UROLOGIC
ONCOLOGY

Laboratory-Bladder cancer

Measurement of Urinary Level of a Specific Competing endogenous RNA network (FOS and RCAN mRNA/ miR-324-5p, miR-4738-3p, /lncRNA miR-497-HG) Enables Diagnosis of Bladder Cancer

Sanaa Eissa, MD, PhD[a],[*], Maheera Safwat, Msc[b], Marwa Matboli, MD, PhD[a], Ashraf Zaghloul, MD[c], Maha El-Sawalhi, PhD[b], Amira Shaheen, PhD[b]

[a] *Medical Biochemistry and Molecular biology Department, Faculty of Medicine, Ain Shams University Research Institute (MASRI), Abbassia, Cairo 11381, Egypt*
[b] *Biochemistry Department, Faculty of Pharmacy, Cairo University, Kasr El -Aini Street, Cairo 11562, Egypt*
[c] *Department of Surgical Oncology, National Cancer Institute, Cairo University, Cairo, Egypt*

Abstract

Objective: To assist in the diagnosis, treatment, and prognostic prediction of bladder cancer, the molecular patterns associated with it should be elucidated. Competing endogenous RNA network: MicroRNA (miRNA), long noncoding RNA (lncRNA), and their target autophagy genes have been strongly implicated in tumor development and metastasis.

Patients and methods: Bioinformatics analysis was performed to retrieve a ceRNA: lncRNA-miRNA-mRNA network linked to autophagy and relevant to bladder cancer. Expression of selected noncoding human RNAs (miR-324-5p, miR-4738-3p, and lncRNA miR-497-HG) and their target genes (*RCAN1* mRNA and *FOSB* mRNA) was examined by qPCR in bladder tissues and urine samples obtained from 196 individuals (98 patients with bladder cancer, 48 patients with benign lesions, and 50 healthy controls).

Results: Expression levels of the selected genes in urine samples in the bladder cancer group were significantly different from those in the control group ($P < 0.001$). Expression in bladder cancer tissue samples correlated with that in urine samples. Urinary expression levels of all biomarkers had high accuracy to distinguish patients with and without bladder cancer, with *FOSB* mRNA and *RCAN1* mRNA having the highest accuracy (99% for *RCAN1* mRNA or *FOSB* mRNA, 87.8% for miR-324-5p, 84.7% for miR-4738-3p, and 90.5% for lncRNA miR-497-HG). *FOSB* mRNA and *RCAN1* mRNA expression showed also a higher accuracy than cytology (77.6%).

Conclusion: The significant differential expression of the ceRNA network: lncRNA-miRNA-mRNA network in bladder cancer as compared to noncancerous controls has revealed the superior accuracy of the chosen biomarkers to cytology, especially *FOSB* mRNA and *RCAN1* mRNA, suggesting their involvement in bladder cancer pathogenesis and promising role for future diagnosis, and targeted therapy. © 2019 Elsevier Inc. All rights reserved.

1. Introduction

Bladder cancer is the ninth most common malignancy worldwide, and the ninth leading cause of mortality among males. Two subtypes of bladder cancer have been recorded: non-muscle-invasive bladder cancer and muscle-invasive bladder cancer. Patients with muscle-invasive bladder cancer generally have poor prognoses, and distant metastasis occurs in many cases. Bladder cancer has a high recurrence rate and an extremely high treatment-cost per person, and therefore places a substantial economic burden on many developed and developing countries, including Egypt [1].

*Corresponding author. fax:+202 26859928
E-mail addresses: dr_sanaa_eissa@yahoo.com, Drsanaa_mohamed@med.asu.edu.eg (S. Eissa).

Cystoscopy is the main diagnostic tool for bladder cancer, but it is an expensive, invasive, and time-consuming procedure. Often, cytology is performed in addition to cystoscopy, to detect the presence of malignant cells in urine samples. Its advantages include high specificity and relatively low expenses, while its main limitation is low sensitivity especially for low-grade tumours [2,3]. Therefore, independent, reliable, and noninvasive biomarkers for the diagnosis and prognostic prediction of bladder cancer are needed to improve monitoring sensitivity and decrease follow-up costs.

Autophagy-related genes and apoptosis play pivotal roles in the progression of many cancers, including bladder cancer. Autophagy ('self-eating') is a highly conserved degradative pathway that promotes survival during metabolic stress. Autophagy is a multifaceted process that has been hypothesized to suppress tumor growth during the early stages of cancer development, but to promote tumor-cell survival in the late stages of tumorigenesis [4]. Although the metabolic reactions of autophagy occur primarily in the cytoplasm, nuclear processes of transcriptional and epigenetic regulation are also important [5]. Epigenetic regulation comprises DNA methylation, histone modifications, and microRNA (miRNA) regulation, which are all transformed in carcinogenesis. Moreover, miRNAs have been associated with cell proliferation, differentiation, and apoptotic processes in tumor tissues. Several oncogenic miRNAs are significantly upregulated in bladder cancer [6]. Numerous studies have addressed the potential role of miRNAs−−single or in combination−−as diagnostic biomarkers in urine samples of tumor patients, with some being downregulated such as miR-125b, miR-140-5p [7,8], miR-141, miR-200a, miR-200c [9], and others upregulated such as miR-18a, miR-92a [8], miR-96 [10,11]. MiR-25b and miR-92a were suggested as indicators of aggressiveness [8] and miR-17-5p as a marker of recurrence [12]. The posttranscriptional regulation of RNA transcripts by miRNAs represents a classic example of RNA−RNA interactions (crosstalk). One type of crosstalk involves 'decoy' mRNAs with multiple base-pairing sites that sequester miRNAs with complementary sequences, hence minimizing the miRNA-dependent repression of other target mRNAs. Similarly, miRNAs can also be sequestered by long noncoding RNAs (lncRNAs) [13] or circular RNAs [14]. The study of such ceRNA: lncRNA-miRNA-mRNA network can aid in the understanding of many oncogenetic pathways and identification of prognosis- and diagnosis-related RNAs. In bladder carcinoma, Huang et al reported that lncRNA H19 and circRNA MYLK could bind competitively with miRNA-29a-3p increasing target gene *DNMT3B*, *VEGFA*, and *ITGB1* expressions, which are involved in the progression and metastasis of bladder cancer [15].

