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Original Article

Considering Blood Samples for Early Diagnosis of Prostate Cancer by Evaluating Prostate Cancer Antigen 3 Expression Values

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Abstract

Background: Prostate cancer antigen 3 (PCA3) is the most specific biomarker for an early diagnosis of prostate cancer (PCa), and it is routinely evaluated in urine samples. Recent investigations consider blood as a reliable source to determine PCA3 expression levels and fast diagnosis of PCa since in addition to bacterial and viral contaminations and the hygienic problems, if the area of the prostatic duct is not involved PCa, the obtained urine samples may not contain any cancerous cells. **Materials and Methods:** This study investigated blood expression values of the PCA3 gene in patients with PCa and multiple endocrine neoplasia compared with normal volunteers in an Iranian population. A total of 150 blood samples from three groups were assessed for PCA3 expression using real-time polymerase chain reaction. Prostate-specific antigen (PSA) serum levels, age, and family history of PCa were also analyzed. **Results:** PCA3 expression analysis showed a significant increase in PCa patients and in patients with PSA serum levels higher than 7 ng/ml ($P = 0.005$ and $P = 0.0011$, respectively). Analysis of blood samples from men with an older age and positive family history also showed significant PCA3 expression values. Results of this study suggests that evaluating PCA3 gene expression in blood samples is an adequate method for an early diagnosis of PCa. The decision to perform a prostate biopsy should be made more cautiously in patients with a PSA serum level between 4 and 7 ng/ml. Patients with a positive PCa family history and higher age should be considered for PCa diagnostic procedures. **Conclusion:** Blood samples can be considered for PCA3 evaluating as a promising alternative of urine samples for PCa diagnosis.

Keywords: Blood, multiple endocrine neoplasia, prostate cancer, prostate cancer antigen 3, prostate-specific antigen

Introduction

Prostate cancer (PCa) is a major public health problem worldwide,[1-3] and various studies have reported its

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epidemiology which varies by the geographical area, nutrition, race, and lifestyle.[1,4-6] Invasive overtreatment of PCa such as radical radiation and surgical therapies as a consequence of deficient diagnostic methods of PCa (e.g., prostate-specific antigen [PSA] testing) and the consequential urinary, sexual, and gastrointestinal side effects of such overtreatment[3,7,8] has led to a high rate of mortality reported since 1992 in the United State, and this has prompted the search for more specific biomarkers and early detection methods for PCa. In 2012, the Food and Drug Administration approved prostate cancer antigen 3 (PCA3) as the golden standard diagnostic biomarker[9-11] along with PSA screening. The PCA3 gene (PCA3-HGNC: 8637) is overexpressed in PCa, and its long noncoding RNA (lncRNA) is known to be the most specific biomarker for PCa.[12] Overexpression of the PCA3 gene is an early event, and it is not expressed in other types of malignant tissues.[11]

Recent investigations have increased interest in the diagnostic value of PCA3 expression in making an early diagnosis of PCa. These studies have shown that testing for PCA3 can significantly prevent a large number of unnecessary prostate biopsies.[13-17] Diagnostic methods based on measuring the PCA3 biomarker can also help prevent aggressive biopsies during follow-up treatment programs in both early stages patients who receive 5-alpha-reductase inhibitors (5ARIs) and in patients with high-grade tumors after surgery.[16]

Most of the studies on PCA3 have compared their results alone or in combination with other diagnostic methods,[15,18] such as the PSA test, and have shown that the accuracy of the PSA test can be significantly improved by the specificity of the PCA3 test.[3,11,17,19] PCA3 has shown promising diagnostic sensitivity and accuracy in comparison with both total PSA and free PSA tests.[3] The PCA3 test can determine PCa in early stages, and it has even been shown to be sensitive enough in patients with low-risk PCa who receive 5ARIs.[16] However, despite the established role of PCA3 in early PCa diagnosis, the exact value of PCA3 expression has yet to be determined.[19]

