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The role of urine prostate cancer antigen 3 mRNA levels in the diagnosis of prostate cancer among Hong Kong Chinese patients

ORIGINAL ARTICLE

Objective   To establish and verify the utility of measuring urine prostate cancer antigen 3 (PCA3) mRNA levels in the diagnosis of prostate cancer among Hong Kong Chinese patients.

Design   Cross-sectional study.

Setting   Urology Unit of a regional hospital in Hong Kong.

Patients   This study was carried out in two parts. In the first part, 102 post-prostatic massage urine samples were collected from patients with known prostate cancer (38 patients) and controls (64 patients, with normal digital rectal examination and serum prostate-specific antigen <4 ng/mL). The urine levels of PCA3 and prostate-specific antigen mRNA were measured and the best cut-off point for differentiating cancer was determined. In the second part of the study, post-prostatic massage urine samples from 47 patients with clinically suspected prostate cancer were collected prior to prostate biopsy. The performance of PCA3 as a diagnostic aid for cancer was then assessed using the aforementioned cut-off value.

Results   In the first part of the study, the best cut-off for the PCA3 ratio (defined as the ratio of the Ct value of PCA3/PSA mRNA) was 1.127. Applying this cut-off to the 47 patients with clinically suspected prostate cancer and no history of previous prostate biopsy, the sensitivity and specificity of PCA3 for diagnosing prostate cancer were 71% and 92%, respectively.

Conclusion   The post-prostatic massage urine PCA3 level shows utility for diagnosing prostate cancer in patients with elevated prostate-specific antigen levels that could facilitate decisions to undertake prostate biopsy and avoid unnecessary biopsies.

New knowledge added by this study
• Prostate cancer antigen 3 (PCA3) determination can aid the diagnosis of prostate cancer in the local Chinese population.
Implications for clinical practice or policy
• By providing additional information, PCA levels can facilitate decision of undertaking a prostate biopsy in patients with elevated serum prostate-specific antigen (PSA) levels.
• PSA level measurements may improve the yield from transrectal ultrasound-guided prostate biopsies and avoid unnecessary recourse to the procedure.

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Introduction

In recent decades, the incidence of prostate cancer (PC) has been rising rapidly throughout Asia, including Hong Kong.[1] Currently, it is the third commonest cancer in Hong Kong Chinese males. Although the serum prostate-specific antigen (PSA) level remains the most often used marker for screening of the disease, its positive predictive value is low. As a result, many patients are subjected to transrectal ultrasound-guided prostate biopsy (TRUSPB) with negative results. There is therefore a need to develop more specific PC markers to improve the diagnostic yield.

The prostate cancer 3 gene was recently identified, and has non-coding messenger RNA referred to as prostate cancer antigen 3 (PCA3), which is overexpressed in PCs.[2] The

尿液中前列腺癌抗原基因3（PCA3）mRNA在診斷前列腺癌華籍患者中的角色

目的 探討及核實尿液中PCA3 mRNA在診斷前列腺癌華籍患者中的角色。

設計 橫斷面研究。

安排 香港一所分區醫院的泌尿科。

患者 本研究分為兩部份。第一部份包括從38名前列腺癌確診患者及64名有正常直腸指檢結果及前列腺特異抗原（PSA）值少於4 ng/mL的對照組中取得共102個前列腺按摩後尿液樣本，並量度樣本的PCA3及PSA mRNA值，以及找出分辨前列腺癌的最佳截取值。第二部份中，47個前列腺癌的懷疑病例在進行活檢前，搜集了他們的前列腺按摩後尿液樣本。再按兩部份得出的結果評估PCA3是否可以作為前列腺癌的一個診斷指標。

結果 第一部份中，PCA3比例（即PCA3的Ct值／PSA mRNA值）的最佳截取值為1.127。利用這截取值來分析第二部份中47個未進行活檢的前列腺癌懷疑病例，發現PCA3作為前列腺癌診斷指標的敏感性為71%，特異性為92%。

