CCBE1 is a Prognostic Biomarker and Contributes to Prostate Cancer Cell Progression.

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Research Article

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CCBE1 is a prognostic biomarker and contributes to prostate cancer cell progression.

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Abstract

Subject: Collagen And Calcium Binding EGF Domains 1 (CCBE1) is a coding protein which plays a significant role in extracellular matrix remodeling and migration and is involved in the development of Hennekam syndrome and lymphangiogenesis. Here, we investigate its prognostic value in prostate cancer based on TCGA database and its antioncogenic role in prostate cancer.

Methods: Wilcoxon rank sum test, Pearson χ^2 test, and logistic regression analysis were utilized to evaluate the correlation between CCBE1 and clinicopathological variables. Kaplan-Meier and Cox regression analysis were used to reveal the relation between CCBE1 and survival rates. The role of CCBE1 in prostate cancer was investigated using CCK-8 assay, EdU assay, and transwell experiments, respectively.

Results: Here, we found that CCBE1 expression is down-regulated in prostate cancer tissue dramatically in TCGA database. Furthermore, high CCBE1 expression predicted a good prognosis in patients with prostate cancer. High expression level of CCBE1 in PRAD cohort was prominently correlated with T classification (OR =0.49 for T3&T4 vs T2, $P<0.001$), Gleason score (OR = 0.42 for 8&9&10 vs. 6&7, $P<0.001$). Kaplan-Meier and Cox regression analysis showed that prostate cancer patients with high CCBE1 expression had a better progression-free interval (hazard ratio [HR]:0.50; 95% confidence interval [CI]: 0.33-0.77; $P = 0.002$) and overall survival (hazard ratio [HR]:0.38; 95% confidence interval [CI]: 0.15-0.92; $P = 0.032$). In vitro experiments indicated that overexpressed CCBE1 inhibited prostate cancer cell proliferation, migration, and invasion.

Conclusion: CCBE1 plays a pivotal role in the progression of prostate cancer and up-regulated CCBE1 expression inhibits prostate cancer tumorigenicity.

Keywords: CCBE1, prostate cancer, prognosis, The Cancer Genome Atlas database, tissue microarray

Introduction

Prostate cancer is one of the most common malignant tumors worldwide [1]. The morbidity and mortality of prostate cancer rank the first in male tumors in the US [2]. Although the morbidity of prostate cancer in China is much lower than that in Europe and US, with the aging population and changes of lifestyle, the morbidity shows an increasing trend in recent years [3]. At present, prostatectomy and radiation are considered to be the standard of care for locally prostate cancer [4]. Many patients, however, experience recurrence after surgery [5,6]. Androgen receptor (AR), a hormone-dependent transcription factor, modulates the carcinogenesis of prostate cancer crucially [7]. Therefore, androgen deprivation therapy (ADT) is recommended as front-line treatment to induce the amount of circulating testosterone to a very low level [8,9,10], which dramatically suppresses the progression of the tumor. However, some

tumors can become hormone independent over a period of 12-36 months of ADT, featured by up-regulation of PSA levels in blood and the increasing AR in cancer cells [11,12]. This state of prostate cancer is called castration resistant prostate cancer (CRPC), considered to be the main cause of death among prostate cancer patients [13]. In addition, prognosis takes into account clinical tumor stage and Gleason scores, patients in early stage have higher survival rate [14], therefore, it is crucial to identify reliable predictors which are associated with tumor stage and Gleason score, and identify new targets for diagnosis and prognostic assessment of prostate cancer. Although multiple biomarkers are proved to be associated with pathogenesis of prostate cancer, such as PSA, CCAT2 [15], MALAT1 [16,17], their reliability remains controversial.

Collagen And Calcium Binding EGF Domains 1 (CCBE1), also known as HKLLS1, is an encoding protein which is widely expressed in numerous tissues and plays a significant role in lymphangiogenesis [18]. Previous study has demonstrated that CCBE1 mutations are involved in lymph vessel dysplasia [19,20] and Hennekam Syndrome [21]. In addition, it is also related to multiple cancers [22]. For example, CCBE1 is overexpressed in colorectal cancer and promotes tumor lymphangiogenesis [23,24,25,26]. In contrast, CCBE1 acts as a tumor suppressor gene in lung cancer [27] and breast cancer [28]. However, its role in prostate cancer has not been elucidated.

