Non-invasive Diagnostic Potential of microRNA-203 in Liquid Biopsy of Urothelial Carcinoma of Bladder

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Abstract

Purpose

Increased CD44 antigen activity has been reported in recurrent cases of UBC. To date, no reliable biomarker is available with high significance and specificity for non-invasive detection of UBC. This study aimed to identify a CD44-linked microRNAs (miRNAs) panel for non-invasive diagnosis of bladder cancer from other urinary tract malignancies.

Methods

The expression of CD44-linked miRNAs was examined in serum, urine, and tissue specimens of Indian UBC patients (N = 25). For this purpose, healthy subjects (N = 25) and benign prostatic hyperplasia (BPH) (N = 10) patients were taken as controls. The relative expression of miRNAs was analysed in serum, urine and tissue samples using real-time quantitative reverse transcription PCR (qRT-PCR). The diagnostic potential of these miRNAs was accessed by plotting ROC curve.

Results

Increased miR-9 expression was observed in serum of UBC patients than healthy and BPH controls. In UBC patients, miR-34a expression was lower than healthy controls but non-significant as compared to BPH. miR-203 expression was considerably higher in serum of UBC patients but non-significant as compared to BPH controls. miR-203 was found to be considerably higher in urine samples from UBC patients as compared to BPH and healthy controls. The diagnostic potential of these miRNAs was evaluated using the ROC curve. Higher miR-203 levels in the urine of Indian UBC patients demonstrates its non-invasive diagnostic ability out of the three miRNAs studied.

Conclusions

Our results characterize the non-invasive diagnostic potential of CD44-linked miR-203 in the urine of Indian UBC patients, which could be utilized in clinical settings in future after validation in larger patient cohort.

Introduction

Urinary bladder cancer (UBC) is the tenth most prevalent type of urogenital malignancy worldwide. In 2020, there were an estimated 573,278 new cases and 212,536 deaths recorded globally [1]. Urothelial carcinoma, also called transitional cell carcinoma (TCC), is the predominant histological type of UBC. It is mainly classified into two types, viz non-muscle invasive (NMIBC) and muscle-invasive bladder cancer (MIBC) [2]. The largest cases of UBC are diagnosed at the NMIBC stage. Patients with NMIBC have minimal risk of tumor-related death besides a very high recurrence rate (more than 75% of patients recur within five years [3]. About 10-30% of NMIBC advances to MIBC with a reduced survival rate and frequent distant metastasis. The prevalence of UBC is four times greater in men than in women [4]. Environmental

or occupational exposure to carcinogens, including tobacco and azo dyes in textile industries, are the critical risk factors for UBC. Most cases of UBC are detected in people over the age of 60 [5].

The most common symptom of UBC is either macroscopic or microscopic hematuria. Currently, UBC diagnosis involves cystoscopy and biopsy, which are obnoxious and expensive procedures [6]. The stage of bladder cancer diagnosis has not changed over the past ten years due to a lack of effective screening. Several FDA-approved urine-based tests (e.g., NMP22, NMP22 BladderChek, UroVysion, BTA-TRAK and BTA-STAT etc.) for UBC diagnosis and monitoring are commercially available [7]. However, the sensitivity, specificity and diagnostic efficacy of these urine-based assays are still sub-optimal; therefore, new molecular markers or assays must be translated into the clinics. Our lab has been working in the area of UBC for the past two decades and the diagnostic potential of certain molecules has also been assessed in UBC [8],[9]. The use of minimally invasive "liquid biopsies" for the classification and characterization of urogenital malignancies is advancing rapidly [10]. Tumor cells release cell-free DNA, RNA (including mRNAs, miRNA and long non-coding RNA), peptides and proteins, which confers potential as a promising biomarker [11].

