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# Elevated CISD2 expression predicts poor diagnosis and promotes invasion and migration of prostate cancer cells

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**Abstract. – OBJECTIVE: To explore roles of CDGSH iron-sulfur domain-containing protein 2 (CISD2) in the progression of prostate cancer (PCa) cells, and relationships between CISD2 expression and the prognosis and clinical pathological parameters in PCa patients.**

**PATIENTS AND METHODS: Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) analysis and Western blot analysis were used to detect the CISD2 expression in PCa tissues and cells. CISD2 siRNA was used to inhibit the CISD2 expression. Kaplan-Meier method and Log rank analysis were performed to determine survival analysis while Chi-square test was performed to analyze the association between CISD2 and clinicopathological parameters of PCa patients. Transwell assay and wound healing assay was conducted to examine the invasion and migration ability of PCa cells, respectively.**

**RESULTS: CISD2 was up-regulated in PCa tissues and cells, and showed positive association with the poor prognosis, T stage, lymphatic invasion, prostate-specific antigen (PSA) level, and distant metastasis of PCa patients. Besides, we found that inhibition of CISD2 significantly impaired the migration and invasion ability of PCa cells.**

**CONCLUSIONS: The paper demonstrated that CISD2 could act as a new target for the diagnosis and treatment of PCa patients.**

*Key Words:*

Prostate cancer (PCa), CDGSH iron-sulfur domain-containing protein 2 (CISD2), Prognosis, Invasion, Migration.

## Introduction

Prostate cancer (PCa) is the most common malignant tumor of the male reproductive system, and its incidence varies greatly among different countries and regions[1]. Generally, the incidence rate in developed countries and regions is considerably higher than that in developing countries and regions, which was closely related to the local economic level, lifestyle, and diet structure. At present, treatment of PCa, including external radiotherapy, prostatectomy, and brachytherapy, and the 5-year survival rate of PCa was acceptable among most countries[2-6]. However, it remains a severe challenge around the world due to its yearly increased incidence rate and recurrence rate[7].

CDGSH iron-sulfur domain-containing protein 2 (CISD2), a member of the iron-sulfur protein family, is located on human chromosome 4q24[8], which is an evolutionarily conserved gene mainly distributed in the mitochondrial outer membrane and endoplasmic reticulum[9,10]. In addition, CISD2 can interact with B-cell lymphoma 2 (Bcl-2) and regulate autophagy and calcium ion homeostasis[11], and modulate the levels of intracellular iron and reactive oxygen species that are important factors of the proliferation of tumor cells and the progression of the diseases[12,13]. The expression level of CISD2 is remarkably upregulated in some tumors such as early cervical cancer[14], esophageal cancer[15], primary hepatocellular cancer[16], and gastric cancer[17], and often predicts poor prognosis[18,19].

In view of the fact that there is still no literature reporting the role of CISD2 in PCa progression, we thus systematically analyzed the expression of CISD2 in PCa tissues and explored the relationship between CISD2 and clinic pathological features of PCa patients, as well as the effect of CISD2 on PCa cells.

## Patients and Methods

### Tissue Collection

A total of 52 pairs of PCa tissue and adjacent normal tissues were obtained from PCa patients undergoing surgery in The First Affiliated Hospi-

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tal, School of Medicine, Zhejiang University, and normal tissues adjacent to PCa were taken as control specimens. The diagnosis was determined by two pathologists according to the relevant diagnostic criteria in the WHO prostate histology classification. The collection of tissue samples and clinical case data was approved by the patient (family) and approved by the Ethics Committee of The First Affiliated Hospital, School of Medicine, Zhejiang University.

### Cell Culture

Human PCa cell lines DU145, PC3, VCaP, and prostate immortalized normal epithelial cells RWPE cells were purchased from the American Type Culture Collection (ATCC) cell bank (Manassas, VA, USA). The cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; HyClone, South Logan, UT, USA) at 37°C with 5% $CO_2$. When the density reached 80%, the cells were gently washed with phosphate-buffered saline (PBS), and digested with 1 ml of trypsin. Then, 2 mL of medium (containing 10% FBS) were added to terminate the digestion. The cell suspension was then centrifuged at 1000 rpm for 5 min. Finally, the cells were suspended with fresh medium, and plated in 6 cm dish with a ratio of 1:3.

