

Urinary exosomal expression of long non-coding RNAs as diagnostic marker in bladder cancer

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Background: Long non-coding RNAs (lncRNAs) and exosomes have been regarded as components of cell signal transmission that modulate indigenous cellular microenvironments. Exosomes also participate in relocation of functional lncRNAs between cells.

Methods: In the present study, we evaluated expression of *LINC00355*, *LINC00958*, *UCA1-201*, *UCA1-203*, and *MALAT1* lncRNAs in urinary exosomes isolated from transitional cell carcinoma (TCC) of bladder, non-malignant urinary disorders, and normal subjects.

Results: *LINC00355*, *UCA1-203*, and *MALAT1* expression was significantly higher in TCC patients compared to controls (non-malignant or normal samples). However, *UCA1-201* expression was significantly decreased in TCC patients compared with controls. *LINC00355* and *MALAT1* expression was significantly lower in cigarette smokers and opium-addicted TCC patients, respectively. On the other hand, *LINC00355* expression tended to be higher in opium-addicted TCC patients. The proposed panel of lncRNAs (composed of *UCA1-201*, *UCA1-203*, *MALAT1*, and *LINC00355*) had 92% sensitivity and 91.7% specificity for diagnosis of bladder cancer from normal samples.

Conclusion: Transcript levels of lncRNAs in urinary exosomes are potential diagnostic biomarkers in bladder cancer.

Keywords: lncRNA, exosome, bladder cancer

Introduction

Recent reports have highlighted the role of long non-coding RNAs (lncRNAs) in the pathogenesis of several human malignancies including genitourinary cancers.^{1,2} This underscored part of human transcripts participates in the pathogenesis of transitional cell carcinoma (TCC) of bladder through alteration of cellular pathways of cell transformation.³ In the meantime, as exosomes naturally produce nano-vesicles, which contain proteins and RNAs, they contribute to urothelial carcinogenesis, possibly through alteration of inter-cellular communications.⁴ Cooperatively, lncRNAs and exosomes have been regarded as components of cell signal transmission that modulates indigenous cellular microenvironments, since exosomes have role in the relocation of functional lncRNAs between cells.⁵ Several lncRNAs have been shown to be involved in the pathogenesis of bladder cancer. The metastasis-associated lung adenocarcinoma transcript 1 (*MALAT1*) upregulation has been detected in bladder cancer samples compared with matched normal urothelium and in high-grade carcinomas compared with low-grade carcinomas. Its roles in enhancement of cell proliferation and motility, while inhibiting apoptosis, have been confirmed in vitro.⁶ Moreover, *MALAT1* has been suggested as an independent prognostic factor in bladder cancer patients and a putative therapeutic target in this kind of malignancy.⁷ In addition,

LINC00958 has an established role in the pathogenesis of bladder cancer, as its silencing decreased cell viability, migration, and invasive potential of these cells. Moreover, *LINC00958* has interactions with proteins that are involved in the regulation and initiation of translation and in post-transcriptional modification of RNA and have recognized participation in bladder cancer.⁸ *LINC00355* has been among the most upregulated lncRNAs in bladder cancer samples compared with normal samples as revealed through RNA sequencing of these samples. Moreover, in vitro assessments have demonstrated its contribution in apoptosis, cell proliferation, and migration processes.⁹ Finally, the urothelial carcinoma-associated (*UCA1*) lncRNA has been cloned from human bladder TCC cell line. Both in vitro and in vivo assays have confirmed its role in cell proliferation, migration, invasiveness, and drug resistance of TCC cells.¹⁰ Several splicing variants have been identified for this lncRNA among which are *UCA1-201* (ENST00000397381.5) and *UCA1-203* (ENST00000600160.3) with 2,025 bp and 998 bp lengths, respectively. Considering the reported roles of these lncRNAs in the pathogenesis of bladder cancer and the importance of urinary exosomes as diagnostic markers in this malignancy,¹¹ we evaluated expression levels of these lncRNAs in urinary exosomes isolated from TCC patients, normal subjects, and patients with non-malignant urinary disorders to evaluate their diagnostic power.