CD44 serves as a receptor for hyaluronic acid (HA, non-sulfated glycosaminoglycan). Our group had previously reported the importance of CD44 and its multiple variants along with HAS (hyaluronic acid synthase) in pathogenesis of UBC [12]. HA-activated CD44 favors tumor cell survival, metastasis and chemoresistance via activation of diverse cellular pathways, including PI3K-Akt, RAS-MAP, JAK-STAT etc. [13], [14]. miRNAs are small (18-22 nucleotide long), non-coding RNAs that regulate gene expression at the transcription and translation level. The sequence-specific pairing of miRNAs with the 3'-untranslated region (UTR) of the target mRNAs results in its degradation or translation suppression [15]. Deregulated miRNA expression has been spotted in many tumors and is associated with invasion, migration, and epithelial-mesenchymal transition (EMT) [16]–[18]. Our group has also reported the diagnostic significance of circulatory microRNAs in renal cell carcinoma (RCC) and multiple myeloma [19], [20].

CD44 associated miRNAs include miR-9, miR-21, miR-34a, miR-130, miR-143, miR-199 and miR-203 etc. [21]–[23]. miRNAs are broadly classified into oncogenic (oncomiR) and tumor-suppressive (Anti-oncomiR) miRNAs. In bladder cancer, upregulated expression of miR-9 and downregulated expression of miR-34a and miR-203 have been reported [24]–[26]. Most of these studies were conducted in-vitro using cell lines. Very limited reports are available on human specimens.

In this study, we investigated the differential expression of CD44-linked miRNAs comprising miR-9, miR-34a and miR-203 in serum, urine, and tissue specimens of UBC patients in the Indian population. Moreover, the non-invasive diagnostic potential of these miRNAs was estimated by plotting the ROC curve. Out of all three miRNAs, miR-203 showed the non-invasive diagnostic potential in urine specimen of Indian UBC patient which require further validation in larger cohorts.

Materials And Methods

Study samples

The study was authorized by Institute's Ethics Committee (IEC No.: IEC-532/02.08.2019, RP-23/2019), and written consent was received from all patients and healthy subjects (N = 25). Histopathologically confirmed BPH (N = 10) and UBC (N = 25) patients were involved in the study. Patients and healthy subjects having urinary tract infections (UTIs) and other malignancy were excluded. Blood and urine specimens were collected from healthy subjects. Blood, urine, and tumor tissue specimens were collected from BPH controls. Blood, urine, tumor tissue and adjacent healthy tissue specimens were collected from UBC patients. Total 3mL of venous blood and 5mL urine were collected from all the participants. Tumor and adjacent healthy tissues were collected from the same UBC patients during surgery.

Total RNA extraction and reverse transcription of microRNA

Blood was collected in serum separator tubes (SST) (BD Vacutainer®) to isolate serum. Total RNA was isolated as per manufacturer's protocol from serum and urine using miRNeasy Serum/Plasma kit (Qiagen; Catalog No. 217184) while miRNeasy mini kit (Qiagen; Catalog No. 217004) was used for tissue specimens. *cel-miR-39a* was used as spike-in control for serum and urine samples (miRCURY LNA RT kit, Qiagen; Catalog No. 339340). RNA was quantified using Nanodrop™ spectrophotometer (ThermoFisher, USA). For reverse transcription, 1µg of purified RNA was utilized to synthesize cDNA using miScript II RT kit (Qiagen; Catalog No. 218160) as per the manufacturer's protocol.

Quantitative microRNA expression by real-time PCR

The cDNA was diluted in 1:9 ratio using nuclease-free water (NFW) supplied in the miScript II RT Kit. The diluted cDNA was amplified using miRNA-specific primers (purchased from Qiagen) and miScript SYBR® Green PCR Kit (Qiagen; Catalog No. 218073) as per the manufacturer's protocol. Relative expression of miRNAs was analysed using Bio-Rad CFX96 real-time PCR machine. *Cel-miR-39a* primer was used as an internal control in serum and urine, while SNORD48 was used to normalize relative expression in tissue samples.

Receiver Operating Characteristic (ROC) Curve

ROC curve was plotted to access the diagnostic potential of these circulatory miRNAs between the true positive rate against the false positive rate for the different possible cut-points of quantitative miRNA expression results to define the optimal cut-off values with maximum sensitivity and specificity. All the data analysis was done using GraphPad Prism 9.