Hence, we sought in this study to identify a novel network of urinary biomarkers (lncRNA-miRNA-mRNA) to aid in the early detection of bladder cancer.

2. Patients and methods

2.1. Patients and clinical samples

All samples were obtained from the Urology departments of the National Cancer Institute Hospital, Cairo University and the Faculty of Medicine, Ain Shams University Hospitals, Cairo, Egypt, between 2014 and 2016. All patients were included in the study on the basis of a primary diagnosis of a suspicious bladder lesion by cystoscopy. According to histopathological examination of tissue specimens, patients with confirmed bladder cancer or benign lesions were included in the study. Patients with past history of bladder cancer, previous chemotherapy, or any other type of cancer in the past 5 years were excluded from the study. All practices and procedures were approved by the Research Ethics Committees of Ain Shams and Cairo Universities. Written informed consent was provided by all participants in accordance with the ethical standards set out in the Helsinki declaration.

Plasma and voided urine samples of 196 participants were assessed: 98 patients had histologically confirmed bladder cancer, 48 patients matched for age and gender had benign urological conditions (benign prostatic hyperplasia or bilharzial cystitis), and a control group of 50 healthy volunteers (recruited during routine check-ups) had no previous history of gross hematuria, urolithiasis, or active urinary tract infections. Clinical and laboratory data of all participants were documented.

For 15 of the 98 patients with bladder cancer, who underwent transurethral resection, bladder carcinoma, and corresponding nontumor specimens ($\approx$ 0.1 g) with a macroscopic safety margin of 0.5 cm to the tumor were collected and confirmed by microscopic examination. From these patients, 40 to 60 ml of urine was also collected before the surgical procedures.

2.2. Sample collection and processing

Tissue specimens were preserved in RNAlater (Qiagen, Germantown, MD), transported in liquid nitrogen and stored at 80°C. Tumor staging was performed according to the American Joint Committee on Cancer−Union Internationale Contre le Cancer tumor-node metastasis classification and histologically graded by a specialist uropathologist [16].

Voided urine samples ($\sim$50 ml) were supplied prior to any surgical intervention. The samples were centrifuged at 4000 rpm for 10 to 20 minutes at room temperature, and the pellets were washed with phosphate-buffered saline [17]. A portion of each pellet was processed for cytology by an experienced examiner. Another portion of the pellet was treated with RNAlater and stored at 80°C prior to further processing.

2.3. Bioinformatics analysis to identify an RNA cancer panel

This network was obtained in 3 main steps. First, 2 relevant protein-coding genes were selected from examination

of the Gene Atlas (http://genatlas.medecine.univ-paris5.fr/) and The Cancer Network Galaxy (TCNG; http://tcng.hgc.jp/) public microarray databases. *RCAN1* mRNA, an autophagy gene, encodes regulator of calcineurin 1, and *FOSB* mRNA, which is highly related to bladder cancer, encodes FBJ Murine Osteosarcoma Viral Oncogene Homolog B. Second, miR-324-5p and miR-4738-3p were identified as epigenetic regulators of both *FOSB* mRNA and *RCAN1* mRNA that are related to bladder cancer using http://www.mirbase.org/cgi-bin/mirna. Finally, lncRNA miR-497-HG, which is thought to control expression of *FOSB* mRNA and *RCAN1* mRNA, was identified by accessing the database of lncRNAs that act as competing endogenous RNAs (http://gyanxet-beta.com/lncedb/index.php).

2.4. Total RNA extraction and reverse transcription

The miRNeasy Kit (Qiagen, Hilden, Germany) was used for extraction of total RNA from tissue samples and urinary pellets, according to the manufacturer's instructions. RNA quality and concentration were evaluated by measuring the absorption at 260 nm and 280 nm with a BioPhotometer Plus (Eppendorf, Hamburg, Germany). An A_{260}/A_{280} ratio of between 1.8 and 2.0 [18] was considered to represent a satisfactory level of RNA quality. Reverse transcription of RNA into cDNA was performed with the miScript II RT Kit (Qiagen, Germany), as per the manufacturer's protocol for serum/tissue samples, in a Hybaid thermal cycler (Thermo Electron, Waltham, MA, USA).

2.5. Real-time quantitative PCR (qPCR) assessment of RNA cancer network

For the determination of miR-324-5p and miR-4738-3p expression levels in urine and tissue samples, the miScript SYBR Green qPCR kit (Qiagen, Hilden, Germany) was used with miRNA-specific forward primers (Hs_miR-324-5p_1 and Hs_miR-4738-3p_1 miScript Primer Assays, cat. no. MS00006825 and MS00039592, accession no. MIMAT0000761 and MIMAT0019867, respectively). Expression of lncRNA-miR-497-HG was assessed with RT^2 SYBR Green ROX qPCR Mastermix (Qiagen, Hilden, Germany) and RT^2 lncRNA qPCR Assay for Human MIR497HG (ENST00000385056; cat. no. LPH25656A; Qiagen). Expression of *FOSB* mRNA and *RCAN1* mRNA was assessed by QuantiTect SYBR Green qPCR (Qiagen, Hilden, Germany) with gene-specific primers; specifically, Hs_FOSB_1_SG QuantiTect Primer Assay (cat. no. QT00013076) and Hs_RCAN1_1_SG QuantiTect Primer Assay (cat. no. QT00029428).

