Improving the diagnosis of prostate cancer by telomerase-positive circulating tumor cells: A prospective pilot study

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Abstract

Background Prostate-specific antigen (PSA) testing is limited in identifying prostate cancer (PCa) with modestly elevated PSA levels. Therefore, a robust method for the diagnosis of PCa is urgently needed.

Methods A total of 203 men with a PSA level of ≥4 ng/ml were eligible for enrollment in this study from July 2018 to May 2021, and randomly divided into a training set (n=78) and a validation set (n=125). Circulating tumor cells (CTCs) were detected using telomerase-based CTC detection (TBCD), and the diagnostic ability was evaluated using receiver operating characteristic (ROC) and logistic regression analyses.

Findings In the training set, the area under the curve (AUC) of CTCs was 0.842 with a sensitivity of 80.33% and specificity of 82.35%. In the validation set, the AUC of CTCs was 0.789, with a sensitivity of 79.31% and specificity of 81.58%. There was no significant difference between CTCs (AUC=0.793) and PSA (AUC=0.697) in the range of 4-50 ng/ml. In the ranges of 4-20 ng/ml and 4-10 ng/ml, the AUC of CTCs were 0.811 and 0.825, respectively, which were superior to the AUC of PSA (0.588 and 0.541). The sensitivity and specificity of CTCs in the three PSA groups were higher than 80%. Moreover, we further established a CTC+PSA combined model, which could significantly improve the diagnostic ability of a PSA level of '4-10 ng/ml'.

Interpretation TBCD could be a valuable method for distinguishing PCa and benign prostatic disease, especially in the PSA diagnostic gray area of '4-10 ng/ml'.

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Keywords: Circulating tumor cell; Prostate cancer; Prostate-specific antigen; Diagnosis; Liquid biopsy

1. Introduction

Prostate-specific antigen (PSA) is the most valuable tumor marker for prostate cancer (PCa) diagnosis and prognosis. PSA testing plays a vital role in the early diagnosis of PCa and can reduce prostate cancer-specific mortality according to European Randomized study of Screening for Prostate Cancer (ERSPC) clinical trial results [1]. However, the cost-effectiveness of PSA testing remains disputed [2]. A meta-analysis of randomized trials has shown that PSA testing causes 20.7% ~ 50.4% of patients to experience overdiagnosis and overtreatment, contributing to their anxiety and extra costs

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Research in context

Evidence before this study

PSA is the most valuable tumor marker for prostate cancer diagnosis and prognosis. However, PSA may be abnormally elevated due to benign prostatic hyperplasia, especially in "gray area" of 4-10 ng/ml, and cause unnecessary prostate biopsy. Therefore, a robust method for the early diagnosis of PCa is urgently needed.

Added value of this study

In this study, a telomerase-based prostate CTC detection method was established and applied in the differential diagnosis of prostate cancer. The data showed that CTCs had robust diagnostic efficiency for prostate cancer, especially for those in the diagnostic gray area, and the CTC plus PSA combined model could further improve diagnostic ability. These data support the use of CTCs for the differential diagnosis of prostate cancer, especially those in the diagnostic gray area of PSA.

Implications of all the available evidence

The available evidence suggests that CTC detection is ideal and promising clinical prostate cancer diagnosis approach, especially applied in "gray area" of 4-10 ng/ml PSA level. This approach could be served as an auxiliary method for prostate cancer diagnosis with high sensitivity and specificity and to decreased unnecessary biopsy. In addition, the potential usage for screening should be tested in future studies.

[3]. In addition, PSA may be abnormally elevated due to benign prostatic hyperplasia, infection, etc. [4, 5]. Thus, men with abnormal elevation of PSA often suffer false-positive results, and only 18% of patients in the diagnostic "gray area" of 4-10 ng/ml are pathologically confirmed to have PCa [6,7]. Multiparametric magnetic resonance imaging (mpMRI) showed 93% sensitivity for clinically significant PCa detection and was recommended by the European Association of Urology as a prior option to determine the need for biopsy [8]. Nevertheless, the diagnostic specificity of mpMRI was only 41% [9]. Patients with elevated PSA and abnormal mpMRI will accept prostate biopsy [8,10]. This means that some patients with benign prostatic diseases will receive an unnecessary prostate biopsy. Therefore, a robust method for the early diagnosis of PCa is urgently needed.

Liquid biopsy is a breakthrough technology for tumor detection and adjuvant therapy that detects circulating tumor cells (CTCs), circulating tumor DNA (ctDNA), and exosomes [11,12]. Compared to tissue biopsy, liquid biopsy provides several advantages: it offers quick and easy detection, is relatively noninvasive, and has a low cost [13]. Accordingly, some studies have identified CTCs in localized PCa and have shown potential clinical value [14–18]. However, most of the studies have investigated the application of CTCs for prognosis. To date, no powerful diagnostic method with high sensitivity and specificity for CTC detection in the early PCa stage has been developed.

In a previous study, we established a telomerase reverse transcriptase (TERT)-based method for CTC detection and exhibited a reliable ability to diagnose benign and malignant pulmonary nodules [19, 20]. This study shows that TERT-based CTC detection (TBCD) can efficiently identify PCa and benign prostatic disease, even among patients with abnormally elevated PSA levels in the gray zone. We further show that CTCs combined with PSA could improve the diagnostic ability for PCa.

