Prostate Cancer Diagnosis Using Urine Sediment Analysis-Based α-Methylacyl-CoA Racemase Score: A Single-Center Experience

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Abstract
To evaluate the diagnostic value of α-methylacyl-CoA racemase (AMACR) score in Han Chinese patients with prostate cancer (PCa) through urine sediment analysis. We collected 292 urine sediment samples after digital rectal examination. Levels of AMACR and prostate-specific antigen (PSA) messenger RNA (mRNAs) were evaluated by quantitative real time-polymerase chain reaction. The diagnostic value of AMACR score was assessed by receiver-operating characteristic analysis (ROC), Mann-Whitney test, logistic regression analysis and decision curve analysis. In all patients (n = 292), the area under the curve (AUC) for serum PSA, AMACR score, and a combinative model of these 2 parameters were 0.745 (95% confidence interval [CI]: 0.691-0.794), 0.753 (95% CI: 0.700-0.802), and 0.784 (95% CI: 0.732-0.830). No statistical difference was found between AMACR score and serum PSA (P = .826), while the combinative model was better than AMACR score (Z = 5.222, P < .001). Among patients with serum PSA level of 4 to 10 ng/mL (n = 121), the AMACR score was significantly higher in patients with PCa (P = 0.0002), while serum PSA showed no difference (P = 0.3023). Alpha-methylacyl-CoA racemase score (AUC = 0.712, 95% CI: 0.623-0.790) and a combinative model (AUC = 0.714, 95% CI: 0.626-0.793) showed a better diagnostic value than serum PSA (AUC = 0.559, 95% CI: 0.466-0.649), (P = .048, P = .042). Decision curve analysis showed a biopsy prediction model including AMACR score have a better net benefit when the threshold probability greater than 20%. The diagnostic model combing serum PSA and AMACR score has a better diagnostic value in patients with abnormal PSA level (including PSA level ranging from 4-10 ng/mL), and could reduce unnecessary prostate biopsy in clinical use.

Keywords
prostate cancer, α-methylacyl-CoA racemase, prostate-specific antigen, biomarkers, clinical diagnosis

Introduction
As with the highest morbidity and the second fatality rate of males in the United States and Europe, prostate cancer (PCa) remains a major health challenge worldwide.¹ Prostate cancer is the most common age-related cancer, which has become a substantial health burden in China with its rapidly aging population. Prostate cancer has the sixth highest morbidity and seventh highest mortality among cancers in China, which has been growing rapidly in recent years.² Early PCa screening has been advocated when curative radical surgery or local radiotherapy is possible.³

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Currently, the mainstay screening modalities for PCa are serum prostate-specific antigen (PSA) and digital rectal examination (DRE), positive results for which generally justifies patients to be recommended for transrectal ultrasonography (TRUSG)-
guided prostate biopsy for further pathological diagnosis.

Prostate-specific antigen is particularly useful as an early
diagnostic biomarker of PCa and it is the only marker that is
clinical available for the diagnosis and prognosis prediction of
PCa currently. It has been reported that PSA screening can
decrease the mortality of PCa patients by 20%. However, PSA
represents the prostate tissue as a whole rather than being
specific for PCa, since cancer cell is not the only cell type that
produces PSA. Some benign diseases like benign prostatic
hyperplasia, or medical procedures such as TRUSG, can also
lead to an elevated PSA level. Particularly, for patients with a
PSA level arrangement grey zone (4-10 ng/mL), the detection
rate of PCa by PSA is only 25%. More importantly, the lack of
specificity of PSA and the consequent high false negative rate
could result in overdiagnosis and unnecessary treatment of PCa.

The proportion of prostate biopsy in the United States each year
after elevated PSA level was detected has been as high as 70-80%,
which is costly and invasive and may result in patient
distress and potential side effects including urinary incontinence,
erectile dysfunction, and patient anxiety. Therefore, molecular
markers with higher sensitivity and specificity are urgently
needed to improve the diagnostic accuracy of PCa, and thus
reduce the social and economic burden brought by the disease.