PCA3 is mostly known as a urine marker for PCa diagnosis. However, the PCA3 urine test is not an independent test, and it should be accompanied with PSA mRNA assay. Normal prostate cells express PCA3 in very small quantities, potentially leading to false-positive results of PCA3 overexpression in urine samples with a high number of normal prostate cells. Therefore, determining PSA mRNA is needed to normalize PCA3 expression results and also to validate that the urine samples contain a sufficient amount of PCA3 mRNA.[20]

It may be preferential to assess PCA3 gene expression in blood samples rather than urine due to impure extraction and bacterial contamination of urine samples which may lead to incorrect results,[21] and also in patients with PCa in whom cancer does not interfere with the area of the prostatic duct, the cancerous cells may not be washed by urine into the obtained samples.[22,23] Moreover, it is known that prostate massage before urine collection is necessary to maintain test sensitivity of PCA3 gene expression analysis by reverse transcription polymerase chain reaction (RT-PCR).[23] Consequently, whole blood would be better for PCa detection.

Because the rate of PCa is increasing in Iran[4] and in underdeveloped countries,[21] the early diagnosis of PCa is necessary to prevent subsequent costs. This article investigated the PCA3 gene expression in blood samples of Iranian PCa patients using molecular techniques for the early diagnosis of PCa and compared the accuracy of PCA3 results with PSA serum levels. Patients with multiple endocrine neoplasia (MEN) were also studied to determine the specificity of PCA3 gene expression in PCa, as the tumors associated with MEN are known to be nonmetastatic.[24] Two important factors of age and genetic background that are known to play an important role in PCa and its diagnosis process[25,26] were also considered in this study.

Materials and Methods

One hundred and fifty volunteers participated in this pilot study from hospitals affiliated to the Tehran Medical University from 2015 to 2017. All of the volunteers signed approval documents to attend this study under ethics committee of Islamic Azad University, Tehran, Iran (IR.IAU.EAST TEHRAN.REC. 1393.18) approved at 1393/08/09. The participants were divided into the three groups as healthy, MEN, and PCa groups. Blood samples were collected from men between 43 and 72 years of age who had no acute or chronic bacterial prostatitis, no previous prostate surgery or biopsy, no PCa, and PSA serum values between 3 and 20 ng/ml. Metastatic or nonmetastatic PCa (PCa and MEN study groups) was classified by sonography examinations and bone scans, or pathological evidence. Each group included fifty patients, and the healthy group included fifty people with no evidence of malignancy. Universal ethylenediaminetetraaceticacid-containing blood sampling tubes were used for collecting 2.5 ml of whole blood from each volunteer, and the samples were immediately used for total RNA extraction.

Total RNA extraction and cDNA synthesis

Total RNA was extracted using a GeneJET RNA Purification Kit (Thermo Scientific, USA) according to the manufacturer's instructions. The quality of RNA was evaluated with spectrophotometry (NanoDrop 2000c) and gel electrophoresis. Total RNA extracted from the blood specimens was then reverse transcribed with Superscript™ III RT (Thermo Fisher Scientific, USA), and the obtained cDNA which used for a PCR reaction was diluted to 10 ng/µl.

Gene selection and design of the primers

To investigate the PCA3 gene expression, specific primers were designed using AlleleID software (Premier Biosoft Intl., Palo Alto, CA, USA) and Primer Express software (Applied Biosystems, Foster City, CA, USA) based on the conserved regions of genes. The primers were designed as exon–exon splice junction to prevent coamplification

of genomic DNA. Primer sequences for the PCA3 gene were F; 5′-CCTGAATCGTTGCTTGTGTT-3′ and R; 5′-TACAATTGATCCTGCACACG-3′ with an 85 bp amplicon size. The Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as an internal control, and its primer sequences were as follows: F; 5′-ATGGAGAAGGCTGGGGCT-3′ and GAPDH R; 5′-ATCTTGAGGCTGTTGTCATACTTCTC-3′ with an amplicon size of 124 bp. The primers were purchased from Thermo Fisher Scientific Co.