Patients and methods

Study participants

The study protocol was approved by the Ethical Committee of Tehran University of Medical Sciences. All methods were performed in accordance with the relevant guidelines and regulations. This study was conducted in accordance with the Declaration of Helsinki. All study participants signed the informed consent forms for participation in the study. A total of 59 TCC patients (age [mean \pm SD]=61.28 \pm 13.01 years), 24 normal subjects (age [mean \pm SD]=68 \pm 13.56 years), eleven patients with bladder stone (age [mean \pm SD]=55.42 \pm 15.55 years), six patients with obstructive uropathy (age [mean \pm SD]=42.5 \pm 8.26 years), and eight patients with benign prostate hyperplasia (age [mean \pm SD]=73 \pm 7.48 years) participated in the study. All study participants were male. A sample of first morning urine samples was obtained from all participants and centrifuged at 800 \times g for 10 minutes at 4°C to remove cell pellets and stored at -20°C until further assessments.

Urine exosome isolation

Norgen's Urine Exosome RNA Isolation Kit (Biotek Corporation, Thorold, ON, Canada) was used for isolation of urine exosomes by spin column method.

Characterization of urine exosomes

Western blotting, dynamic light scattering (DLS) assessments, and electron microscopy were used for assessment of size and morphology of the isolated exosomes.

Western blotting

The commercial antibody against exosomal marker protein CD63 was used for probing exosomes after assessment of the protein concentration using Bradford protein assay. BSA was used as the standard sample. Samples were kept at 37°C for 5 minutes and separated on 10% pre-casted gel. Then, they were transferred to nitrocellulose membranes and blocked overnight (5% milk and 0.05% Tween-20 in PBS). Subsequently, they were incubated with primary antibody (Santa Cruz Biotechnology, Dallas, Texas, USA) for 1 hour, washed with PBS, and lastly incubated with secondary horseradish peroxidase-conjugated antibody (SinaClon, Tehran, Iran). The resultant immunoreactive bands were envisioned using chemiluminescent detection system. The prestained protein ladder (SinaClon, Tehran, Iran) was used for assessment of protein size.

Dynamic light scattering assessments

The size of exosomes was also assessed by DLS using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) based on the company guidelines.

Electron microscopy

After addition of the slurry b1 solution (Norgen's Urine Exosome RNA Isolation Kit; Biotek Corporation), a portion of the precipitated exosomes was dissolved in PBS and visualized under scanning electron microscopy (SEM) (QUANTA SEM system; FEI Company, Hillsboro, OR, USA).

Exosomal RNA isolation

Urine Exosome RNA Isolation Kit (Norgen; Biotek Corporation) was used for isolation of RNA from exosomes. This kit uses an all-in-one method for the concentration and isolation of exosomal RNA from urine samples. After binding of the urinary exosomes to a patented resin, RNA is refined from the exosomes using a column-based system.

Quantitative real-time PCR analysis

PrimeScript™ RT reagent Kit (Takara, Tokyo, Japan) was used for cDNA synthesis. Expression levels of lncRNA genes in urine exosomes were measured in the rotor gene 6,000 corbett Real-Time PCR System using RealQ Plus 2x Master Mix Green (Ampliqon, Herlev, Denmark). 5S rRNA was used as normalizer. All experiments were per-

formed in duplicate in 30 μ L total volumes. The nucleotide sequence of primers used in expression analyses are shown in Table 1.

Statistical methods

All statistical analyses were performed using SPSS v18.0 (SPSS Inc., Chicago, IL, USA). The expression level of each gene was measured by using the following formula: $\text{Efficiency}^{\text{cycle threshold (CT)}}_{\text{normalizer gene}} / \text{Efficiency}^{\text{CT}}_{\text{target gene}}$. The magnitude of expression of each gene between cancerous and control samples was described as fold change value and was calculated by dividing the mean of expression level values in cancerous samples to the matching value of control samples. The differences between relative expressions of genes in defined subcategories of TCC patients were evaluated using independent *t*-test. Correlation between expression levels of genes in urine samples was assessed using Pearson's correlation coefficient. The level of significance was set at $P < 0.05$ in all analyses. The receiver operating characteristic (ROC) curve was depicted to assess the suitability of expres-