Alpha-methyl CoA-racemase (AMACR) is a peroxisomal and
mitochondrial enzyme, which contributes to catalyze β-oxidation
of branched fatty acids and catabolism of bile acid metabolites.
The AMACR gene, encoding 382 amino acids, is located on chro-
mosome 5p13. Previous studies have reported that AMACR is
overexpressed at both protein and mRNA levels in cancerous pro-
static tissue, with specificity and sensitivity of 97% and 100%,
respectively, suggesting AMACR to be an excellent immunohis-
tological biomarker for Pca. As an important source of prostatic
secretions and tumor shedding cells, urine sample is an essential
tool in researches on noninvasive diagnostic markers of PCa.

Materials and Methods

This study was approved by the Ethics Committee of Shanghai
Hospital, Naval Medical University (Second Military Medical
University; NO. CHEC2013-115). To conduct a retrospective anal-
ysis, we collected 292 urine samples of patients with elevated
serum PSA who visited the urology clinic from March 2011 to
April 2017 (Supplementary Table S1). Informed consents were
obtained from all patients. All patients underwent ultrasound-
guided prostate biopsy with 6 to 12 needles, and the biopsy samples
and the pathological diagnosis were confirmed by 2 senior pathol-
ologists. The study cohort consisted of both patients with positive
needle biopsy (PCa, n = 138) and non-PCa patients (n = 154).

Sample Collection and Preparation

First morning urine was collected after prostate massage before
prostate examination in all patients. The massage maneuver:
Press from both sides of the prostate to the central line for 3
times, followed by massaging the central line from top to bot-
tom for 3 times. The initial urine after micturition was collected
(at about 50 mL) and stored at 4°C, which was then centrifuged at
× 4000 rpm for 15 minutes at 4°C within 3 hours. After remov-
ing the supernatant, urine sediment was washed again with
cold PBS (× 1) and centrifuge at × 5000 rpm for 15 minutes at
4°C. Discard the supernatant, homogenize the sediment in 1.5
mL centrifuge tubes for RNA extraction or further use. Extract
the total RNA of urine sediment with the manufacturer’s
instructions (HiPure Total RNA Mini Kit, Magen, China). The
RNA concentration and purity were measured using Nanodrop
2000 (Thermo Scientific, US) and those with an RNA concen-
tration lower than 5 ng/μL were excluded. Amplify the com-
plementary DNA with TransPlex Complete Whole Transcriptome Amplification Kit (WTA2, SIGMA, China)
according to the manufacturer’s instructions.

Quantitative RT-PCR Analysis

Quantitative real-time-polymerase chain reaction (RT-PCR)
was performed to detect the expressions of AMACR and PSA
mRNAs in urinary sediment using SYBR Green Realtime PCR
Master Mix (QPK-201, Toyobo, Japan) with Applied Biosys-
tem QuantStudio 3 Real-Time PCR System (Thermo Fisher,
US) according to the manufacturer’s recommended cycling
conditions. Quantitative real-time-polymerase chain reaction
primers were designed as follow: AMACR forward primer
5’-CTGGGTGTGCGCTTTATGCTT-3’, AMACR reverse pri-
mer 5’-CCAAAGTCTTTTGATCATAGACGC-3’; PSA forward
primer 5’-GTCGACTGATTTGATGCTG-3’, PSA reverse pri-
mer 5’-GAAGCTGTGCGTACCTGAA-3’. All experiments
were performed in triplicate wells. The data were analyzed
using QuantStudio Design & Analysis Software (Thermo
Fisher, US). The expression of AMACR was represented by
the AMACR score (AMACR score = AMACR mRNA/PSA
mRNA × 1000 = Ct[PSA]−Ct[AMACR] × 1000).

Statistical Analysis

Statistical analyses were performed using SPSS Software ver-
ison 21.0 (IBM, US). To analyze the difference between patients
with positive and negative biopsy results, we performed the Mann-Whitney U test. We assessed the relationships between AMACR score and clinical variables by the Spearman rank correlation test. The regression model of AMACR score and serum PSA was established using logistic regression analysis. We also constructed the receiver-operating characteristic (ROC) curves among different groups of patients, and the predictive power of AMACR score, serum PSA and the combination of the two was evaluated by the ROC curve and area under the curve (AUC). The AUCs of different diagnostic models were compared by Pairwise comparison. The patient’s benefit threshold was assessed by the decision curve. All P values were 2-sided, and P < .05 was considered statistically significant.

