Volume 22, No 4 | International Journal of Radiation Research, October 2024

Significance of long-noncoding RNA ATB and SChLAP1 expression in liquid biopsy of bone scan-confirmed metastatic prostate cancer patients

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► ***Original article***

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Received: May 2023
Final revised: February 2024
Accepted: February 2024

Int. J. Radiat. Res., October 2024; 22(4): 941-946

DOI: 10.61186/ijrr.22.4.941

Keywords: *Prostate cancer, lncRNA, liquid biopsy, ATB, SChLAP1.*

ABSTRACT

Background: Long non-coding RNAs (LncRNAs) play an important role in the biological and pathological processes of many cancers. LncRNA SChLAP1 and ATB have been shown to be overexpressed in a variety of cancers and may be involved in tumor cell invasion and metastasis. The goal of this study was to investigate the significance of lncRNA ATB and SChLAP1 expression in liquid biopsy of metastatic prostate cancer (PCa) relative to routine investigations. ***Materials and Methods:*** urine samples from 65 PCa patients we collected to assess ATB and SChlap1 by realtime PCR, serum samples were collected to assess PSA. Bone scan and clinicopathological data including Gleason sum, clinical stage, tumor size and lymph node involvement were collected. ***Results:*** A significant elevation in lncRNA SChLAP1 and ATB expression in bone scan-diagnosed metastatic PCa patients. Both markers were significantly associated with advanced clinical stage, Gleason sum and tumor size. SCHLAP1 expression has high specificity (100%) and moderate sensitivity (68%) at a cutoff point of 2.528. ATB expression has a high sensitivity (93.75%) and specificity (75.76%) at a cutoff value of 4.55. In univariate analysis, Gleason score (> 8), tumor size (> 2), lncRNA ATB express (>4.55), lncRNA SCHAP (>2.53), and PSA (> 35 pg/ml) were independently predictive of a positive bone scan. Only ATB was significant, regardless of the other adjusted factors. ***Conclusions:*** Expression levels of LncRNA SCHALP1 and ATB in PCa patients' urine samples are promising, non-invasive markers that are associated with advanced clinicopathological parameters, including advanced clinical stage, high grade (Gleason sum) and larger tumor size.

INTRODUCTION

The second most frequent malignancy in males and the fifth major cause of cancer mortality is prostate cancer (PCa) [1]. While the overall five-year survival rate is 98%, this drops dramatically when distant metastases are diagnosed. The skeletal system is the most prevalent location for distant metastases, and over 85% of individuals with lethal prostate cancer have bone metastasis [2].

A bone scan is the most popular and cost-effective method for diagnosing bone metastases in clinical practice [3]. However, bone scans are known to be non-specific and insensitive for assessing treatment response at an early enough stage to be clinically meaningful, and they have low sensitivity for disease detection [4].

The prostate-specific antigen (PSA) is used as a biomarker for prostate cancer detection, but it also serves to monitor prostate cancer development [5]. Although PSA is expressed exclusively in the prostate, it is not uniquely expressed in prostate cancer, and increased levels of serum PSA are detected in a variety of noncancerous diseases including benign prostatic hyperplasia, prostatitis, infections, trauma, and urinary retention [6]. As a result, PSA screening has a positive predictive value of only about 25%-40% [7]. As a result, novel predictive indicators must be identified in order to enhance patient outcomes and clinical care in prostate cancer patients.

Long non-coding RNAs (lncRNAs) are 200 nucleotide non-protein-coding RNA molecules that play key roles in many malignancies, as they control proliferation and invasion among other biological and pathological processes [8]. Several studies have revealed that lncRNAs can act as oncogenes or tumour suppressors in distinct forms of cancer [9]. Increasing data suggests that lncRNAs may emerge as fresh and improved indicators or therapeutic targets in cancer diagnosis and therapy [10]. The lncRNA-ATB is found in greater concentrations in hepatocellular carcinoma tissues and is associated with a worse prognosis in hepatocellular carcinoma patients [11]. However, nothing is known regarding the clinical importance and role of lncRNA-ATB in human prostate cancer so far.

[DOI: 10.61186/ijrr.22.4.941]

A novel lncRNA called SChLAP1 may contribute to tumor cell invasion and metastasis because it exhibits abnormal expression in a small proportion of prostate cancers [12]. Previous studies have reported an elevated expression levels of SCHLAP 1 in tumor tissues using in situ hybridization which raises the possibility that it could be used a tissue-specific prostate cancer biomarker [13].