結論 前列腺按摩後尿液樣本中的PCA3水平可為PSA值較高的病人提供前列腺癌的診斷指標。利用這指標不但可加快決定病人是否應進行活檢，更可避免病人進行不必要的活檢。

measurement of PCA3 in post-prostatic massage urine (PPMU) has become a new screening test for PC, and was shown to have better sensitivity and specificity than serum PSA alone.[3,4] However, despite supporting evidence for the beneficial role of PCA3 in diagnosing PC in western populations, information regarding its utility in the Chinese is scanty. During the design of this study, the commercial test from Gen-Probe was still unavailable in our region. We therefore aimed to establish an assay for PCA3 in our local laboratory and assess the role of PCA3 in the diagnosis of PC in our local Chinese population.

Methods

This study comprised two parts. Part 1 entailed construction of the receiver operating characteristic (ROC) curve of urine PCA3 and its optimal cut-off point for the diagnosis of PC. Part 2 entailed verification of the cut-off point by applying it in patients planned for TRUSPB. The study was approved by our institutional review board, and conformed to the provisions of the Declaration of Helsinki. Signed consent was obtained from all patients before specimen collection.

For the first part of the study, patients were recruited from the urology clinic, Prince of Wales Hospital, Hong Kong. The inclusion criteria were (1) age ≥50 years, (2) histologically proven PC, and (3) not having received any treatment for PC. The exclusion criteria were (1) TRUSPB performed within 4 weeks, (2) having symptoms suggesting active urinary tract infection, (3) presence of gross haematuria during assessment, (4) having a urethral catheter in situ. The PPMU specimen was collected at least 4 weeks after the initial diagnosis so as to avoid the effects of TRUSPB on PCA3 measurement. At the same time, a group of patients with no clinical evidence of PC were also recruited from our urology clinic as controls. The inclusion criteria for these controls were: (1) age ≥50 years, (2) normal digital rectal examination (DRE), and (3) serum PSA level <4.0 ng/mL. Their exclusion criteria were (1) having symptoms suggesting active urinary tract infection, (2) presence of gross haematuria during assessment, (3) having a urethral catheter in situ. The PCA3 mRNA level was measured and the best cut-off level was determined using the ROC curve approach.

For the second part of the study, male patients scheduled for TRUSPB in our centre were recruited. The inclusion criteria are (1) age ≥50 years, (2) clinical suspicion of PC and planned for TRUSPB, (3) no previous history of TRUSPB. Their exclusion criteria were: (1) having a urethral catheter in situ, (2) already known to have PC by transurethral resection of prostate. Informed consent was obtained prior to TRUSPB. Blood (for serum PSA measurement) and PPMU were collected before TRUSPB. The TRUSPB was performed by ultrasound guidance so as to obtain 10 cores (base, middle, apical, upper lateral, and lower lateral biopsies for each lobe). If a lesion was seen on ultrasound and could not be included in the standard 10 core biopsies, an additional core of biopsy from the lesion was obtained. The pathology result, serum PSA level, and PCA3 level were then analysed. The pathologists involved were blinded to these urine and blood test results and reported their findings independently. Similarly, the research staff involved in the measurement of urine markers were blinded to the clinical and pathological information.

Collection of post-prostatic massage urine

Digital rectal examination was performed by applying firm pressure (enough to depress the prostate surface of 0.5 to 1 cm) from base to apex and from the lateral to the median line for each lobe, using three strokes per lobe.[5] The patient was then asked to pass the first 30 to 50 mL of urine into a collection bottle for PCA3 measurement. Urine samples were placed in ice immediately after collection and processed within 4 hours. This entailed centrifugation at 2000 x g for 30 minutes at room temperature, and discarding of the supernatants. The urine sediments were suspended in 1 mL ice-cold phosphate-buffered

saline, and transferred to 1.5-mL centrifuge tubes. The tubes were then centrifuged at 16 000 x g for 10 minutes, and the supernatants discarded. To stabilise the RNA in the specimens, urine sediments were suspended with RNA later (Ambion Inc, Austin [TX], US) with overnight incubation at 4°C. The tubes were then centrifuged at 16 000 x g for 10 minutes, and the supernatants discarded. Processed specimens were stored at -80°C until further analysis.