In this study, we explored CCBE1 expression of patients with prostate cancer adapted from The Cancer Genome Atlas (TCGA) database and a tissue microarray containing 291 prostate cancer patients. Then, we investigated the relationship between CCBE1 expression and prognostic indicators of prostate cancer patients, including progression-free interval (PFI) and overall survival (OS). Gene set enrichment analysis (GSEA) was performed to reveal related biological process. In addition, we investigated the correlation between CCBE1 expression and tumor-infiltrating immune cell levels in prostate tumor

microenvironment via GSVA package in R. To further elucidate the function of CCBE1 in prostate tumor, a series of experiments were performed in vitro.

Materials and methods

RNA-sequencing data and bioinformatics analysis

A total of 495 cases with both gene expression data (HTSeq-Counts) and clinical information from PRAD project were collected from TCGA for further analysis. Next, we transformed HTSeq-Counts data into TPM (transcripts per million reads). Considering the fact that the relation between CCBE1 expression and the process of prostate cancer is independent of the follow-up days, 495 data of cases were utilized for survival analysis. In addition, the characteristics of patients consists of age, race, TNM stage, Gleason score, PSA level and TP53 status. Several of them which were not available were treated as missing value. This study satisfied the publication requirement stated by TCGA (<http://cancergenome.nih.gov/publications/publication-guidelines>).

Gene set enrichment analysis

In order to elucidate the significant function and pathway difference between high- and low-expression groups of CCBE1, GSEA was carried out by R package clusterProfiler (3.8.0) [29]. Gene set permutations were performed 1000 times for each analysis. CCBE1 expression level was regarded as a phenotype label. The adj.P value < 0.05, FDR q-value < 0.25, and normalized enrichment score were conducted to sort the pathway enrichment in each phenotype.

Immune infiltration analysis by ssGSEA

The immune infiltration analysis of prostate cancer was performed by ssGSEA (single-sample Gene Set Enrichment Analysis) method using GSVA package [30] in R (3.8.0) for 24 types of immune cells in

prostate cancer samples containing NK cells, Mast cells, Th1 cells, Neutrophils, Tgd, Eosinophils, Tem, iDCs, Macrophages, B cells, DCs, T cells, Tfh, Tcm, T helper cells, Cytotoxic cells, Th17 cells, NK CD56dim cells, CD8 T cells, pDCs, Th2 cells, aDCs, NK CD56bright cells, Treg [31]. The correlation between CCBE1 expression and these immune cells was evaluated by Spearman correlation, and the infiltration of immune cells between high- and low- CCBE1 groups was analyzed by Wilcoxon rank sum test.

Cell lines and transfections

Prostate cancer cell lines PC-3, DU145 were purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China), and had been validated by short tandem repeat DNA profiling analysis. Cell lines PC-3, DU145 were cultured in MEM (Gibco, A4192201) media with 10% Fetal Bovine Serum (FBS) (Gibco) and at 37 °C under a humidified atmosphere of 5% CO₂.

Plasmid vectors (pcDNA3.1, pcDNA3.1-CCBE1) were obtained from Miaolingbio (Wuhan, China). PC-3 and DU145 cells were transfected with a mixtures of plasmid pcDNA3.1-CCBE1 and Lipo2000 (Invitrogen) in 6-well plates, using cell lines transfected with pcDNA3.1 as a negative control group.

Western blotting analysis

Whole cell extracts were generated using RIPA lysis buffer (Sangon Biotech, China) with 1% protease inhibitor cocktail (Bimake). Protein samples were boiled with SDS/PAGE sample buffer for 10 minutes. After subjecting on SDS-polyacrylamide gel electrophoresis, protein samples were transferred to VEDF membranes (Millipore). The membranes were blocked in TBST with 5% BSA and then were probed with the CCBE1 antibody (Infinity, DF10092) and the GAPDH antibody (CST, D16H11) overnight respectively. The membranes were incubated with HRP-linked secondary antibodies anti-rabbit IgG (CST) for one hour at room temperature and were visualized with ECL Prime Western Blotting

Detection Reagent.

Cell Counting Kit-8 assay

PC-3 and DU145 cells transfected with plasmid vectors were seeded in 96-well plates at a density of 3,000 cells per well. Then, cell viability was measured at 0, 24, 48, 72 hours with a CCK-8 assay kit. The 450nm OD value in each well was measured with a spectrophotometer.

