Translational Oncology 14 (2021) 100963

Contents lists available at ScienceDirect

Translational Oncology

journal homepage: www.elsevier.com/locate/tranon

Prostatic aspirated cellular RNA analysis enables fast diagnosis and staging of prostate cancer

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ARTICLE INFO

Keywords:
Prostate cancer;Aspirated cellular RNA analysis;Cancer diagnosis

ABSTRACT

Objective: The aim of this study is to investigate the potential application of prostatic aspirated cellular RNA analysis technique for fast diagnosing and staging prostate cancer.

Methods: Prostatic aspirated cells were obtained immediately after transrectal ultrasound (TRUS)-guided needle biopsy. Cellular RNA such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, prostate specific antigen (PSA) mRNA and prostate-specific RNA (PCA3) mRNA were analyzed by using Reverse Transcription-Polymerase Chain Reaction (RT-PCR). PCA3 score was calculated as the ratio of PCA3 mRNA to PSA mRNA expression. Diagnostic performance of the fast-aspirated cellular RNA analysis technique for determining prostate cancer and metastatic status were evaluated by developing receiver operating characteristic curves (ROC), and the correlation between aspirated cellular RNA mRNA expressions and risk grouping was calculated, to investigate the underlying potential for PCa staging.

Results: PCA3 score was significantly higher in prostatic aspirated cells obtained from cancerous tissues than noncancerous tissues. The median aspirated cellular PCA3 score was higher in patients with PCa compared to BPH, and presenting an area under the ROC curve (AUC) of 0.87 (95%CI: 0.79–0.94) for PCa diagnosis. Multivariate regression analysis revealed that baseline median aspirated cellular PCA3 score (OR=9.316, 95%CI: 1.045–83.033, $P<0.05$) was an independent predictive factor for metastatic status in PCa patients.

Conclusion: The ease of use and minimal complexity of fast prostatic aspirated cellular RNA analysis may be a valuable diagnostic technique, providing urgent diagnostic information for accurate PCa diagnosing and staging.

Introduction

Prostate cancer (PCa) is one of the most common male malignancies in the worldwide, and become the leading cause of cancer-related mortality globally [1, 2]. Over the decades, serum prostate specific antigen (PSA) has been a main biomarker for PCa screening, risk stratification and monitoring owing to its non-invasion and cost-effectiveness. Evidences gathered by European Randomized Study of Screening for Prostate Cancer (ERSPC) suggest that population-based PSA screening reduces mortality rates of prostate cancer by twenty percent with the ages ranging from 55 to 69 years [3], but the deficiency of cancer specificity of PSA leads to overdiagnosis at an estimated rate of 23 to 42% [4].

Practically, when an elevated serum PSA levels or an abnormal digital rectal examination (DRE) is observed, transrectal ultrasound (TRUS)-guided needle biopsy will be recommended for PCa diagnosis. Whereas, it encounters an inevitable consequence that about 50% of small tumors may be missed [5]. Therefore, the “saturation biopsy” technique employing 8~22 cores in a single biopsy session was adopted [6, 7]. However, a downstream histopathological examination may still fail to reveal cancers in up to 30% in men [5, 8], thus resulting in repeated prostate biopsy procedures and increasing the risk of infection after the biopsy procedure especially by transrectal approach [9]. Some informative tissue may also be lost or destroyed during histological sample preparation that limits the reproducibility and predictive power of pathological diagnosis [10, 11]. Additionally, the time-consuming procedures in later histological examination hampers efficient pathological identification of a suspected PCa patient, and increases healthcare costs [12]. Therefore, efficient and precise diagnostic technique is quite needed as an auxiliary approach in revealing PCa patient. Ideally, such technique should be rapid and non-destructive to tissue structure, en-

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https://doi.org/10.1016/j.tranon.2020.100963
Received 8 September 2020; Received in revised form 15 November 2020; Accepted 16 November 2020

abling efficient differentiating PCa that is also synchronous to histological examination protocols.

