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RESEARCH ARTICLE

Promoter hypermethylation of Y-chromosome gene *PRKY* as a potential biomarker for the early diagnosis of prostate cancer

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ABSTRACT

Aim: To develop a methylation marker of Y-chromosome gene in the early diagnosis of prostate cancer (PCa).

Materials & methods: We utilized bioinformatics analysis to identify the expression and promoter methylation of Y-chromosome gene *PRKY* in PCa and other common malignancies. Single-center experiments were conducted to validate the diagnostic value of *PRKY* promoter methylation in PCa.

Results: *PRKY* expression was significantly down-regulated in PCa and its mechanism may be related to promoter methylation. *PRKY* promoter methylation is highly specific for the diagnosis of early PCa, which may be superior to prostate-specific antigen, mpMRI and other excellent molecular biomarkers.

Conclusion: *PRKY* promoter methylation may be a potential marker for the early and accurate diagnosis of PCa.

TWEETABLE ABSTRACT

Developing excellent diagnostic methylation markers for #prostate cancer! Bioinformatics analysis and experimental verification revealing promoter methylation of Y-chromosome gene *PRKY* is helpful to identify early prostate cancer, which may be superior to other molecular biomarkers.

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KEYWORDS
Early diagnosis; *PRKY*; Promoter methylation; Prostate cancer; Y-chromosome

1. Background

Prostate cancer (PCa) is the second leading cause of cancer death among men in USA, with nearly 260,000 new cases and 30,000 deaths, according to cancer statistics in 2022 [1]. PCa patients have no apparent symptoms in the early stage and most are in the middle or late stages at the initial diagnosis. Some patients quickly develop metastatic castration-resistant prostate cancer after treatment, which is highly invasive, incurable and has a poor prognosis [2]. Therefore, early diagnosis and treatment are important for PCa patients. Currently, the routine screening methods for PCa are serum prostate-specific antigen (PSA) and digital rectal examination (DRE) [3]. DRE can only palpate the part of the prostate adjacent to the rectum and the results are affected by the subjective judgment of clinicians; consequently, approximately 20% of PCa patients have a positive [4]. PSA screening is the primary method for detecting PCa early, but its low specificity frequently leads to unnecessary biopsies and overtreatment [5]. Previous studies have confirmed that multiparameter MRI (mpMRI) has high accuracy for diagnosing PCa, especially for clinically significant PCa, but for T1 or T2 tumors and patients in the PSA gray zone, mpMRI still has a certain rate of missed diagnosis [6]. The gold standard for PCa diagnosis is a prostate biopsy, but as an invasive procedure, it causes significant suffering for patients, including rectal bleeding, pain in the operating area, sepsis and other complications [7]. Therefore, it is warranted to search for a noninvasive, highly sensitive biomarker that can be used to diagnose early PCa.

DNA methylation plays an important role in the biological behavior of human malignant tumors. DNA methylation status of certain genes can be used as potential tumor biomarkers for risk prediction, diagnosis, prognosis and efficacy assessment of cancer [8]. Aberrant DNA methylation primarily includes promoter hypermethylation of the tumor suppressor gene, hypomethy-

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lation of the oncogene promoter and genome-wide DNA hypomethylation in advanced malignant tumors [9]. Studies have demonstrated that hypermethylation of gene promoters is one of the most common somatic genomic changes during the occurrence and development of PCa, frequently leading to loss of gene expression or function. Silencing of these genes is related to the initiation, progression, invasion and metastasis of PCa, with *GSTP1* being the most frequently silenced gene [10]. Aberrant DNA methylation is believed to occur in the early stages of PCa. Since these changes can be evaluated in several body fluid samples, liquid biopsy based on DNA methylation is a promising method for detecting precancerous lesions or early cancer cells and assessing prognosis [11]. Paziewska *et al*. [12] discovered that the methylation of *APC*, *TACC2*, *RARB*, *DGKZ* and *HES5* gene promoters had high sensitivity and specificity for diagnosing PCa, with an area under the curve (AUC) of 0.95–1 for the receiver operating characteristic (ROC) analysis. It is superior to gene expression (*HOXC6*, *AMACR* and *PCA3*) in distinguishing PCa from BPH, indicating that DNA methylation is promising and even more reliable than gene expression in prostate biopsy cancer detection. However, even the most thoroughly studied *GSTP1* gene has yet to be used in clinical practice, highlighting the critical need for further development of DNA methylation biomarkers [13].

Based on The Cancer Genome Atlas (TCGA) database, some researchers have also reported that there exist extreme down-regulation (11.1%) of chromosome-Y gene expression and loss of chromosome-Y (7.7%) in cancer tissue compared with normal tissue in PCa samples [14]. These changes may promote tumor progression, leading to the disappearance of the anticancer effect of these genes. PCa is a male-specific malignant tumor and the Y-chromosome exists only in males. Therefore, we speculated that DNA methylation biomarkers on the Y-chromosome might be related to the PCa diagnosis and analyzed them using various databases. We found that methylation levels in *PRKY* promoter are highly associated with PCa through a series of analyses, analyzed the correlation between methylation levels and clinicopathological characteristics and further explored the value of PRKY promoter methylation in the early diagnosis of PCa.

2. Materials & methods

2.1. Data sources & processing

In this study, list of 253 genes on the Y-chromosome was obtained from the University of California Santa Cruz Xena (https://xenabrowser.net/datapages/). These gene expression data (version 2017.10.13; Platform: IlluminaHiSeq_RNASeqV2) in prostate adenocarcinoma (PRAD) and eight common malignancies in men were downloaded from TCGA database (https://portal.gdc.cancer.gov/) and analyzed using the Gene Expression Profiling Interacting Analysis (GEPIA; http://gepia.cancer-pku.cn). The gene expression data of *PSA*, *AMACR*, *PCA3* and *PRKY* in PCa tissues and adjacent normal tissues were downloaded from TCGA, International Cancer Genome Consortium (ICGC: https://dcc.ICGC.org/releases) and Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/): GSE32571 (including Illumina HumanHT-12 V3.0 expression beadchip data of 59 PCa and 39 matched benign tissue samples), GSE60329 (including Agilent-028004 SurePrint G3 Human GE 8 × 60 K Microarray data of 14 benign prostate tissues and 54 PCa tissues), GSE70770 (including Illumina HumanHT-12 V4.0 expression beadchip data of 220 PCa tissues, 73 normal prostate tissues) and GSE88808 (including Illumina HumanHT-12 WG-DASL V4.0 expression beadchip data of 49 benign prostate tissues and 49 PCa tissues).

The methylation data (Infinium 450K Methylation Array; Platform: GPL13534) of *PRKY* promoters for nine common malignancies in males was downloaded from TCGA database was processed with University of Alabama at Birmingham Cancer (UALCAN) data analysis Portal (http://ualcan.path.uab.edu/index.html). *PRKY* promoter regions were defined as 1500 bp upstream from the transcriptional start site (TSS) and methylation probes cg05163709, cg08045599, cg05618150, cg20401549 and cg09546548 were used as the representative probes to study *PRKY* promoter methylation status, respectively. Methylation data of CpG sites (cg05163709, cg08045599 and cg05618150) located in the *PRKY* promoter (TSS1500) and clinical data of patients were downloaded from the UCSC Xena database (Infinium 450K Methylation Array; Platform: GPL16304) and the GEO database: GSE76938 (including Infinium 450K Methylation Array data of 63 benign prostate tissues and 73 PCa tissues), GSE112047 (including Infinium 450K Methylation Array data of 16 benign prostate tissues and 31 PCa tissues), GSE73549 (including Infinium 450K Methylation Array data of 57 PCa tissues, 14 normal prostate tissues, 18 tumor-positive lymph nodes, two prostatic intraepithelial neoplasia and one tumor-negative lymph node) and GSE101908 (including Infinium Methylation EPIC Array data of 21 benign prostate tissues and 21 PCa tissues).

