Section A-Research paper

Prostatic Cancer Antigen 3 Role in Development of Cancer Prostate

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Abstract

Background: prostatic cancer antigen 3 was firstly identified the DD3 (later called PCA3) gene, functioning as non-coding RNA.Using a reverse transcriptase polymerase chain reaction (RT-PCR) method, they detected that PCA3 was overexpressed in cancerous tissue and low expressed in benign prostatic tissue and not measurable in the normal tissue of numerous organs such as the testis, bladder, kidney, seminal vesicles, brain and lung. PCA3 is highly prostate specific and was overexpressed in 95 % of tumor lesions, but in only 1 of 7 human PCa cell lines (lymph node carcinoma of the prostate) and in none of 18 non-malignant prostate samples**.** A multitude of studies further implicated significantly higher PCA3-mRNA expression in prostatic tumors in comparison to non-malignant prostatic tissue. These findings promoted the idea of developing a PCA3 diagnostic test. It was reported that transient knockdown of PCA3 transcripts reduced cell growth and viability, in addition to inducing apoptotic cell death .These data reinforced the hypothesis that PCA3 could modulate PCa cell survival. It was also reported an association between PCA3 and the androgen-receptor (AR) signaling pathway,and it was found that cells treated with AR agonist dihydrotestosterone (DHT) induced significant upregulation of PCA3 expression, which was reversed by AR antagonist flutamide. In addition, it was also observed upregulation of androgen-responsive genes (ARGs) (TMPRSS2, NDRG1, GREB1, PSA, AR, FGF8, CdK1, CdK2, and PMEPA1) in response to DHT treatment. Interestingly, these findings were reversed when silencing PCA3 using RNA interference

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Introduction

By comparing PCa tissue with non-malignant prostatic tissue, firstly identified the DD3 (later called PCA3) gene, functioning as non-coding RNA.Using a reverse transcriptase polymerase chain reaction (RT-PCR) method, they detected that PCA3 was overexpressed in cancerous tissue and low expressed in benign prostatic tissue and not measurable in the normal tissue of numerous organs such as the testis, bladder, kidney, seminal vesicles, brain and lung. PCA3 is highly prostate specific and was overexpressed in 95 % of tumor lesions, but in only 1 of 7 human PCa cell lines (lymph node carcinoma of the prostate) and in none of 18 non-malignant prostate samples**.** A multitude of studies further implicated significantly higher PCA3-mRNA expression in prostatic tumors in comparison to non-malignant prostatic tissue. These findings promoted the idea of developing a PCA3 diagnostic test and **(1).**

PCA3 gene structure

The first description of PCA3 gene unit reported its location on human chromosome 9q21–22 and its 25 kb length containing four exons**.** The primary PCA3 transcript can be submitted to alternative splicing, alternative polyadenylation and produces different sized transcripts. The classical isoform contains exons 1, 3, 4a and 4b. Moreover, the high frequency of stop codons detected in all PCA3 reading frames further evidenced it as a non-coding RNA and no protein or peptide was found to be coded by PCA3 transcripts. The nuclear localization of PCA3 polyadenylated transcripts was demonstrated **(2).**

However, later reports also showed PCA3 detection into the cytoplasm**.** A further detailed description of the PCA3 gene structure was performed, presenting a more complex transcriptional unit, including novel additional exons. Exon 1 was found to be 1150 bp longer, with 5 possible transcription start sites. Three variants were also described in exon 2 (2a, 2b and 2c) and four additional polyadenylation sites in exon 4 were observed, bringing the total number of polyadenylation sites to **(3).**

Additional PCA3 isoforms have also been reported, named as PCA3 isoforms 1-4, with transcription start sites respectively located at 1150 bp, 699 bp, 640 bp and 136 bp upstream from the original PCA3 start site **(3).**PCA3-4 corresponds to only 1% of total PCA3 transcripts, whereas the PCA3-5 is the major transcript found in PCa tissue samples **(4).**

Further investigations on the organization and evolution of the PCA3 gene locus demonstrated that PCA3 is an intronic antisense transcript, mapped in the opposite orientation of the Prune homolog 2 coding (PRUNE2) genewithin its intron 6 **(3).**

