Prostate Cancer Antigen 3 Gene Expression in Peripheral Blood and Urine Sediments from Prostate Cancer and Benign Prostatic Hyperplasia Patients versus Healthy Individuals

Hemen Moradi Sardareh,[1,2] Mohammad Taghi Goodarzi,[1,2] Reza Yadegar-Azari,[2,3] Jalal Poorolajal,[4] Seyed Habibollah Mousavi-Bahar,[5] Massoud Saidijam[2,3*]

Purpose: To determine the expression of prostate cancer antigen 3 (PCA3) gene in peripheral blood and urine sediments from patients with prostate cancer (PCa) and benign prostatic hyperplasia (BPH) and normal subjects.

Materials and Methods: A total number of 48 patients [24 with biopsy proven prostate cancer (PCa) and 24 with benign prostate hyperplasia (BPH)] were studied. Twenty-four healthy individuals were also recruited as control group. After blood and urine sampling, total RNA was extracted and cDNA was synthesized. Expression of PCA3 gene was assessed by quantitative reverse transcription polymerase chain reaction.

Results: Comparison of PCA3 gene expression between control and BPH groups indicated no statistically significant differences in both urine and blood samples. Patients with PCa demonstrated an increased PCA3 gene expression rate compared to control and BPH groups (10.64 and 7.17 folds, respectively). The rate of fold increased PCA3 gene expression in urine was 20.90, 20.90, and 20.35 in patients with PCa, BPH and normal subjects, respectively.

Conclusion: Evaluation of PCA3 gene expression can be considered as a reliable marker for detection of PCa. Increased level of this marker in urine sediments is more sensitive than blood for distinguishing between cancerous and non-cancerous groups.

Keywords: prostatic neoplasms; diagnosis; tumor markers; biological; blood; urine; gene expression regulation; oncogene proteins.

INTRODUCTION

Prostate cancer (PCa) is a common neoplasm in developed countries and its incidence has increased in the past few years in Iran, too.[1,2] The available information in recent years shows that among various neoplasms, PCa has the highest estimated new cases in men. After lung and bronchus cancers, the most estimated deaths rate belong to PCa in the same years.[3,4]

For the first time in 1986, US Food and Drug Administration (FDA) approved the prostate specific antigen (PSA) test for monitoring PCa patients.[5] Subsequently in 1994, it was accepted as a screening tool for detection of PCa.[6] Today, the benefits of PSA screening are accepted but its usage has some limitations such as low specificity.[7] In this respect, Ghafoori and colleagues concluded that results obtained from serum PSA test solely is not valid for detection of PCa since it lacks enough sensitivity and specificity.[8] Also, Yoon and colleagues studied the serum PSA levels in men for detecting PCa. However, no significant difference was observed in cancer detection rates according to serum PSA levels between their studied groups.[9]

Despite of 3 ng/mL value[7] as threshold of serum PSA level to differentiate healthy men from PCa patients, the rate of negative biopsy is 70-80%.[7] In a 7-year study on healthy men less than 55 years old based on serum PSA level less than 3 ng/mL and normal digital rectal examination (DRE), Thompson and colleagues concluded that there was no threshold of serum PSA levels to differentiate between healthy individuals and PCa patients. Also, they demonstrated that there was a risk of PCa in all levels of serum PSA.[10] Moreover, Mehrabi and colleagues evaluated the serum PSA levels in an Iranian population and compared it with studies in United States and Japan. They observed that the serum reference PSA level in Iranian men is significantly lower.[11] Therefore, more specific tests are needed to detect PCa, particularly in men with a previous negative biopsies.[12] Prostate cancer antigen 3 (PCA3) gene is located on 9q21.2 chromosome and expresses exclusively in prostate tissue.[13] Recent studies have shown that PCA3 gene expresses in prostate epi-

