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CTHRC1 and PD-1/PD-L1 expression predicts tumor recurrence in prostate cancer

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Abstract. Collagen triple helix repeat containing 1 (*CTHRC1*) is a gene that has been associated with tumor progression in human prostate cancer (PC). The tumor immune microenvironment has been linked with disease outcome in PC. In the present study, the correlation between *CTHRC1* with PC recurrence and the tumor immunological microenvironment was investigated. Using the data supplied by the Tumor Immune Estimation Resource (TIMER), the expression of *CTHRC1*, programmed cell death protein 1 (*PD-1*), and programmed cell death 1 ligand 1 (*PD-L1*) were analyzed. Immunohistochemical staining of *CTHRC1*, PD-1 and PD-L1 was performed using a tissue microarray construction of prostate adenocarcinoma (PRAD) specimens. In PRAD, an association was reported between the *CTHRC1* expression and the disease free survival (DFS) rate (P=0.022). Overexpression of *CTHRC1* was correlated with increased levels of PD-1 (R=0.272, P=0.021) and PD-L1 (R=0.298, P=0.016), elevated levels of infiltrating B cells (P=9.51e^{-11}), CD4$^+$ cells (P=1.51e^{-11}), macrophages (P=8.25e^{-5}), neutrophils (P=2.17e^{-9}) and dendritic cells (P=3.13e^{-13}). Bioinformatics analysis revealed that *CTHRC1* was correlated with the expression levels of matrix metalloproteinase-9, mucin 1 and solute carrier organic anion transporter family member 2B1 genes, which exert an influence in PRAD. The occurrence of this condition is most likely to be associated with regulation of the tumor microenvironment. Taken together, we demonstrated that the prognosis and immunity of PC are closely linked to *CTHRC1* upregulation. Furthermore, these results suggest that the immune function of PC may be suppressed by *CTHRC1*-targeting therapy.

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Introduction

Prostate cancer (PC) remains the most commonly diagnosed malignant disease in males (1). Increasing evidence has revealed that the tumor microenvironment exerts a crucial role in the progression of PC (2,3). Furthermore, the tumor microenvironment exhibits a high degree of heterogeneity in different individuals (4). In PC, inflammatory cells, immune cells, the vasculature, stromal cells, extracellular matrix and immune cells constitute the tumor microenvironment (5). In terms of its involvement in PC, the essential function of the tumor microenvironment manifests via the recurrence of the disease, metastasis and castration resistance (6).

In previous studies, the protein, collagen triple helix repeat containing 1 (CTHRC1), has been proposed as a pivotal tumor promoter (7-9). Additionally, CTHRC1 has been revealed to activate the planar cell polarity pathway via stabilization of the Wnt-receptor complex (10). In certain solid tumors, including those of pancreatic ductal adenocarcinoma (11), gastric cancer (12), hepatocellular carcinoma (13) and esophageal squamous cell carcinoma (14), CTHRC1 has been demonstrated to be markedly upregulated, although its role in PC is yet to be fully elucidated.

Currently, therapeutic applications of the immunological microenvironment in the treatment of PC have been extensively studied (15). Among them, immune checkpoint therapy has achieved impressive results in certain types of neoplasms. In this case, typical immunotherapeutic antibodies targeting programmed cell death protein 1 (PD-1)/programmed cell death 1 ligand 1 (PD-L1) have been applied in clinical trials for a number of different types of cancer, including PC (16,17). However, not all patients react favorably towards the treatment; thus, further research is required to elucidate the underlying mechanism.

At present, to the best of our knowledge, the association between the expression levels of *CTHRC1*, PD-1 and PD-L1 in PC has not been studied. The premise of the present study was therefore to use the PC dataset in the Tumor Immune

Estimation Resource (TIMER) database to determine the association between the CTHRC1 network and PC recurrence. Additionally, the expression levels of PD-1/PD-L1 and *CTHRC1* in a cohort of PC patients was analyzed, and their potential association with recurrence was investigated.

Materials and methods

Data mining of the cancer genome Atlas (TCGA)-prostate adenocarcinoma (PRAD) dataset. The correlation between the disease-free survival (DFS) rate and the expression level of *CTHRC1* in PRAD was computed using the Gene Expression Profiling Interactive Analysis (GEPIA) online database (18). The TIMER web server was utilized to examine the correlation between CTHRC1 mRNA expression and its clinical impact in dif-ferent immune cells in PRAD (19), thereby acquiring the immunity profile of TCGA-PRAD dataset.

Patient selection and tissue microarray (TMA) construction. Prostate tissue samples and the associated clinical pathology data from a total of 122 patients with PC were retrieved from the archives of the Department of Pathology, Xiangya Hospital, Central South University. All the enrolled patients underwent radical prostatectomy between January 2003 and December 2010, and they did not receive chemotherapy or radiation prior to surgery. For the use of clinical materials for research purposes, written informed consent was obtained from each patient prior to surgery. The research program was approved by the Ethics Committee of The First Affiliated Hospital of Hunan University of Chinese Medicine. The clinical characteristics of patients enrolled in our study were presented in Table I. The modified Gleason and 2010 pathological tumor-node-metastasis classification grading systems (20) were applied to categorize the hematoxylin and eosin-stained tumor sections of each sample (21). Ultimately, TMA blocks containing the PRAD tissues from the 122 cases were structured.

