Identification of Hub Gene and Potential Drugs for Enzalutamide Resistant Castration-Resistant Prostate Cancer Depend On Bioinformatics Analysis

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Research Article

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Abstract

Background

Androgen deprivation therapy (ADT) is still the first line method to treat PCa. However, after a period of therapy, primary PCa will inevitably progress into castration-resistant prostate cancer (CRPC). Enzalutamide (MDV3100) is an androgen receptor (AR) signal inhibitor which can delay the progression of CRPC and increase survival of patients with metastatic CRPC. However, the mechanisms of enzalutamide resistant castration-resistant prostate cancer (EnzR CRPC) are still controversial.

Methods

We collected RNA-seq data of enzalutamide resistant castration-resistant prostate cancer cell line LNCaP (EnzR LNCaP) from GSE44905, GSE78201 and GSE150807. We found hub gene from the three datasets. Then we tested the expression of hub gene in TCGA database and potential drug that has effect on hub gene. Finally, we verified the hub gene expression and drug function.

Results

From GSE44905, GSE78201 and GSE150807, we found 45 differently expressed genes (DEGs) between LNCaP and EnzR LNCaP. Ten hub gene were found in protein-protein interaction (PPI) network. The hub gene expression and survival analysis were analyzed by GEPIA depend on TCGA database. We found that CDK6 was highly expressed in both EnzR LNCaP cell and prostate cancer patients. Ten potential small molecules can suppress CDK6 expression from CMap. Finally, we verified the expression of CDK6 in both CRPC patients' samples and prostate cancer cell lines. The function of three potential CDK6 inhibitors, apigenin, chrysin and fisetin, was tested in prostate cancer cell lines.

Conclusions

The study proved that the mutation of CDK6 maybe a reason to the occurrence of CRPC and suppress CDK6 expression may be a potential way in treating CRPC.

Background

Prostate cancer(PCa)has the highest incidence among all type of cancers in elder men in America. Its incidence is rapidly increasing. The estimated incidence rate of prostate cancer is 174,650 in United States in 2019, which means that 1 of 5 men newly diagnosed with cancer would suffer from prostate cancer. In addition, the mortality rate of prostate cancer has risen recent years. In America, the greatest number of deaths are from cancers of the lung, prostate and colorectum in men.^[1] Prostate cancer also have a higher and higher incidence and death rate in China. According to National Cancer Center of China, there were 72 thousand new patients suffered from PCa in China in 2015, with an estimated incidence ration of 10.23/100 000.^[2] Androgen deprivation therapy (ADT) is still the first line therapy for treating locally advanced and even metastatic PCa. ^[3] However, after a period of treatment, nearly all

patients will inevitably develop into castration-resistant prostate cancer (CRPC).^[4] The treatment of CRPC is still a difficult problem.

Patients with CRPC will still have disease progression when the serum testosterone < 50ng/dl or 1.7nmol/l.^[5] The progression of CRPC is usually due to the response of androgen receptor (AR).^[6] Enzalutamide (Enz) as a second-generation AR inhibitor is approved by the Food and Drug Administration (FDA) to treat CRPC patients.^[7] It inhibits the activated AR nuclear translocation and prevent its combination to androgen response elements (ARE) and coactivator recruitment, which promotes apoptosis while suppresses proliferation of the CRPC cells.^[8, 9] Studies have been proven that Enz can improve the survival time of patients with CRPC compared to placebo or bicalutamide.^[10, 11] Despite Enz have therapeutic effect initially, CRPC patients will finally resistant to Enz. So, it may have significant clinical value to find the mechanism of Enz resistant in treating CRPC.

Bioinformatic analysis has been widely used in biofunctional analysis. It may help find the mechanism of Enz resistant (EnzR) in CRPC patients. In this study, we found differently expressed genes (DEGs) in EnzR prostate cancer cell line LNCaP (EnzR LNCaP) depend on GEO database. Then we analyzed the significant functional modules, pathways which the DEGs enriched. We also constructed the protein-protein interaction (PPI) network to find the hub gene. Then, we tested the expression of hub gene in normal prostate tissues and prostate cancer tissues depend on TCGA and CPGEA database. We also analyzed the influence of hub gene on survival status. Next, we found the potential drugs which can affect the function of hub gene. Finally, we validated the expression of hub gene, CDK6, in tumor tissues and para-cancerous tissues. Three potential small molecules which are CDK6 inhibitors were used to treat two EnzR PCa cell line and normal control prostate cancer cell line LNCaP and C4-2 to test its effect on treating CRPC. We found that these inhibitors can decrease the proliferation of LNCaP and C4-2. At the same time, the cell proliferation ability of EnzR LNCaP and C4-2 was suppressed, too. Therefore, these small molecules may be potential therapy targets to treat EnzR CRPC.

