Parsian Hadi (Orcid ID: 0000-0002-3965-7566)

Evaluation of the plasma level of long non-coding RNA PCAT1 in prostatic hyperplasia and newly diagnosed prostate cancer patients

Setareh Rezatabar[1, 2]. Emadoddin Moudi[3, 4]. Farzin Sadeghi[5]. Soraya Khafri[6]. Tayebeh Azramezani Kopi[2]. Hadi Parsian[5*]

[1]Student Research Committee, Babol University of Medical Sciences, Babol, Iran

[2]Department of Clinical Biochemistry, Babol University of Medical Sciences, Babol, Iran

[3]Department of Urology, Shahid Beheshti Hospital, Babol University of Medical Sciences, Babol, Iran

[4]Cancer Research Center, Health Research Institute, Babol University of Medical Sciences, Babol, Iran

[5]Cellular and Molecular Biology Research Center, Health Research Institute, Babol University of Medical Sciences, Babol, Iran

[6]Department of Epidemiology, Babol University of Medical Sciences, Babol, Iran

*Corresponding authors: Hadi Parsian, Cellular and Molecular Biology Research Center, Health Research Institute, Babol University of Medical Sciences, Babol, Iran. Tel: +98-1132199936, Fax: +98-1132199936, E-mail: hadiparsian@yahoo.com

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Abstract

Background: Prostate cancer (PCa) is generally detected by prostate-specific antigen (PSA) as one of the most widely applied tumor markers over decades for its high sensitivity. Nevertheless, it causes overtreatment or an unnecessary biopsy because of its limited specificity. Prostate cancer-associated ncRNA transcript1 (PCAT1), the newly identified long non-coding RNA (lncRNA) has been reported to associate with the progress of prostate cancer. In *vitro* studies proposed that PCAT-1 may be an appealing candidate for diagnostic accuracy improvement regard to its notable overexpression in prostate cancer cells. The purpose of the present study was to evaluate the diagnostic potential of the plasma PCAT1 expression levels in prostate cancer patients in comparison to benign prostatic hyperplasia (BPH) patients and healthy controls.

Methods: The plasma lncRNA PCAT1 level was measured by real-time quantitative reverse transcription PCR in 40 men newly diagnosed with PCa, 20 patients with BPH and 20 healthy subjects. The results were analyzed statistically by SPSS software 25.

Results: The expression of PCAT1 was significantly higher in healthy subjects compared to BPH patients (P=0.03). The diagnostic accuracy of the plasma lncRNA PCAT-1 for discrimination of the healthy subjects than BPH patients was reasonable (AUC=0.799; sensitivity=71%; specificity=74%; NPV=74%; PPV=71%).

Conclusions: It seems that the plasma levels of PCAT1 expression have reasonable diagnostic accuracy for the discrimination of healthy individuals than BPH. While no significant difference of PCAT1 expression levels in comparisons between prostate cancer group with BPH and normal groups was observed.

Keywords: long non-coding RNA PCAT1; Prostatic Neoplasms; Prostatic Hyperplasia; Plasma

Introduction

Prostate cancer (PCa) is the most prevalent malignancy in males and the second-highest death rate associated with cancer among men, with an estimated more than 160,000 new cases and 29,400 deaths in 2018 in the United States[1] . Although the incidence of PCa in Asian countries, including Iran, has been lower, in recent years, the number of affected people has risen worldwide [2,3]. This growing trend over the last decades can be largely attributed to the widespread use of serum prostate-specific antigen (PSA) testing, introduced in the late 1980s. PSA, a kallikrein serine protease, is a tissue-specific biomarker which secreted nearly exclusively via the epithelial cells of the prostate and small amounts of it present in the blood of healthy men [4]. Despite the high sensitivity of PSA (70-90 %), due to insufficient specificity (20-40%), the diagnosis of PCa upon PSA blood levels is not definite. Sometimes people with normal PSA, (approximately 15% of men with low serum PSA (<4ng/ml)), have prostate cancer. Also, PSA levels can increase in cases of inflammation and in excess of 50% benign prostatic hyperplasia (BPH) in men older than 50 years [5,6]. Now, PSA has been measured as a continuous parameter in combination with a digital rectal examination (DRE) and transrectal ultrasound (TRUS) for the diagnosis of PCa [4]. Recent studies have focused on the essential function of protein-coding genes [7]. The human genome consists of wide and complex nucleotide sequences, which can generate over 100000 proteins by transcription and translation. Whereas, just approximately 2% of genomic transcripts have protein-coding potential; the rest 98% of genome transcripts do not have a protein-encoding role called non-coding RNAs (ncRNAs) [8,9]. ncRNAs are categorized in terms of their size to small ncRNAs (<200 bp) and long ncRNAs (>200 bp) [10]. A wide range of long ncRNAs (lncRNAs) is characterized to have great roles in cellular