Cell culture and western blot assay. Human prostate cancer cell lines LNCaP and PC-3 cells, and human prostate normal cells RwPE-2 were obtained from the American Type Culture Collection. All cells were propagated standard cell culture conditions (5% CO_2, 37˚C) with Dulbecco's Modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc.) and 10% fetal calf serum. After 3 days in culture, cells were harvested and lysed. Cell extracts were prepared in a lysis buffer (150 mM NaCl, 20 mM HEPES, 1% Triton X-100, 2 mM EGTA, 20 mM glycerol phosphate, 1 mM EDTA, and 10% glycerol with protease). Protein levels of cells were determined by a Bradford's assay. A total of 50 μg protein were denatured and loaded on 10% sodium dodecyl sulfate polyacrylamide gels. PVDF membranes (EMD Millipore). After blocking with 5% milk in TBST, the membranes were blocked for 1 h in Blocking Buffer (5% 1X TBST) for 90 min before incubation overnight (at 4˚C) Buffer (5% 1X TBST) for 90 min before incubation overnight (4˚C). The proteins were transferred to PVDF membranes, and incubated with the corresponding primary and secondary antibodies for 90 min at 37˚C before incubation then overnight at 4˚C. The following primary antibodies were used: Anti-CTHRC1 (polyclonal antibody 1:1,000; Novus Biologicals, LLC, cat. no. AF5960).

Table I. Summary of clinical characteristics of PC (n=122).

Clinical characteristics	Value
Age (years)	
Mean	59.7
Range	40-72
Pre-operative prostate-specific antigen (ng/ml)	
<4	2
4-10	86
>10	33
Unknown	1
Gleason grade	
5	-
6	18
7 (3+4)	96
8	7
9	1
10	0
Pathology stage	
pT2	37
pT3a	75
pT3b	8
pT4	2
Positive surgical margins	43
Extra-capsular extension	85

Anti-actin (cat. no. SAB4200248; monoclonal antibody, 1:3,000; Sigma-Aldrich; Merck KGaA) antibody was used as protein loading control. A horseradish peroxidase-conjugated secondary antibody (polyclonal antibody 1:10,000, Santa Cruz Biotechnology, Inc.) was used for the assay. The protocols of western blot assay were employed as described previously (22).

Immunohistochemistry (IHC) analysis. IHC was performed as previously described (23). Sections as thin as 4 μm were sliced from paraffin-embedded tissue blocks. Antibody dilutions used were 1:500 for the CTHRC1 polyclonal antibody (cat. no. AF5960; Novus Biologicals, LLC), 1:1,000 for the PD-L1 polyclonal antibody (cat. no. PA5-18337; Thermo Fisher Scientific, Inc.), and 1:1,000 for the PD-1 polyclonal antibody (cat. no. PA5-32543; Thermo Fisher Scientific, Inc.) for 30 min at 37˚C. The samples were incubated with horseradish peroxidase-labeled polymer conjugated to goat anti-rabbit immunoglobulin G (IgG; cat. no. K5007; the DAKO EnVision™+ system; Agilent Technologies, Inc.) for 30 min at room temperature, and subsequently developed using 3,3'-diaminobenzidine as the color substrate for 1 min at room temperature.