sion levels of each gene or combinations of their expression levels for diagnosis of TCC from normal or non-malignant urinary disorders. The Youden index (*j*) was applied to obtain the maximum difference between sensitivity (true-positive rate) and 1–specificity (false-positive rate). The diagnostic power of transcript levels was assessed through calculation of the area under curve (AUC) values. AUC values were judged using the following method: 0.90–1= excellent (A), 0.80–0.90= good (B), 0.70–0.80= fair (C), 0.60–0.70= poor (D), and 0.50–0.60= fail (F).

Ethical approval

Ethical approval was obtained from the ethical committee of Tehran University of Medical Science (IR.TUMS.1395.525). Informed consent was obtained from the patients.

Results

General data of patients

The general demographic and clinical data of study participants are summarized in Table 2.

Table 1 Nucleotide sequence of primers used for expression analysis of lncRNAs in urinary exosomes

Gene name	Forward primer	Reverse primer
LINC00355	TGGGTCTCCTCTGAGCTGTT	TGTCCTGTGTCCAGGATGAA
LINC00958	AGAGAGGAGGAGAAGCAA	TGTGAAGTGCAGGGAGGA
UCA1-201	GCTTAGTGGCTGAAGACTGATG	TCATATGGCTGGGAATCCTC
UCA1-203	GCATCCAGGACAACACAAAG	ACCCTTTTCCCATAGGTGTG
MALAT1	CTTCCCTAGGGGATTTTCAGG	GCCACAGGAACAAGTCCTA
5s rRNA	GCCCGATCTCGTCTGATCT	AGCCTACAGCACCCGGTATT

Abbreviation: lncRNA, long non-coding RNA.

Table 2 The general characteristics of study participants

Study groups	Number of samples	Variables	Values	
TCC patients	59	Age (mean \pm SD [range]), years	61.28 \pm 13.01 (33–84)	
		Cigarette smoking	No	13 (22%)
			Yes	37 (62.7%)
			Not available	19 (32.2%)
		Opium addiction	No	21 (35.6%)
			Yes	20 (33.9%)
			Not available	18 (30.5%)
		Grade	High grade	28 (47.4%)
			Low grade	20 (33.9%)
Not available	11 (18.6%)			
Controls	49	Age (mean \pm SD [range]), years	64.42 \pm 15.53 (31–88)	
		Cigarette smoking	No	25 (51%)
			Yes	5 (10.2%)
			Not available	19 (38.8%)

Abbreviation: TCC, transitional cell carcinoma.

Confirmation of exosome size and morphology

Assessment of isolated particles with SEM confirmed that we could effectively isolate exosomes with acceptable quality regarding their size range and morphology (Figure 1). The results were also confirmed by DLS assessment (Figure 2).

Western blot

We subsequently validated the results of SEM imaging through Western blot assessment of protein lysates acquired from the isolated exosomes. Western blot analysis of samples

demonstrated the expression of CD63 as a shared marker of exosomes (Figure 3).

Expression of lncRNA genes in urinary exosomes

We assessed expression of lncRNAs in isolated exosomes of all study participants and compared their expressions between TCC and controls. As demonstrated in Figure 4, there are significant differences in $-\Delta\text{CT}$ values of *UCA1-203*, *UCA1-201*, and *MALAT1* between TCC patients and total controls.