Measurement of prostate cancer antigen 3 level

RNA isolation and cDNA synthesis

Total RNA was extracted from the centrifuged sediment by the RNeasy Mini Kit (Qiagen) in accordance with the manufacturer's instructions. Immediately after RNA extraction, 12 µL of the RNA extraction product was subjected to cDNA synthesis using the QuantiTect Reverse Transcription Kit (Qiagen). The cDNAs were stored at -20°C until analysis.

Real-time quantitative polymerase chain reaction data acquisition

The mRNA expression of PCA3, PSA, and a housekeeping gene (ie β-actin) were quantified by real-time quantitative polymerase chain reaction (RT-QPCR). The primer and probe were designed for PCA3 and the sequences were: Forward 5'- AAA GGA AGC ACA GAG ATC CCT G -3' (located in exon 3, nucleotides 287-308, GenBank accession number NR_015342); Reverse 5'-GGG CGA GGC TCA TCG AT-3' (located in exon 4a, nucleotides 338-354); Probe 5'-6FAM-AGA AAT GCC CGG CCG CCA TC- Black Hole Quencher1-3'. For PSA and β-actin, both primers and probes have been published previously, and were designed to span intron-exon junctions in order to avoid genomic DNA amplification.[6] The primer and probe sequences of PSA were: Forward 5'-GAC CAC CTG CTA CGC CTC A-3'; Reverse 5'-GGA GGT CCA CAC ACT GAA GTT TC-3'; Probe 5'-HEX-CAG CAT TGA ACC AGA GGA GTT CTT GAC CC- Black Hole Quencher1-3'. The primer and probe sequences of β-actin were: Forward 5'-GGC ACC CAG CAC AAT GAA G-3'; Reverse 5'-GCC GAT CCA CAC GGA GTA CT-3'; Probe 5'-Cy3-TCA AGA TCA TTG CTC CTC CTG AGA GCG C- Black Hole Quencher2-3'. The primers and probes were synthesised at 1st BASE (Singapore) and Integrated DNA Technologies (US), respectively.

The RT-QPCR was conducted by the iQ5 Multicolour Real-Time PCR Detection system (Bio-Rad). The 20 µL final volume mixture contained primers and probe at optimised concentrations; 10× PCR buffer II, $MgCl_2$, AmpliTaq Gold (Applied Biosystems), and 1 µL of cDNA. Thermal cycler parameters included 10 minutes at 95°C and 40 cycles involving denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 1 minute. Results were collected and analysed with the RT-PCR ABI Prism 7500 software v2.0.3.

The PSA mRNA levels were used to normalise PCA3 to the total amount of prostate RNA present in the sample and ensure that the RNA yield was sufficient for analysis. The housekeeping gene served as an internal control for RNA extraction and cDNA synthesis procedure. Samples with Ct values of β-actin larger than 30 or PSA greater than 36 were considered 'non-informative', where the RNA content was too low for accurate determination of the PCA3 score. The PCA3 ratio was calculated by dividing the Ct value of PCA3 by that of PSA.

Statistical analysis

Statistical analyses were conducted using the Statistical Package for the Social Sciences (Windows version 15.0; SPSS Inc, Chicago [IL], US). For the first part of the study, the PCA3 ratio cut-off was derived from the ROC curve by the Youden's J statistic approach. For the second part of the study, the sensitivity and specificity of the PCA3 ratio was derived using another group of patients. Associations between PCA3 ratio with the Gleason score, number of positive cores, and clinical T-staging were calculated using Spearman's correlation coefficient. The association between PCA3 test result (above or below cutoff) and bilateral lobe involvement were assessed using Chi squared tests.

Results

From June 2008 to September 2009, 149 patients were involved in this study.