Cellular RNA analysis yields additional diagnostic information beyond conventional morphological examination [13, 14]. Due to the unique molecular profiles revealed by cancerous cells, rational detection of cellular RNA allows specific identification of a certain cancer, as reported by varieties of practical studies. For instance, cellular androgen receptor (AR) splice variant V7 (AR-V7) mRNA can be used as an indicator of resistance to pharmacotherapy [15, 16], and a crop of miRNAs were discovered as valuable biomarkers for diagnosis and prognosis in prostate cancer (PCa) [17, 18]. In this work, we carried out experiments to analyze aspirated cellular RNA obtained from prostatic fine needle aspiration (FNA) procedures, and aimed to provide a proof of concept that using aspirated cellular RNA as novel biomarker for fast PCa diagnosis and cancer assessment.

Method and materials

Materials

All the oligonucleotide including primers and probes were synthesized by Sangon, and the detailed sequences were listed in Table S1. All the oligonucleotides were dissolved by DEPC-treated water and diluted to 10 μM, then stored at −20 °C until used. DEPC-treated water was purchased from Shanghai Sangon Biological Engineering Technology & Services (Shanghai, China). PrimeScript™ RT reagent Kit (with gDNA Eraser) was provided by Takara Biotechnology (Dalian) Co., Ltd.

Patients recruitment and sample preparation

Adult patients from the second affiliated hospital of Zhejiang university from December 2014 to December 2015 who underwent transrectal ultrasound (TRUS)-guided prostate needle biopsy were enrolled. Exclusion criteria were the following: (1) incomplete clinical examination results or medical history; and (2) serious infectious disease, other tumor or serious conditions. The study protocol was approved by the Human Ethics Review Committee of the second affiliated hospital of Zhejiang university (IR2020001450). Following case finding and informed consent, participants provided venous blood samples prior to prostate needle biopsy. The blood samples were stored at 4 °C for intraday measurement, otherwise frozen at −80 °C until used.

Prostatic aspirated cellular RNA analysis and serum PSA evaluation

Prostatic tissues from 12-core needle biopsy were immediately smeared onto sterile slides to obtain aspirated cells, and subsequently transferred to formaldehyde solution for further histopathological examination. Aspirated cells were then thoroughly eluted from the slides using sterile 0.9% saline solution. Following 3 times of washing steps, total RNAs from aspirated cells were extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions [19]. cDNA was then synthesized using PrimeScript™ RT reagent kit with gDNA Eraser (Takara, Dalian, China) following the manufacturer's protocols and stored at −80 °C for further analysis. The reactions were performed using the STEP ONE Plus Real-Time PCR (RT-PCR) system (Applied Biosystems, Foster City, CA, USA). The PCR run protocol was as follows: Holding stage: 95 °C for 20 s, pre-Cycling stage (10 cycles): 95 °C for 5 s and 62 °C for 20 s, Cycling stage (50 cycles, signal collection): 95 °C for 5 s and 60 °C for 20 s. Practically, due to the potential DNA contamination in RT-PCR, a cycle threshold value (Ct) of less than 40 was defined as a positive test, while a Ct value of 40 or more was defined as a negative test. The automated chemiluminescent microparticle immunoassay analyzer ARCHITECT i2000 (Abbott Laboratories, Abbott Park, Illinois, United States) was used following the manufacturers protocols to determine the concentrations of PSA protein in serum samples.