5-ethynyl-2'-deoxyuridine (EdU) assay

Cells were inoculated in 96-well plates at a density of 10,000 cells per well. Then cells were cultured using media with 10μmol EdU (KeyGEN, Nanjing, China) for 1 hour. Then, cells were fixed in 4% PFA for 15 min and subsequently permeabilized in PBS with 0.3% Triton X-100 for 30 min. According to the assay, cells were incubated in Click-iT kfluor488 mixture for 30min and were counterstained with DAPI for 15min. In order to measure the proliferation rate of cells, the images were obtained under a fluorescence microscopy and the proliferation rate was calculated at a ratio of EdU-positive cells/ DAPI-positive cells.

Colony formation assay

Cell clone formation ability was calculated by plate clone formation assay. Cells were inoculated in 6-well plates at 200 cells per well. Half of the medium was then changed every 3 days for a further 2 weeks and then cell clones were fixed with 4% PFA and examined with crystal violet staining.

Migration and invasion assay

For transwell migration assay, a total of 20,000 cells were suspended in 200μl MEM media without FBS, seeded into the upper chamber of transwell inserts (Corning Falcon). The lower chambers contained MEM media with 20% FBS. After incubated for 18 hours, cells were fixed and stained with crystal violet for 15 minutes. For transwell invasion assay, 30,000 cells were seeded on the upper chamber of transwell

inserts, which had been coated with Matrigel (BD Biosciences). After incubated in MEM media with 20% FBS for 24 hours, the inserts were fixed and then stained with crystal violet staining solution. Cells were counted using ImageJ according to the random fields captured by 100× microscope.

Statistical analysis

In this study, the plots were produced using R (3.8.0) and all statistical analysis were conducted by SPSS (22.0). Pearson χ^2 test was utilized to determine the correlation between CCBE1 expression and clinicopathologic variables. The t-test $p < 0.05$ was used to determine the significant statistical difference between two groups. We compared the progression-free interval (PFI) of prostate cancer patients separated by the expression level of the specific gene. Kaplan-Meier curves were utilized to compare the survival time differences and the log-rank test $p < 0.05$ suggested the significance of survival time differences. Univariate and multivariate survival analysis were generated by using Cox logistic regression model to find independent factors, including age, T stage, N stage, M stage, Gleason score, PSA level, TP53 status, and CCBE1 expression. The hazard risk of the individual indicators was estimated by hazard ratio (HR) with 95% confidence interval (CI). All reported P-values were two-sided and P-values less than 0.05 was considered to be significant. * represents $P < 0.05$, ** represents $P < 0.01$ and *** represents $P < 0.001$.

Results

Association between CCBE1 expression and clinicopathologic features

As shown in Table 1, the data, which was collected from TCGA in October 2019, contained 495 tumor samples with both clinical information and gene expression data. The clinical information of patients consisted of race, age, PSA level, TNM stage, Gleason score, and TP53 status. To further analyze

the significance of CCBE1 expression, a total of 495 prostate cancer patients were divided into two cohort according to expression level of CCBE1. According to TNM stage, T2 stage was recognized in 187 patients (73 with low CCBE1 expression and 114 with high CCBE1 expression), T3 stage in 291 (166 with low expression and 125 with high expression), T4 stage in 10 (5 with low expression and 5 with high expression). 78 cases (53 with low CCBE1 expression and 25 with CCBE1 high expression) had regional lymph node invasion and 4 cases had distant metastasis. Correlation analysis indicated that CCBE1 expression was significantly associated with T stage ($P = 0.001$), N stage ($P = 0.003$), Gleason score ($P < 0.001$), TP53 status ($P = 0.001$). No correlation was observed between CCBE1 expression and other clinicopathologic features (Table 1).

As shown in Fig. 1A, CCBE1 expression was down-regulated in prostate cancer than in normal tissue with P-value less than 0.001. By comparing with 50 pairs cancer and adjacent tissue, results suggested that CCBE1 was low expressed in prostate cancer prominently (Fig. 1B). Furthermore, expression level of CCBE1 was lower in T III/IV stage than in T I/II stage (Fig. 1C), and similarly, CCBE1 expression of patients with regional lymph node invasion tumor was lower than those without lymph node invasion significantly (Fig. 1D). As shown in Fig. 1F, the expression of CCBE1 in prostate cancer was lower in patients with higher PSA levels with a P value less than 0.001. In addition, prostate cancer with a higher gleason score was correlated with lower expression of CCBE1 (Fig. 1G). As shown in Fig. 1H, patients with TP53 mutations had lower expression of CCBE1.

