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Two long non-coding RNAs, CAT179 and CAT1796, differentiate between benign prostate hyperplasia and prostate cancer

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Abstract: Several long non-coding RNAs (lncRNAs) have recently emerged as potential biomarkers in cancer biology. In the present study, we examined the expression of four lncRNAs (CAT179, CAT1796, PRCAT47, and CAT1066) to evaluate their ability to discriminate prostate tumors from benign prostate hyperplasia (BPH). Expression of these four lncRNAs was examined in 20 prostate cancer and 20 benign prostate hyperplasia (BPH) samples, as well as in urine samples (11 BPH, and 11 cancer). Total RNA was extracted for cDNA syntheses. The expression of the candidate lncRNAs was evaluated by quantitative real-time PCR (qRT-PCR). The lncRNAs CAT1796 and CAT179 were both upregulated in prostate cancer compared to BPH clinical samples ($P<0.05$). ROC curve analysis showed that CAT1796 had high sensitivity and specificity for diagnosis of prostate cancer (AUC=0.8151[95%CI 0.65-0.97]), suggesting that CAT1796 lncRNA could be a prostate cancer biomarker.

Keywords: prostate cancer; long non-coding RNAs (lncRNAs); benign prostate hyperplasia (BPH); CAT1796; biomarker

INTRODUCTION

Prostate cancer (PCa) is the second most prevalent cancer among men worldwide [1]. It has been predicted that by 2025 the incidence of prostate cancer could increase by 45% in developing countries [2]. During the past ten years, PCa has become the most prevalent hormone-related cancer in Iran [3]. The lack of any confirmed biomarkers for the early diagnosis of PCa, contributes to an overall low patient survival [4]. Although advances have been made in treatment strategies, the prognosis of patients with progressive PCa remains disappointing [4]. PSA (prostate-specific antigen) is a commonly applied biomarker for PCa screening, but it has numerous drawbacks and is not completely specific for PCa. Therefore the discovery of new specific biomarkers for early diagnosis of PCa is important [5].

Long non-coding RNAs (lncRNAs) are transcribed from genomic intergenic regions, and have attracted much interest in transcriptomic studies in cancer [6, 7]. LncRNAs have been shown to play roles in many biological processes through a range of different mechanisms [4]. They can have either oncogenic or tumor-suppressive functions which have been explored in tumorigenesis and tumor biology [8,9]. The dysregulation of lncRNAs has been reported in numerous types of cancers, including PCa, while some lncRNAs have been shown to be PCa specific, including PCGEM1 [10], PRNCR1 [11], PCAT1 [12] and SChLAP1 [13]. Many lncRNAs show tissue- and cell type-specific expression patterns and therefore have the potential to be used as diagnostic biomarkers for cancer [14, 15].

The recent application of RNA sequencing (RNA-Seq) in different human cancer types has revealed

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several dysregulated lncRNAs that could be used for discrimination of tumor versus non-tumor samples. Moreover, lncRNAs can be used to determine the clinical significance of various tumor subclasses. Although the putative roles of lncRNAs in many cellular processes have been reported, their role in the initiation and progression of cancer and usefulness for cancer diagnosis are not completely understood. Based on data available in the MiTranscriptome project [16], we selected four putative PCa-associated lncRNA genes (CAT179, CAT1796, PRCAT47.1 and CAT1066), and evaluated the sensitivity and specificity of their expression levels to distinguish between tissue samples from PCa and benign prostate hyperplasia (BPH).

MATERIALS AND METHODS

Bioinformatics studies

To select the lncRNAs to be used for the expression analysis, RNA seq data from the MiTranscriptome project were analyzed. After comparing the mean expression levels of a panel of lncRNAs, four putative PCa-associated lncRNA genes (CAT179, CAT1796, PRCAT47.1 and CAT1066) with the highest expression levels were selected.

Cell culture

PC3 and LNCaP PCa cell lines were used. The cells were obtained from the National Cell Bank of Iran (Pasteur Institute, Tehran, Iran). Cell lines were cultured at a density of 4×10^5 cells in 12 well plates in RPMI 1640 medium (Gibco, CA, USA), with 10% fetal bovine serum and 1% penicillin/streptomycin and incubated at 37°C in a 5% CO_2 humidified atmosphere.

