Original Article

Diagnostic value of DACT-2 methylation in serum of prostate cancer patients

Ting Lan[1,2#], Lingyu Yin[2#], Haoliang Zhang[2,3#], Bing Gu[1,2,3], Ping Ma[1,2,3], Shibao Li[1,2,3], Hongchun Li[2,3]

[1]Xuzhou Key Laboratory of Laboratory Diagnostics, Xuzhou Medical University, Xuzhou, China; [2]School of Medical Technology, Xuzhou Medical University, Xuzhou, China; [3]Department of Laboratory Medicine, Affiliated Hospital of Xuzhou Medical University, Xuzhou, China

Contributions: (I) Conception and design: S Li, H Li; (II) Administrative support: S Li, P Ma; (III) Provision of study materials or patients: H Zhang, L Yin; (IV) Collection and assembly of data: T Lan, L Yin; (V) Data analysis and interpretation: T Lan, L Yin; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

[#]These authors contributed equally to this work.

Correspondence to: Shibao Li, PhD, MD. Department of Laboratory Medicine, Affiliated Hospital of Xuzhou Medical University, 99 West Huaihai Road, Xuzhou 221002, China. Email: sdjnshlb@xzhmu.edu.cn; Hongchun Li, MD. Department of Laboratory Medicine, Affiliated Hospital of Xuzhou Medical University, Xuzhou 221002, China. Email: 13775891123@163.com.

Background: Currently, prostate cancer (PCa) remains a hard nut to crack for the medical community. Therefore, the identification and development of novel biomarkers that can accurately diagnose disease and predict prognosis are of paramount importance. The objective of this study was to examine the clinical value of DACT-2 promoter methylation in serum of patients with PCa, to discover a potential diagnostic marker for PCa.

Methods: We investigated the methylation status of DACT-2 in the serum of 64 patients with PCa, 22 patients with benign prostatic hyperplasia (BPH), and 47 healthy subjects by methylation-specific PCR (MSP) and real-time methylation-specific PCR (QMSP). Further, we evaluated the relationship between DACT-2 methylation and clinic pathological parameters. Receiver operating characteristic (ROC) curve analysis was applied to assess the sensitivity, specificity, and diagnostic value of DACT-2 methylation and PSA levels.

Results: The results of MSP and QMSP showed that the level of methylation of DACT-2 promoter in patients with PCa was significantly higher than that in patients with BPH and healthy subjects. The PCa patients Gleason score and tumor node metastasis (TNM) positively correlated with promoter methylation level of serum DACT-2. The DACT-2 methylation rate was 0.745 with a sensitivity of 81.8%, and a specificity of 75.0%, the sensitivity, and specificity of PSA was 80.1% and 59.4%. ROC curve results displayed that the diagnostic value of DACT-2 is superior to PSA.

Conclusions: Our study confirms that the level of methylation of the DACT-2 promoter in patients with PCa is much higher than that in patients with benign prostatic hyperplasia (BPH) and healthy subjects, suggesting that DACT-2 methylation in serum is a potential biomarker of PCa.

Keywords: DNA methylation; DACT-2; prostate cancer

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Introduction

Prostate cancer (PCa) is one of the most emergent health issues globally, accounting for the second cause of cancer deaths in males (1,2). Recently, the incidence of PCa is increasing in numerous countries at pace with the steadily ceaseless development of society, especially the rapid expansion of the economy (3,4). Moreover, there has been evidence that the lifestyle and living environment of the public are two potential risk factors for PCa (5). Currently,

PCa screening remains to rely on serum prostate-specific antigen (PSA) testing and random biopsy (6,7). However, the biopsy is an invasive test, which is not an appropriate screening. Meanwhile, PSA has low specificity, with false-positive results in patients with benign prostatic hyperplasia (BPH) and biopsy is only positive in around 25% of patients with PSA in the range between 2 and 10 μg/L. PSA screening and random biopsy strategies may induce that they diagnosed the other diseases and missing the PCa. The US Preventive Health Task Force issued a "D" grade for PSA-based screening in 2012 because they both over-diagnose low-risk disease and under diagnose high-risk cancers (8). Therefore, it is demanding to explore new biomarkers for the diagnosis and prognosis of PCa.