2. Methods

2.1. Participants and study design

We enrolled a total of 203 Chinese participants with PSA levels ≥4 ng/ml from the Cancer Hospital, Chinese Academy of Medical Sciences from July 2018 to May 2021. None of the participants had a family history of PCa. One hundred eighty-four biopsy-naÿve participants had mpMRI-detected abnormalities. The other 19 biopsy-naÿve participants only received CT due to the installation of metal devices in bodies or other contraindications and found abnormalities in prostate tissues. MRI results were scored according to PIRADS version 2. All participants were randomly divided into the training set (n=78) and the validation set (n=125) and underwent CTC detection before biopsy. Then, 184 participants who received MRI underwent ultrasound-guided cognitive fusion transrectal biopsy with 12-needles systemic biopsy, and other 2-needles were performed in the tumor-suspected area according to the MRI findings. Other 19 participants who received CT underwent 12-needles systemic biopsy, and 2-needles were performed in the tumor-suspected area guided by ultrasound. All biopsy samples were evaluated by experienced pathologists based on the ISUP 2014 modified Gleason score (GS) system. A receiver operating characteristic (ROC) curve was constructed after morphologic identification of CTCs via flow imaging and FISH to determine the best cutoff value in the training set. Then, we applied this value to evaluate and compare the diagnostic performance of CTCs and PSA (Figure 1). This manuscript adheres to the reporting guidelines of the TRIPOD Checklist for Prediction Model Validation.

2.2. Virus

The oHSV1-hTERTp-GFP virus with an endogenous ICP4 promoter replaced with the hTERT promoter and with genes encoding cell protein 34.5 (ICP34.5) replaced

with the GFP gene was described in our previous work (Figure 2) [20, 21]. The purified viruses were divided into aliquots and stored at −80°C until use. We confirmed that oHSV1-hTERTp-GFP had high accuracy in various mimic CTC models and could effectively identify CTCs with 78.95% sensitivity and 96.00% specificity in patients with solid tumors in previous work [19, 20]. Graham et al. concluded that telomerase might be useful in PCa diagnosis [22], thus we applied oHSV1-hTERTp-GFP for PCa diagnosis among patients with abnormal elevated PSA.

2.3. Blood sampling and CTC detection

Four milliliters of prebiopsy peripheral blood (PB) was collected in 4 ml EDTA tubes and kept at 4°C until separation, carried out within one hour of collection. The blood samples were centrifuged at 500 g for 5 min, and the upper plasma layer was discarded. Then, red blood cell lysis buffer (0.15 M NH_4Cl, 0.1 mM EDTA, 10 mM $KHCO_3$; pH = 7.2) was added to the samples at a blood cell: red blood cell lysis buffer ratio of 1:10. The samples were centrifuged at 500 g for 5 min, the supernatant was discarded, and 5 mL sterile 1× PBS was added to resuspend and wash the cells gently. The cells were centrifuged again at 500 g for 5 min, and the supernatant was discarded. Finally, 2 mL serum-free medium was added to resuspend the cells for seeding.

Cells transduced with oHSV1-hTERTp-GFP (MOI=1) were incubated in a humidified atmosphere of 5% CO_2 at 37°C for 24 hours. The transduced cells were collected and stained with an anti-CD45 antibody. After washing with PBS, flow cytometry was used to identify CTCs. The 'CD45-/GFP+' cells were recorded as CTCs for TBCD (Figure 2, Supplementary Figure S1).

2.4. Identification of CTCs using ImageStreamX® and FISH

Two tubes of PB were treated with a standard CTC detection process. For FlowSight imaging, one tube of treated blood cells was incubated with an eFluor 450-labeled anti-CD45 antibody and APC-labeled anti-PSMA antibody and detected using the ImageStreamX® Mark II system (Amnis, USA). 'CD45-GFP+PSMA+' cells were recorded as CTCs (Supplementary Figure S2). For FISH, MACS magnetic beads were used to isolate CD45- cells. After fixation, cells were labeled with a dual-color FISH probe set consisting of a Spectrum Red-labeled probe specific for the PTEN gene on the chromosome 10q23.3 region and a spectrum Green-labeled centromere 10 (CEP10) probe (CELNOVTE FISH system). DAPI was used to stain the nuclei. Heterozygous deletion of PTEN was defined as the presence of fewer PTEN signals than CEP10 probe signals.

2.5. Study approval

This study was carried out in accordance with the recommendations of the Ethics Committee of the Cancer Institute and Hospital of the Chinese Academy of Medical Sciences with written informed consent from all subjects.

2.6. Statistics

Statistical analysis of the study data was performed with standard software (IBM SPSS Statistics 26.0, Prism GraphPad version 8.2, MedCalc Software). The non-parametric Mann-Whitney U test was used to compare the patient groups (asymmetric and continuous data). Pearson's chi-squared test was used to compare the expected and observed frequencies in two categories of a contingency table. The random selection procedure in SPSS Version 26 was used for replicating the randomization of training set and validation set 50 times to evaluate the diagnostic performance. Logistic regression was used to calculate the predictive probability for the CTC plus PSA combined method. ROC curves were constructed based on CTCs and PSA diagnostic efficiency, and the area under the curve (AUC) represented the diagnostic performance. Clinical usefulness was evaluated by decision curve analysis (DCA). Data are expressed as the mean ± SD. All P values were two-sided, with $P<0.05$ considered statistically significant.

2.7. Role of funding sources

The funder of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

3. Results

3.1. Patient characteristics

The characteristics of the participants enrolled in this study are provided in Table 1. A total of 203 patients were enrolled, among whom 148 had malignant disease and 55 had benign disease. There were 55 participants with a PSA level >10 ng/ml and 23 with a PSA level ≤10 ng/ml in the training set, 75 with a PSA level >10 ng/ml and 50 with a PSA level ≤10 ng/ml in the validation set. The mean PSA levels of the participants are provided in supplementary Table S1. There were no significant differences in age, pathological results, PSA distribution, Gleason score, or MRI between the two sets ($P>0.05$).