Tissue specimens and RNA isolation

A total of 20 BPH and 20 PCa tissue samples were collected from Ayatollah Kashani Hospital (Shahrekord, Iran). Informed consent was obtained from all patients participating in the present study. None of the patients had undergone radiation or chemotherapy before surgery. The Ethics Committees of Shahrekord University of Medical Science (Shahrekord, Iran) approved the experimental protocol. The fresh tissue specimens were snap-frozen in liquid nitrogen and stored at -80°C until use for RNA extraction. Total RNA from tumor and non-tumor tissues and cell lines was extracted using TRIzol reagent (Invitrogen, USA) based on the manufacturer's protocol. To eliminate any genomic DNA contamination, RNase-free DNaseI (Fermentas, Lithuania) was used to treat the extracted RNA samples. RNA quality was evaluated by gel electrophoresis, and its quantification was measured by optical density at 260 nm. Twenty-two urine samples were collected from patients with PCa or BPH. Urinary sediments were obtained by centrifugation of a 20 mL sample volume at 4°C, 16000×g for 10 min. The obtained sediments from urine were washed twice with ice-cold phosphate-buffered saline (PBS) and centrifuged each time under the same conditions. These urinary sediments were used for RNA extraction using TRIzol reagent (Invitrogen, USA). The clinical pathological features of the studied population are presented in Supplementary Tables S1 and S2.

cDNA synthesis, and RT-PCR

One µg of total RNA from prostate tissue samples (PCa or BPH) and cell lines, and 1.5 µg of RNA form the urine sediment were used for complementary DNA (cDNA) synthesis. Random hexamer and oligo dT primers plus MMLV reverse transcriptase (Fermentas, Lithuania) were used for reverse transcription following the manufacturer's instructions. The RT-PCR reaction was carried out for 35 cycles for all the cDNAs with 1 µg of synthesized cDNA plus 6 µL of 10× PCR Master Mix (Amplicon, Denmark), 0.75 µL of each primer and deionized distilled water in a 15 µL PCR reaction volume.

qRT-PCR

qRT-PCR was carried out using a Rotor-Gene 6000 instrument (Corbett Life Science, Sydney, Australia). Optimized conditions of qRT-PCR were as follows: 15 µL total volume containing 1 µL of cDNA template, 0.25 µL of each 10 pmol primers and 5 µL of 2X SYBR Green PCR Mix (Takara, Japan). The PCR reaction conditions were as follows: 5 min initial denaturation at 95°C followed by 35 cycles including 5 s denaturation at 95 °C, annealing temperature at 62°C for 30 s and extension at 72°C for 18 s. The

sequences of the primers designed by Gene Runner software, ver. 4.0 are as follows: (listed 5'-3'): CAT179 forward primer: CATAGACTTACTCTTCAGAATTAAAGTGC, reverse primer: ACAAGTTCTCTTGACTCTAGCAAGG with a 196 bp PCR product. The CAT1796 forward and reverse primers are TGAAGGTAGAGGTGGCCACTGGAG and GGGGAGATCCTTATTCTGCCTACG, respectively (137 bp). The sequences of forward primer and reverse primers for β-actin as the internal control are ACCACCTTCAACTCCATCATG and CTCCTTCTGCATCCTGTCG, with 120 bp length of the PCR-product. All PCR experiments were repeated 3 times for each sample. The effectiveness of PCR for each sample was determined using the LinRegPCR program. β-actin was used as an endogenous control to normalize gene expression. The Livak method was used to calculate the relative expression of each gene by comparing the threshold cycle number[17]. Briefly, the comparative cycle threshold (CT) was the base to calculate the gene expression levels. ΔCt values were defined by subtracting the average β-actin Ct value from the average target gene Ct value. Comparative expression of each gene was computed by the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

All experiments were carried out as three replicates. Statistical analysis was performed by Graph Pad Prism software (GraphPad Software, San Diego, CA, USA). The differences in the RNA levels between the two groups (BPH and cancer patients) were assessed by the independent unpaired sample t-test. The efficiency of each gene as a biomarker was determined by receiver operating characteristic curve (ROC) analyses. The Kolmogorov-Smirnov and Pearson's correlation method was used to determine the normal distribution of data and correlation tests, respectively. $P<0.05$ was considered as significant.

RESULTS

Bioinformatics

Based on the results of MiTranscriptome data, 4 lncRNAs with the highest differential expression level in PCa were selected. The differential expression of the lncRNAs is shown in Figs. 1A-D.