Using liquid biopsy-based markers provides a reliable, minimally invasive tool that can be used to assess and monitor PCa patients. The current study aimed to confirm the role of SCHLAP1 and to our knowledge is the first to address the role of ATB in urine of mPCa in comparison to conventional investigations such as bone scans and PSA values.

MATERIALS AND METHODS

Patients

This study was conducted on sixty-five male prostate cancer patients referred for bone scintigraphy at Ayady E-Mostaqbal Hospital, Alexandria, Egypt between August 2019 and April 2020. This study was approved by the Institutional Human Ethics Committee for conducting research on human subjects, Medical Research Institute, University of Alexandria, and in accordance with the Helsinki Declaration of 1975, as revised in 2000 (#E/C. S/N. T50/2019, Jul 2019) According to its instructions, signed consent was obtained from all the participants in the study. Clinicopathological data was obtained from the pathology reports including; Gleason sum, clinical stage, tumor size and lymph node involvement. The mean age of enrolled patients was 70.08 ± 4.56 years and their clinicopathological characteristics are summarized in table 1.

Table 1. Clinicopathological data of patients.

Clinical Data		No.	%
Bone Scan Assessed Metastasis	Negative	33	50.8
	Positive	32	49.2
Gleason Sum	£6	28	43.1
	7	23	35.4
	≥8	14	21.5
Tumor Size (T)	T1	31	47.7
	T2	16	24.6
	T3	18	27.7
Lymph Node Involvement (N)	N0	62	95.4
	N1	3	4.6
Clinical Stage	I	3	4.6
	II	17	26.2
	III	13	20.0
	IV	32	49.2

Bone scintigraphy

Bone scintigraphy (BS) examination was carried out using Symbia SPECT/CT (Siemens, Germany). Moybdenum-99 was purchased from the Egyptian Atomic Energy Authority and used to produce Technichium-99m by inhouse generator. ^{99m}Tc-labeled diphosphonate with activity 740 to 1110 MBq (20 to 30 mCi) was injected intravenously. Planar imaging was taken in the anterior and posterior planes of the whole body without regional spot views or SPECT reconstructions. The skull, clavicle, scapulae, rib, vertebrate, pelvis and bones of the limbs were among the seven areas of the systema skeletal that were seen. The classification of lesions included benign, normal, positive and ambiguous. Two senior nuclear medicine physicians separately categorized the results of BS into two categories: (1) benign or negative and (2) positive [14].

Sampling

From each patient, 50 of milliliters morning urine samples were collected and processed within 2–3 hours after voiding. Urine sample were sedimented by centrifugation at 2,000 g for 10 min. The pellet was then washed in phosphate-buffered saline (PBS) followed by another 10 min centrifugation. The supernatant was discarded and the pellet was re-suspended in approximately 200 ml of PBS. Urine sediments were then stored at –80 ˚C until further processing [15].

Quantitative estimation of lncRNAs ATB and SCHLAP1 gene expression

Total RNA was extracted from urine sediments using RNeasy mini Kit (Qiagen Group, USA). The purity and concentration of extracted RNA were evaluated by NanoDrop(R) ND-1000 UV-Visible Spectrophotometer (Thermo Fischer Scientific, USA). Total RNA was reverse transcribed using high-capacity cDNA reverse transcription Kit with RNase Inhibitor (Applied Biosystems, USA) for lnc ATB and SChLAP1, according to the manufacturer's instructions, on a GeneAmp PCR System 9700 N8050200 thermal cycler (Applied Biosystems, USA). Reaction conditions were incubation for 10 minutes at 25°C, then for 120 minutes at 37°C, 5 minutes at 85°C. Real-time PCR was the performed using SYBR™ Green PCR Master Mix (Thermo Fischer Scientific, USA) on a CFX real-time PCR system (BioRad, USA). Primer sequence for ATB, F: GGTGACCTGTCTG-TATTTGCG, R: CATACTGCCCCTCCCGTTTG, SChLAP1: F: GAGCGGGATGGAGAAAGGAG, R: GCCTCTTGGGTTC ACCATCT, GAPDH, F: GCTCTCTGCTCCTCCTGTTC, R: TTCCCGTTCTCAGCCTTGAC. Reaction tubes were incubated at 95°C for 10 min, followed by 45 cycles of 94°C for 30 sec, 60°C for 40 sec and 72°C for 30 sec. After the reactions were completed, the CT values were determined by setting a fixed threshold. The relative amount of lncRNA ATB and SCHLAP was normalized to GAPDH. Results were presented as average fold change of target gene in test to control group using $2^{-\Delta\Delta CT}$ formula.