3.2. TBCD effectively detected CTCs in PCa

First, we analyzed the number of 'CD45-/GFP+' cells between the enrolled patients with PCa and those with benign lesions. As shown in Figure 3a, there was a statistically significant difference in the number of 'CD45-/GFP+' cells between the malignant group and

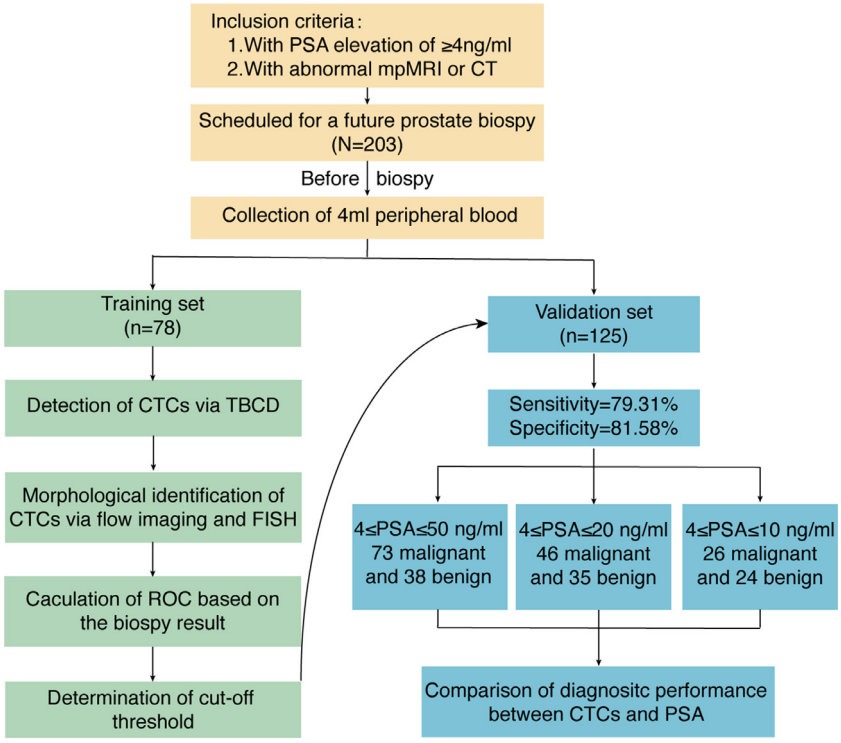

Figure 1. Study design flowchart. A total of 203 Chinese participants with PSA levels ≥4 ng/ml were enrolled in this study. All participants were randomly divided into the training set (n=78) and the validation set (n=125) and underwent CTC detection before biopsy. A receiver operating characteristic (ROC) curve was constructed after morphologic identification of CTCs via flow imaging and FISH to determine the best cutoff value in the training set. Then, we applied this value to evaluate and compare the diagnostic performance of CTCs and PSA. PSA: prostate specific antigen, mpMRI: multiparametric magnetic resonance imaging, CT: computed tomography, TBCD: TERT-based CTC detection, CTC: circulating tumor cell, FISH: fluorescence in situ hybridization, MRI: magnetic resonance imaging.

benign group (4.12 vs. 1.15 cells/4 ml PB, p<0.001). Then, we randomly assigned 38% of the enrolled patients (61 malignant, 17 benign) to the training set and 62% to the validation set (87 malignant, 38 benign) (Table 1). There were also significant differences between the malignant group and benign group in the training set and the validation set (4.59 vs. 0.82 cells/4 ml PB, p<0.001, and 3.79 vs. 1.29 cells/4 ml PB, p<0.001, respectively). The linear correlation coefficient showed that the number of CTCs did not correlate with the serum PSA level in the total PCa patients (R^2=0.06, P=0.004, Figure 3b). In addition, as shown in Figure 3c, there was no statistically significant difference between the different MRI PIRADS scores among the PCa patients. This finding suggests that CTCs could be an independent factor for the diagnosis of PCa. In this study, 69 patients underwent radical prostatectomy after biopsy, and the post-RP pathological diagnosis was consistent with biopsy results, especially in the PSA gray area of 4-10 ng/ml (Supplementary Table S3). With the disease progress, the CTC positive rate was raised. The positive rate of CTC was 79.48% (31/39), 81.48% (22/27), and 100% (3/3) for pT2, pT3 and pT4 in patients underwent radical prostatectomy, respectively. Similarly, the positive rate of CTC was 79.66% (47/59) and 90% (9/10) for pN0 and pN1 in those patients, respectively (Supplementary Figure S3). It indicated that CTCs can spread into the peripheral blood in the early stage of PCa, and can be used as a promising auxiliary diagnostic indicator. To further verify whether CTCs were derived from the primary PCa site, we performed FlowSight imaging and PTEN FISH. We first introduced prostate-specific membrane antigen (PSMA), which is expressed on the membrane of some prostate cells, to identify CTCs using FlowSight images. As shown in Figure 3d, CTCs were slightly larger than white blood cells (WBCs) and highly expressed GFP and PSMA but not CD45 ($CD45^-/GFP^+/PSMA^+$). Moreover, WBCs expressed only CD45 ($CD45^+/GFP^-/PSMA^-$). As shown in Figure 3e, PTEN FISH images (60×) were captured from the same four patients. The results showed that CTCs exhibited the typical signal configuration for heterozygous deletion of the PTEN gene with one red signal, and chromosome 10, where the deleted gene for PTEN is located, had two normal green fluorescence signals.

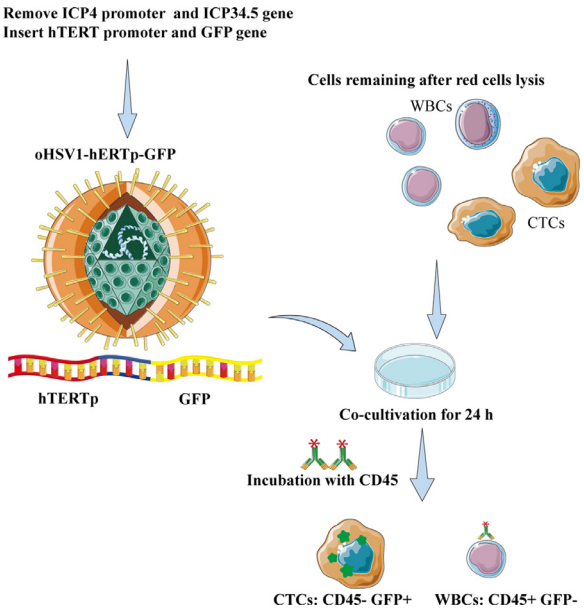

Figure 2. Flowchart of oHSV1-hTERTp-GFP construction and CTCs detection. The endogenous ICP4 promoter of oHSV1-17+ was replaced with the hTERT core promoter. Additionally, the ICP34.5 site was replaced with the GFP expression box. ICP4: immediate early protein, hTERT: human telomerase reverse transcriptase, WBCs: white blood cells, CTC: circulating tumor cell, GFP: green fluorescent protein, CD45: leukocyte common antigen, oHSV1-hERTp-GFP: oncolytic herpes simplex virus-hTERT promoter-GFP.