Stained IHC sections were evaluated by two expert pathologists using uniform criteria from the Department of Pathology of Xiangya Hospital in Central South University; ~100 cells were randomly selected, and the positive cells were filtered from five average isolated fields of each section. Protein expression was evaluated according to the extent and intensity of staining. The percentage of positive cells was measured on a scale of 0-3: <5% scored 0; 6-25% scored 1; 25-50% scored 2;
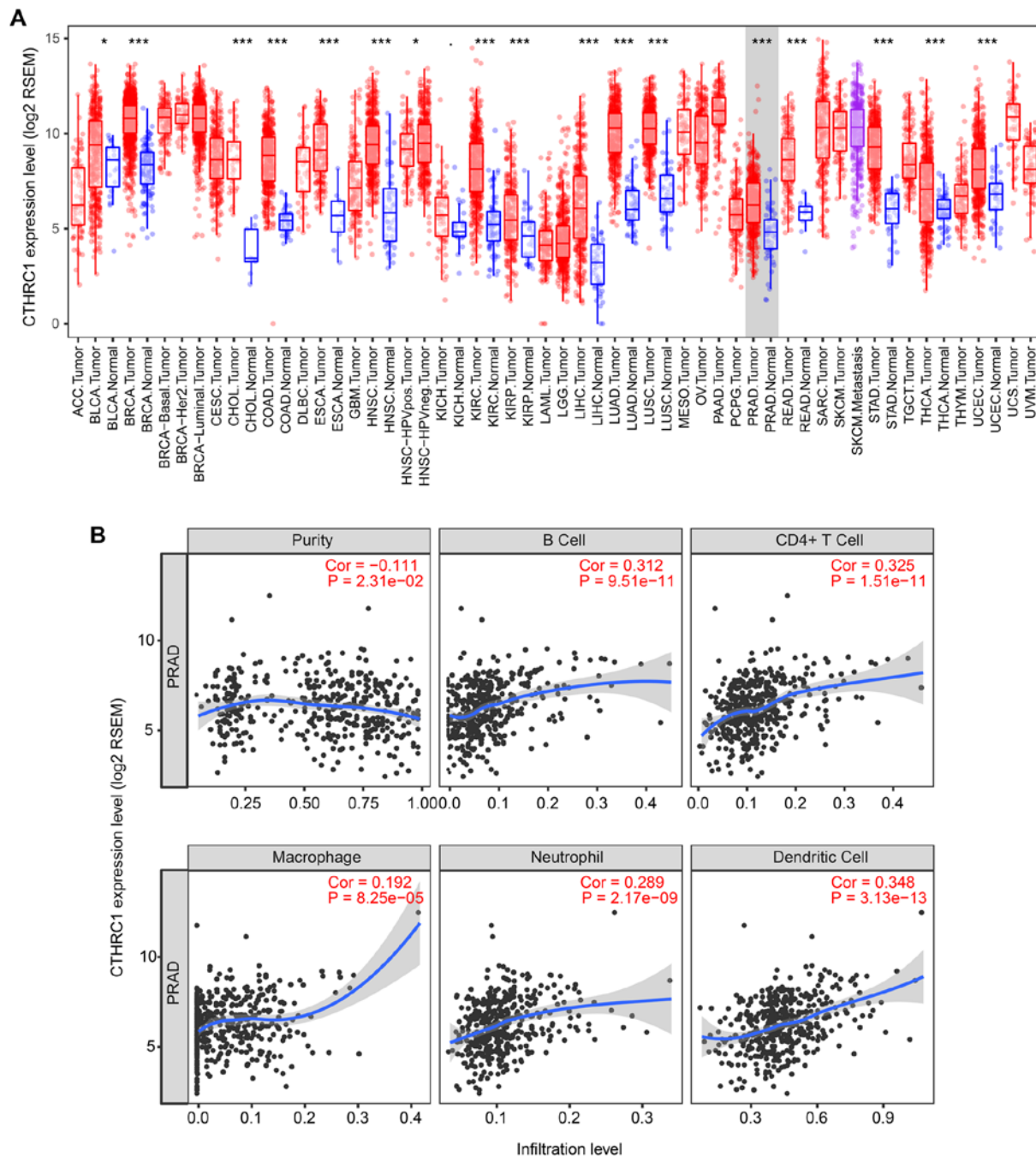
Figure 1. CTHRC1 mRNA levels are significantly reduced in tumors of PRAD and are associated with immunity. (A) Differential expression of CTHRC1 between tumor and adjacent normal tissues from various types of cancer. Data were extracted from the Tumor Immune Estimation Resource web server (*P<0.05, ***P<0.001 tumor vs. normal tissue). (B) Correlation between CTHRC1 expression and immunity in PRAD, reproduced from The Cancer Genome Atlas-PRAD cohort; the correlation between CTHRC1 expression and tumor purity, and between CTHRC1 expression and the count of B cells, CD4+ cells, macrophages, neutrophils and dendritic cells were presented. CTHRC1, collagen triple helix repeat containing 1; PRAD, prostate adenocarcinoma.

>50% scored 3. The intensity of staining was measured on a scale of 0-3: 0, no staining; 1, weak staining; 2, moderate staining; 3, strong staining. A median score was employed as the cut-off value to differentiate between low and high expression by using the score of positive staining multiplied by the grade of intensity staining.

Statistical analysis. The categorical vari-ables were compared using a χ^2 test. Continuous parametric variables were analyzed by Student's t-test or a Mann-Whitney test and data were presented as the mean ± standard error of the mean. Correlation

between two groups was determined using Spearman corre-lation coefficient analysis. In multivariate analysis, Cox's regression model was employed to test the contribution of individual factors to survival. P<0.05 was considered to indi-cate a statistically significant value. GraphPad Prism software (GraphPad Software, Inc.) was used for all statistical analyses.