For each reaction, cDNA was mixed with SYBR Green Mastermix, RNAse-free water and gene-specific primers. qPCR was carried out with a Step One Plus System (Applied Biosystems, Foster, CA) with the following cycling conditions: initial activation at 95˚C for 15 minutes, followed by 40 cycles of denaturation at 94˚C for 15 seconds, annealing at 55˚C for 30 seconds and extension at 70˚C for 30 seconds for the miRNA assay; or initial activation at 95˚C for 10 minutes, followed by 40 cycles of denaturation at 95˚C for 15 seconds, annealing and extension at 65˚C for 1 minute for the assay of lncRNA miR-497-HG; or initial denaturation at 95˚C for 15 minutes, followed by 35 to 45 cycles of denaturation at 94˚C for 15 seconds, annealing at 50 to 60˚C for 30 seconds and extension for 30 seconds at 72˚C for *FOSB* mRNA and *RCAN1* mRNA assays.

The $2^{-\Delta\Delta Ct}$ method was employed to calculate the relative quantities of RNAs in each sample [19]. *RNU6* (Hs_RNU6-2_11 miScript Primer Assay, cat. no. MS00033740) served as the housekeeping gene (normalization control) for the miRNAs, and *ACTB* (β-actin, RT^2 lncRNA qPCR Assay, cat. no. LPH28471A) was the control for lncRNA miR-497-HG, *FOSB* mRNA, and *RCAN1* mRNA. In addition, the raw data were compared with reference control samples.

The threshold cycle (Ct) value for each assay in each sample was calculated with Step One Plus software v2.2.2 (Applied Biosystems). Ct values >36 were regarded as negative results. The specificities of PCR amplification were verified by identification of a single peak in each melting curve. Each sample was analyzed in duplicate.

2.6. Statistical analyses

Analyses (Mann-Whitney, Kruskal−Wallis, and chi-square) were conducted with the Statistical Package for the Social Sciences (SPSS) version 20 (IBM, Armonk, NY). The receiver operating characteristic (ROC) curve was also plotted, to analyze the optimal cut-off value of the selected RNA cancer panel in differentiating bladder cancer from noncancerous conditions. Additionally, Spearman rank correlation test was applied to evaluate the relationships between RNA levels in different samples. A 2-tailed *P* value of ≤0.05 was considered to indicate a statistically significant difference. The nonparametric type of testing was used here, as the data did not seem to follow a normal distribution.

3. Results

3.1. Characteristics of the study population

No statistically significant differences were identified in age, sex or smoking status among the 3 groups (*P* >0.05), as shown in Supplementary Table 1. A significant difference was observed by chi-square test in the proportions of individuals in the 3 groups who were positive for the presence of bilharzial antibodies (*P* = 0.016).

3.2. Urinary levels of ceRNA network in the 3 study groups

Individual relative quantity (RQ) values of each RNA in urine samples in the 3 groups are shown in Fig 1 (P <0.05),
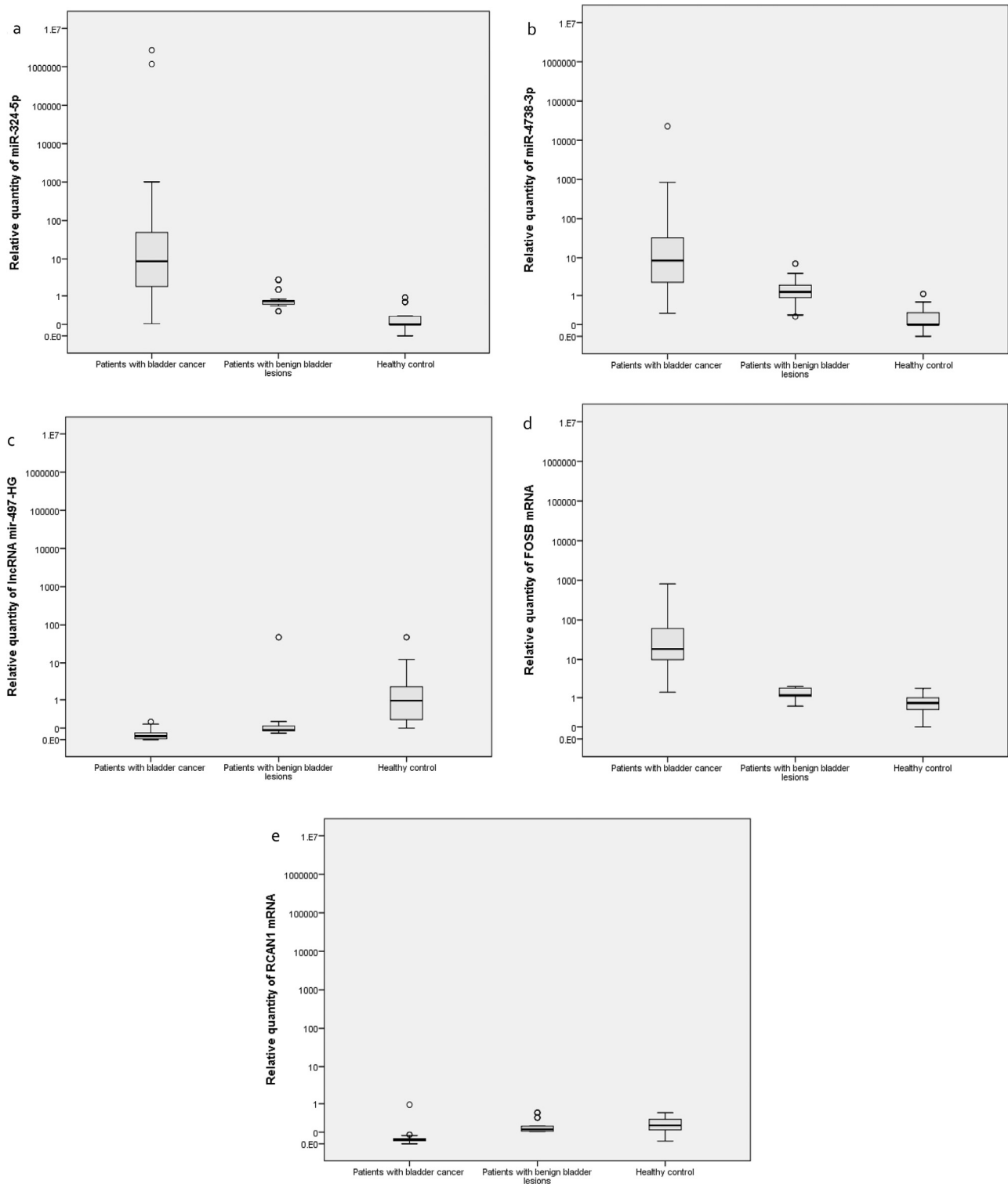
Fig 1. Urinary levels of RNAs in the bladder-cancer panel. Boxplots show medians, interquartile ranges and maximum and minimum values for relative quantities of 5 RNAs in urine samples from 98 patients with bladder cancer, 48 patients with benign lesions and 50 healthy controls. RNA levels were determined by qRT-PCR (A) miR-324-5p; (B) miR-4738-3p; (C) lncRNA miR-497-HG; (D) *FOSB* mRNA; (E) *RCAN1* mRNA,).