Results

The Predictive Value of AMACR Score for Prostatic Biopsy Result

Among all 292 patients undergoing TRUS-guided prostate biopsy, 138 had positive and 154 had negative results with a positive rate of 47.3%. The patients had a mean age of 66 ± 7.29 years old and the mean prostate volume of them was 62.55 mL (interquartile range [IQR]: 34.31-75.12). The mean serum PSA level, fPSA level, and fPSA/PSA ratio of the patients were 26.65 ng/mL (IQR 7.31-21.93), 3.22 ng/mL (IQR 0.97-3.04), and 0.21 (IQR 0.11-0.21), respectively. Digital rectal examination revealed positive results in 76 (26%) cases.

The AMACR scores in all patients were not normally distributed, we used Mann-Whitney test to evaluate the diagnostic values of AMACR score and serum PSA level. Both parameters were found to be elevated in patients with PCa (Figure 1A, P < .001, Figure 1B, P < .001). Spearman test showed that the AMACR score was correlated with age (P = .014), serum PSA level (P < .001), fPSA/tPSA ratio (P = .047), DRE (P = .021) and PSA density (P < .001). However, serum fPSA level, prostate volume, and Gleason score were unrelated to the AMACR score (Supplementary Table S2).

We used logistic regression analysis to identify the risk factors for PCa diagnosis. However, except for AMACR score and serum PSA, other indexes showed no statistical significance in the logistic regression analysis (age, P = .185, fPSA/tPSA ratio, P = .180, DRE, P = .454, PSA density, P = .35). Therefore, a regression model of serum PSA and AMACR score was established accordingly. Among all patients, the odds ratios (ORs) of serum PSA and AMACR score were 15.78 and 5.16, which discriminated patients with
PCa from patients who had a negative biopsy result. Receiver-operating characteristic analysis (Figure 1E) was then used to evaluate the AUCs for serum PSA, AMACR score, and the combinative model of these 2 parameters (logit(\(P\)) = -1.993316 + 0.06779 × PSA + 0.00385 × AMACR score) for the discrimination between PCa and non-PCa samples. The AUCs for serum PSA, AMACR score, and the combinative model were 0.745 (95% confidence interval [CI]: 0.691-0.794), 0.753 (95% CI: 0.700-0.802), and 0.784 (95% CI: 0.732-0.830), respectively. No statistical difference (\(Z = 0.22, P = 0.826\)) was found in the comparison of diagnostic value between AMACR score and serum PSA. Nevertheless, the combinative model showed a significantly better diagnostic value compared to AMACR score alone (\(Z = 5.222, P < .001\)), which indicates that the combination of AMACR score and serum PSA can enhance the accuracy of prediction of prostate biopsy results in all patients.

The Predictive Role of AMACR Score in Patients With PSA Level of 4 to 10 ng/mL

One hundred twenty-two patients who underwent TRUS guided prostatic biopsy had PSA levels of 4 to 10 ng/mL (grey zone). Among which, 37 (47.26%) were confirmed to have positive PCa and 85 patients were negative. The mean age of these patients was 64 ± 8.19 years and the mean prostate volume was 57.35 mL (IQR: 33.12-72.10). Mean serum PSA levels, fPSA levels, and fPSA/PSA ratio in these patients were 6.57 ng/mL (IQR: 5.35-8.16), 1.39 ng/mL (IQR: 0.71-1.56), and 0.18 ng/mL (0.12-0.22), respectively. Meanwhile, DRE was positive in 30 (24.59%) cases. Serum PSA was positive in 37 (47.26%), fPSA was positive in 25 (31.7%), and fPSA/PSA was positive in 32 (39.5%) respectively. The ORs of serum PSA, AMACR score, and the combinative model were 0.559 (95% CI: 0.466-0.649), 0.712 (95% CI: 0.623-0.790), and 0.714 (95% CI: 0.626-0.793), respectively. Alpha methylacyl-CoA race- 