Relative real-time polymerase chain reaction

Real-time RT-PCR was performed using a real-time PCR 7500 system (Applied Biosystems, USA). A total volume of 20 µl of the reaction mixture containing 100 ng of cDNA (10 ng/µl), 12.5 µl of SYBR Green PCR Master Mix (Applied Biosystems, USA), 0.5 µl of each forward and reverse primer (10 mmol/µl), and 5.5 ml nuclease-free water were used; initial denaturation at 95°C for 10 min, followed by forty cycles at 95°C for 10 s, and annealing/extension for 30 s at 59°C. PCA3 and GAPDH were examined simultaneously. To minimize experimental variation of the Ct values, the threshold cycle in which the fluorescence signal was substantially exacerbated above the background stage and by the second derivative maximum method was determined. Melting curve analysis was used to detect any nonsequence-specific amplified products that could generate a false-positive signal. Negative controls were included in each run. The PCR for each sample was performed in triplicate.

Statistical analysis

Relative gene expression levels were determined using the ΔΔct method using the ABI system. Statistical differences in PCA3 gene expressions and clinical pathologic data among the study groups were assessed with GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA, USA). Two-way ANOVA and Tukey posttest were used to analyze the parametric data. For data which did not follow Gaussian distribution, the Kruskal–Wallis test and Dunn's multiple comparison posttest were used for comparisons of two groups of data. The nonparametric Kolmogorov–Smirnov, D'Agostino and Pearson tests, and Mann–Whitney posttest were used for comparisons of more than two groups of nonparametric data. $P < 0.05$ was considered to be statistically significant.

Results

Table 1 shows the results of statistical analysis of the data in four parts as described below. Figure 1 also shows four graphs that are related to parts of Table 1. The methods of statistical analysis of the original data for each part are different as described in the Materials and Methods section.

Part (A) of Table 1 shows the age data in each group. The mean ± standard deviation age of the PCa patients (65.10 ± 4.501) was higher than that of the two other groups, and there was a significant difference ($P < 0.0001$) between the mean age of the PCa patients with MEN and the normal group. In addition, the mean age of the MEN group (54.44 ± 4.833) was higher than that of the normal (52.76 ± 5.995) group, but the difference was not significant ($P > 0.05$) [Figure 1a].

The statistical analysis of PCA3 gene expression considering the mean relative quantification (RQ) of PCA3 gene expression in the three groups is shown in Part (B) of Table 1. The PCA3 expression in the PCa group was significantly higher ($P = 0.005$) than the normal and MEN groups, whereas

Table 1: Data statistics; (A) age, (B) prostate cancer antigen 3 expression, (C) prostate cancer antigen 3 expression in patients with various prostate-specific antigen levels, (D) family history and its related prostate cancer antigen 3 expressions

	PCa	MEN	Normal	Total	PCA3 mean RQ	Mean different
A. Age						
Number of persons	50	50	50	150		
Minimum	52	43	44	43		
Maximum	72	61	67	72		
B. PCA3 expression						
PCA3 mean RQ	2.860	2.252	1.099			
Max PCA3 RQ	5.265	4.118	1.865			
Min PCA3 RQ	0.9651	0.9727	0.7352			
C. PSA levels ng/ml						
0-2	0	0	50	50	1.0990	-
3-6	6	21	-	27	2.4857	−1.387
7-10	18	14	-	32	2.8354	−1.736
11-20	26	15	-	41	2.8865	−1.787
D. Family history						
Yes	32	11	8	51	3.2689	
No	13	28	23	64	1.8434	
Unknown	5	11	19	35	-	

Each part is analyzed by different method which described in details in materials and methods. PCA3: Prostate cancer antigen 3, PCa: Prostate cancer, MEN: Multiple endocrine neoplasia, RQ: Relative quantification, PSA: Prostate-specific antigen