development, differentiation, and various other biological processes. Moreover, it is illustrated that the aberrant expression of lncRNAs is related to cancer progression, proliferation, and metastasis through transcriptional regulation of target genes [11]. Analysis of the lncRNA transcriptome with genomic data, epigenomic profiles, and genetic predispositions have shown a range of lncRNAs that are involved in vulnerability to prostate cancer, including PCAT1 **(**prostate cancer-associated transcript 1), a lncRNA located in the 8q24.21 gene desert [12]. PCAT1 repress BRCA2 expression, control MYC stabilization in prostate cancer, and also interacts with AR (androgen receptor) and LSD1 (lysine-specific demethylase 1, known as AR cofactors), after prolonged androgen therapy [12-14]. In addition, PCAT1 leads to an increase in the expression of cMyc, thereby causing cancer cell proliferation. It also reduces the expression of BRCA2 and disrupts the process of double-stranded DNA repair, which can have an oncogenic role [15]. In *vitro* studies illustrated that PCAT-1 likely would be an appealing candidate for biomarker improvement due to its notable overexpression in prostate cancer and spread role in cell proliferation [13]. Regarding multiple roles of PCAT1 in the pathogenesis of prostate cancer and the importance of early diagnosis in the efficacy of prostate cancer treatment, this study was done. Therefore, we aimed to evaluate the potential diagnostic value of the plasma PCAT1 in patients with prostate cancer and BPH compared to normal controls.

Materials and methods

Study participants and sample collection

Samples in this study were obtained from 40 men newly diagnosed PCa patients, 20 BPH patients and 20 healthy volunteers who referred to the department of urology of hospitals affiliated to our university. The healthy volunteers were who referred to the annual checkup; their serum levels of PSA were normal and according to the clinical examination by an expert physician did not show any abnormal associated clinical symptoms, and also had no abnormalities in the digital rectal examinations (DRE). Benign prostatic hyperplasia (BPH) patients were referred to the physician with specific urinary symptoms such as difficulty starting urination, poor flow of urine that stops and starts. Their increased serum PSA was detected before rectal examination by an enzyme immunoassay method in the hospital, and also the abnormality in their transrectal ultrasonography of the prostate (TRUS) was found. All cancerous patients were diagnosed according to histopathological examination and were not subjected to any therapy such as surgery, preoperative radiotherapy or chemotherapy, and also without other malignant diseases before sample collection. Following a complete explanation of the study process, written informed consent was obtained from each participant prior to blood sample collection, and this study was confirmed by the Ethics Committee of Babol University of Medical Sciences. From each individual, a 5ml blood sample was taken in an EDTA anticoagulant vacuum tube. Then the plasma of the blood samples was immediately separated and stored at -80˚C until analysis. Also, the demographic information on the participated subjects in the study, including age, weight, height, waist circumference, hip circumference, history of the disease and clinicopathological features such as serum PSA level, TNM stage, and Gleason score received and was recorded in the form provided for this purpose. The BMI and body fat% of subjects calculated by following equations; BMI = Weight (kg)/ Height^2 (m^2); body fat% = (BMI (kg/m2) ×1.2) + (0.23×age (year)) – (G×10.8) – 5.4; in this equation the value of G for the men is equal to one and for women is equal to zero.

RNA isolation and cDNA synthesis

Total RNA was extracted from 250µl plasma samples by the means of the TRIzol reagent (Bio Basic, Canada), using high pure filter tubes (Roche, Germany), plus treatment with recombinant DNase I, RNase free (Takara Bio Inc, Japan) in accordance with manufacturer's instructions. The purity and concentration of purified RNA were evaluated using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, USA). PrimeScript™ RT Reagent Kit, (Takara, Japan) for synthesis of cDNA was applied according to the manual. Briefly, RNA was reverse transcribed to cDNA with the following conditions: Heat Lid 110ºC, 10 min at 30°C, 60 min at 42°C, 5 min at 95°C, store 30 min at 8°C, and then cDNA was stored at −20°C.