### Cell Transfection

Cells were digested and counted, and a total of 1 x $10^4$ cells were planted in a 6-well plates. When the density reached 50%, the transfection system (8 μL lipo3000 + 16 μL CISD2-siRNA/NC+100 μL medium) was mixed, allowed to stand at room temperature for 10 min-15 min, and then added to the plate. After 6 h, the complete medium was replaced, and cells underwent transfection were used for following assay.

### Wound Healing Assay

After 48 h of transfection, the cells in the 6-well plate were quickly scratched with a 100 μL tip perpendicular, then washed with PBS for three times. Thereafter, the cells were replaced with serum-free medium and cultured in a 37°C, 5% $CO_2$ incubator for 24 h. Later, the scribe region was photographed, the width was measured, and statistical analysis was performed.

### Cell Invasion Assay

The cells were digested and counted, resuspended in 200 μL of serum-free medium, then added to a normal transwell chamber/pre-coated chamber, and 500 μL of complete medium was added to the small outdoor side. After culture for 24 h, cells in the small outdoor side hole were fixed with 500 μL of 10% paraformaldehyde solution at room temperature for 15 min, then stained with crystal violet solution for 5 min. After drying, the number of cells was counted and observed under an inverted microscope.

### RNA Extraction and Analysis

The cells were washed with pre-cooled PBS and lysed with 500 ml TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The purity and concentration of RNA samples were measured by absorbance at 260 nm using Nanodrop-2000. Primers in the study were as follows: CISD2 Forward: 5'-GCAAGGTAGCCAAGAAGTGC-3', Reverse: 5'-CCCAGTCCCTGAAAGCATTA-3', GAPDH Forward: 5'-TTGAGGTCAATGAAGGGGTC-3', Reverse: 5'-GAAGGTGAAGGTCGGAGTCA-3'. The PCR procedure was performed according to the SYBR Green reagent instructions: pre-denaturation: 95°C for 10 min; PCR reaction: denaturation: 95°C 15 s, annealing: 60°C 60 s, extension: 72°C 30 s, for 40 cycles. Three replicate wells were set for each sample, and the expression level of GAPDH was set as an internal reference, using the $2^{-\Delta\Delta CT}$ method for data processing.

### Western Blot Analysis

For Western blot, 200 μL of cell lysis buffer (prepared with radioimmunoprecipitation assay (RIPA) and phenylmethylsulfonyl fluoride (PMSF) at a ratio of 100:1) (Beyotime, Shanghai, China) was added to each well and lysed on ice for 15 min. Then, the lysate was collected and centrifuged at 12,000 rpm and 4°C for 15 min. The protein concentration was detected according to the instructions of a bicinchoninic acid (BCA) protein quantification kit (Pierce, Rockford, IL, USA). Thereafter, 30 μg of protein samples were successively added to the sample wells and transferred to a polyvinylidene difluoride (PVDF) membrane that was appropriately sized and activated by methanol (Millipore, Billerica, MA, USA) at 4°C and constant current (217 mA) for 2 h. After that, the membrane was blocked with 5% skim milk powder at room temperature for 1 h, washed once with tris-buffered saline with Tween®20 (TBST), incubated with diluted primary antibodies on a shaker at 4°C overnight. Thereafter, the PVDF membrane was washed with TBST

on the shaker for 10 min ×3 times, incubated with secondary antibodies (diluted with 5% skim milk powder) in an incubator at 37°C for 30-40 min. Lastly, the membrane was added with enhanced chemiluminescence (ECL) liquid and exposed in an imaging system.

### Statistical Analysis

Statistical analysis was performed using GraphPad Prism 7.0 software (La Jolla, CA, USA). Differences between two groups were analyzed by Student's *t*-test. Comparison among multiple groups was analyzed using One-way ANOVA test followed by post-hoc test (Least Significant Difference). Survival analysis was performed using the Kaplan-Meier method and Log rank analysis. Chi-square test was performed to analyze the association between CISD2 and clinicopathological parameters of PCA patients. $p<0.05$ was considered statistically significant.

## Results

### CISD2 was Highly Associated with PCa Progression

Tumor tissues and adjacent tissues in PCa patients were collected for determining CISD2 mRNA expression. The qRT-PCR results showed that CISD2 was notably increased in PCa tissues and DU145, PC3, and VCaP cell lines compared to non-PCa tissues and RWPE cell lines, respectively (Figure 1A, 1B). Next, we performed Kaplan-Meier survival analysis and log-rank testing, results showed that PCa patients with higher CISD2 expression had a poor overall survival compared with those in low-CISD2 group (Figure 1C). Collectively, these results indicated that CISD2 was highly involved in the progression of PCa.