Univariate analysis using logistic regression showed that higher CCBE1 expression regarded as an independent variable was correlated with better prognostic characteristics (Table 2). High expression level of CCBE1 in PRAD cohort was significantly associated with T classification (OR =0.49 for T3&T4 vs. T2, $P < 0.001$), N classification (OR =0.45 for N1 vs. N0, $P=0.01$), Gleason score (OR =0.42 for

8&9&10 vs. 6&7, $P=0.01$). These results revealed that prostate cancer with high CCBE1 expression is more likely to be in a primitive stage than those with low CCBE1 expression.

Association between CCBE1 expression and prognosis and diagnosis of patients

Kaplan Meier curves were used to check the significant difference in survival rates between high expression level and low expression level of CCBE1. Results appeared that patients with high CCBE1 expression identified a higher survival rate of PFI than those with low CCBE1 expression in PRAD cohort ($P = 0.002$, Fig. 2A). In addition, as shown in Fig. 2B, a ROC curve was constructed to examine the diagnose value of CCBE1 in prostate cancer, and the result indicated that the area under the curve (AUC) of CCBE1 for diagnose was up to 0.885.

To confirm the result of prognosis in TCGA dataset, we performed a tissue-microarray to access the expression of protein CCBE1 in prostate tumors. According to the immunohistochemical test results, the prostate cancer tissues were divided into high CCBE1 expression group and low CCBE1 expression group (Fig. 2C). As shown in Fig. 2D, high expression of CCBE1 cohort identified a better overall survival (OS) than low expression cohort (HR = 0.38 (0.15-0.92); CI: 0.15-0.92; $P = 0.032$). The correlation between CCBE1 protein expression and clinicopathologic features was shown in table 3 and the immunohistochemical test results of tissue micro-array were shown in Supplementary Materials.

Next, univariate Cox regression analysis was performed to explore the correlation between CCBE1 expression and prognosis of prostate cancer patients. The results demonstrated that high CCBE1 expression correlated with a fine PFI (hazard ratio [HR]: 0.505; 95% confidence interval [CI]: 0.330-0.772; $P < 0.01$), and other clinical variables including advanced T stage, N stage, PSA level, and Gleason score remained associated with a good prognosis (Table 3). Multivariate analysis suggested that high CCBE1 expression was independently associated with a better PFI (HR = 0.582; CI: 0.357-0.949; $P =$

0.03). The details were shown in Table 4.

CCBE1 expression related signaling pathways obtained by GSEA

As a number of pathways contribute to tumor pathogenesis, high expression of CCBE1 may be closely correlated with multiple signaling pathways activated in prostate cancer. Therefore, GSEA was performed to identify the significant signaling pathways involved in prostate cancer between different expression level of CCBE1. According to NES, adjusted P-value, and FDR value, the most significant differential enrichment pathways in CCBE1 high expression phenotype were selected. Results indicated that hallmark myc targets, G2M checkpoint, E2F targets, GO cell cycle checkpoint, DNA replication initiation, KEGG ribosome were prominently enriched in high-risk group. The details were shown in Fig. 3A-3F and Table 5.

The correlation between CCBE1 expression and immune infiltration

In order to further show the microenvironment of prostate cancer tissue, ssGSEA was conducted to explore the association between CCBE1 expression and immune cell infiltration level, which was shown using spearman correlation. As shown in Fig. 4B, Treg cells had a positive correlation with CCBE1 expression dramatically with Spearman r up to 0.598 with a P-value less than 0.001. In addition, Mast cells were positively correlated with expression level of CCBE1 significantly with Spearman r up to 0.532 with a P-value less than 0.001. Other immune cells, including Th1 cells, Neutrophils, Tgd, Eosinophils, Tem, iDCs, Macrophages were moderately correlated with CCBE1 expression (Fig. 4A, 4D-4J).