with mean ranks of RQ in Supplementary Figure 1 and Table 1. The Kruskal−Wallis test results indicated that the mean ranks of RQ were not equivalent in the 3 groups for any of the RNAs (all $P <0.001$). Compared with the control

and benign-lesion groups, the bladder cancer group demonstrated higher urinary levels of miR-324-5p, miR-4738-3p, and *FOSB* mRNA, and lower levels of lncRNA miR-497-HG and *RCAN1* mRNA.

Table 1
Positivity and differential expression of the 5 urinary biomarkers across the investigated groups expressed as RQs (relative quantities)

Groups	miR-324-5p		miR-4738-3p		lncRNA miR-497-HG		FOSB mRNA		RCAN1 mRNA	
	Mean rank of RQ[a]	Cases above cut-off (≥7.382)[b], n (%)	Mean rank of RQ[a]	Cases above cut-off (≥14.311)[b], n (%)	Mean rank of RQ[a]	Cases below cut-off (≤0.743)[b], n (%)	Mean rank of RQ[a]	Cases above cut-off (≥31.826)[b], n (%)	Mean rank of RQ[a]	Cases below cut-off (≤0.857)[b], n (%)
Patients with malignant primary tumors	139.27	86 (87.8%)	140.9	83 (84.7%)	53.23	86 (90.5%)	142.32	97 (99%)	51.24	97 (99%)
Patients with benign bladder lesions	85.33	11 (22.9%)	85.56	19 (39.6%)	114.08	16 (33.3%)	63.43	0 (0%)	139.65	0 (0%)
Healthy controls	31.24	0 (0%)	27.82	0 (0%)	161.35	0 (0%)	29.88	1 (2.2%)	146.91	1 (2.2%)
χ^2	123.774	119.95	135.168	99.11	128.927	99.11	149.917	183.124	132.393	183.124
P<	0.001***	0.001***	0.001***	0.001***	0.001***	0.001***	0.001***	0.001***	0.001***	0.001***

RQ = relative quantity
Significant differences were detected between the investigated groups at $P < 0.001$ (***) using the non-parametric Kruskal−Wallis test[a] and chi-square test[b]

3.3. Accuracy of urinary parameters for prediction of bladder cancer

To assess the diagnostic relevance of the proposed biomarkers, receiver operating characteristic ROC analysis was performed, and area-under-the-curve (AUC) values were obtained (Supplementary Figure 2). For comparison of patients with bladder cancer and individuals without cancer, the most suitable RQ cut-off values for differentiation between the 2 groups were 7.4, 14.3, 0.7, 31.8, and 0.9 for miR-324-5p, miR-4738-3p, lncRNA miR-497-HG, FOSB mRNA, and RCAN1 mRNA, respectively. For miR-324-5p, miR-4738-3p, and FOSB mRNA, an RQ greater than or equal to the cut-off value indicated a diagnosis of bladder cancer, whereas for lncRNA miR-497-HG and RCAN1 mRNA, an RQ less than or equal to the cut-off value indicated the presence of cancer. With these cut-off values, the sensitivities were 87.8%, 84.7%, 90.5%, 99%, and 99%, and the specificities were 86.7%, 80.6%, 83%, 98.9%, and 98.9%, respectively, for miR-324-5p, miR-4738-3p, lncRNA miR-497-HG, FOSB mRNA, and RCAN1 mRNA (Supplementary Figure 3).

3.4. Urinary RNA levels in relation with clinicopathological factors in patients with bladder cancer

Relationships between urinary RNA levels and clinicopathological parameters in patients with bladder cancer were evaluated by nonparametric Kruskal−Wallis tests. For RCAN1 mRNA, the mean rank of RQ was significantly higher in patients with positive urine cytology (54.65) than in patients with negative cytology (43.18; $P = 0.047$) (Supplementary Table 2). A significant association was also identified between the mean rank of RQ of miR-4738-3p and urine cytology, with mean rank values of 41.4 for patients with positive cytology and 59.44 for patients with negative cytology ($P = 0.002$). No significant difference between different stages, grades nor histologic type of the tumor (Supplementary Figures 4−6)

3.5. Correlations between the RQs of the RNAs among the 3 different groups

Spearman's correlation test was used to explore the correlations between the RQs of the 5 biomarkers. Significant positive correlations were noted between expression levels of pairs of RNAs, including miR-324-5p and miR-4738-3p ($P < 0.001$), miR-324-5p, and FOSB mRNA ($P < 0.001$), and lncRNA miR-497-HG and RCAN1 mRNA ($P < 0.001$). Significant negative correlations were observed between expression levels of miR-324-5p and lncRNA miR-497-HG, miR-324-5p and RCAN1 mRNA, miR-4738-3p and lncRNA miR-497-HG, and miR-4738-3p and RCAN1 mRNA (all at $P < 0.001$) (Supplementary Table 3).