### Relationships of CISD2 with Clinical Pathological Features of PCa

To figure out the association between CISD2 and clinic pathological parameters of PCa. We

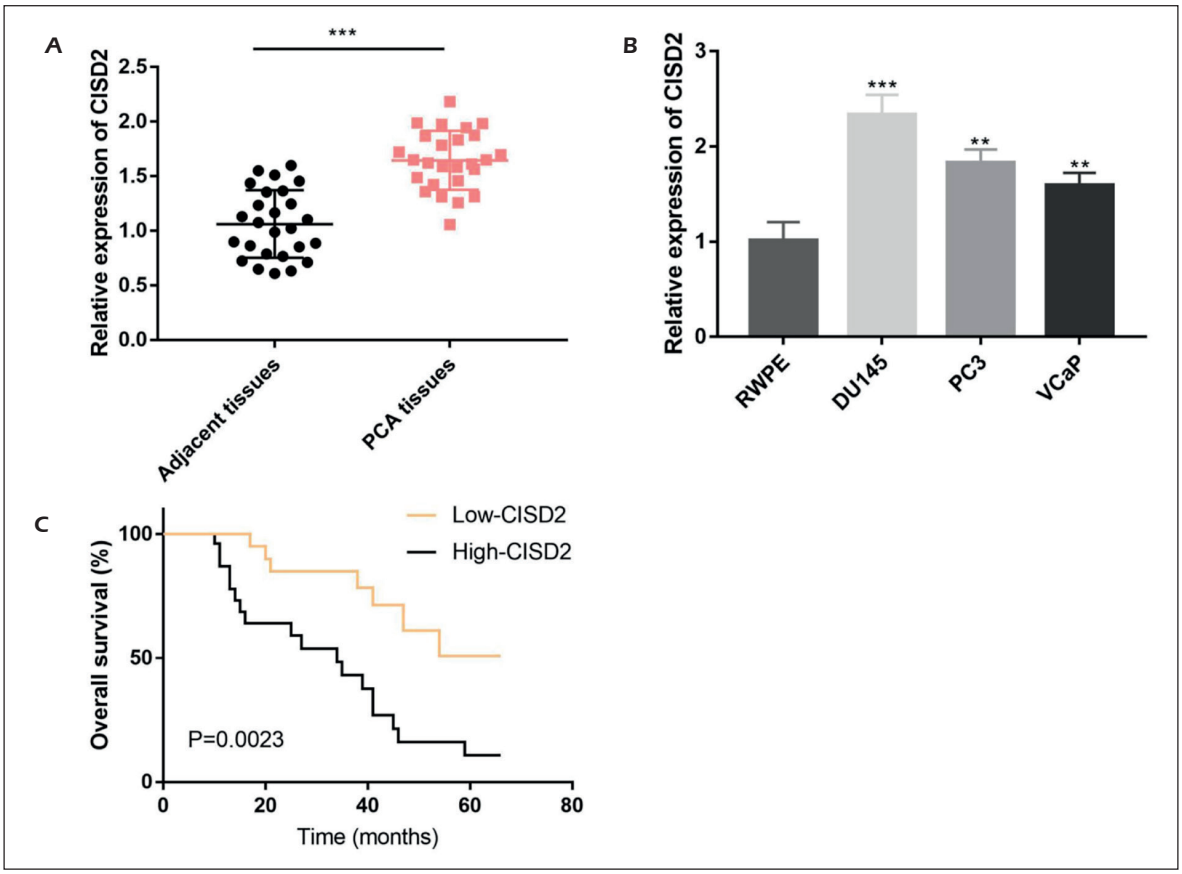


**Figure 1.** CISD2 was over-expressed in PCa tissues and cells. **A,** CISD2 was upregulated in PCa tissues compared to adjacent normal tissues. **B,** CISD2 was significantly upregulated in DU145, PC3 and VCaP cell lines than that in RWPE cell lines. **C,** High expression of CISD2 in PCa patients predicted a poor prognosis. (**$p<0.01$, ***$p<0.001$).

divided the patients into two groups: CISD2 high-expression group (n=26) and CISD2 low-expression group (n=26) according to the middle expression level of CISD2. Using Chi-square test, we then found that CISD2 was positively associated with the T stage, lymphatic invasion, PSA level, and distant metastasis of PCa (Table I).