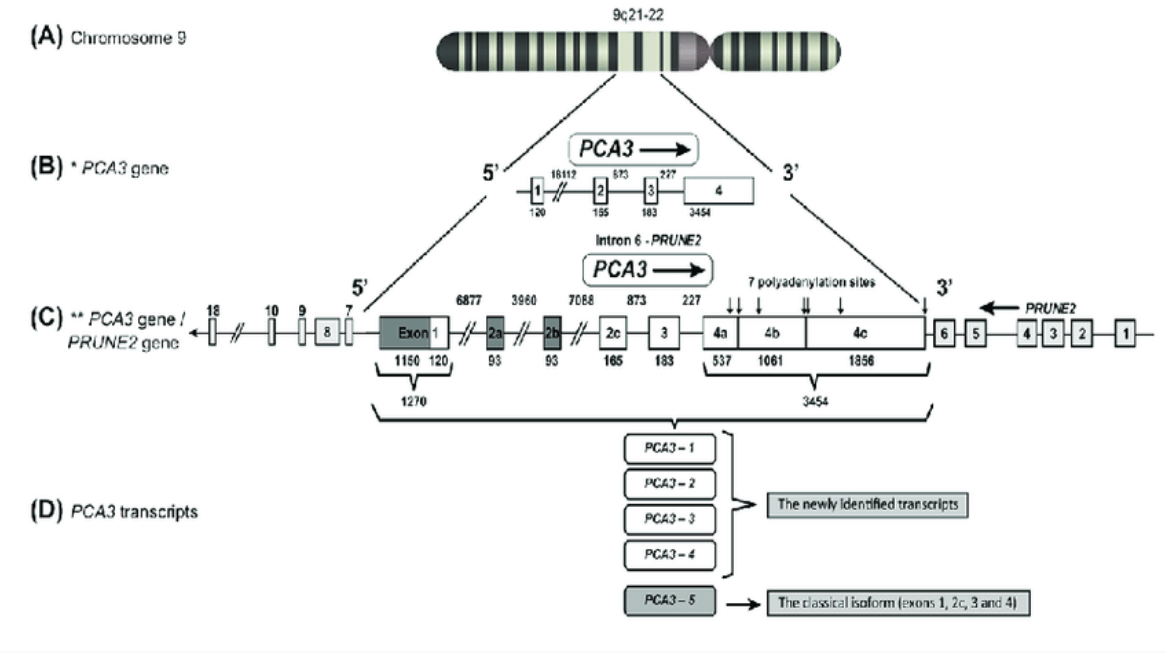

Figure 1: PCA3 gene localization, structure and transcripts (4).

PCA3 origin

It was suggested that PCA3 originated from an ancient virus sequence that was incorporated into the human genome and therefore could be regulated by virus-specific patterns**.** According to this report, the presence of additional features in the PCA3 gene could corroborate this hypothesis. First, PCA3 initial gene portion is included in a long interspersed nuclear element type 2 (LINE-2) repeat, a retro transposon element derived from an ancient virus **(5).**

PCA3 localization in the opposite strand of PRUNE2 is also similar to the case of Epstein–Barr virus bidirectional transcription, with the opposite direction mainly associated with the transcription of noncoding and regulatory genes **.** Moreover, PCA3 adenosine deaminases acting on RNA (ADAR) mediated editing is also a post-transcriptional mechanism largely employed in the cellular responses to viruses **(6).**

PCA3 role in developmet of cancer prostate

It was reported that transient knockdown of PCA3 transcripts reduced cell growth and viability, in addition to inducing apoptotic cell death .These data reinforced the hypothesis that PCA3 could modulate PCa cell survival. It was also reported an association between PCA3 and the androgen-receptor (AR) signaling pathway,and it was found that cells treated with AR agonist dihydrotestosterone (DHT) induced significant upregulation of PCA3 expression, which was reversed by AR antagonist flutamide. In addition, it was also observed upregulation of androgen-responsive genes (ARGs) (TMPRSS2, NDRG1, GREB1, PSA, AR, FGF8, CdK1, CdK2, and PMEPA1) in response to DHT treatment**.** Interestingly, these findings were reversed when silencing PCA3 using RNA interference **(7).**

Clarke *et al*. (3) showed that PRUNE2 levels were induced by AR stimulation**,** whereas, **Salameh *et al*. (8)** observed that androgen stimulation decreased PRUNE2 levels, besides inducing a concomitant increase in PCA3 expression. Thus, PRUNE2 PCA3 regulation appears to be sensitive to AR activation.