Statistical analysis

The ΔCt model was applied as mathematical model for mRNA analysis in this study (See Text S1 for mathematical details). PCA3 score is calculated as the ratio of PCA3 mRNA to PSA mRNA expression. The analytical results of PSA mRNA and PCA3 scores from all the biopsy sites and corresponding coefficient of variations were visualized in Fig. 1. The primary comparisons for this study were defined as PCa cases (all stages) versus BPH cases, and metastatic cases versus localized cases. Intergroup comparison was assessed by Student's T-test or Wilcoxon's test or Chi square test. Kendall's Tau or Spearman's rank correlation coefficients were calculated to explore the relationships between different variables. Univariate and multiple logistic regression models were developed to consider PCA3 scores alone and combined with serum PSA. The response variable was coded as 1 to indicate PCa or metastatic status, 0 for BPH or localized cancer. Patients with smoking history was entered as categorical variable of 1, or 0 for patient without smoking history. Patients' ages and candidate markers such as serum PSA concentrations and aspirated cellular RNA expression were regarded as continuous variables, unless otherwise stated. For determining metastatic status, PCA3 score was dichotomized as 0=negative ($<8.08\times10^{-2}$) or 1= positive ($\geq 8.08\times10^{-2}$). Risk grouping was based on the NCCN Clinical Practice Guidelines in Oncology: Prostate Cancer (Version 2.2017). In this study, patients with very low and low risk were classified as group Low, patients with intermediate and high risk were classified as group Intermediate and patients with very high risk or in metastatic status were classified as the group High, respectively. Adjusted PCA3 score was calculated by taking age and smoking history into account for risk grouping (Adjusted PCA3 score = 2.232*[PCA3 score] + 0.236*[smoke history] + 0.111*[age] − 10.48). The area under the ROC curve (AUC) was calculated for each model considered. $P<0.05$ was regarded as statistically significant. All statistical analyses were performed using SPSS software 20.0 (SPSS, Chicago, Illinois, United States), and graphics were created using Prism for Windows 10, GraphPad Software, Inc.

Results

Baseline clinical characteristics and laboratory data in study population

According to the final histopathological results, eligible patients were divided into two groups: 31 of prostate cancer patients (PCa) and 50 of benign prostatic hyperplasia patients (BPH), respectively. None of the total 81 patients have ever received anti-cancer therapy. Patients with PCa are much older than patients with BPH ($P<0.05$). Serum PSA levels were significantly higher in PCa patients compared with BPH patients (Table 1). The receiver operating characteristic curve (ROC) based on serum PSA for predicting PCa showed optimal sensitivity and specificity of 70.97% and 66.0%, respectively (Fig. S1A), and serum PSA higher than 10.4 ng/mL strongly indicated a PCa regardless of age and smoke history (OR=3.785, 95%CI:1.398–10.252, $P<0.01$). 7 in 31 PCa patients were diagnosed with metastatic status at the time of biopsy confirmed by positron emission tomography/computed tomography (PET/CT) or emission computed tomography (ECT).

Basic analytical evaluation in aspirated cellular RNA analysis

We conducted 8–12 biopsy cores in each single biopsy session and 972 resected specimens were finally obtained. Cellular internal reference glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA analysis showed that up to 76.9% of the total 972 resected specimens were above the detection limit, indicating acceptable sampling quality by our approach. No significant difference in detection rates of aspirated cellular GAPDH mRNA was observed between cancerous aspirated specimens and noncancerous ones ($P>0.05$, data not shown). We further evaluated the relative expression of prostate specific antigen (PSA) mRNA in aspirated specimens for prostate cell identification. It showed that almost all