Methods

Data Source

The GEO database (<http://www.ncbi.nlm.nih.gov/geo/>) at the National Center for Biotechnology Information (NCBI) is a public database that provides a genomics data repository of gene expression, chip, and microarray data^[12]. There gene expression datasets, GSE44905, GSE78201 and GSE150807, were downloaded from GEO. Due to clinical samples was hard to collected, we downloaded RNA-seq data from EnzR LNCaP. The three datasets were included the high-throughput sequencing of enzalutamide resistant LNCaP cell and normal control LNCaP cell. GSE44905 (relied on the GPL570 Affymetrix Human Genome U133 Plus 2.0 Array) included 3 control LNCaP samples and 6 enzalutamide resistant LNCaP samples. GSE78201(relied on GPL10558 Illumina HumanHT-12 V4.0 expression beadchip) included 4 untreated LNCaP samples and 4 enzalutamide resistant LNCaP samples. GSE150807(relied on GPL9052

Illumina Genome Analyzer (Homo sapiens)) included 3 parental LNCaP samples and 3 enzalutamide resistant LNCaP samples.

Identification of DEGs

The raw microarray expression data of mRNAs downloaded as Series Matrix files from the GEO database were mapped to the corresponding genes according to the SOFT formatted family files from the GEO database. The primary data were normalized by R software Limma package.^[13] The genes with an adjusted P-value < 0.05 and $|\log_2\text{fold change (FC)}| > 1$ were deemed as DEGs.^[14] Then, we used the online web tool, bioinformatic (<http://www.bioinformatics.com.cn/>), to draw Venn map and find the DEGs. The upregulated and downregulated DEGs were saved and for further analysis.

GO and KEGG analysis

GO term included a frame of biological processes (BP), molecular functions (MF) and cellular components (CC). In addition, KEGG pathways is widely utilized to analyze biological pathways that DEGs enriched. Both GO annotation and KEGG pathway enrichment analyses were finished by Metascape (<http://metascape.org/>). Then, the Scatter plots were drawn by online web tool, bioinformatic (<http://www.bioinformatics.com.cn/>). P value < 0.05 was considered statistically significant.

Construct PPI network and identify hub gene

The evaluate the potential PPIs among the DEGs identified, STRING online database (<http://string-db.org/>) was used to build PPI network. Then, Cytoscape software was used to find the correlation among DEGs. We defined the hub gene as which gene has connection with other genes.

Detection of hub gene expression

The Cancer Genome Atlas (TCGA) is one of the largest tumor databases included 499 prostate cancer samples and 52 normal prostate samples^[15]. A website tool, GEPIA (<http://www.gepia.cancer-pku.cn/>), can help finding the expression of hub gene in TCGA samples and GTEx. Then, Chinese patients' data from Chinese Prostate Cancer Genome and Epigenome Atlas (CPGEA) (<http://www.cpgea.com/>) were downloaded and analyzed the expression of hub gene in Chinese population, too.

Survival analysis

Besides expression data, GEPIA website can help finishing the survival analysis. We next carried on survival analysis based on GEPIA. The expression of hub gene on overall survival (OS) and disease-free survival (DFS) was analyzed.

Clinical value analysis

To find hub gene expression in different tumor stage, we downloaded the clinical data of PCa patients from TCGA. Combined the gene expression and clinical data, we analyzed the hub gene expression in different Gleason Score, Tumor-Node-Metastasis (TNM) tumor classification of malignance tumor and ACJJ tumor classification of malignance tumor.

Screening of potential small molecules

CMap (<https://portals.broadinstitute.org/cmap/>) collected the genome-wide transcriptional expression data that can help finding functional connections among drugs, genes, and diseases.^[16] We downloaded all potential small molecules that may affect the function of the hub gene.

Tissue samples

Prostate cancer samples and para-cancerous samples were collected at Tongji Hospital, School of Medicine, Tongji University. The methods used for collecting samples were agreed by the Ethic Committee of Tongji Hospital, School of Medicine, Tongji University. Patients who provided samples known the process of the experiment and informed consent.