Results

Patient characteristics. The validation cohort included 122 cases who underwent radical prostatectomy, aged 45-73 years
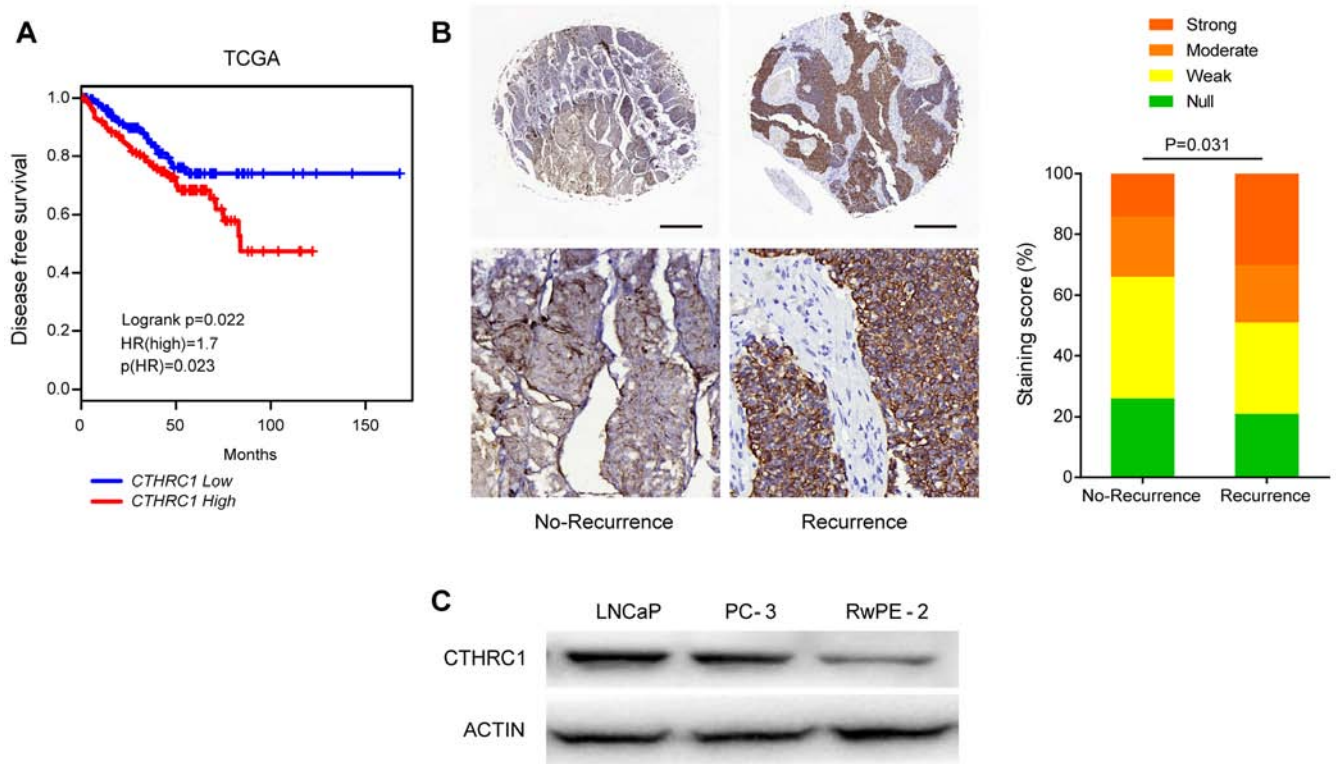
Figure 2. CTHRC1 protein expression levels are elevated in PRAD tumors, and this is associated with tumor recurrence and poor clinical outcome. Scale bar, 100 μm; original magnification, x400. (A) DFS rates were compared between the CTHRC1^{high} and CTHRC1^{low} groups. Analysis of DFS was accomplished using The Cancer Genome Atlas cohort. (B) Increases in CTHRC1 protein expression in PRAD generated from the validation cohort, followed by immunohistochemistry straining for CTHRC1. Typical images of CTHRC1 staining in tumors without recurrence (n=80) or with recurrence (n=42). (C) CTHRC1 protein expression levels were markedly increased in prostate cancer LNCaP and PC-3 cell lines compared with human prostate normal cells RwPE-2. CTHRC1, collagen triple helix repeat containing 1; HR, hazard ratio; PRAD, prostate adenocarcinoma; DFS, disease free survival.

(median age, 64 years). A total of 42 patients experienced cancer recurrence, and 86 patients were determined to have a high preoperative serum prostate-specific antigen levels (>5 ng/ml; 70.4%). In light of the combined Gleason score, patients were assigned either to group A (score ≤7; 79.7%) or B (score >7; the remaining 21.3%). The patients' tumor stage was distributed either in pT2 (99/122; 81.4%) or pT3 (23/122; 18.6%).

Upregulation of CTHRC1 expression in PRAD. As shown in Fig. 1A, the mRNA expression levels of *CTHRC1* were significantly decreased in PRAD compared with normal tissues by using the TIMER web server (P<0.001). *CTHRC1* has been shown to serve an immune modulatory role (18). It is noteworthy that the increase in tumor purity (i.e., the percentage of cancer cells in a solid tumor sample) was inversely correlated with the expression of *CTHRC1* (Fig. 1B). Specifically, this may have resulted from an increase in the numbers of infiltrating B cells, CD4^{+} cells, macrophages, neutrophils and dendritic cells.