Expression levels of lcRNA candidate genes in PC3 and LNCaP cell lines, prostate tumor and BPH samples

RT-PCR was performed to determine the optimal conditions for the amplification of candidate lncRNAs. We first evaluated the expression level of these genes in two human PCa cell lines, PC3 and LNCaP. The chromosomal locations of the CAT179 and CAT1796 were 15q26.3 (Fig. 2A), 1q42.13 (Fig. 3A), respectively. RT-PCR analysis showed that the lncRNAs were expressed in PC3 and LNCaP cells (Figs. 2B and 3B, respectively). Having confirmed the expression of candidate lncRNAs in PCa cell lines, further analysis was carried out on clinical samples.

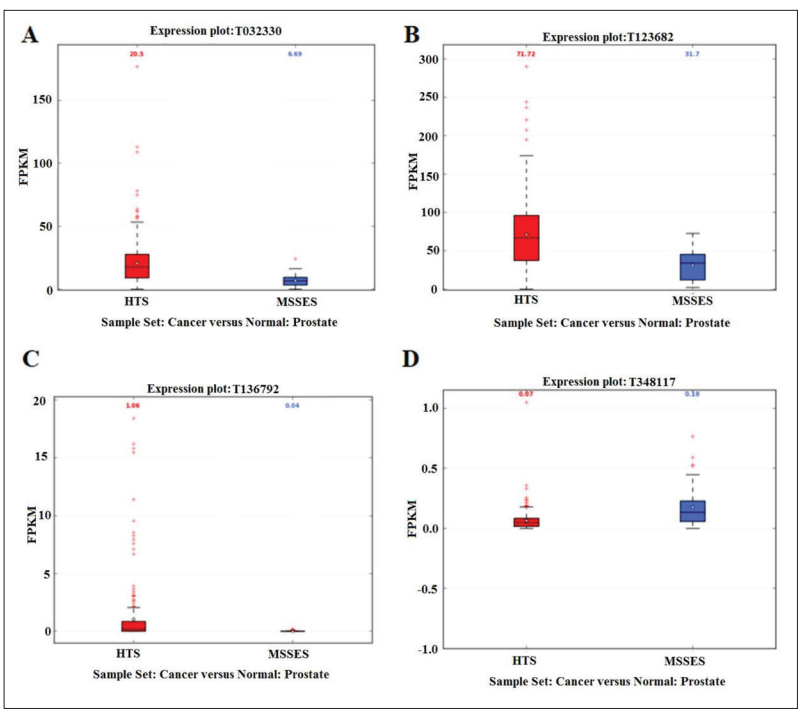

Fig. 1. Bioinformatics analysis. Differential expression of CAT179 (**A**), CAT1796 (**B**), PRCAT47.1 (**C**), and CAT1066 (**D**) in PCa samples vs. BPH samples. (Color Code: Cancer (Left); Normal (Right)).

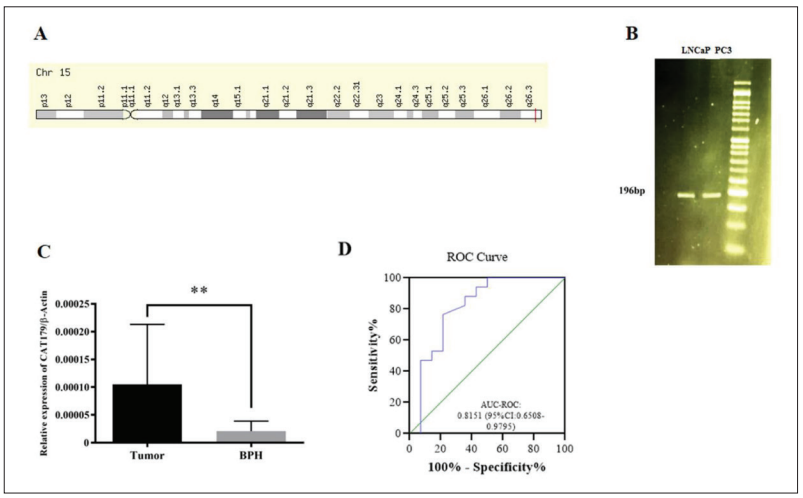

Fig. 2. Use of CAT1796 as a PCa biomarker. **A** – Chromosomal location of CAT1796 lncRNA **B** – RT-PCR analysis of CAT1796 expression in PC3 and LNCaP cell lines. **C** – Relative gene expression of CAT1796 in tumor and BPH samples. Gene expression was normalized against the β-actin reference gene and obtained by the $2^{-\Delta\Delta Ct}$ method. **D** – ROC curve analysis showing the sensitivity and specificity of CAT1796 for diagnosis of prostate cancer in tissue specimens (AUC=0.8151[95%CI 0.65-0.97]). All data are presented as the mean±SD.

