70%) patients, 1B (high-grade squamous cell carcinoma) in 2 (10%) patients, 2A (low-grade TCC) in 3 (15%) patients, 2B (low-grade SCC) in 1 (5%) patient. In agreement with our findings, Gaber *et al*.[13] reported that high-grade transitional cell carcinoma was the predominant type, representing 40 (80%) of cancer cases in their study, followed by high- and low-grade SCC. Close to our findings, El-Shal *et al*.[22] reported similar grading of bilharzial BC: G1, G2 (low grade) in 42 (82.3%) patients and G3 (high grade) in 9 (17.7%) patients.

Our study revealed that *Sh*-miRNA-71a was significantly higher in the bilharzial BC group than in the chronic bilharziasis group. MAPK3 was higher significantly in the bilharzial BC group than in the chronic bilharziasis group and the controls and was significantly higher in the chronic bilharziasis group than in the controls. In agreement with our findings, Gaber *et al*.[13] found that *Sh*-miRNA-71a and MAPK3 were significantly higher in the bilharzial BC group than in bilharzial cystitis.

In the present study, regarding the relation between MAPK3 and *Sh*-miRNA-71a and histopathological grades of bilharzial BC, *Sh*-miRNA-71a and MAPK3 were significantly higher in patients with high grades than in patients with low grade malignancies. In agreement with our findings, Gaber *et al*.[13] found that cases with high-grade malignancy varied with a statistically significant difference in MAPK-3 gene expression when compared to cases with low-grade cancer. Supporting our findings, Mamdouh *et al*.[23] found that miRNAs were significantly higher in high-grade bilharzial BC than in low-grade cases.

In our study, there was also positive correlation between MAPK3 and *Sh*-miRNA-71a, and both were higher in cases who had TCC than in those with SCC. On the other hand, results reported by Gaber *et al*.[13] indicated insignificantly higher MAPK3 and *Sh*-miRNA-71a in cases with TCC than in cases with SCC. There was also a potent correlation between MAPK-3 and *Sh*-miRNA-71a in the bilharzial cystitis and BC groups.

Our study showed that *Sh*-miRNA-71a and MAPK3 can significantly predict BC. Similarly, Leija-Montoya *et al*.[24] reported that *Sh*-miRNA-71a was a potential biomarker in BC. Additionally, both *Sh*-miRNA-71a and MAPK3 significantly differentiated between high- and low-grade BC. Gaber *et al*.[13] reported similar findings concerning the significantly high sensitivity, specificity of *Sh*-miRNA-71a and MAPK3 for the diagnosis of high-grade malignancy. El-Shal *et al*.[22] also reported that miRNA-183-5p and miRNA-96-5p can significantly differentiate between high and low grades of BC. Moreover, Wang *et al*.[25] found that miRNA-20a-5p, miRNA-17-5p, and miRNA-92a-3p can differentiate between high and low grades of BC. Supporting our findings, Abd El Gayed *et al*.[1] carried out a study on the role of miRNAs in BC. They showed that miRNA-130a-3p and miRNA-301a-3p expression can differentiate BC patients from healthy controls.

In conclusion, urinary *Sh*-miRNA-71a and its target gene MAPK3 can be used as non-invasive diagnostic markers to diagnose BC and differentiate between high- and low-grade cases, with a positive significant correlation.

Author contribution: Ali EM proposed the study topic, and performed the practical and statistical methods. Alhady HA contributed in the study design and in the performance of the practical methods. Abdl-Wahab AG, and Ali MM performed urologic practical part and revision of urologic data of the study. Osman HE contributed in the performance of the practical methods and wrote the manuscript. All authors revised and accepted the final version for publication.

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