[1]Department of Biochemistry and Nutrition, School of Medicine, Hamadan University of Medical Sciences, Hamadan, Iran.
[2] Research Center for Molecular Medicine, Hamadan University of Medical Sciences, Hamadan, Iran.
[3] Department of Genetics and Molecular Medicine, Hamadan University of Medical Sciences, Hamadan, Iran
[4] Research Center for Modeling of Non-communicable Diseases, Department of Epidemiology and Biostatistics, School of Public Health, Hamadan University of Medical Sciences, Hamadan, Iran.
[5] Urology and Nephrology Research Center, Hamadan University of Medical Sciences, Hamadan, Iran.
*Correspondence: Research Center for Molecular Medicine, Hamedan University of Medical Sciences, Hamadan, Iran.
Tel: + 98 81 38380462. Fax: + 98 81 38380464. E-mail: sjam110@yahoo.com.
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thelial cells. However, no cytoplasmic protein is created from its translation. PCA3 expresses significantly in more than 95% of primary and metastatic PCa samples,[14] but no significant correlation has been observed between the expression of PCA3 and tumor stage or Gleason score.[15] PCA3 expression was surveyed primarily in prostate tissue samples,[15] then the expression of this marker was also checked in urine[13,16] and blood.[15] Several studies have shown different sensitivity and specificity for PCA3 tests. The aim of this study was to assess the expression of PCA3 in urine and blood simultaneously and to compare the results in the same conditions. To achieve this goal the laboratory methods were modified to get more accurate results.

MATERIALS AND METHODS

This was a frequency matched case-control study. Among the patients who were referred to our urology center in Hamadan city, Iran, during January 2011 to December 2012, 48 cases were selected and categorized into two groups: PCa (group 1, n = 24 and benign prostatic hyperplasia (BPH), group 2, n = 24). All participants had pathologic diagnosis. Twenty-four healthy men (group 3) were also included as control group. The participants of the three groups had no significant differences based on age. Blood and urine samples were collected from PCa and BPH patients one month after the pathologic examination confirmation.

All participants in PCa group were diagnosed less than one year before the beginning of the study. The excluding criteria were doing hormone therapy, chemotherapy and radiation therapy. Patients whose PCa was definitively ruled out according to tissue pathology were included in the BPH group. For this group the excluding criteria were having a history of cancer, taking finasteride or the other anticancer drugs more than one month or having prostate tissue pathology results suspected being prostatic intraepithelial neoplasia. All participants were aware of their participation and had willingly signed a consent form. The study protocol was approved by the Medical Ethics Committee of Hamedan University of Medical Sciences.

Blood Sampling

Immediately after blood sampling, 2.5 mL blood was added to ethylenediaminetetraacetic acid (EDTA) containing tubes and kept at 4°C in the box of ice. Then, RNA extraction and cDNA synthesis were performed in less than 2 hours later. For RNA extraction from whole blood, lysis solution was prepared containing 10 mM Tris-HCl, 320 mM sucrose, 5 mM $MgCl_2$ and Triton X100 1% solution. The pH was adjusted to 8.

Based on the protocol,[17] lysis solution was added to the blood about 4 times of the volume of the sample. After centrifugation for 20 min at 4000 rpm, supernatant was discarded. Then the sediment was mixed with phosphate buffered saline and was stirred in order to unfold the clot completely. Subsequently, the samples were centrifuged for 10 min at 3000 rpm and supernatant was discarded again. The prepared sediment contained white blood cells along with the cancer cells. To ensure complete removal of hemoglobin, an inhibitor of the polymerase chain reaction (PCR), lysis buffered solution and phosphate buffered saline washing solution were added and the process was repeated. Finally, the sediment (which should have been transparent as much as possible) entered the main phase of RNA extraction which was carried out according to the manufacturer protocol.

Urine Sampling

Urine cells integrity is affected by pH, temperature and concentration of urine and the time interval between sampling and testing. On the other hand, urine cells contain a great amount of ribonuclease that can degrade the urine RNA rapidly if these cells are destroyed. To harvest high-quality RNA and prevent its degradation in urine, the key-points are protecting urine cells from destruction in pre-extraction RNA, decreasing the temperature and reducing the time between sampling and extraction.