Cell culture and drug treating

The human normal prostate epithelial cell RWPE-1 and prostate cancer cell LNCaP and C4-2 were cultured in Roswell Park Memorial Institute (RPMI) 1640 (sigma Darmstadt, Germany) with 10% fetal bovine serum (FBS) (gibco, Thermo Fisher Scientific, Waltham, MA, USA). All the cell lines were cultured in a humid environment containing 5% CO₂ and 95% air at 37°C. The enzalutamide resistant LNCaP and C4-2 cell line were treated with enzalutamide with 10, 20, 30, and then to 40 µM until 20 days. Then, 10 µM enzalutamide was used to maintain. When enzalutamide resistant (EznR) cell cultured, we treated the cell lines with different small molecules. Adding small molecules to the medium to deal with the cells according to the manufacturer's instructions.

RNA extraction and qRT-PCR

Total tissue and cell RNA was isolated using a TRIzol reagent in accordance with the manufacturer's instructions. The mRNA was reversed transcription to cDNA by reverse transcription kit (Advantage® RT-for-PCR Kit, Takara Bio Inc., Kusatsu, Japan). The reverse transcription procedure was finished by the manufacturer's instructions respectively. The qRT-PCR was performed by Applied Biosystems 7500 Sequence Detection system. The volume of cDNA using qRT-PCR reagents and a kit (TB Green® Premix Ex Taq™ II, Takara Bio Inc.) according to the manufacturer's instructions. The β-tubulin were used as normal control. The expression of RNA was calculated according to $2^{-\Delta\Delta Ct}$ method. The following primers were used: cyclin-dependent kinases 6 (CDK6)(Forward: 5'-CAAGGTCAGGTCTACTCAAAGTCTCAC-3', Reverse: 5'-CTGCCAACGATTGAATGCCAGAATG-3')β-Tubulin (Forward:5'- TGGACTCTGTTCGCTCAGGT - 3', Reverse: 5'- TGCCTCCTTCCGTACCACAT - 3').

Western blot

Tissue sample and cell line protein was extracted with RIPA lysis buffer. Protein samples were treated with Dual Color Protein Loading Buffer (Thermo Fisher Scientific, Waltham, MA, USA). SDS-PAGE gels (7.5% and 10%) were utilized to separate proteins, then transferred to nitrocellulose membranes (NC) (Merck KGaA, Darmstadt, Germany). Protein-Free Rapid Blocking Buffer (Thermo Fisher Scientific) was utilized to block the membranes. Then the membranes were incubated at 4°C overnight with primary

antibodies against CDK6 (1:1000) and β -Tubulin (1:1000) (Abcam UK, Cambridge, UK). In the second day, the membranes were washed by 1×TBST three times (10 minute. each). Then, the membranes were incubated at normal temperature for 1.5 h with a matched secondary antibody (HRP-labeled Goat Anti-Human IgG (H + L), Beyotime Biotechnology, Shanghai, China). Finally, the membranes were exposed to X-ray film.

Cell proliferation assay

The cell proliferation ability was detected by CCK-8 kit (Dojindo, Japan). In brief, cells were put in 96-well plates (3000 cells/well), cultured with 200 μ L 1640 + 10%FBS for 0h, 24h, 48h, or 72h. After culture, the cells were detected by CCK-8 kit according to the manufacturer's illustration and absorbance at 450 nm was meterage by a spectrophotometer (LD942, Beijing, China).

Statistical analysis

Data represent all repeated at least three times. The data were represented as means $\pm$ standard deviation (SD). Data were analyzed by Student's t-test for two groups and one-way analysis of variance (ANOVA) for three groups and more. The chip data from GEO and CPGEA database were analyzed by R software with different packages (R Version 4.0.3). A P value no more than 0.05 was considered statistically significant.

Results

DEGs identification

We detected GEO database to find CRPC samples. Finally, cell samples treated by Enz were included in the study. To find DEGs in CRPC, we analyzed three gene expression datasets: GSE44905, GSE78201 and GSE150807, in total which included 10 untreated LNCaP cell samples and 13 EnzR LNCaP cell samples. In these samples we identified 43 upregulated DEGs (Fig. 1A) and 2 downregulated DEGs (Fig. 1B). In total, 45 DEGs in all 3 datasets.

