



## Research article

## Verification of cell cycle-associated cyclin-dependent kinases facilitated prostate cancer progression by integrated bioinformatic analysis and experimental validation

Yean Huang<sup>a,1</sup>, Shuo Lu<sup>a,1</sup>, Yi Chen<sup>b,1</sup>, Yunhao Qing<sup>a</sup>, Ruji Wu<sup>a</sup>, Tan Ma<sup>a</sup>, Zixiao Zhang<sup>a</sup>, Yu Wang<sup>a,\*\*</sup>, Ke Li<sup>a,\*</sup><sup>a</sup> Department of Urology, The Third Affiliated Hospital of Sun Yat-sen University, Tianhe Road 600, Guangzhou 510630, China<sup>b</sup> Department of Surgery, Su Fengxi Clinic, Zhongshan 2nd Rd 54th and 56th, Yuexiu District, Guangzhou 510000, China

## HIGHLIGHTS

- ccCDKs were expressed at higher levels in prostate cancer tissues than in normal tissues.
- High expression of ccCDKs was associated with poor disease-free survival in prostate cancer patients.
- ccCDKs were enriched in the IL-18 signaling pathway and correlated with the infiltration of immune cells in prostate cancer.
- CDK3 promoted tumor progression in PCa cells and could be used to predict biochemical recurrence in PCa patients.

## ARTICLE INFO

## Keywords:

Prostate cancer  
Cell cycle-associated cyclin-dependent kinases  
Bioinformatic analysis  
Biochemical recurrence  
Prognosis

## ABSTRACT

**Introduction:** Cell cycle-associated cyclin-dependent kinases (ccCDKs) are essential regulators known to control cell division and facilitate tumorigenesis and progression. However, there is currently no comprehensive study of distinct ccCDKs in prostate cancer (PCa). The purpose of this study was to determine the value of ccCDK expression in predicting the prognosis of patients with PCa and to identify the gene functions of ccCDK in PCa.

**Methods:** The UALCAN databases were analyzed to examine the expression of CDKs in prostate cancer. The Human Protein Atlas was used to verify the expression of CDKs online. Then, we assessed the prognostic values of CDKs using GEPIA. GeneMANIA and Metascape analyses were used to predict biological functions. We analyzed the mutation of CDKs by cBioPortal. The TIMER database was used to evaluate the correlation of CDKs and immune infiltration. The expression of CDKs in tissue was examined through quantitative real-time polymerase chain reaction. After that, we focused on CDK3 and identified the expression of CDK3 by immunohistochemistry and western blot. The functions of CDK3 in C4-2 cell proliferation were determined by CCK-8 assays. C4-2 cells were tested for their ability to invade and migrate through transwell and wound healing assays.

**Results:** The results showed that CDK1/3/4/5/6/16 was expressed at relatively higher levels in PCa tissues than in normal tissues. Patients with low expression of CDK1/3/5/16 exhibited significantly better disease-free survival than those with high expression. ccCDKs were enriched in the IL-18 signaling pathway and correlated with the infiltration of immune cells in PCa. Moreover, our cohort study data verified that there were significantly higher expression of CDK1/3/5/16 in PCa tissues compared to benign prostate hyperplasia tissues, and CDK3 was remarkably associated with a shorter progression-free survival for biochemical recurrence in PCa patients. CDK3 was positively expressed in PCa cells and tissues, and functional experiments demonstrated that silencing CDK3 inhibited PCa cell proliferation, migration, and invasion.

**Conclusions:** Our study provides new evidence of ccCDKs in promoting PCa progression and implies that CDK3 may serve as an oncogene in PCa and may be valuable in the prognosis of biochemical recurrence in PCa patients.

\* Corresponding author.

\*\* Corresponding author.

E-mail addresses: [wangyu27@mail2.sysu.edu.cn](mailto:wangyu27@mail2.sysu.edu.cn) (Y. Wang), [like35@mail.sysu.edu.cn](mailto:like35@mail.sysu.edu.cn) (K. Li).<sup>1</sup> These authors contributed equally to this article.

## 1. Introduction

Prostate cancer (PCa) diagnosis is among the second most common among developed countries [1]. Even though PCa is often a slow-progressing disease, it remains one of the top three causes of cancer-related deaths in men [2]. Previous studies have identified common genetic drivers of PCa, such as the fusions of ETS genes, amplification of androgen receptor (AR) [3], and loss of TP53 and PTEN [4]. Recently, Wu et al. [5] showed that genomic instability is caused by biallelic CDK12 loss, resulting in tandem duplications increase, which form a specific subgroup of PCa, namely, Cyclin-dependent kinases (CDKs) are cyclin-dependent protein kinases whose activity and substrate selectivity are regulated by cyclin subunits [6]. Each CDK bound to cyclin is essential to controlling the cell cycle, division and transcription. CDKs can be categorized into two types: transcription-associated CDKs and cell cycle-associated CDKs (ccCDKs). The latter include CDK1, CDK2, CDK3, CDK4, CDK5, CDK6, CDK14, CDK15, CDK16, CDK17 and CDK18 [7]. There are precedents for using CDK inhibitors for clinical treatment, particularly against breast cancer, non small cell lung cancer, melanoma, and head and neck squamous cell carcinoma [8]. However, it is unknown how the ccCDK family of genes may affect the prognosis and treatment of PCa patients. Consequently, it is necessary for us to examine the underlying mechanisms regarding PCa therapy as well as progression and identify ccCDKs as biomarkers that have higher specificity and sensitivity.

In the present study, we surveyed the landscape of ccCDKs across PCa to identify the highest prevalence of ccCDK oncogenic alterations and described the clinicopathologic characteristics and clinical outcomes for PCa patients harboring ccCDK alterations. In addition, we verified these computational results in a further cohort study. Finally, the function of the ccCDK genes was further confirmed via functional analyses. These findings provide new ideas for elucidating the critical role of ccCDKs in PCa.

## 2. Methods

### 2.1. Patients and samples

A total of 60 PCa and 60 benign prostate hyperplasia (BPH) patients from December 2018 to December 2020 were eligible for inclusion at the Third Affiliated Hospital of Sun Yat-sen University. Based on histopathology and clinical history, the patients were diagnosed with PCa or BPH, and tissues were stored in RNAlater (Invitrogen Life Technologies, CA, USA) at  $-80^{\circ}\text{C}$  until RNA extraction. There was no chemotherapy, hormonal treatment, or preoperative radiotherapy for PCa patients. PCa patients received surgery, and biochemical recurrence was defined as 2 sequential instances of increasing PSA values  $>0.2\text{ ng/mL}$ . An informed consent form was received from all subjects and approved by the Third Affiliated Hospital of Sun Yat-sen University's Ethical Committee.

### 2.2. RNA isolation and qRT-PCR

We extracted the cellular total RNA from tissues using TRIzol reagent (Invitrogen Life Technologies, CA, USA), and the cDNA was reverse transcribed using PrimeScript™ RT reagent kit (TAKARA, Japan) according to the manufacturer's instructions. After mixing cDNA, TB Green, Dye II and primers, qRT-PCR was carried out on a Fast Real-Time PCR System 7500 (Applied Biosystems). We calculated relative difference in gene expression level by applying the  $2^{-\Delta\Delta\text{CT}}$  formula.

### 2.3. UALCAN

UALCAN (<http://ualcan.path.uab.edu>) is an online platform that analyzes cancer data derived from The Cancer Genome Atlas (TCGA). Using this database, the gene expression analysis may be carried out to compare the transcriptional expression of potential genes among tumors, normal

samples, tumor subgroups, as well as the association of transcriptional expression with clinical parameters [9]. UALCAN was used to analyze mRNA expression data for CDK family members of clinicopathologic parameters in this study.

### 2.4. GEPIA

The GEPIA analysis tool (<http://gepia.cancer-pku.cn/index.html>) provides data regarding RNA sequence expression. In this database, survival analysis is performed using gene expression analysis, based on sample selections and methods that are defined by the user [10]. In our study, we explored differential mRNA expression and correlative prognostic analysis of CDK family members.

### 2.5. cBioPortal

Online tool cBioPortal (<http://www.cbioportal.org>) visualizes and analyzes multidimensional data from cancer genomics [11]. PRAD (prostate adenocarcinoma) was analyzed for its genomic mutation types and alteration frequency. There are various genomic mutations of CDKs, including amplifications, deep deletions, mRNA upregulation, and unknown missense mutations.

### 2.6. GeneMANIA

GeneMANIA (<http://www.genemania.org>) is a popular website that provides information on interactions of gene and protein, gene enrichment analysis, coexpression, colocalization, and as well as predicting the function of favorite genes [12]. GeneMANIA enabled the prediction of CDKs function and visualization of gene networks.

### 2.7. Metascape

The Metascape data analysis website (<http://metascape.org>) is designed to provide experimental biologists with comprehensive database for gene annotation and analysis resource [13]. A pathway analysis of genes relating to CDKs was performed with Metascape using Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG). Analysis was conducted on GO terms with regard to cellular components (CC), biological processes (BP), and molecular functions (MF).

### 2.8. TIMER

TIMER2.0 (<http://timer.cistrome.org/>) evaluates immune infiltration degree for TCGA or user-supplied tumor profiles based on six state-of-the-art algorithms [14]. The "Gene module" was utilized in our study to examine the relationship between CDK levels and immune cell infiltration.