The scientific and practical findings led us to prepare a mixed solution, including chaotropic agents, detergent, phosphate buffered saline and 2-mercaptoethanol. Then the pH of solution was checked and adjusted on 7. The mixed solution was kept at 4°C for protection from deterioration. Immediately after the DRE, 20-25 mL of the primary urine samples was collected in sterile containers.[16] Then the collected urine was added to the equal volume of prepared cold mixed solution. RNA extraction and cDNA synthesis were accomplished within 2 hours.

RNA Extraction and cDNA Synthesis

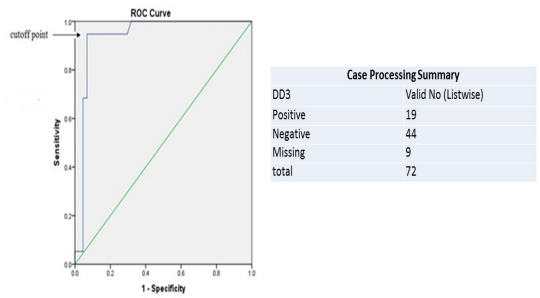
Total RNA was isolated from peripheral blood and urine in accordance to standard procedures and manufacturer manuals using RNeasy Mini Kit (Qiagen, Hilden, Germany). The purity and quantity of all extracted RNA were evaluated by nanodrop spectrophotometer (Epoch, BioTek, Winooski, Vermont 05404-0998, USA), and the ratio of A260/A280 was measured. Then RNA integrity was checked by agarose gel electrophoresis (1%), 1× Tris-Borate-EDTA (TBE). Subsequently, 1µg of extracted RNA was subjected to cDNA synthesis kit (Qiagen, Hilden, Germany). cDNA was stored at -80°C after production.

To confirm the success of reverse transcription and absence of contamination before performing quantitative PCR, 18s rRNA gene expression was checked as an internal control. Real-time reverse transcription polymerase chain reaction (RT-PCR) test was performed using CFX96 real time Thermocycler system (BioRad, Hercules, CA, USA) and QuantiFast SYBR Green PCR Kit (Qiagen, Hilden, Germany). The reaction was incubated at 95°C for 5 min, followed by 40 cycles of 15s at 94°C, 30s at annealing temperature, 30s at 72°C and then fluorescence was measured. Primers, designed by software AlleleID 7.6, were:

PCA3-F (5'-CAATATAATGTCTAAGTAGT-3'),
PCA3-R (5'-TTAAGGAACACATCAAT-3'),
18s rRNA-F (5'-GTAACCCGTTGAACCCCATT-3'),
18s rRNA-R (5'-CCATCCAATCGGTAGTAGCG-3').

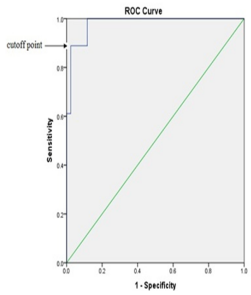
Relative expression of the studied genes was calculated by measuring the delta threshold cycle value (ΔCt) for each sample. Delta threshold cycle value for each sample was determined as the average of a triplicate assay.

Statistical analysis was done by STATA 11 software with 95% confidence intervals (CI). Receiver operating characteristic (ROC) curve was drawn using the sensitivity and specificity to compare PCA3 expression in blood and urine. In this analysis, the specificity (true negative) and



Case Processing Summary	
DD3	Valid No (Listwise)
Positive	19
Negative	44
Missing	9
total	72

Figure 1. Receiver operating characteristic (ROC) analysis of the DD3 gene expression in blood: Area under the ROC curve (AUR): 0.937.