Statistical analysis

Data were presented as Median (Range) for circulatory levels of miRNA. Comparison between patients and different control groups were performed using the Wilcoxon rank-sum test and one way ANOVA test (Kruskal Wallis test). All the data analysis was done using GraphPad Prism 9. Statistical significance was defined at a p-value less than 0.05 ($p < 0.05$).

Results

Study subjects

Newly diagnosed 25 UBC patients, 10 BPH patients, along with 25 healthy controls, were introduced in the study. The demographic details of the participants are given in Table 1.

Table 1
Demographic and clinical data for the UBC patients and the control subjects

Patient Sample		Control Samples		
		C1	C2	C3
Serum	25	25	10	--
Urine	15	15	10	--
Tissue	25	--	10	12
Male/Female	21/4	20/5	10/0	10/2
Age (in yrs)	59 ± 8	49 ± 8	60 ± 9	55 ± 9
**C1: Healthy subjects; C2: BPH patients; C3: Adjacent healthy tissue from same patient				
Values are represented as mean ± SD				

Relative microRNA expression in serum

The relative expression of CD44 associated miRNAs (miR-9, miR-34a and miR-203) has been examined in serum of UBC, BPH and healthy subjects using qRT-PCR. The relative expression of miR-9 in serum of UBC patients was significantly upregulated in contrast to healthy ($p < 0.001$) and BPH controls ($p < 0.01$) (Fig. 1A, Table 2, 3). The relative expression of miR-34a in serum of UBC patients was significantly downregulated in UBC patients in opposition to healthy controls ($p < 0.05$) but was non-significant in BPH controls (Fig. 1B, Table 2, 3). The relative expression of miR-203 was significantly upregulated in UBC patients in contrast to healthy controls ($p < 0.05$) but was non-significant in BPH controls (Fig. 1C, Table 2, 3).

Table 2

Relative miRNA expression in blood urine and tissue samples of UBC patients and their respective BPH and healthy controls

Relative microRNA expression					
Median (Range)					
		Control	BPH	UBC	
miR-9	Serum	5.097e-005 (8.745e-007 – 0.008)	1.274e-005 (3.035e-006 – 2.479e-005)	0.001 (0.0001 – 0.939)	
		0.0002 (4.069e-005 – 0.004)	0.0007 (1.053e-005 – 0.003)	0.003 (0.0005 – 0.048)	
	Tissue	—	0.153 (0.039 – 0.303)	UBC NT 0.088 (0.008 – 0.88)	UBC T 0.468 (0.013 – 8.056)
miR-34a	Serum	0.006 (0.0002 – 0.646)	0.011 (0.005 – 0.175)	0.002 (2.968e-005 – 0.036)	
		0.011 (0.0008-0.982)	0.001 (0.0001– 0.008)	0.005 (0.001 – 0.282)	
	Tissue	—	2.034 (0.384 – 14.93)	UBC NT 1.159 (0.013 – 8.574)	UBC T 0.406 (0.025– 3.106)
miR-203	Serum	6.867e-005 (1.066e-006 – 0.009)	1.025e-005 (4.146e-006 – 0.0001)	0.001 (1.168e-007 – 0.216)	
	Urine	0.001 (6.726e-005 – 0.010)	0.0004 (1.079e-005– 0.009)	0.045 (0.002 – 3.434)	

UBC T: UBC tumor tissue; UBC NT: UBC adjacent normal tissue

Relative microRNA expression				
Median (Range)				
Tissue	—	0.271 (0.039- 2.694)	UBC NT 0.243 (0.011 – 2.723)	UBC T 2.021 (0.016– 11.75)
UBC T: UBC tumor tissue; UBC NT: UBC adjacent normal tissue				

Table 3

The level of significance of microRNAs between different groups in serum, urine, and tissue samples

miR-9		miR-34a		miR-203	
Control/UBC	BPH/UBC	Control/UBC	BPH/UBC	Control/UBC	BPH/UBC
Serum	<0.0001	<0.001	<0.05	<0.05	ns
Urine	<0.05	ns	ns	<0.001	<0.05
BPH/UBC T	UBC NT/UBC T	BPH/UBC T	UBC NT/UBC T	BPH/UBC T	UBC NT/UBC T
Tissue	Ns	<0.05	<0.05	Ns	ns
UBC T: UBC tumor tissue; UBC NT: UBC adjacent normal tissue; ns: non-significance					