### Knockdown of CISD2 Inhibited the Migration Ability of PCa Cells

To further investigate the relationship between CISD2 and PCa migration, we transfected CISD2-siRNA in DU145 and PC3 cell lines. As shown in Figure 2A, B, DU145, and PC3 cells underwent CISD2-siRNA treatment showed significantly reduced CISD2 expression detected by qRT-PCR and Western blot, respectively. Then, we performed transwell assay and observed that both the DU145 and PC3 cells in si-CISD2 group showed an impaired migration ability than that in NC group (both $p<0.001$, Figure 2C-2F).

### Inhibition of CISD2 Reduced the Invasion Ability of PCa Cells

To determine whether CISD2 influenced the invasion ability in DU145 and PC3 cell lines, the wound healing assay was conducted. As shown in Figure 3A-3D, when CISD2 was inhibited in DU145 and PC3 cells, the wound healing rate in both of them significantly decreased, with 64.33 ± 4.91% in DU145 NC group compared to 27.67 ± 1.202% in DU145 si-CISD2 group ($p<0.01$) and 71 ± 1.732% in PC3 NC group compared to 34.33 ± 3.383% in PC3 si-CISD2 group ($p<0.01$).

## Discussion

PCa is regarded as a common non-skin malignant tumor, which is the leading cause of cancer-related mortality all over the world[20]. It should be noted that the development and progression of PCa vary in individuals, which is one of the reasons for its high heterogeneity[21,22]. Given this, comprehensively and deeply analyzing the molecular basis of the development and progression of PCa, as well as combining gene molecular type with clinical pathological grade and stage may accurately classify the subtypes of PCa, thus improving targeted therapy, treatment response prediction, and prognosis evaluation.

As a member of the iron-sulfur protein family, CISD2 is a 2Fe-2S dimer mainly expressed in the endoplasmic reticulum and mitochondrial membrane[9]. It is capable of keeping the integrity of mitochondria and modulating autophagy, calcium ion, and iron ion homeostasis and reactive oxygen species level[23-25]. Several studies have illustrated that CISD2 was wildly participated in cancer progressions. Li et al[18] have revealed that CISD2 was highly expressed in lung cancer cells and promoted the proliferation of lung cancer cells and the growth of the tumor by maintaining mitochondrial structure and function stability. Kim et al[15] reported that inhibiting CISD2 protein expression was able to reduce c-Myc protein expression and Bcl-2/Bax ratio, thereby repressing the proliferation ability of squamous-cell carcinoma. Besides, Sun et al[26] demonstrated that CISD2 was in-

**Table I.** CISD2 expression and clinic pathological parameters of patients with PCa.

| Variable | CISD2 mRNA expression | | *p*-value |
|---|---|---|---|
| | Low | High | |
| Age (years) | | | 0.573 |
| < 60 | 9 | 12 | |
| ≥ 60 | 17 | 14 | |
| T stage | | | 0.023* |
| T1-T2 | 15 | 6 | |
| T3-T4 | 11 | 20 | |
| Lymphatic invasion | | | 0.005** |
| No | 19 | 8 | |
| Yes | 7 | 18 | |
| PSA level | | | < 0.001*** |
| < 4 | 20 | 7 | |
| ≥ 4 | 6 | 19 | |
| Distant metastasis | | | 0.004** |
| No | 16 | 5 | |
| Yes | 10 | 21 | |