Statistical analysis

Data were analyzed using statistical analysis in social science (SPSS) software package version 20.0 (IBM Corporation, Chicago, Illinois, USA).

[DOI: 10.61186/ijrr.22.4.941]

Quantitative data were described using mean ± standard deviation. The distributions of quantitative variables were tested for normality using Kolmogorov-Smirnov test. Mann-Whitney test was used to compare between two studied groups and Kruskal Wallis test was used to compare between more than two groups. Receiver operating characteristic (ROC) curve was done to interpret the sensitivity and specificity of ATB and SCHLAP against bone scan for predicting BC metastasis. Cox regression logistic analysis was used to identify parameters that can predict metastasis. At all statistical analyses, p value was considered significant at ≤ 0.05.

RESULTS

Bone scintigraphy assessment of metastasis in PC patients

Of the 65 patients included in the current study, visual analysis of bone scan images detected skeletal lesions in 32 patients either in single or multiple sites. The sites of metastasis are illustrated in table 2 and a sample of bone scan images indicating metastasis sites is illustrated in figure 1. The results of bone scan were used as a basis for patients' stratification into the study groups (metastatic – non-metastatic) and all the subsequent statistical analyses.

Table 2. Sites and number of bone metastases in PCa patients.

Sites of bone metastasis	Number of the case of bone metastasis
Shoulder	5
Thoracic cadge	7
Spine	9
Iliac bone	6
Femur	1
Knee	2
Skull	1
Hip	1

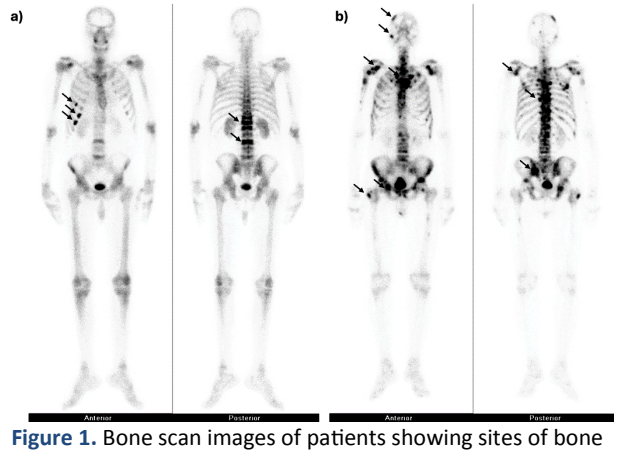

Figure 1. Bone scan images of patients showing sites of bone metastasis in mPCa patients including **a)** rips and spine and **b)** skull, shoulder joint, iliac bone, ribs and spine.

Quantitative determination of urine lncRNA ATB and SCHLAP1 expression in prostate cancer patients

Our results indicated that the median ATB expression level in urine samples of PCa patients was significantly upregulated in metastatic group compared with that in non-metastatic group (U = 106, p < 0.001). The median fold change was 8.56 (6.42-10.53) in metastatic group while in non-metastatic group it was 4.0 (1.30 - 4.55). Similarly, the median SCHLAP1 expression level also showed a significant upregulation in metastatic PCa patients compared to non-metastatic group (U = 88.0, p < 0.001). The median fold change of SCHLAP1 in metastatic PCa urine samples was 2.92 (2.40 - 3.91) while in non-metastatic group it was 1.23 (1.05-2.23) (figure 2).

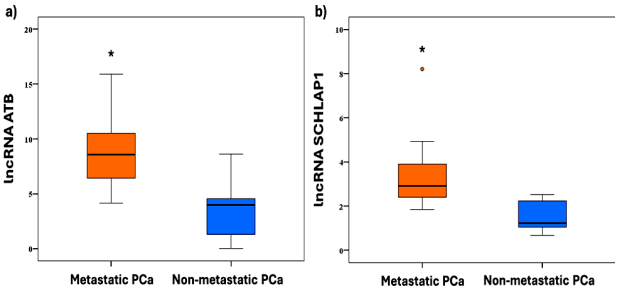

Figure 2. Expression of lncRNA **(a)** ATB and **(b)** SCHLAP1 in metastatic and non-metastatic PC patients.