Association between CTHRC1, recurrence and poor survival. Furthermore, survival analysis based on the data from the GEPIA web server also revealed that high levels of *CTHRC1* expression were associated with a lower DFS rate in PRAD (P=0.022; Fig. 2A). In the IHC cohort, *CTHRC1* protein expression was detected in the present study. These results indicated that *CTHRC1* expression was markedly higher in recurrent PRAD tumor tissues compared with recurrence-free

tissues (P=0.031; Fig. 2B). Western blotting verified the similar trend in elevated CTHRC1 protein expression in PRAD cell lines (Fig. 2C).

Association of CTHRC1 with PD-1/PD-L1 expression in PRAD. Similarly to *CTHRC1*, high levels of expression of PD-1/PD-L1 were associated with tumor progression, including PRAD (24); therefore, we proposed that our contradictory findings were due to the presence of an increased number of tumor-infiltrating leukocytes, with concomitantly increased levels of PD-1/PD-L1 expression in PRAD. The association between *CTHRC1* expression and immune infiltrates was analyzed, as indicated by the decreased tumor purity. *CTHRC1* mRNA expression was positively correlated with increased levels of PD-1 and PD-L1 expression in the TCGA cohort (Fig. 3). In our validation cohort, *CTHRC1*-positive staining was detected in 43.6% (53/122) of the tumor samples. PD-1 was assessed to have positive staining reactivity in 30.3% (36/122) of tumors, whereas PD-L1 was assessed to have positive staining reactivity in 38.5% (47/122) of the tumors. Of note, cases with higher levels of *CTHRC1* expression exhibited significantly increased levels of PD-1/PD-L1 in PRAD (Fig. 4A). IHC staining score analysis showed that PD-1/PD-L1 expression was notably higher in patients with recurrence of PRAD than those with no-recurrence (Fig. 4B). Furthermore, *CTHRC1* expression did not only correlate with PD-1 (R=0.272, P=0.021), but also with PD-L1 expression (R=0.298, P=0.016) (Fig. 4C).
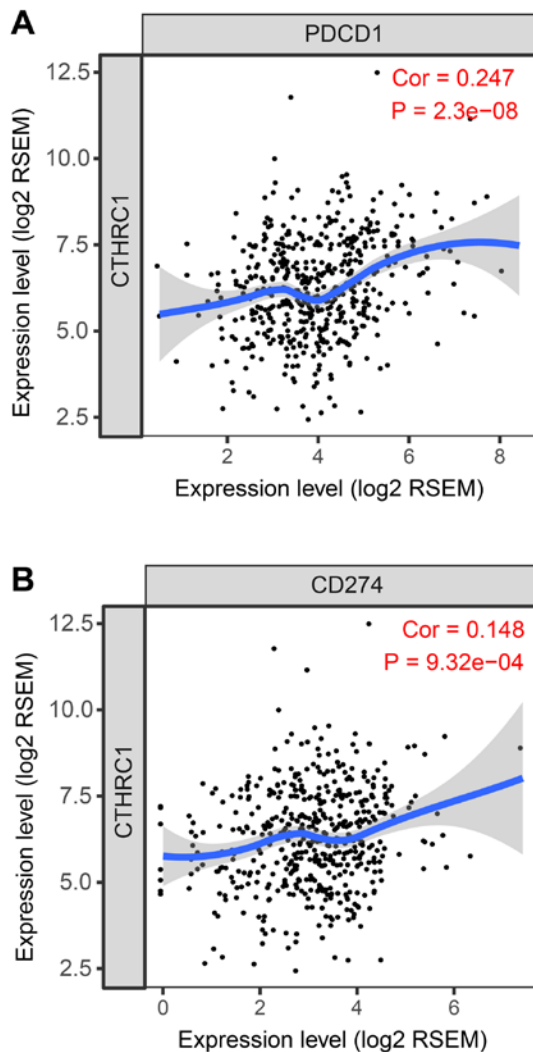
Figure 3. Correlation between the expression levels of (A) CTHRC1 and PD-L1 (CD274) and (B) CTHRC1 and PD-1 (PDCD1). RSEM, RNA sequencing by expectation-maximization; CTHRC1, collagen triple helix repeat containing 1; PD-1/CD274, programmed cell death protein 1; PD-L1/PDCD1, programmed cell death 1 ligand 1; RSEM, RNA-Seq by expectation-maximization.

CTHRC1 expression is associated with the signaling molecules of the tumor microenvironment. As presented in Fig. 5, in the TIMER web server, the mRNA expression of *CTHRC1* was positively correlated with certain genes in PRAD, including the expression of matrix metalloproteinase (MMP)9 (25), mucin 1 (26), solute carrier organic anion transporter family member 2B1 (27), phosphatase and tensin homolog (28,29), E-cadherin and CD44 (30). The expression of these genes has been suggested to be associated with functions that support tumor growth, invasion and the microenvironment of PRAD (31-33). However, further investigation in *CTHRC1* in PRAD is required to determine its critical role in tumor growth and invasion.