Functional and enrichment analysis

To find the DEGs biology function, we used Metascape to construct KEGG pathways and GO functional analysis. Then, we utilized a webtool bioinformatic to draw scatter plot map. GO enrichment indicated that the DEGs mainly enriched in negative regulation of cell differentiation and cell morphogenesis involved in differentiation. KEGG pathway enrichment denoted that the most enrichment pathways are Pathways in cancer and Cytokine-cytokine receptor interaction. The significant KEGG pathways which DEGs enriched are shown in scatter plot map (Fig. 2A). In addition, the BP, MF and CC pathways were also shown in scatter plot map, respectively (Fig. 2B).

PPI network construction and hub gene finding

We next used STRING to draw PPI network of DEGs. We made the network to find the connection among DEGs and find the hub gene (Fig. 3). We defined that the gene which have connection with other genes are hub gene. We found that *GRIP2*, *EPHB2*, *CDK6*, *PAX6*, *BMP7*, *ITGA1*, *LAMB1*, *IGFBP5*, *LY6K* and *MDGA2* have connection with other genes and we considered them as hub gene.

Hub gene expression in TCGA samples

Due to the finding of hub gene depending on EnzR prostate cancer cell line LNCaP, we tested the hub gene expression on TCGA data. We used GEPIA which included all the 492 prostate cancer patients' samples and 152 normal control samples' sequencing information from TCGA and GTEx. We found that *EPHB2*, *PAX6*, *LY6K* and *MDGA2* expressed no difference, all other genes expressed differently in patients' tissues. (Fig. 4) However, only *CDK6* expressed highly in cancer tissues which in according with the result from cell line samples. Other genes, which highly expressed in EnzR LNCaP cell line, are lower expressed in cancer tissues.

Hub gene expression in Chinese patients

Although TCGA data provided large sequencing information of prostate cancer patients, it is mainly contained Western populations' data. Try to find the expression of hub gene in Chinese population, we downloaded the sequencing data from CPGEA, which comprised sequencing data from Chinese patients of PCa. Different from the result from TCGA, the hub genes, *LY6K* and *MDGA2*, expressed no significant in Chinese patients, other genes include *SLC2A3* and *CDK6* expressed differently between Chinese cancer tissues and normal tissues. Hub genes, *CDK6* and *MDGA2*, are expressed highly in cancer tissues. (Fig. 5). All the results we found indicated that *CDK6* expressed higher in EnzR prostate cancer cell line and tumor tissues. It indicated that *CDK6* may be a key gene leading to PCa and EnzR CRPC.

Survival analysis

To find whether the hub gene can affect patients' survival status, we further used GEPIA to find the affection of hub gene on survival status. The overall survival (OS) and disease-free survival (DFS) were contained in the study. Due to the sample size is insufficient as requested, we did not analyze MDGA2. The result shown that all the hub gene do not affect OS (Fig. 6). However, *ITGA1* and *LAMB1* can affect DFS (Fig. 7). The result shown that ITGA1 and LAMB1 may play a key role in PCa occurrence and may affect the disease progression.

The expression of CDK6 in different PCa stage

Next, we try to find CDK6 expression in different tumor stage. First, we used UALCAN (<http://ualcan.path.uab.edu/home>) to find CDK6 expression in different Gleason score patients from TCGA. We found that there is a significant difference between patients with a Gleason score 6 and Gleason score 7 (Fig. 8A). Then, we downloaded the clinical information of PCa patients from TCGA database. TNM tumor stage is a common international staging standards to access the tumor condition. It accesses the condition of tumor from situation of primary tumor focal (T), regional lymph node metastasis status (N) and tumor metastasis in the distance (M). We analyzed the expression of CDK6 in

different T and N tumor stage. However, we found that the expression of CDK6 was different in T tumor stage (Fig. 8B) and N tumor stage (Fig. 8C). Furthermore, the expression of CDK6 in different T tumor stage was analyzed, too. We found that there is no different in T2 tumor stage (Fig. 8D) and T3 tumor stage (Fig. 8E). The results reflects that though CDK6 may be a reason causing to PCa and CRPC, there was no association between the tumor severity and CDK6.