Association between urine lncRNA ATB and SCHLAP1 and clinicopathological parameters

Stratification analysis revealed that urine levels of ATB and SCHLAP1 in all prostate cancer patients (n = 65) with clinicopathological parameters are represented in figure 3. Statistical analysis showed that the increase in ATB and SCHLAP1 levels was significantly associated with advanced clinical stage of PCa patients (H = 31.875*, p < 0.001 and H = 38.694*, p < 0.001 respectively). The results also showed a significant association between both markers and the Gleason score of PCa patients (H = 10.363, p = 0.006, H = 13.233, p = 0.001 for ATB and SCHLAP1 respectively) and with tumor size as well (H = 16.660*, p = 0.001 and H = 10.319*, p = 0.006*).

Receiver operator characteristic (ROC) analysis of urine lncRNAs ATB and SCHLAP1 expression

To investigate the sensitivity and specificity of urine lncRNA ATB and SCHLAP1 in predicting PC metastasis, a receiver operating characteristic (ROC) curve was constructed against bone scan positive imaging results as a gold standard (figure 4). The ROC curves for lncRNA ATB and SCHLAP1 are represented in figure 3. For ATB, the ROC curve has an area below the curve of 0.900 (p < 0.001) with confidence interval (0.827- 0.972). The cut-off point, or Youden Index, was > 4.55. The model has high specificity (75.76%) and sensitivity (93.75%). SCHLAP1 had an

[DOI: 10.61186/ijrr.22.4.941]

area below the curve of 0.917* (p < 0.001) with confidence interval (0.853 - 0.980). The cut-off point or Youden Index, was > 2.528. The model has high specificity (100.0%) and sensitivity (68.75%).

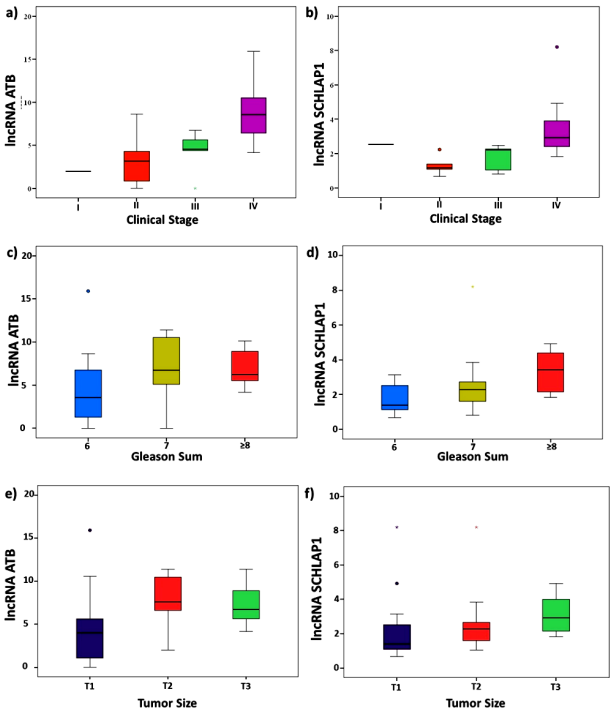

Figure 3. The association of PCa patients' clinical stage **(a, b)**. Gleason sum **(c, d)** and tumor size **(e, f)** with urine lncRNA ATB and SCHLAP1 expression respectively.

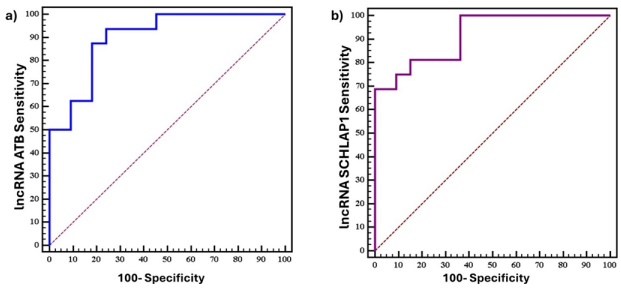

Figure 4. ROC curve of lncRNA a) ATB and b) SCHLAP1 expression in metastatic and non-metastatic PCa patients.

Value of lncRNAs ATB and SCHLAP1 and clinical parameters in predicting bone metastasis

Univariate and multivariate analysis were performed on PCa cases to identify factors that correlate with prognosis using Cox proportional hazards regression model analysis (table 3). In univariate analysis, Gleason score (> 8), tumor size (> 2), lncRNA ATB express (> 4.55), lncRNA SCHAP (> 2.53) and PSA (> 35 pg/ml) were independently predictive of positive bone scan, while lymph node involvement was not.