### 2.9. The Human Protein Atlas

The Human Protein Atlas (<https://www.proteinatlas.org>) is a database of immunohistochemistry (IHC)-based protein expression profiles for nearly 20 highly common kinds of cancers, normal tissue and cell lines [15]. Using this database, we identified the CDK family members of human normal prostate tissue and human PCa tissues. In details, antibody staining was observed on samples obtained from 144 individuals representing 44 various kinds of normal tissue by using tissue microarrays, and a total of 216 cancer samples representing 20 types of cancer. Every images of tissues stained by IHC undergo manual annotation by a specialist, followed by the other specialist's verification of the results. Among the basic annotation parameters are staining intensity (negative, weak, moderate or strong), proportion of stained cells ( $<25\%$ ,  $25\text{--}75\%$  or  $>75\%$ ), and subcellular distribution (nuclear and/or cytoplasmic/membranous). SNOMED is used to classify both topography and

morphology. In addition to the given original diagnosis, SNOMED is also used to describe the normal samples as well as the cancer samples.

2.10. Cell culture and transfection

American Type Culture Collection (ATCC, America) provided the human PCa cell lines LNCaP, C4-2, DU145, 22Rv1 and PC3, as well as the normal prostate epithelial cell line RWPE1. 10% fetal bovine serum supplement was used as a growth medium for PCa cells, and keratinocyte serum-free medium (K-SFM) (Thermo Fisher Scientific) was used for RWPE1 cells. We grew all cell lines in 37 °C incubators with 5% CO2. As in previous studies, small interfering RNAs (siRNAs) were used to knock down CDK3 mRNA levels [16, 17]. In details, si-CDK3 (sense, 5'-TTTGTGAGTTGGGTGCCATCAAGTTCAAGAGACTTGATGGCACCCA-ACTCATTTT-3', and antisense 5'-CTAGAAAAATGAGTTGGGTGCCA TCAAGTCTCTTGAACCTTGATGGCACCCA-3') was commercially

constructed by RIBOBIO (Guangzhou, China). A Lipofectamine 3000 Transfection kit (Invitrogen, United States) was used to transfect si-CDK3 into C4-2 cells in accordance with the instructions of manufacturer. 48 h after transfection, the following experiment and assays were conducted.

2.11. Western blot

Whole-cell extracts were prepared by lysing the cells in RIPA buffer supplemented with 1% PMSF. The BCA reagent (KeyGen Biotech, China) was applied in quantitation of the protein concentration, then 10% SDS-PAGE was used to separate equal amounts of total proteins. Then, PVDF membranes (Millipore, USA) were used to transfer the proteins to membranes. We blotted the membranes with an CDK3 antibody (ab197297, Abcam, 1:500), and the protein levels were normalized with GAPDH (2118, CST, 1:1000). Second antibodies (Affinity Biosciences, USA) and ECL reagents (Advanta, USA) were then used to visualize.

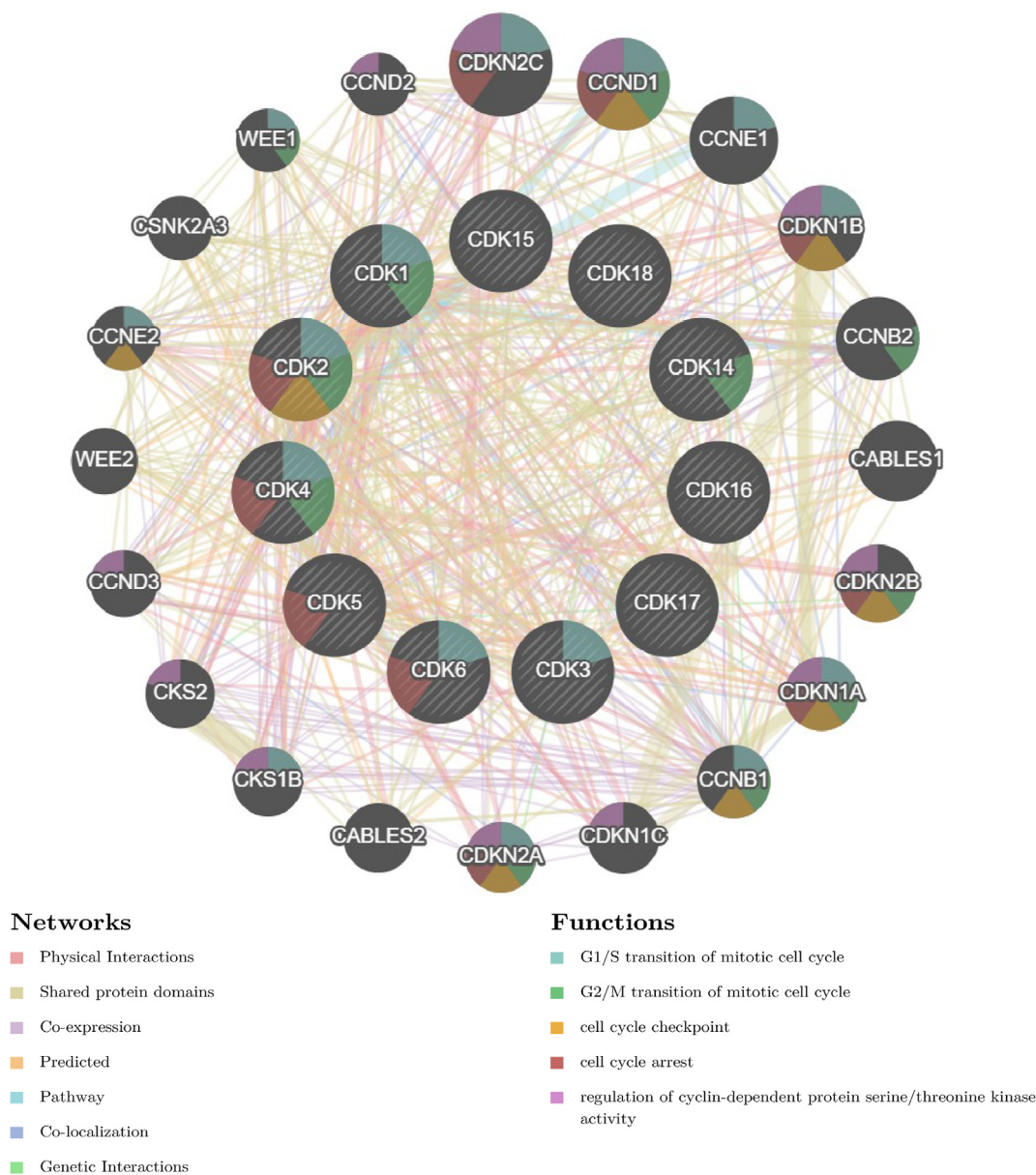
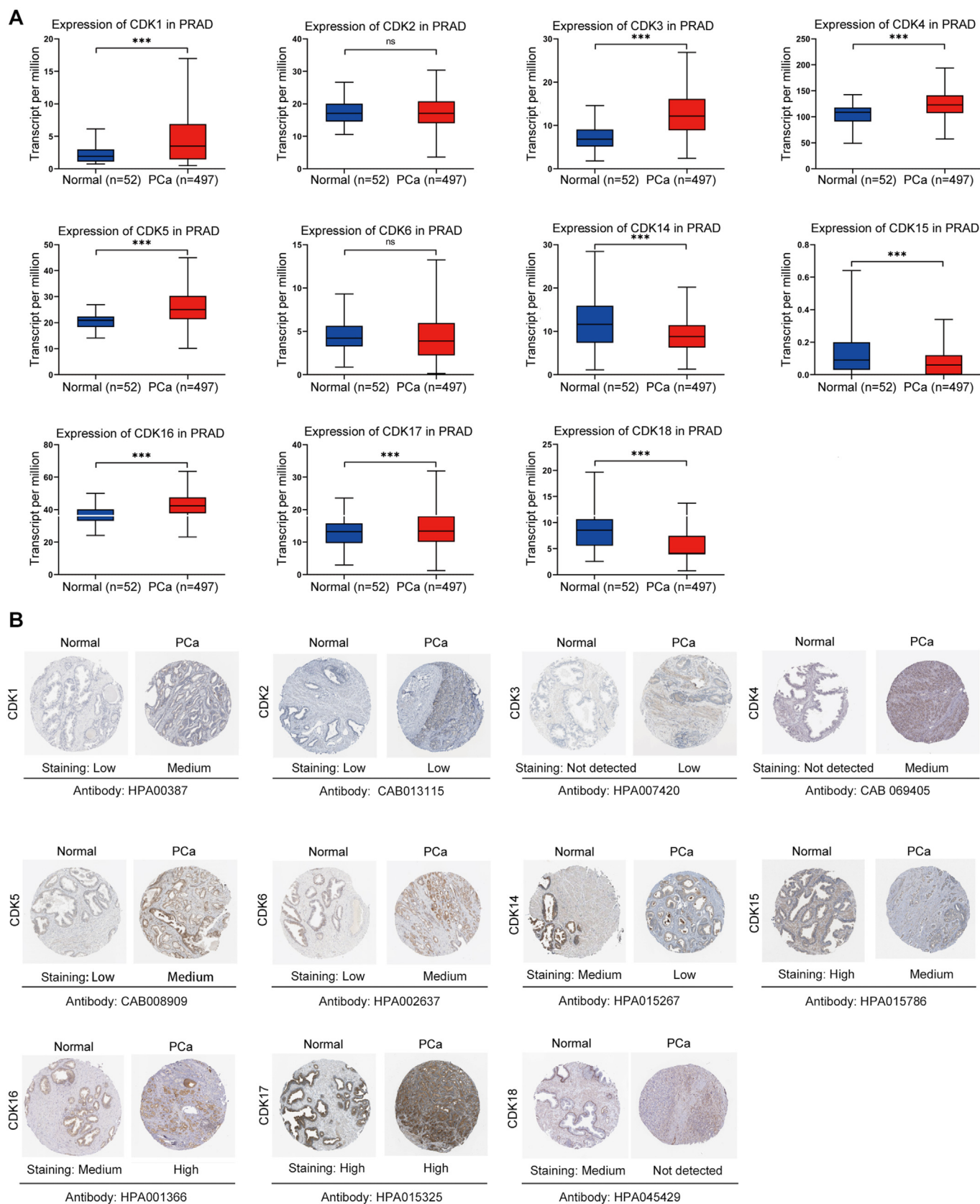
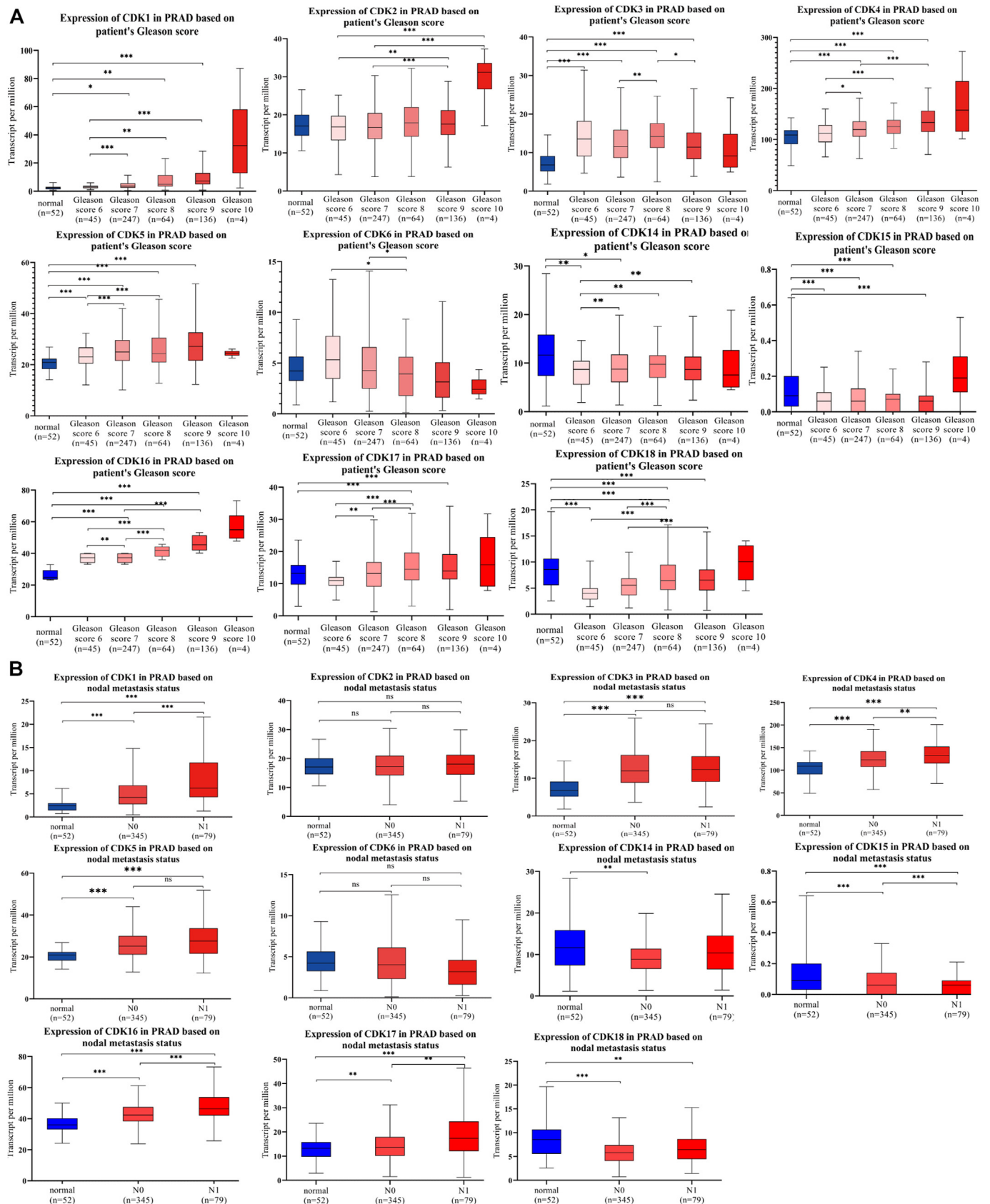