2.2. Clinical samples

Tissue samples used in this study were collected in The Second Affiliated Hospital of Soochow University. Inclusion Criteria Patients who meet the following criteria

will be included in the study: (1) Patients with clinically suspected PCa were scheduled to undergo a prostate biopsy. (2) All patients had no other malignant tumors. (3) Baseline clinical variables were collected from the electronic medical record system, including age, total PSA (tPSA), free PSA (fPSA), the ratio of fPSA to tPSA (f/tPSA), DRE results, prostate volume, prostate Imaging Reporting and Data System (PI-RADS) and pathology reports.

tissue specimens were obtained under transrectal ultrasonography guidance by experienced urologists. All patients underwent an ultrasound-guided, systematic prostate biopsy. Meanwhile, we examined the promoter methylation level in two punctured tissues, which was further obtained by prostate needle biopsy from each patient. One puncture tissue was highly suspected PCa lesions under ultrasound and the other was considered benign. If there is a diffuse lesion in the prostate under ultrasound imaging or no suspected lesion can be found, we only puncture one needle of prostate tissue, which is highly suspected to be cancerous or normal tissue. A total of 20 patients were enrolled. Among them, a total of 26 prostate needle biopsy tissues were obtained from 13 patients and 7 tissues were obtained from 7 patients, for a total of 33 needles. The collected tissue samples were embedded in paraffin and then 10–15 pieces of 5-um-thick tissue were cut and put into a 1.5-ml centrifuge tube.

2.3. DNA extraction & bisulfite conversion

We extract DNA from the centrifuge tube according to the instructions of the DNA Rapid Extraction Kit (Qiagen Company, Hilden, Germany), pack the collected DNA samples into 3 ul for quality inspection and repackage the DNA samples that meet the standard.

2.4. Quantitative PCR

Based on the DNA sequence after bisulfite conversion, gene-specific primers and probes were designed and synthesized by Wuxi Regular Precision Medicine Testing Company. We determined the methylation level of cg05163709, cg08045599 and cg05618150 in PRKY promoter and the ACTB gene was used as a methylation reference gene. The quantitative PCR (qPCR) Master Premix was prepared according to the number of samples to be tested, including positive quality control, negative quality control and RNase-Free ddH2O. Take a 96-well plate and PCR Master Premix was divided into each well. There are two methylation qPCR reaction systems: the cg05163709/cg05618150 reaction system (15 ul premix and 10 ul template are added to each well) and the cg08045599/cg05618150 reaction system (15 ul premix and 10 ul template), then seal the cap and mix the tube gently. The premixed solution was made according to Table 1. The real-time fluorescence quantitative PCR detection was performed using an ABI 7500 real-time PCR amplification instrument. The PCR conditions consisted of pre-denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 15 s and fluorescence data were collected during the 56°C annealing/elongation step (cg05163709 and cg05618150 were input into the FAM channel, cg08045599 was the CY5 channel and ACTB was the VIC channel). Fluorescence data were analyzed by the ABI 7500 real-time PCR system and expressed as Ct, the number of cycles needed to generate a fluorescent signal above a predefined threshold. An increase in the fluorescence signal was detected when methylation occurred at the CpG site and the number of PCR cycles correlated with the CpG methylation level. The results were valid only when the methylation Ct values of cg05163709, cg08045599 and cg05618150 were less than 45 and the Ct values of the internal reference gene (ACTB) were less than 25. The ROC curve for PCa diagnosis was drawn according to the ΔCt value, which is the difference between the Ct values of the target and reference gene (ACTB) normalized to the amount of DNA and the best cutoff point for sensitivity and specificity was selected by the Jorden index.

2.5. Statistical analysis

Continuous variables were analyzed using unpaired *t*-test or nonparametric Mann–Whitney U test after testing for normality distribution using Shapiro–Wilk's test. The mRNA expression data of all genes in cancers were normalized using $\log_2$ transformation. Correlation analysis was evaluated using Pearson correlation analysis and $|R| > 0.2$ or $P < 0.01$ indicated a significant correlation. The ROC curve was established to evaluate the diagnostic value of CpG methylation in PCa patients. Graph design and statistical analyses were performed using GraphPad Prism, version 8.2 (GraphPad Software, CA, USA), MedCalc Software, version 14 (MedCalc Software bvba, Ostend, Belgium) and R Studio v1.2.5033 package ggplot2 (RStudio Inc., MA, USA).

3. Results

3.1. Identification of DEGs on the Y-chromosome in PCa

Based on the UCSC Xena database, we obtained 253 genes on the Y-chromosome, including protein-coding genes, pseudogenes and long noncoding RNA genes. An online analysis of the GEPIA database revealed that five genes (*LINC00106*, *ASMTL-AS1*, *AKAP17A*, *DDX3Y* and *PRKY*) were differentially expressed between cancer and normal tissues in PRAD and other eight types of malignant

Table 1. Elements of the premixed solution in qPCR.

cg05163709/cg05618150 reaction system	Volume (ul)	Name	Volume (UI)
qPCR Mix	12.5	qPCR Mix	12.5
PPmix1	2.5	F-cg05163709	0.3
		R-cg05163709	0.3
		P-cg05163709	0.2
		F-cg05618150	0.3
		R-cg05618150	0.3
		P-cg05618150	0.2
		F-ACTB	0.25
		R-ACTB	0.25
		P-ACTB	0.15
		ddH2O	0.25
Total	15	Total	15
cg08045599/cg05618150 reaction system	Volume (ul)	Name	Volume (UI)
qPCR Mix	12.5	qPCR Mix	12.5
PPmix2	2.5	F-cg05618150	0.3
		R-cg05618150	0.3
		P-cg05618150	0.2
		F-cg08045599	0.3
		R-cg08045599	0.3
		P-cg08045599	0.2
		F-ACTB	0.25
		R-ACTB	0.25
		P-ACTB	0.15
		ddH2O	0.25
Total	15	Total	15

tumors with a higher incidence in males than females: PRAD, stomach adenocarcinoma, lung adenocarcinoma (LUAD), lung squamous cell carcinoma (LUSC), liver hepatocellular carcinoma (LIHC), colon adenocarcinoma (COAD), rectal adenocarcinoma (READ), esophageal adenocarcinoma (ESCA) and bladder urothelial carcinoma (BLCA) (Figure 1A). Further analysis demonstrated that the *ASMTL-AS1* and *PRKY* gene expressions were significantly lower in PCa tissues than in normal prostate tissues, with *PRKY* being the best distinguishing between PCa and normal tissues (Figure 1B). We downloaded four raw data sets (GSE32571, GSE60329, GSE70770 and GSE88808) from the GEO database and drew box diagrams for verification. The results revealed that *PRKY* was dramatically downregulated in PCa tissues, consistent with the analysis in the TCGA database (Figure 1C). Finally, we selected *PRKY*, the most differentially expressed gene, for further study.

3.2. PRKY promoter is hypermethylated in PCa

The expression of *PRKY* gene is significantly decreased in PCa tissues and the most common reason for its low expression may be associated with hypermethylation of gene promoter. To evaluate the methylation level of the PRKY promoter in PCa, the UALCAN was used to process the data from TCGA database. The promoter methylation level of *PRKY* was significantly higher in PCa than in normal tissues (Figure 2A). Detailed results exhibited that the methylation levels of CpG sites cg08045599 and cg05168150 differed significantly between PCa and normal tissues (Figure 2B & C) and *PRKY* expression negatively correlated with the methylation of these two CpG sites (Figure 2D & E). According to the literature, there was a significant difference of methylation level of another CpG site (cg05163709) located in the *PRKY* promoter differed significantly between PCa and normal tissues [15]. Thus, we further obtained methylation data of cg05163709, cg08045599 and cg05618150 from the GEO database (GSE76938, GSE112047 and GSE73549), analyzed the methylation levels of these three sites in PCa and normal tissues. The results from three different laboratories revealed that the methylation level of CpG sites was higher in PCa than in normal tissues (Figure 2F–H).