In multivariate analysis, only ATB was significant regardless of the other adjusted factors. Neither SCHLAP1 nor PSA could independently predict a positive bone scan in multivariate analysis. In general, the multivariate analysis showed a decrease in significance levels and in the odds ratios (OR) with the exception of ATB which had an increase in OR.

Table 3. Univariate and multivariate logistic regression analysis for different parameters affecting metastasis in patients with prostate cancer.

	Univariate		Multivariate	
	p	**OR (95%C. I)**	**p**	**OR (95%C. I)**
Gleason Sum	**<0.001***	6.401 (2.377-17.238)	**0.380**	0.588 (0.180-1.923)
T2 + T3	**<0.001***	9.524 (3.060-29.645)	**0.416**	0.252 (0.009-6.984)
ATB	**<0.001***	2.036 (1.444 – 2.871)	**0.011***	3.555 (1.333-9.486)
SCHLAP	**<0.001***	19.475 (4.062 – 93.365)	–	–
PSA value	**0.001***	1.022 (1.009 – 1.035)	**0.278**	1.057 (0.957-1.167)

DISCUSSION

PCa is a major health problem affecting males all over the globe [1]. Clinical and pathological data such as serum prostate-specific antigen (PSA), Gleason score (GS) and TNM stage can be used to determine predictive and prognostic markers. However, patients with similar TNM stages, GS and PSA may have different outcomes due to PCa heterogeneity [16]. Because PSA testing lacks specificity for prostate cancer diagnosis and can lead to needless prostate biopsies, the development of new accurate biomarkers for prostate cancer and/or high-risk prostate cancer is crucial. A good starting point for the development of new prostate cancer biomarkers is urine. Prostatic secretions contain prostate cancer indicators, which eventually find their way into the urine. Following prostatic alteration, urine is enriched in prostate cancer biomarkers like prostate cancer cells, DNA, RNA, proteins and other small molecules [17].

The results of the current study revealed a significant median fold increase in lncRNA SCHLAP1 expression in urine samples of bone scan-confirmed metastatic PC patients compared to non-metastatic ones (p < 0.001). SChLAP1 expression has been reported to be extremely specific for prostate tissue, being present at modest levels in bladder, kidney, and testis samples [12]. Previous publications have also linked the lncRNA SChLAP1 to metastatic progression backed up these findings. Presenter *et al* in 2014 have linked a 2.45-fold to a greater risk of metastatic progression in males who underwent radical prostatectomy [18]. SCHLAP1 has been hypothesized to increase prostate cancer invasion and metastasis by a variety of pathways, including altering the SWI/SNF complex (SWItch/Sucrose Non-Fermentable) complex's metastasis inhibiting action, which functions as a chromatin remodeler and tumor suppressor [12]. However, Schap1 tumorigenic effects were also found to be independent of SWI/SNF in a recent study [19]. Furthermore, by binding miR-198

[DOI: 10.61186/ijrr.22.4.941]

and activating the MAPK signaling pathway, SChLAP1 might promote PCa cell proliferation and metastasis [20]. SChLAP1 may also boost the proliferation, migration, and tumorigenicity of prostate cancer cells by mediating promoter methylation modification of miR-340-5p/miR-143-3p/miR-145-5p via a DNMT3a -feedback loop, according to Huang *et al.* [21]. Apoptosis, reduced cancer invasiveness and metastasis have been reported as a result of SChLAP1 knockdown [22].

In our study, statistical analysis also showed that the increase in lncRNA SCHLAP1 in metastatic PCa patients was significantly associated with advanced clinical stage, high grade (Gleason sum) and larger tumor size of PCa patients. These results are consistent with previous reports associating increased risk with high-grade prostate cancer (Gleason 8–10) vs. low- to intermediate-grade illness (Gleason 7 on histology) [18]. lncRNAs with specificity for prostate cancer initial tumor stage or related with lymph node metastases have also been found in other investigations [23].

LncRNA-ATB, which is found on chromosome 14, was initially shown to be overexpressed in hepatocellular carcinoma (HCC) and to have several regulatory roles [24]. In comparison to non-metastatic PC patients, our findings revealed a significant 8.56-fold increase in lncRNA-ATB expression in urine samples from bone scan-confirmed metastatic PC patients compared to a 4.0-fold increase in non-metastatic group ($p < 0.001$). *In vitro* studies have shown that the expression of lncRNa-ATB is associated with cancer cell proliferation and cell cycle regulatory proteins including cyclins E and D1. Furthermore, blocking down lncRNA-ATB expression have been linked to signaling pathways regulating epithelial mesenchymal transition [25].