The first part of the study was carried out from June 2008 till March 2009, and entailed 38 patients with known PC and 64 with no clinical evidence of PC (the controls). These patients were identified in the urology clinic and recruited after giving their informed consent. The median ages of the PC and control patients were 73 (range, 56-86) years and 69 (range, 51-84) years, respectively. The mean serum PSA level of the control group was 1.55 (range, 0.27-3.52) ng/mL. Regrettably, some of the urine samples developed precipitates during the freezing process that affected the extraction of microRNAs, with the result that the levels of microRNAs were very low. These samples were therefore excluded from the analysis. Finally, 74 informative specimens (30 from cancer patients and 44 from controls) were available for analysis. From the results available from these samples, a ROC curve was constructed and the optimal cut-off level for the PCA3 ratio was determined to be 1.127. The corresponding value for sensitivity was 73% (95% confidence interval [CI], 56-86) and for specificity was 77% (95% CI, 63-87) [Fig.

The derived cut-off was then applied in the second part of the study.

In the second (validation) part of the study, 47 consecutive patients with suspected PC but no history of a previous prostate biopsy were recruited between April and September 2009. Their mean serum PSA level was 16.6 (range, 0.6-85.6) ng/mL. Among these patients, 41 had informative specimens—27 had elevated serum PSA levels (>4.0 ng/mL), five had

FIG. The receiver operating characteristic curve constructed from the first part of the study

TABLE 1. Results of the second part of the study*

	Prostate cancer	No evidence of cancer	Total
PCA3† +ve	12	2	14
PCA3 –ve	5	22	27
Total	**17**	**24**	**41**

* Sensitivity = 71%, specificity = 92%, and positive predictive value = 86%
† PCA3 denotes prostate cancer antigen 3

abnormal DRE findings, and nine had both elevated serum PSA and abnormal DRE findings. In all, 17 (41%) of the patients were diagnosed as having PC (based on prostate biopsies), so that the sensitivity and specificity of PCA3 for diagnosing PC were 71% and 92%, respectively (Table 1). Applying these results to the validation sample, for PCA3 the positive predictive value was 12/14 (86%) and the negative predictive value was 81%. Among the five patients with negative PCA3 results and a positive prostate biopsy, only one had advanced disease (serum PSA 68 ng/mL, clinically T2 disease, and Gleason 4+4 cancer in all the 10 core biopsies). The other four patients had localised disease with serum PSA levels of 6-12 ng/mL and 1-3 cores positive for cancer (out of 10 core biopsies). Regarding the five subjects with abnormal DRE findings but negative serum PSA level results (<4 ng/mL), they all had negative biopsies, whilst the PCA3 test yielded a specificity of 80% (only one case had positive PCA3 test). These findings were comparable to the overall specificity of 92% derived from the second part of our study. A summary of the sensitivity and specificity of various combinations of DRE, serum PSA level, and PCA3 results is listed in Table 2.

Correlation of the PCA3 ratio with other clinical or pathological parameters was assessed by combining all patients with a diagnosis of PC in both the first part (27 patients) and second part (17 patients) of this study. The clinical information for all of the PC patients is listed in Table 3. No significant association was found between the PCA3 ratio and Gleason scores, number of positive cores, bilateral lobe disease, or clinical T staging.
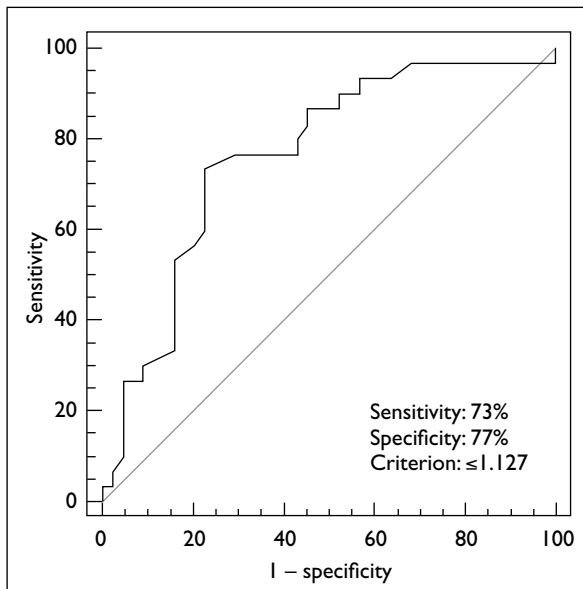
Discussion