Case Processing Summary	
DD3	Valid No (Listwise)
Positive	18
Negative	43
Missing	11
Total	72

Figure 1. Receiver operating characteristic (ROC) analysis of the DD3 gene expression in urine: Area under the ROC curve (AUR): 0.981.

sensitivity (true positive) was plotted on the X and Y axes, respectively. ROC curve was used to determine a cutoff point to differentiate between cancerous patients from non-cancerous one (control subjects and BPH group).

RESULTS

The mean of participants' age was 66.17 ± 12.72 years (range, 47-87 years) in control group, 66.62 ± 6.39 years (range, 58-79 years) in BPH group and 64 ± 5.11 years (range, 56-75 years old) in PCa group. There were no significant differences between the mean age of the three groups (P = .542). Also, the three groups showed no significant differences in terms of body mass index (BMI) (P = .396).

Evaluation of 18s rRNA Gene Expression as an Internal Control

Average threshold cycle values (Ct) of 18s rRNA in the blood was 25.63 ± 0.93 in the control group, 25.37 ± 1.35 in BPH group and 25.55 ± 0.91 in the PCa group that showed no statistically significant differences (P = .713). Also, average threshold cycle values in the urine were 25.92 ± 0.53, 25.71 ± 1.46 and 25.91 ± 1.02 in the control, BPH and PCa groups, respectively (P = .798).

The participants were divided into two groups based on the mean age (< 66 and ≥ 66 years old) and mean BMI (< 23 and ≥ 23 kg/m^2). Expression of the internal control in blood and urine of these groups showed no statistically significant differences (**Table 1**). For more documentation, all participants were divided into 5 subgroups based on their age (41-50, 51-60, 61-70, 71-80 and more than 81 years old). Analysis of variance among these five groups in both blood and urine samples showed no statistically significant differences in gene expression rate.

PCA3 Gene Expression in the Urine and Blood

The ΔCt value of PCA3 marker was determined in urine and blood samples using the formula [Ct value of marker - Ct value of internal control]. The mean ΔCt in blood was 7.18 ± 1.02, 6.61 ± 2.09 and 3.77 ± 1.0 in control, BPH and PCa groups, respectively. There was no statistically significant difference between control and BPH groups (P = .273). However, in cancer group the difference was statistically significant compared to the control (P = .001) and BPH (P = .001) groups.

The mean ΔCt in control, BPH and PCa groups were 7.40 ± 0.76, 7.41 ± 2.51 and 3.20 ± 1.10, respectively. Again there was no statistically significant differences between control and BPH groups, but differences between control and BPH groups compared with PCa group was significant (P = .001). Then, PCA3 expression was compared among the five age subgroups (41-50, 51-60, 61-70, 71-80 and more than 81 years old). There was no statistically significant difference, however the 51-60 years old men

Table 1. Comparison of Ct value for 18s RNA in different age and body mass index (BMI) groups.

Sample		Risk Factors	No.	Mean	Standard Deviation	*P*
Urine	BMI	< 23	29	25.6476	1.17550	.134
		≥23	23	26.0870	0.81795	
	Age	< 66	30	25.6510	1.43299	.839
		≥66	27	25.5789	1.20965	
Blood	BMI	< 23	41	25.4437	1.10123	.528
		≥23	25	25.6192	1.07195	
	Age	< 66	34	25.5691	1.05127	.694
		≥66	33	25.4642	1.12007	

Table 2. Sensitivity and specificity of the DD3 gene expression in blood and urine.

Sample		Cancerous		Non-cancerous		*P*
		Number	Percent	Number	Percent	
Blood	Present	18	94.74	08	18.18	.001
	Absent	01	05.26	36	81.82	
Urine	Present	18	100.00	06	13.64	.001
	Absent	00	000.00	38	86.36	

had the highest expression rate. We did not observed any relationship between the PCA3 expression in urine and blood with weight.