3.6. Expression of RNAs differs in matched bladder cancer tissues and adjacent cancer-free tissues

From the 98 patients with bladder cancer, RNA levels were compared in matched tumor and nontumor tissue samples and urine samples from 15 patients (12 malignant and 3 nonmalignant). Levels of miR-324-5p, miR-4738-3p and *FOSB* mRNA were higher in tumor tissues than in nontumor tissues, whereas levels of lncRNA miR-497-HG and *RCAN1* mRNA were lower in tumor tissues than in normal tissues. Notably, significant positive correlations were found between the urine and tissue levels of all 5 biomarkers (all $P < 0.05$) (Supplementary Table 4).

3.7. The urinary RNA panel has high diagnostic accuracy

The RNA urine biomarkers were able to discriminate bladder cancer from benign lesions with high accuracy, reaching 80.1% (miR-324-5p), 81.5% (miR-4738-3p), 78.3% (lncRNA miR-497-HG), 99.3% (*FOSB* mRNA), and 99.3% (*RCAN1* mRNA)). So, in this respect each RNA was superior to cytology, which had an accuracy of 69.9% (Table 2). Both *RCAN1* mRNA and *FOSB* mRNA had very high accuracy (99 %) and specificity (98.9%) and thus may serve as discriminating markers for bladder cancer.

4. Discussion

In an attempt to identify novel, reliable, noninvasive biomarkers for diagnosis of bladder cancer, we have combined a bioinformatics-based selection procedure with clinical validation. The proposed ceRNA network of 5 RNA biomarkers included a combination of miRNA, lncRNA, and mRNA, to maximize the representation of different regulatory processes. (Fig. 2). Studying the interactions between the noncoding RNAs and mRNAs in a ceRNA network might give insights into the pathogenesis and diagnosis of bladder cancer . Zhu et al identified 3 lncRNAs with a central role in the progression of bladder cancer; MAGI2-AS3, ADAMTS9-AS2, and LINC00330. They regulate the expression of numerous bladder cancer related mRNAs via interactions with miR-200, miR-143, miR-141, miR-195, and miR-145, which are implicated in many pathways and processes such as apoptosis, cell proliferation, tumor angiogenesis, and metastasis [20].

We found that urinary levels of miR-324-5p, miR-4738-3p, and *FOSB* mRNA were upregulated in bladder cancer, whereas lncRNA miR-497-HG and *RCAN1* mRNA were downregulated, compared with patients with benign lesions and healthy controls. The sensitivities and accuracies of the RNAs were markedly higher

Table 2
Overall sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy of investigated urinary biomarkers and cytology in detecting bladder carcinoma (vs. all non-cancer cases and vs. benign cases), superficial bladder cancer and low-grade bladder cancer.

| Biomarker	Sensitivity	Specificity	PPV	NPV	Accuracy
Bladder cancer cases vs. all non-cancer cases					
miR-324-5p	87.8%	86.7%	88.7%	87.8%	88.3%
miR-4738-3p	84.7%	80.6%	81.4%	84%	82.7%
lncRNA miR-497-HG	90.5%	83%	84.3%	89.7%	86.8%
FOSB mRNA	99%	98.9%	99%	98.9%	99%
RCAN1 mRNA	99%	98.9%	99%	98.9%	99%
Cytology	55.1%	100%	100%	69.0%	77.6%
Bladder cancer cases vs. benign cases					
miR-324-5p	76.5%	89.6%	92.6%	64.6%	80.1%
miR-4738-3p	76.5%	93.8%	96.1%	65.2%	81.5%
lncRNA miR-497-HG	80%	87.5%	86.4%	65.5%	78.3%
FOSB mRNA	99%	100%	100%	98%	99.3%
RCAN1 mRNA	99%	100%	100%	98%	99.3%
Cytology	55.1%	100%	100%	52.2%	69.9%
NMIBC vs. MIBC (early vs. late stage)					
miR-324-5p	49.4%	46.7%	83.7%	14.3%	49%
miR-4738-3p	48.2%	46.7%	83.7%	14.3%	49%
lncRNA miR-497-HG	49.4%	42.9%	81.6%	10.9%	47.4%
FosB mRNA	63.9%	46.7%	86.9%	18.9%	61.2%
Rcan mRNA	51.8%	46.7%	82.8%	12.5%	54.1%
Cytology	56.6%	53.3%	87%	18.2%	56.1%
Low-grade bladder cancer					
miR-324-5p	58.9%	40%	72.9%	23.1%	53.1%
miR-4738-3p	58.9%	44%	75.4%	26.8%	55.1%
lncRNA miR-497-HG	64.3%	48%	76.3%	30.6%	58.9%
FosB mRNA	54.8%	48%	75.5%	26.7%	53.1%
Rcan mRNA	50.7%	44%	72.4%	22.5%	52%
Cytology	53.4%	40%	72.2%	22.7%	50%
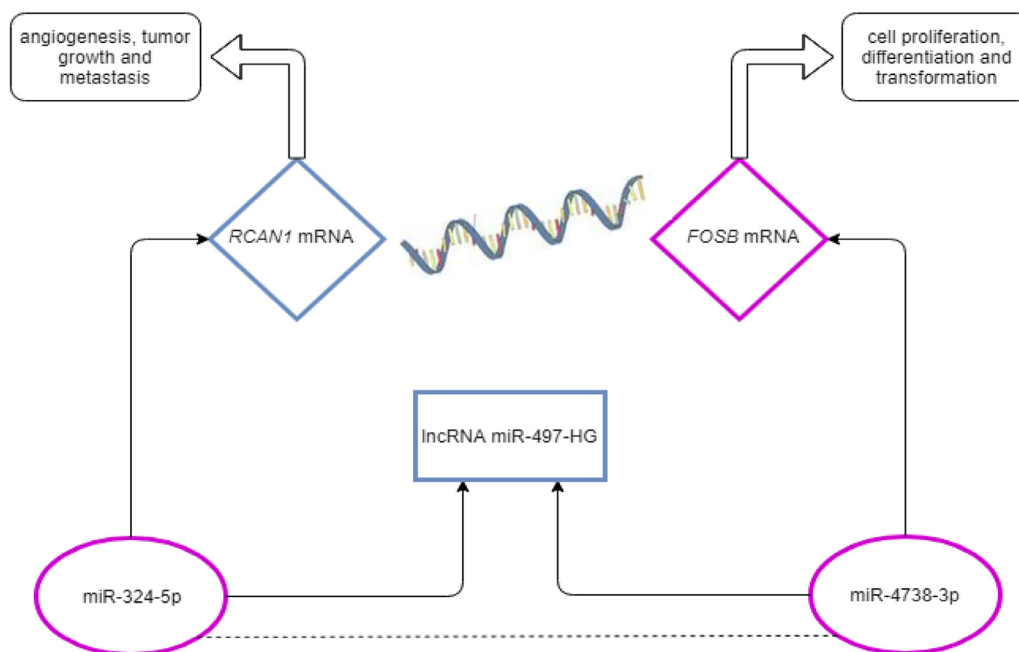
Fig 2. Illustration of the proposed miRNA−lncRNA−mRNA network implicated in bladder cancer. Ovals, rectangles and rhombuses represent miRNAs, lncRNA and mRNAs respectively with blue color indicating upregulation and purple color indicating downregulation in case of bladder cancer. The solid lines indicate interactions of miRNAs with mRNAs on one hand and with lncRNA on the other hand. The dashed line indicates the co-expression of the 2 miRNAs.