Screening of potential small molecular drugs

According to the results shown above, we tried to find potential drugs that may treat EnzR CRPC by inhibiting the function of CDK6. From CMap, we found ten compounds can inhibiting the activity of CDK6 (Fig. 9). Three of them has been used in clinical to treat breast cancer. Two molecular has been in the preclinical phase, two in the phase II and three in the phase I. The detailed information of the ten drugs were shown in Table 1. We found that only apigenin, chrysin and fisetin do not affect other numbers of CDK except CDK6. So, we choose apigenin, chrysin and fisetin for further study.

Table 1
The detailed information of 10 CDK6 inhibitors from CMap

Name	Mechanism of Action (MOA)	Target	Phase
Abemaciclib	CDK inhibitor	CDK4, CDK6	Launched
Alvocidib	CDK inhibitor	CDK1, CDK2, CDK4, CDK5, CDK,6 CDK7, CDK8, CDK9, EGFR, PYGM	Phase2
AMG-925	CDK inhibitor, FLT3 inhibitor	CDK4, CDK6, FLT3	Phase1
Apigenin	Casein kinase inhibitor, cell proliferation inhibitor	AKR1B1, AR,CDK6, CFTR, CYP19A1, CYP1B1, HSD17B1	Preclinical
AT-7519	CDK inhibitor	CDK1, CDK2, CDK4, CDK5, CDK6, CDK9	Phase2
Chrysin	Breast cancer resistance protein inhibitor	AKR1B1, CDK6, CYP19A1, CYP1B1	Phase1
Fisetin	Aurora kinase inhibitor	CDK6, FASN	Preclinical
Palbociclib	CDK inhibitor	CDK4, CDK6	Launched
RGB-286638	CDK inhibitor	CDK1, CDK2, CDK3, CDK4, CDK5, CDK6, CDK7, CDK9, FLT3, GSK3B, JAK2, MAP3K7, MAPK9	Phase 1
Ribociclib	CDK inhibitor	CDK4, CDK6	Launched

CDK6 expression in PCa samples and EnzR prostate cancer cell line

As CDK6 may be important in the appearance of PCa and EnzR CRPC, we collected PCa patients' tumor tissues and para-cancerous tissues and EnzR PCa cell lines to compare the CDK6 expression. We found the expression of CDK6 was highly in prostate cancer tissues at mRNA and protein level (Fig. 10A-B). In addition, CDK6 expressed highly in PCa cell LNCaP and C4-2 than RWPE-1 (Fig. 10C-D). Further, CDK6 expressed highly in EnzR LNCaP and C4-2 than normal LNCaP and C4-2 (Fig. 10E-F). The result reflects that CDK6 may truly play a critical role in the occurrence of PCa even EnzR CRPC.

CDK6 inhibitors' function in PCa and CRPC

To examine whether the drugs we mentioned above truly have the ability to treat CRPC, we treated the LNCaP, C4-2, EnzR LNCaP and EnzR C4-2 with them. We found that when treated with CDK6 inhibitors the expression of CDK6 truly downregulated in both mRNA and protein level in PCa cell lines (Fig. 11A-D). The result reflects that these drugs have the ability to suppress CDK6. In addition, when treated with CDK6 inhibitors, the progression of EnzR LNCaP and C4-2 will decrease. At the same time CDK6 inhibitors can decrease the proliferation of LNCaP and C4-2, too (Fig. 11E-F). The result proves that CDK6 inhibitors have the ability to treat CRPC in vivo and may be a protein drug to treat PCa even CRPC in the future.

Discussion

Millions of old men in the world suffered from prostate cancer (PCa). In America, PCa has the highest incidence in men. The incidence of PCa is nearly 20% in new patients diagnosed with cancer. That means one of five new of diagnoses with cancer have PCa. In addition, the mortality of PCa is also increased rapidly too. It is estimated that PCa is the second dead reason for men with cancer only lower than lung cancer in 2019.^[1] However, due to the prostate-specific antigen (PSA) has been extensive tested in previously unscreened men, prostate cancer patients was diagnosed early and treated promptly.^[17] So, PCa patients have the highest 5-year relative survival rate.^[1] At the same time, PCa also has a higher and higher incidence in Chinese population and seriously threaten the patients' health. According to National Cancer Center of China, there were 72 thousand new prostate cancer patients found in China in 2015. The estimated probability of occurrence is about 10.23/100 000. In addition, the crude number of prostate cancer death was 3.07 thousand in China in 2015, with an estimated death rate of 4.36/100 000.^[2] The current treatment methods for localized PCa are comprised of surgical prostatectomy, chemotherapy, immune therapy, and androgen deprivation therapy (ADT).^[18] Until now, ADT is still first line therapy for treating localized advanced and metastatic PCa.^[3] As an effective therapy, ADT has effectively improved patients survival.^[19] However, after a period of therapy, the primary PCa will inevitably relapsed into castration-resistant prostate cancer (CRPC).^[4] When patients progressed in CRPC stage, the disease will expand even the serum androgen in the body is already at an extremely low level.^[20] It is important to find effective methods to treat CRPC.