Epigenetic changes play a vital role in the development and progression of different cancers, including PCa. In particular, hypermethylation of CpG islands of the gene promoter region leads to reduced expression of gene (9). The original study has demonstrated that circulating tumor DNA (ctDNA) has the potential function for prediction, molecular analysis, and monitoring. Thus, ctDNA in the blood of tumor patients can be used as biomarkers (10). So far, circulating tum75 or DNA methylation as a biomarker of cancer has been used in the early detection of numerous cancers (11). Several studies have confirmed that abnormal methylations of serum EphA5, PITX3, PD-L1, DEFB1, PITX2, CRMP4 genes are involved in the malignant evolution of PCa, which can be used as a biomarker for assessing the risk of PCa (12-17). Accordingly, hypermethylation of the gene promoter may be a potential biomarker for the diagnosis of PCa.

The human DACT gene family contains three members, namely dact-1, dact-2, and dact-3, with 2, 1, and 5 CpG islands in the promoter region, respectively. Therefore, the presence of aberrant methylation of the DACT promoter may lead to a lower expression of mRNA, which in turn affects its normal biological function, including its role in the development of the tumor (18,19). Recent studies have shown that DACT-2 is down-regulated by its promoter methylation in various tumors, including colon cancer, gastric cancer, thyroid cancer, and esophageal cancer (20-23). It is hypothesized that DACT-2 may be a potential tumor suppressor gene involved in tumor progression. Our previous studies have also confirmed that DACT-2 is down-regulated by methylation in both prostate cancer cells and prostate cancer tissues (24). However, it is unclear whether the presence of DACT-2 gene methylation is present in the serum of patients with PCa. In this study we investigated the methylation status of DACT-2 promoter in serum of patients with PCa. We analyzed the relationship between the serum DACT-2 methylation and clinic pathological parameters in PCa for the better of the development of a novel and potential biomarker during diagnosis in PCa.

We present the following article in accordance with the STARD reporting checklist (available at http://dx.doi.org/10.21037/apm-20-1496).

Methods

Eligibility criteria

Between January 2015 and December 2016, 135 participants, including 66 patients with PCa, 22 patients with benign prostatic hyperplasia and 47 healthy control, were consecutively enrolled in this prospective observational study conducted in the Affiliated Hospital of Xuzhou Medical University. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013) and was approved by the Ethics Committee of the Affiliated Hospital of Xuzhou Medical University (XYFY2019-KL113-01). Written informed consent was obtained from all participants.

Inclusion criteria of PCa were as follows: (I) male that was pathologically diagnosed with PCa were included in the PCa group; (II) male that was diagnosed with benign prostatic hyperplasia (BPH) were used color Doppler ultrasound examinations as benign group; (III) men with normal findings both at physical and color Doppler ultrasound examinations were included in the healthy group.

Exclusion criteria of PCa were as follows: (I) history of other primary or secondary tumors; (II) history of other comorbidities (e.g., hypertension, diabetes, heart disease, renal disease, etc.); (III) pre-operative chemotherapy or radiotherapy; (IV) hormonal treatment before surgery.

After color Doppler ultrasound examinations, blood samples were collected from patients with BPH and healthy controls, blood samples of patients with PCa were obtained daily before treatment, including radical prostatectomy, pre-operative chemotherapy, and radiotherapy. The pathological parameters, Gleason scores were taken from biopsies or prostatectomies. All samples were separated from serum and rapidly frozen at –80 ℃. According to the 7th edition of the AJCC staging system, PCa patients were evaluated for the pathological stage. Clinical and biological data from the patients with PCa and BPH are listed in *Table 1*.