3.3. Promoter methylation of PRKY does not change in other malignancies

Based on the UALCAN database, We analyzed the difference in *PRKY* promoter methylation levels between normal and cancer tissues in eight common malignancies with a higher incidence in males than females, including LUAD, LUSC, LIHC, COAD, READ, ESCA, BLCA, pancreatic adenocarcinoma. Because Y-chromosome DNA methylation data should only be analyzed in samples possessing a Y-chromosome, we only analyzed Y-chromosome *PRKY* methylation data in male samples of additional tumors to reduce the interference of the *PRKX* gene [16]. The results revealed that the promoter methylation level of *PRKY* was increased in LUSC than in normal tissues, but the difference was not statistically significant ($p > 0.01$ or Δmedian <0.1). In contrast, the methylation level

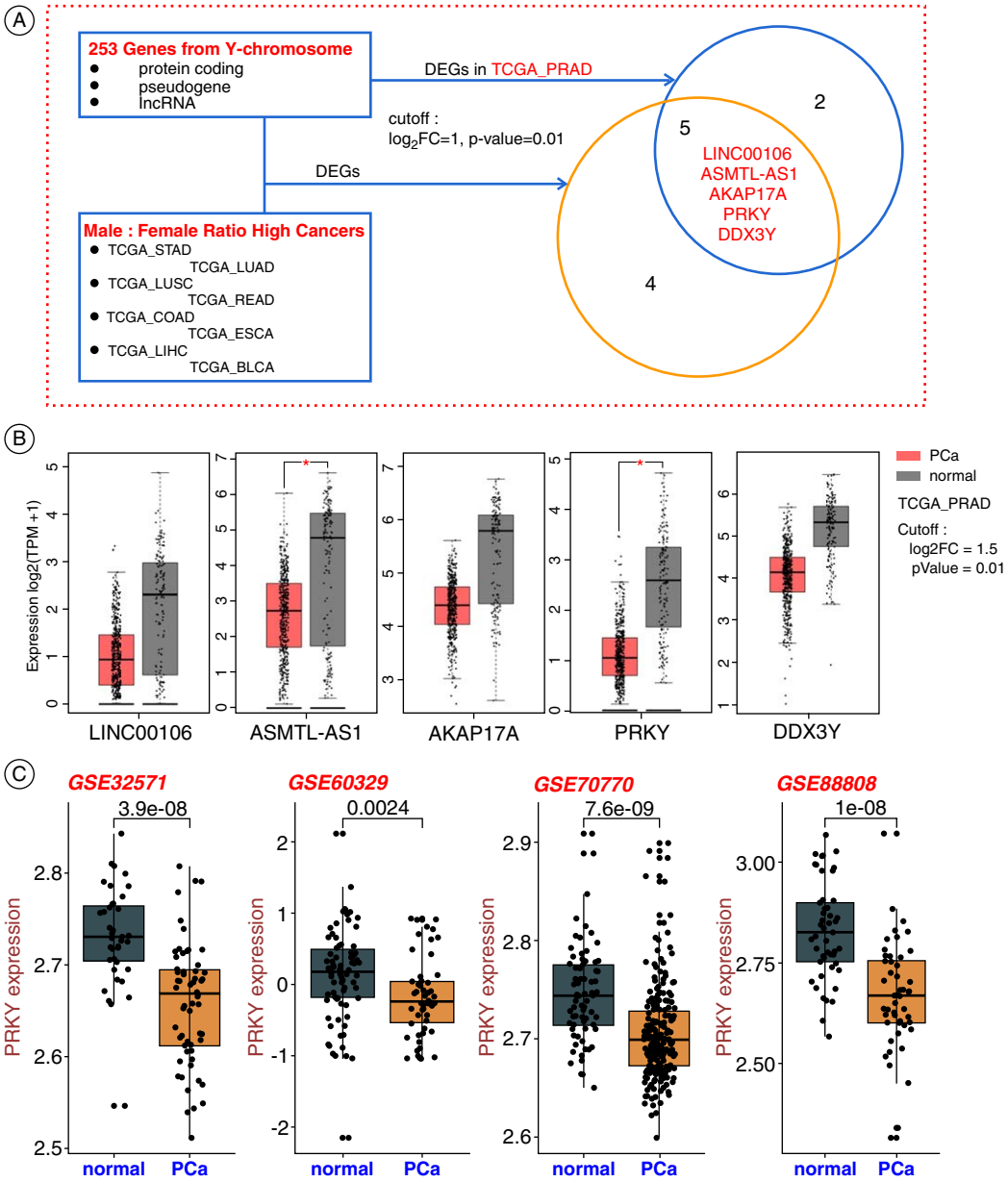

Figure 1. Identification of DEGs on the Y-chromosome in PCa. **(A)** Venn diagram of the intersections of DEGs in nine cancer types from the TCGA database. $|\log_2 FC| = 1$ and p-value = 0.01 were set as the cut-off criteria. **(B)** The GEPIA database revealed five candidate gene expressions between PCa and normal tissues, including *LINC00106*, *ASMTL-AS1*, *AKAP17A*, *DDX3Y* and *PRKY*. $|\log_2 FC| = 1.5$ and p-value = 0.01 was set as the cut-off criteria. **(C)** *PRKY* gene expression differences between PCa and normal tissues in the GEO database (GSE32571, GSE60329, GSE70770 and GSE88808).
DEG: Differentially expressed gene; GEO: Gene Expression Omnibus; PCa: Prostate cancer; TCGA: The Cancer Genome Atlas.

of the *PRKY* promoter was lower in the other seven malignant tumors than that in normal tissues and the difference was statistically significant only in COAD and READ. ($p < 0.01$ and median >0.1). Overall, there was no significant increase of promoter methylation level of *PRKY* in male samples of LUAD, LUSC, LIHC, COAD, READ, ESCA, BLCA and pancreatic adenocarcinoma (Figure 3A–H).

3.4. *PRKY hypermethylation can be detected in very early stage of PCa*

Based on TCGA database, we compared the CpG methylation level of *PRKY* promoter in normal tissues to in PCa tissues of different clinicopathological features. The results demonstrated that the methylation levels of cg08045599 and cg05618150 sites of PRKY promoter