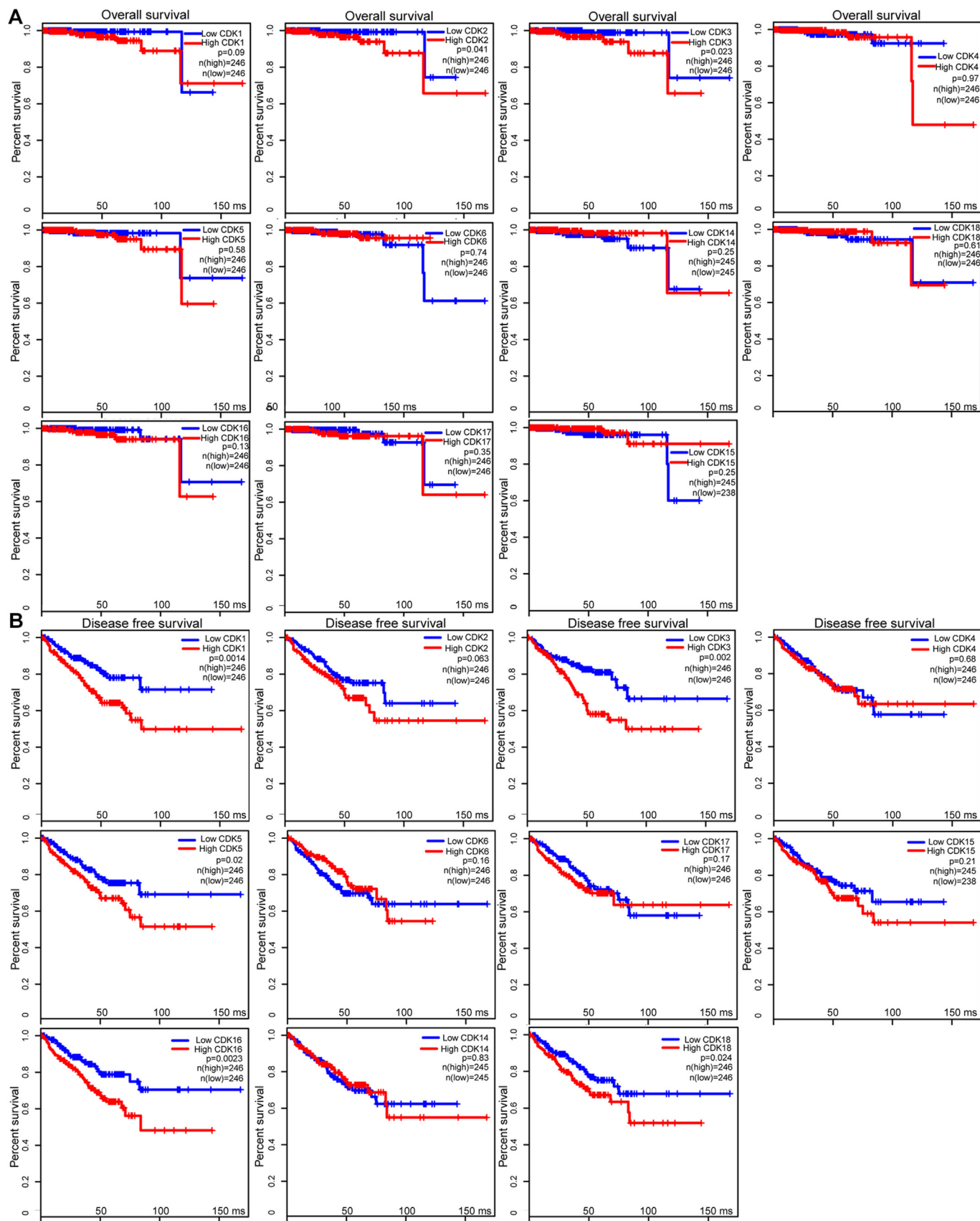
Figure 1. Protein-protein interaction network of ccCDK genes. Each node represents a gene. The node size represents the strength of interactions. The internode connection lines represent the types of gene-gene interactions, and the line color represents the types of interactions. The node color represents the possible functions of the respective genes. ccCDKs: cell cycle-associated CDKs.



**Figure 2.** ccCDKs are differentially expressed between PCa and normal tissues. A. qRT-PCR detection of the mRNA expression of ccCDKs in normal and PCa tissues from the UALCAN database. B. Immunohistochemical staining for the protein expression of ccCDKs in normal and PCa tissues from the Human Protein Atlas database. ccCDKs: cell cycle-associated CDKs. PRAD: Prostate adenocarcinoma. ns: not significant. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .



**Figure 3.** The relationship of ccCDK mRNA expression to clinicopathological parameters in PCa by UALCAN analysis. A. Correlation between mRNA expression of ccCDKs and Gleason score. B. Correlation between the mRNA expression of ccCDKs and lymph node metastasis status. PRAD: Prostate adenocarcinoma. ns: not significant. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .



**Figure 4.** The correlation between ccCDK expression and prognosis in PCA patients. A. GEPIA analysis showed the significance of ccCDK mRNA expression in predicting the prognosis of OS for PCA patients. High expression of CDK2/3 was significantly correlated with poor OS ( $p = 0.041$  and  $0.023$ , respectively). B. Survival analyses of differential ccCDK expression groups with DFS in TCGA PCA patients. High expression of CDK1/3/5/16 was significantly correlated with poor DFS ( $p = 0.0014$ ,  $0.002$ ,  $0.02$ ,  $0.0023$ , respectively). ccCDKs: cell cycle-associated CDKs. OS: overall survival. DFS: disease-free survival.

**Table 1.** Cell cycle CDKs coexpressed genes from GeneMANIA.

Genes											
CDK1	CDK3	CDK5	CDK16	CCNB1	CCNB2	CCNA2	CKS1B	SPAG6	CKS2	CCNB1	CKS1B
CCNE2	CCNE1	CDK5R2	NUBP2	FIBP	CCNB1	DUSP7	UBA1	CSNK2B	MLF2	BSG	TP11
EIF4G1											

**2.12. Cell proliferation assay**

C4-2 cells transfected with siRNA were seeded into 96-well plates at a density of 3000/well for 24 h. A time-course of 24 h, 48 h, 72 h, 96 h and 120 h after transfection was put into practice by adding 10 μL of CCK-8 (Dojindo, Japan) and incubating for 1 h at 37 °C. Microplate readers (BioTek, USA) were used to determine absorbance at 450 nm.

**2.13. Cell invasion, migration and wound healing assays**

Transwell inserts with pores of 8 μm (EMD Millipore Inc., United States) were used to determine cell invasion and migration according to a prior study. In addition, a scratch wound healing assay was also performed to assess cell migration after siRNA transfection. Briefly, we seeded PCa cells in a six-well plate. A scratch wound was created with a

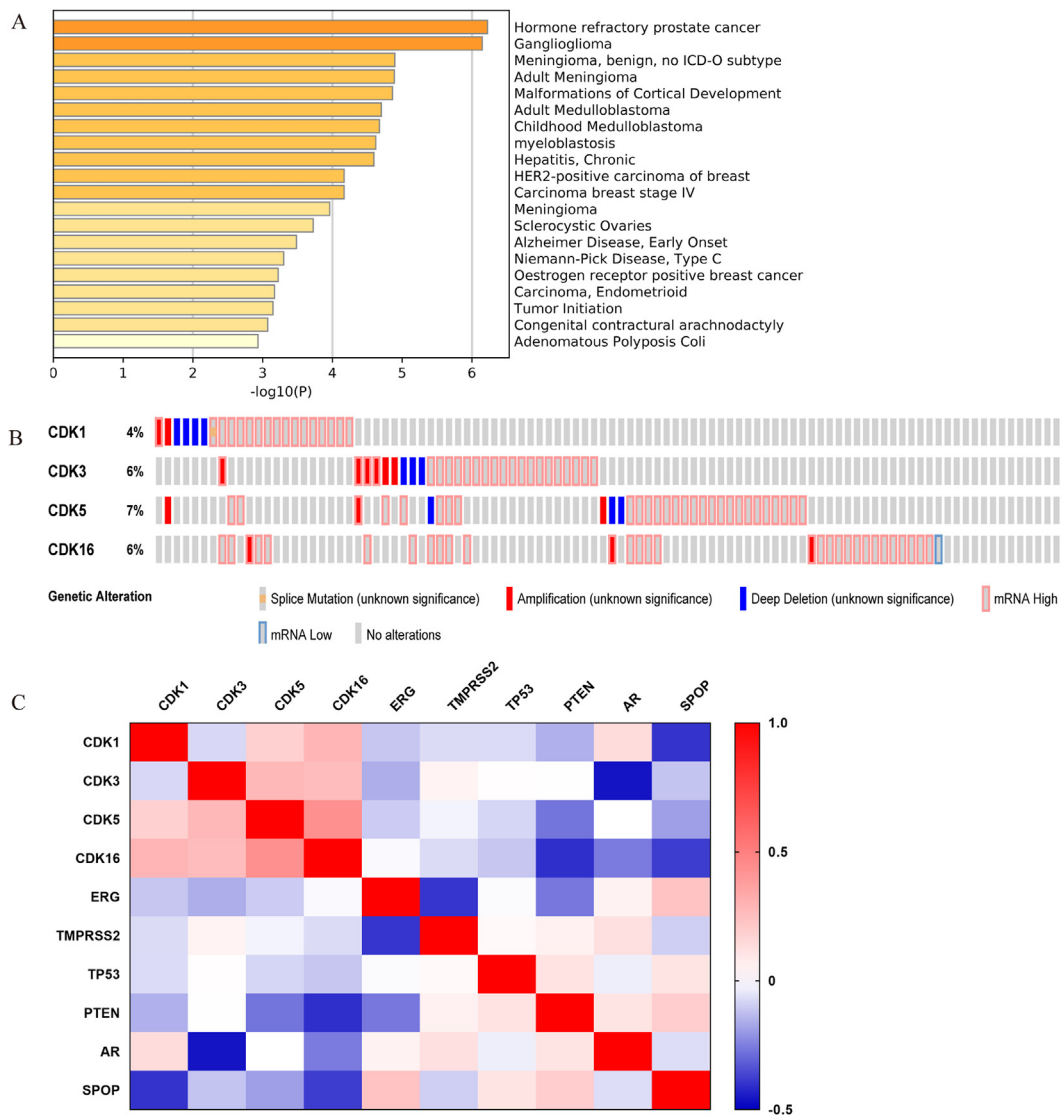
sterile micropipette tip when the cells reached subconfluence, and the 10% FBS medium was substituted with serum-free RPMI-1640 medium. We observed the scratch width every 24 h and photographed it under a microscope. The results are expressed in percent scratch closure.

**2.14. Tissues immunohistochemistry**

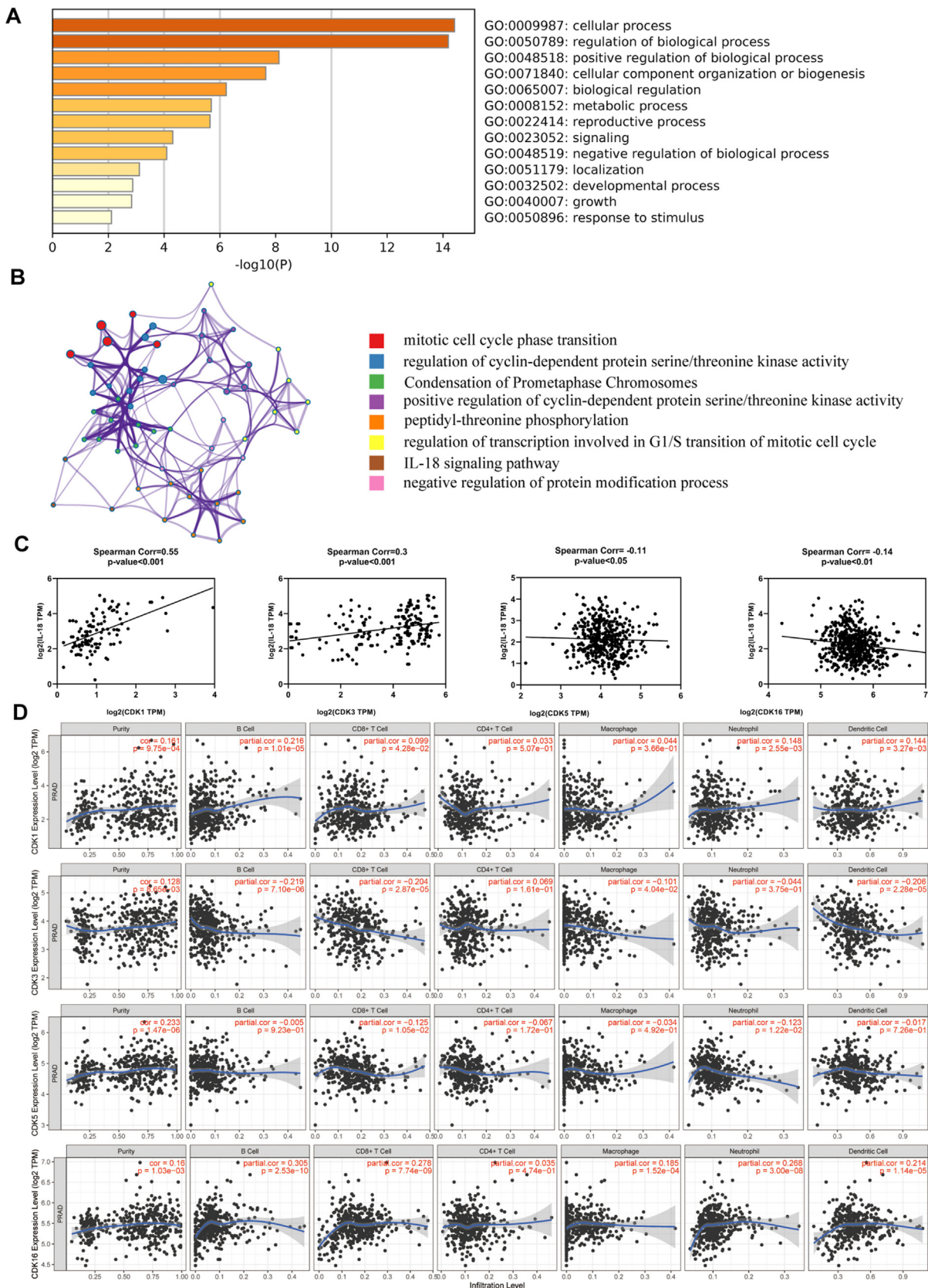
Formalin-fixed, paraffin-embedded tissue specimens from PCa patients were cut into 4 μm slices. Immunostaining was carried out with an antibody against CDK3 (ab197297, Abcam, 1:100). Antigen retrieval was conducted in citrate buffer (10 mmol/L, pH 6.0) at 100 °C for 15 min in a microwave oven, followed by endogenous peroxidase blocking with methanol containing 0.3% hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) for 15 min. We incubated primary antibodies at 4 °C overnight, and second antibodies at 37 °C for 30 min. Finally, sections were treated with 3,3'-diaminobenzidine (DAB) and counterstained with hematoxylin.