Discussion

Although our understanding of the prognostic role of cancer-associated *CTHRC1* gene expression in solid tumors has improved in light of several recently studies (34,35), *CTHRC1*

function in cancer immune modulation remains unclear. The present study investigated the aberrant expression of *CTHRC1* in PC and its potential role. Higher levels of expression of *CTHRC1* were shown to be associated not only with poor DFS, but also with poor immune response in a TCGA-PRAD dataset and validation cohort in the present study. A previous study has also confirmed the potential role of *CTHRC1* in cancer cells (36). However, little is currently known about the role of this gene in human immune cells. The most important finding of the current study was the identification of a positive correlation between *CTHRC1* and increased numbers of infiltrating B cells, CD4^{+} cells, macrophages, neutrophils, and dendritic cells. This is a potential novel feature of PRAD in the context of the immune response, driven by *CTHRC1*.

Immune checkpoint therapies have demonstrated broad antitumor activity in cancer treatment. Monoclonal antibodies targeting PD-L1/PD-1 have also been approved as antitumor immunotherapy in clinical trials (NCT00730639) for PC (37). Since blocking PD-1/PD-L1 should lead to an improvement in anticancer immunity and immunosuppression of the tumor microenvironment, predictive biomarkers to test the efficacy are urgently needed. Data from the aforementioned clinical trials of 122 patients with PC revealed that the expression of PD-L1 as determined by IHC and suppressed immunity were able to predict more effective therapies of anti-PD-1/PD-L1 in patients of numerous cancer types (38). Recently, Shalapour *et al* (39) revealed that eradication of the immunosuppressive IgA^{+} IL-10^{+} PD-L1^{+} plasmocytes reactivated the killing capacity of CD8^{+} cytotoxic T cells to tumor cells in aggressive mouse prostate cancer models. Another intriguing finding of the present study was that *CTHRC1* expression was increased in PRAD, and PD-1/PD-L1 expression were also upregulated. PD-L1, together with *CTHRC1*, an inflammation-associated factor, have been demonstrated to be an independent factor for predicting prognosis in tumors and autoimmune diseases (35). However, the R value of correlation analysis on the CTHRC1 and PD-1/PD-L1 was <0.3. As genes highly expressed in the microenvironment are expected to have negative associations with tumor purity, the opposite is expected for genes highly expressed in the tumor cells; the abundance of immune cells in tumor tissues is extremely low (40). As the TIMER database contains microarray expression values of glioblastoma multiforme/ovarian serous cystadenocarcinoma) for calculation; the results have statistical significance and correlation although R is <0.3. In addition, one study meets the criteria (40); patients with high expression of *CTHRC1* and PD-L1 had poor clinical outcome. The findings of the present study were consistent with those studies, and are, to the best of our knowledge, the first to propose a predictive role for *CTHRC1* and PD-L1 in the prognosis of PC.