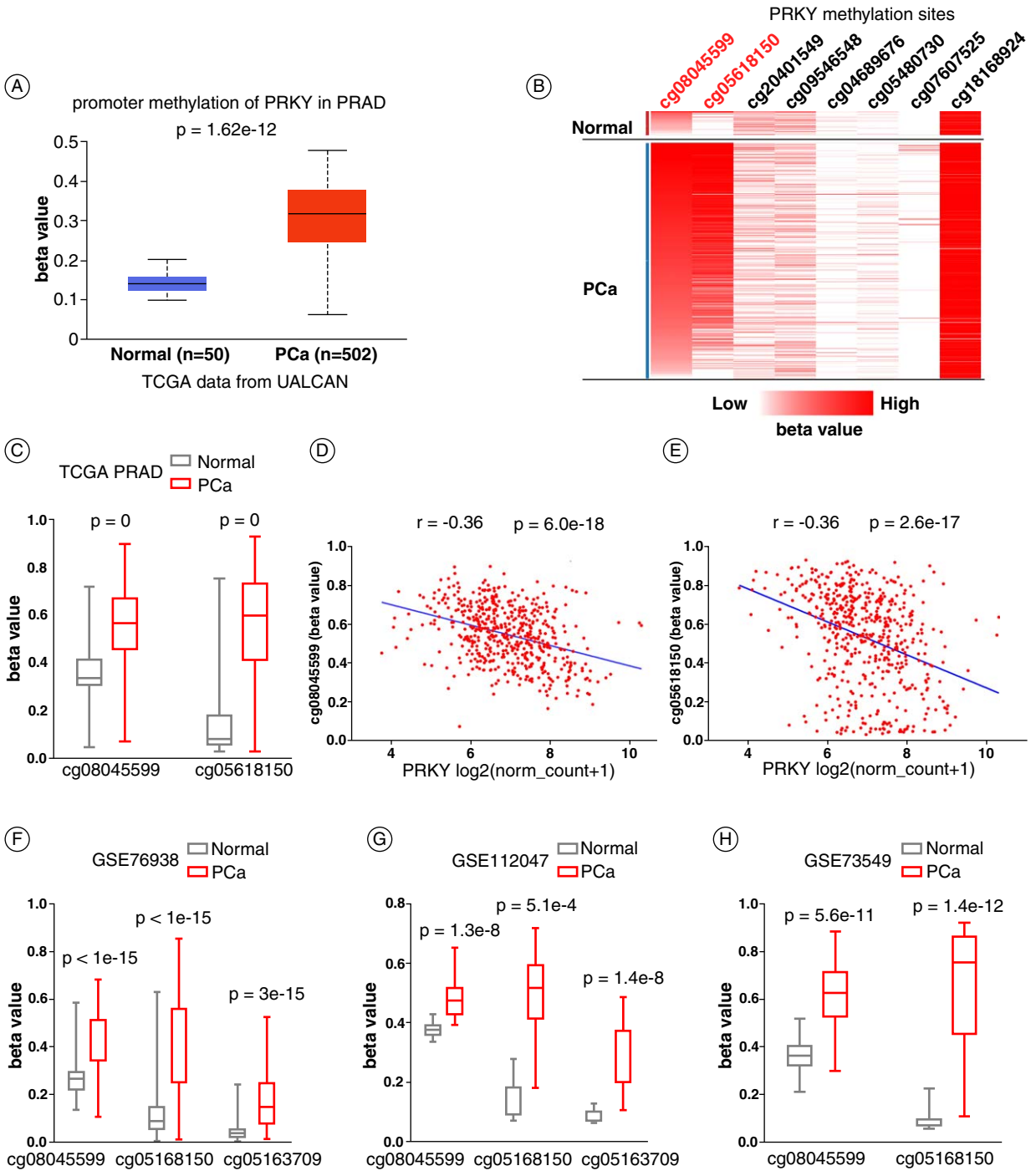

Figure 2. *PRKY* promoter is hypermethylated in PCa. **(A)** Methylation level of *PRKY* promoter between PCa and normal tissues from the UALCAN database. **(B)** Methylation levels of eight CpG sites in *PRKY* promoter between PCa and normal tissues from the UCSC Xena database. **(C)** Methylation levels of two CpG sites (cg08045599 and cg0516150) in PCa and normal tissues from the TCGA database. **(D)** Correlation between *PRKY* gene expression and cg08045599 methylation. **(E)** Correlation between *PRKY* gene expression and cg05618150 methylation. **(F–H)** Methylation levels of three CpG sites (cg08045599, cg0516150 and cg0516709) in PCa and normal tissues from the GEO database (GSE76938, GSE112047 and GSE73549).
GEO: Gene Expression Omnibus; PCa: Prostate cancer; UALCAN: University of Alabama at Birmingham Cancer.

were significantly higher in PCa tissues than in normal tissues ($p < 0.0001$), regardless of whether patients were in early TNM stage (T1& T2, N0 and M0) or late Tumor-Node-Metastasis (TNM) stage (T3 & T4, N1 and M1). The methylation levels of cg08045599 and cg05618150 sites were significantly higher in PCa tissues of patients with different PSA value ranges (PSA <4, 4 < PSA <10, PSA >10) than in normal tissues ($p < 0.0001$). The

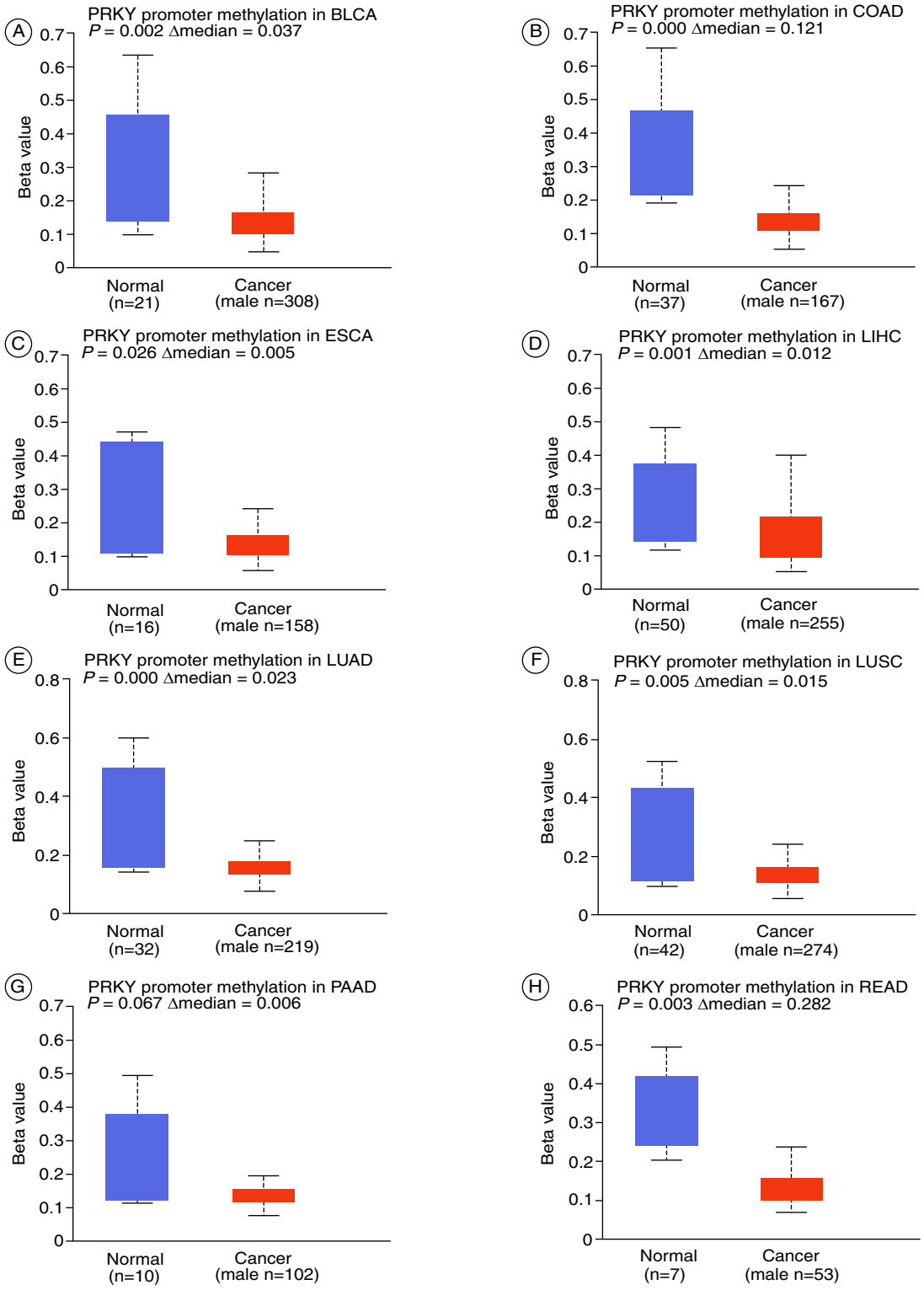

Figure 3. Promoter methylation of *PRKY* does not change in other malignancies. **(A–H)** The UALCAN database revealed methylation levels of *PRKY* between normal and cancer tissues in male samples of eight malignant tumors (LUAD, LUSC, LIHC, COAD, READ, ESCA, BLCA and PAAD). $p < 0.01$ and Δmedian > 0.1 indicated a significant difference.
BLCA: Bladder urothelial carcinoma; COAD: Colon adenocarcinoma; ESCA: Esophageal adenocarcinoma; LIHC: Liver hepatocellular carcinoma; LUSC: Lung squamous cell carcinoma; PAAD: Pancreatic adenocarcinoma; READ: Rectal adenocarcinoma; UALCAN: University of Alabama at Birmingham Cancer.

methylation levels of cg08045599 and cg05618150 sites were significantly higher in PCa tissues with different Gleason scores (Gleason = 6, Gleason = 7, Gleason = 8–10) than in normal tissues ($p < 0.0001$, Figure 4A–E).