PCA3 expression was also investigated in the PCa group based on Gleason Score. Patients with PCa was classified into two subcategories according to Gleason Score (< 7 and ≥ 7). The mean ΔCt of PCA3 in patients' blood with Gleason < 7 and ≥ 7, were 5.05 and 6.57, respectively, which did not show statistically significant differences (*P* = .14). Also, in the urine samples this difference was not significant.

Based on ΔCt value of PCA3 marker, the cut-off point in urine and blood samples were determined with CI of 95%. Based on these data, the sensitivity and specificity of the PCA3 marker were 94.74% and 81.82% in blood, and 100% and 86.36% in urine, respectively.

Comparing the rate of increasing PCA3 expression between the three groups revealed that the mean of urinary PCA3 expression in PCa group vs. control group was 20.90 fold and for the BPH group, was 20.35 fold while the expression of this marker in urine of BPH group was only 1.02 fold vs. control group.

Increasing rate of PCA3 expression in blood of PCa group was 10.64 and 7.17 fold compared to control and BPH groups, respectively. Furthermore, the expression level of this gene in BPH vs. control participants was only 1.48. The sensitivity and specificity of PCA3 marker in blood and urine is shown in **Table 2**.

According to these results, ROC curve was plotted and area under the curve (AUC) was calculated (**Figures 1 and 2**). ROC curve was used to determine a cutoff level. The cutoff values were 4.81 and 4.46 in blood and urine samples, respectively (arrows in **Figures 1 and 2**). However with greater cutoff values the sensitivity did not increase.

According to the achieved data from PCA3 primers-BLAST (basic local alignment search tool), they were exclusively attached to the various variants of PCA3 mRNA that are in Genbank. The PCR product produced by these primers had 430bp lengths. Also, the length of the PCR product for 18s rRNA was 151 bp. To confirm these results the generated products by reverse transcription PCR (RT-PCR) were subjected on agarose gel electrophoresis 1% (**Figure 3**).

DISCUSSION

PCa diagnosis is currently based on abnormal PSA test following biopsy. However, a number of factors affect srum PSA levels.[18,19] Therefore this test has so many false results.

PSA production is controlled by androgenic and some non-androgenic factors such as obesity and prostate disease. Factors such as age and prostatitis affect PSA production, too. Likely, obesity is associated with low levels of PSA.[18,19] On the other hand, obesity leads to increasing the size of the prostate.

Assuming 3 ng/mL as the cutoff value for PSA, the rate of negative biopsies will increase up to 70-80%.[7] According to Thomson and colleagues, a borderline could not be considered for PSA test in PCa diagnosis.[20] In second half of 1990s, PCA3 gene was detected in a collaborated study by the Johns Hopkins Hospital in Baltimore and Radboud University in the Netherlands,[21] which was called DD3.[22] At first, this gene was used in differential diagnosis between BPH and PCa, particularly in the prostate tissue, and then it was isolated from urine sediments and yielded acceptable results.[14-16]

Hessels and colleagues and van Gils and colleagues studied PCA3 gene in urinary sediments after DRE and concluded that the presence of PCA3 in urine sediment is beneficial as a diagnostic test for PCa.[22,23] These studies also revealed that uses of this test might raise the specificity of diagnosis of PCa. So it can prevent unnecessary prostate biopsies.[16,23]

Therefore, in addition to better detection, using this test

Table 3. Sensitivity and specificity of the PCA3 marker in some studies.

References	No.	PCa (%)	Sensitivity (%)	Specificity (%)	AUC
van Gils et al[23]	534	33.0	65	66	0.66
Marks et al[29]	226	26.5	58	72	0.68
Hessels et al[16]**	108	28.0	67	83	0.72
Tinzel et al[13]**	201	39	82	76	0.87
Present study*	72	33.3	94.74	81.82	0.93
Present study**	72	33.3	100	86.36	0.98

Abbreviations: PCA3, prostate cancer antigen 3; PCa, prostate cancer; AUC, area under the curve.
*Blood **Urine