The definition of CRPC is when the serum testosterone < 50ng/dl or 1.7nmol/l, the disease with progression: three consecutive rises in PSA interval of one week, leading to 100% ascends over the nadir, and PSA > 2 ng/mL.^[21] One reason of the progression of CRPC is due to the restart of androgen receptor (AR).^[6] So, depress the activity of AR can delay the progression of CRPC. Enzalutamide (Enz) as a second-generation AR inhibitor is consented by the Food and Drug Administration (FDA) to remedy CRPC patients.^[7] It inhibits the activated AR nuclear translocation and prevent its combination to androgen response elements (ARE) and coactivator recruitment, which promotes apoptosis while suppresses proliferation of the CRPC cells.^[8, 9] Although EnZ prolong OS of CRPC patients in a certain extent, patients will inevitably develop into Enz-resistant (EnzR) CRPC. The mechanisms of EnzR CRPC have been widely studied. Genetic change may be a key factor leading to EnzR CRPC. Study had proven that AR will express and transcriptionally active in EnzR CRPC.^[22] Some genes such as kallikrein-related peptidase 3 (KLK3) and transmembrane protease serine 2 (TMPRSS2) which can active AR will increase expression in EnzR CRPC. The level of AR target genes change can be a reason to EnzR CRPC.^[23] AR will change into splice variants in CRPC patients. AR can carry out selective shear to form hypotypes that consist exons 1 to 3 with LBD deletion. The AR without LBD will fail to respond to Enz.^[24] Many genes expression will change and effect the expression of AR-Vs mainly refers to AR-V7 in EnzR CRPC. The splicing factor hnRNPA1, the long non-coding RNA Malat1 (LncRNA Malat1), arginine vasopressin receptor 1a (AVPR1A) and monoamine oxidase-A (MAO-A) will change and play an impact on the expression of AR-V7.^[25-27] In addition, some genes which regulate Wnt signalling such as SOX9 and PRKAR2B can also cause EnzR CRPC.^[28-30] All the results reflect that gene mutation is important in the occurrence of EnzR CRPC. Moreover, a review deem new methods like high-throughput next-generation sequencing can promote the drug resistant genes and find the mechanisms of Enz resistant.^[31]

Bioinformatic analysis is a method that can analyze the results of multiple high-throughput sequencing comprehensively and find more accurate differential genes expression in disease occurrence.^[32] In this study, we used bioinformatic analysis to help find the key genes and their function in EnzR CRPC. First, we found 45 DEGs include 43 upregulated and 2 downregulated DEGs depend on EnzR LNCaP. The GO and KEGG analysis were used to search for the enrichment pathways. We found that DEGs enriched in Pathways in cancer and Cytokine-cytokine receptor interaction. Pathways in cancer and Cytokine-cytokine receptor interaction have proven in the occurrence of cancer.^[33] The PPI network was constructed to find the hub gene. When hub gene founded, we tested the expression of hub gene in TCGA and CPGEA database and found that cyclin-dependent kinases6 (CDK6) is highly expressed in EnzR LNCaP and prostate cancer patients while other hub genes have the verse trend between cells and patients' data. The result reflects that CDK6 may be a critical factor in causing EnzR CRPC. The expression of CDK6 in different clinical stage tumor was analyzed, too. However, the CDK6 expression level cannot reflect the PCa progression. Finally, we tested the results in clinical samples and EnzR LNCaP and C4-2 PCa cell. We found that CDK6 expressed highly in tumor tissues than paracancerous tissues in each paired patient. At the same time, CDK6 was also expressed highly in EnzR LNCaP and C4-2. Finally, we proved that three potential small molecules, apigenin, chrysin and fisetin, can truly decrease the expression of CDK6 and

suppress cell proliferation. These results indicated that CDK6 may be a reason in causing CRPC and may be a potential target for treating CRPC.