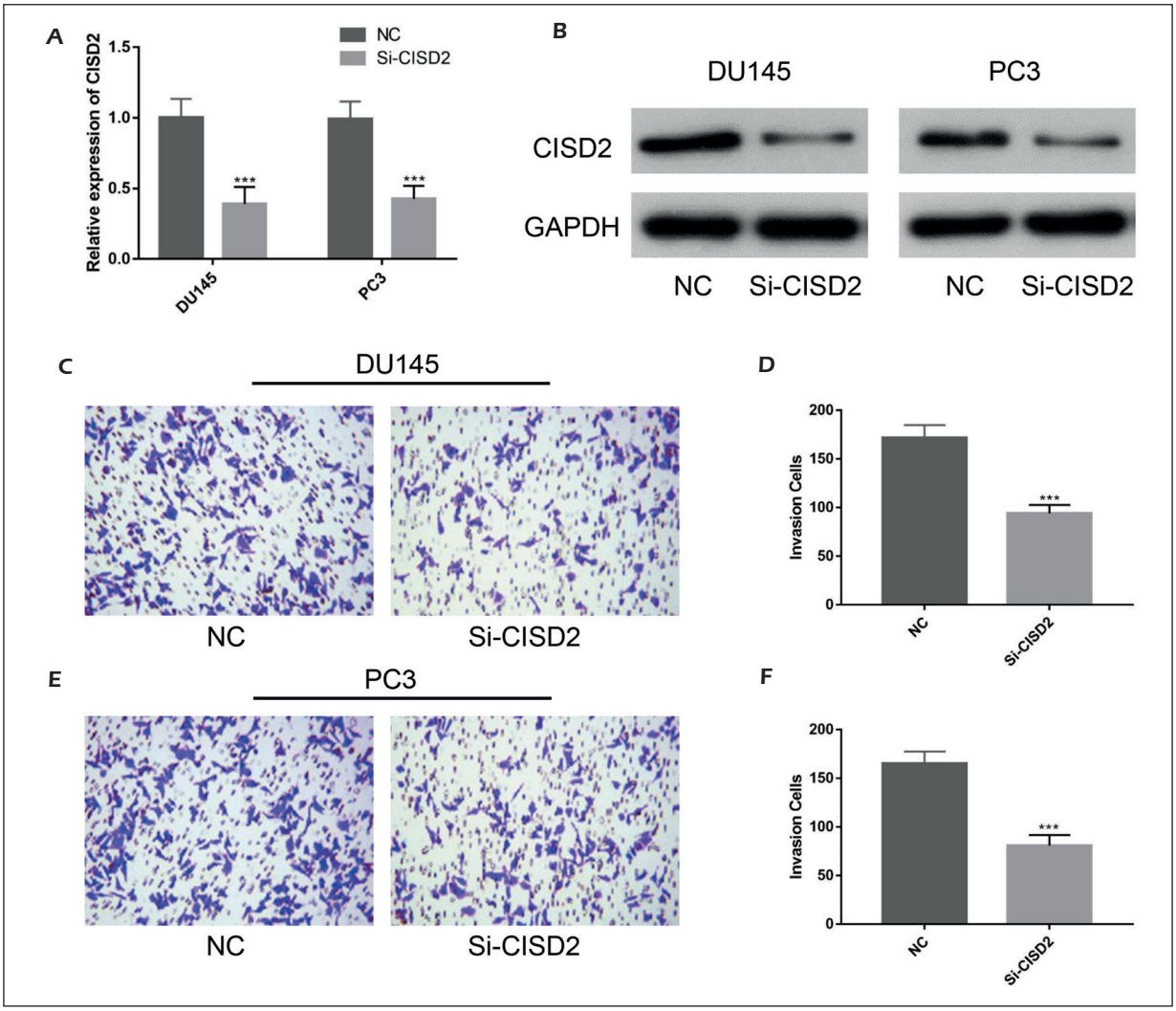


**Figure 2.** Knockdown of CISD2 inhibited the migration ability of PCa cells. **A-B,** DU145 and PC3 cells underwent CISD2-siRNA treatment showed significantly reduced CISD2 expression detected by Western blot. **C-D,** DU145 cells transfected with CISD2-siRNA showed a decreased migration ability compared to un-treated DU145 cells (magnification: 40x). **E-F,** PC3 cells under CISD2-siRNA treatment exhibited reduced migration ability compared to normal PC3 cells (magnification: 40x). (***$p<0.001$).

creased in glioma cancers and predicted poor prognosis and aggressive tumor behavior.

Considering the limited literature on the association between CISD2 and PCa, in this paper, we firstly determined the CISD2 expression in PCa tissues and the adjacent normal tissues. Similar with other reports, CISD2 was found notably up-regulated in cancer tissues[26,27]. In view of these, we collected patients' information and analyzed their prognosis with different expression manner of CISD2 by using Kaplan-Meier survival analysis and log-rank testing. Not surprisingly, we found a significantly poor overall survival in patients with higher CISD2 expression than those with low-CISD2 expression. Besides, results analyzed by Chi-square test even showed that CISD2 was positively associated with the T stage, lymphatic invasion, PSA level, and distant metastasis of PCa patients, indicating its tightly involvement in the PCa progression.