than those associated with cytology, suggesting that these RNAs have potential as diagnostic tools.

To the best of our knowledge, no study has previously demonstrated a diagnostic role for miR-324-5p in bladder cancer, although it has been implicated in other types of cancer. Notably, miR-324-5p is upregulated in colon cancer [21] and ovarian cancer [22], and downregulated in hepatocellular carcinoma [23] and osteosarcoma [24]. While the previously mentioned studies estimated the level of miR-324-5p in cell lines, serum, tissue, or tumor exosomes, our study was concerned particularly with its urinary level. Urine samples are a reflection of the urinary tract and its associated malignancies [25]. *MIR-497-HG* is a human cluster host RNA gene that encodes several noncoding RNAs, including a miRNA (miR-497) that is associated with Parkinson's disease, and a lncRNA transcript [26]. Being abnormally expressed in cancer, many lncRNAs may serve as useful diagnostic biomarkers of bladder cancer such as MALAT1 and UCA1 [27,28]. To the best of our knowledge, no study has previously addressed the potential diagnostic role of lncRNA miR-497-HG in malignancy, although miR-497 has been linked to many types of cancer, including bladder cancer (in which it is downregulated), suggesting possible roles for miR-497 as a tumor suppressor or prognostic marker [29,30]. Here, we found that urinary levels of miR-324-5p were significantly higher in patients with bladder cancer than in those with benign lesions or in healthy controls ($P < 0.001$), suggesting that it has oncogenic functions. By contrast, urinary levels of the lncRNA transcript of miR-497-HG were significantly lower in patients with bladder cancer ($P < 0.001$), and levels of

miR-324-5p and lncRNA miR-497-HG were significantly negatively correlated ($P < 0.001$). These results validate our bioinformatics network suggesting that lncRNA miR-497-HG is modulated by miR-324-5p, that their expression is altered in patients with bladder cancer and that the upregulation of miR-324-5p leads to the downregulation of lncRNA miR-497-HG, contributing to tumorigenesis. We also identified upregulation of miR-4738-3p in patients with bladder cancer, which is consistent with previous findings of upregulation of this miRNA in solid tumors [31]. Similar to miR-324-5p, levels of miR-4738-3p were negatively correlated with those of lncRNA miR-497-HG. Notably, in contrast to miR-324-5p, few studies have previously addressed the role of miR-4738-3p in oncogenesis.

Our bioinformatics analysis identified *RCAN1* mRNA and *FOSB* mRNA as targets of miR-324-5p and miR-4738-3p. Members of the regulator of calcineurin family are endogenous proteins that interact with calcineurin and control its function by hindering calcineurin binding to the nuclear factor of activated T cells, thereby affecting its function as a transcription regulator. Although its role in bladder cancer has not previously been studied, *RCAN1* mRNA is thought to inhibit angiogenesis in other types of cancer, suppressing tumor growth and metastasis in thyroid cancer [32]. Moreover, dysregulation of RCAN1 mRNA expression has been identified in many cancer types, such as hepatocellular carcinoma [33]. RCANs have also been reported to act as metastasis suppressors via modulation of PPAR-γ-regulated colon-cancer-cell invasion [34].

FOSB is a member of the *FOS* family, and encodes a leucine-zipper protein (FosB proto-oncogene, AP-1 transcription

factor subunit) that can dimerize with proteins of the JUN family, thereby forming the transcription factor complex AP-1. FOS proteins have been implicated as regulators of cell proliferation, differentiation, and transformation. Oncogenic roles have been demonstrated for *FOSB* mRNA in promotion of the invasiveness of tumor cells and in downregulation of tumor-suppressor genes [35]. *FOSB* has been shown to be overexpressed in tissues in hemangioendothelioma [36], and to have an oncogenic role in MCF-7 breast cancer cells [37]. We have now shown that *FOSB* mRNA levels are upregulated in the urine and tissues of patients with bladder cancer. Notably, other members of the FOS family, c-FOS and FRA-1 (FOS-related antigen), have also been linked to bladder cancer [38,39].

