



Effect of upregulation of DD3 on early detection and prognosis in prostate cancer

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Background: Expression of prostate cancer antigen 3 (PCA3 OR *DD3*) in the blood has been reported to be significantly higher in prostate cancer (PCa) than in benign prostate hyperplasia (BPH). To confirm whether *DD3* expression is significantly different between PCa and BPH tissues, *DD3* expression was tested in the blood both preoperatively and postoperatively and in the paired tissues of PCa patients.

Methods: Expression levels of *DD3* mRNA in the blood of patients who did not undergo surgery (PCa, n=102; BPH, n=53), those underwent surgery (preoperative, n=35; postoperative, n=35), and in PCa tissue specimens (tumor, n=41; adjacent normal, n=21) were determined by real-time quantitative PCR. Sensitivity and specificity for *DD3* in PCa patients were validated by receiver operating characteristic (ROC) curve analysis.

Results: Our data suggest that expression level of *DD3* in blood samples was significantly higher in PCa patients than in BPH patients (P=0.005). Expression of *DD3* mRNA was also significantly elevated in PCa tissues compared with adjacent normal tissues (P=0.013). The increase in *DD3* expression in PCa patients was further validated using a dataset from The Cancer Genome Atlas (n=549). Postoperative *DD3* expression decreased following surgical intervention (P<0.001). Moreover, low *DD3* expression was associated with improved overall survival (OS). Using gene set enrichment analysis, *DD3* expression was correlated with specific PCa target genes including carcinogenesis-related and cancer proliferation-related genes.

Conclusions: This study demonstrated that expression of *DD3* was upregulated in blood and PCa tumor tissues and was associated with prognosis. The oncogenic role of *DD3* was further validated in the TCGA database, indicating that *DD3* is a potential therapeutic target for PCa. Furthermore, this study suggests that *DD3* expression could be considered as a prognostic biomarker for PCa.

Keywords: *DD3*; prostate-specific antigen (PSA); postoperative prognosis; prostate cancer (PCa); benign prostate hyperplasia (BPH)

Submitted Dec 23, 2019. Accepted for publication May 25, 2020.

doi: 10.21037/tau-19-899

View this article at: <http://dx.doi.org/10.21037/tau-19-899>

Introduction

Prostate cancer (PCa) is the second leading cause of cancer-related death among men in the United States (1). Its incidence has increased at a tremendous rate among Asian male populations in the past decade (2). Most PCa patients are diagnosed at an advanced stage, requiring curative surgery with extended lymphadenectomy. Traditional treatment for PCa primarily consists of radical surgery and radiotherapy, and is only effective for early-stage PCa without metastasis. Presently, no curative therapy is available for PCa with local or distant metastases, resulting in poor prognosis for patients with advanced disease (3). Therefore, there is a pressing need to discover effective diagnostic biomarkers for the detection of early-stage PCa. Biopsies, digital rectal examination, and transrectal ultrasound are current techniques for PCa detection, but they perform poorly as early detection tools.

Prostate-specific antigen (PSA) is the most widely used biomarker for malignancy screening in prostate epithelium biopsy analysis and is considered the gold standard for the diagnosis of PCa in clinical practice (4). In the routine initial screening procedures for PCa, a high PSA level (≥ 4 ng/mL) indicates the requirement of a prostatic puncture biopsy with histopathological analysis for the differential diagnosis of PCa from other prostatic diseases (5). As elevated PSA levels are also observed in patients with benign prostate hyperplasia (BPH) and prostatitis, unnecessary puncture biopsies are often performed. In one study, approximately 15% of men with PSA levels below the cut-off value of 4 ng/mL were reported to be at risk for PCa (6). Additionally, two randomized trials evaluated the effects of PSA-based screening on patient mortality, but produced conflicting results (7,8). These data overwhelmingly reveal that PSA is not a PCa-specific biomarker. Moreover, PSA level does not indicate PCa risk or distinguish indolent from aggressive disease, particularly when the level is below 20 ng/mL. Because PSA is an inadequate tumor biomarker, there is an urgent need to identify novel high-fidelity PCa biomarkers.