It has been demonstrated that PCA3 and PRUNE2 display opposite roles in both in vitro and in vivo models of PCa and it was described that PCA3 silencing or PRUNE2 ectopic overexpression decreased cell proliferation and transformation in vitro. In addition, PCa cells overexpressing PRUNE2 presented decreased cell adhesion, spreading and migration, while PCa cells in which PRUNE2 was stably silenced presented larger tumor xenografts. These data showed the tumor suppressor activity of PRUNE2 **(8).**

Lemos *et al*. (9) presented further comprehensive mechanisms for LNCaP cell line survival rates modulated by PCA3. It was found that LNCaP cells in which PCA3 was knocked-down induced the expression from 16 out of 84 tested tumor markers, including those involved in transcription control, cell signaling, angiogenesis, apoptosis, cell senescence, invasion, metastasis, cell adhesion and DNA damage repair .These data further proposed that the upregulation of AR cofactor transcripts could be one of the possible mechanisms by which ARGs are negatively modulated in response to PCA3 silencing . Further evidencing the key role of PCA3 on PCa cell survival, it was reported that PCA3 silencing sensitized PCa cells to enzalutamide-induced loss of cell growth, reinforcing the link between PCA3 and modulation of AR signaling **(10).**

Clinical Applicability of Prostate Cancer Antigen 3

1. **Early Detection of Prostate Cancer: Initial and Repeat Prostate Biopsy:**

A main limitation of early PCa detection due to elevated PSA levels remains the high proportion of men detected with non-malignant findings at first or subsequent prostate biopsy (BX). One of the most important clinical rationales of PCA3 application therefore is the reduction of potentially unnecessary BXs **(11).**

Marks *et al.* (12) evaluated the diagnostic ability of PCA3 in 226 men subjected to repeat BX. They demonstrated PCA3's superiority over PSA in predicting positive BX outcome.Using 35 as PCA3 score cut-off (The PCA3 Score is calculated as the ratio of PCA3 RNA copies to PSA RNA copies, multiplied by 1000). But, compared to earlier studies **de Kok *et al*.(1)**,median PCA3 scores in aggressive PCa (Gleason score (GS) < 7 vs. GS > 7) were not significantly different**.** In contrast, **De la Taille *et al*. (13)** have shown in the initial BX setting that the PCA3 score was significantly higher in men with GS 7 or greater vs. GS less than 7.

Consequently, prospective U.S. and European multicentre trials were conducted in patients undergoing initial or repeat BX **(14).** As a result comparable diagnostic accuracies of U.S. and European men at first and repeat BX were reported.Despite some conflicting results, both studies demonstrated that a combination of PCA3 with established BX risk factors such as age, PSA ,DRE, prostate volume and %fPSA improved the predictive accuracy in multivariable regression models. **Ploussard *et al*. (15)** performed a subgroup analyses of the European multicenter study and confirmed the superiority of PCA3 over %fPSA in univariable analysis as a predictor of repeat BX outcome**.**

The incorporation of PCA3 in the Prostate Cancer Prevention Trial risk calculator (PCPT-RC) improved the diagnostic accuracy compared with the established BX risk factors**.** In a large mixed BX patient cohort from Europe and Northern America (n = 809), **Chun *et al*. (16)**following Kattan criteria **(17),** demonstrated that PCA3 independently predicted PCa, and its addition to established risk factors (age, PSA

, DRE, prostate volume, BX history) significantly improved predictive AUC of the base model between 2 % and 5 %.

Perdona *et al*. (18) compared the updated PCPT-RC, including PCA3 and Chun's PCA3 based nomogram. A significantly better discriminative power (AUC: 0.80 vs. 0.72, p = 0.04) and superior calibration was demonstrated. Decision curve analysis revealed a higher net benefit for Chun's nomogram, resulting in up to 21 % of avoided unnecessary repeat BXs.

Regarding health care expenses in different European countries, Urinary PCA3 measurement is more expensive than PSA measurement. Up-to-date costs for urinary PCA3 testing may be up to 15-fold higher. But, due to PCA3's use to avoid up to 67 % of repeat BXs compared with PSA**.** The avoided BX expenses and further follow-up diagnostic interventions should be considered. Moreover, BX-related anxiety, discomfort and complications may be spared **(19).**

Predictive accuracy estimates of biopsy outcome predictions were quantified using the AUC of the receiver operator characteristic analysis in models with and without PCA3. PCA3-based nomogram has been presented by **Chun *et al*.,(16)** that identifies men at risk of harboring PCa and it assists in deciding whether further evaluation is necessary. When PCA3 is combined with serum PSA and biopsy GS, it increased diagnostic accuracy to 90% to predict extracapsular extension or clinically low volume tumor**.** Patients with a biopsy GS of 6, low PSA and a low PCA3 could be excellent candidates for active surveillance. The incorporation of PCA3 data into the Prostate Cancer Prevention Trial (PCPT) risk calculator showed additional benefit**(20).**