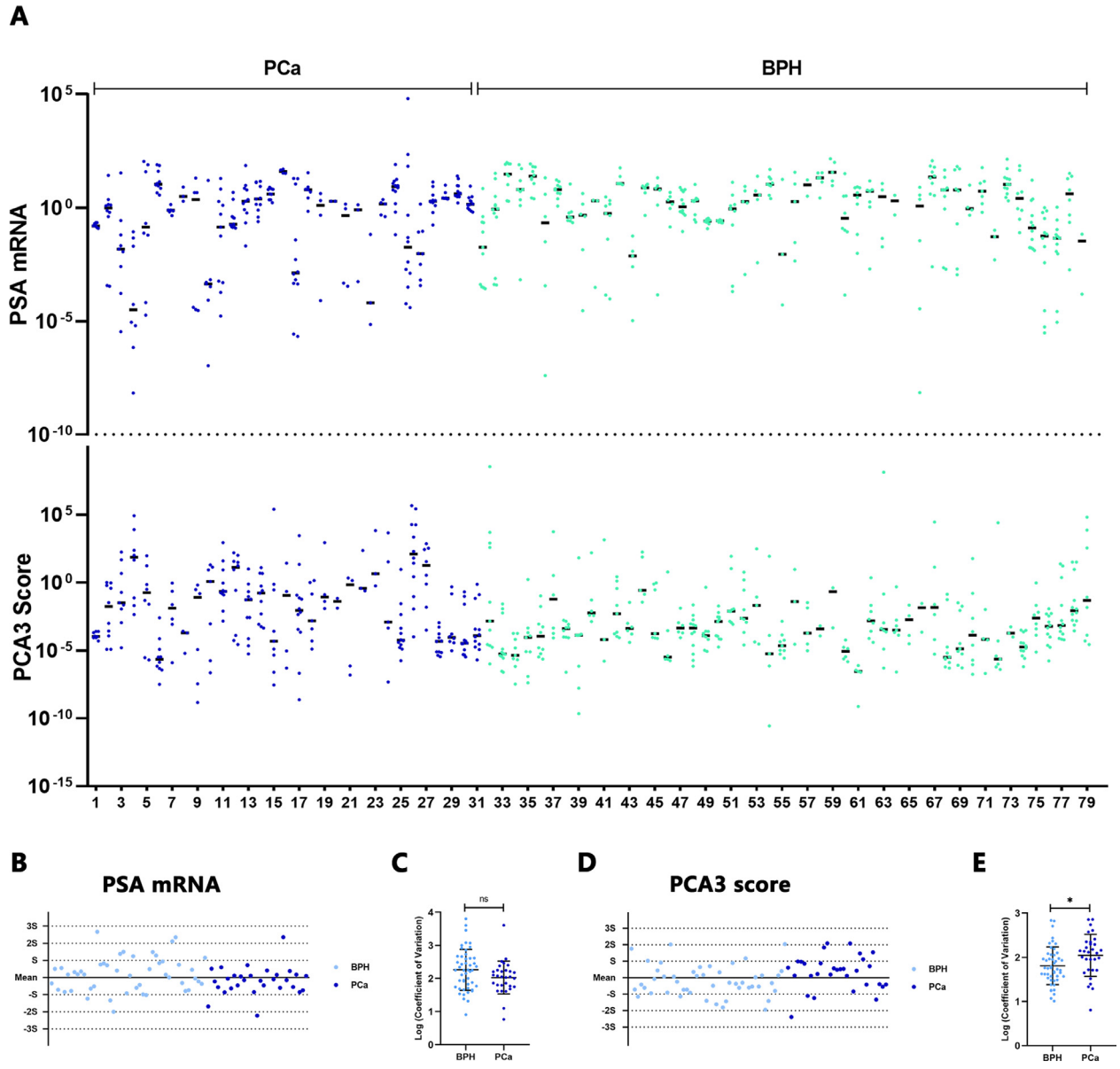

Fig. 1. A: Visualization of PCA3 score and PSA mRNA plots from each biopsy site. The blue plots and green plots correspond to biopsy site from PCa and BPH patients, respectively. The black bars are the median values in each patient. CV_{intra} of PSA mRNA (B) and PCA3 score (D) in PCa were plotted in dark blue, and plotted in light blue for BPH, respectively. Comparison of CV_{intra} of PSA mRNA (C) and PCA3 score (E) between PCa and BPH were analyzed using unpaired Student's T-test. *: $P<0.05$, ns: no significance. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Comparison of baseline data in PCa patients and benign prostatic hyperplasia patients.