Evaluating the Diagnostic Performance of Established Models With Decision Curve Analysis

Decision curve analysis was conducted for biopsy prediction in base model (PSA, age, prostate volume, fPSA/PSA, and DRE) and the optimized model combining the base model and the AMACR score showing the optimized model had a better performance when the threshold probability was greater than 20% (Figure 2). Furthermore, after analyzing for different threshold to reduce unnecessary prostate biopsies, the combinative model was found to have a better net benefit when the threshold probability was greater than 20% in both the whole cohort and patients with grey-zone PSA level (Table 1), and the combinative model could avoid 12 unnecessary prostate biopsies at the predicted probability threshold value of 15% in the entire cohort (Table 2). However, no significant difference in this respect was found between these 2 models in patients with grey-zone PSA level.

Discussion

New molecular biomarkers are particularly needed in clinical practice to distinguish PCa from benign disease. Due to the limitations of PSA, the diagnostic rate of PCa in patients, especially a specific group of patients, that is, those with PSA level of 1 to 4 ng/mL, is between 16% and 39%,5,18 which results in unnecessary prostate biopsies in a great number of patients with benign diseases which increases patients’ social and economic burdens. Since prostate secretion products and shedding tumor cells are present in the urine, the discovery of potential urine molecular biomarkers for PCa is of vital importance as a non-invasive diagnostic approach for PCa. Several urine PCa biomarkers have been reported, such as fusion gene TMPRSS2: ETS, prostate cancer antigen 3 (PCA3), glutathione S-transferase P1, vascular endothelial growth factor (VEGF), matrix metalloproteinases-9, and annexin A3, among which PCA3 is the most widely used in current practice. As a long noncoding RNA, PCA3 is located on chromosome 9 (9q21-22) which has optimal diagnostic potential because it only expresses in PCa tissues. Previous studies19 have shown that detecting patients’ urine PCA3 using RT-PCR can help avoid unnecessary biopsies. Additionally, a study20 showed that the combination of PCA3 and TMPRSS2: ERG or PCA3 and PSA21 can improve the detecting rate of PCa. Notably, there are studies22,23 claiming that the diagnostic efficacy of PCA3 could be significantly different in different populations. Therefore, PCA3 is not necessarily the most suitable for the Chinese population, which warrants the need to identify molecular markers that are more suitable for the Chinese population.

Alpha-methylacyl-CoA racemase is highly expressed in PCa tissues, which has been demonstrated with good diagnostic value7,24 with a sensitivity between 82% and 100% and a specificity between 79% and 100%. However, most of the studies about AMACR were conducted on the basis of tumor tissue samples, and the relationship between AMACR expression and
PCa cannot be studied precisely due to the subjective limitation of histochemical staining score. Rogers et al. found that patients with negative prostate biopsy result could be diagnosed by elevated urine AMACR protein level; while Sroka et al. reported that the expression of AMACR protein in urine could not distinguish benign prostate diseases from PCa. In the present study, we used AMACR score firstly proposed by Ziehl et al. to evaluate its diagnostic efficacy for PCa in Chinese population.

In this study, we used 198 samples in the preliminary experiment and other 94 samples for internal validation. In the total 292 urine samples, we found that both AMACR score and serum PSA level could be used as diagnostic markers for PCa (P < .001). However, ROC analysis indicated that there was no statistical difference (Z = 0.22, P = .826) between AMACR score and serum PSA, indicating that serum PSA is still of great value in the diagnosis of PCa in Chinese population, and single usage of AMACR score has no superiority over serum PSA in PCa diagnosis.