Overexpressed CCBE1 inhibited the progression of prostate cancer in vitro

To further investigate the role of CCBE1 expression in prostate cancer, we selected PC-3 and DU145 cell lines for subsequent research. Western blot showed that protein expression of CCBE1 was effectively

up-regulated in PC-3 and DU145 cell lines transfected with pcDNA3.1-CCBE1 (Fig. 5A). As shown in Fig. 5B-5C, high expression of CCBE1 impaired the ability of proliferation in prostate cancer cells. EdU proliferation assay also demonstrated that up-regulated CCBE1 decreased the proliferation index of PC-3 and DU145 cells (Fig. 5D). As shown in Fig. 5E, CCBE1 overexpression inhibited the progression of colony formation in prostate cancer. For further studies, we conducted transwell assay to clarify the role of CCBE1 in prostate cancer migration and invasion. Results showed that the abilities of migration and invasion were significantly weakened in CCBE1 over-expressed prostate cancer cells with a P value less than 0.01 (Fig. 5F-5G). Taken together, CCBE1 expression was involved in the progression of prostate cancer cell and inhibits the abilities of proliferation, migration and invasion in prostate cancer in vitro.

Discussions

In the current research, we explored the expression of CCBE1 in prostate cancer and assessed the feasibility of CCBE1 acting as a prognostic biomarker. All 495 patients with prostate cancer from TCGA-PRAD were collected to access the correlation between CCBE1 expression and clinical variables. Among the late stage of prostate cancer patients in TCGA database, expression levels of CCBE1 were observed to down-regulated, which indicated that CCBE1 gene acted as a tumor suppressor gene affecting prostate cancer progression.

Previous studies have focused attention on CCBE1 mutations in inherited disease such as lymph vessel dysplasia [19,20] and Hennekam Syndrome [21]. Recent research, however, discovered that CCBE1 plays an important role in multiple carcinomas. For instance, Song's research has proved that CCBE1 is crucial to vascular endothelial growth factor C (VEGFC) proteolysis in colorectal cancer (CRC) and high CCBE1 expression promotes cell-induced lymphangiogenesis in CRC.

Contrary to the results of expression in CRC, CCBE1 expression was detected to be decreased in breast cancer, lung cancer and ovarian cancer, suggesting its role of tumor suppressor gene. However, CCBE1 expression has not been detected in prostate cancer and the mechanism of CCBE1 in prostate cancer has remained unclear. In this study, our results showed that high CCBE1 expression had good predictive value for prostate cancer and overexpressed CCBE1 contributed to prostate cancer progression.

In our current study, a remarkable decrease of the CCBE1 expression level in prostate tumor tissues was observed in high stage of TNM compared with tumor tissues in low stage. In addition, Clinical relevance study results suggested that high expression of CCBE1 was correlated with low Gleason scores, PSA levels, and mutations in TP53. However, there is no significant difference of CCBE1 expression between metastasis tumor and non-metastasis tumor because there are only 3 metastasis prostate tumor samples in TCGA-PRAD database. Besides, high expression of CCBE1 is connected with good prognosis of prostate cancer. As the Kaplan Meier curves shown, prostate cancer patients with a higher expression of CCBE1 had a higher survival rate of PFI and OS. Furthermore, multivariate Cox proportional hazard analysis implicated that CCBE1 was an independent biomarker in prostate cancer.

From previously published studies, tumor infiltrating immune are considered to be crucial to the prognosis of tumor patients and response to immunotherapy [32,33]. Multiple types of immune cells are proved to be related to tumor progression [32,33,34] and tumor-infiltrating immune may contribute to anti-tumor effects in Prostate cancer [35]. In our research, we discovered that expression of CCBE1 had a positive correlation with some types of immune cells, such as NK cells, Neutrophils, which are reported to be crucial to antitumor activities. These results were consistent with what we discovered of CCBE1 in Prostate cancer. To further analyze the role of CCBE1 in prostate cancer development,

progression and prognosis, GSEA was performed and indicated that high CCBE1 expression phenotype was negative correlated with multiple significant biological functions which were critical to tumorigenesis, such as DNA replication, G2M checkpoints, transcriptional initiation etc. But the specific mechanism of CCBE1 impacting DNA replication requires further clarification.