Variable	PCa patient ($n=31$)	BPH ($n=50$)	P value
Age, years	70.35±10.67	66.12±8.27	**0.049**
Smoking history (%)	18 (58.04)	26 (52.00)	0.818
Serum PSA, ng/mL	16.62 (7.83–35.58)	8.34 (6.19–12.70)	**0.001**
Prostate volume, mL	36.11±28.29	49.63±25.71	**0.032**
PSAD, ng/mL^2	0.65 (0.27–1.34)	0.18 (0.13–0.25)	**0.000**
PSA mRNA relative expression	0.97 (0.10–6.32)	1.88 (0.26–5.65)	0.455
PCA3 score	4.06×10^{-2} (1.21×10^{-4}–3.79×10^{-1})	3.83×10^{-4} (6.35×10^{-5}–4.38×10^{-3})	**0.000**

Continuous variables were presented as mean ± standard deviation (SD) or median (inter-quartile range) (IQR). $P < 0.05$ were considered as statistically significant. PSA: Prostate Specific Antigen, PSAD: Prostate Specific Antigen Density, PCA3 score: Prostate Cancer Antigen 3.

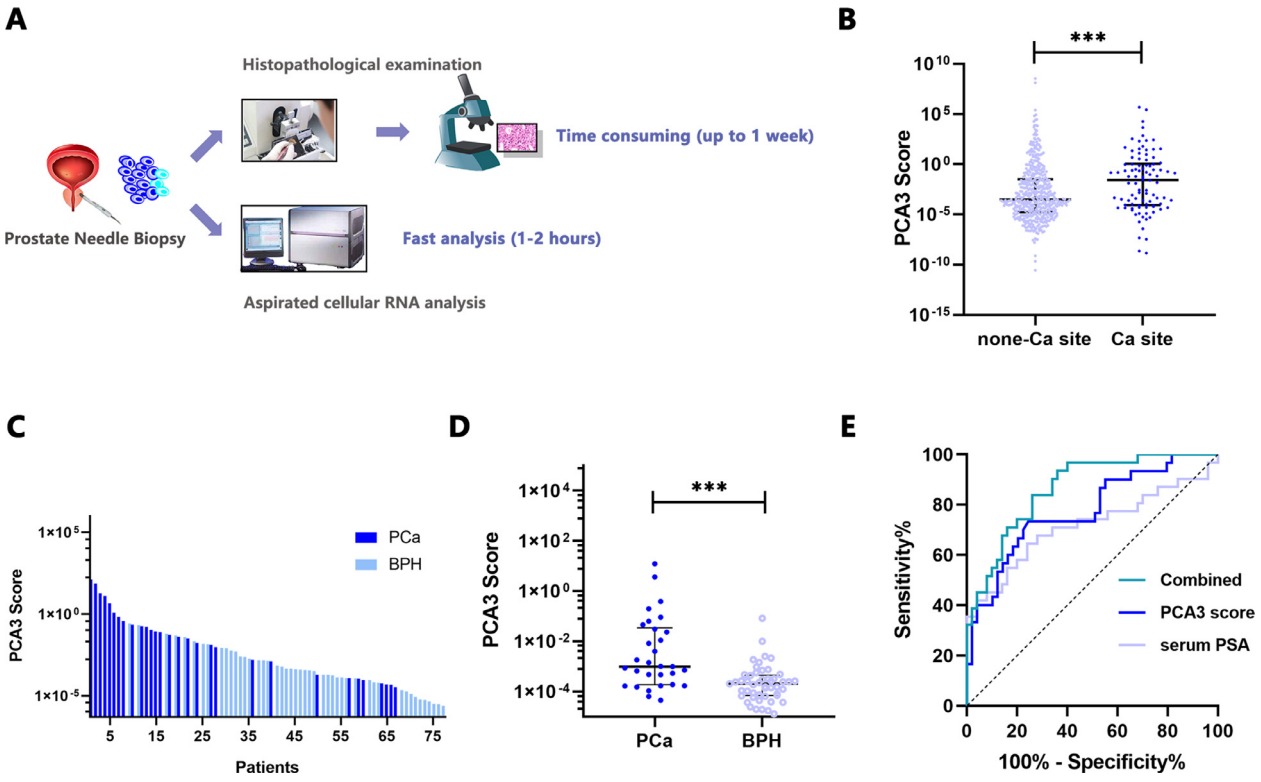

Fig. 2. A: General scheme of prostatic aspirated cellular RNA analysis compared with traditional pathological diagnostic technique. B: Comparison of aspirated cellular PCA3 score levels between cancerous biopsied tissues and noncancerous tissues. C: The distribution of median aspirated cellular PCA3 score levels from all patients. D: Comparison of median aspirated cellular PCA3 score levels between PCa patients and BPH patients. E: Receiver operating characteristic curve (ROC) of median aspirated cellular PCA3 scores and combined indicator (with serum PSA) for prediction of PCa. *** $P<0.001$.

of patients were detectable in PSA mRNA, with a median detection rate of 95.83% in each patient (Fig. S1B), revealing the successful acquisition of prostatic cells from biopsies. Rationally, since PSA mRNA expresses ubiquitously in prostatic tissues regardless of cancerization, and yielded minimal variance in all the patients (Fig. 1A–C), PSA mRNA can be arguably considered as an internal quality control marker. As a result, it shows significant capability to obtain prostatic cells with great sampling quality using our protocols. The general scheme of technique versus traditional histopathological examination was presented in Fig. 2A.

Aspirated cellular RNA analysis for PCa determination