Based on the above results, PRKY promoter hypermethylation can be detected in PCa tissues in the early stages. Therefore, we further explore the clinical value of *PRKY* promoter methylation and mpMRI in the early diagnosis of PCa. We downloaded the GSE101908 [17] dataset from the GEO database. The study included 42 prostate needle biopsy tissues from six men with PCa who underwent radical prostatectomy and these tissues were confirmed to be benign or malignant by pathological examination, with a total of 21 PCa tissues and 21 normal tissues. Postprostatectomy, the prostate was inked and then serially sectioned from apex to base. A slice ~1 cm thick was obtained from the mid-prostate. This slice was randomly sampled via punch biopsy to yield 8–12 cores, with core locations marked and photographed. Each core underwent standard hematoxylin and eosin (H&E) staining and was submitted for pathological review. A consultant pathologist reviewed the H&E slides and marked the tumor area for the dissection process. Meanwhile, mpMRI correlation to whole-mount histopathology was performed by identifying the axial plane in T2W images corresponding to the histopathology section from which the cores were obtained. The location of each core was mapped on T2W axial scans and their visibility or invisibility was determined. In the end, 42 cores and 6 H&E whole-mount sections with tumor-marked areas were identified. All patients had mpMRI and methylation chip data. The data showed that many lesion areas were nonvisible in mpMRI and diagnosed as PCa after a subsequent systematic needle biopsy. Only 16 of the 21 positive biopsy tissues could be identified using mpMRI, while the remaining five could not be recognized. We analyzed the promoter methylation of the cg05618150 site in these prostate needle biopsy tissues. Figure 4F illustrates one typical patient with eight prostate needle biopsy tissues, of which three were PCa tissues and five were normal tissues confirmed by pathology. Only one of the three PCa lesions could be identified using mpMRI. We integrated mpMRI, biopsy pathology and cg05618150 methylation data to draw bar graphs. The results revealed that the methylation level of cg05618150 in PCa tissues was significantly higher than that in normal prostate tissues and significant hypermethylation of cg05618150 could be detected in PCa lesions that could not be recognized by mpMRI. Figure 4G integrates the statistics of all 42 prostate needle biopsy tissues, including mpMRI, biopsy pathology and methylation (cg05163709 and cg08045599) data. The results showed that the methylation levels of cg05618150 and cg05163709 in PCa tissues were significantly higher than those in normal prostate tissues and significant hypermethylation of cg05618150 and cg05163709 could be detected in PCa lesions that could not be recognized by mpMRI.

3.5. Diagnostic efficacy of PRKY methylation

To evaluate the efficacy of CpG site methylation at *PRKY* promoter for PCa diagnosis, we compared *PRKY* methylation with *PCA3* and *AMACR*. These two genes have been extensively studied in recent years and are considered as potential markers for the early diagnosis of PCa. Based on TCGA database, we drew the ROC curve, which compared the sensitivity and specificity of *PSA* mRNA, *AMACR* mRNA, *PCA3* mRNA, cg08045599 methylation and cg05618150 methylation for the diagnosis of PCa. Results revealed that the sensitivity, specificity and AUC of *PSA* were 80.9%, 45.7% and 0.659; the *AMACR* were 82.5%, 88.6% and 0.897; and PCA3 were 68.9%, 91.4% and 0.840, respectively. Simultaneously, the sensitivity, specificity and AUC of the cg08045599 methylation in diagnosing PCa can reach 84.9%, 85.7% and 0.897 and the cg05618150 methylation can reach 71.3%, 91.4% and 0.856, respectively (Figure 5A).

Except for the *PSA*, the *AMACR*, *PCA3*, cg08045599 methylation and cg05618150 methylation demonstrated high diagnostic efficiency, with cg05618150 having a higher AUC than cg08045599. ROC analysis of *AMACR*, *PCA3* and cg05618150 methylation was performed again using the ICGC and GEO databases for further confirmation. Meanwhile, we also analyzed several widely studied methylation biomarkers, such as *GSTP1*, *APC* and *RARB*. ROC curve analysis revealed that the AUC of *AMACR* was 0.870 (GSE60329), 0.929 (GSE70770) and 0.915 (ICGC), the AUC of *PCA3* was 0.864 (GSE60329), 0.886 (GSE70770) and 0.869 (ICGC), the AUC of *GSTP1* methylation was 0.672 (GSE101908), 0.624 (GSE112047) and 0.929 (GSE73549), the AUC of *APC* methylation was 0.659 (GSE101908), 0.562 (GSE112047) and 0.886 (GSE73549) and the AUC of *RARB* methylation was 0.681 (GSE101908), 0.611 (GSE112047) and 0.938 (GSE73549) . The curve exhibited great fluctuation and the AUC value was unstable enough (Figure 5B–F).

Based on the GSE datasets, the AUC of cg05618150 for diagnosing PCa can reach 0.971 (GSE101908), 0.984 (GSE112047) and 0.986 (GSE73549) and the fluctuation of the ROC curve was not apparent and the AUC value was stable and close to 1. Overall, the above results demonstrated that PRKY methylation site cg05678150 might be a novel biomarker for diagnosing PCa.

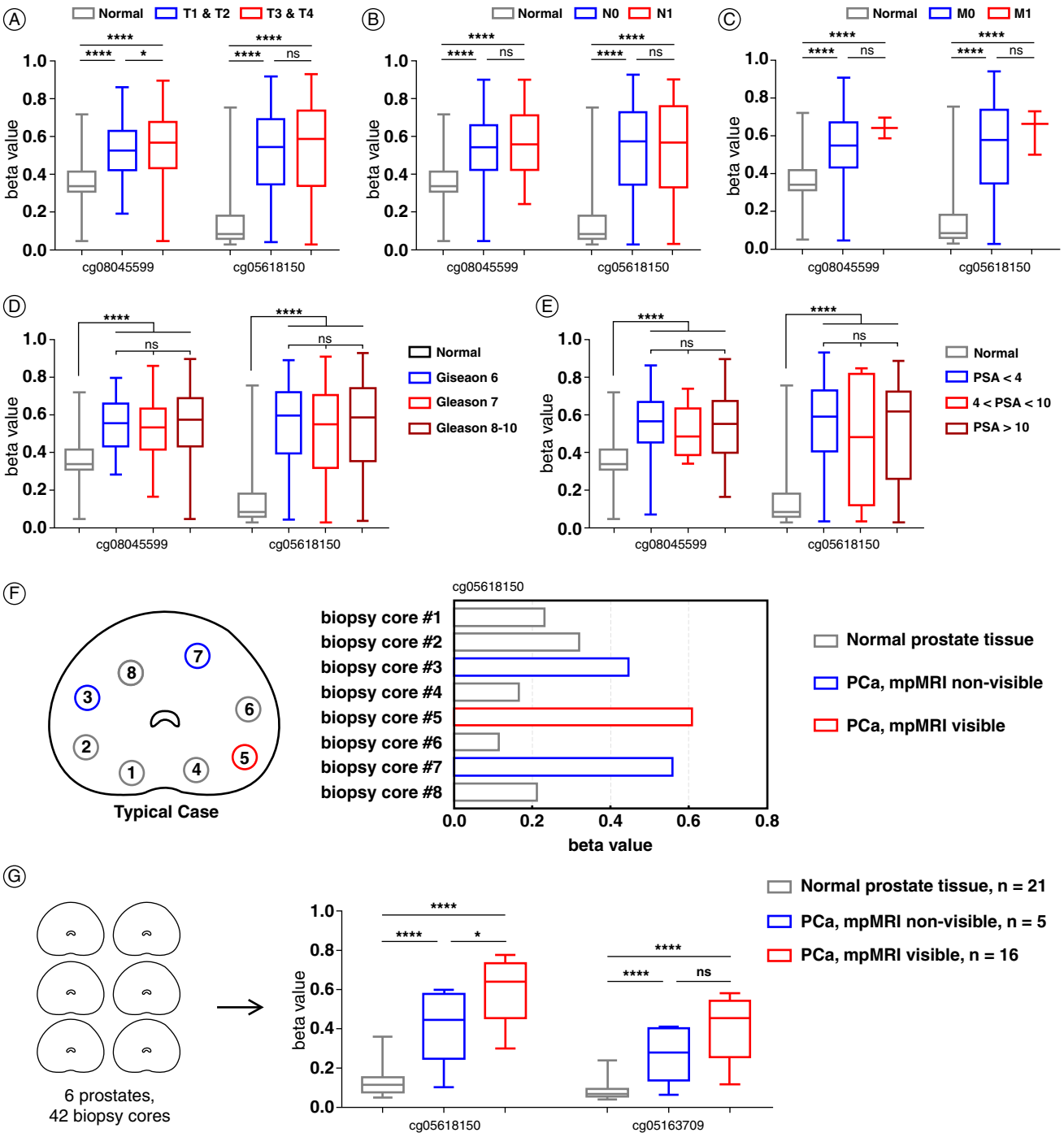

Figure 4. *PRKY* hypermethylation can be detected in very early stage of PCa. **(A)–(C)** Boxplot graph showed the methylation levels of cg08045599 and cg05618150 in PCa tissues with different TNM stages and normal tissues. **(D)** Boxplot revealed the methylation levels of cg08045599 and cg05618150 in PCa tissues with different Gleason scores and normal tissues. **(E)** Boxplot exhibited the methylation levels of cg08045599 and cg05618150 in PCa tissues with different PSA ranges and normal tissues. **(F)** The bar graphs presented the cg05618150 methylation level in PCa biopsy tissues with mpMRI visible or nonvisible and normal tissues. **(G)** Boxplot presented the methylation levels of cg05618150 and cg05163709 in PCa biopsy tissues with mpMRI visible or nonvisible and normal tissues.
*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$ and ns represents not significant.
mpMRI: Multiparameter MRI; PCa: Prostate cancer.