3.3. Determining the positive CTC threshold value in the training set

As shown in Figure 4a, ROC curve analysis showed that the AUC was 0.842 (95% CI= 0.749-0.935) in the training set. The Youden index showed that the best threshold value was ≥2 CTCs/4 ml PB, with a diagnostic sensitivity of 80.33% and diagnostic sensitivity of 82.35%, respectively (Figure 4b). As an independent test, we applied this threshold to the validation set samples (Figure 4c) and achieved an AUC of 0.789 (95% CI= 0.697-0.880), with a sensitivity of 79.31% and a specificity of 81.58% for the diagnosis of PCa from the enrolled patients.

Furthermore, to verify the reliability of the diagnostic threshold, we performed 50 random groupings and confirmed a consistent diagnostic threshold and stable diagnostic efficiency in both training and validation sets (Supplementary Table S3). As shown in Figure 4d and Table S3, the mean AUC of the training set was 0.811± 0.042, and the mean AUC of the validation set was 0.803± 0.026.

3.4. Comparison of the diagnostic ability of CTCs vs. PSA in the validation set

Subsequently, we compared the diagnostic ability of CTCs vs. PSA in the validation set. ROC analysis showed that for CTCs, the AUC was 0.789 (95% CI=0.697-0.880), with a sensitivity of 79.31% and specificity of 81.58%, and for PSA, the AUC was 0.746 (95% CI=0.659-0.832), with a sensitivity of 70.11% and specificity of 63.16%. The results showed no significant difference between CTCs and PSA in the total validation set (p=0.50) (Figure 5 a, Table 2, Supplementary Table S4). Then, we further subdivided participants into three PSA ranges, 4-50 ng/ml, 4-20 ng/ml and 4-10 ng/ml. In the PSA range of 4-50 ng/ml, the AUC of CTCs was 0.793 (95% CI=0.698-0.887), with a sensitivity of 80.82% and specificity of 81.58%, and the AUC of PSA was 0.697 (95% CI=0.598-0.796), with a sensitivity of 64.38% and specificity of 63.16%, respectively, with no significant difference observed between the two detection methods (p=0.16) (Figure 5 b, Table 2, Supplementary Table S4). In the PSA range of 4-20 ng/ml group, the AUC of CTC was 0.811 (95% CI=0.709-0.912), with a sensitivity of 80.43% and specificity of 82.86%, and the AUC of PSA was 0.588 (95% CI=0.463-0.713), with a sensitivity of 65.21% and specificity of 51.43%, respectively (p=0.0068). Notably, for the patients in the diagnostic gray area of 4-10 ng/ml, the AUC for CTCs was 0.825 (95% CI=0.703-0.946), with a sensitivity of 80.77% and specificity of 83.33%, while the AUC for PSA was 0.541 (95% CI=0.378-0.704), with a sensitivity of 57.70% and specificity of 50.00% (p=0.0061) (Figure 5 c, d, Table 2, Supplementary Table S4). These results suggest that CTCs can effectively diagnose PCa, especially those in the diagnostic gray area, and the diagnostic efficiency of CTCs was significantly better than that of PSA.