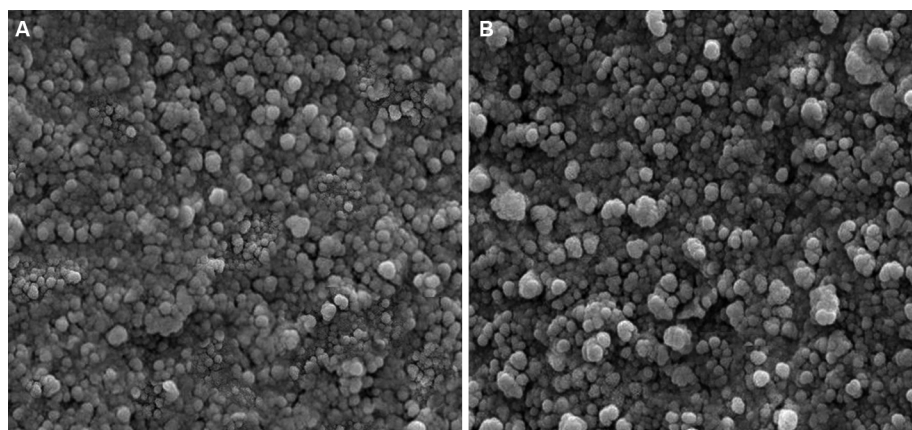


Figure 1 Scanning electron micrographs of exosomes isolated from urine samples of TCC patients (A) and control (B). The size of exosomes was not different between study groups.

Abbreviations: TCC, transitional cell carcinoma.

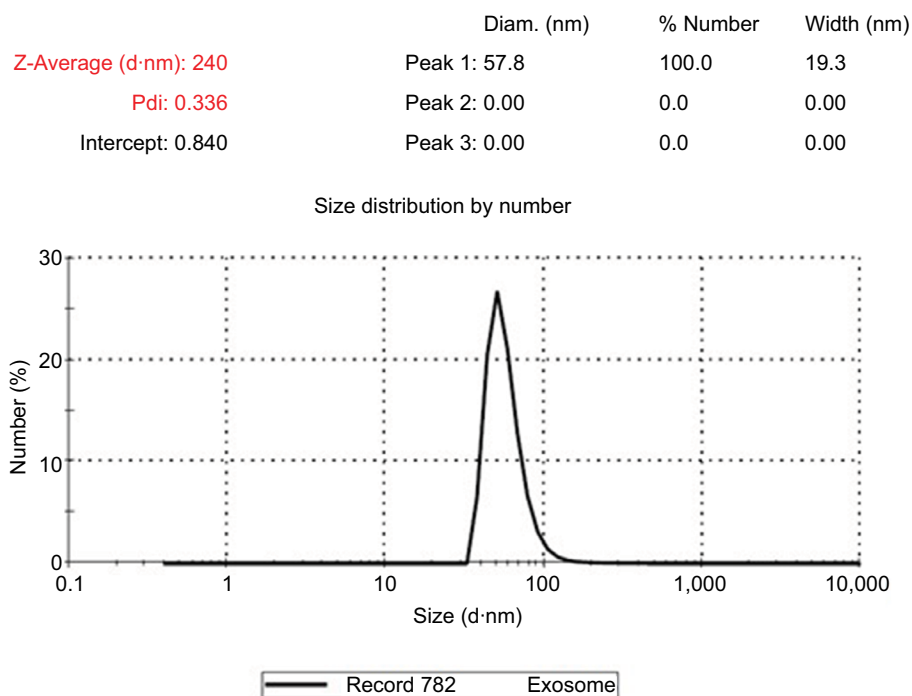


Figure 2 Size distribution of exosomes by volume as demonstrated by Zetasizer Nano ZS.

LINC00355, *UCA1-203*, and *MALAT1* expression was significantly higher in TCC patients compared to controls (non-malignant urinary disorders or normal samples). However, *UCA1-201* expression was significantly decreased in TCC patients compared with controls. Table 3 shows the relative expression of lncRNAs in urinary exosomes of TCC patients compared with controls.