In summary, higher expression of CCBE1 was identified with a better prognosis in prostate cancer and it can even be served as a potential therapeutic target in prostate cancer by affecting DNA replication and cell cycle. Meanwhile, in order to promote the clinical utility of CCBE1 in the evaluation of prostate cancer prognosis, more comprehensive research need to be performed to confirm our findings.

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Contributions

PL substantially contributed to conception or design and drafted the manuscript for important content.

PL and HX did the experiment and contributed to acquisition, analysis, or interpretation of data. MZ,

YC, DZ, MG, ZW, YT, QC participated in the collection of clinical specimens. MZ, YC and MG

critically revised the manuscript for important content. ZW and MG approved the final manuscript. All

authors read and approved the final manuscript.

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Ethics declarations

Ethics approval and consent to participate

The study was approved by the ethical committee of Ninth People's Hospital, Shanghai Jiaotong

University School of Medicine. Informed written consent was obtained from the patients.

Consent for publication

Written informed consent for publication of the clinical details was obtained from the patients.

Consent.

Competing Interests

The authors declare that the research was conducted in the absence of any commercial or financial

relationships that could be construed as a potential conflict of interest.

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Figure 1 Association between CCBE1 expression and clinicopathologic characteristics

CCBE1 expression in tumor tissue and in adjacent tissue (A, B); expression level of CCBE1 was correlated with clinicopathologic characteristics, including TNM stage (C, D, E), PSA level (F), Gleason scores (G), and P53 status (H).

Figure 2 Association between CCBE1 expression and prognosis and diagnosis of patients

Kaplan Meier curves were performed to show the significant difference in survival rate of PFI between high expression level and low expression level of CCBE1 (A); ROC curve was used to examine the diagnose value of CCBE1 in prostate cancer (B). Representative IHC result of prostate cancer tissues from the CCBE1 high expression group and low expression group (C). Kaplan Meier curves showed the significant difference in OS between CCBE1 high-exp group and low-exp group (D).

Figure 3 CCBE1 expression related signaling pathways obtained by GSEA

Enrichment of genes in the hallmark myc targets by GSEA (A); Enrichment of genes in the G2M checkpoint by GSEA (B); Enrichment of genes in the E2F targets by GSEA (C); Enrichment of genes in

the GO cell cycle checkpoint by GSEA (D); Enrichment of genes in the GO DNA replication initiation by GSEA (E); Enrichment of genes in the KEGG ribosome by GSEA (F).

Figure 4 The correlation between CCBE1 expression and immune infiltration

The correlation between CCBE1 expression and 24 types of immune cell infiltration levels (A); expression of CCBE1 was positive correlated with Treg cells (B), Mast cells (C), Th1 cells (D), Neutrophils (E), Tgd (F), Eosinophils (G), Tem (H), iDCs (I), Macrophages (J).

Figure 5 overexpressed CCBE1 inhibited prostate cancer progression in vitro

Western blot results showed CCBE1 protein expression in PC-3 and DU145 cells after transfected with plasmids (A). The proliferation of PC-3 and DU145 cells that were transfected with pcDNA3.1-CCBE1 or negative control was assessed by CCK-8 assay (B, C). EdU assay was performed to examine the influence of CCBE1 overexpression on the proliferation of prostate cancer (D). Colony formation assay was used to measure the role of CCBE1 on proliferative capacity of PC-3 and DU145 cells (E). The migration and invasion of PC-3 and DU145 cells after the transfection of pcDNA3.1-CCBE1 or NC were measured by transwell assay (F, G).

Table 1 Patients' information in the TCGA

Characters	level	Low expression of CCBE1	High expression of CCBE1	p
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n		248	247	
T stage (%)	T2	73(29.9%)	114(46.7%)	0.001
	T3	166(68.0%)	125(51.2%)	
	T4	5(2.0%)	5(2.0%)	
N stage (%)	N0	168(76.0%)	176(87.6%)	0.003
	N1	53(24.0%)	25(12.4%)	
M stage (%)	M0	230(99.1%)	223(99.6%)	1
	M1	2(0.9%)	1(0.4%)	
Gleason score (%)	6	13(5.2%)	32(13.0%)	<0.001
	7	107(43.1%)	139(56.3%)	
	8	35(14.1%)	28(11.3%)	
	9	90(36.3%)	47(19.0%)	
	10	3(1.2%)	1(0.4%)	
Primary therapy outcome (%)	CR	155(73.5%)	182(81.6%)	0.121
	PD	18(8.5%)	10(4.5%)	
	PR	20(9.5%)	20(9.0%)	
	SD	18(8.5%)	11(4.9%)	
Residual tumor (%)	R0	146(63.5%)	168(71.5%)	0.139
	R1	82(35.7%)	64(27.2%)	
	R2	2(0.9%)	3(1.3%)	
TP53 status (%)	Mut	40(16.2%)	16(6.5%)	0.001
	WT	207(83.8%)	229(93.5%)	
Age (%)	<=60	101(40.7%)	121(49.0%)	0.079
	>60	147(59.3%)	126(51.0%)	
PSA(ng/ml) (%)	<4	199(91.7%)	212(95.9%)	0.101
	>=4	18(8.3%)	9(4.1%)	