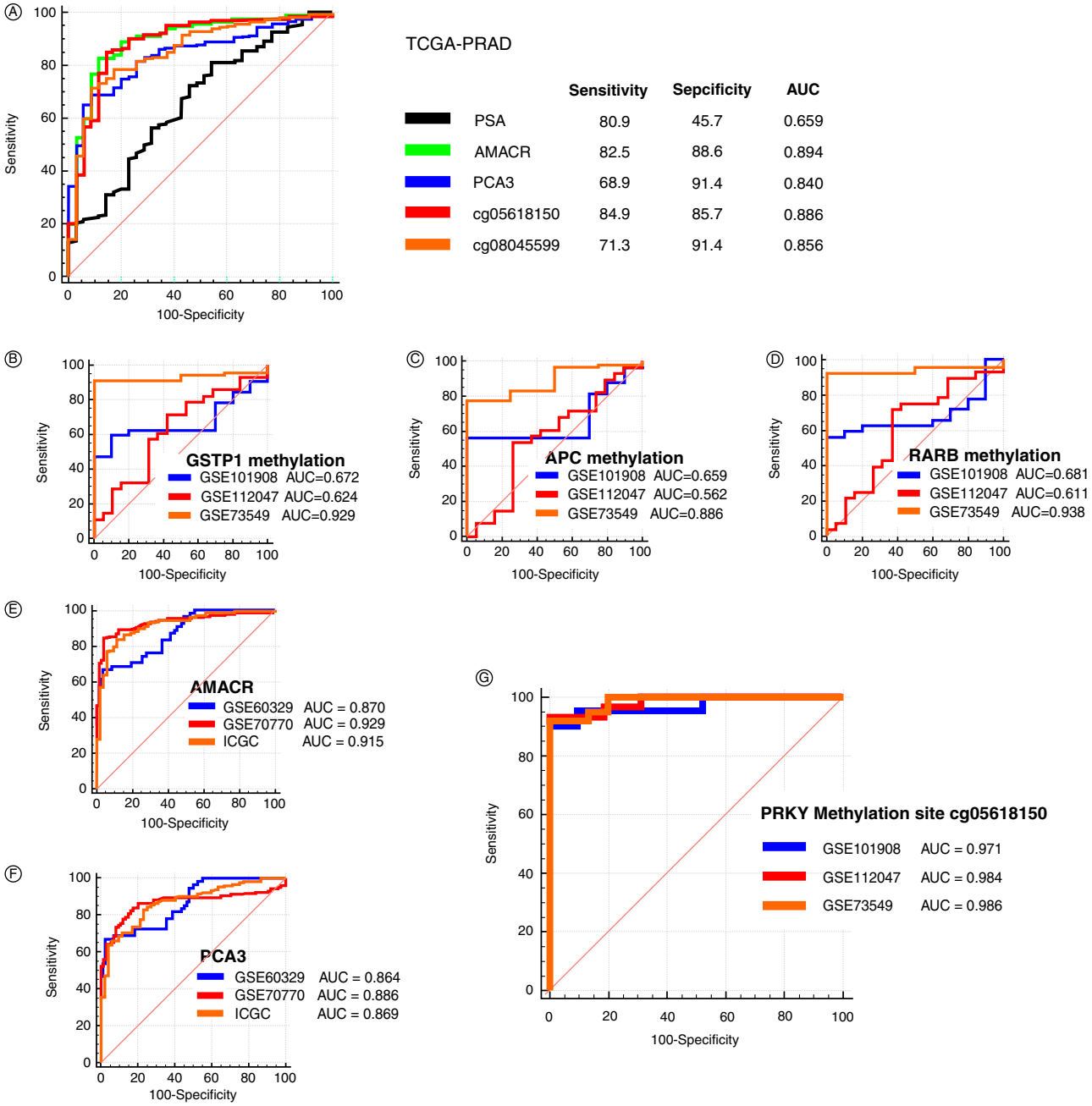

Figure 5. Diagnostic efficacy of *PRKY* methylation. **(A)** The ROC curves compared the sensitivity and specificity of *PSA*, *AMACR*, *PCA3*, cg08045599 methylation and cg05618150 methylation to diagnose PCa from the TCGA database. **(B–D)** The ROC revealed the AUC of *GSTP1*, *APC* and *RARB* methylation for diagnosing PCa in the GEO (GSE101908, GSE112047 and GSE73549) databases. **(E & F)** The ROC revealed the AUC of *AMACR* and *PCA3* for diagnosing PCa in the ICGC and GEO (GSE60329 and GSE70770) databases. **(G)** The ROC exhibited the AUC of cg05618150 methylation for diagnosing PCa in the GEO (GSE101908, GSE112047 and GSE73549) databases. AUC: Area under the curve; GEO: Gene Expression Omnibus; PCa: Prostate cancer; TCGA: The Cancer Genome Atlas; ROC: Receiver operating characteristic.

3.6. Validation of the diagnostic value of PRKY promoter hypermethylation in PCa from single-center clinical samples

To verify the findings from the above databases studies, we conducted a series of experimental validations. First of all, we detected the CpG sites (cg05163709, cg08045599 and cg05618150) methylation level of *PRKY* promoter in prostate needle biopsy tissues of 20 patients by qPCR (Supplementary Table S1). Based on the pathological results and Δct value of qPCR in 33 prostate needle biopsy tissues, we plotted the bar plot and ROC curve. The results demonstrated that the methylation

level of cg08045599 (Δct-cg08045599) has no significant differences between cancer tissues and normal tissues and the AUC of cg08045599 hypermethylation in the diagnosis of PCa was 0.563 (95%CI: 0.380–0.734, $P > 0.05$). In addition, the methylation levels of cg05163709 and cg05618150 are significantly higher in cancer tissues than in normal tissues, hence a lower Δct. The sensitivity and specificity of cg05163709 methylation in the diagnosis of PCa were 93.75% and 82.35% and the AUC was 0.941 (Supplementary Table S2). The sensitivity and specificity of cg05618150 methylation were 100% and 88.2%, respectively and the AUC was 0.963 (Supplementary Table S2). The diagnostic efficacy of CpG methylation in PCa was significantly higher than that of tPSA (AUC = 0.625) and PI-RADS (AUC = 0.825). The results are presented in Figure 6.

4. Discussion

Since 1994, Lee *et al.* [18] first reported that promoter hypermethylation of *GSTP1* is one of the most common epigenetic changes in patients with PCa. An increasing number of relevant studies have demonstrated that promoter DNA methylation plays a vital role in carcinogenesis. Some researchers [19] have confirmed that DNA hypermethylation of gene promoters leading to the silencing of gene expression is an important cause of prostate tumor formation, with *GSTP1* being the most thoroughly studied hypermethylated gene. The *GSTP1* gene is located on chromosome 11q13. Over 90% of PCa cases may have inhibition or silencing of *GSTP1* gene expression, which is closely related to the CpG island hypermethylation in the promoter region of *GSTP1*. In addition to *GSTP1*, *RASSF1*, *APC*, *RARB* and other genes were also widely studied in the diagnosis and prognosis of PCa [20]. Although past studies have identified numerous differentially methylated genes associated with diagnosing PCa, not even the *GSTP1* gene has been implemented into clinical practice. The only commercially available test based on DNA methylation is Confirm MDx, which depends on the methylation status of three genes (*GSTP1*, *APC* and *RASSF1*) in the prostate needle biopsy tissues. However, ConfirmMDx only assesses whether a patient needs repeat needle biopsies [21]. Therefore, there is an urgent need to develop new methylation biomarkers to diagnose PCa.