Prostate cancer antigen 3 (DD3), a long non-coding RNA also known as differential display code 3, is expressed 10- to 100-fold in PCa tissues compared with adjacent non-cancerous tissues. It locates on the long arm of chromosome 9, containing 3 introns and 4 exons (9). In normal tissues and other prostatic lesion tissues, DD3 levels were found below detection range by Northern blot analysis (9), making it an ideal biomarker for detecting PCa (10). Several studies

have reported that DD3 expression can be detected across multiple biological sample types, such as blood and urine, serving as a specific tumor marker for PCa (11). One study showed that DD3 was highly expressed in 95% of PCa tumor cells with a median 66- to 100-fold upregulation compared with adjacent non-neoplastic cells (12).

It is feasible to understand the variable expression of DD3, specifically as it relates to tumorigenesis and the development of PCa. For example, expression of DD3 mRNA was demonstrated to be androgen-regulated via the activation of androgen receptor (AR)-mediated signaling, with the potential to regulate PCa cell survival (13). Another study reported that DD3 silencing resulted in the loss of PCa cell viability with regulatory effects on multiple tumor-related genes, the majority of which were linked to epithelial-mesenchymal transition markers and AR cofactors (14). As a pro-cancer marker, DD3 was observed to play a role in increase of cell proliferation, invasion, migration, and inhibition of apoptosis (15). The wealth of evidence supporting DD3 as a biomarker of PCa led to the development of a commercial kit named Progenesa[®]. This DD3 assay was manufactured by GenProbe and employs a PSA mRNA internal control for the quantification of DD3 in urine samples (16). In urine, several cancer products including DD3 that are released through prostate ducts act as cell-free markers (17). Salagierski *et al.* (18) also supported DD3 mRNA transcripts as being detectable and quantifiable in urine. However, the detection of DD3 gene expression in urine is prone to error due to the different degrees of prostatic massage among patients and the unhygienic extraction of urine samples. Thus, detecting DD3 in the blood may be a more reliable strategy for biomarker detection and PCa screening.

In this study, the SYBR Green assay was used to detect copies of the DD3 gene in blood. Detection efficacy was then further validated in PCa tissues. For the first time, DD3 expression levels in patients were compared before and after surgical intervention. The Cancer Genome Atlas (TCGA) database, DD3 mRNA expression, and PCa patient mortality data were used to elucidate the role of DD3 in tumorigenesis and progression.

Methods

Patients and clinical specimens

Forty-one human prostate tumor tissue samples and 21 adjacent normal tissues were collected from PCa patients

who underwent curative resection of the tumor at Yantai Yuhuangding Hospital (Shandong, China) between 2012 and 2016 immediately after surgery and stored at -80°C for further use. Tissue sample size was verified by a pathologist and the paired peritumoral samples were confirmed to be free of tumor deposits. Blood samples were collected in BD PAXgene Blood RNA tubes (Qiagen, Hilden, Germany) and stored in aliquots at -80°C . Peripheral blood samples were obtained from 53 patients with BPH and 102 patients with PCa, and also from 35 PCa patients before and after operation. PCa was confirmed by prostatic biopsy and histopathological evaluation to distinguish benign epithelial cells (in BPH samples) from adenocarcinoma cells (in PCa samples). Each tumor was classified by two pathologists and graded using the Gleason scoring system. All blood and tissue specimens were obtained from consenting subjects. This study strictly followed protocols approved by the Institutional Review Board and Research Ethical Committee of Yantai Yuhuangding Hospital of Qingdao University.