While amounts of evidences have suggested that cellular PSA mRNA was higher in PCa cells compared with normal cells [20, 21], we didn't find any statistically significant difference of aspirated cellular PSA mRNA levels between cancerous and noncancerous specimens (data not shown). Moreover, the aspirated cellular PSA mRNA levels in PCa patients were also indistinguishable from benign ones (Table 1 and Fig. 1A). This may result from a "dilution effect" that the elevation of PSA mRNA expression by tiny cancerous cells might be concealed by a massive number of normal prostatic cells using fine-needle prostatic aspiration.

We analyzed another prostate-specific RNA (PCA3), which is reported highly expressed in prostate cancerous tissues rather than in benign prostatic hypertrophy tissues or normal prostatic glands [22–24]. Varieties of studies have indicated its roles in prostate cancer development [25], cell survival and modulating androgen receptor signaling [26], and the PCA3 score (defined as the ratio of PCA3 mRNA to PSA mRNA) may provide potential utility as predictor for PCa. We thus supposed that aspirated cellular PCA3 score can be a candidate molecular biomarker in PCa diagnosing and staging. As a proof of concept, we initially investigated its capability in discriminating cancerous tissues. As shown in Figs. 1A and 2B, PCA3 score in cancerous tissue sites was higher than benign tissue sites with a statistically significance, but the performance for prostatic cancerous sites identification was quite limited with positive predictive value only of 28.57%. Interestingly, we found that the analytical variance of PCA3 scores in PCa patients was significantly larger compared to BPH patients (See Text S2, Fig. 1A, D and E), which indicated that the elevation of PCA3 scores from some portion of biopsy tissues may account for such variance in PCa patients. However, there was no significant difference of PCA3 scores in cancerous sites compared to noncancerous sites. Accordingly, we supposed that such elevation of PCA3 scores in PCa may be resulted from cancer cells that were independent from the cancerous biopsies, and also reflect the cancerization of some prostatic cells in PCa patients.