Table 1 Patient clinical and histological characteristic

	Prostate cancer (%)	BPH (%)	Health Persons (%)
Case, n	64	22	47
Age (mean ± SD, years)	71.1±6.7	70.8±7.3	69.5±10.3
PSA (ng/mL)			
<4	4 (6.3)	21 (95.5)	46 (97.9)
4–10	15 (23.4)	0 (0)	1 (2.1)
>10	45 (70.3)	1 (4.5)	
TNM			
T1	9 (14.1)		
T2	12 (18.8)		
T3	13 (20.3)		
T4	30 (46.8)		
Gleason score			
6–7	18 (28.1)		
8–10	46 (71.9)		

BPH, benign prostatic hyperplasia; PSA, prostate specific antigen; TNM, tumor node metastasis.

Serum DNA extraction and sodium bisulfite modification

According to the manufacturer's instructions, 200 μL of serum was used to extract DNA from each sample with the aid of Serum/Plasma Circulating DNA Kit (TIANGEN, Beijing, China). Then, the eluted 100 μL of serum DNA was stored at –20 ℃ for subsequent experiments. The extracted DNA was modified based on the operating manual for the EZ DNA Methylation-Gold Kit (ZYMO Research Co, Orange, CA) kit, and the modified DNA was stored at –20 ℃.

Methylation-specific polymerase chain reaction (MSP)

The MSP primer sequences applied to amplify the modified DNA in this study were derived from the previous research (20). The sequences of MSP primers are as follows: 5'-GCGCGTGTAGATTTCGTTTTTCGC-3' (MF) and 5'-AACCCCACGAACGACGCCG-3' (MR); 5'-TTGGGGTGTGTG TAGATT TTG TTT TTTGT-3' (UF) and 5'-CCCAAACCCCACAAA CAA CAC CA-3' (UR). The MSP amplification system used in this experiment was 20 μL, consisting of 10 μL of Taq PCR MasterMix, 10 μL of nuclease-free water, 1 μL of each upstream and downstream primers and 1 μL of modified DNA. The amplification conditions involved in this reaction are as follows: 95 ℃ 5 min, (95 ℃ 30 s, 58 ℃ 30 s, 72 ℃ 30 s) ×38, 72 ℃ 7 min. The size of methylated and unmethylated products was about 152 and 161 bp, respectively, separated on a 3% agarose gel and visualized under UV illumination.

Real-time methylation-specific PCR

As above, the DNA of the serums of 47 healthy subjects, 22 prostatic hyperplasia subjects and 64 PCa subjects was extracted and subjected to bisulfite modification. The level of methylation of the DACT-2 promoter was examined on an ABI 7500 system (Applied Biosystems) using the MSP primers, and amplification conditions described above. The methylation level of the DACT-2 gene was calculated according to the following formula: M%= 100%× [number of copies of methylated DNA/(number of copies of methylated + unmethylated DNA)]. In this study, the levels of DACT-2 methylation rate at 0.745 were defined as positive according to the ROC (receiver operating characteristic) curves. The levels of PSA >7.56 ng/mL were defined as positive based on the reference range provided by the kit.

Statistical analysis

The experimental data were analyzed using SPSS 16.0 and GraphPad Prism 5.0 software. Differences between groups were compared by *t*-test. The differences between the three groups were assessed by the one-way ANOVA test and the relationship between DACT-2 methylation level and clinic pathological parameters was compared by the chi-square test. A receiver operating characteristic (ROC) curve was established to evaluate the diagnostic value of DACT-2 in PCa patients. When the P value is less than 0.05, the difference is considered statistically significant.