Relative microRNA expression in urine

The relative expression of CD44 associated miRNAs (miR-9, miR-34a and miR-203) has been studied in the urine of UBC patients along with healthy and BPH controls using qRT-PCR. The relative expression of miR-9 in the urine of UBC patients was significantly upregulated in relation to healthy control ($p < 0.05$) but was non-significant in BPH controls (Fig. 2A, Table 2, 3). The relative expression of miR-34a in the urine of UBC patients was non-significant as opposed to BPH and healthy controls (Fig. 2B, Table 2, 3). The relative expression of miR-203 was significantly upregulated in UBC patients in relation to healthy ($p < 0.01$) and BPH controls ($p < 0.05$) (Fig. 2C, Table 2, 3) which indicates its non-invasive diagnostic ability.

Relative microRNA expression in tissue

The relative expression of CD44 associated miRNAs (miR-9, miR-34a and miR-203) has been studied in the tissue specimens of UBC patients along with BPH controls using qRT-PCR. The relative expression of miR-9 in tumor tissue of UBC patients was significantly upregulated as opposed to adjacent non-tumor tissue of UBC patients ($p < 0.05$) but was non-significant in relation to BPH controls (Fig. 3A, Table 2, 3).

The relative expression of miR-34a in tumor tissue of UBC was downregulated in comparison to BPH ($p < 0.05$) but non-significant as opposed to the adjacent non-tumor tissue of same UBC patients (Fig. 3B, Table 2, 3). The relative expression of miR-203 in tumor tissue of UBC patients was non-significant in comparison to adjacent non-tumor tissue of UBC patients and BPH controls (Fig. 3C, Table 2, 3).

Diagnostic potential of microRNAs in serum, urine, and tissue of UBC

The diagnostic potential of CD44 associated miRNAs (miR-9, miR-34a and miR-203) was evaluated by plotting the ROC curve, which provides sensitivity, specificity, area under the curve (AUC) and cut-off values of these biomolecules. miR-203 showed optimum sensitivity and specificity in the serum (Fig. 4. A-F), urine (Fig. 5A-F) and tissue specimen (Fig. 6A-F) of UBC patients in contrast to miR-9 and miR-34a.

Correlation

The expression of microRNAs (miR-9, miR-34a and miR-203) in serum, urine and tissue specimen of UBC patients was correlated by Spearman correlation analysis. It has been observed that expression of miR-203 in urine showed significant positive correlation with expression in tumor tissue of UBC patients ($r = 0.5832$, $p = 0.0393$). This finding, thus, suggests that urinary miR-203 could be exploited further for diagnosis of UBC in future.

Discussion

Urothelial carcinoma of bladder is characterized by excessive growth in the inner lining of the bladder, i.e., urothelium. The cases of UBC are steadily rising due to changing lifestyle (smoking) and environmental (and occupational) conditions. Epigenetic modifications including DNA methylation, histone modifications and post-transcriptional alterations mediated by non-coding RNA transcripts such as miRNAs plays a crucial role in tumor development and progression

microRNAs are short, non-coding RNAs that affect gene expression at the post-transcriptional level. A variety of miRNAs differentially expressed in bladder cancer have been reported [27]. Catto *et al.* found altered miRNA expression in the urothelium of bladder cancer patients as opposed to healthy controls. They also observed a pattern of decreased miRNA expression in low-grade tumors, including loss of miRNAs which normally suppresses FGFR3 expression. In high-grade tumors, they found upregulation of a number of miRNAs, including one that affects p53 [28].

The CD44 antigen is a cell-surface glycoprotein that serves as hyaluronic acid receptor and involved in cell-cell interactions, cell adhesion and migration. Higher CD44 expression in cells provides stem-cell-like properties to the cancer cells. Previously our group reported that CD44 splice variants support the progression of urothelial carcinoma of bladder through, ERK, Akt, p53 and STAT pathways [12].