CDK6 is a number of cyclin-dependent kinases (CDKs) consist of 13 different serine/threonine kinases that become periodic activation when bound to the cyclins, their respective regulatory subunits. CDKs adjust various important cellular processes including cell-cycle progression and transcription. When abnormal kinase activation, cell cycle disordered regulation occurred and then causing uncontrolled cell proliferation. So, CDKs can lead to cancer occurrence.^[34] CDK6 and CDK4, the highly homologous enzyme, are regarded as classic cell cycle kinases that promote cell proliferation through forming complexes with D-type cyclins at the early G1 phase of cell cycle.^[35] Until now, it has been proven that CDK6 plays role in the occurrence of many tumors. CDK6 malfunction can lead to the occurrence of hematologic malignancies like acute myeloid leukemia (AML) and affect T-cell lymphoblastic lymphoma.^[36, 37] In addition the dysfunction of CDK6 can cause the occurrence of breast cancer and melanoma, too.^[38, 39]

The function of CDK6 in prostate cancer has been reported, too. A study found that Baicalin can decrease prostate cancer cell proliferation and the effect would reverse when upregulate expression of CDK6, which means that CDK6 can promote cell proliferation.^[40] Another study also found that CDK6 inhibitors (G1T28 and G1T38) played a role in treating CRPC.^[41] In addition, a research also found that CDK6 may have function in the occurrence of EnzR CRPC. The study found that a CDK6 inhibitor, Palbociclib, can promote EnzR LNCaP cell death.^[42] Though all these reflect that CDK6 can cause the occurrence and even development of PCa, there was no direct evidence reflect the expression of CDK6 in PCa and EnzR CRPC. In our study, we found that CDK6 was indeed expressed highly in tumor tissues than normal tissues. At the same time, it is also expressed highly in EnzR LNCaP and C4-2 compared to LNCaP and C4-2. Furthermore, we found that three CDK6 inhibitors, apigenin, chrysin and fisetin, can decrease the expression of CDK6 and can also decrease the cell proliferation. Our results directly illustrate that the mutation of CDK6 may be a reason in the occurrence of CRPC. In addition, the result reflected that these small molecules may be potential drugs in treating EnzR CRPC in the future.

However, our study had some shortcomings, too. First, the DEGs and hub gene we found in EnzR CRPC were from EnzR LNCaP. Despite we tested the expression of hub gene in TCGA and CPGEA database, the result may be different from EnzR CRPC patients' samples. Second, except *CDK6*, *GRIP2*, *PREX2*, *ITGA1* and *LAMB1* also expressed differently in cells samples and database. Due to other genes expressed reversed between cell samples from GEO and clinical samples from TCGA, we did not research their function. They may also be important factors in leading to PCa and EnzR CRPC. However, the expression of them may change due to the use of Enz. Third, the small molecules we found which are CDK6 inhibitors also have the other functions that may cause the occurrence of tumors. Their function in suppressing EnzR cells proliferation may come from other reasons to some extent. So, further studies need to verify the phenomenon we found.

Conclusion

In conclusion, we found 45 DEGs and 10 hub genes in EnzR LNCaP. The hub gene, CDK6, maybe an important factor in the occurrence of PCa and even EnzR CRPC. Three small molecules, apigenin, chrysin and fisetin, can decrease the expression of CDK6 in EnzR prostate cancer and suppress cell proliferation. They may also be potential drugs in treating CRPC in the future.

Abbreviations

PCa Prostate cancer

ADT Androgen deprivation therapy

DEGs Differentially expressed genes

AR Androgen receptor

CRPC Castration-resistant prostate cancer

GEO Gene Expression Omnibus

GO Gene oncology

KEGG Kyoto Encyclopedia of Genes and Genomes

PPI Protein-protein interaction

TCGA The Cancer Genome Atlas

GEPIA Gene Expression Profiling Interactive Analysis Platform

CPGEA Chinese Prostate Cancer Genome and Epigenome Atlas

OS Overall survival

DFS Disease-free survival

TNM Tumor-Node-Metastasis

CDK6 Cyclin-dependent kinases6

Declarations

Acknowledgments

Not applicable

Authors' contributions

Xi Chen and Yechen Wu put forward the idea of the article, wrote the manuscript and analyzed the data. Licheng Wang and Yicong Yao collected the data from public database. Xinan Wang and Chengdang Xu finished the RT-qPCR, Western blot and CCK-8 experiments. Denglong Wu and Gang Wu help collecting the clinical specimens and revised the manuscript.