The role served by *CTHRC1* in cancer, and the underlying mechanism, at present remain unknown. *CTHRC1* has often been shown to be aberrantly expressed in human solid tumors, promoting cancer cell invasion and metastasis (41). Recent studies have suggested that it functions in modulating the tumor microenvironment, particularly the extracellular matrix via the E6/E7-p53-POU2F1 axis or focal adhesion kinase signaling (7,8). In addition, numerous mRNAs have been revealed to target CTHRC1, and directly regulate cell proliferation and metastasis (42-44). CTHRC1 also promoted the infiltration of M2-like tumor-associated macrophages by
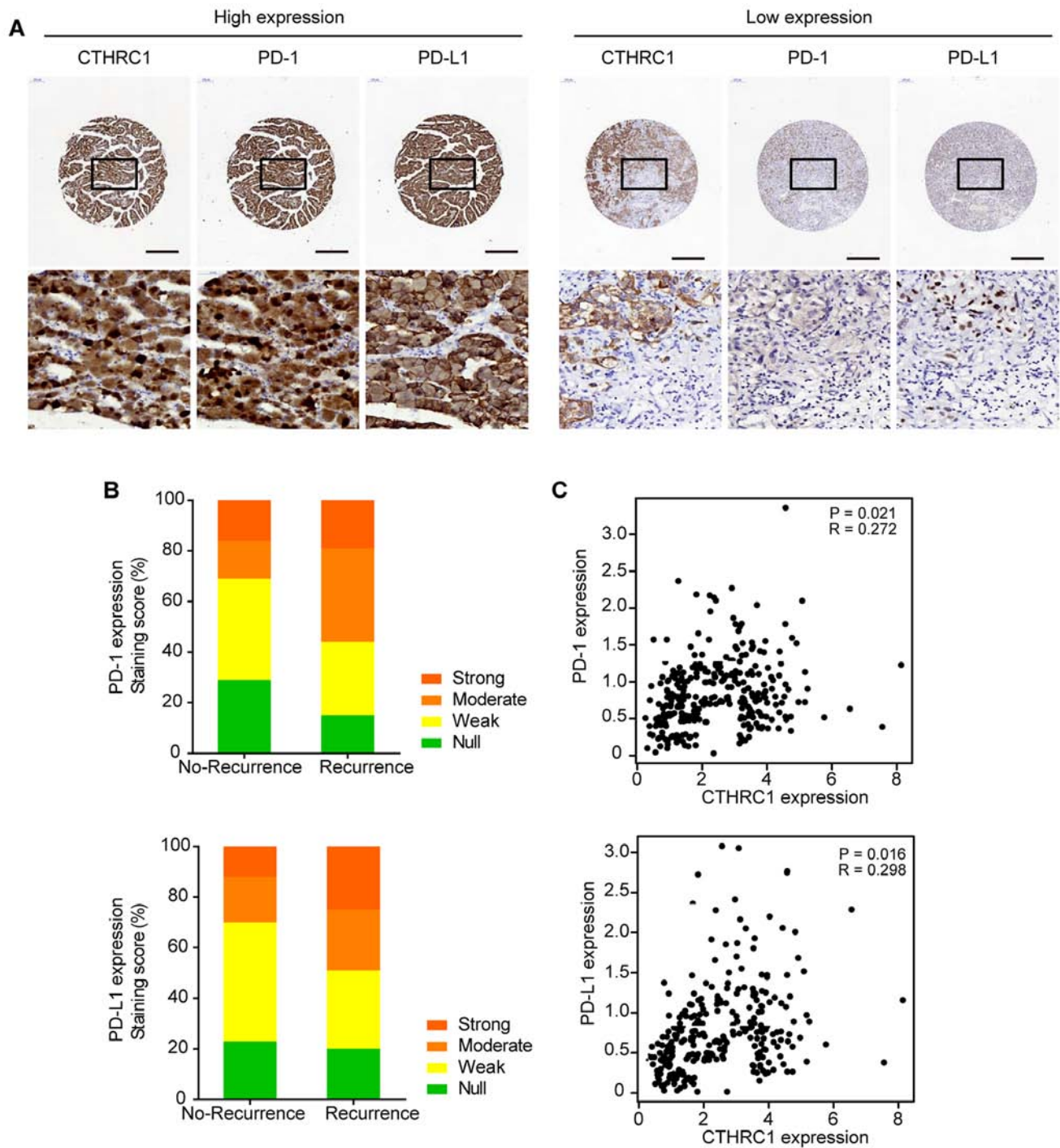
Figure 4. Correlation between CTHRC1 and tumor immune checkpoint PD-1/PD-L1 protein expression. (A) Representative IHC staining characteristics of CTHRC1, PD-1 or PD-L1 expression in a same set of PRAD tissue samples (magnification: Top panel x5; bottom panel x400, scale bar 100 μm). (B) IHC staining score analysis showed that PD-1/PD-L1 expression was notably higher in patients with recurrence of PRAD than those without. (C) Spearman's correlation analysis showed the positive association between CTHRC1 expression, and PD-1 and PD-L1. CTHRC1, collagen triple helix repeat containing 1; PD-1, programmed cell death protein; PD-L1, programmed cell death 1 ligand 1; PRAD, prostate adenocarcinoma; IHC, immunohistochemistry.

upregulating Fractalkine chemokine receptor expression (45). The precise mechanism underlying the *CTHRC1*-mediated tumor progression of PC, however, remains to be elucidated. Recent studies have indicated that B cells are likely to have a dual role in regulating cancer immunity (46,47). The tumor microenvironment may enable a population of tumor cells to escape the immune response by impairing the migration and homing of dendritic cells (48). Since tissue invasion and metastasis are both associated with cancer cell migration (49),

it is possible that *CTHRC1* contributes to these processes by increasing tumor cell migration. The present study suggests that an increased number of B cells may not be a contributory factor towards prognosis in patients overexpressing *CTHRC1*. Although it appears that B cells do exert a pro-tumorigenic role in PRAD, the results of the present study only fulfil an observational capacity in a cross-sectional perspective.