PCA3, PSA and DRE data from 521 men undergoing prostatic biopsy were included in the original PCPT risk calculator. The AUC for the updated PCPT calculator was 0.7, which was statistically superior to the AUC (0.61) of original PCPT calculator without the PCA3. Further more, the sensitivity, positive and negative predictive value were improved with the incorporation of the PCA3. Lastly, patients with precursor lesions of PCa (prostatic intraepithelial neoplasia,atypical small acinar proliferation) had a significant higher mean PCA3 score, in comparison with men with negative biopsy results**.** Nearly one third of patients with a negative biopsy and a PCA3 score > 35 had high-grade prostatic intraepithelial neoplasia **(21).**

2. Screening and Active Surveillance(AS):

PCA3 was assessed as a first-line screening test within the European Randomised Study of Screening for Prostate Cancer (ERSPC) trial. A PCA3 score ≥10 demonstrated a positive predictive value of 17.1 compared with 18.8 for a PSA value ≥3.0 ng/ml. Interestingly, PCA3 versus PSA missed substantially fewer cancers (32 % vs. 65 %) and serious cancers (26 % vs. 58 %).Because this unique study evaluated a PSA-prescreened cohort (third round or more; 33 % had a negative first BX), a consecutive study in unscreened patients, avoiding attribution bias,should be conducted to further assess PCA3 as a potential screening marker **(22).**

Tosoian *et al*. (23) assessed PCA3's ability to rule out clinically significant PCa in men undergoing AS according to the criteria for clinically significant PCa defined by **Epstein *et al*. (24).** A trend towards higher median PCA3 scores in patients with GS upgrading at follow-up BX was recorded. However, at adjusted multivariable Cox regression analysis, PCA3 did not represent an independent risk factor of BX progression (p = 0.15) **(23).** Considering the limitations that the number of events was small (n = 38) and that PCA3 was assessed only once at the time of first diagnosis but not repeated during the follow up biopsies, so far no evidence for the usefulness of PCA3 in AS programs has been presented. Since PCA3 does not appear to represent a useful marker to monitor PCa aggressiveness at biopsies , its role in risk assessment during AS needs to be tested in larger studies with repeated PCA3 score measures **(12).**

3. Prediction of Pathological Tumour Volume, Stage and Grade:

Due to the fact that PCA3 is highly overexpressed in PCa tissue and improves the prediction of BX outcome, several studies have focused on its potential ability to predict pathological PCa stage and aggressiveness, **Bostwick *et al*. (25)** at first reported on 24 patients undergoing radical prostatectomy (RP) for PCa.The assessed RP specimens demonstrated no difference in cancer volume, location,stage, and GS compared with RP specimens of men diagnosed with PCa based on PSA or suspicious DRE findings.

It was confirmed That PCA3's correlation to TV and identified it as an independent predictor ($p < 0.01$) of extracapsular extension (ECE) resulting in a multivariable AUC of 0.90 when combined with PSA and BX GS. It was demonstrated that neither a significant correlation of PCA3 to pathologic grading nor to TV and pathologic stage in a cohort combining 132 patients**.(26)**

The largest (n = 305) published series on urinary PCA3 's correlation to clinicopathologic features demonstrated that the multivariable AUC of low-volume disease and insignificant PCa models improved when PCA3 was added to standard clinical risk factors.On the other side, there was no significant correlation between PCA3 and adverse features such as ECE and seminal vesicle invasion and its significance on aggressive PCa was reported to be limited **(27).** Similar results were reported on 106 consecutive men undergoing RP due to clinically low-risk disease (PSA < 10 ng/ml, T1c–T2a, and biopsy GS < 7). Low urinary PCA3 scores and favourable BX criteria independently predicted small TV (<0.5 ml) and insignificant PCa.Again, the urinary PCA3 score, combined with established risk factors in multivariable logistic regression models, was not significantly associated with high-grade and locally advanced disease **(15).**

Higher PCA3 scores are supposed to be associated with more aggressive cancer, which is based on the hypothesis that with increasing differentiation, PCa cells become more invasive and could therefore more easily be shed into the ductal system of the prostatic gland after DRE or that larger tumours simply have more surface area left to shed PCA3 **(15).**