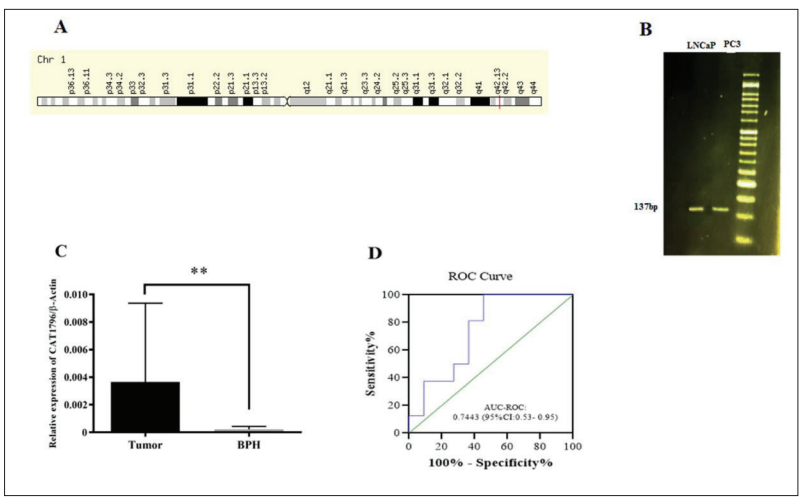

Fig. 3. Use of CAT179 as a PCa biomarker. **A** – Chromosomal location of CAT179 lncRNA. **B** – RT-PCR analysis of CAT179 expression in PC3 and LNCaP cell lines. **C** – Relative gene expression of CAT1796 in tumor and BPH samples. Gene expression was normalized against the β-actin reference gene and obtained by $2^{-\Delta\Delta Ct}$ method. **D** – ROC curve analysis demonstrating that CAT179 had an AUC=0.7443 (95%CI 0.53-0.95) in prostate cancer tissue samples. All data are presented as the mean±SD.

qRT-PCR analysis was carried out to measure the expression levels of the candidate lncRNAs in 20 PCa tumor samples and 20 BPH specimens. The relative expression of CAT1796 and CAT179 in PCa specimens was significantly higher than in BPH samples (P<0.01 and P<0.05 respectively; Figs. 2C and 3C). The expression level of the other two lncRNAs (PRCAT47, and CAT1066) was not significantly different between the samples and therefore no further analysis was performed on these lncRNAs. ROC curve analysis demonstrated that the area under curve-receiver operating characteristic (AUC-ROC) values for CAT1796 and CAT179 were 0.8151 and 0.7443, respectively (Figs. 2D and 3D). This result suggested that CAT1796 would be a better diagnostic marker for PCa than CAT179.

CAT1796 expression in urine specimens

To test whether the studied lncRNAs were detectable in the urine samples of patients, we carried out RT-PCR using the total RNA extracted from urine sediments obtained from BPH and PCa patients. The results showed that CAT1796 expression was significantly higher in PCa urine samples compared to the BPH urine samples (P<0.05) (Fig. 4A). ROC curve analysis also showed that the AUC-ROC value for CAT1796 was 0.7521 (Fig. 4B), which suggested that it could be a suitable biomarker for diagnosing PCa and BPH patients in non-invasive urine samples.

Expression correlation of candidate lncRNAs in tumor samples

To determine the relationship between the expressions of candidate lncRNAs, Pearson's correlation was computed based on the expression levels of CAT179 and CAT1796 in PCa samples. The results showed a weak positive correlation between the expression levels of the lncRNAs mentioned above, with a correlation coefficient of 0.04, but this was not statistically significant (P-value: 0.93; Fig. 4C).