**2.15. Statistical analysis**

UALCAN and GEPIA gene expression data were analyzed using Student's t test. Spearman's correlation analysis was used for evaluation of correlation analysis. Database-derived tools were applied to all statistical



**Figure 5.** Gene-associated diseases, genetic alterations, and coexpression analyses of ccCDKs among PCa patients. A. Summary of enrichment disease analysis in DisGeNET of Metascape. B. Summary of alterations in differentially expressed ccCDKs in PCa from cBioPortal analysis. C) Correlation heatmap of differentially expressed ccCDKs and common genetic drivers in PCa. ccCDKs: cell cycle-associated CDKs.



**Figure 6.** Functions of ccCDKs as well as those showing significant association with ccCDK alterations. A. Heatmap of GO enriched terms colored by P values. B. Network of GO and KEGG enriched terms colored by P values. C. Scatter plot showing the correlations between (log2-transformed) the expression levels of IL-18 and CDK1/3/5/16 in PRAD. A linear regression line was included for better visualization. D. Associations between CDK1/3/5/16 and immune infiltration levels in PCA patients from TIMER analysis. ccCDKs: cell cycle-associated CDKs. PRAD: Prostate adenocarcinoma. TIMER: tumor immune estimation resource.



tests. We generated scatter plots and histograms using GraphPad Prism 8.0 (GraphPad Software, Inc., La Jolla, CA, USA). The statistical analysis was performed using IBM's Statistical Program for Social Sciences 20.0 (Armonk, NY, USA). Student's t test, paired t test, or Mann-Whitney U test were used to compare the differences between the two groups. To compare two or more groups, we used ANOVA or Kruskal-Wallis tests. Statistical significance was defined as  $P < 0.05$ .

### 3. Results

#### 3.1. The role of ccCDKs in cell cycle regulation

First, the GeneMANIA database was used to confirm the role of ccCDKs in cell cycle regulation. In the results, a total of 20 nodes, which represent genes that are correlated with the family based on their physical interaction, coexpression, prediction, colocalization and genetic interaction, surround the central nodes representing ccCDK family members. These ccCDKs were further analyzed for their relationship with the cell cycle. Additionally, these protein-protein interactions were correlated with regulation of the cell cycle G1/S transition, cell cycle G2/M transition, cyclin-dependent protein serine-threonine kinase activity, cell cycle checkpoint and cell cycle arrest (Figure 1).

#### 3.2. Expression of ccCDKs in patients with PCa

Then, screening and analysis were performed on the UALCAN database to determine the differential expression pattern of ccCDKs between tumor samples and paired normal samples. As shown in Figure 2A, in contrast to normal prostate tissues, transcriptional levels of CDK1 ( $P = 1.82e-7$ ), CDK3 ( $P = 1.62e-12$ ), CDK4 ( $P = 3.13e-7$ ), CDK5 ( $P = 1e-12$ ), CDK16 ( $P = 6.03e-11$ ) and CDK17 ( $P = 2.36e-4$ ) in PCa tissues were obviously elevated, but it's the opposite in CDK14 ( $P = 3.88e-03$ ), CDK15 ( $P = 6.04e-4$ ) and CDK18 ( $P = 3.00e-3$ ).

In order to determine if ccCDK proteins are differentially expressed in PCa tissues, Human Protein Atlas database analyzed IHC staining images of ccCDK proteins between normal prostate and PCa samples. As shown in Figure 2B, compared with various expression atlas in normal prostate tissues, the protein expression of CDK1, CDK3, CDK4, CDK5, CDK6 and CDK16 showed relatively higher level in PCa tissues, while CDK15 and CDK18 displayed relatively lower expression in PCa tissues than in normal prostate tissues ( $P < 0.05$  for all).

#### 3.3. Correlation between ccCDK expression and clinicopathological parameters in patients with PCa

UALCAN was used to determine whether ccCDK mRNA expression was relative to clinicopathological parameters. The results indicated that patients in the Gleason score  $\geq 8$  groups expressed remarkably higher mRNA levels of CDK1, CDK2, CDK4, CDK5, and CDK16 than those in the other Gleason score  $< 8$  groups. However, there was significantly lower expression of CDK3 and CDK6 in patients with higher Gleason scores ( $\geq 8$ ) than in patients with other low Gleason scores ( $< 8$ ) ( $P < 0.05$  for all, Figure 3A). However, due to the limitation of very few samples in the Gleason score 10 groups (number = 4 for all), we could not analyze any ccCDK expression profile in these groups. In addition, patients with N<sub>1</sub> (lymph node metastasis) stage displayed a tendency to have higher CDK1, CDK4, CDK8, CDK9 and CDK13 expression than those with N<sub>0</sub> (no lymph node metastasis) stage, which implied that the above ccCDK expression was significantly correlated with patients' lymphocytic metastasis status ( $P < 0.05$  for all, Figure 3B).

#### 3.4. Clinical outcomes of patients with PCa according to the presence of ccCDKs

We conducted GEPIA analysis to evaluate the prognostic value of ccCDK expression in PCa patients. According to the results, patients with

high levels of transcription of CDK2 or CDK3 have significantly worse overall survival (OS) ( $P = 0.041$  and  $0.023$ , respectively, Figure 4A). Besides, we analyzed the relationships between ccCDK expression and disease-free survival (DFS) in PCa patients. Similarly, patients with high transcriptional levels of CDK1, CDK3, CDK5 or CDK16 displayed a significantly poorer DFS than those with a low level ( $P = 0.0014$ ,  $0.002$ ,  $0.02$  and  $0.0023$ , respectively, Figure 4B).

#### 3.5. Genetic alteration and correlation analyses of ccCDKs among PCa patients

Because patients with high expression of CDK1, CDK3, CDK5 or CDK16 displayed poor DFS, we focused on these ccCDKs and further explored their features in PCa. Next, we gathered and shifted through ccCDK coexpressed genes from GeneMANIA (Table 1) and then predicted these gene-associated diseases via DisGeNET of the Metascape database. Intriguingly, disease analysis revealed that ccCDKs (CDK1, CDK3, CDK5 and CDK16) and their coexpressed genes might be related to the progression of hormone refractory PCa (Figure 5A). Then, we comprehensively analyzed the transcriptome sequencing data from cBioPortal and delineated the signatures of structural genomic instability across the different ccCDKs in cBioPortal. As a result, CDK1, CDK3, CDK5 and CDK16 were altered by 4%, 6%, 7% and 6% in the queried PCa samples, respectively. Among all types of mutations, enhanced mRNA expression accounted for the largest proportion, and the rest of the mutations included splice mutations, amplifications, and deep deletions (Figure 5B).