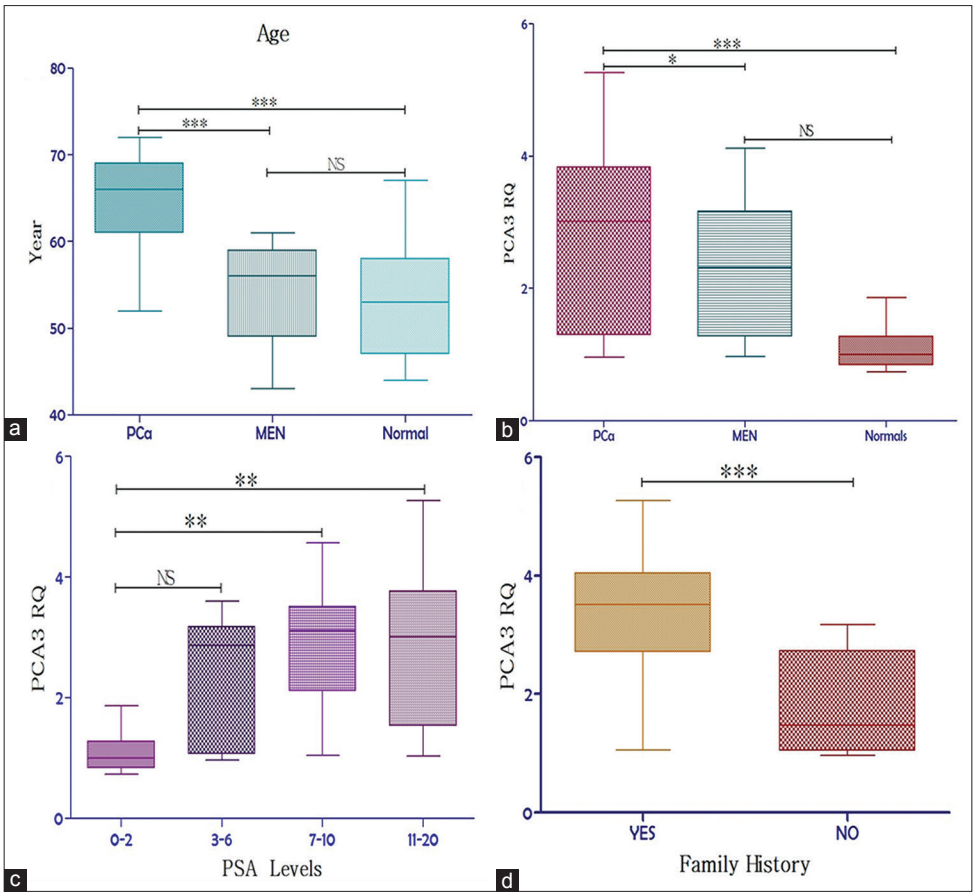

Figure 1: Graphs of statistical analysis: (a) Mean age of three groups of study. (b) The mean of relative quantification of prostate cancer antigen 3 gene expression. (c) Prostate cancer antigen 3 relative quantification mean in volunteers with various prostate-specific antigen levels. (d) Prostate cancer antigen 3 relative quantification mean in patients with positive/negative prostate cancer family history. NS: non significant, *: $P<0.01$, **: $P<0.001$ and ***: $P<0.0001$

the PCA3 mean expression in the MEN group did not differ statistically ($P > 0.05$) from the normal group [Figure 1b].

The number of participants with various PSA serum levels and also the mean RQ of PCA3 expression for each level of serum PSA are shown in Part (C) of Table 1. There was a significant increase ($P = 0.0011$) in PCA3 gene expression in the participants with a PSA serum level of more than 7 ng/ml than in those with a PSA level of 0–2 ng/ml [Figure 1c].

We also compared the PCA3 gene expression in the participants with positive and negative family histories of PCa [Table 1 Part [D]]. The results showed a significantly higher expression of the PCA3 gene in the group with a positive family history ($P < 0.0002$) [Figure 1d].

Discussion

It has been reported that an increased serum level of PSA is not an independent variable for PCa diagnosis.[18] Several studies have also indicated that an elevation in PSA serum level in patients with prostatic hyperplasia, prostatitis, prostate irritation or urethral manipulations, and even recent ejaculation can interfere with the diagnostic accuracy of PCa. It has also been reported that nearly 15% of patients with PCa (detected by biopsy) had a PSA level below 4 ng/ml. Moreover after more than 25 years of PSA testing in PCa patients, the PSA cutoff value for a prostate biopsy has yet to be independently determined, and the decision to perform a biopsy is based on factors including the patient's age and prostate size. The PCA3 test has been demonstrated to effectively reduce PSA false results with a considerable percentage of 85% lives saved, including 50% of false positives and 25% of overdiagnoses, especially in persons with a PSA serum level between 4 and 10 ng/ml.[11,15,19,21-23] Our results revealed that the PCA3 expression was statistically higher ($P = 0.0011$) in the patients with a PSA serum level of more than 7 ng/ml. However, the mean expression of PCA3 in the patients with a PSA level 3–6 ng/ml was also higher than those with a PSA level of 0–2 ng/ml, although there was no statistically significant difference ($P > 0.05$) between these two groups. Along with other investigations, our results suggest that PSA serum level alone, especially in the range of 4–7 ng/ml, cannot be a reliable criterion for physicians to decide about whether to perform a prostate biopsy, unless the PCA3 expression is significantly increased in a blood sample.