Quantitative real-time polymerase chain reaction

Real-time PCR was performed using SYBR Premix Ex Taq™ II (Takara, Japan) to detect the PCAT1 expression as a target gene and 5srRNA was used as endogenous control, implemented in the real-time PCR cycler Rotor-Gene Q (Qiagen). The qRT-PCR was carried out in a volume of 20µl containing: 10µl of SYBR Premix Ex Taq, 1µl of forward primer (5pmol/µl), 1µl of reverse primer (5pmol/µl), 2µl of cDNA product and 6µl of RNase-free dH2O. The real-time PCR cycling situation was as follows: at first hold at 95°C for 10 min, then 40 amplification cycles of melting at 95°C for 15 seconds, annealing and extension one min at 60°C. The sequence of LncPCAT1 and 5srRNA was obtained from ncbi.nlm.nih.gov/PubMed. Then the primer design and evaluation of parameters and characteristics of primers were performed by PubMed /primer-blast, Allele ID software, and idtdna site. To confirm amplification specificity, the melting curve analysis, and gel electrophoresis were performed. Nonspecific products were not found. PCR efficiency (E) values were calculated by Rotor-Gene Q software and in accordance with Pfaffl method the relative expression ratio of a target gene was assessed according to E and the C_T deviation of an unknown sample against the control and compared to a reference gene as the following equation [16]:

$$\text{Ratio}= [(E_{\text{ref}})^{C_{T\ \text{Sample}}} / (E_{\text{target}})^{C_{T\ \text{Sample}}}] / [1/n\sum [(E_{\text{ref}})^{C_{T\ \text{Calibrator}}} / (E_{\text{target}})^{C_{T\ \text{Calibrator}}}]$$

Statistical analysis

The normality of the distribution of variables was tested by the Shapiro-Wilk test. Subsequent for further analysis, the Kruskal-Wallis test, Chi-square and Spearman correlation was carried out. In addition, the receiver operating characteristic (ROC) curve was performed to evaluate the diagnostic accuracy of the PCAT1 for discriminating against the study groups. Furthermore, the log-transformation of the relative expression ratio was applied to reduce the variance of data to increase the validity of the associated statistical analyses. Moreover, we removed four outlier data with the drawing box plot. All statistical analysis was carried out by the SPSS software package, version 25.0 and a p-value <0.05 was considered statistically significant.

Results

The expression level of lncRNA PCAT1 in 40 newly diagnosed patients with PCa, 20 patients with BPH and 20 healthy controls was evaluated by qRT-PCR. The primer sequences for quantitative real-time PCR are demonstrated in Table 1. The detailed demographic features of the studied participants are indicated in Table 2. To understand the relationship between the expressions of lncRNA PCAT1and the clinic pathological characteristics, we collected and analyzed the patients' clinical data as shown in Table 3.

Comparison of plasma PCAT1 expression level among PCa patients, BPH patients, and healthy controls

The results showed that the expression level of PCAT1were significantly differed between studing groups (P=0.03) (Fig. 1). The pairwise comparison demonstrated that PCAT1 was significantly higher in healthy subjects than BPH patients, whereas no significant difference of PCAT1expression levels was found in comparisons between prostate cancer group with BPH and normal groups. As shown in table 2, the lowest plasma PCAT1 expression levels were related to the BPH group and a slightly down-regulation of plasma PCAT1 expression levels in the healthy subjects toward PCa patients was observed, but it's not appeared significant. In addition, to the subsequent investigation, the demographic characteristics of the studied groups were collected and analyzed. As shown in Table 2, PSA levels had significant differences between groups, and no significant differences were found in BMI and body fat % between these groups. Moreover, we analyzed the association between the expressions of PCAT1 with aging, the body mass index (BMI) and %body fat. To determine the effect of these variables on the plasma PCAT1 expression levels, participants of the study were classified in two different age groups, i.e. ≥65 years old and <65 years old, three different groups for BMI: healthy weight (18.5-24.9 kg/m^2), overweight (25-30 kg/m^2) and obese (>30kg/m^2); and two classes for %body fat include normal (less than 25%) and obese (more than 25%). As it is illustrated in Table 3, no significant association was found between PCAT1 expressions with aging, BMI, and %body fat. The relationship between the expression of plasma level of lncRNA PCAT1 and serum PSA was also examined. No significant correlation was observed between the plasma PCAT1 expression levels and serum PSA levels of participants. According to Spearman's test the cancer group, BPH, and the healthy individuals correlation coefficient= -0.052, -0.233, 0.277; and P-value= 0.749, 0.368, 0.25 were shown respectively.