Associations between exosomal expression of lncRNAs and clinicopathological data

LINC00355 and *MALAT1* expression was significantly lower in cigarette smokers and opium-addicted TCC patients,

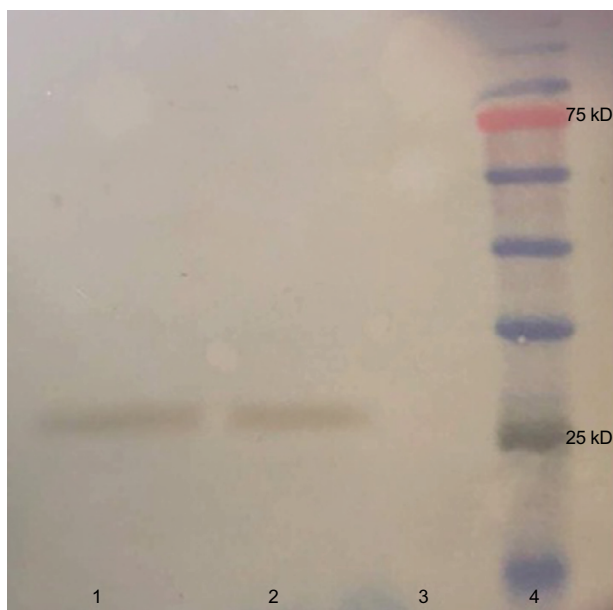


Figure 3 Exosome fractions were evaluated by Western blotting for CD63. Lanes 1 and 2: two representative isolated exosomes from TCC and controls, respectively; lane 3: negative control; lane 4: prestained protein marker.

Abbreviation: TCC, transitional cell carcinoma.

respectively. On the other hand, *LINC00355* expression tended to be higher in opium-addicted TCC patients. No significant difference was found in expression levels of lncRNAs between different subcategories of TCC patients (Table 4).

Correlations between exosomal expressions of lncRNAs

Significant correlations were found between exosomal expressions of certain lncRNAs in both TCC patients and controls (Table 5). The most significant correlations were found between *MALAT1* and *LINC00355* in both TCC samples and controls. Moreover, expressions of *LINC00985* and *UCA1-203* were significantly correlated in TCC samples.

ROC curve analysis

We assessed the diagnostic power of urinary exosome lncRNAs levels in differentiation of TCC from normal samples/total controls (Table 6). Based on the calculated AUC values, *UCA1-201* transcript levels had the best performance in differentiation of TCC from normal samples/total controls (AUC values of 0.73 and 0.93, respectively). Of note, the proposed panel of lncRNAs (composed of *UCA1-201*, *UCA1-203*, *MALAT1*, and *LINC00355*) had 92% sensitivity and 91.7% specificity for diagnosis of bladder cancer from normal samples (Figure 5).

Discussion

Direct contact of cancerous cells with urine makes this bio-fluid a potential source of biomarkers. Urine collection is noninvasive and cost-effective. Different fractions of urine such as urinary sediment, supernatant, and exosome have been assessed to find the most informative biomarkers for genitourinary malignancies.¹¹ Exosomes are nano-vesicles produced by almost all cell types and released into the

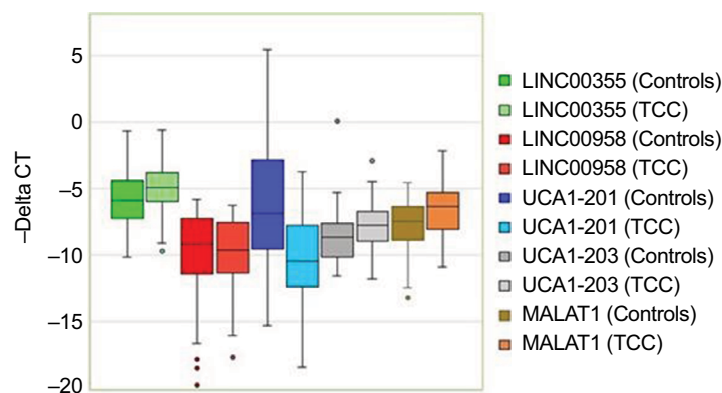


Figure 4 The $-\Delta\text{CT}$ values of lncRNAs in isolated exosomes of TCC patients and controls.

Abbreviations: CT, cycle threshold; lncRNAs, long non-coding RNAs; TCC, transitional cell carcinoma.