The Y-chromosome is the sex-determining chromosome in many species [22]. Deletion of different regions of the Y-chromosome can lead to certain diseases, such as blood diseases, acute myeloid leukemia and myelodysplastic syndrome [23,24]. The copy number loss of YP11.2 was associated with *TSPY* gene cluster in PCa [25]. Kido *et al.* [26] discovered that the *TSPY* gene in YP11.2 plays a key role in forming PCa, during which *TSPY* may be abnormally activated, resulting in high cancer heterogeneity. Similarly, Jobling *et al.* [27] discovered that the most common deletion of the Y-chromosome appeared to be caused by *TSPY*-mediated recombination in the study of 45 males from 12 different populations. The *PRKY* gene in Yp11.2 is a pseudogene that can be detected in skin, prostate and other tissues [28]. Based on the TCGA website (https://portal.gdc.cancer.gov/v1/genes/ENSG00000099725), we found that the most frequent somatic mutations in *PRKY* gene are substitution and deletion, which occur in tumor tissue of the skin, colon, rectal, stomach and lung, but not in PCa tissue. Meanwhile, there are no *PRKY* copy number gains or losses were found in the above patients screened for CNVs. Therefore, we speculated that down-regulation of *PRKY* expression on the Y-chromosome is strongly associated with the occurrence of PCa and the mechanism may be connected to aberrant DNA methylation.

Based on TCGA data, we found that in cancers with a higher incidence in men than women, the expression of *PRKY* in cancer tissues was significantly lower than that in normal tissues. The promoter methylation level of *PRKY* was significantly higher in PCa tissues than in normal tissues. However, the level of *PRKY* promoter methylation did not show a significant increase in other common malignant tumor tissues, except PRAD. This is completely different from the *GSTP1* gene and the promoter methylation level of *GSTP1* is significantly increased in various cancer tissues [29], especially LUAD, LUSC, LIHC, BLCA and gastrointestinal malignancies. Based on the above results, it can be concluded that *PRKY* methylation is highly specific in PCa and is not easily disturbed by other common male malignancies. Regrettably, our results only apply to male patients, as only in-sex comparisons (i.e., sex-stratified analyses) are biologically valid for X- and Y-chromosome DNA methylation data [16]. The Y-chromosome *PRKY* gene is located in Yp11.2, which is not in pseudoautosomal regions (PAR). But in our study, when using the probes of CpG sites (cg08045599), there will be a strong signal from *PRKX* as well. The current chip technology is unable to detect signals on the X- and Y-chromosomes, respectively. From this, we only analyzed male samples for the methylation levels of additional tumors to reduce the interference of the *PRKX* gene.

In the human genome, 70–80% of CpG dinucleotides are in the methylated state, while unmethylated CpG dinucleotides are unevenly distributed, exhibiting a tendency for local aggregation and eventually forming some regions with high GC content, namely CpG islands [30]. CpG islands remain unmethylated in embryonic and other normal tissues, with a few exceptions. However, the mechanism remains unclear as to why CpG islands remain

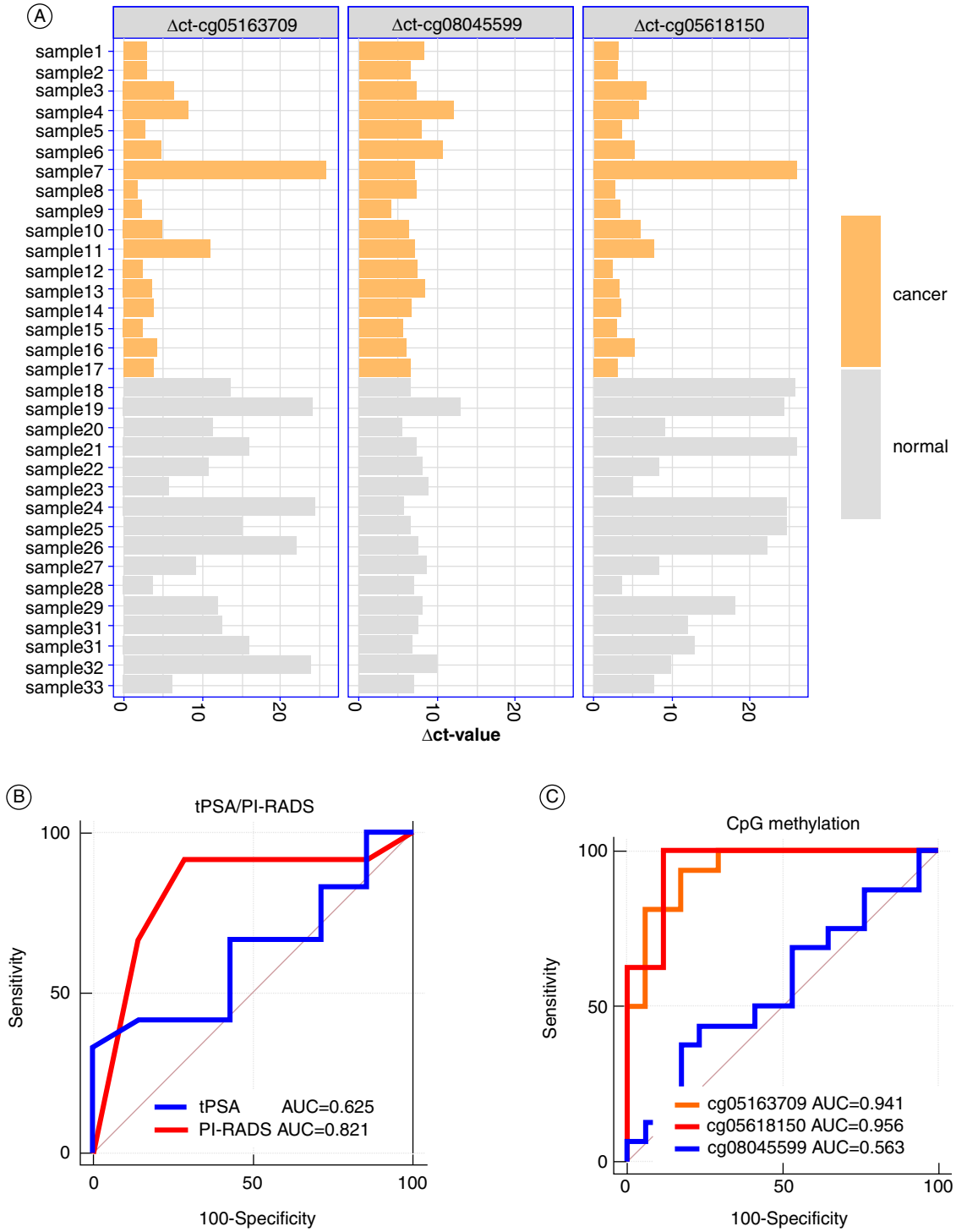

Figure 6. Validation of the diagnostic value of *PRKY* promoter hypermethylation in PCa from single-center clinical samples **(A)** the CpG sites (cg05163709, cg08045599 and cg05618150) methylation level of *PRKY* promoter in 33 prostate needle biopsy tissues **(B)** The ROC revealed the AUC of clinical indicators (tPSA and PI-RADS) for diagnosing PCa. **(C)** The ROC indicated the AUC of CpG methylation for diagnosing PCa.
AUC: Area under the curve; PCa: Prostate cancer; ROC: Receiver operating characteristic.

unmethylated in normal cells. CpG islands are often located in the gene promoter around the transcriptional start site (TSS). The hypermethylation of CpG island in the gene promoter may be an important cause of the development of PCa, which has been studied extensively. However, the CpG sites we studied are located in TSS1500, which is not on the CpG island (chrY: 7273481–72751835). This result indicates that the methylation of CpG sites

(cg05163709, cg08045599 and cg05618150) in *PRKY* promoter is associated with PCa and may not be restricted to the CpG island of the promoter region. On the one hand, all methylation data is basically from the Illumina Human Methylation 450K Chip, which the probe contained in the 450K chip cannot cover the CpG site of the CpG island in the *PRKY* promoter region. On the other hand, the CpG dinucleotides we studied are unmethylated in normal tissues and significantly methylated in PCa tissues, which is entirely possible, although it is not common. Thus, how CpG dinucleotide methylation located in *PRKY* promoter affects transcription and PCa development needs further study.