Comparison of PCAT1 expression and clinicopathologic features

In an effort to explore the relationship between the expression of plasma levels of PCAT1 and clinicopathologic features, patients were classified regarding these variables. As shown in Table 3, the plasma PCAT1expression level was significantly associated with the T stage (P-value= 0.048). Further, the pairwise comparisons were shown a significant difference only between T1 and T2 in T stage groups (P-value= 0.048). However, no significant association was found between the plasma PCAT1expression levels with the TNM stage (P-value = 0.36) and the Gleason score (P-value = 0.301).

The diagnostic value of PCAT1 for discrimination of study groups

ROC curves and the area under the ROC curve (AUC) were performed to investigate the diagnostic accuracy of the PCAT1 for discriminating against the study groups. Regarding the ROC analysis, the cutoff value of 0.31 PCAT1 folds change showed the best diagnostic accuracy for discriminating the BPH patients than healthy subjects; (Fig. 2). As shown in Table 4, the diagnostic accuracy of the plasma lncRNA PCAT1 for discrimination of the healthy subjects than BPH patients were reasonable: AUC=0.799 (95%CI=0.646-0.951); sensitivity=%71; specificity=%74; negative predictive value (NPV) = %74; positive predictive value (PPV) = %71.

Discussion

Several studies suggest that lncRNAs can serve as diagnostic or prognostic biomarkers and likely attractive therapeutic targets in PCa. Long non-coding RNA PCAT1 has been described as an oncogenic lncRNA in some solid tumors, including prostate cancer. Nevertheless, the molecular mechanism of PCAT1 involved in PCa is

still not fully defined [17]. An effective biomarker for clinical use in PCa management should be able to discriminate tumor tissue from benign tissue, and aggressive tumors from indolent tumors, with high specificity and sensitivity [13]. Notably, biomarkers that can be obtained non-invasively, are more attractive to employ as a screening or diagnostic tool [18]. In *vitro* studies propose that PCAT1 likely can be an appealing candidate for biomarker development in regard to its notable overexpression in prostate cancer and functional role in cancer proliferation [13]. To the best of our knowledge, this study may represent the first analysis of PCAT-1 in body *fluids in prostate cancer and BPH patients.* This study represented to investigate how plasma levels of PCAT1 expression change in patients with prostate cancer versus those with BPH and healthy individuals. The results indicated that PCAT1 expression levels were significantly higher in normal subjects compared to BPH patients, but no significant difference was found with the cancerous group. As shown in Table 2, the lowest plasma PCAT1 expression levels were related to the BPH group and a slightly down-regulation of plasma PCAT1 expression levels in the healthy subjects toward prostate cancer patients was observed but it's not appeared significant. It seems that the PCAT1 could discriminate the BPH group from the cancerous and healthy groups, although this discrimination was only significant between the two BPH and healthy groups. Prensner et al. applied transcriptome sequencing (RNA-Seq) in a group of 102 prostate tissues and cell lines to identify unannotated ncRNAs. They introduced PCAT1 as a novel prostate-specific regulator of cell proliferation and the target of the Polycomb Repressive Complex 2 (PRC2), among 121 PCATs with cancer- particular expression patterns [19]. In the other study, Prensner et al. demonstrated that PCAT1 suppress the BRCA2 tumor repressor gene, resulting in downstream impairment of homologous recombination. The homologous recombination is one of the mechanisms employed to uncontrolled double-stranded DNA breaks (DSB) repair. Functional impairment of homologous recombination induces high sensitivity to small molecule inhibitors of base excision repair PARP1 [14]. In addition, this group in another study found that PCAT1 post-transcriptionally upregulates cMyc protein, result in cell proliferation and specific gene expression programs, comprising accelerated expression of cMyc target programs like protein translation [13]. Xu et al. illustrated that PCAT1 serves as an oncogenic factor in PCa cells partially suppressing by the expression of miR-145-5p. Furthermore, miR-145 was previously shown to suppress cell proliferation, migration and invasion by targeting Fascin-1 (FSCN1), an actin-binding protein which is closely related to invasion and migration in cancer, including PCa. In other words, PCAT1 increased PCa cell proliferation, migration, and invasion via up-regulation of FSCN1 that mediated by miR-145-5p [17]. In another study, the result of exploring the associations of four tagging single nucleotide polymorphisms (tagSNPs) and PCa risk showed that genetic variant of rs1902432 in PCAT1 was significantly associated with accelerated risk of PCa [20]. Furthermore, PCAT1 as a novel biomarker in lncRNA family is indicative of poor prognosis in esophageal squamous carcinoma, hepatocellular carcinoma and multiple myeloma cancer patients [21-23]. Recent studies demonstrated that obesity is associated with an accelerated risk of various cancers, but inconsistent results have been observed between BMI and PCa risk. However, some other anthropometric indicators have been shown to be associated with the risk of PCA. [24]. In the present study, significant differences between the study groups were not found in terms of BMI and % body fat. Moreover, significant associations between the plasma PCAT1 expression levels with BMI and % body fat were not observed. Previous studies demonstrated that the incidence of prostate cancer accelerates with age. Prostate cancer rarely occurs in men less than 40 years of age; of newly diagnosed cases in the United States, 64% are over 65 years and 23% older than 75 years old [25]. Nevertheless, no significant association was observed between PCAT1