Founding

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Availability of data and materials

The datasets supporting the conclusions of this article are available in the TCGA-PRAD (<https://portal.gdc.cancer.gov/>) CPGEA (<http://www.cpgea.com/>) and CMap (<https://portals.broadinstitute.org/cmap/>) database.

Ethic approval and consent participate

The study was approved by the ethic committee of Tongji Hospital, School of Medicine, Tongji University. Each participate volunteered to join and signed the informed consent form.

Consent for publication

Not applicable

Competing interests

All authors declare that they have no conflict of interest regarding the publication of the paper.

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Figures

Figure 1

The different expression genes (DEGs) from GSE44905, GSE78201 and GSE150807. (A) The upregulated DEGs from GSE44905, GSE78201 and GSE150807. (B) The downregulated DEGs from GSE44905, GSE78201 and GSE150807.

A

B

Figure 2

The scatter map of enrichment pathways. (A) The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways of DEGs was shown in scatter map. (B) The Gene Oncology (GO) analysis of DEGs was shown in scatter map.

Figure 3

The Protein-protein interaction (PPI) network of DEGs.

Figure 4

The expression of hub genes in GEPIA database (normal tissue include data from TCGA and GTEx database). * represents statistical differences. Red color represents Tumor samples and gray color represents normal samples.

Figure 5

The expression of hub genes in Chinese patients with prostate cancer. (The sequence data from CPGEA database)

Figure 6

The overall survival (OS) of hub genes from GEPIA database depend on TCGA database. (A) GRIP2 (B) EPHB2(C) CDK6 (D) PAX6 (E) BMP7(F) ITGA1 (G) LAMB1 (H) IGFBP5 (I) LY6K

Figure 7

The disease-free survival (DFS) of hub gens from GEPIA database depend on TCGA database. (A) GRIP2 (B) EPHB2 (C) CDK6 (D) PAX6 (E) BMP7 (F) ITGA1 (G) LAMB1 (H) IGFBP5 (I) LY6K

Figure 8

The expression of CDK6 in different tumor stage of PCa depend on TCGA database. (A) CDK6 expression in different Gleason score patients. (B) The expression of CDK6 in different Tumor (T) stage rely on TNM classification of malignant tumors. (C) The expression of CDK6 in different Node (N) stage rely on TNM classification of malignant tumors. (D) The expression of CDK6 in different ACJJ Tumor (T) stage. (E) The expression of CDK6 in different T2 tumor stage on TNM classification of malignant tumors. (F) The expression of CDK6 in different T3 tumor stage on TNM classification of malignant tumors.

Figure 10

CDK6 expressed highly in PCa and EnzR CRPC. (A) The mRNA expression of CDK6 in Prostate cancer samples and paracancerous samples from 14 paired patients. (B) The expression of CDK6 protein between tumor tissues and paracancerous tissues from 6 paired patients. (C) The mRNA expression of CDK6 among normal prostate epithelial cell RWPE-1 and prostate cancer cell LNCaP and C4-2. (D) The expression of CDK6 protein between normal cell and cancer cell. (E) The mRNA expression of CDK6 in paternal LNCaP and C4-2 and EnzR LNCaP and C4-2. (F) The expression of CDK6 protein in paternal prostate cancer cell lines and EnzR prostate cancer cell lines. * represents $P < 0.05$, ** represents $P < 0.01$.

Figure 11

CDK6 inhibitors can decrease the expression of CDK6 and suppress cell proliferation. (A) The mRNA expression of CDK6 after LNCaP and EnzR LNCaP treated with CDK6 inhibitors. (B) The expression of CDK6 in mRNA level after C4-2 and EnzR C4-2 treated with CDK6 inhibitors. (C) CDK6 inhibitors can suppress CDK6 expression at protein level in LNCaP and EnzR LNCaP. (D) The CDK6 protein expression decreased in C4-2 and EnzR C4-2 after CDK6 inhibitors treated. (E) The cell proliferation ability was

decreased after LNCaP and EnzR LNCaP treated by CDK6 inhibitors. (F) C4-2 and EnzR C4-2 cell proliferation ability was decreased after CDK6 inhibitors treated the cell. * represents $P < 0.05$, ** represents $P < 0.01$, *** represents $P < 0.001$.

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