Table 3 Expression ratio of lncRNAs in urinary exosomes of TCC patients compared with controls, as described by dividing the mean of Efficiency^A CT_{normalizer gene} / Efficiency^A CT_{target gene} values in cancerous samples to the matching value of control samples

		TCC (n=59) vs controls (n=49)	TCC (n=59) vs normal subjects (n=24)	TCC (n=59) vs obstructive uropathy (n=6)	TCC (n=59) vs bladder stone (n=11)	TCC (n=59) vs BPH (n=8)
LINC00355	Expression ratio	1.45	3.4	1.15	1.41	0.29
	P-value	0.11	0.001	0.63	0.71	0.1
LINC00958	Expression ratio	1.52	2.48	0.65	1.68	0.61
	P-value	0.51	0.19	0.49	0.5	0.39
UCA1-201	Expression ratio	0.05	0.01	0.44	0.5	0.08
	P-value	<0.001	<0.001	0.44	0.36	0.07
UCA1-203	Expression ratio	6.51	21.17	1.08	1.14	0.66
	P-value	0.002	<0.001	0.95	0.54	0.39
MALAT1	Expression ratio	2.22	3.57	1.33	1.74	1.27
	P-value	0.003	<0.001	0.64	0.3	0.86

Abbreviations: BPH, benign prostate hyperplasia; CT, cycle threshold; lncRNAs, long non-coding RNAs; TCC, transitional cell carcinoma.

Table 4 Associations between relative expression of lncRNAs in urinary exosomes of TCC patients and clinicopathological data (relative expression levels were calculated using Efficiency^A CT_{normalizer gene} / Efficiency^A CT_{target gene} formula and data are presented as mean ± SD)

	LINC00355	P-value	LINC00985	P-value	UCA1-201	P-value	UCA1-203	P-value	Malat1	P-value
Age (years)		0.14		0.68		0.23		0.11		0.44
<60	0.1±0.22		0.002±0.004		0.004±0.01		0.01±0.01		0.02±0.04	
≥60	0.03±0.05		0.003±0.007		0.01±0.02		0.005±0.006		0.01±0.03	
Cigarette smoking		0.04		0.44		0.3		0.18		0.82
Yes	0.04±0.06		0.003±0.006		0.01±0.02		0.008±0.01		0.02±0.03	
No	0.16±0.35		0.002±0.003		0.003±0.006		0.003±0.004		0.02±0.04	
Opium addiction		0.06		0.18		0.51		0.48		0.02
Yes	0.06±0.08		0.001±0.002		0.008±0.01		0.009±0.009		0.009±0.01	
No	0.02±0.01		0.004±0.007		0.01±0.02		0.006±0.01		0.03±0.05	
Grade		0.38		0.15		0.84		0.53		0.8
High	0.07±0.19		0.004±0.007		0.009±0.01		0.008±0.01		0.02±0.04	
Low	0.03±0.04		0.001±0.003		0.008±0.02		0.006±0.007		0.02±0.03	

Abbreviations: CT, cycle threshold; lncRNAs, long non-coding RNAs; TCC, transitional cell carcinoma.

Table 5 Pearson's correlation coefficient values between relative expression of lncRNAs in urinary exosomes of TCC patients and controls (relative expression levels were calculated using Efficiency^A CT_{normalizer gene} / Efficiency^A CT_{target gene} formula)

		Malat1	UCA1-203	UCA1-201	LINC00985
LINC00355	TCC	0.4**	0.13*	0.01	0.13*
	Control	0.44**	0.04	0.005	0.09*
LINC00985	TCC	0.08*	0.34**	0.01	
	Control	0.13*	0.01	0.14*	
UCA1-201	TCC	0.006	0.09*		
	Control	0.07	0.008		
UCA1-203	TCC	0.06*			
	Control	0.001			

Note: *P<0.05, **P<0.01.