Urinary levels of noncoding RNAs were correlated with those of their target genes, with significant positive correlations between the levels of miR-324-5p and miR-4738-3p with *FOSB* mRNA, and between lncRNA miR-497-HG and *RCAN1* mRNA, and significant negative correlation between miR-324-5p and miR-4738-3p with *RCAN1* mRNA. Urinary levels of these RNA biomarkers correlated with their expression levels in tumor tissues. Notably, all 5 biomarkers demonstrated higher sensitivity, negative predictive value and accuracy than cytology for detection of patients with bladder cancer. *FOSB* mRNA and *RCAN1* mRNA scored the highest sensitivity, negative predictive value and accuracy among all biomarkers with a specificity of 98.9%, almost equivalent to cytology. Our results suggest that *RCAN1* mRNA and *FOSB* mRNA may serve as discriminating markers for diagnosis of bladder cancer.

5. Conclusion

We assessed the differential expression of a urinary ceRNA: lncRNA-miRNA-mRNA network for diagnosis of bladder cancer. The network consisted of 5 RNAs, 3 of which were noncoding, and all of which showed superior sensitivity to cytology. The 2 mRNAs (*FOS B* and *RCAN1*) displayed the highest accuracy and potential as biomarkers for the detection of bladder cancer.

Conflict of interest

The authors have no conflicts of interest.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.1016/j.urolonc.2018.12.024.

References

[1] Chavan S, Bray F, Lortet-Tieulent J, Goodman M, Jemal A. International variations in bladder cancer incidence and mortality. Eur Urol 2014;66:59–73.

[2] Eissa S, Swellam M, Shehata H, El-Khouly IM, El-Zayat T, El-Ahmady O. Expression of HYAL1 and survivin RNA as diagnostic molecular markers for bladder cancer. J Urol 2010.

[3] Maas M, Walz S, Stühler V, Aufderklamm S, Rausch S, Bedke J, Stenzl A, Todenhöfer T. Molecular markers in disease detection and follow-up of patients with non-muscle invasive bladder cancer. Expert Rev Mol Diagn 2018;18.

[4] Marx J. Autophagy: is it cancer s friend or foe? Science 2006;312:1160–1.

[5] Füllgrabe J, Lynch-Day M, Heldring N, Li W, Struijk R, Ma Q, Hermanson O, Rosenfeld M, Klionsky D, Joseph B. The histone H4 lysine 16 acetyltransferase hMOF regulates the outcome of autophagy. Nature 2013;500:468–71.

[6] Gottardo F, Liu C, Ferracin M, Calin G, Fassan M, Bassi P, Sevignani C, Byrne D, Negrini M, Pagano F, Gomella L, Croce C, Baffa R. Micro-RNA profiling in kidney and bladder cancers. Urol Oncol Semin Orig Investig 2007;25:387–92.

[7] Ingelmo-Torres M, Lozano JJ, Izquierdo L, Carrion A, Costa M, Gómez L, Ribal MJ, Alcaraz A, Mengual L. Urinary cell microRNA-based prognostic classifier for non-muscle invasive bladder cancer. Oncotarget 2017;8:18238–47.

[8] Mengual L, Lozano JJ, Ingelmo-Torres M, Gazquez C, Ribal MJ, Alcaraz A. Using microRNA profiling in urine samples to develop a non-invasive test for bladder cancer. Int J Cancer 2013;133:2631–41.

[9] Wang G, Chan E, Kwan B, Li P, Yip S, Szeto C, Ng C. Expression of microRNAs in the urine of patients with bladder cancer. Clin Genitourin Cancer 2012;10:106–13.

[10] Yamada Y, Enokida H, Kojima S, Kawakami K, Chiyomaru T, Tatarano S, Yoshino H, Kawahara K, Nishiyama K, Seki N, Nakagawa M. MiR-96 and miR-183 detection in urine serve as potential tumor markers of urothelial carcinoma: Correlation with stage and grade, and comparison with urinary cytology. Cancer Sci 2011;102:522–9.

[11] Urquidi V, Netherton M, Gomes-Giacoia E, Serie D, Eckel-Passow J, Rosser C, Goodison S. A microRNA biomarker panel for the non-invasive detection of bladder cancer. Oncotarget 2016;7:86290–9.

[12] Mearini E, Poli G, Cochetti G, Boni A, Eegidi MG, Brancorsini S. Expression of urinary miRNAs targeting NLRs inflammasomes in bladder cancer. Onco Targets Ther 2017;10:2665–73.

[13] Cesana M, Cacchiarelli D, Legnini I, Santini T, Sthandier O, Chinappi M, Tramontano A, Bozzoni I. A long noncoding RNA controls muscle differentiation by functioning as a competing endogenous RNA. Cell 2011;147:358–69.

[14] Hansen TB, Jensen TI, Clausen BH, Bramsen JB, Finsen B, Damgaard CK, Kjems J. Natural RNA circles function as efficient microRNA sponges. Nature 2013;495:384–8.

[15] Huang M, Zhong Z, Lv M, Shu J, Tian Q, Chen J. Comprehensive analysis of differentially expressed profiles of lncRNAs and circRNAs with associated co-expression and ceRNA networks in bladder carcinoma. Oncotarget 2016;7:47186–200.

[16] Sobin L, Wittekind C. International Union against Cancer. TNM classification of malignant tumours. TNM classification of malignant tumours; 2002. p. 6th ed. 2002239.

[17] Lucchesi PMA, Arroyo GH, Etcheverría AI, Parma AE, Seijo AC. Recommendations for the detection of Leptospira in urine by PCR. Rev Soc Bras Med Trop 2004;37:131–4.

[18] Fleige S, Pfaffl MW. RNA integrity and the effect on the real-time qRT-PCR performance. Mol Aspects of Med 2006;27:126–39.