The prognosis of the disease is still debatable. Currently, there is no diagnostic tool with optimum sensitivity and specificity for the non-invasive detection of bladder cancer. Biopsy, which is an invasive method, is the only confirmatory test for bladder cancer. Hence, in this maiden attempt, we studied the

relative expression of CD44 associated miRNAs (miR-9, miR-34a & miR-203) in biological fluids and tissue specimens of Indian UBC patients along with BPH and healthy controls and assessed their diagnostic potential as non-invasive biomarker for early detection of bladder cancer.

In the present study, relative miR-9 expression was significantly upregulated in serum samples of UBC patients in relation to their BPH and healthy controls. Urine samples of UBC patients showed significant expression of miR-9 in contrast to healthy controls but not BPH controls. However, significant expression of miR-9 was reported in tumor tissue specimens of UBC patients in comparison to adjacent non-tumor tissues but not BPH tissues. This is the first study showcasing the circulatory levels of miR-9 in serum of UBC patients and the results are in harmony with the reports of its higher levels in serum and tissue samples in other cancers like ovarian cancer and lung cancer [29], [30]. ROC curve analysis of miR-9 in serum samples of UBC patients and healthy controls showed optimum sensitivity and specificity proving its less invasive diagnostic potential to differentiate UBC patients from healthy controls through serum samples.

Relative miR-34a expression in serum and tissue samples was lower in UBC patients as compared to healthy controls but non-significant in contrast to BPH controls. Expression of miR-34a expression was non-significant in urine of UBC patients as compared to healthy and BPH controls. It may be due to the small sample size of the study subjects. In concordance with this observation, we previously observed downregulation of miR-34a in circulation and tumor site of RCC [19]. Similar reports of downregulation of miR-34s were also observed in breast cancer, prostate cancer and mice models of bladder cancer [25], [31], [32]. ROC curve analysis of miR-34a in showed only about 60% specificity and sensitivity which is very less to use it as a biomarker.

We found significantly increased relative miR-203 expression in urine samples of Indian UBC patients as compared to healthy and BPH controls. Similar reports of higher miR-203 expression are also available for tissue specimens of pancreatic adenocarcinoma and breast cancer [33], [34]. miR-203 expression is downregulated in cell lines of several cancers like cutaneous squamous cell carcinoma and prostate cancer including urinary bladder cancer showing its dual role in cancer progression [26], [35], [36]. The ROC curve analysis of miR-203 in urine showed maximum sensitivity and specificity as compared to other miRNAs which indicate its non-invasive diagnostic potential for early diagnosis of bladder cancer using urine specimens. Further, urinary expression of miR-203 correlated significantly with tissue levels in UBC affirming the utilization of urine specimen as an in-situ representation of tumor milieu, hence, could be exploited for diagnosis of UBC in clinical settings.

This study is a maiden attempt that provides a brief insight into the potential role of CD44 linked miRNAs (miR-9, miR-34a and miR-203) as a non-invasive biomarker for the diagnosis of urothelial carcinoma of bladder. Dysregulated expression of these microRNAs might contribute to disease pathogenesis which needs to be validated in future. Out of three miRNAs, miR-203 showed potential of non-invasive biomarker for urine-based detection of UBC from other urinary tract malignancies. Furthermore, study of

these microRNAs in larger cohort might validate the clinical utility as non-invasive diagnostic biomarker in early detection of urinary bladder cancer.

Conclusion

Urothelial carcinoma of the bladder becomes a significant concern these days. Most of the cases are detected at advanced stages. Confirmation of UBC patients is done mainly by invasive methods such as TURBT due to the lack of effective non-invasive biomarkers. Identification of new potential biomarkers in biological fluids can help diagnose bladder cancer at an early stage. We found that miR-203 showed high sensitivity and specificity in urine that can serve as a non-invasive biomarker for UBC detection, which needs to be validated in a larger cohort.