Abbreviations: CT, cycle threshold; lncRNAs, long non-coding RNAs; TCC, transitional cell carcinoma.

extracellular microenvironment. These membrane-bound sacs are carriers of major biomolecules including DNAs, mRNAs, miRNAs, lncRNAs, proteins, and lipids which collectively convey a specific message for neighboring or

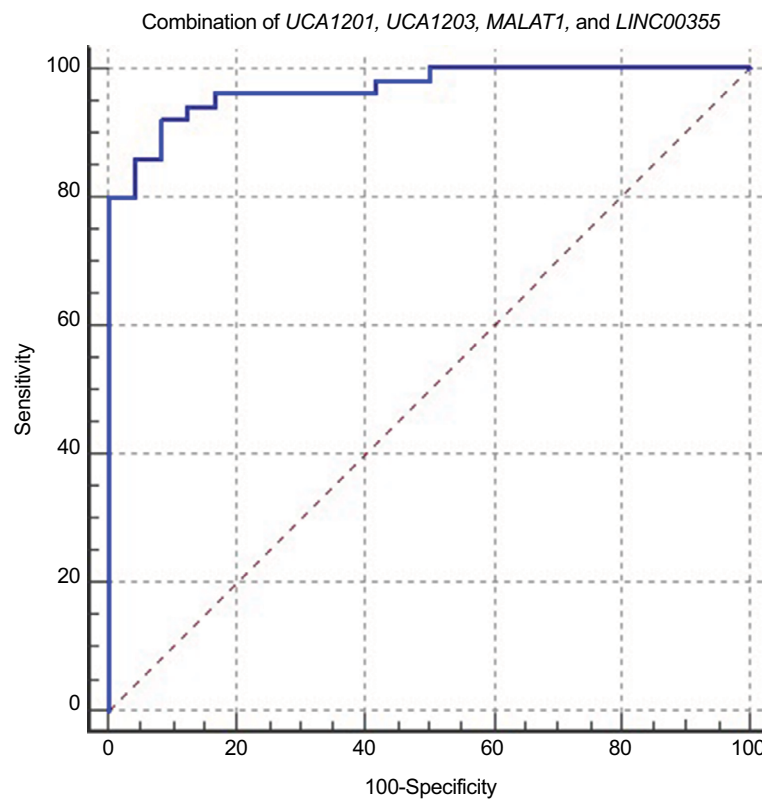
distance cells.^{4,12} Deciphering the altered messages in certain diseased conditions such as TCC would facilitate recognition of informative diagnostic or prognostic biomarkers. In the current study, we assessed expressions of five lncRNAs

Table 6 The results of ROC curve analysis

		Estimate criterion	AUC	J ^a	Sensitivity	Specificity	P-value ^b
TCC vs total controls	UCAI-201	>7.01	0.73	0.41	86	55.1	<0.0001
	UCAI-203	≤7.85	0.66	0.26	73.5	53.4	0.002
	MALAT1	≤6.86	0.65	0.31	62.1	69.4	0.002
	Combination of UCAI-201, UCAI-203, and MALAT1	≤0.59	0.77	0.47	94.7	53.1	<0.0001
TCC vs normal samples	UCAI-201	>7.69	0.93	0.75	75.4	100	<0.0001
	UCAI-203	≤8.66	0.8	0.47	72.4	75	<0.0001
	MALAT1	≤6.97	0.73	0.47	63.8	83.3	0.0001
	LINC00355	≤5.53	0.75	0.47	68	79.2	<0.0001
	Combination of UCAI-201, UCAI-203, MALAT1, and LINC00355	>0.58	0.96	0.83	92	91.7	<0.0001

Notes: ^aYouden index; ^bsignificance level P (area =0.5), estimate criterion: optimal cut-off point for gene expression.

Abbreviations: AUC, area under curve; ROC, receiver operating characteristic; TCC, transitional cell carcinoma.

**Figure 5** The results of ROC curve analysis of performance of lncRNA transcript levels in urinary exosomes for diagnosis of bladder cancer from normal samples.