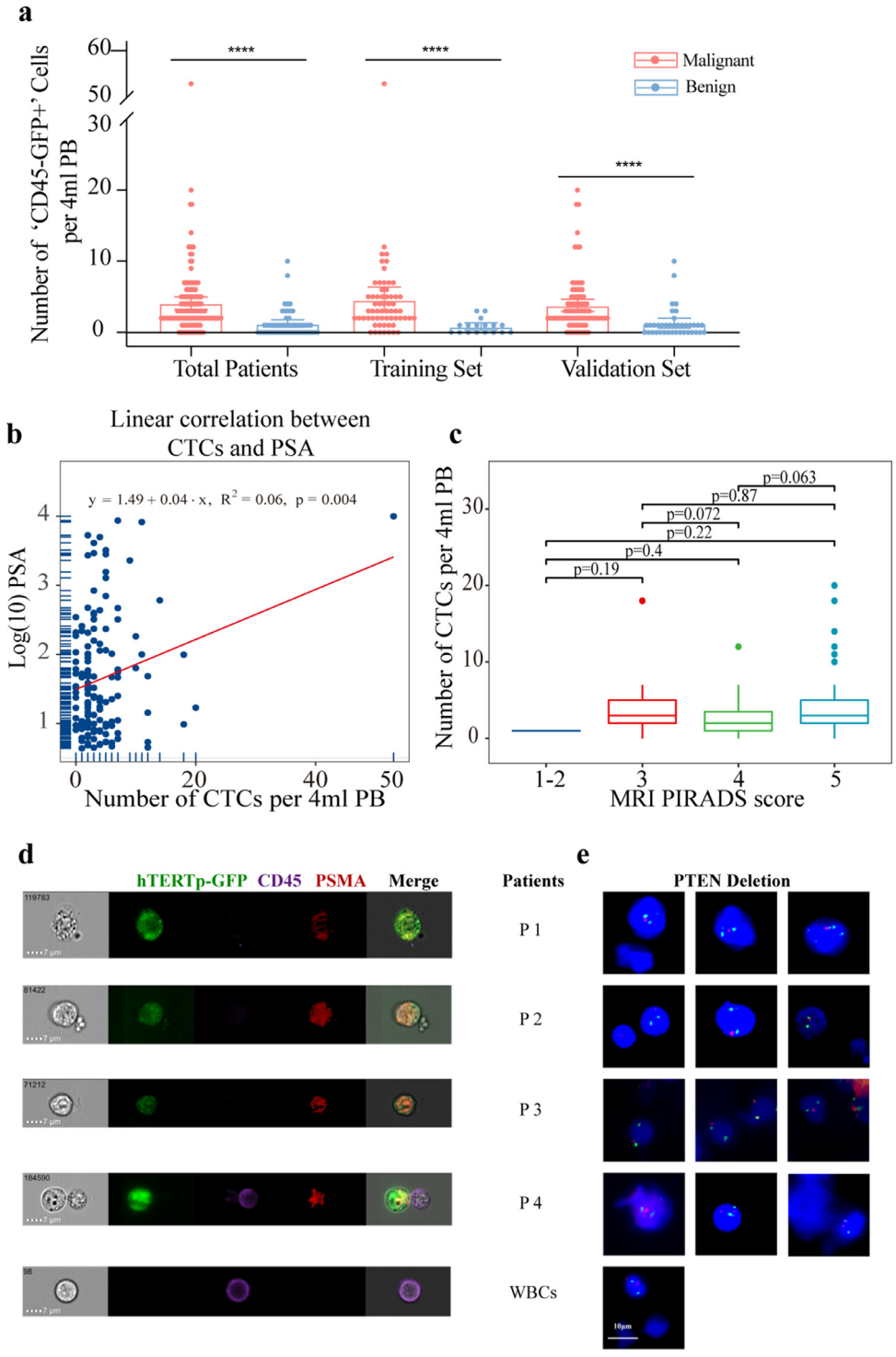

Figure 3. Effective detection and validation of CTCs in patients with PSA elevation. a. Comparison of the average number of CTCs between the malignant and benign groups. Total patients: the average number of 'CD45-GFP+' cells was 4.12±5.41 per 4 ml PB in the malignant group, and the average number of 'CD45-GFP+' cells was 1.15±1.87 per 4 ml PB in the benign group ($p<0.001$). Training Set: the average number of 'CD45-GFP+' cells was 4.59±6.98 per 4 ml PB in the malignant group, and the average number of 'CD45-GFP+' cells was 0.82±1.02 per 4 ml PB in the benign group ($p<0.001$). Validation Set: the average number of 'CD45-GFP+' cells was 3.79±3.97 per 4 ml PB in the malignant group, and the average number of 'CD45-GFP+' cells was 1.29±2.14 per 4 ml PB in the benign group ($p<0.001$). Data were shown as mean ± SD. The nonparametric Mann−Whitney U test was used to test

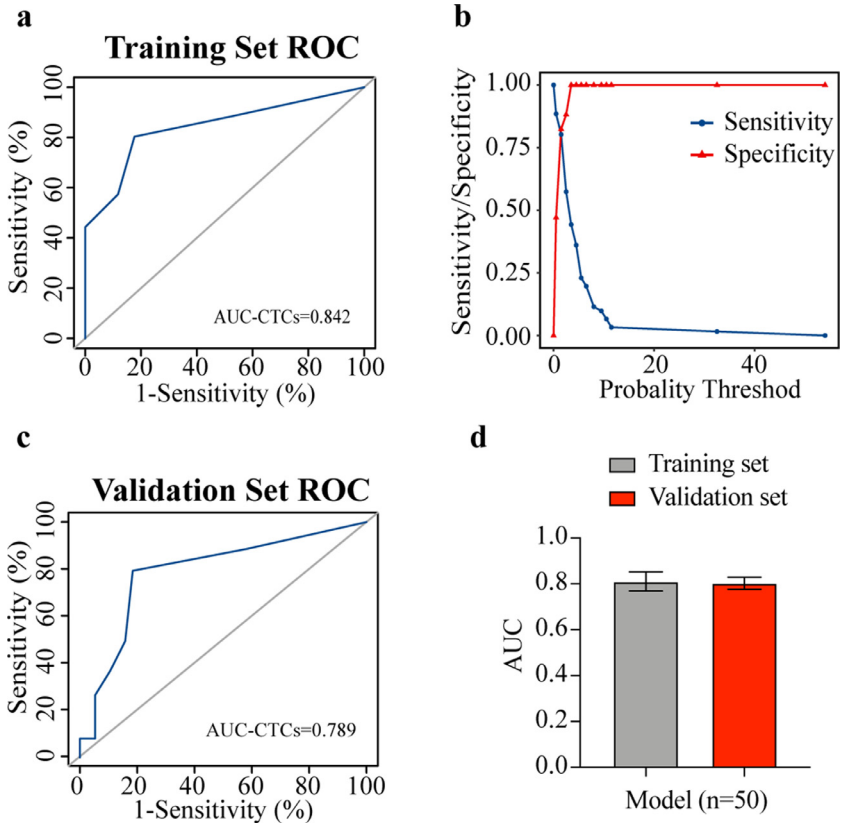

Figure 4. ROC curves of CTCs in the training set and the validation set. a. ROC curve for CTC detection in the training set. AUC=0.842; b. Youden index of TBCD-CTC in the training set. Sensitivity=80.33% and specificity=82.35% at best threshold value 2; c. ROC curve for CTC detection in the validation set. AUC=0.789; d. Diagnostic performance of the TBCD was evaluated 50 times repeats. Data were shown as mean ± SD. The nonparametric Mann−Whitney U test was used to test (categorical and continuous data). ROC curves were constructed based on the diagnostic efficiency of CTCs. ROC: receiver operating characteristic, CTC: circulating tumor cell, AUC: area under curve, TBCD: TERT-based CTC detection.