Therefore, we defined the median aspirated cellular PCA3 scores (mPCA3 score) from biopsies in each patient as a novel biomarker to distinguish PCa from BPH (see Text S1). As is shown in Fig. 2C and D, the mPCA3 scores were significantly higher in PCa compared to benign prostatic hyperplasia patients, exhibiting diagnostic specificity of 97.96% upon the cut-off value of 1.07×10^{-2}. The diagnostic performance can be significantly improved when combined with basic clinical and laboratory data such as age, smoking history and serum PSA (Fig. 2E). Moreover, we observed that when the serum PSA values were within the range of 4 ng/mL to 10 ng/mL (PSA gray zone), the mPCA3 score higher than 1.24×10^{-1} showed a greater predictive capability for PCa incidence (OR=12.0, 95%CI: 0.871–165.402, $P=0.06$) compared with serum PSA. Results above strongly revealed that prostatic aspirated cellular RNA analysis can be a valuable tool for PCa diagnosis.

Aspirated cellular RNA analysis for PCa stage assessment

To investigate whether aspirated cellular RNA analysis can help assessing PCa and play significant roles in the clinical decision-making process, we further evaluated mPCA3 score levels in 7 patients with metastatic PCa versus 24 patients with localized PCa. In this case,

Table 2
Comparison of baseline data in patients with metastatic and localized cancer.

Variable	Metastatic cancer ($n=7$)	Localized cancer ($n=24$)	P value
Age, years	69.04±9.53	74.86±13.80	0.210
Smoking history (%)	3 (42.86)	15 (62.50)	0.413
Serum PSA, ng/mL	137.2±138.4	24.52±33.57	**0.001**
Prostate volume, mL	27.72±10.15	38.67±31.59	0.380
PSAD, ng/mL^2	3.17 (0.61–10.06)	0.50 (0.22–0.81)	**0.011**
PCA3 score, >8.08×10^{-2}	5 (71.43)	8 (33.33)	**0.072**

Continuous variables were presented as mean ± standard deviation (SD) or median (interquartile range) (IQR). $P < 0.05$ were considered as statistically significant. PSA: Prostate Specific Antigen, PSAD: Prostate Specific Antigen Density, PCA3 score: Prostate Cancer Antigen 3.

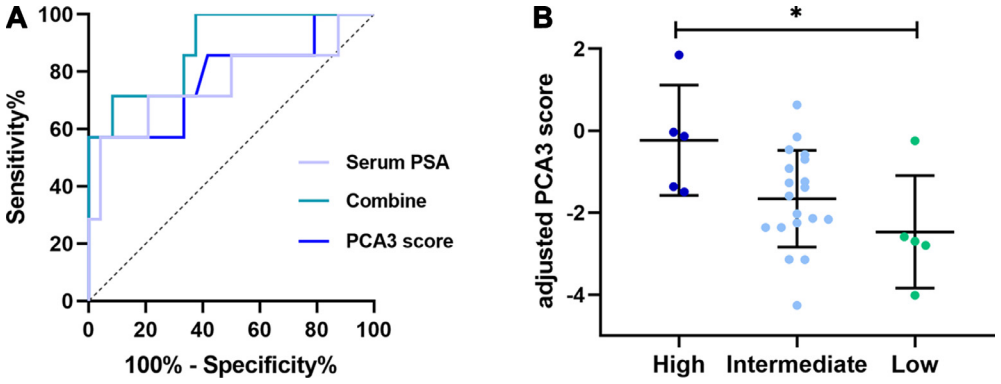

Fig. 3.. A: Receiver operating characteristic curve (ROC) of aspirated cellular PCA3 scores and the combination in differentiating metastatic PCa. B: Comparison of adjusted PCA3 scores between different risk groups. *: $P<0.05$.

mPCA3 score higher than 8.08×10^{-2} (obtained from ROC curve of mPCA3 score for differentiating metastatic PCa, Fig. S1C) was defined as positive, so that a positive mPCA3 score should indicate a higher risk of metastasis. Results showed that metastatic patients have a higher positive rate in mPCA3 score compared with localized patients with a reasonable significance (Table 2). When adjusted by age and smoking history, mPCA3 score exhibited significant capability for metastasis prediction (OR=9.316, 95%CI: 1.045–83.033, $P<0.05$), revealing that aspirated cellular mPCA3 score may be a strong indicator for metastatic status in PCa patients. Moreover, this capability can be further improved when combining with serum PSA (Fig. 3A). Results also showed that the adjusted mPCA3 score significantly correlated with risk grouping, which underscored the potential clinical significance of aspirated cellular RNA analysis for assessing PCa (Fig. 3B).