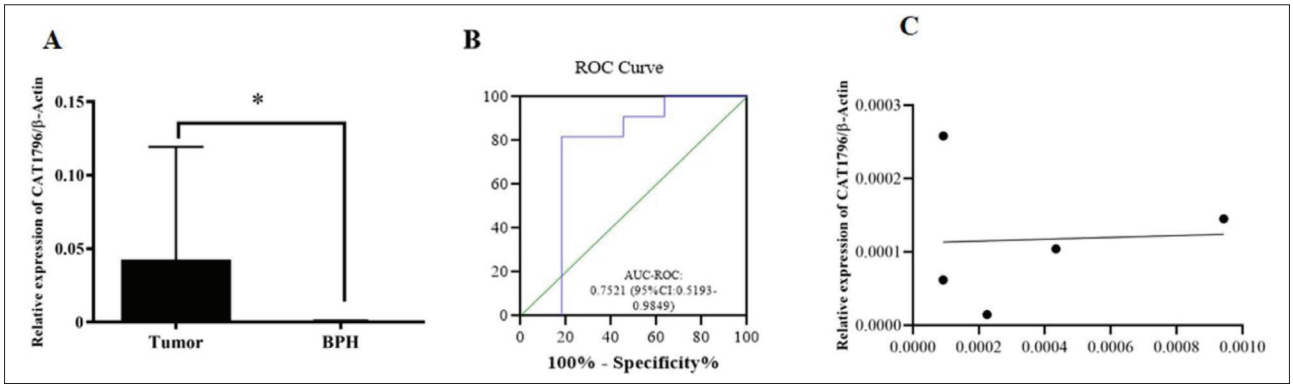

Fig. 4. CAT1796 expression in urine samples. **A** – Expression of CAT1796 quantified by real-time PCR in 11 BPH and 11 prostate cancer urine samples; β-actin was used as the reference gene. All data are represented as mean ± SD. **B** – ROC curve analysis showing the sensitivity and specificity of the CAT1796 as a biomarker in urine sediment samples (AUC=0.7521; 95% CI 0.51-0.98). **C** – Decreased expression of miR-27b-3p and increased expression of ZEB1 in prostate cancer samples compared to BPH samples. **D** – Correlation (r=0.04) between CAT179 and CAT1796 expression.

DISCUSSION

PCa is a type of cancer that can be managed and controlled with proper treatment if diagnosed early [18]. Prostate specific antigen (PSA) is a well-known marker used for screening PCa but it is not exclusively expressed in prostate cancer and its use results in a negative biopsy rate of 70-80% [19]. The PCa antigen 3 gene (PCA3) is now considered as one of the most successful molecular diagnostic biomarkers for PCa. Its use has dramatically reduced the number of biopsy surgeries required for accurate diagnosis [20]. Although PCA3-based diagnosis has reduced the number of unnecessary biopsies (67%), it does not identify 21% of cases that go on to be clinically proven as cancerous [21]. Additionally, there is no good correlation between the PCA3 level and the clinicopathological status of patients [21]. Therefore, the discovery of new biomarkers for the diagnosis of PCa patients is urgent and essential.

Increasing evidence has shown that lncRNAs can function as crucial regulators in cellular processes acting through various mechanisms. Although only a few lncRNAs have been functionally characterized, lncRNAs are known to play a role in the induction, development and progression of several types of cancers, such as prostate [22], ovarian [23], breast [24] and colorectal [25]. It is important to further understand the function of the uncharacterized lncRNAs that could lead to novel therapeutic targets for the treatment of cancer. Some lncRNAs are expressed in a tissue-specific manner and could act as potential biomarkers for the treatment of many diseases [26, 27].

In the present study, candidate lncRNAs were selected using the RNA-seq data of the MiTranscriptome project and the correlation between the expression levels of these lncRNAs and PCa was examined. The MiTranscriptome project was started in 2015 by Iyer et al.[28] who provided a genome-wide analysis of changes in the expression of lncRNAs for different cancer types; 7,256 RNA-seq libraries from tumors, normal tissues and cell lines were analyzed, and finally 7942 lineage or cancer-associated lncRNA genes were confirmed[28]. The data in our study showed that the expression levels of two lncRNAs, CAT1796 and CAT179, were significantly elevated in human PCa tissues compared to the BPH samples.

To date, several different lncRNAs have been reported to be dysregulated in PCa [29]. The results of a deep sequencing study from the transcriptome of PCa revealed that lncRNAs and other unannotated transcripts constituted about one quarter of the transcripts involved in prostate tumorigenesis [11]. Accordingly, defining the exact role of these lncRNAs in the initiation and progression of PCa could be a milestone towards the discovery of new therapeutic targets in this disease.