3.5. CTCs combined with PSA could enhance the diagnostic efficiency and clinical effects for PCA diagnosis in the gray area

To further verify whether CTCs could be used as a diagnostic marker for PCa clinical diagnostic application in the gray area, we combined CTCs with PSA to establish a diagnostic model in the validation set. The AUC of the CTC plus PSA combined model was 0.837 (95% CI=0.723-0.950), with a sensitivity of 76.90% and specificity of 83.33%, for CTCs, while the AUC of PSA was 0.541 (95% CI=0.378-0.704), with a sensitivity of 57.70% and specificity of 50.00% in the PSA range of 4-10 ng/ml (p=0.0035, Figure 6a, Supplementary Table S5). The FDA has approved FPSA/TPSA as an auxiliary index for detecting prostate cancer in patients with PSA levels in the range of 4-10 ng/mL. Thus, we compared the diagnostic performance of CTCs and FPSA/TPSA in that range. The results showed that the AUCs of the CTC plus PSA combined model (AUC=0.837, 95% CI=0.723-0.950) were better than that of FPSA/TPSA (AUC=0.503, 95% CI=0.335-0.671, p=0.0013) (Figure 6a, Supplementary Table S5). Finally, DCA was

(categorical and continuous data). b. Linear correlation between CTCs and PSA levels in PCa patients. R^2=0.06, p=0.004; c. Comparison of the number of CTCs between different MRI PIRADS score groups. Data were shown as mean ± SD. The nonparametric Mann−Whitney U test was used to test (categorical and continuous data). d. Images of CTCs from four representative PCa patients acquired by FlowSight imaging. CD45-eFluor 450 (purple), hTERTp-GFP (green), PSMA (red), and bright-field digital images are shown for CTCs and white blood cells (WBCs). CTCs were defined as CD45-/GFP+/PSMA+. WBCs were only marked with CD45. Scale bar, 7 μm; e. Images of CTCs from the same four PCa patients acquired by FISH. Dual-color FISH results for heterozygous PTEN (10q23) deletion in CTCs and WBCs. Scale bar, 10 μm. CD45: leukocyte common antigen, GFP: green fluorescent protein, CTC: circulating tumor cell, PSA: prostate specific antigen, PB: peripheral blood, MRI: magnetic resonance imaging, PIRADS: prostate imaging reporting and data system, hTERTp-GFP: human telomerase reverse transcriptase promoter-GFP, PSMA: prostate specific membrane antigen, PTEN: phosphatase and tensin homolog deleted on chromosome ten, WBCs: white blood cells.

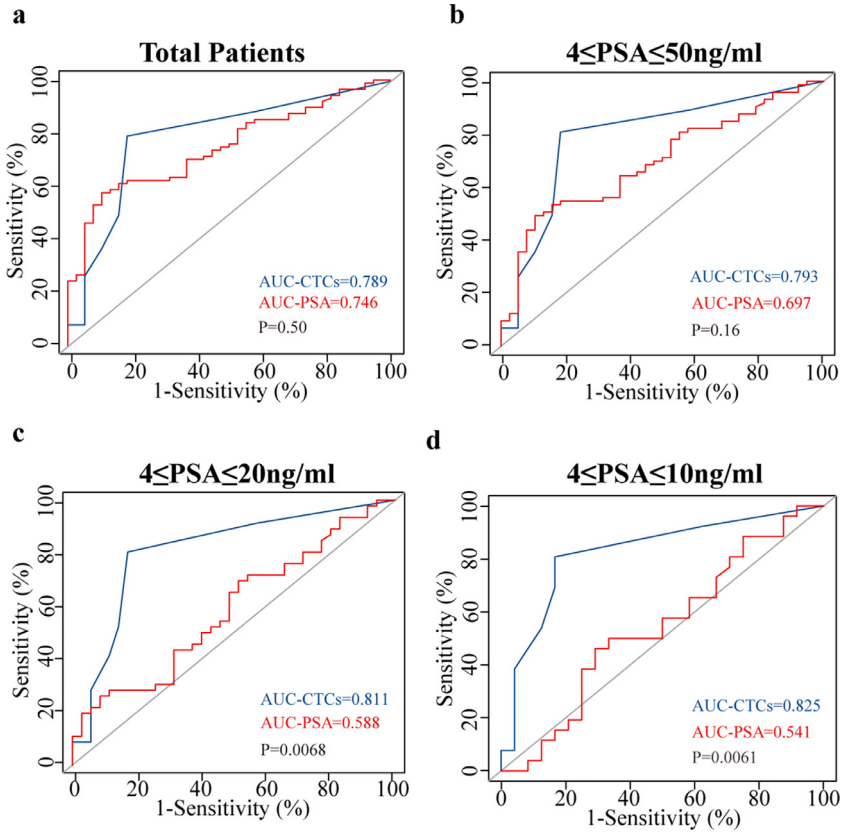

Figure 5. Comparison of ROC for CTCs and PSA within different PSA ranges in the validation set. a. ROC curves and AUC of total patients in the validation set (AUC-CTCs vs. AUC-PSA=0.789 vs. 0.746, p=0.50); b-d. ROC curves and AUC of patients with 4 ng/ml≤PSA ≤50 ng/ml (AUC-CTCs vs. AUC-PSA=0.793 vs. 0.697, p=0.16), 4 ng/ml≤PSA ≤20 ng/ml (AUC-CTCs vs. AUC-PSA=0.811 vs. 0.588, p=0.0068), and 4 ng/ml≤PSA≤10 ng/ml (AUC-CTCs vs. AUC-PSA=0.825 vs. 0.541, p=0.0061) in the validation set. ROC curves were constructed based on the diagnostic efficiency of PSA and CTCs. PSA: prostate specific antigen, ROC: receiver operating characteristic, CTC: circulating tumor cell, AUC: area under curve.

performed to compare the clinical effects of CTCs combined with PSA and PSA alone (Figure 6b, c). While PSA testing did not benefit patients, CTCs and the combined model showed good clinical benefits for patients in the gray area. Interestingly, although CTCs showed that patients could benefit clinically when the threshold was 0.2, CTCs plus PSA provided clinical benefit to patients at a lower threshold. These results show that the CTC plus PSA combined model can achieve a higher diagnostic ability and potentially benefit patients by reducing unnecessary clinical biopsies in the gray area.