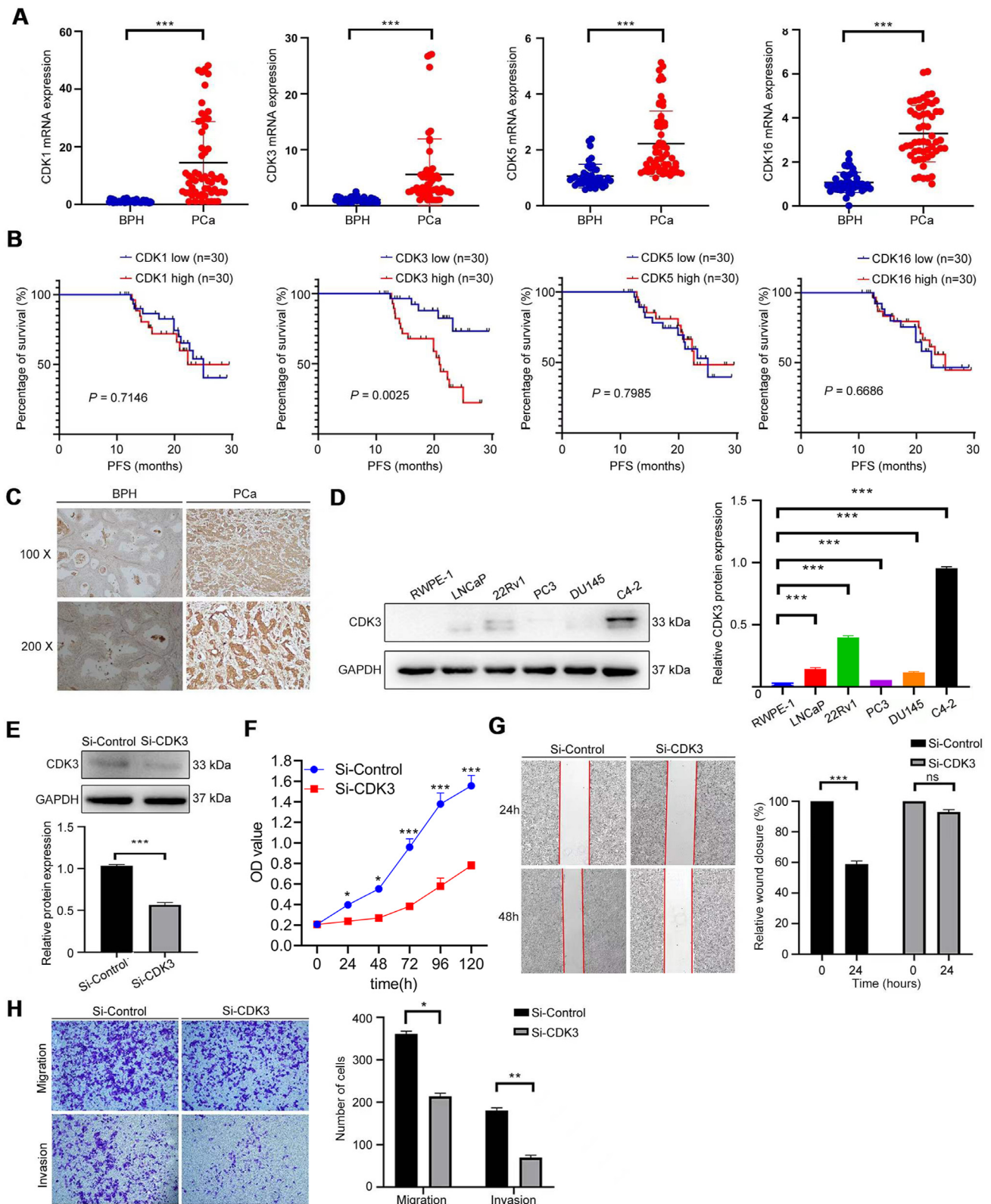
In addition, previous studies have identified aberrations of AR, ETS genes, TP53, PTEN and SPOP that are frequent in PCa [4, 18]. Thus, we next performed correlation analysis of ccCDKs and PCa common genetic drivers. The results indicated that there was a negative correlation between SPOP and CDK1, CDK3, CDK5 or CDK16, while other common genetic drivers were inconsistently correlated with ccCDKs (Figure 5C).

#### 3.6. ccCDKs are enriched in the IL-18 signaling pathway and correlated with infiltration of immune cells in patients with PCa

We intended to investigate the potential molecular mechanisms for the expression of ccCDKs (1, 3, 5, 16) related to poorer DFS in PCa patients. To this end, GO was conducted to predict cellular processes, biological regulation, cellular component organization, and metabolic processes were remarkably regulated by CDK1, CDK3, CDK5 and CDK16 in PCa (Figure 6A). Additionally, KEGG enrichment analysis suggested that the IL-18 signaling pathway was one of the related pathways (Figure 6B, Table 2). Then, we incorporated IL-18 and CDK1, 3, 5, and 16 into a correlation analysis for further validation. The scatter plot showed that IL-18 had a strong positive correlation with CDK1 and CDK3 expression ( $P = 2.8e-9$  and  $1.5e-4$ , respectively). IL-18 was negatively

Table 2. Top 9 clusters with their representative enriched terms.

GO	Description	Count	%
GO:0044772	mitotic cell cycle phase transition	12	54.55
GO:0000079	regulation of cyclin-dependent protein serine/threonine kinase activity	8	36.36
R-HSA-2514853	Condensation of Prometaphase Chromosomes	4	18.18
GO:0045737	positive regulation of cyclin-dependent protein serine/threonine kinase activity	4	18.18
GO:0018107	peptidyl-threonine phosphorylation	4	18.18
GO:0031400	negative regulation of protein modification process	4	18.18
WP4754	IL-18 signaling pathway	4	18.18
GO:0000083	regulation of transcription involved in G1/S transition of mitotic cell cycle	3	13.64



**Figure 7.** Expression levels of DFS-related ccCDKs in clinical patients and silencing CDK3 suppress PCa cell proliferation, migration, and invasion. A. CDK1/3/5/16 mRNA expression in 60 PCa specimens and 60 benign prostate specimens analyzed by qRT-PCR. B. Survival analyses of CDK1/3/5/16 expression with biochemical recurrence-free survival in PCa patients. Sixty PCa specimens were divided into two groups according to the median mRNA expression level of ccCDKs. All of the data are presented as the means  $\pm$  SD. C. The protein level of CDK3 was higher in PCa tissues than in BPH tissues. D. The protein level of CDK3 was higher in PCa cell lines than in the normal prostate epithelial cell line RWPE-1. E. CDK3 protein expression was repressed in PCa cells after CDK3-specific siRNA transfection. F. Silencing CDK3 suppressed cell proliferation in C4-2 cells. G. Silencing CDK3 suppressed cell wound healing in C4-2 cells. H. Silencing CDK3 inhibited the migration and invasion of C4-2 cells. DFS: disease-free survival. ccCDKs: cell cycle-associated CDKs. PCa: prostate cancer. BPH: benign prostate hyperplasia. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

correlated with CDK5 and CDK16 expression ( $P = 1.4e-2$  and  $1.1e-3$ , respectively, Figure 6C).

Since immunotherapy was introduced, the treatment paradigm for several solid tumors has changed, and the therapeutic algorithm is expected to improve in the future [19]. Therefore, we assessed the correlation between DFS-related ccCDKs (CDK1, 3, 5, 16) and immune scores in PCa and evaluated the presence of infiltrating immune cells in tumor tissues through the TIMER database. As shown in Figure 6D, Positive correlation was found between CDK1, CDK16 and the infiltration of immune cells (B cells, CD4+ T cells, CD8+ T cells, macrophages, neutrophils or dendritic cells). However, CDK3 and CDK5 were negatively related to B cell, CD8+ T cell, macrophage or dendritic cell infiltration.

### 3.7. Expression levels of DFS-related ccCDKs in clinical patients and silencing CDK3 suppress proliferation, migration, invasion of PCa cell

After screening out the above ccCDK (1, 3, 5, 16) genes, we verified their different expression in tumors and normal specimens. Sixty PCa tissue specimens and 60 BPH specimens were collected. Consistent with the above bioinformatics analysis results, in our cohort, cancer tissues expressed significantly more CDK1, CDK3, CDK5 and CDK16 compared to BPH tissues ( $P < 0.05$  for all, Figure 7A). Furthermore, during a median follow-up period of 21.2 months (range: 7.7–30.2 months), 22 patients (36.7%) experienced disease progression. The survival curve and log-rank (Mantel–Cox) test analysis revealed that the higher mRNA expression of CDK3 was remarkably related to a shorter progression-free survival (PFS) of biochemical recurrence in patients with PCa ( $P = 0.0025$ ). However, the mRNA levels of CDK1, CDK5 and CDK16 weren't markedly associated with PFS in our cohort ( $P = 0.7146$ ,  $0.7985$  and  $0.6686$ , Figure 7B).

Further experiments focused on CDK3 function in PCa were carried out. We first observed stronger immunostaining of CDK3 in PCa tissues than in BPH tissues (Figure 7C). And then, we compared the expression of CDK3 in different PCa cell lines (22Rv1, LNCaP, C4-2, PC3 and DU145) and a normal prostate epithelial cell RWPE1. Western blot analysis showed that CDK3 was positively expressed among all PCa cell lines, and with the highest expressing in C4-2 cell. However, there was almost no expression of CDK3 in RWPE1 cell (Figure 7D). Therefore, we selected C4-2 cell for subsequent experiments. Successfully, the expression of CDK3 was downregulated at the protein level in C4-2 cells by transfection with si-CDK3 (Figure 7E). Subsequently, according to the cell proliferation assay, silencing CDK3 weakened the ability of PCa cells to proliferate (Figure 7F). Furthermore, the absence of CDK3 decreased the migration ability of C4-2 cells. Additionally, CDK3 silencing repressed PCa cell invasion, as shown by transwell assays (Fig. 7G-H).

## 4. Discussion

The cell cycle is the mainstay of cell proliferation, but it can also lead to PCa progression [20]. ccCDKs are key regulators of cell cycle progression and transcription. Previous studies also provided evidences supporting the oncogenic potential of CDK3 and CDK5 in skin tumorigenesis and pancreatic neuroendocrine tumors, while an increase in CDK7 expression is related to a poorer overall survival and disease-free survival in squamous cell carcinomas of the head and neck [21, 22, 23]. At present, there are no systematic and comprehensive studies on ccCDK family genes. We first confirmed the role of ccCDKs, which regulate the cell cycle in different processes, and analyzed ccCDK expression in PCa by utilizing online expression databases and bioinformatics tools. In particular, we showed that distinct ccCDKs were differentially expressed between cancer and normal tissues and may modulate tumor progression, clinical outcomes, and immune cell infiltration levels. The differences between mRNA expression in UALCAN and protein expression in HAP of ccCDKs might be due to posttranscriptional modification or posttranslational modification, which needs further verification. On the basis of clinicopathological parameter analysis,

ccCDKs may be related to Gleason score and lymphocytic metastasis status. The prognostic prediction value of ccCDKs could group PCa patients into different risk grades or therapeutic responses, which could be conducive to the individualization and precision of medical treatment.