Table2 CCBE1 expression associated with clinical pathological variables (logistic regression).

Characteristics	Total (n)	Odds Ratio in CCBE1	P value
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T stage (T3&T4 vs. T2)	488	0.49(0.33-0.70)	<0.001
N stage (N1 vs. N0)	422	0.45(0.26-0.75)	0.003
M stage (M1 vs. M0)	456	0.52(0.02-5.42)	0.59
Gleason score (8&9&10 vs. 6&7)	495	0.42(0.29-0.60)	<0.001
PSA(ng/ml) (>=4 vs. <4)	438	0.47(0.20-1.04)	0.072
TP53 status (Mut vs. WT)	492	0.36(0.19-0.65)	0.001

Table3 Correlation between CCBE1 expression and clinicopathological characteristics of prostate cancer in the cohort

Characters	level	Low expression of CCBE1	High expression of CCBE1	P
n		178	113	
age	<=60	17(9.6%)	15(13.3%)	0.322
	>60	161(90.4%)	98(86.7%)	
T stage	T2	67(37.6%)	44(38.9%)	0.049
	T3	103(57.9%)	64(56.6%)	
	T4	8(4.5%)	5(4.4%)	
N stage	N0	171(96.1%)	109(96.5%)	0.864
	N1	7(3.9%)	4(3.5%)	
Gleason scores	6	20(11.2%)	17(15.0%)	0.516
	7	112(62.9%)	68(60.2%)	
	8	28(15.7%)	13(11.5%)	
	9	17(9.6%)	15(13.3%)	
	10	1(0.6%)	0	

Table4 Associations with PFI and clinicopathologic characteristics in PRAD cohorts using univariate and multivariate Cox regression analysis

Characteristics	Total(N)	Univariate analysis		Multivariate analysis	
		HR (95% CI)	P value	HR (95% CI)	P value
T stage (T3&T4 vs. T2)	488	3.716(2.100-6.575)	<0.001	1.553(0.734-3.283)	0.249
N stage (N1 vs. N0)	422	1.854(1.137-3.026)	0.013	0.763(0.443-1.315)	0.33
Gleason score (8&9&10 vs. 6&7)	495	4.603(2.909-7.284)	<0.001	2.828(1.503-5.319)	0.001
Primary therapy outcome (PD&SD&PR vs. CR)	434	6.793(4.430-10.416)	<0.001	3.910(2.316-6.602)	<0.001
PSA(ng/ml) (>=4 vs. <4)	438	4.246(2.119-8.510)	<0.001	1.764(0.789-3.941)	0.167
TP53 status (Mut vs. WT)	492	2.086(1.258-3.461)	0.004	0.953(0.549-1.657)	0.866
CCBE1 (High vs. Low)	495	0.505(0.330-0.772)	0.002	0.582(0.357-0.949)	0.03

Table 5 Gene set enrichment analysis in high-expression and low-expression group

Name	NES	pvalue	p.adjust	FDR
HALLMARK_MYC_TARGETS_V1	-1.957	0.001	0.007	0.002
HALLMARK_G2M_CHECKPOINT	-2.055	0.001	0.007	0.002
HALLMARK_E2F_TARGETS	-2.309	0.001	0.007	0.002
GO_CELL_CYCLE_CHECKPOINT	-1.678	0.001	0.019	0.011
GO_DNA_REPLICATION_INITIATION	-1.876	0.002	0.019	0.011
KEGG_RIBOSOME	-2.468	0.002	0.014	0.007

Figures

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