Results

The methylation status of DACT-2 in serum

First of all, we detected the levels of methylated DACT-2 in the serum of 64 patients with PCa, 22 patients with BPH, and 47 healthy controls by methylation-specific PCR (MSP-PCR). Interestingly, methylated DACT-2 appeared in the serum of 34 (53.1%) patients with PCa. In contrast, the frequency of the methylation of DACT-2 in serum was

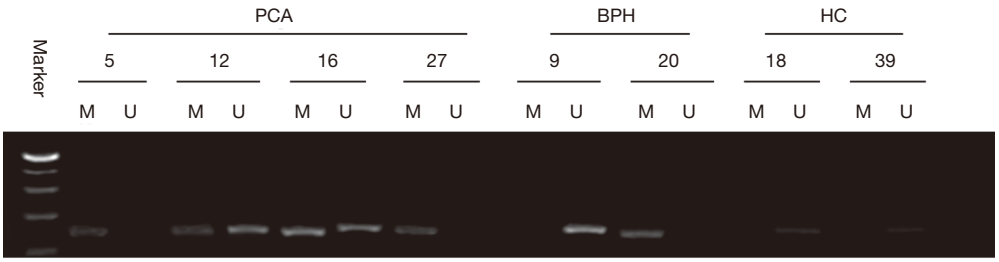

Figure 1 DACT-2 promoter methylation status in serum samples from prostate cancer (PCa) patients, benign prostatic hyperplasia (BPH) patients and healthy subjects controls (HCs) by methylation-specific PCR (MSP). M and U represent the amplification products of the methylated and unmethylated primers, respectively.

lower in BPH (9.1%) and healthy subjects (4.3%) (*Figure 1*).

Relationship between the serum of DACT-2 methylation status and clinical pathological-parameters

To elucidate the clinical significance of DACT-2 methylation in serum of patients with PCa, we used Methylation-specific polymerase chain reaction (MSP) to analyze the association between DACT-2 methylation and clinic pathological parameters of PCa. The results showed a significant association between DACT-2 aberrant methylation and advanced pathological stage (P=0.027) and high Gleason score (P=0.011). However, there was no correlation between DACT-2 methylation and age (P>0.05) or preoperative PSA levels (P>0.05). The results of the statistical analysis are listed in *Table 2*.

The rate of DACT-2 methylation in serum

The rate of DACT-2 methylation in all serum samples was determined using real-time fluorescence MSP. We discovered that the methylation rate of the DACT-2 gene in serum DNA in patients with PCa was higher than that in BPH patients and healthy subjects (*Figure 2A*). To clarify the diagnostic value of PSA and serum DACT-2 promoter methylation rates in PCa, the ROC curve was used to analysis the preoperative serum PSA level and DACT-2 promoter methylation rate in serum. The ROC curve revealed a more favorable diagnostic performance of serum DACT-2 methylation rate than serum PSA concentrations in these samples. When the optimal cut-off point of DACT-2 methylation rate was 0.745 with a sensitivity of 81.8% and a specificity of 75.0%, the serum PSA value cut-off was 7.56 (ng/mL), with a sensitivity of 80.1% and a specificity of 59.4% (*Figure 2B*).

Correlation between the methylation rate of DACT-2 and clinicopathologic features

By analyzing histopathological parameters of DACT-2 methylation rate, DACT-2 methylation rate was independent of median age (P>0.05) and PSA serum concentration (P>0.05). However, the methylation rate of the DACT-2 promoter in PCa patients is related to the Gleason score and the TNM stage. By the Real-time methylation-specific PCR our results exhibited that among PCa patients, the higher Gleason score [8–10] has a higher methylation rate of the DACT-2 promoter than the low Gleason score [6–7] (*Figure 3A*). Consistent with a higher rate of methylation of the serum DACT-2 promoter appeared in PCa with an advanced pathological stage (*Figure 3B*).