RNA isolation and reverse transcription

Total blood RNA was isolated using the PAXgene Blood RNA Kit (Qiagen, Germany), and total tissue RNA was isolated from PCa specimens using the ABgene Total RNA Isolation Reagent (Advanced Biotechnologies Ltd., Epsom, Surrey, UK). RNA concentration and quality were analyzed with the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, RRID:SCR_018042). Following the manufacturer's instructions, cDNA was generated from total RNA using the Quantitect Reverse Transcription Kit (Qiagen, Germany). Quantified analysis of DD3 mRNA was conducted using real-time quantitative polymerase chain reaction (RT-qPCR) (Funglyon Biotech Co., Ltd, Shanghai, China) and the QuantiNova SYBR Green PCR Kit (Qiagen, Germany). The primer sequences were as follows: DD3 forward primer (5'-ACACAGGAAGCACAAAAGG-3') and reverse primer (5'-GATGACCCAAGATGGCGGC-3'), and β -actin forward primer (5'-CTGGACTTCGAGCAAGAGATG-3') and reverse primer (5'-GAGTTGAAGGTAGTTTCGTGGA-3'). RT-qPCR reaction was conducted at 95°C for 2 min, 95°C for 5 s and 60°C for 10 s for 40 cycles, followed by melt curve analysis. $2^{-(\Delta\text{ct})} \times 10^6$ was used as the quantitative threshold for gene expression.

TCGA data

Dataset of mRNA levels (RNASeq v2) in PCa patients were downloaded from the TCGA database (www.synapse.org, The Cancer Genome Atlas, RRID:SCR_003193), and the DD3 mRNA levels and whole transcriptome data were used. Clinical data were obtained from the cBioPortal database (www.cbioportal.org, RRID:SCR_014555).

Gene set enrichment analysis

The associations between DD3 mRNA expression level and biological processes and signaling pathways were analyzed using gene set enrichment analysis software (GSEA v2.2, software.broadinstitute.org/gsea/, RRID:SCR_003199). GSEA calculated an estimated gene set enrichment score as a function of a predefined gene set of high- and low-ranked PCa genes distributed randomly (Molecular Signatures Database, software.broadinstitute.org, gene sets: TUMORIGENESIS, PROSTATE_CANCER). Default settings of the software were utilized. Significance was determined by permutation analysis (1,000 permutations) and false discovery rate (FDR). A gene set was considered significantly enriched when the FDR was <0.05 .

Statistical analysis

The results from the RT-qPCR of paired samples (i.e., blood from a patient before and after surgery) were analyzed by the paired *t*-test. Results of RNA-seq v2 and the qPCR of unpaired samples were analyzed by the independent samples *t*-test. Statistical Package for Social Sciences (version 19.0; SPSS Inc., Stanford, CA, USA, RRID:SCR_002865) and GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA, USA, RRID:SCR_002798) were used for statistical analyses. To assess the sensitivity and specificity of various biomarkers associated with the diagnosis of prostate abnormalities, receiver operating characteristic (ROC) curve analysis was applied using an estimation of the area under the curve (AUC). The log-rank test for the generated Kaplan-Meier curve was performed to evaluate the association between the DD3 mRNA expression level and patient mortality. Overall survival (OS) was defined as the time between the first surgery for primary PCa and death. Disease-free survival was defined as the time between the first surgery and disease recurrence. Logistic regression analysis was conducted to confirm the