Declarations

Author contributions

AS¹ conceived and designed the study. PS¹ provided patient sample; PS² AS² KDR performed experiments, acquired and analyzed data; AS¹ and NG drafted the manuscript; NG helped in analyzing data; AS¹, SA, PS², AS² and NG edited the final manuscript. **[AS¹ : Alpana Sharma; AS²: Aishwarya Singh; PS¹: Prabhjot Singh; PS²: Pradeep Singh]**

Data Availability

All data generated or analyzed during this study are included in this published article.

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This research did not receive any specific grant from funding agency.

Compliance with Ethical Standards

Conflict of Interest

The authors declare no conflict of interest.

Ethical Approval

This research involving human patients and healthy individuals had provided written informed consent. This study has been approved by Institutional Human Ethics Committee of All India Institute of Medical Sciences, New Delhi, India (IEC No.: IEC-532/02.08.2019, RP-23/2019) prior to its commencement.

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Figures

Figure 1

Circulatory levels of microRNAs of UBC patients. **A.** Relative miR-9 expression in serum **B.** Relative miR-34a expression in serum and **C.** Relative miR-203 expression in serum.. (n= 25 healthy controls; n= 10 BPH controls; n= 25 UBC patients). The levels were represented as arbitrary units (AU). The data was represented as median (range). The bars of significance not mentioned between some groups depicts non-significance. [Healthy controls (control); BPH controls (BPH); UBC patients (UBC); * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns: non-significant]

Figure 2

Relative microRNAs expression in urine. **A.** Relative miR-9 expression in urine **B.** Relative miR-34a expression in urine and **C.** Relative miR-203 expression in urine. (n= 15 healthy controls; n= 10 BPH controls; n= 15 UBC patients). The levels were represented as arbitrary units (AU). The data was represented as median (range). The bars of significance not mentioned between some groups depicts non-significance. [Healthy controls (control); BPH controls (BPH); UBC patients (UBC); * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns: non-significant]

Figure 3

Relative microRNAs expression in bladder tissue specimen. Box whisker plot depicting the relative microRNA expression of **A.** miR-9 expression in tissue **B.** miR-34a expression in tissue and **C.** miR-203 expression in tissue. (n= 10 BPH controls; n= 12 UBC NT; n=25 UBC T). The levels were represented as arbitrary units (AU). The data was represented as median (range). The bars of significance not mentioned between some groups depicts non-significance. [BPH controls (BPH); UBC adjacent normal tissue (UBC NT); UBC tumor tissue (UBC T); *p<0.05; **p<0.01; ***p<0.001; ns: non-significant].

Figure 4

The ROC curve for diagnostic potential of microRNAs in serum. miR-9 serum (A, B); miR-34a serum (C, D); miR-203 serum (E, F). Graph **A, C and E** depicts the comparison between the serum samples of healthy controls and UBC patients. Graph **B, D and F** depicts the comparison between serum samples of BPH control and UBC patients. The graphs showcase the optimum sensitivity and specificity, area under curve along with cut-off values for serum levels [Healthy controls (control); BPH controls (BPH); UBC patients (UBC); AUC – area under the curve]

Figure 5

The ROC curve for diagnostic potential of microRNAs in urine. miR-9 urine (A, B); miR-34a urine (C, D); miR-203 urine (E, F). Graph A, C and E showcase the comparison between the urine samples of healthy controls and UBC patients. Graph B, D and F showcase the comparison between urine samples of BPH control and UBC patients. The graphs showcase the optimum sensitivity and specificity, area under curve along with cut-off values for serum levels [Healthy controls (control); BPH controls (BPH); UBC patients (UBC); AUC – area under the curve]

Figure 6

The ROC curve for diagnostic potential of microRNAs in tissue. miR-9 tissue (A, B); miR-34a tissue (C, D); miR-203 tissue (E, F). Graph A, C and E depicts the comparison between the tissue of BPH and UBC patients. Graph B, D and F depicts the comparison between adjacent normal tissue and tumor tissue of UBC patients. BPH controls (BPH); UBC adjacent normal tissue (UBC NT); UBC tumor tissue (UBC T). The graphs showcase the optimum sensitivity and specificity, area under curve along with cut-off values for

serum levels [BPH controls (BPH); UBC adjacent normal tissue (UBC NT); UBC tumor tissue (UBC T); AUC
– area under the curve]