Discussion

Currently, the early diagnosis of PCa mainly relies on prostate-specific antigen PSA screening. Due to its low specificity for PCa, the detection of enhanced PSA levels not only results in the false positive diagnosis of PCa but also leads to a large number of unnecessary and invasive prostate biopsies (6,25). Therefore, it is reasonably critical to identify diagnostic biomarkers with high sensitivity, specificity, and non-invasion for early diagnosis, improvement of prognosis, and survival rate of patients with PCa.

Increasing evidence has shown that circulating tumor DNA (ctDNA) is a more appropriate and non-invasive liquid biopsy biomarker, which is easily detectable in a patient's plasma at an early stage of the disease (26-28).

Table 2 Relationship between the serum of DACT-2 methylation status and clinical pathological-parameters

Parameters	Case	DACT-2		P
		Methylated (N=34)	Unmethylated (N=30)	
Age (years)				0.862
≤70	27	14	13	
>70	37	20	17	
PSA (ng/mL)				0.251
≤10	19	8	11	
>10	45	26	19	
Stage (TNM)				0.027
T1-T2	21	7	14	
T3-T4	43	27	16	
Gleason score				0.011
6–7	18	5	13	
8–10	46	29	17	

PSA, prostate specific antigen; TNM, tumor node metastasis.

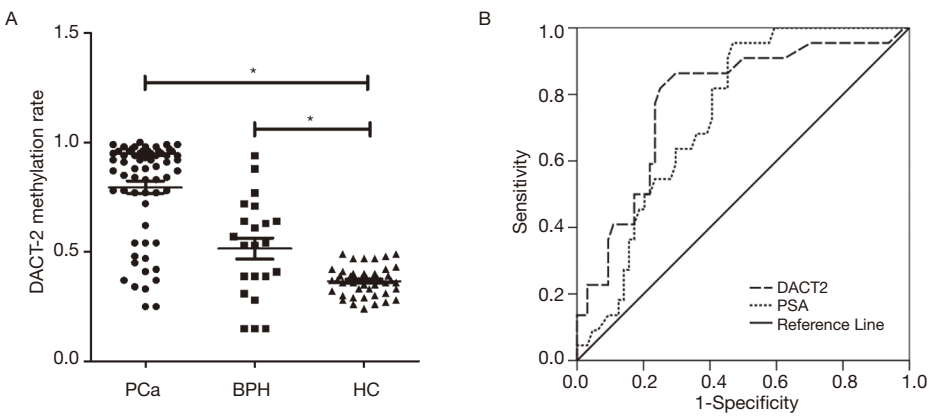

Figure 2 Methylation rate of DACT-2 in serum of prostate cancer (PCa) patients, benign prostatic hyperplasia (BPH) patients and healthy subjects controls (HCs). (A) The distribution of DACT-2 methylation rate in PCa patients, benign prostatic hyperplasia (BPH) patients and healthy subjects controls (HCs). (B) The receiver operating characteristic (ROC) curve of *DACT-2* gene methylation rate (cutoff value of 0.745) and prostate specific antigen (PSA) level (cutoff value of 7.56 ng/mL). *P<0.05.

In particular, tumor-specific DNA methylation can be reflected in the ctDNA of cancer patients, demonstrating its potential for widespread clinical application in cancer detection (29,30). Given this general idea, we mainly probed into the methylated expression of DACT-2 in patients with PCa. Results of MSP showed that compared with BPH and healthy subjects, the methylated DACT-2 was highly present in the serum of patients with PCa. The above results are consistent observed in prostate cancer cells and prostate cancer tissues. Also, the discovery of this study is accordant with previous findings regarding the performance of DACT-2 in other tumors, suggesting that DACT-2 exhibits highly methylated in a variety of tumors (20-23). Guo *et al*. have validated that the hypermethylation