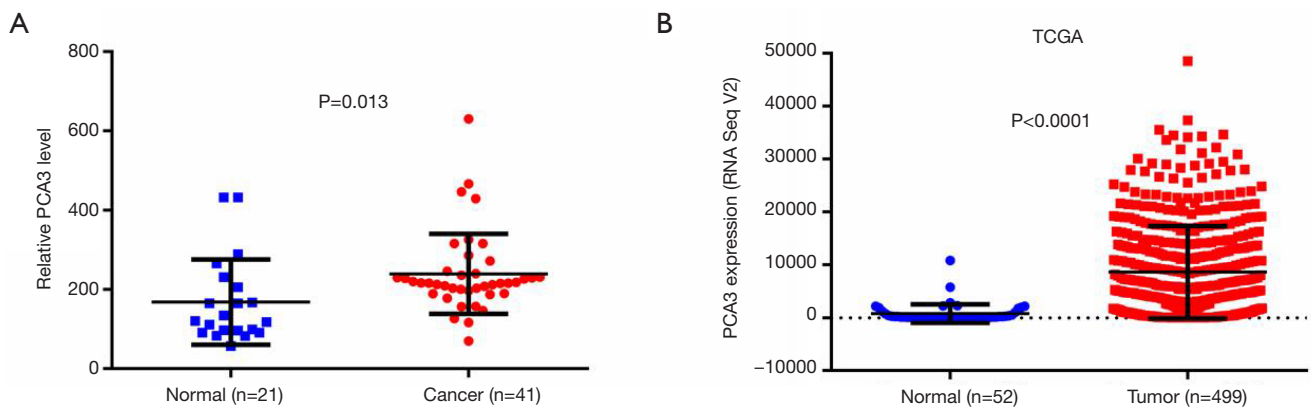


Figure 1 DD3 mRNA was upregulated in PCa tissues. (A) Upregulated expression of DD3 mRNA in PCa tissues compared with adjacent normal tissues, as determined by RT-qPCR analysis of tissue samples. (B) Increased expression of DD3 mRNA in PCa tissues compared with adjacent normal tissues from TCGA dataset. DD3, prostate cancer antigen 3; PCa, prostate cancer; TCGA, The Cancer Genome Atlas.

analysis results. Observations were considered statistically significant when two-tailed P values <0.05 . Data values are reported as the mean \pm standard error of the mean unless otherwise noted.

Results

Elevated DD3 mRNA expression in PCa tissues

DD3 mRNA contains four exons. We selected an appropriate exon of DD3 mRNA for amplification according to a recent report (19). The expression patterns of DD3 were examined across 62 tissue specimens using the described SYBR Green RT-qPCR assay. A total of 41 PCa tissues and 21 adjacent normal tissues were analyzed. DD3 expression was significantly increased ($P=0.013$) in PCa tumors compared with paired adjacent prostate tissues (Figure 1A). By analyzing data from the publicly available TCGA database we further validated the significant increase of DD3 expression in PCa tissues ($P<0.0001$, Figure 1B).

DD3 upregulation associated with poor outcome

To evaluate the value of DD3 expression level as a prognostic marker of PCa, patients from TCGA dataset were divided into high DD3 expression and low DD3 expression groups by the median level of DD3. The Kaplan-Meier curve suggested a trend linking shorter OS with high DD3 expression than with low DD3 expression ($P=0.36$, Figure 2A). Further analysis of patients who survived longer than 5 years showed that patients with low DD3 expression

had longer OS ($P=0.02$, Figure 2B). The Gleason score and TNM stage of PCa were important manifestation of disease progression. We analyzed the relationship between DD3 expression and Gleason score and TNM stage of PCa from TCGA dataset. The expression of DD3 in patients with Gleason score ≥ 8 was higher than in those with Gleason score ≤ 7 ($P<0.01$, Figure 2C). Also the expression levels of DD3 in PCa was correlated with TNM status ($P<0.05$, Figure 2D).

Different blood DD3 mRNA level between PCa and BPH patients

RNA was isolated *in vitro* and purified from the peripheral blood samples of a total of 155 patients, including 102 (65.81%) and 53 (34.19%) cases of biopsy-confirmed PCa and BPH, respectively (Table 1). The optical density at 260 nm (OD_{260}) over that at 280 nm (OD_{280}) of RNA ranged from 1.8 to 2.0. Patients' age ranged from 52 to 89 years. The relative mRNA expression of DD3 was significantly lower in BPH patients (2.87 ± 6.88) than in PCa patients (6.77 ± 8.49) ($P=0.005$). However, there was no significant difference in patient's age ($P=0.12$), free PSA (fPSA) levels ($P=0.546$) or total PSA levels ($P=0.659$) between patients with BPH and patients with PCa (Table 1).