A previous study demonstrated that prostaglandin E2 also led to an increase in MMP9 expression, and activated Notch1
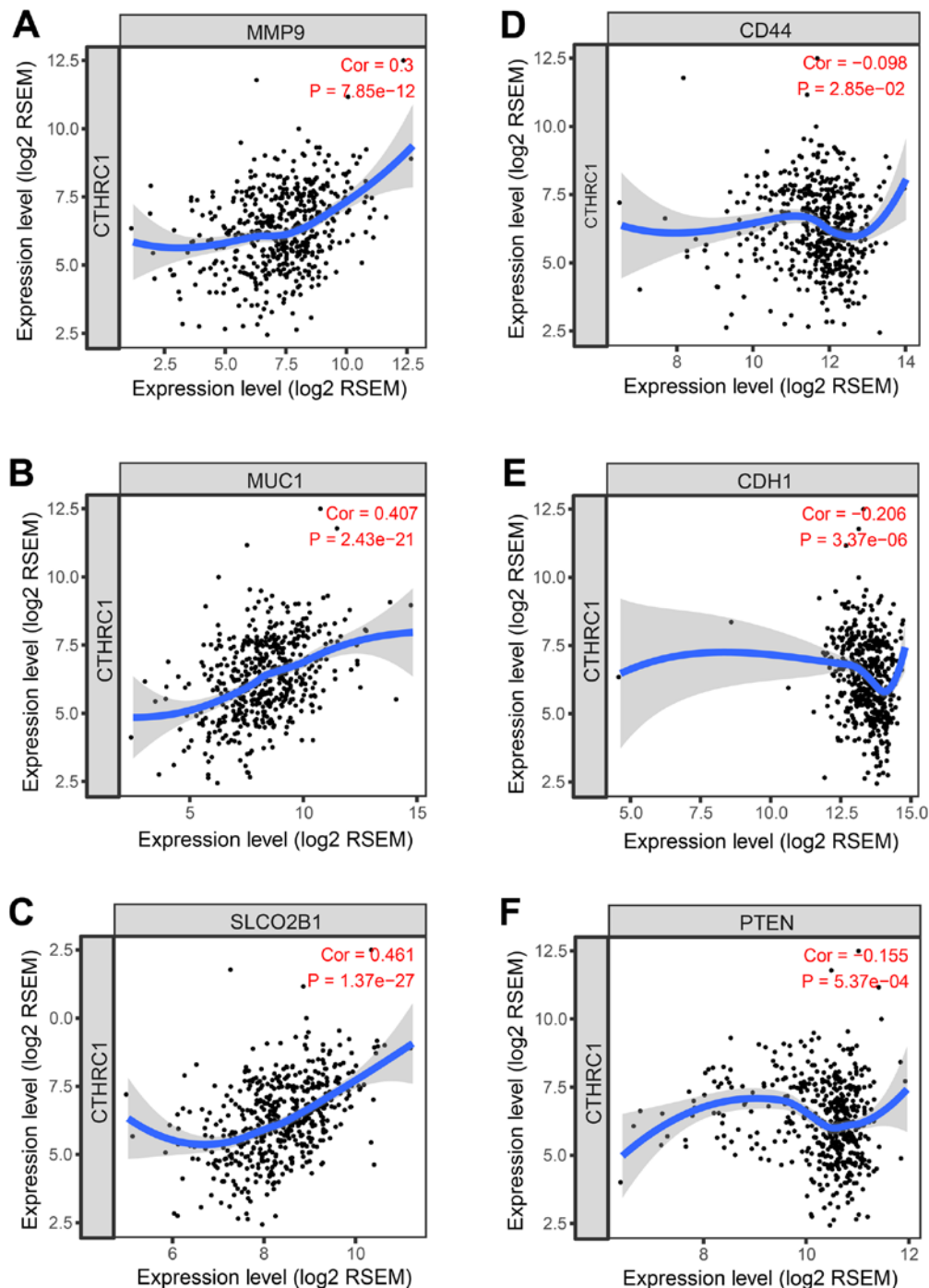
Figure 5. Spearman's correlation analysis of the tumor markers, (A) MMP9, (B) MUC1, (C) SLCO2B1, (D) CD44, (E) CDH1, and (F) PTEN with CTHRC1 expression in the PRAD profiles of The Cancer Genome Atlas dataset. MMP9, matrix metalloproteinase 9; MUC1, mucin 1; SLCO2B1, solute carrier organic anion transporter family member 2B1; CTHRC1, collagen triple helix repeat containing 1; CDH1, E-cadherin; PTEN, phosphatase and tensin homolog; RSEM, RNA-Seq by expectation-maximization.

signaling on dendritic cells (50). In the present study, *CTHRC1* was revealed to correlate with MMP9 expression. Therefore, our findings may suggest a novel perspective for therapeutic interventions in terms of PC immune escape. There are limitations of the present study. First, a novel prognostic marker for the pro-tumorigenic pathway with *CTHRC1* upregulation in PRAD has been proposed, although how *CTHRC1* is associated with the mechanism of immune escape requires further investigation. Secondly, the association between *CTHRC1* and immunity supports the application of appropriate PD-1/PD-L1

inhibitors in PRAD; however, further investigation is required to provide further insight into the role of *CTHRC1* in PC cell proliferation and growth *in vitro* and *in vivo* for PRAD.

In conclusion, the present study has identified a link between *CTHRC1* and PD-1/PD-L1 expression in PRAD. CTHRC1 may serve as a candidate target in treating PRAD. Our study has an additional limitation. The characteristics of CTHRC1 expression were found in PC samples from a Chinese population; thus, validation is required from prostate cancer patient cohorts from other regions.

Acknowledgements

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

The manuscript was written through contributions of all authors. XFT conceived and designed the study. QZ, WX, XZ, RSG, QFL, HYL and JNL performed the experiments. XFT and QZ reviewed and edited the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of The First Affiliated Hospital of Hunan University of Chinese Medicine; patients provided written informed consent.

Patient consent for publication

No applicable.

Competing interests

The authors declare that they have no competing interests.

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