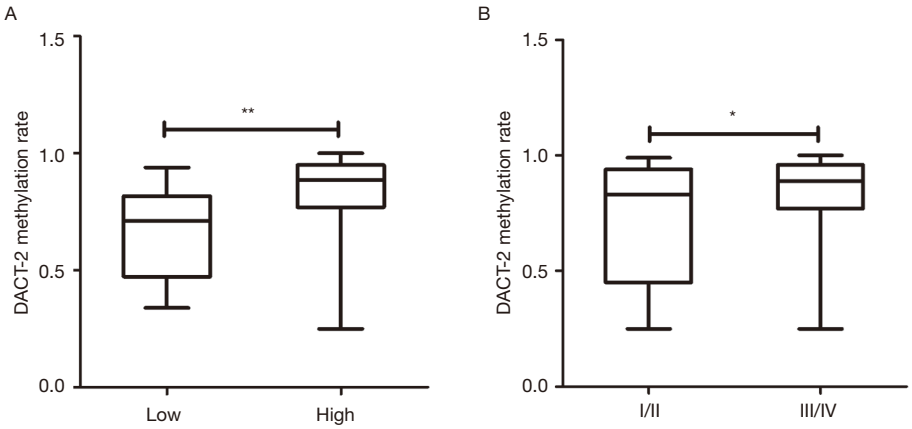

Figure 3 Clinicopathological features and DACT2 methylation rate in prostate cancer patients. (A) The Real-time methylation-specific PCR analysis correlation between DACT-2 methylation rate and Gleason Score, **represents a statistically significant difference of P<0.01. (B) the Real-time methylation-specific PCR analysis correlation between DACT-2 methylation rate and tumor node metastasis (TNM) stage, *shows a statistically significant difference of P<0.05.

of DACT-2 effectively promoted the progression of esophageal cancer (23). Moreover, several other studies have demonstrated that methylated DACT-2 can contribute to the development of tumors, including colon cancer, thyroid cancer, and breast cancer (21,22,31). All the above findings validate that DNA methylation plays a crucial role in the carcinogenesis of tumors, so we further explored the clinical value of DACT-2 methylation in patients with PCa. Our analysis suggested that methylated DACT-2 was correlated with the Gleason score and TNM stage, and DACT-2 methylation expressed more frequently in high Gleason scores and patients with advanced PCa. Moreover, no correlation between the methylated DACT-2 and median age (P=0.862), preoperative PSA levels (P=0.251) was observed in prostate carcinoma samples. In conclusion, the above results indicated that DACT-2 might act as a tumor suppressor for PCa.

To analyze the correlation between methylation of DACT-2 and clinic pathological parameters of PCa, we further explored the significance of the DACT-2 methylation rate in PCa by QMSP. We observed that the methylation rate of DACT-2 in the serum of patients with PCa was significantly higher than that in patients with BPH and healthy subjects. Moreover, we investigated the clinical significance of DACT-2 methylation rates in PCa, with a view to better understanding the diagnostic value of DACT-2 in PCa. Consistent with previous research, the experimental results displayed that the DACT-2 methylation rate of patients with high Gleason scores and advanced PCa was much higher than that of BPH and healthy subjects, which means the DACT-2 methylation plays an extraordinary role in the development and progression of PCa. In light of the fact that PCa screening is still dependent on PSA screening, we compared the diagnostic value of DACT-2 with PSA to detect PCa. ROC curve stated clearly that the sensitivity and specificity of DACT-2 were slightly higher than that of PSA, which proved that DACT-2 possessed a preferable diagnostic ability for PCa. Therefore, the above findings are sufficient to authenticate that DACT-2 methylation is a potential and clinically significant biomarker for the diagnosis of PCa.

Our findings demonstrate that methylation of DACT-2 is involved in the evolution of PCa, which could potentially make DACT-2 a valuable biomarker for PCa diagnosis. The specific mechanism about how DACT-2 promotes the development of PCa needs to be further explored.

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Footnote

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/apm-20-1496). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013) and was approved the Ethics Committee of the Affiliated Hospital of Xuzhou Medical University (XYFY2019-KL113-01). Written informed consent was taken from all the patients.

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