expressions with aging. In the simultaneous evaluation of the effects of age and the relative PCAT1 expression on the incidence of prostate cancer, we were not able to perform the multi-variable advanced model because of the small sample size. It is suggested that in further studies with increasing sample size, simultaneously evaluate the effects of age and the relative PCAT1 expression on the incidence of prostate cancer and also the severity of age interfering effect. Our result indicates that there is a significant association between PCAT1 expression levels and T stage, but, no significant association with TNM stages and Gleason score of the disease were found. Shi et al demonstrated that upregulation of PCAT1 expression levels in esophageal squamous carcinoma was significantly related with progressive clinical stage and poor prognosis [21]. Zhang et al. reported that PCAT1 not only improperly overexpressed in hepatocellular carcinoma tissues and cell lines, but also correlated with TNM stage, metastasis, and histological grade [26]. Shen et al. had illustrated that overexpression of serum PCAT1as predictive biomarker is associated with a clinical diagnosis of multiple myeloma with %71.7 sensitivity and %93.8 specificity [22]. In vitro studies have illustrated that PCAT-1 may be an appealing candidate for biomarker development in regard to its notable overexpression in PCa [13] but to our knowledge, there is no report regarding the diagnostic accuracy of PCAT1 in prostate cancer. It seems that PCAT1 with %71sensitivity and %74specificity has reasonable diagnostic accuracy for discriminating BPH patients than healthy subjects. Although, there was a difference in plasma PCAT1 expression levels between the BPH group in comparison with cancer and healthy groups, only significant difference was found between the two groups of BPH and healthy. Also, we did not observe any correlation between PCAT1 expression levels with PSA levels. It seems that PSA and PCAT1 are two different tools. Serum PSA levels can be used as a discriminator between cancer and BPH groups, as well as between cancerous and healthy groups, Due to the insignificance of the changes in the expression of PCAT1 between the cancer group compared to the BPH and healthy groups, it may not be possible to combine these two tools or factors. Surly further studies for determining the accuracy of combining the PCAT1with PSA as a probabilistic diagnostic tool is needed. However, why PCAT-1 upregulation was not observed in cancer cases and the exact molecular mechanisms behind the altered expression of PCAT-1 in BPH are still unclear and need to be further large-scale studied. Probably, the result can be affected by sample size, clinical and individual features, ethnic group, and analytical methods.

Conclusions

In conclusion, it seems that plasma PCAT-1 expression levels in the PCa group in comparison of the normal subjects are lower in crude data. This means a slight down-regulation of PCAT1 levels in the healthy group toward PCa patients occurred, but it's not appears significant. In addition, the lowest plasma PCAT-1 expression levels were related to BPH subjects. It seems that the sensitivity of 71% and a specificity of 74% have an appropriate diagnostic accuracy to diagnose healthy individuals than BPH. Surely another study is needed for highlighting these results.