To deeply investigate the effect of CISD2 on PCa cells, we transfected CISD2 siRNA in DU145 and PC3 cell lines to inhibit the expression of CISD2, and then performed transwell assay and wound healing assay. Results in the transwell assay showed that the migration ability in DU145 and PC3 cell lines after CISD2-siRNA treatment was remarkably reduced than those without

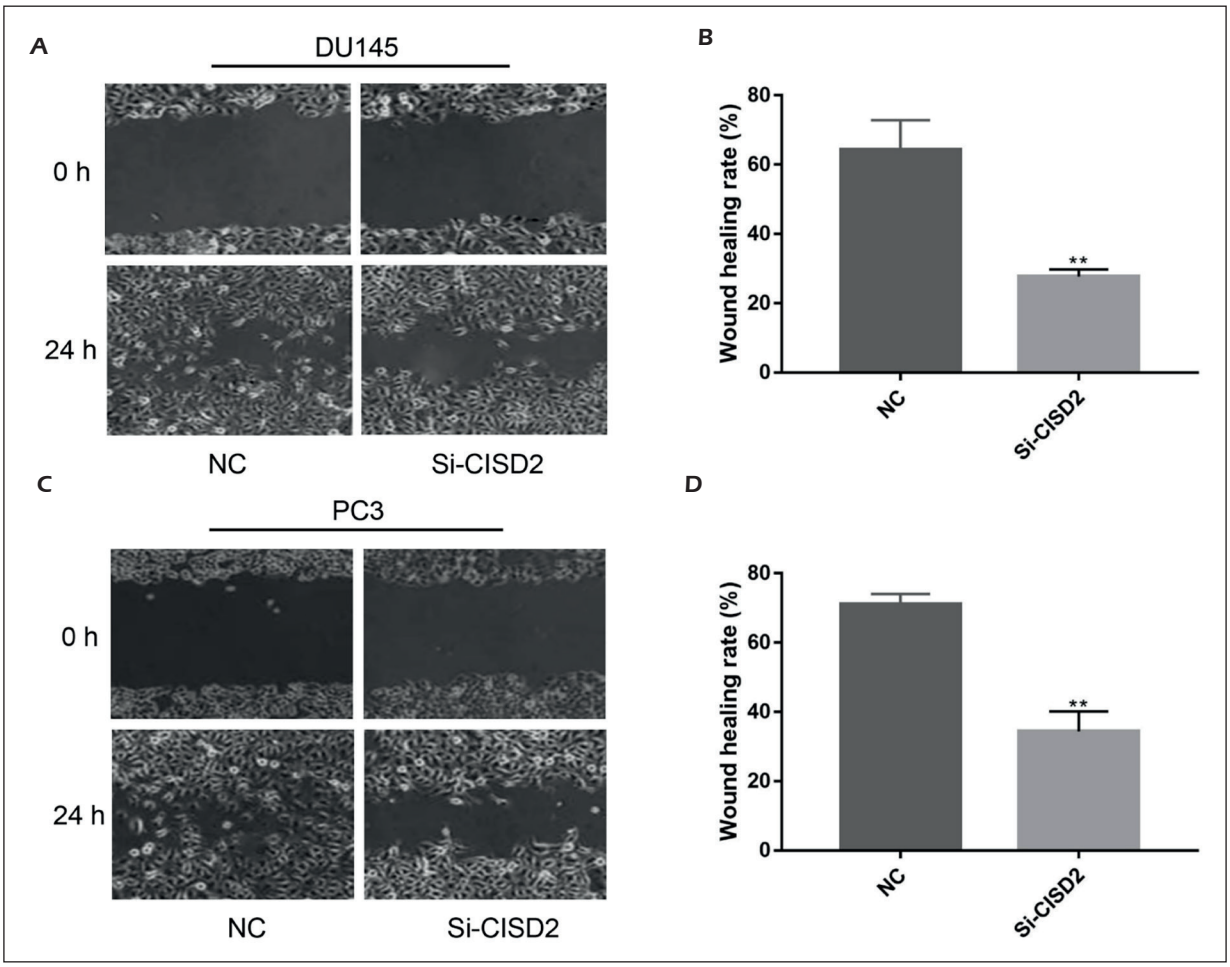


**Figure 3.** Inhibition of CISD2 impaired the invasion ability of PCa cells. **A-B,** The invasion ability of DU145 decreased significantly when expression of CISD2 was inhibited (magnification: 10x). **C-D,** PC3 cells with low expression of CISD2 showed a decreased invasion ability compared to normal PC3 cells (magnification: 10x). (**$p$<0.01).

treatment. Also, when the CISD2 expression was suppressed in DU145 and PC3 cell lines, both of their invasion ability decreased significantly, which was proofed by the wound healing assay. Collectively, these results further illustrated the role of CISD2 in the invasion and migration of PCa, consistent with the above analysis of the clinical and pathological features of PCa patients.