We found that for patients with suspected PC, measurement of the PCA3 level in PPMU had a high specificity (92%) and high sensitivity (71%) for making the diagnosis in accord with the first biopsy. The combination of serum PSA and PCA3 may help

TABLE 2. A summary of the sensitivity, specificity, and positive predictive value using various combinations of digital rectal examination (DRE), serum prostate-specific antigen (PSA) level, and prostate cancer antigen 3 (PCA3) results in the diagnosis of prostate cancer in the 41 patients in the second part of the study

	Sensitivity (%)	Specificity (%)	Positive predictive value (%)
Abnormal DRE	53	79	64
Serum PSA level >4 ng/mL	100	21	47
PCA3 result +ve	71	92	86
Abnormal DRE with serum PSA level >4 ng/mL	53	100	100
Abnormal DRE and PCA3 result +ve	41	96	88
Serum PSA level >4 ng/mL and PCA3 result +ve	71	96	92
Abnormal DRE with serum PSA level >4 ng/mL and also PCA3 result +ve	41	100	100

TABLE 3. The characteristics of patients with prostate cancer

	Patients from part I	Patients from part II	Overall
No.	27	17	44
Mean age (range) [years]	71 (56-86)	70 (59-84)	71 (56-86)
Serum PSA* level: mean / median (range) [ng/dL]	16 / 9 (2-127)	26 / 12 (5-86)	20 / 10 (2-127)
Clinical staging			
T1c	14	9	23
T2	7	6	13
T3	6	2	8
Metastatic disease on presentation	3	3	6
Gleason score <7	18	7	25
Gleason score ≥7	9	10	19

* PSA denotes prostate-specific antigen

to improve the diagnostic yield of patients with elevated serum PSA and planned for TRUSPB (from 47% to 92%) [Table 2].

Following the introduction of serum PSA measurement as a purported tool for screening and early diagnosis of PC in the 1980s, it still remains the most commonly used marker. For patients with raised PSA, TRUSPB is necessary for histological confirmation of the diagnosis of PC. Although PSA is an organ-specific marker, it is not disease-specific and raised serum levels can occur in many benign conditions, such as benign prostate hyperplasia, and acute retention of urine. The proportion of patients with an elevated serum PSA level due to benign conditions is particularly high in patients with only mildly elevated serum PSA levels (4-10 ng/mL). The incidence of PC in Asian countries is relatively low compared to that in Caucasians.[1,7] From the data in a local centre, the frequency of PC diagnosed by TRUSPB in patients with normal DRE findings and serum PSA levels between 4 and 10 ng/mL was only 7%.[8] Thus, the vast majority of patients subjected to TRUSPB have negative results, so there is clearly a need for better means to select whom to biopsy.

There are two potential ways to resolve this issue—improve the performance of PSA assessment or develop better disease markers. There have been several modifications to serum PSA assessment to improve the test's performance, including recourse to age-specific PSA levels, PSA density, and free-to-total PSA ratios.[9] Meanwhile, new biomarkers are being explored for diagnosis of PC, and include the fusion gene (TMPRSS2-ERG), GSTP-1 hypermethylation, and PCA3.[10] Among them, PCA3 has shown promise,[3,11] and is already available for clinical use in some parts of the world. Currently, it is mainly recommended for patients with a negative TRUSPB in whom a clinical suspicion of PC persists. The added information from PCA3 determination could help decide the need for a repeat biopsy.

In our region however, the incidence of PC is relatively low and the low diagnostic yield of first TRUSPB may be a more critical problem than repeat TRUSPBs. We therefore concentrated on patients having their first biopsy. Currently, other studies attempting to investigate the role of PCA3 in patients having their first biopsy have shown some promise.[12] Our findings indicate a very high specificity (92%) and good sensitivity (71%) for the test in patients with clinically suspected PC. In which case, PCA3 determination might help ascertain the need for TRUSPB in patients with an elevated serum PSA level, since only 19% of those who tested negative would be expected to yield a PC from a TRUSPB. Thus, for patients who were tested PCA3-negative, further discussions/counselling would be necessary to take account of other clinical information (eg PSA level) before resorting to invasive investigation in the form of a biopsy. This would entail explaining the potential of missing some cancers and the risk of delaying the management from opting out of TRUSPB. These patients would nevertheless continue to be monitored with respect to their serum PSA and regular DREs. Any further clinical suspicion of PC would then be grounds for proceeding to TRUSPB.