List of abbreviations

Prostate Cancer (PCa); Benign Prostatic Hyperplasia (BPH); long non-coding RNA (lncRNA); Prostate Cancer-Associated ncRNA Transcript1 (PCAT1)

Funding

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Availability of data and materials

The datasets applied and/or analyzed in the current research are available from the corresponding author on reasonable request**.**

Authors' contributions

Setareh Rezatabar, Emadoddin Moudi, and Farzin Sadeghi performed the research; Tayebeh Azramezani Kopi and Hadi Parsian designed the research study; Hadi Parsian contributed essential reagents or tools; Setareh Rezatabar and Soraya Khafri analysed the data; Setareh Rezatabar wrote the paper

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Competing interests

All authors declare that have no conflict of interests.

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Table 1. The primer sequences for quantitative real-time PCR

NCBI accession no.	Primer Name	Sequence (5′-3′)
NR_045262.2	LncPCAT1-forward	GAGAGCTGACATAGGCACCC
	LncPCAT1-reverse	TCTCCACTGGTGTTCATGGC
NR_023371.1	5srRNA-forward	GCCCGATCTCGTCTGATCT
	5srRNA-reverse	AGCCTACAGCACCCGGTATT

Table 2. Comparison of clinical and demographic characteristics of the studied groups

Variables	**Cancer Group n=40**	**BPH Group n=20**	**Healthy Group n=20**	**P-value**
Age (Year)	70.3 ±10.4	66.2 ±5.9	59.9 ±8.3	<0.001 [a]
BMI (kg/m^2)	25.6 ±3.3	26.2 ±3.8	27.4 ±3.1	0.098
Body fat %	30.7 ±4.1	30.5 ±5.1	30.4 ±3.8	0.887
PSA (ng/ml)	18.5 ±16.9	2.2 ±1.3	1.5 ±1.1	<0.001 [b]
PCAT1	5.64 ±12.9	0.87 ±2.8	7.39 ± 13.6	0.037 [c]

Results are reported as mean ± SD;

P-value of pairwise comparisons for normal-BPH; normal-cancer; BPH-cancer as following respectively: **a**: 0.069, <0.001, 0.395; **b**: 1, <0.001, <0.001; **c**: 0.99, 0.039, 0.149.

Table 3. The relationship between levels of lncRNA PCAT1 with clinicopathological parameters and demographic data

variables		No	Mean ranks	Mean± Std. Error(median)	P -value
Age status					
< 65 years	Cancerous	11	16.36	7.595 ± 2.82 (1.96)	0.103
	BPH	6	9.67	0.173 ± 0.05 (0.18)	
	Healthy	15	19.33	5.616 ± 1.92 (3.55)	
≥ 65years	Cancerous	29	22.28	4.899 ± 2.62 (0.09)	0.619
	BPH	11	21	1.258 ± 1.03 (0.14)	
	Healthy	4	28.25	14.06 ± 13.89 (0.25)	
BMI status					
healthy weight (18.5-24.9)		31	35.48	4.343 ± 1.96 (0.13)	0.56
over weight (25-30)		34	41.38	6.400 ± 2.40 (0.35)	
obese (> 30-40)		11	38.09	2.612 ± 1.11 (0.45)	
% Body fat					
≤ 25%		4	17.75	0.045 ± 0.03 (0.02)	0.054
> 25%		72	39.65	5.289 ± 1.41 (0.33)	
T stage					
T1		18	16.61	6.79 ± 4.33 (0.027)	0.048
T2		16	26.06	6.08 ± 1.59 (5.141)	
T3		6	17.33	0.97 ± 0.59 (0.038)	
I		15	17.40	8.178± 5.14 (0.09)	0.36
II		19	22.47	5.112 ± 1.43 (1.96)	
III		5	18.40	0.972 ± 0.59 (0.04)	
Gleason score					
≤ 6		24	22.25	8.43 ± 3.28 (2.38)	0.301
7		10	20.20	2.04 ± 1.00 (0.08)	
8-9		6	14	0.49 ± 0.47 (0.02)	