PCA3 is only expressed in the prostate and is only slightly expressed in normal prostate tissue. It is not expressed in other normal tissues or blood, and it is not altered by prostate inflammation, enlargement, or manipulation. Thus, in persons with normal prostate, no false-positive result is found in blood or urine samples. Furthermore, in patients with PCa, the PCA3 mRNA expression has a remarkable increase of up to 34–66 fold in prostate tissue, which allows for the accurate detection of PCa from blood and urine samples.[21,22,27] Despite the high specificity and sensitivity of the PCA3 test for PCa, the cutoff value for PCA3 in urine samples is still under discussion.[23,27] It has been demonstrated that blood samples are more valid than urine for performing the new molecular-based PCa diagnostic methods such as miRNA evaluation,[28,29] and that blood samples are more hygienic and appropriate than urine for RNA analysis methods such as RT-PCR. In addition, the PCA3 values in peripheral blood are sufficiently increased to be promisingly sensitive for an early diagnosis of PCa.[21] Our statistical analysis of RT-PCR data showed a significant increase ($P = 0.005$) in PCA3 gene expression in the PCa patients compared with the MEN and normal groups, whereas there was no significant difference ($P > 0.05$) between the MEN and normal groups. This suggests that whole blood samples and RT-PCR techniques may be sufficient to evaluate the PCA3 expression in PCa patients. As our PCa volunteers did not undergo a prostate biopsy before entering this study, they were in the early stages of PCa. Our results indicated that the early stages of PCa can be immediately detected using blood samples rather than urine because, in the early stages of PCa, the related malignant cells may not have shed into the urine samples.

A large retrospective population-based study on a family history of PCa including 635, 443 men showed that a history of PCa even in third-degree maternal or paternal relatives significantly contributed to the risk of PCa.[25] Various population-based studies have emphasized the importance of a family history in the risk of PCa and related diagnosis programs.[30-32] Our statistical analysis of PCA3 expression rates showed that the men with a positive PCa family history had a significantly ($P < 0.0002$) higher expression of PCA3 gene than those with a negative family history of PCa. Therefore, the patients' family records may be a good decision-making indicator when other data are not sufficiently reliable.

As mentioned, age is considered to be a very important factor in the diagnosis of PCa.[15,19] Statistical analysis of age data in this study also showed a significantly higher ($P < 0.0001$) age in the PCa patients compared with the MEN and normal groups.

This study validates the RT-PCR technique for evaluating PCA3 gene expression to allow for the early diagnosis of PCa from whole blood samples. Therefore, the mRNA extraction of whole blood specimens can be an alternative to urine samples, especially in men with problems which affect the hygiene and compound of the urine samples. Although the RT-PCR technique is quite a specific and sensitive method for detecting gene expressions, in patients with a PSA serum level in the gray zone (4–7 ng/ml), more extended studies are needed to clinically define the cutoff value of PCA3 in blood samples. Our study also shows the importance and efficiency of considering other informative factors such as family history and patient's age for PCa diagnosis and performing a prostate biopsy.

Conclusion

Evaluating PCA3 gene expression in whole blood samples showed promising results to considering blood samples for accurate PCa diagnosis specially in cases of urinary tract infection or obstruction, or during early screening for PCa detection in which the cancerous cells may not have shed into the urine sample. The patient's family history and age are important factors in diagnosing PCa and preventing unnecessary biopsies.

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Conflicts of interest

There are no conflicts of interest.

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