Better diagnostic value for DD3 than total and free PSA

A ROC curve was used to illustrate the diagnostic performances of DD3 mRNA and PSA for the detection of PCa (Table 2). In particular, the diagnostic performance

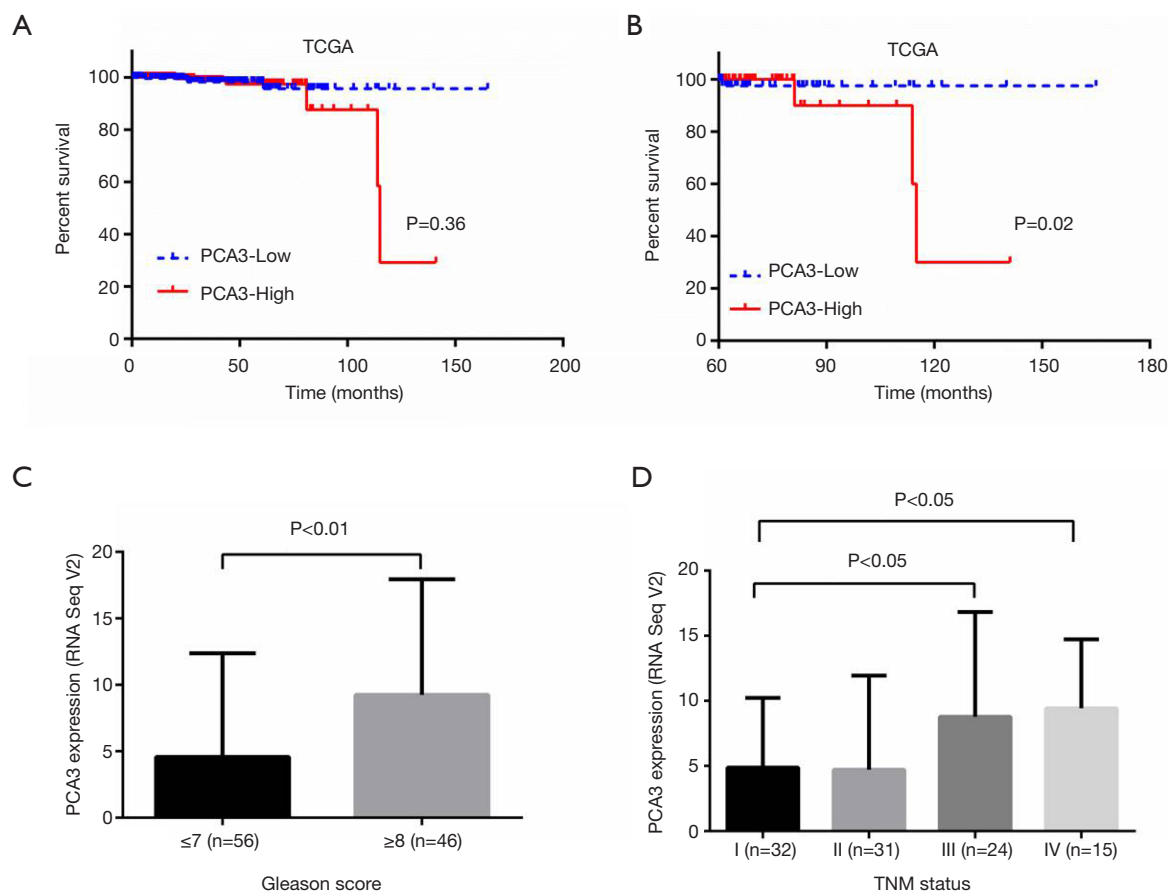


Figure 2 DD3 expression correlated with a poor prognosis in PCa patients. (A) Kaplan-Meier analysis of OS based on DD3 expression level in 496 PCa patients from the TCGA dataset. (B) Kaplan-Meier analysis of OS based on DD3 expression level in 85 cases with survival time longer than 5 years from the TCGA dataset. The median level of DD3 was used as the cutoff. (C) Expression of DD3 in Gleason score ≥ 8 was higher than in Gleason score ≤ 7 ($P < 0.01$). (D) Expression levels of DD3 in PCa in connection with TNM status ($P < 0.05$). Error bars indicated standard deviation. DD3, prostate cancer antigen 3; PCa, prostate cancer.