## Conclusions

We revealed that CISD2 was upregulated in PCa tissues and cells, and its high level of expression not only predicted a poor prognosis in PCa patients, but also implied worse T stage, lymphatic invasion, PSA level and distant metastasis. Besides, we demonstrated that CISD2 could promote the migration and invasion of PCa cells. Thus, CISD2 may expect to become a new target for the diagnosis and treatment of PCa.

### Conflict of Interest

The Authors declare that they have no conflict of interests.

## References

1) Livingstone TL, Beasy G, Mills RD, Plumb J, Needs PW, Mithen R, Traka MH. Plant bioactives and the prevention of prostate cancer: evidence from human studies. Nutrients 2019; 11: 2245.

2) Pomykala KL, Czernin J, Grogan TR, Armstrong WR, Willliams J, Calais J. Total-body 68Ga-PSMA-11 PET/CT for bone metastasis detection in prostate can-

cer patients: potential impact on bone scan guidelines. J Nucl Med 2019: pii: jnumed.119.230318. doi: 10.2967/jnumed.119.230318. [Epub ahead of print].

3) Brand DH, Tree AC, Ostler P, van der Voet H, Loblaw A, Chu W, Ford D, Tolan S, Jain S, Martin A, Staffurth J, Camilleri P, Kancherla K, Frew J, Chan A, Dayes IS, Henderson D, Brown S, Cruickshank C, Burnett S, Dufton A, Griffin C, Hinder V, Morrison K, Naismith O, Hall E, van As N. Intensity-modulated fractionated radiotherapy versus stereotactic body radiotherapy for prostate cancer (PACE-B): acute toxicity findings from an international, randomised, open-label, phase 3, non-inferiority trial. Lancet Oncol 2019: 1531-1543.

4) Laursen EB, Fredsoe J, Schmidt L, Strand S, Kristensen H, Ildor RA, Daugaard TF, Mouritzen P, Hoyer S, Kristensen G, Stroomberg HV, Brasso K, Roder MA, Borre M, Sorensen KD. Elevated miR-615-3p expression predicts adverse clinical outcome and promotes proliferation and migration of prostate cancer cells. Am J Pathol 2019: 2377-2388.

5) Qu HW, Jin Y, Cui ZL, Jin XB. MicroRNA-373-3p inhibits prostate cancer progression by targeting AKT1. Eur Rev Med Pharmacol Sci 2018; 22: 6252-6259.

6) Aghdam RA, Amoui M, Ghodsirad M, Khoshbakht S, Mofid B, Kaghazchi F, Tavakoli M, Pirayesh E, Ahmadzadehfar H. Efficacy and safety of177 Lutetium-prostate-specific membrane antigen therapy in metastatic castration-resistant prostate cancer patients: first experience in West Asia - A prospective study. World J Nucl Med 2019; 18: 258-265.

7) Feagins LA, Kim J, Chandrakumaran A, Gandle C, Naik KH, Cipher DJ, Hou JK, Yao MD, Gaidos J. Rates of adverse IBD-related outcomes for patients with IBD and concomitant prostate cancer treated with radiation therapy. Inflamm Bowel Dis 2019: pii: izz175. doi: 10.1093/ibd/izz175. [Epub ahead of print].

8) Chen YF, Kao CH, Chen YT, Wang CH, Wu CY, Tsai CY, Liu FC, Yang CW, Wei YH, Hsu MT, Tsai SF, Tsai TF. Cisd2 deficiency drives premature aging and causes mitochondria-mediated defects in mice. Genes Dev 2009; 23: 1183-1194.

9) Chen YF, Kao CH, Kirby R, Tsai TF. Cisd2 mediates mitochondrial integrity and life span in mammals. Autophagy 2009; 5: 1043-1045.

10) Chen YF, Wu CY, Kirby R, Kao CH, Tsai TF. A role for the CISD2 gene in lifespan control and human disease. Ann N Y Acad Sci 2010; 1201: 58-64.

11) Chang NC, Nguyen M, Shore GC. BCL2-CISD2: an ER complex at the nexus of autophagy and calcium homeostasis? Autophagy 2012; 8: 856-857.

12) Wang CH, Chen YF, Wu CY, Wu PC, Huang YL, Kao CH, Lin CH, Kao LS, Tsai TF, Wei YH. Cisd2 modulates the differentiation and functioning of adipocytes by regulating intracellular Ca2+ homeostasis. Hum Mol Genet 2014; 23: 4770-4785.