Most studies failed to confirm this hypothesis **(15,26).** But, following GS system ,some authors suggest that tumours with pattern 4 and 5 increasingly lose their glandular differentiation and lumina, disabling cells to be shed into urine after DRE in correlation with their TV. Therefore, potentially higher PCA3 mRNA tissue levels, resulting from larger tumour masses might not be adequately measured by the urinary test **(27).**

4. Prostate Cancer Antigen 3 Score Alterations Over Time and Consequence for Bioptic or Medical Intervention:

Within the placebo arm of the Reduction by Dutasteride of Prostate Cancer Events (REDUCE) trial, urinary PCA3 , PSA and %fPSA were available at the year 2 and year 4 follow-up BX in 1072 men. On univariable analyses for the prediction of year 4 BX outcome based on year 2 biomarker values, PCA3 score was exclusively found as a significant predictor for a positive follow-up BX at year 4. Interestingly, PCA3 scores in BX-positive men only slightly increased (+15.7 %) within the study period **(28).**

Urinary PCA3 scores before and 2 h after BX, showed no significant difference of measured PCA3 scores, neither in all men (18 %, $p > 0.05$) nor in PCa-positive men (1.5 %, $p > 0.05$) **(28).**

Sokoll *et al*. (29) suggested a certain robustness of PCA3 towards interventional effects on the prostatic tissue. In this context, the influence of Dutasteride (5a-reductase inhibitor [5-ARI]) on prostatic markers was assessed by **van Gils *et al*. (30) ,** in 16 men with BPH and 9 men with clinically localised PCa (all treated with 5-ARI), PSA, testosterone, dihydrotestosterone (DHT), and urinary PCA3 were measured at baseline and after1, 2 and 3 months. As expected, Dutasteride reduced DHT (>90 %), halved PSA levels,decreased prostate volume (10–16 %), and increased testosterone (20–30 %). In contrast, 5-ARI treatment had a widely variable effect on PCA3 scores, which increased (75–284 %) and decreased (14–77 %) over time, irrespective of whether patients with or without PCa were observed . This needs to be taken into account when counselling patients on Dutasteride who are designated for a PCA3 test **(30).**

5. Combination of Prostate Cancer Antigen 3 with New Biomarkers:

Since PCA3 is highly PCa specific and a clinically useful marker to predict BX outcome, its combined use with other new tumour markers may further improve its diagnostic accuracy.Therefore, transcripts of a fusion between the transmembrane-serine protease gene (TMPRSS2) and the vets erythroblastosis virus E26 oncogene (ERG) were evaluated in combination with PCA3 in the post-DRE urine of 108 patient undergoing BX. In this study TMPRSS2-ERG fusion transcripts were only found in 59 % of the primary PCa tissue specimens and the included patients did not represent a typical BX because PCa detection rate was quite high with 72 % due to PSA levels ranging from 1.1 to 1.619 ng/ml. Urine sediments of men diagnosed with PCa were positive for TMPRSS2-ERG fusion transcripts and PCA3 (cutoff: 48) in 37 % and 62 %, respectively.

Combining both markers improved the sensitivity to 73 %, yet a considerable decreased specificity of 63 %, compared with 93 % of TMPRSS2-ERG fusion alone **(31).**

Laxman *et al*. (32) further evaluated Golgi membrane protein 1 (GOLM1), serine peptidase inhibitor Kazal type 1 (SPINK1), PCA3 and TMPRSS2-ERG fusion in sedimented urine of men before BX (n = 216) or RP (n = 60). A multi variable regression model for the detection of PCa including these four biomarkers improved the diagnostic AUC from 0.66 (for PCA3 alone) to 0.76, respectively.

Rigau *et al*. (33) using PCA3 together with prostate specific demonstrated comparable findings G-protein coupled receptor in urine sediments after prostatic massage from 215 patients presented for BX. An increased specificity of 44 % at an assumed sensitivity of 90 % was reported for the combined test compared with each biomarker used as a stand alone test.

6. Detection of Prostate Cancer Antigen 3 in Circulating Tumour Cells:

In PCa patients, the presence of circulating tumour cells (CTCs) appears to be correlated with a poor prognosis**.** For this reason detection of specific biomarkers found in prostatic CTCs could potentially indicate an advanced and aggressive stage of disease. In 2008, It was described a quantitative RT-PCR assay for the detection of PCA3 mRNA in peripheral blood and evaluated 67 patients with locally advanced (n = 23) and metastatic disease (n = 9), respectively. Interestingly, only two patients were found positive for PCA3 mRNA in peripheral blood samples.In contrast, **Marangoni *et al*. (34)** detected PCA3 mRNA expression in 25 (62.5 %) of 40 patients with PCa compared with 15 (37.5 %) of 40 BPH patients by evaluating preoperative peripheral blood samples.