Table 1 Clinical characteristics of men with PCa and BPH

Characteristics	PCa	BPH	P value
Subjects (n)	102	53	
Age (years)	72.63 \pm 8.07	70.34 \pm 9.61	0.12
DD3 mRNA	6.77 \pm 8.49	2.87 \pm 6.88	0.005
fPSA (ng/mL)	10.00 \pm 10.05	11.15 \pm 12.81	0.546
PSA (ng/mL)	22.55 \pm 12.58	23.83 \pm 23.32	0.659

PCa, prostate cancer; BPH, benign prostatic hyperplasia; PSA, prostate-specific antigen; fPSA, free PSA; DD3, prostate cancer antigen 3.

Table 2 AUC of DD3 log copy number, PSA, and fPSA in patients

Variable	AUC	P value	95% CI
DD3 mRNA	0.738	<0.0001	0.661–0.805
fPSA (ng/mL)	0.554	0.3243	0.472–0.634
PSA (ng/mL)	0.570	0.1941	0.489–0.650

AUC, area under the curve; CI, confidence interval; PSA, prostate-specific antigen; fPSA, free PSA; DD3, prostate cancer antigen 3.

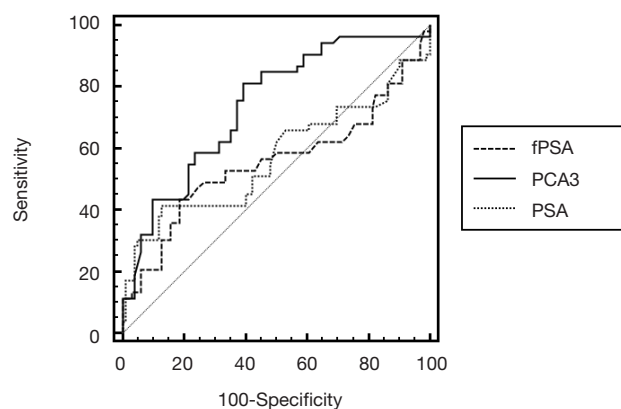


Figure 3 ROC curve of peripheral blood DD3 mRNA, PSA, and fPSA as prostate cancer detection markers. DD3, prostate cancer gene 3; PSA, prostate specific antigen; fPSA, free PSA; ROC, receiver operating characteristic.

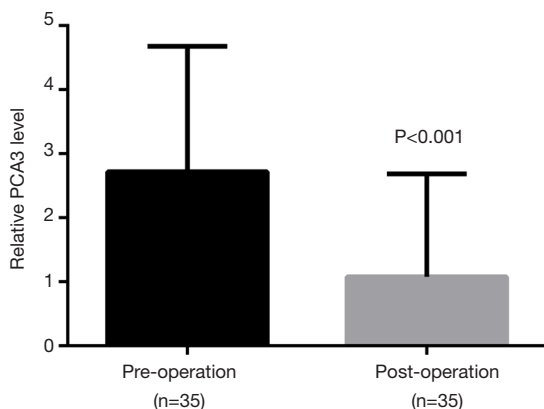


Figure 4 DD3 expression in blood was significantly decreased following surgical intervention (n=35). Measurements were made from preoperative and postoperative paired tissue samples. Error bars indicated standard deviation. DD3, prostate cancer gene 3.

of DD3 mRNA, fPSA, and PSA with regard to sensitivity and specificity was demonstrated (Figure 3). The AUC of both PSA and fPSA was smaller than that of DD3 mRNA.

The DD3 mRNA showed a significant correlation with the clinical diagnosis of PCa ($P < 0.0001$).