Numerous pieces of evidence demonstrated the role of more well-known lncRNAs, such as GAS5,

HOTAIR, and PCA3, in the occurrence and progression of prostate cancer. Many of these lncRNAs are overexpressed in cancerous tissues and prostate cancer cells compared to healthy tissues. These lncRNAs act as oncogenes, leading to the promotion of cell proliferation, invasion, migration and epithelial to mesenchymal transition [30]. LncRNAs such as MALAT1[31], prostate cancer-associated intergenic noncoding RNA transcript 1(PCAT1) [32], and HOTTIP [33] are mainly known for their oncogenic role in prostate cancer. On the other hand, a small proportion of known lncRNAs in prostate cancer act as tumor suppressors, and their reduced or suppressed expression has been associated with prostate cancer [30]. PCAT29 for example, is the first reported lncRNA with a tumor suppressor role in prostate cancer [34]. H19 [35] and GAS5 [36] play the role of suppressive tumors in prostate cancer, which have been well studied, and their mechanism of action in this cancer has been elucidated. Identifying the lncRNAs involved in prostate cancer and understanding their mechanism of action provides an opportunity to use them as effective therapeutic targets or high-performance diagnostic biomarkers in clinical studies[37,38]. Thus, several lncRNAs associated with prostate cancer affect tumorigenesis by affecting androgen receptor (AR) signaling. AR suppresses HOTAIR and in AR deficiency increased expression indicates that it leads to increased prostate cancer cell division and invasion [37, 39]. The present study also introduces some lncRNAs associated with prostate cancer for the first time; however, finding their mechanism of action could bring scientists closer to therapeutic purposes in future studies. In the present study, four hitherto uncharacterized lncRNAs, including CAT1796, CAT179, PRCAT47.1 and CAT1066 were examined. Among these four molecules, CAT1796 and CAT179 were expressed differentially in PCa and BPH tissues. Moreover, based on AUC-ROC values of 0.81 for CAT1796 and 0.74 for CAT179, it appeared that CAT1796 would be a better diagnostic marker than CAT179. These two lncRNAs could be considered as potential biomarkers for distinguishing PCa from normal prostate tissue.

Our findings of elevated expression levels of CAT1796 and CAT179 in PCa tumor tissue compared to BPH samples are in line with RNAseq data deposited in the MiTranscriptome database. Although expression analysis of PRCAT47.1 and CAT1066 showed no significant difference between PCa and BPH samples, a positive correlation between CAT1796 and CAT179 in PCa was observed.

In order to be used for large scale screening for the early detection of PCa, biomarkers should be detectable in a non-invasive manner within body fluids, such as urine samples. The expression data obtained from sediments of urine samples showed that CAT1796 was significantly different between PCa patients and urine from control groups, with a sensitivity and specificity value of around 63.63 and 63.27, respectively. In contrast, the CAT179 expression levels exhibited no significant change between the urine samples of the two groups. PCA3 is a specific diagnostic biomarker in PCa and its sensitivity and specificity range has been shown to be from 47-69% and 66-83%, respectively [40], indicating that the sensitivity index of CAT1796 is at a similar level as PCA3. Therefore, in this study we describe two novel lncRNAs with a potential use in prostate cancer diagnosis. Collectively, CAT1796, which plays an oncogenic role in PCa, could be used as a diagnostic biomarker in PCa either in tumor samples or in patient urine, alone or combined with PCA3. The mechanism by which CAT1796 is regulated in PCa needs more study.

There were some limitations in this study; for example, this study's sample sizes were relatively insufficient and that can adversely affect our results' representativeness. Similar studies with larger sample sizes and accurate determination of the lncRNAs mechanisms of action introduced in this study can be conducive for biomarker-based research in prostate cancer.

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Author contributions: NA and FA contributed equally to this work; they conceived the presented idea and carried out a part of the experiment. M.A. A.M and B.H. performed the analytical methods and carried out the experiment. All authors contributed in manuscript writing. M.R.H. developed the theory, supervised the findings of this work and edited the final version of the manuscript.

Conflicts of interest disclosure: The authors declare no conflicts of interest.

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Supplementary Material

The Supplementary Material is available at: http://www.serbiosoc.org.rs/NewUploads/Uploads/Ebrahimi%20et%20al_6734_Supplementary%20Material.pdf