Abbreviations: lncRNA, long non-coding RNA; ROC, receiver operating characteristic.

in urinary exosomes of TCC patients, urogenital disorders, and normal subjects to appraise their diagnostic power. Of note, we found significant difference in the expression of four lncRNAs including *UCAI-201*, *UCAI-203*, *MALAT1*, and *LINC00355* between TCC and normal samples. Bladder cancer-derived exosomes have been previously demonstrated to contain substances that promote angiogenesis

and migration of bladder cancer cells and endothelial cells.⁴ Based on the observed elevated levels of *MALAT1* in urinary exosomes of TCC patients compared with normal samples in the current study and the previous reports of participation of *MALAT1* in proliferation and motility of bladder cancer cells,⁶ exosomal transfer of *MALAT1* might mediate bladder carcinogenesis. Several previous studies have pointed to the

role of *UCA1* in the pathogenesis of different cancers and assessed its expression without specification of the transcript variant. For instance, plasma levels of this lncRNA have been suggested as diagnostic markers in gastric¹³ and bladder cancers.¹⁴ *UCA1*-containing exosomes derived from hypoxic bladder cancer cells have been shown to alter tumor micro-environment in favor of tumor growth and development.¹⁴ In the present study using two sets of primers which specifically amplify two separate transcripts, we demonstrated upregulation of *UCA1-203* while downregulation of *UCA1-201* in TCC samples compared with normal subjects. Such different pattern of expression of these two variants might imply specific roles for them which should be assessed in future studies. Evidence from protein coding genes has shown that alternative splicing can change oncogenic role of a protein to tumor suppressor role.^{15,16} However, data regarding the presence of such contrary functions of splice variants of lncRNA are scarce due to the significant difference in splicing dynamics between protein coding and lncRNA genes and lower completed splicing index for lncRNAs in the total chromatin fraction.¹⁷ Meanwhile, different splice variants of lncRNAs have distinct secondary structure which can define function of the lncRNA isoform.¹⁸

Despite the established oncogenic role of *LINC00958* in bladder cancer,⁸ we could not detect any difference in its urinary exosome expression between TCC, normal, and urogenital disorder samples. Such observation might be explained by different expression levels of this lncRNA in secreted exosomes vs cells as has been previously reported for some lncRNAs such as lincRNA-p21, HOTAIR, and ncRNA-CCND1.¹⁹

We also demonstrated higher *LINC00355* exosomal expression in TCC patients with no history of cigarette smoking compared with smokers and higher *MALAT1* expression in TCC patients with no history of opium addiction compared with addicted patients. Few previous reports have highlighted the role of ncRNAs in smoking exposure responses.²⁰ From our observations, one might speculate that opium/cigarette smoking might decrease the level of certain lncRNAs. A previous study hypothesized that smoking might lead to the chromosome damage and deep deletion leading to significant downregulation of some lncRNAs in the context of lung cancer.²¹ However, another study reported upregulation of lncRNAs in heroin abusers consistent with the anticipated role of lncRNAs as facilitators of extensive alterations in gene expression as happens in drug abuse.²²

Pearson's correlation analyses revealed significant correlations between exosomal expressions of certain lncRNAs in both TCC patients and controls. The most significant correlation was found between *MALAT1* and *LINC00355* in control samples followed by the correlation between *LINC00985* and *UCA1-203* in TCC samples. Totally, our data revealed different patterns of correlations between lncRNAs in cancerous and noncancerous samples which might imply dys-regulation of their expression in the context of cancer or a disease-dependent release of lncRNAs in exosomes which warrants further assessments.

We also demonstrated high specificity and sensitivity of the proposed panel of lncRNAs in the diagnosis of bladder cancer. Current diagnosis of bladder cancer relies on cystoscopy which is troublesome for patients and costly for health system.²³ Few US Food and Drug Administration-approved urinary biomarkers including protein assays and exfoliated cell tests suffer from imperfect sensitivity and/or specificity values.²³ Consequently, establishment of diagnostic or prognostic panels is of critical significance in this malignancy, especially considering the heterogeneous nature and high recurrence rate of this cancer which obligate cystoscopic investigations.²⁴ The current study provides the first clues for application of urinary exosome transcript levels of lncRNAs as diagnostic markers in bladder cancer. The possible application of this panel as prognostic markers needs further studies.

Conclusion

Transcript levels of lncRNAs in urinary exosomes are potential diagnostic biomarkers in bladder cancer.

Consent for publication

The manuscript does not contain any individual person's data in any form.

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Author contributions

All authors contributed to data analysis, drafting and revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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