[19] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method.. Methods 2001;25:402–8.

[20] Zhu N, Hou J, Wu Y, Liu J, Li G, Zhao W, Ma G, Chen B, Song Y. Integrated analysis of a competing endogenous RNA network reveals key lncRNAs as potential prognostic biomarkers for human bladder cancer. Medicine (Baltimore) 2018;97:e11887.

[21] Chen Y, Wang SX, Mu R, Luo X, Liu ZS, Liang B, Zhuo HL, Hao XP, Wang Q, Fang DF, Bai ZF, Wang QY, Wang HM, Jin BF, Gong WL, Zhou T, Zhang XM, Xia Q, Li T. Dysregulation of the MiR-324-5p-CUEDC2 axis leads to macrophage dysfunction and is associated with colon cancer. Cell Rep 2014;7:1982–93.

[22] Taylor DD, Gercel-Taylor C. MicroRNA signatures of tumor-derived exosomes as diagnostic biomarkers of ovarian cancer. Gynecol Oncol 2008;110:13–21.

[23] Cao L, Xie B, Yang X, Liang H, Jiang X, Zhang D, Xue P, Chen D, Shao Z. MIR-324-5p suppresses hepatocellular carcinoma cell invasion by counteracting ECM degradation through post-transcriptionally downregulating ETS1 and SP1. PLoS One 2015;10.

[24] Dai N, Zhong ZY, Cun YP, Qing Y, Chen Ch, Jiang P, Li MX, Wang D. Alteration of the microRNA expression profile in human osteosarcoma cells transfected with APE1 siRNA. Neoplasma 2013;60:384–94.

[25] Eissa S, Swellam M, Labib RA, El-Zayat T, El Ahmady O. A Panel of Angiogenic Factors for Early Bladder Cancer Detection: Enzyme Immunoassay and Western Blot. J Urol 2009;181:1353–60.

[26] Gennarino VA, Sardiello M, Avellino R, Meola N, Maselli V, Anand S, Cutillo L, Ballabio A, Banfi S. MicroRNA target prediction by expression analysis of host genes. Genome Res 2009;19:481–90.

[27] Fan Y, Shen B, Tan M, Mu X, Qin Y, Zhang F, Liu Y. TGF-b-induced upregulation of malat1 promotes bladder cancer metastasis by associating with suz12. Clin Cancer Res 2014;20:1531–41.

[28] Su G, He Q, Wang J. Clinical values of long non-coding RNAs in bladder cancer: a systematic review. Front Physiol 2018;9:652.

[29] Ding Q, He K, Luo T, Deng Y, Wang H, Liu H, Zhang J, Chen K, Xiao J, Duan X, Huang R, Xia Z, Zhou W, He J, Yu H, Jiao X, Xiang G. SSRP1 contributes to the malignancy of hepatocellular carcinoma and is negatively regulated by MIR-497. Mol. Ther. 2016;24:903–14.

[30] Zhang Y, Zhang Z, Li Z, Gong D, Zhan B, Man X, Kong C. Micro-RNA-497 inhibits the proliferation, migration and invasion of human bladder transitional cell carcinoma cells by targeting E2F3. Oncol Rep 2016;36:1293–300.

[31] Shi B, Gao H, Zhang T, Cui Q. Analysis of plasma microRNA expression profiles revealed different cancer susceptibility in healthy young adult smokers and middle-aged smokers. Oncotarget 2016;7:21676–85.

[32] Wang C, Saji M, Justiniano SE, Yusof AM, Zhang X, Yu L, Fernández S, Wakely P Jr, La Perle K, Nakanishi H, Pohlman N, Ringel MD. RCAN1-4 is a thyroid cancer growth and metastasis suppressor. JCI Insight. 2017;2:e90651.

[33] Jin H, Wang C, Jin G, Ruan H, Gu D, Wei L, Wang H, Wang N, Arunachalam E, Zhang Y, Deng X, Yang C, Xiong Y, Feng H, Yao M, Fang J, Gu J, Cong W, Qin W. Regulator of Calcineurin 1 gene isoform 4, down-regulated in hepatocellular carcinoma, prevents proliferation, migration, and invasive activity of cancer cells and metastasis of orthotopic tumors by inhibiting nuclear translocation of NFAT1. Gastroenterology 2017;153:799–811:e33.

[34] Iizuka M, Abe M, Shiiba K, Sasaki I, Sato Y. Down syndrome candidate region 1, a downstream target of VEGF, participates in endothelial cell migration and angiogenesis. J Vasc Res 2004;41:334–44.

[35] Bakin AV. Role of DNA 5-Methylcytosine transferase in cell transformation by fos. Science 1999;283:387–90.

[36] Sugita S, Hirano H, Kikuchi N, Kubo T, Asanuma H, Aoyama T, Emori M, Hasegawa T. Diagnostic utility of FOSB immunohistochemistry in pseudomyogenic hemangioendothelioma and its histological mimics. Diagn Pathol 2016;11:75.

[37] Li H, Li L, Zheng H, Yao X, Zang W. Regulatory effects of ΔFosB on proliferation and apoptosis of MCF-7 breast cancer cells. Tumor Biol 2016;37:6053–63.

[38] Huhe M, Liu S, Zhang Y, Zhang Z, Chen Z. Expression levels of transcription factors c-Fos and c-Jun and transmembrane protein HAb18G/CD147 in urothelial carcinoma of the bladder. Mol Med Rep 2017;15:2991–3000.

[39] Sayan AE, Stanford R, Vickery R, Grigorenko E, Diesch J, Kulbicki K, Edwards R, Pal R, Greaves P, Jariel-Encontre I, Piechaczyk M, Kriajevska M, Mellon JK, Dhillon AS, Tulchinsky E. Fra-1 controls motility of bladder cancer cells via transcriptional upregulation of the receptor tyrosine kinase AXL. Oncogene 2012;31:14931503.