Blood DD3 decreased after prostatectomy

To evaluate prognostic value of blood DD3 mRNA level in PCa, blood specimens from 35 PCa patients were collected before and after the operation. Total RNA from whole blood was isolated and subjected to SYBR Green-based RT-qPCR to measure DD3 expression. The results showed that DD3 expression in the blood was significantly decreased postoperatively compared to preoperative samples ($P = 0.0016$), suggesting that it might act as an effective tumor prognostic marker (Figure 4).

DD3 associated with PCa tumorigenesis and proliferation

To address the mechanisms of DD3-driven tumorigenesis, TCGA PCa samples were analyzed with GSEA. Intriguingly, GSEA showed significant enrichment of tumorigenesis-related genes in samples with high DD3 expression, suggesting that DD3 might participate in the tumorigenesis of PCa (Figure 5A). Then, GSEA was used to further explore the correlation of DD3 expression level with cell proliferation in PCa patients. PCa patient data from the TCGA dataset were divided into high and low DD3 expression groups by the median level of DD3. Correlation between DD3 expression level and cell proliferation gene set was analyzed (Figure 5B). The gene set correlating with cell proliferation was highly enriched in the DD3 high expression group, suggesting that DD3 expression level was positively correlated with cell proliferation.

Discussion

Currently, serum PSA levels are widely used to screen patients for PCa. According to the European Association of Urology and the National Comprehensive Cancer Network guidelines, a digital rectal examination followed

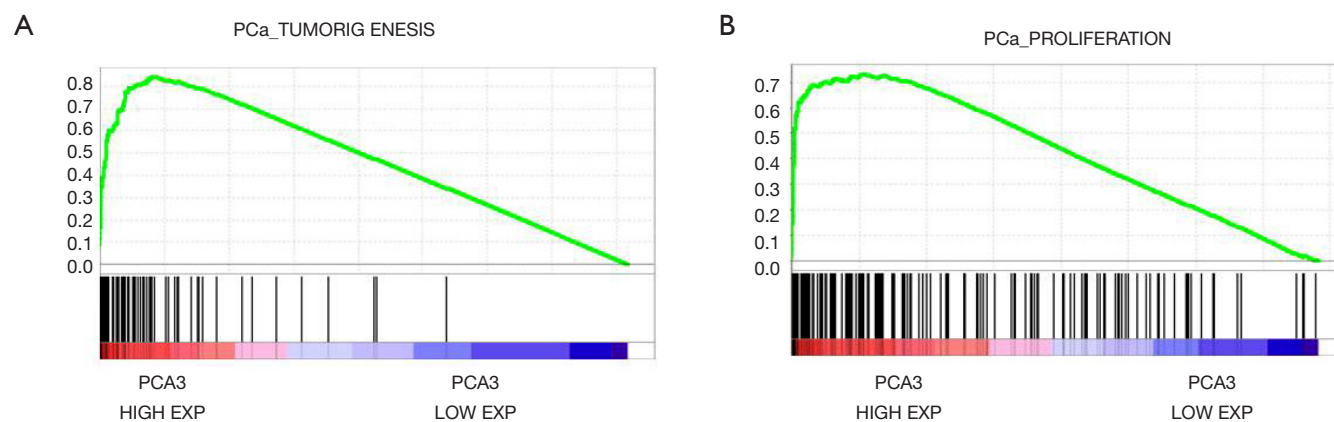


Figure 5 DD3 might be associated with the tumorigenesis and proliferation of PCa. High DD3 expression was positively correlated with (A) PCa tumorigenesis and (B) cell proliferation in PCa patients. DD3, prostate cancer antigen 3; PCa, prostate cancer.

by histological analysis of prostate biopsy is the golden standard for PCa diagnosis (20,21). However, the specificity of PSA detection for PCa is low, and approximately 75% of men who are clinically suspected of PCa have a negative biopsy result (22,23). Thus, it remains a challenge to explore and develop a diagnostic signature of PCa through the application of novel high-fidelity biomarkers. Diagnosis of PCa has improved with the identification of new biomarkers, and data suggested that implementation of DD3 testing is reducing the number of unnecessary biopsies.