Discussion

Herein we present a novel use of fast aspirated cellular RNA analysis technique that enables rapid and accurate identification of PCa who underwent TRUS-guided needle biopsy. To our knowledge, this is the first demonstration of using unfixed biopsy specimens for aspirated cellular molecular analysis, whilst keeping the completeness of tissue structure in downstream pathologic diagnosis. The presented technique was able to distinguish between cancerous and benign features of prostate biopsies. However, the relatively poor accuracy might hinder its application in specimen identification. This may be the fact that the analytical procedure only obtained exfoliated cells from the surface of biopsies tissues, which inevitably lost some molecular information from cancerous cells, especially when the small tumor was entirely located inside the tissues. Therefore, our technique may be more suitable for RNA analysis related to cancer microenvironment, of which the pathological process occurs independently from tumor cells. Multiple studies have revealed that molecular signatures related to extracellular microenvironment rather than tumor cells themselves show great diagnostic capabilities for cancer at early stage [27–30], which may be associated with short-distance or long-distance intercellular communication that result in specific molecular alterations in other cells [31, 32]. Our further study will focus on such aspects to obtain better diagnostic performance.

The preliminary diagnostic results presented in our study (AUC 0.79 to 0.94) demonstrate the potential of fast aspirated cellular RNA analysis as an intermediate non-destructive diagnostic assessment of biopsies, providing urgent diagnostic information for clinicians to proceed with additional treatments. Currently, identification of PCa is highly relied upon the “gold standard” pathologic diagnosis. Unfortunately, the diagnostic and grading conclusions provided by histopathological techniques are often varied due to the inter-observer and intra-observer variability between pathologists [33, 34]. In this case, our results would also be of prime importance that the diagnostic results afforded by aspirated cellular RNA analysis could assist pathologists to make a more accurate pathologic result especially when the single histopathological diagnosis is quite uncertain.

While the current study was relatively simple in design and only analyzed a well reported RNA biomarker (PCA3 mRNA), more analytical systems may be developed to explore varieties of aspirated cellular molecular signatures, achieving high throughput identification and prognosis assessment of PCa. Another limitation is the small number of patients enrolled in this study, which may result in analytical bias. Therefore, further validation studies will employ a larger number of participates to more accurately and robustly determine the diagnostic performance of aspirated cellular RNA analysis. Notwithstanding, our results are encouraging, and they presented significant potentials for fast whole-biopsy review without disrupting the follow up histopathological diagnosis

Conclusion

In conclusion, our study suggests that using fast prostatic aspirated cellular RNA analysis technique may provide additional information over traditional histopathological examination, helping clinicians and pathologists improving decisions regarding cancer diagnosis and therapy.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Yiwen Sang: Methodology, Formal analysis, Writing - original draft. **Xuchu Wang:** Formal analysis, Visualization, Writing - original draft. **Pan Yu:** Formal analysis, Writing - original draft. **Luyan Zhang:** Methodology, Formal analysis. **Yibei Dai:** Methodology. **Lingyu Zhang:** Methodology. **Danhua Wang:** Methodology. **Zhenping Liu:** Methodology. **Yao Wang:** Conceptualization, Methodology. **Zhihua Tao:** Conceptualization, Methodology, Writing - review & editing, Supervision, Resources, Project administration, Funding acquisition.

Acknowledgments

Financial support for this work was provided by National Natural Science Foundation of China (Nos. 81271917 and 81902156).

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