| Probability Cut-Off (%) | Model       | PCa Missed, No (%) | Unnecessary Biopsies Spared, No (%) |
|-------------------------|-------------|--------------------|-----------------------------------|
| 15                      | Base model  | 0                  | 12 (4.1)                          |
|                         | Base model + AMACR | 0                  | 18 (6.2)                          |
| 20                      | Base model  | 2 (0.7)            | 21 (7.2)                          |
|                         | Base model + AMACR | 5 (1.7)            | 42 (14.4)                         |
| 25                      | Base model  | 7 (2.4)            | 30 (10.3)                         |
|                         | Base model + AMACR | 11 (3.8)           | 65 (22.3)                         |
| 30                      | Base model  | 19 (6.5)           | 56 (19.2)                         |
|                         | Base model + AMACR | 17 (5.8)           | 80 (27.4)                         |
| 35                      | Base model  | 27 (9.2)           | 82 (28.1)                         |
|                         | Base model + AMACR | 26 (8.9)           | 100 (34.2)                        |
| 40                      | Base model  | 35 (11.9)          | 95 (32.5)                         |
|                         | Base model + AMACR | 33 (11.3)          | 106 (36.3)                        |

Abbreviation: AMACR, α-methylacyl-CoA racemase.
Kanyong et al\textsuperscript{13} stated that the application of AMACR was limited by its low specificity on PCa diagnosis when used alone. Meanwhile, an appropriate serum PSA level to be included in the diagnostic criteria for the Chinese population is to be determined.\textsuperscript{27} Consistently, we investigated a diagnostic model combining AMACR score and serum PSA level in the Chinese population, this model was superior to the single use of serum PSA or AMACR score. Similarly, Ouyang et al\textsuperscript{15} has measured the transcription levels of PCA3, AMACR, and PSA in urine sediments in 92 patients indicating combing AMACR and PCA3 could reach a more superior diagnostic efficiency with a sensitivity of 81\% and specificity of 84\%. Jamaspishvili et al\textsuperscript{15} also reported that the combined use of 4 markers (TRPM8, MSMB, AMACR, and PCA3) could complement the limitations of single markers. Therefore, the combination of multiple indicators would be a better solution to improve the diagnostic efficiency for PCa.

Previous studies have reported AMACR was the only biomarker that could play a diagnostic role for PCa in patients with serum PSA levels among 3 to 15 ng/mL, with an AUC of 0.645.\textsuperscript{5} In this study, data on 122 patients with grey zone PSA levels (4-10 ng/mL) revealed that patients with positive biopsy results had significantly higher AMCAR scores ($P = 0.0002$), while serum PSA showed no differences for positive biopsy result, which is consistent to conclusions in previous studies. Furthermore, ROC analysis indicated that both AMACR score alone (AUC = 0.712, $P = .048$) and the combinative model of serum PSA and AMACR score (AUC = 0.714, $P = .042$) had better predictivity in grey-zone PSA patients compared to serum PSA alone. Therefore, this study suggests a limited diagnostic value of serum PSA in PSA grey zone patients while AMACR score alone or in combination with serum PSA can be more effective in the diagnosis of PCa. On the basis of the above evidence, we thus recommend AMACR score to be adopted as a clinical diagnostic marker for PCa, patients with gray-zone PSA levels.

In this study, we first demonstrated the novel model proposed in this study provided better prediction in the whole patient cohort to avoid unnecessary prostate biopsies than the basic model (18 vs 12). These results indicate that the combined use of AMACR score and basic model in all patients can reduce unnecessary biopsy without increasing the chances of missed diagnosis thereby reducing potential injuries to patients and lowering their financial burden. However, in patients with gray-zone PSA level, the optimized model was not significantly more superior than the basic model, which may be due to the limited sample size in this study.

There are several limitations in this study: Firstly, the study is limited by its single-centered data with a small number of patients; secondly, there is a lack of comparison of urine AMACR with either tissue and blood samples, or other biomarkers already reported elsewhere. Previous studies have shown that combination of multiple clinical markers can improve the diagnostic efficiency, more diagnostic indicators, such as PCA3, should be included in further studies to improve the diagnostic efficiency of this model. Therefore, the urine AMACR score needs to be verified by future studies with multicenter nature and large-scale clinical samples.

**Authors’ Note**

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This study was approved by the Ethics Committee of Changhai Hospital, Naval Medical University (Second Military Medical University). All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

**Declaration of Conflicting Interests**

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**Supplemental Material**

Supplemental material for this article is available online.

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