One limitation of our study was the relatively small sample size. Further studies to confirm or refute our findings should be on a larger scale. Nevertheless, these results suggest that PCA3 measurement can provide additional information for deciding on whether to carry out a first TRUSPB in our local population.

Another limitation of our study was that our definition of control patients differed from the one used in other studies.[3,4] According to such literature, PCA3 tests were mainly performed in patients before they had a TRUSPB,[3,4] the relationship of test scores and biopsy results was then assessed. Therefore, the cut-off score definition was mainly determined by the presence of a positive and negative biopsy.

From our own experience, however, about 20% of the patients whose first biopsy was negative subsequently had positive biopsies (unpublished data of our centre). Therefore, the control group (ie patients with a negative biopsy) might actually include patients with PC not diagnosed in the first biopsy. Consequently, in the first part of our study we decided to use patients with a low clinical suspicion of PC (normal DRE findings and serum PSA level <4 ng/mL) as our controls. We understood that it would be impossible to be absolutely certain that a subject did not have the cancer, and that in our locality we could use controls based on 'low suspicion of having PC'. Hopefully a cut-off for PCA3 ratio developed by this means will have higher accuracy in clinical settings. Moreover, we would not offer TRUSPB in such patients who were deemed to be normal, and so it would be unethical to perform biopsies in them just to exclude PC. Thus, owing to the definition of our controls, our data and cut-off point might differ from those in the literature and direct comparison of results might not be feasible.

Attempts have also been made to explore the role of PCA3 in the prediction of staging and prognosis of PC. However, in a review by Vlaeminck-Guillem et al,[13] there was still no conclusive evidence to support its value for predicting Gleason grading, tumour staging, tumour volume, or cancer aggressiveness. In our data, there was no clear correlation between clinical and pathological parameters, except for a negative association with metastatic disease. Recent reports nevertheless suggest that the PCA3 level may help identify patients with less-aggressive disease that might be more suitable for active surveillance.[14] This possibility requires further study.

As we are still in the initial development of the PCA3 test from PPMU samples in our local setting, we had to contend with several technical problems. Some samples (about 20%) yielded very low PCA3 levels due to precipitation in the urine after freezing, which we consider to be uninformative. Similar issues with uninformative specimens have also been reported in other publications, especially in earlier studies.[15,16]

Moreover, to minimise the chance of degradation of mRNA, the freshly collected urine samples need to be stored at 4°C until they were processed. Regrettably, precipitate formed in some of the urine samples and ideally they should have been repeated. We are currently trying to collect urine in close proximity to our laboratory, so that the specimens can be handled immediately without the need of storage, which may help to decrease the number of uninformative samples. Further developments of better specimen transport systems may improve the utility of the test in areas outside our laboratory.

The small sample size and the inclusion of results from only one centre may affect the generalisation of our results. The frequency of positive biopsies (42%) seemed to be quite high in our series and was probably related to the relatively high serum PSA levels when the patients first presented. This is common in our region as many patients present quite late and routine serum PSA screening is still not a common practice in our locality. Nevertheless, our study has demonstrated the additional benefit of PCA3 measurement in the diagnosis of PC in the Hong Kong Chinese population. Further studies of the application of PCA3 in this region may be beneficial.

Conclusion

The measurement of PCA3 mRNA in PPMU was a reasonable diagnostic test with acceptable sensitivity and high specificity. In future, combining clinical information, serum PSA, and PPMU PCA3 levels could help in the counselling of patients with suspected PC, whenever the possibility of TRUSPB is under consideration.

Acknowledgements

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