We also observed that patients with higher CDK1, 3, 5, and 16 expression in PCa may have poor prognosis, except for CDK18, because the expression of CDK18 is contradictory to prognosis. DisGeNET of the Metascape database also indicated that ccCDK coexpressed genes associated with human diseases were enriched in hormone refractory PCa, which could be the cause of poor prognosis. Then, mutations in ccCDKs were frequent in PCa patients. Interestingly, men with germline mutations also constitute a large proportion of those with metastatic PCa [24]. Genetic mutation has potential importance in treatment, and tumor-directed somatic mutation may guide treatment decision-making [25]. These results indicate a potential role of ccCDKs in identifying drug targets, understanding the biological underpinnings of PCa, and personalizing cancer treatments. Our results demonstrated that canonical genetic drivers of PCa, such as AR, ERG, PTEN, TMPRSS2, and TP53, were inconsistently correlated with ccCDKs, but there was a negative correlation between SPOP and CDK1, 3, 5, 16. Several works have shown that SPOP acts as a tumor inhibitor in PCa by promoting its degradation of various oncoproteins, including AR [26], MYC [27] and ERG [28, 29], which play a key role in lots of cellular progression, such as cellular metabolism and apoptosis [30]. We therefore hypothesize that expressing ccCDKs in the prostate would reveal a poor prognosis in natural cancer triggered by SPOP. The association of SPOP with hormone resistance has been reported [31, 32]. Considering the correlation of ccCDKs with SPOP, it is believed that the regulation of ccCDK expression through SPOP may be the cause of tumor hormone resistance and poor prognosis.

In addition, we explored whether the expression of ccCDKs was significantly related to the infiltration of immune cells (such as B cell, macrophage, neutrophil or dendritic cell, CD4+ T cell, CD8+ T cell), and the enrichment pathway of KEGG analysis was the IL-18 signaling pathway. In previous studies, IL-18 was found to regulate CD8 cytotoxic cells and neutrophils depending on the microenvironment of the host. The production of IL-18 by many types of hematopoietic cells takes place, including the production by dendritic cells and macrophages [33]. We deduced that ccCDKs may regulate immune infiltration through regulation of IL-18 pathways. Furthermore, researchers are actively exploring the tumor microenvironment as a prognostic or diagnostic biomarker or therapy target [34]. According to several studies, the immune system plays an important role in the progression and development of PCa [35]. We previously identified a common mutation of ccCDKs in PCa, and ccCDK mutant tumors are immunogenic and can influence immune cell infiltration. Consequently, we deem that mutations of ccCDKs are involved in the process of tumor immunity. In recent years, advances in immune checkpoint research have led to long-lasting antitumor responses that weren't previously possible, where a key role is played by the tumor-infiltrating cells, immune microenvironment and immune biomarkers. Taken together, our results indicate that PCa patients harboring ccCDK mutations could benefit from immunotherapy.

However, the importance of CDKs in cancer progression remains open for testing through multicenter, large-sample studies rather than online databases. Despite these limitations, we first investigated the differential expression of ccCDKs in PCa and their potential as prognostic markers. Taken together, we highlight CDK1/3/5/16 as potential diagnostic and prognostic biomarkers as well as potential targets to treat PCa although the IL-18 signaling pathway.

## 5. Conclusions

In this study, the value of ccCDK expression for PCa patients' prognosis was analyzed, as well as the biofunctions of ccCDK genes in PCa. We found that ccCDKs were expressed at relatively higher levels in prostate cancer tissues than in normal tissues. In addition, a high expression of ccCDKs was related to poor disease-free survival in prostate cancer

patients. Furthermore, our analysis also demonstrated that ccCDKs were enriched in the IL-18 signaling pathway and correlated with the infiltration of immune cells in prostate cancer. Indeed, through functional experiments, CDK3 was shown to promote proliferation, migration, and invasion in PCa cells. In conclusion, we speculated that ccCDKs could modulate tumor progression and serve as prognostic biomarkers for PCa patients.

## Declarations

### Author contribution statement

Ke Li and Yu Wang: Conceived and designed the experiments; Wrote the paper.

Yean Huang and Shuo Lu: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Yi Chen: Contributed reagents, materials, analysis tools or data.

Yunhao Qing and Tan Ma: Analyzed and interpreted the data; Wrote the paper.

Ruji Wu: Contributed reagents, materials, analysis tools or data; Analyzed and interpreted the data.

Zixiao Zhang: Contributed reagents, materials, analysis tools or data; Wrote the paper.

### Funding statement

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

### Data availability statement

Data included in article/supp. material/referenced in article.

### Declaration of interest's statement

The authors declare no conflict of interest.

### Additional information

Supplementary content related to this article has been published online at <https://doi.org/10.1016/j.heliyon.2022.e10081>.