Table 4. The diagnostic value of PCAT1 for discrimination of study groups

Group	AUC	Sensitivity	Specificity	Positive predictive value (PPV)	Negative predictive value (NPV)
BPH -normal	0.799	%71 %CI=49-92	%74 %CI=54-93	%71 %CI=49-92	%74 %CI=54-93
Cancer-BPH	-	%53 %CI=37-68	%29 %CI=8-51	%64 %CI=47-80	%21 %CI=5-37
Cancer-normal	-	%53 %CI=37-68	%74 %CI=54-93	%81 %CI=66-96	%42 %CI=26-59

%CI= confidence interval %95

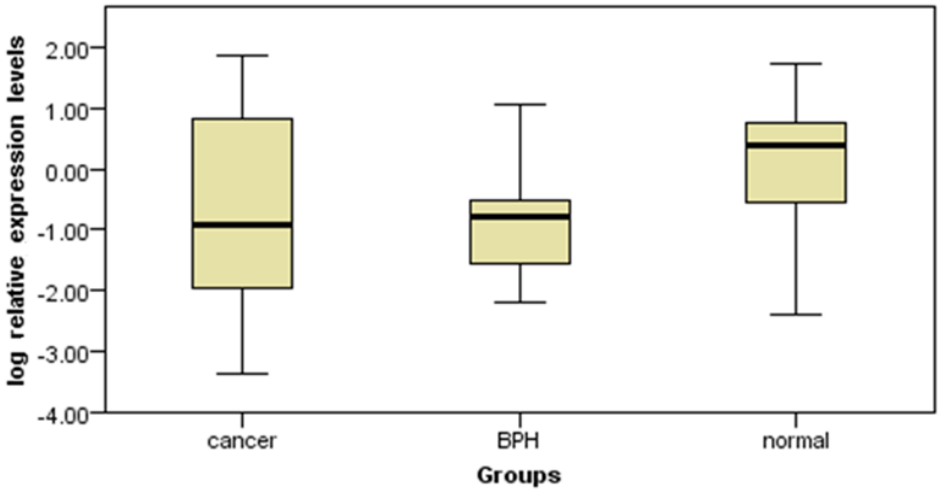

Fig. 1 The expression of lncRNA PCAT-1. The expression levels of lncRNA PCAT1 relative to 5SrRNA in the Plasma of prostate cancer patients versus BPH patients and healthy controls was determined by real-time PCR. P = 0.03

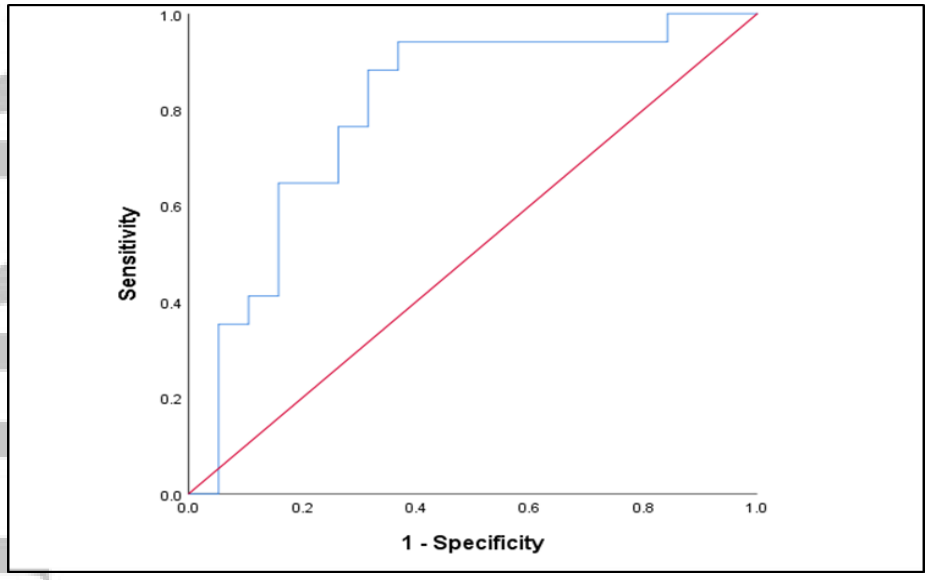

Fig. 2 Potential diagnostic accuracy of plasma lncRNA PCAT1 for discriminating of BPH patients from healthy controls (AUC=0.799; %95 CI= 0.646 – 0.951)