4. Discussion

We conducted this prospective study to explore whether CTC detection could be used as a supplemental method for the diagnosis of PCa. This study utilized TBCD to detect CTCs among the enrolled subjects before a scheduled prostate biopsy. Our results showed that TBCD had higher and more stable diagnostic efficiency in individuals with modestly elevated PSA levels than PSA testing, especially in the PSA diagnostic gray area of 4 to 10 ng/ml. Furthermore, we constructed a combination CTC plus PSA model to further improve the clinical role of diagnosing PCa in the gray area.

PSA testing is one of the critical diagnostic methods for PCa. Previous studies have shown that PSA testing has low sensitivity when the PSA cutoff value is set at ≥4 ng/ml, and the diagnostic rate among patients with a PSA level of 4-10 ng/ml was much lower [6]. The NCCN guidelines recommend MRI combined with PSA to improve the early diagnosis of PCa among suspected cases [10]. In our cohort, since all included subjects had MRI or CT suspected of PCa, the diagnostic rate of prostate cancer was 54% in the PSA range of 4-10 ng/ml. However, 46% of patients in this range still receive unnecessary prostate puncture; thus, reducing this unnecessary biopsy is necessary. The PROMIS study also proved that MRI has superior sensitivity for early PCa diagnosis (93%) but poor specificity (41%) [9]. Interestingly, TBCD could strike a balance with

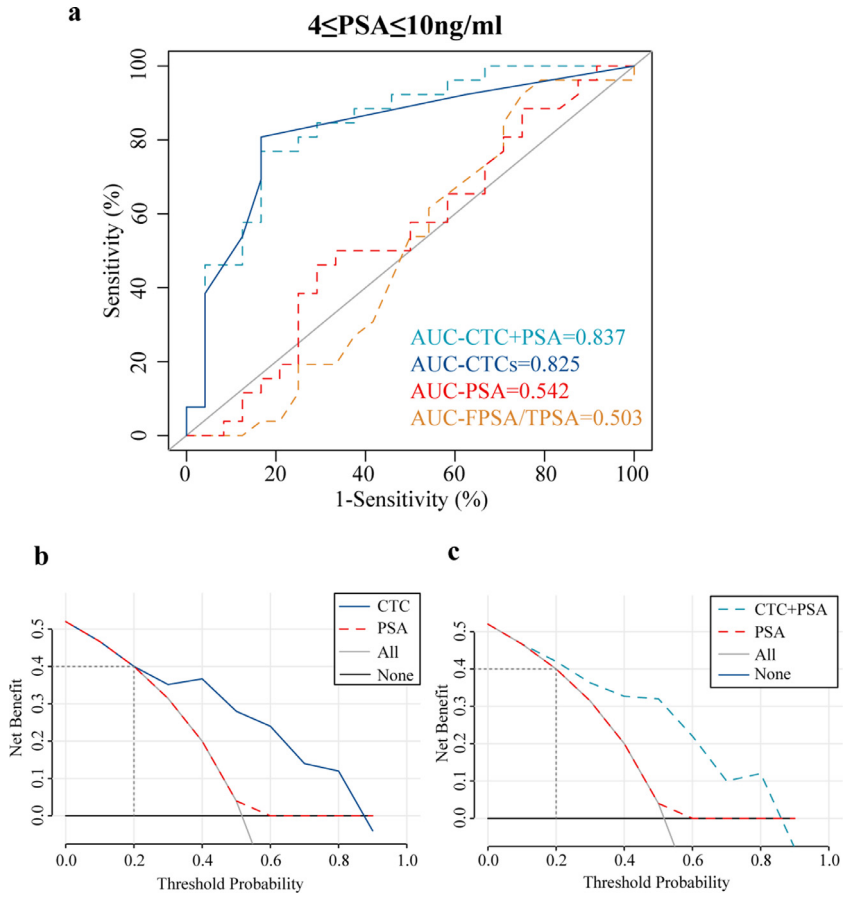

Figure 6. Comparison of ROC and DCA for the combined model and PSA in the gray area. a. ROC curves for CTC, PSA, CTC+PSA and FPSA/TPSA in patients with 4 ng/ml≤ PSA ≤10 ng/ml in the validation set; b. DCA for CTCs and PSA in patients with 4 ng/ml≤ PSA ≤10 ng/ml in the validation set; c. DCA for CTC plus PSA combined model and PSA in patients with 4 ng/ml≤ PSA ≤10 ng/ml in the validation set. ROC curves were constructed based on the diagnostic efficiency of PSA and CTCs. DCA were used for evaluating prediction models. PSA: prostate specific antigen, ROC: receiver operating characteristic, CTC: circulating tumor cell, AUC: area under curve, DCA: decision curve analysis, FPSA/TPSA: free prostate specific antigen/total prostate specific antigen.

diagnostic efficiency, showing a sensitivity of 79.31% and specificity of 81.58% in the validation set. A Chinese cohort study showed that prostate biopsy had lower positive diagnostic rates at PSA levels of <50 ng/ml [23]. Thus, we further evaluated the diagnostic ability in three PSA range subgroups, 4-10, 4-20, and 4-50 ng/ml, and showed that the sensitivity and specificity were both higher than 80%.