## References

- [1] R.L. Siegel, K.D. Miller, A. Jemal, Cancer statistics, *CA A Cancer J. Clin.* 70 (2020) 7–30.
- [2] M.S. Litwin, H.J. Tan, The diagnosis and treatment of PCa: a review, *JAMA* 317 (2017) 2532–2542.
- [3] S.A. Tomlins, D.R. Rhodes, S. Perner, S.M. Dhanasekaran, R. Mehra, X.W. Sun, S. Varambally, X. Cao, J. Tchinda, R. Kuefer, C. Lee, J.E. Montie, R.B. Shah, K.J. Pienta, M.A. Rubin, A.M. Chinnaiyan, Recurrent fusion of TMPRSS2 and ETS transcription factor genes in PCa, *Science* 310 (2005) 644–648.
- [4] D. Robinson, E.M. Van Allen, Y.M. Wu, N. Schultz, R.J. Lonigro, J.M. Mosquera, B. Montgomery, M.E. Taplin, C.C. Pritchard, G. Attard, H. Beltran, W. Abida, R.K. Bradley, J. Vinson, X. Cao, P. Vats, L.P. Kunju, M. Hussain, F.Y. Feng, S.A. Tomlins, K.A. Cooney, D.C. Smith, C. Brennan, J. Siddiqui, R. Mehra, Y. Chen, D.E. Rathkopf, M.J. Morris, S.B. Solomon, J.C. Durack, V.E. Reuter, A. Gopalan, J. Gao, M. Loda, R.T. Lis, M. Bowden, S.P. Balk, G. Gaviola, C. Sougnez, M. Gupta, E.Y. Yu, E.A. Mostaghel, H.H. Cheng, H. Mulcahy, L.D. True, S.R. Plymate, H. Dvinge, R. Ferraldeschi, P. Flohr, S. Miranda, Z. Zafeiriou, N. Tunariu, J. Mateo, R. Perez-Lopez, F. Demichelis, B.D. Robinson, M. Schiffman, D.M. Nanus, S.T. Tagawa, A. Sgaras, K.W. Eng, O. Elemento, A. Sboner, E.I. Heath, H.I. Scher, K.J. Pienta, P. Kantoff, J.S. de Bono, M.A. Rubin, P.S. Nelson, L.A. Garraway, C.L. Sawyers, A.M. Chinnaiyan, Integrative clinical genomics of advanced PCa, *Cell* 161 (2015) 1215–1228.
- [5] Y.M. Wu, M. Cieslik, R.J. Lonigro, P. Vats, M.A. Reimers, X. Cao, Y. Ning, L. Wang, L.P. Kunju, N. de Sarkar, E.I. Heath, J. Chou, F.Y. Feng, P.S. Nelson, J.S. de Bono, W. Zou, B. Montgomery, A. Alva, P.S.C.I.P.C.D. Team, D.R. Robinson, A.M. Chinnaiyan, Inactivation of CDK12 delineates a distinct immunogenic class of advanced PCa, *Cell* 173 (2018) 1770–1782, e1714.
- [6] M. Malumbres, Cyclin-dependent kinases, *Genome Biol.* 15 (2014) 122.
- [7] H. Liu, K. Liu, Z. Dong, Targeting CDK12 for cancer therapy: function, mechanism, and drug discovery, *Cancer Res.* (2020).
- [8] T. Otto, P. Scicinski, Cell cycle proteins as promising targets in cancer therapy, *Nat. Rev. Cancer* 17 (2017) 93–115.
- [9] D.S. Chandrashekar, B. Bashel, S.A.H. Balasubramanya, C.J. Creighton, I. Ponce-Rodriguez, B. Chakravarthi, S. Varambally, UALCAN: a portal for facilitating tumor subgroup gene expression and survival analyses, *Neoplasia* 19 (2017) 649–658.
- [10] Z. Tang, C. Li, B. Kang, G. Gao, C. Li, Z. Zhang, GEPIA: a web server for cancer and normal gene expression profiling and interactive analyses, *Nucleic Acids Res.* 45 (2017) W98–W102.
- [11] J. Gao, B.A. Aksoy, U. Dogrusoz, G. Dresdner, B. Gross, S.O. Sumer, Y. Sun, A. Jacobsen, R. Sinha, E. Larsson, E. Cerami, C. Sander, N. Schultz, Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal, *Sci. Signal.* 6 (2013) p11.
- [12] D. Warde-Farley, S.L. Donaldson, O. Comes, K. Zuberi, R. Badrawi, P. Chao, M. Franz, C. Grouios, F. Kazi, C.T. Lopes, A. Maitland, S. Mostafavi, J. Montojo, Q. Shao, G. Wright, G.D. Bader, Q. Morris, The GeneMANIA prediction server: biological network integration for gene prioritization and predicting gene function, *Nucleic Acids Res.* 38 (2010) W214–220.
- [13] Y. Zhou, B. Zhou, L. Pache, M. Chang, A.H. Khodabakhshi, O. Tanaseichuk, C. Benner, S.K. Chanda, Metascape provides a biologist-oriented resource for the analysis of systems-level datasets, *Nat. Commun.* 10 (2019) 1523.
- [14] T. Li, J. Fu, Z. Zeng, D. Cohen, J. Li, Q. Chen, B. Li, X.S. Liu, TIMER2.0 for analysis of tumor-infiltrating immune cells, *Nucleic Acids Res.* 48 (2020) W509–W514.
- [15] A. Asplund, P.H. Edqvist, J.M. Schwenk, F. Ponten, Antibodies for profiling the human proteome—The Human Protein Atlas as a resource for cancer research, *Proteomics* 12 (2012) 2067–2077.
- [16] Y.Y. Cho, F. Tang, K. Yao, C. Lu, F. Zhu, D. Zheng, A. Pugliese, A.M. Bode, Z. Dong, Cyclin-dependent kinase-3-mediated c-Jun phosphorylation at Ser63 and Ser73 enhances cell transformation, *Cancer Res.* 69 (1) (2009) 272–281.
- [17] J. Lu, Z.L. Zhang, D. Huang, N. Tang, Y. Li, Z. Peng, C. Lu, Z. Dong, F. Tang, Cdk3-promoted epithelial-mesenchymal transition through activating AP-1 is involved in colorectal cancer metastasis, *Oncotarget* 7 (6) (2016) 7012–7028.
- [18] C.E. Barbieri, S.C. Baca, M.S. Lawrence, F. Demichelis, M. Blattner, J.P. Theurillat, T.A. White, P. Stojanov, E. Van Allen, N. Stransky, E. Nickerson, S.S. Chae, G. Boysen, D. Auclair, R.C. Onofrio, K. Park, N. Kitabayashi, T.Y. MacDonald, K. Sheikh, T. Vuong, C. Guiducci, K. Cibulskis, A. Sivachenko, S.L. Carter, G. Saksena, D. Voet, W.M. Hussain, A.H. Ramos, W. Winckler, M.C. Redman, K. Ardlie, A.K. Tewari, J.M. Mosquera, N. Rupp, P.J. Wild, H. Moch, C. Morrissey, P.S. Nelson, P.W. Kantoff, S.B. Gabriel, T.R. Golub, M. Meyerson, E.S. Lander, G. Getz, M.A. Rubin, L.A. Garraway, Exome sequencing identifies recurrent SPOP, FOXA1 and MED12 mutations in PCa, *Nat. Genet.* 44 (2012) 685–689.
- [19] M. Tsiatas, G. Mountziou, G. Curigliano, Future perspectives in cancer immunotherapy, *Ann. Transl. Med.* 4 (2016) 273.
- [20] S. Sommariva, R. Tarricone, M. Lazzeri, W. Ricciardi, F. Montorsi, Prognostic value of the cell cycle progression score in patients with PCa: a systematic review and meta-analysis, *Eur. Urol.* 69 (2016) 107–115.
- [21] T. Xiao, J.J. Zhu, S. Huang, C. Peng, S. He, J. Du, R. Hong, X. Chen, A.M. Bode, W. Jiang, Z. Dong, D. Zheng, Phosphorylation of NFAT3 by CDK3 induces cell transformation and promotes tumor growth in skin cancer, *Oncogene* 20 (2017) 2835–2845.
- [22] A.M. Carter, N. Kumar, B. Herring, C. Tan, R. Guenter, R. Telange, W. Howse, F. Viol, T.R. McCaw, H.H. Bickerton, P. Gupta, F. Gillardon, E.A. Woltering, D. Dhall, J. Totenhagen, R.R. Banerjee, E.M. Kurian, S. Reddy, H. Chen, J. Schrader, J.A. Bibb, Cdk5 drives formation of heterogeneous pancreatic neuroendocrine tumors, *Oncogenesis* 10 (12) (2021), 83.
- [23] T. Jagomast, C. Idel, L. Klapper, P. Kuppler, A. Offermann, E. Dreyer, K.L. Bruchhage, J. Ribbat-Idel, S. Perner, CDK7 predicts worse outcome in head and neck squamous-cell cancer, *Cancers* 14 (3) (2022) 492.
- [24] C.C. Pritchard, J. Mateo, M.F. Walsh, N. De Sarkar, W. Abida, H. Beltran, A. Garofalo, R. Gulati, S. Carreira, R. Eeles, O. Elemento, M.A. Rubin, D. Robinson, R. Lonigro, M. Hussain, A. Chinnaiyan, J. Vinson, J. Filipenko, L. Garraway, M.E. Taplin, S. Aldubayan, G.C. Han, M. Beightol, C. Morrissey, B. Nghiem, H.H. Cheng, B. Montgomery, T. Walsh, S. Casadei, M. Berger, L. Zhang, A. Zehir, J. Vijai, H.I. Scher, C. Sawyers, N. Schultz, P.W. Kantoff, D. Solit, M. Robson, E.M. Van Allen, K. Offit, J. de Bono, P.S. Nelson, Inherited DNA-repair gene mutations in men with metastatic PCa, *N. Engl. J. Med.* 375 (2016) 443–453.
- [25] H.H. Cheng, A.O. Sokolova, E.M. Schaeffer, E.J. Small, C.S. Higano, Germline and somatic mutations in PCa for the clinician, *J. Natl. Compr. Cancer Netw.* 17 (2019) 515–521.
- [26] C. Geng, K. Rajapakse, S.S. Shah, J. Shou, V.K. Eedunuri, C. Foley, W. Fiskus, M. Rajendran, S.A. Chew, M. Zimmermann, R. Bond, B. He, C. Coarfa, N. Mitsiades, Androgen receptor is the key transcriptional mediator of the tumor suppressor SPOP in PCa, *Cancer Res.* 74 (2014) 5631–5643.
- [27] C. Geng, S. Kaochar, M. Li, K. Rajapakse, W. Fiskus, J. Dong, C. Foley, B. Dong, L. Zhang, O.J. Kwon, S.S. Shah, M. Bolaki, L. Xin, M. Ittmann, B.W. O'Malley, C. Coarfa, N. Mitsiades, SPOP regulates prostate epithelial cell proliferation and promotes ubiquitination and turnover of c-MYC oncoprotein, *Oncogene* 36 (2017) 4767–4777.
- [28] J. An, S. Ren, S.J. Murphy, S. Dalangood, C. Chang, X. Pang, Y. Cui, L. Wang, Y. Pan, X. Zhang, Y. Zhu, C. Wang, G.C. Halling, L. Cheng, W.R. Sukov, R.J. Karnes, G. Vasmataz, Q. Zhang, J. Zhang, J.C. Cheville, J. Yan, Y. Sun, H. Huang, Truncated ERG oncoproteins from TMPRSS2-ERG fusions are resistant to SPOP-mediated proteasome degradation, *Mol. Cell* 59 (2015) 904–916.
- [29] W. Gan, X. Dai, A. Lunardi, Z. Li, H. Inuzuka, P. Liu, S. Varmeh, J. Zhang, L. Cheng, Y. Sun, J.M. Asara, A.H. Beck, J. Huang, P.P. Pandolfi, W. Wei, SPOP promotes

- ubiquitination and degradation of the ERG oncoprotein to suppress PCa progression, *Mol. Cell* 59 (2015) 917–930.
- [30] J. Cheng, J. Guo, Z. Wang, B.J. North, K. Tao, X. Dai, W. Wei, Functional analysis of Cullin 3 E3 ligases in tumorigenesis, *Biochim. Biophys. Acta Rev. Canc* 1869 (2018) 11–28.
- [31] K. Nikhil, M. Kamra, A. Raza, H.S. Haymour, K. Shah, Molecular Interplay between AURKA and SPOP Dictates CRPC Pathogenesis via Androgen Receptor, *Cancers* 12 (2020).
- [32] K. Nikhil, H.S. Haymour, M. Kamra, K. Shah, Phosphorylation-dependent regulation of SPOP by LIMK2 promotes castration-resistant PCa, *Br. J. Cancer* 124 (2021) 995–1008.
- [33] S. Wawrocki, M. Druszczynska, M. Kowalewicz-Kulbat, W. Rudnicka, Interleukin 18 (IL-18) as a target for immune intervention, *Acta Biochim. Pol.* 63 (2016) 59–63.
- [34] N.K. Altorki, G.J. Markowitz, D. Gao, J.L. Port, A. Saxena, B. Stiles, T. McGraw, V. Mittal, The lung microenvironment: an important regulator of tumour growth and metastasis, *Nat. Rev. Cancer* 19 (2019) 9–31.
- [35] A. Strasner, M. Karin, Immune infiltration and PCa, *Front. Oncol.* 5 (2015) 128.