DD3 was the first PCa-related non-coding RNA shown to be specifically expressed in prostate tissues and only overexpressed in PCa tissues (24). The clinical application of DD3 has been established as an independent diagnostic biomarker for the prediction of PCa in initial and repeated biopsy environments, primarily through urine-based and biopsy analyses (25). Studies have reported that DD3 expression was high in 95% of PCa tissues compared with paired adjacent tissues (26,27). DD3 has been shown to be a poor marker in the initial biopsy setting with a high false negative rate and low sensitivity across several studies, leading to the underdiagnosis of high-grade disease (28,29). However, DD3 mRNA transcripts can be detected and quantified as a cell-free marker in urine samples (17,18). In addition, urinary DD3 mRNA provides a more accurate PCa diagnostic target than PSA RNA and serum tPSA (19,30,31). Given the limitations inherent to sample acquisition of biopsy tissue and urine, specifically puncture biopsy, and lack of sample collection standardization, the downstream evaluation of DD3 is limited. Unlike urine, blood sample acquisition is clean and standardized, resulting

in dramatically improved sample reliability.

In this study, DD3 mRNA expression was significantly higher in PCa tissues than in paired adjacent tissues, as determined by RT-qPCR analysis. The expression of DD3 was verified through analysis of the TCGA database. Ferreira *et al.* (13) reported DD3 might modulate PCa cell survival. This study examined DD3 as it relates to PCa patient prognosis and survival. The Kaplan-Meier curve suggested that low DD3 expression led to longer OS than did high DD3 expression. ROC curve has been well documented as an effective way to assess the sensitivity and specificity of multiple biomarkers for diagnostic applications. Thus, in this study, ROC curve analysis was used to compare the diagnostic efficacy of DD3 comparing with PSA and fPSA in PCa. The AUC of DD3 mRNA was larger than both PSA and fPSA, suggesting that DD3 is a better tumor biomarker for the effective diagnosis of PCa. Previous data have demonstrated increased sensitivity and specificity when using DD3 copy number as a PCa diagnostic marker compared with PSA copy number, as determined by TaqMan-based RT-qPCR (32). Furthermore, the overall diagnostic efficacy of DD3 RNA is higher than that of PSA RNA (19).

There has been contradiction in the AUC data of DD3 in the literature (33,34), which might be an artifact of their small sample sizes. Following this expanded sample number analysis, the difference of AUC between DD3 and PSA in our study was closer to what had been previously reported in the literature. Interestingly, DD3 expression in the blood was significantly decreased in postoperative samples compared with preoperative samples, further suggesting

DD3 as an effective tumor prognostic marker, a finding that has not been previously reported.

The signaling pathway of DD3 in PCa is gradually being elucidated. Pro-survival signals mediated by AR are key factors in the development of PCa due to the known impacts on PCa cell proliferation, differentiation, and survival (35). In addition, DD3 promotes cell proliferation, invasion, and migration and inhibits apoptosis (15). Our GSEA analysis showed that high DD3 expression was correlated with PCa proliferation-associated genes.

Acknowledgments

Funding: This study was funded by the National Natural Science Foundation of the People's Republic of China (Grant No. 81372367 and 81402292), the Yantai Key Research and Development Project (Grant No. 2017YD005), and the Shandong Provincial Natural Science Foundation, China (Grant No. ZR2017PH047).

Footnote

Data Sharing Statement: Available at <http://dx.doi.org/10.21037/tau-19-899>

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/tau-19-899>). 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). The study was approved by the institutional research board of ethical committee at the Affiliated Yantai Yuhuangding Hospital of Qingdao University (No.: 2020-168) and informed consent was taken from all the patients.

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Cite this article as: Wang K, Zhao H, Wang W, Zhu Y, Zhang X, Ma J, Tan H, Zhang Y, Lin C. Effect of upregulation of DD3 on early detection and prognosis in prostate cancer. *Transl Androl Urol* 2020;9(4):1550-1558. doi: 10.21037/tau-19-899