Based on the TCGA and GEO databases, we determined that the cg08045599 and cg05168150 site methylation levels of *PRKY* promoter were significantly higher in PCa tissues than in normal tissues and the *PRKY* expression level was negatively correlated with the methylation level of the site. Similarly, Yao *et al.* [15] identified the differentially methylated CpG site cg05163709 in *PRKY* promoter in PCa tissue samples using the Illumina Methylation 450K array. We further verified the cg05163709 methylation difference between PCa and normal tissues using the three data sets (GSE76938, GSE112047 and GSE73549). The results are consistent with the cg08045599 and cg05168150 methylation states in PCa. We further analyzed the correlation between the methylation of these three sites (cg05163709, cg08045599 and cg05168150). The clinicopathological features of PCa patients revealed that their methylation levels significantly differed between cancer and noncancer tissues. In contrast to normal tissues, early TNM stages and low-risk PCa (low PSA level or low Gleason score) tissues displayed hypermethylation at these three sites. This result demonstrated that the promoter methylation of *PRKY* can help identify early PCa.

Based on the comprehensive data of prostate needle biopsy tissues from Parry *et al.* [17], it was determined that the methylation levels of cg05163709 and cg05618150 sites were significantly higher in mpMRI nonvisible PCa tissues than in normal tissues. Thus, the above results suggest that promoter methylation of *PRKY* may be easier to detect early PCa than MRI and we may be able to use a combination of mpMRI and *PRKY* methylation to diagnose PCa in the future.

Currently, *AMACR* and *PCA3* have been widely studied in diagnosing PCa and have good potential for clinical application [31–33]. We drew ROC curves of cg08045599 and cg05618150 methylation in diagnosing PCa. We compared them with the diagnostic efficacy of *PSA*, *PCA3* and *AMACR* gene expression in PCa to further verify the diagnostic efficacy of *PRKY* methylation. The analysis of the TCGA dataset revealed that the methylation of cg08045599 and cg05168150 had high sensitivity and specificity in diagnosing PCa, the sensitivity of cg05618150 was better than *AMACR*; and the diagnostic efficacy of cg05618150 and cg08045599 was better than *PCA3*. Meanwhile, we also included three widely studied methylation biomarkers (*GSTP1*, *APC* and *RARB*), which have been essentially confirmed to have the most potential. The results demonstrated that cg05618150 methylation had a higher and more stable AUC value (0.971–0.986) than *GSTP1* methylation (0.672–0.929), *APC* methylation (0.659–0.886), *RARB* methylation (0.681–0.938), *PCA3* (0.864–0.886) and *AMACR* (0.870–0.929) gene expression in the diagnosis of PCa. In conclusion, CpG methylation of *PRKY* promoter is a better indicator for the diagnosis of PCa than gene expression and other methylation markers. Finally, we carried out a preliminary single-center validation and the results were consistent with the results of the bioinformatics analysis. Based on the detection of CpG methylation in tissue samples, we found that the methylation of cg05163709 (AUC = 0.941) and cg05618150 (AUC = 0.956) is a promising biomarker for distinguishing PCa tissue from normal tissue, which was significantly better than tPSA (AUC = 0.625) and PI-RADS (AUC = 0.821). Moreover, the methylation of cg05163709 and cg05618150 was not detected in the prostate needle biopsy tissues of 2 patients with significantly elevated PSA (>90 ng/ml), PI-RADS scores of 3 and 5 and negative biopsies, suggesting that CpG methylation may be useful for the identification of BPH patients with highly suspected PCa. Regrettably, all patients underwent an ultrasound-guided, systematic 12-core prostate biopsy and the possibility of a false negative could not be ruled out. In summary, *PRKY* promoter methylation may be a potential marker for the early and accurate diagnosis of PCa.

In PRAD, low expression of *PRKY* may be closely related to DNA methylation. However, the mechanism of low *PRKY* expression in other tumor tissues is unclear and there may be other nonmethylation factors. Our previous research indicated that serum *PRKY* promoter methylation combined with magnetic resonance imaging is helpful in predicting clinically significant PCa [34]. However, relying solely on serum *PRKY* promoter methylation to diagnose PCa remains challenging. Serum *PRKY* promoter hypomethylation cannot exclude PCa, especially in patients with early stages of PCa. Similarly, serum *PRKY* promoter hypermethylation cannot be completely identified as PCa and may be combined with other tumors. Thus, the clinical value of serum *PRKY* promoter methylation in the diagnosis of early PCa may be limited. We think that urinary *PRKY* promoter methylation may be more promising; after all, most cases only need to take into account the interference of uroepithelial

tumors. In this regard, we will collect urine samples from patients with PCa and further explore the value of *PRKY* methylation in the diagnosis of PCa.

5. Conclusion & future perspective

In this study, *PRKY* expression was significantly down-regulated in PCa tissues and its mechanism may be related to the hypermethylation of promoter region CpG sites (cg05163709, cg08045599 and cg05618150). *PRKY* promoter methylation is highly specific for the diagnosis of PCa and is not easily disturbed by common malignant tumors in men. The detection of *PRKY* promoter methylation is helpful to identify early PCa, which may be superior to PSA, mpMRI and other excellent molecular biomarkers. In the future, prospective and multicenter studies are required to validate the diagnostic value of *PRKY* promoter methylation in PCa, especially in the field of liquid biopsies.

Article highlights

- Y-chromosome gene deletion and down-regulation may promote prostate cancer (PCa) progression. Based on various databases, we finally screened out the Y-chromosome gene *PRKY*.
- *PRKY* expression was significantly down-regulated in PCa tissues and its mechanism may be related to the hypermethylation of promoter region CpG sites (cg05163709, cg08045599 and cg05618150).
- There was no significant increase of promoter methylation level of *PRKY* in other common male malignancies (LUAD, LUSC, LIHC, COAD, READ, ESCA, BLCA and pancreatic adenocarcinoma), which indicates that *PRKY* promoter methylation is highly specific in PCa.
- *PRKY* promoter methylation can be detected in very early stages of PCa (T1 & T2, N0, M0, PSA <4 and Gleason $= 6$), which can also be detected in PCa lesions that could not be recognized by multiparameter MRI.
- PRKY promoter methylation is a better indicator for the diagnosis of PCa than gene expression (*PSA*, *AMACR* and *PCA3*) and other methylation markers (*GSTP1*, *APC* and *RARB*).
- The preliminary single-center validation showed that the methylation of cg05163709 and cg05618150 is a promising biomarker, which was significantly better than tPSA and PI-RADS.

Author contributions

ZD, HC, DY, BX and JZ conceived and designed the study. ZD, KF, TL, WL and YZ analyzed the data and prepared the figures. ZD, HC and KF wrote and revised the manuscript. All authors have read and agreed to the final version of the manuscript.

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Competing interests disclosure

The authors have no competing interests or relevant affiliations with any organization or entity with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

Writing disclosure

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

All experiments in this study was performed in accordance with relevant guidelines and regulations. This study was approved by the Ethics Committees of the Second Affiliated Hospital of Soochow University. The approval number is JDLK202205901 (*date* June 20, 2022). All the patients were informed about and provided consent for the study and written informed consent was obtained from each participant.

Data availablity statement

Data used in this study can be downloaded from The Cancer Genome Atlas (https://tcga-data.nci.nih.gov/tcga/), University of California Santa Cruz Xena (https://xenabrowser.net/datapages/), Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/), International Cancer Genome Consortium (https://dcc.ICGC.org/releases), Gene Expression Profiling Interacting Analysis (http://gepia.cancer-pku.cn) and University of Alabama at Birmingham Cancer data analysis Portal (http://ualcan.path.uab.edu/index.html).

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