Patients presenting with progressive castrate-resistant PCa demonstrated significantly overexpressed levels of PCA3 in CTCs from peripheral blood**.** Similar findings have been reported by **Jost *et al*. (35)** using an immuno-magnetic CTC enrichment method to assess peripheral blood from 67 PCa patients. Although none of the androgen dependent patients has been tested positive for PCA3, 5 (31 %) of 16 androgen-independent patients were found positive for CTC-PCA3.

7. Prostate Cancer Antigen 3 as a Novel Gene Therapy Target:

Van *et al*. (30) have demonstrated the high PCa specificity of PCA3 and highlighted its potential use as a precursor to suicide gene therapy by using a specific diphtheria toxin model**.** A combination of PCA3's promoter region driving the expression of a suicide gene could be used to process novel PCa therapies. In theory,this combined therapeutic construct would bind, interact and finally induce cell death in PCa tissue, and non-malignant and non-prostatic cells would not be affected by this highly specific therapeutic cascade. Based on this concept, **Fan *et al*. (36)** developed an oncolytic adenovirus in which replication is driven by the PCA3 promoter, carrying the therapeutic gene interleukin (IL)-24. Its in vitro and in vivo effects have been investigated in DU-145 cell lines(cell originates from the prostatic carcinoma) and in DU-145 xenograft tumours in nude mice. In five of six treated mice, tumours have been completely eliminated within 50 days. Most remarkably, all mice have survived until the end of observation.

Assays of PCA3

There are several assays measuring PCA3 mRNA, which is highly upregulated in neoplastic prostate tissue **(31).**The assays measure PCA3 mRNA out of prostate cells shed into urine after (DRE). **Hessels *et al*. (31)** were the first to report of PCA3 mRNA measurement in sedimented urine. PSA mRNA was used to normalise for the amount of prostate specific RNA in the molecular test sample. Although PSA expression is constant in normal cells and 1.5 fold lower in PCa cells, the ratio between PCA3 mRNA over PSA mRNA multiplied by 1000, was presented as a new diagnostic tool the "PCA3 score".

In 2006, the prototype of a new quantitative, validated PCA3 -based urine test using post-DRE whole-urine specimens further processed in a single-tube format, was presented by **Groskopf *et al*. (37).** Urine samples were stored at either 4 °C or 30 °C. The PCA3-to- PSA ratio at 4 °C remained within a 20 % range of the initial values after 2 weeks, but at 30 °C a significant degradation of PCA3 reflected its instability at room temperature. Comparing 52 healthy, 52 BX-negative and 16 BX-positive men, again, median PCA3 mRNA to PSA mRNA ratio values showed significant differences (4.5 vs. 27.0 vs. 81.8; $p < 0.01$).

A systematic review with meta-analysis was performed by **Zhiqiang *et al*. (38)** for Diagnostic accuracy of urinary PCA3 level in patients with prostate cancer using ELISA and RT-PCR In this study, the

summary diagnostic odds ratio (DOR) and 95%confidence intervals(CIs) for PCA3 was 5.44 (4.53-6.53), and AUC and 95% CIs was 0.76 (0.72-0.79). Thus, the above results revealed that PCA3 could be acceptable as a valuable biomarker to distinguish PCa patients from healthy individuals.

Askari *et al*. (39) assessed Serum and urine levels of PCA3 in patients with benign prostatic hyperplasia and prostate cancer using ELISA technique,38 patients with prostate cancer and 52 patients with BPH participated in this study. Mean age in prostate cancer group was significantly higher than BPH group (P=0.01). Also mean PCA3, and total PSA in patients with prostate cancer was significantly higher than patients with BPH (P<0.05), the most frequent Gleason score (GS) was 7.This study showed no significant relationship between PCA3 with age and total PSA level (P>0.05),no significant relationship between Gleason score and PCA3 (P=0.83) and a significant relationship between prostate cancer with PCA3 level. The area under the ROC curve (AUC) for PCA3 with cutoff of 3.25 was 0.95, with sensitivity and specificity of 76% and 11%, respectively.

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