Clinically, the expression of lncRNA ATB has been studied in a variety of malignancies. In comparison to normal brain tissue, Ma *et al*. (2016) discovered that lncRNA-ATB was substantially expressed in glioma tissue and cell lines. Furthermore, elevated lncRNA-ATB expression in glioma patients was associated with a worse overall survival rate [26]. The expression of lncRNA-ATB increased in osteosarcoma tissues, which was validated. The suppression of lncRNA-ATB dramatically reduced osteosarcoma cell proliferation, migration, and invasion [27]. On the contrary, the expression of lncRNA-ATB was discovered to be low in pancreatic cancer tissue and cell lines in this investigation [28].

In our study, statistical analysis also showed that the increase in lncRNA-ATB was significantly associated with advanced clinical stage, high grade (Gleason sum), and larger tumor size in PCa patients. These results are consistent with those reported by Xu *et al*. (2016) that ATB high expression correlates with preoperative PSA levels, pathological stage, GS, lymph node metastasis, angiolymphatic invasion, and biochemical recurrence when the knockdown of lncRNA ATB and PI3K/Akt signaling pathways is inhibited. Meanwhile, the roles of lncRNA ATB in the invasion, migration and tumor growth remain unclear [29]. In general, elevated ATB expression has been linked to clinicopathological characteristics of a variety of malignancies. Reduced levels of lncRNA-ATB expression were associated with pancreatic cancer lymph node metastases, invasion and worse overall survival rate [28].

ROC curve analysis have demonstrated that lncRNA SCHLAP1 expression in urine samples of PC patients has high specificity and moderate sensitivity (68%) at cutoff point of 2.528 pg/ml while lnc ATB showed a high sensitivity (93.75%) and lesser specificity (75.76%) at a cutoff value of 4.55 pg/ml. both markers have shown to be predictive of positive bone scan metastasis and thereby lethal progression of the disease using univariate analysis. However, only ATB was significant in multivariate analysis of the investigated markers, independently of the other adjusted covariates.

These results are in accordance with previous studies that have shown that SChLAP1 expression is an independent predictor of PC aggressiveness with highly significant hazard ratios for predicting BCR, clinical progression to systemic disease, and PC-specific mortality compared to other clinical factors like advanced clinical stage and the GS [12]. In the group of non-advanced clinical tumor stage and in the group with a 6–7 GS, scientists found a sensitivity of roughly 24% and a specificity of 94 percent utilizing SChLAP1 as a predictive test [29]. In a recent study, SCHALP1 high expression primary prostate cancer was reported to be an independent predictor of biochemical recurrence [30].

The findings of Xu *et al*, who found that increased lncRNA-ATB expression is an independent predictive factor for biochemical recurrence-free survival in prostate cancer patients, back up these findings. Through the modulation of cell cycle regulatory protein expression levels, overexpression of lncRNA-ATB stimulated and knockdown of lncRNA-ATB prevented the proliferation of prostate cancer cells [31].

In conclusion, our data indicated that quantitative determination of lncRNA SChLAP1 and ATB expression levels in the urine of PC patients may complement current diagnostic tests. The results of the current study sheds light on promising role of measuring expression levels of LncRNA SCHALP1 and ATB in PCa patients' urine samples as specific, sensitive, non-invasive markers that are associated with advanced clinicopathological parameters including advanced clinical stage, high grade (Gleason sum), and larger tumor size.

ACKNOWLEDGMENT

Not applicable.

[DOI: 10.61186/ijrr.22.4.941]

Funding: No funding was received.

Availability of data and materials: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions: Conceptualization, S.E.E., M.I.M and O.S.Z; Methodology, S.E.E and M.N.S.; Validation, M.I.M and O.S.Z.; Investigations, S.E.E., M.N.S.; data analysis: S.E.E.; Original draft preparation, S.E.E; Manuscript final revision M.I.M.

Ethics approval and consent to participate: This study was approved by the Institutional Human Ethics Committee for conducting research on human subjects, Medical Research Institute, University of Alexandria and in accordance with the Helsinki Declaration of 1975, as revised in 2000 (#E/C. S/N. T50/2019, Jul 2019). Enrolled patients were asked to provide a written consent before enrolment.

Competing interests: The authors declare that they have no competing interests.

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[DOI: 10.61186/ijrr.22.4.941]