13) Mozzillo E, Delvecchio M, Carella M, Grandone E, Palumbo P, Salina A, Aloi C, Buono P, Izzo A, D'Annunzio G, Vecchione G, Orrico A, Genesio R, Simonelli F, Franzese A. A novel CISD2 intragenic deletion, optic neuropathy and platelet aggregation defect in Wolfram syndrome type 2. BMC Med Genet 2014; 15: 88.

14) Liu L, Xia M, Wang J, Zhang W, Zhang Y, He M. CISD2 expression is a novel marker correlating with pelvic lymph node metastasis and prognosis in patients with early-stage cervical cancer. Med Oncol 2014; 31: 183.

15) Kim EH, Shin D, Lee J, Jung AR, Roh JL. CISD2 inhibition overcomes resistance to sulfasalazine-induced ferroptotic cell death in head and neck cancer. Cancer Lett 2018; 432: 180-190.

16) Chen B, Shen S, Wu J, Hua Y, Kuang M, Li S, Peng B. CISD2 associated with proliferation indicates negative prognosis in patients with hepatocellular carcinoma. Int J Clin Exp Pathol 2015; 8: 13725-13738.

17) Wang L, Ouyang F, Liu X, Wu S, Wu HM, Xu Y, Wang B, Zhu J, Xu X, Zhang L. Overexpressed CISD2 has prognostic value in human gastric cancer and promotes gastric cancer cell proliferation and tumorigenesis via AKT signaling pathway. Oncotarget 2016; 7: 3791-3805.

18) Li SM, Chen CH, Chen YW, Yen YC, Fang WT, Tsai FY, Chang JL, Shen YY, Huang SF, Chuu CP, Chang IS, Hsiung CA, Jiang SS. Upregulation of CISD2 augments ROS homeostasis and contributes to tumorigenesis and poor prognosis of lung adenocarcinoma. Sci Rep 2017; 7: 11893.

19) Yang L, Hong S, Wang Y, He Z, Liang S, Chen H, He S, Wu S, Song L, Chen Y. A novel prognostic score model incorporating CDGSH iron sulfur domain2 (CISD2) predicts risk of disease progression in laryngeal squamous cell carcinoma. Oncotarget 2016; 7: 22720-22732.

20) Cattrini C, Rubagotti A, Zinoli L, Cerbone L, Zanardi E, Capaia M, Barboro P, Boccardo F. Role of circulating tumor cells (CTC), androgen receptor full length (AR-FL) and androgen receptor splice variant 7 (AR-V7) in a prospective cohort of castration-resistant metastatic prostate cancer patients. Cancers (Basel) 2019; 11: 1365.

21) Qiu Y, Yang S, Pan T, Yu L, Liu J, Zhu Y, Wang H. ANKRD22 is involved in the progression of prostate cancer. Oncol Lett 2019; 18: 4106-4113.

22) Tullio A, Magli A, Moretti E, Valent F. Why we should take care of the competing risk bias in survival analysis: A phase II trial on the toxicity profile of radiotherapy for prostate cancer. Rep Pract Oncol Radiother 2019; 24: 511-519.

23) Wang CH, Kao CH, Chen YF, Wei YH, Tsai TF. Cisd2 mediates lifespan: is there an interconnection among Ca(2)(+) homeostasis, autophagy, and lifespan? Free Radic Res 2014; 48: 1109-1114.

24) Jones MA, Amr S, Ferebee A, Huynh P, Rosenfeld JA, Miles MF, Davies AG, Korey CA, Warrick JM, Shiang R, Elsea SH, Girirajan S, Grotewiel M. Genetic studies in Drosophila and humans support a model for the concerted function of CISD2, PPT1 and CLN3 in disease. Biol Open 2014; 3: 342-352.

25) Cheng YH, Chavez E, Tsai KL, Yang KC, Kuo WT, Yang YP, Chiou SH, Lin FH. Effects of thermosensitive chitosan-gelatin based hydrogel containing glutathione on Cisd2-deficient chondrocytes under oxidative stress. Carbohydr Polym 2017; 173: 17-27.

26) Sun AG, Meng FG, Wang MG. CISD2 promotes the proliferation of glioma cells via suppressing beclin1mediated autophagy and is targeted by microRNA449a. Mol Med Rep 2017; 16: 7939-7948.

27) Sun Y, Jiang Y, Huang J, Chen H, Liao Y, Yang Z. CISD2 enhances the chemosensitivity of gastric cancer through the enhancement of 5-FU-induced apoptosis and the inhibition of autophagy by AKT/mTOR pathway. Cancer Med 2017; 6: 2331-2346.