To further explore and improve the diagnostic efficiency of TBCD in the PSA gray area, we established a CTC plus PSA diagnostic combined model. ROC analysis showed that the combined method had a significantly higher diagnostic performance than PSA alone for patients with elevated PSA levels of 4-10 ng/ml. Although the FDA has approved the use of FPSA/TPSA for the early detection of PCa in the PSA range of 4-10 ng/mL, our results showed that the AUC of FPSA/TPSA was only 0.503, which is consistent with Sun Yinhao's results [10,24]. Moreover, the AUC of CTCs alone and the combined model were both significantly better than the AUCs of FPSA/TPSA. In addition, DCAs were also performed to confirm that TBCD and the combined model can provide valuable clinical benefit to patients. These results suggested that using telomerase-positive CTCs as an auxiliary diagnostic factor could further improve diagnostic ability and reduce unnecessary prostate biopsy.

Approaches that are noninvasive, fast, and efficient with few side effects are ideal for the early diagnosis of tumors. Therefore, CTC detection is ideal and has shown promising clinical application prospects [11,12]. The CellSearch system was approved by the FDA for CTC detection to aid in PCa prognosis. Some studies have detected CTCs using the CellSearch system in patients with localized PCa and showed that the CTC positivity rate ranged from 11% to 73% [15,25-27]. However, methods for detecting CTCs based on epithelial characteristics exclude some CTCs undergoing

Characteristics	Training set	Validation set	p
Participants, n	78	125	
Mean age, y(range)	67.53(50-84)	66.94(32-89)	0.74
Pathological results, n(%)			
malignant	61(78.2)	87(69.6)	0.18
benign	17(21.8)	38(30.4)	
PSA range, n(%)			0.13
10<PSA	55 (70.5)	75 (60)	
4≤PSA≤10	23 (29.5)	50 (40)	
PIRADS of MRI, n(%)			0.49
1-2	1(1.5)	1(0.8)	
3	15(22.7)	37(31.4)	
4	23(34.8)	43(36.4)	
5	27(40.9)	37(31.4)	
Gleason score, n(%)			
6	6(9.8)	12(13.8)	0.48
7	14(23.0)	25(28.7)	
≥8	41(69.5)	50(57.5)	

***Table ? 1*: Characteristics of the participants enrolled in this study.**
PSA: prostate specific antigen, MRI: magnetic resonance imaging, y: years, PIRADS: prostate imaging reporting and data system.

epithelial-mesenchymal transition (EMT) [28]. Our previous work established a sensitive technique for efficient CTC capture based on TERT transcriptional activity, an important pantumor hallmark highly prevalent in PCa [28,29]. Our approach achieved a CTC positivity rate of 79.73% among 148 PCa patients and showed efficient and stable detection in relatively large populations and a CTC positivity rate of 80.77% in patients in the PSA diagnostic gray zone of 4-10 ng/ml. In addition, most of these studies mainly focused on PCa prognosis or curative effects rather than diagnosis, while our study focused on applying CTCs in the diagnosis of PCA and achieved high technical sensitivity.

TERT transcriptional activity is a pantumor hallmark, which means that it is highly expressed on various malignant tumors [29, 30]. Therefore, our study enrolled patients with elevated PSA and abnormal MRI signals who were highly suspected to be diagnosed with malignant PCa. In a previous study, we modified and established a reliable oHSV1 virus as described above and used this virus to infect cells after red blood cell lysis at a multiplicities of infection (MOI) of 1 (cells: virus = 1:1), ensuring that our method has high detection sensitivity(19, 20). However, some circulating cells, such as activated T cells, can also transcribe hTERT, which may cause a high signal-to-noise ratio, limiting the specificity of TERT-based CTC capture methods. Therefore, we used the common leukocyte antigen CD45 antibody to exclude such cells and eliminate background noise. In a previous study, we tested 178 healthy donors and proved that the average number of CD45−/GFP+ cells was 0.63 per 4 ml PB for the healthy donor, which means that the 'GFP+ CD45-' background cells do not contaminate the CTCs [20]. However, some

Serum PSA level (ng/ml)	Pathology	TBCD CTC+	CTC-	Sensitivity(%)	Specificity(%)
Total	Malignant	63	17	78.75	82.86
	Benign	6	29		
4≤PSA≤50	Malignant	54	13	80.60	82.86
	Benign	6	29		
4≤PSA≤20	Malignant	34	8	80.95	84.38
	Benign	5	27		
4 ≤PSA≤10	Malignant	20	5	80.00	85.71
	Benign	3	18		

***Table ? 2*: Sensitivity and specificity of CTCs within different PSA ranges in the validation set.**
CTC: circulating tumor cell, PSA: prostate specific antigen, TBCD: TERT-based CTC detection.

patients who were pathologically diagnosed with benign lesions were positive for CTCs by TBCD, so these patients may suffer from other undetermined malignancies and need close follow-up in the future.

In addition, we acknowledge that this study has some limitations and needs further improvement: 1. Our research is only a single-center study and requires validation with larger sample sizes at more independent research centers. 2. TBCD mainly depends on the pan-tumor hallmark of telomerase transcriptional activity and lacks specificity for prostate cancer. We plan to implement virus modification tools to replace the TERT promoter with a PCA-specific promoter, such as the PSA promoter, to further improve the diagnostic specificity of this method in the future. 3. The combined CTC+PSA model could be used to further screen for the early diagnosis of prostate cancer in the future.

In conclusion, we established a reliable CTC detection method as an auxiliary indicator for PCa diagnosis with high sensitivity and specificity, especially for patients in the PSA diagnostic gray area.

Author contributions

ZRY, CYZ, DFK and GLL performed the experiments. All authors participated in designing various parts of the study and in discussion and interpretation of the results. ZRY, HSB and LJH collected the samples and clinical information. ZRY and WZ performed the data analysis. ZRY and WZ accessed the raw data. ZRY and WZ wrote the manuscript with input from all authors. WZ, JZS and KTZ supervised the study.

Data sharing statement

The data associated with this study are present in the paper or the Supplementary Materials. The CTCs raw data is also available from the corresponding authors upon reasonable request.

Declaration of Competing Interest

All authors declare no conflicts of interest relating to this study.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.eclinm.2021.101161.

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