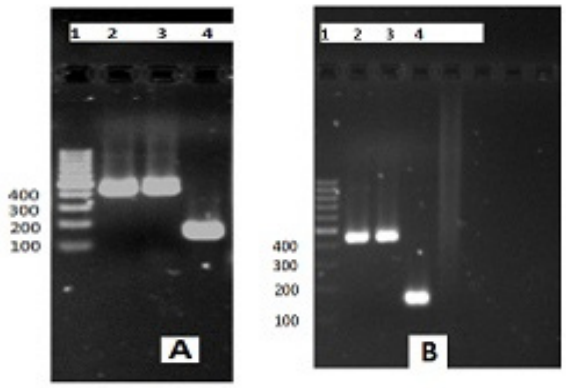


Figure 3. Agarose gel electrophoreses of reverse transcription polymerase chain reaction products: (A) lane 1, molecular weight standards; lanes 2 and 3, Prostate cancer antigen3 products of blood sample from two cancer patients; lane 4, 18s RNA product of blood; (B) lane 1, molecular weight standards; lanes 2 and 3, prostate cancer antigen3 products of urine sample from the same two cancer patients; lane 4, 18s RNA product in urine.

may prevent invasive diagnostic procedures such as prostate biopsy. Hara and colleagues concluded that prostate biopsy may lead to spread of prostate cells in the blood. So biopsy leads to tumor metastasis in PCa patients probably.[24]

Bussemakers and Hessels`s reports indicated that unlike PSA, PCA3 value is not affected by patients' age, the size of prostate and other prostate diseases.[14,22] Our results on age and PCA3 expression in blood and urine confirmed these previous findings. This result suggests that although age is a risk factor for PCa, it has no effect on PCA3 expression.

Previously, the correlation between gene expression and Gleason score has been studied. For instance, Mofid and colleagues investigated the association of HER-2 gene expression and the Gleason score but they did not find any correlation between them.[25] So we studied a novel marker. The PCA3 expression was evaluated in cancer patients based on Gleason score as an indicator of cancer development and progression. This comparison did not show any significant differences in blood and urine. These results confirmed the finding of Hessels and colleagues in which they did not observe a significant association between PCA3 expression (after DRE) and prognostic parameters such as Gleason score or tumor size.[26] Their results was also confirmed by van Gils and colleagues.[27] However, the results obtained by Vlaeminck-Guillem and colleagues don't agree with our results.[12] They observed meaningful correlation between PCA3 gene expression, Gleason score and tumor volume.[12]

Nakanishi and colleagues also observed a significant correlation between PCA3 and tumor volume.[28] These findings supported by Marks and colleagues who stated that, higher levels of PCA3 are seen in men with Gleason score more than seven.[29] Also, Whitman and colleagues found that the rate of PCA3 is associated with pathological finding such as tumor size.[30]

Over 30% of American adults are obese. Obesity is associated with a number of cancers such as breast and colon cancers,[31] but its association with PCa is not known. Although a number of studies have found that increasing BMI is related to more detection of PCa,[32] other researchers have not found any relationship.[33,34]

Interestingly, about one-third of PCa patients are obese. In most cases, when the disease is detected, it is in an advanced stage. Hence, obese men are the greatest victims of this disease.[35] In the present study no significant relationship was observed between the PCA3 expression in blood and urine with weight.

With the changes done in the laboratory method of this study, a significant increase in the sensitivity and specificity of the used test was provided. The sensitivity and specificity of the PCA3 marker obtained in our study (in blood 94.74, 81.82%, respectively, and in urine 100, 86.36% respectively) have been compared to the other reports (**Table 3**).

The limitations of this study were small sample size and lack of uniformity in stage of cancer.

CONCLUSION

Evaluation of PCA3 gene expression could be considered as a reliable marker for detection of PCa. Increased expression of this marker in urine sediments is more sensitive than blood for differentiating subjects with PCa and non-cancerous subjects.

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CONFLICT OF INTEREST

None declared.

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