CCN3 increases cell motility and ICAM-1 expression in prostate cancer cells

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Introduction

Prostate cancer is the most commonly diagnosed malignancy in the USA and other western countries (1). In early stages of prostate cancer, surgery is the most frequent therapeutic intervention. In advanced disease states, however, more systemic interventions are required to inhibit the growth and spread of secondary metastases. Bone metastasis is a common complication associated with advanced prostate cancer, often causing acute pain and bone fractures. Bone metastasis has prognostic value in prostate cancer because the extent of disease in the bone significantly affects survival (2).

Nephroblastoma overexpressed (NOV or CCN3) is a secreted matrix-associated protein that belongs to the CCN gene family and is involved in many cellular functions, including growth, differentiation and adhesion. The effect of CCN3 on human prostate cancer cells, however, is unknown. Here, we have shown that CCN3 increased cell migration and intercellular adhesion molecule-1 (ICAM-1) expression in prostate cancer cells. In addition, expression of CCN3 was positively correlated with both cell migration and ICAM-1 expression in human prostate cancer cells. CCN3 activated a signal transduction pathway that included αvβ3 integrin, integrin-linked kinase (ILK), Akt and nuclear factor-kappaB (NF-κB). Reagents that inhibit specific components of this pathway each diminished the ability of CCN3 to effect cell migration and ICAM-1 expression. Moreover, CCN3 increased binding of p65 to an NF-κB-binding element in the ICAM-1 promoter. Finally, knockdown of CCN3 expression markedly inhibited cell migration, tumor growth in bone and bone metastasis. Taken together, our results indicate that CCN3 enhances the migration of prostate cancer cells by increasing ICAM-1 expression through a signal transduction pathway that involves αvβ3 integrin, ILK, Akt and NF-κB. CCN3 thus represents a promising new target for treating prostate cancer.

Materials and methods

Materials

Protein A/G beads and rabbit polyclonal antibodies specific for NOV were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal antibody specific for αvβ3 integrin was purchased from Chemicon (Temecula, CA). Akt inhibitor, pyrrolidine dithiocarbamate (PDTC) and N’-tosylphenylalaninechloromethylketone (TPCK) were purchased from Calbiochem (San Diego, CA). The recombiant human CCN3 was purchased from R&D Systems (Minneapolis, MN). The Akt (Akt K179A) dominant-negative mutant was a gift from Dr W.M.Fu (National Taiwan University, Taipei, Taiwan), and the IKKα/IKKβ mutants were gifts from Dr H.Nakano (Juntendo University, Tokyo, Japan). pSV-β-galactosidase vector and luciferase assay kit were purchased from Promega (Madison, MA). All other chemicals were purchased from Sigma–Aldrich (St Louis, MO).

Cell culture

Human prostate cancer cell lines (PC-3, DU145 and LNCaP) were obtained from the American Type Culture Collection. Cells were maintained at 37°C in 5% CO2 in RPMI-1640 medium supplemented with 20 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid, 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. CCN3 short hairpin RNA (shRNAs) plasmids were purchased from National RNAi Core Facility Platform (Taipei, Taiwan). CCN3 shRNAs plasmids were transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and CCN3 shRNA-expressing cells were puromycin selected. Surviving cells were picked and expanded to make clonal cell populations. For monolayer growth curves, 104 cells were plated in six-well plates and grown for 1–6 days. Cells were trypsinized, and cell numbers were counted.

Migration assay

The migration assay was performed using Transwell inserts (Costar, NY; 8 mm pore size) in 24-well dishes. Before performing the migration assay, cells were...
pretreated for 30 min with different concentrations of inhibitors (KP-392, Akt, PDTC or TPCK) or vehicle control (0.1% dimethyl sulfoxide). Approximately 1 × 10^6 cells in 200 μl of serum-free medium were placed in the upper chamber, and 300 μl of the same medium containing different concentrations of CCN3 were placed in the lower chamber. The cells were incubated for 24 h at 37°C in 5% CO_2, then fixed in 3.7% formaldehyde for 5 min. The plates were blocked with 0.05% bovine serum albumin for 1 h at room temperature. The plates were incubated with primary antibodies overnight at 4°C. After a final rinse, cells were analyzed by a FACSCalibur flow cytometer and CellQuest software (BD Biosciences).

Flow cytometric analysis

Human prostate cancer cells were grown in six-well dishes and then washed with PBS and detached using trypsin at 37°C. Cells were fixed for 10 min in PBS containing 3.7% paraformaldehyde, rinsed in PBS and incubated with mouse anti-human-ICAM-1 (1:100) (BD Biosciences, CA) for 1 h at 4°C. Cells were washed in PBS and incubated with fluorescein isothiocyanate-conjugated goat anti-mouse secondary IgG (1:100; Leinco Technologies, St Louis, MO) for 45 min at 4°C. After a final rinse, cells were analyzed using a FACSCalibur flow cytometer and CellQuest software (BD Biosciences).

Western blot analysis

Cellular lysates were prepared and proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (24,25). Proteins were transferred to Immobilon polyvinylidene fluoride membranes. The blots were blocked with 4% bovine serum albumin for 1 h at room temperature and probed with rabbit anti-human antibodies against P-glycoprotein synthesis kinase (GSK3β), GSK3β, ILK, p-Akt, Akt, p-IKK, IKK, p-IκBα, IKK-β, p-65, p65, ICAM-1, CCN3 or β-actin (1:1000) for 1 h at room temperature (Santa Cruz Biotechnology). After three washes, the blots were incubated with peroxidase-conjugated donkey anti-rabbit secondary antibody (1:10000) for 1 h at room temperature. The blots were visualized with enhanced chemiluminescence using X-Omat LS film (Eastman Kodak, Rochester, NY).

ILK kinase activity assay

ILK enzymatic activity was assayed in PC-3 cells lysed in Nonidet P-40 buffer (0.5% sodium deoxycholate, 1% Nonidet P-40, 50 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid, pH 7.4 and 150 mM NaCl) as reported (26). Briefly, ILK was immunoprecipitated from 250 μg of lysate using an ILK antibody overnight at 4°C. After immunoprecipitation, beads were resuspended in 30 μl kinase buffer containing 1 μg recombinant substrate (a GSK3β fusion protein) and 200 μM adenosine triphosphate. The reaction was allowed to proceed for 30 min at 30°C. Phosphorylated substrate was visualized by western blot analysis using an antibody against phospho-GSK3β. Total GSK3β was also detected using the appropriate antibody. Anti-ILK was used as a loading control.

Quantitative real-time PCR

Total RNA was extracted from prostate cancer cells using a TRizol kit (MDBio, Taipei, Taiwan). Reverse transcription was performed using 1 μg total RNA and an oligo(deoxythymidine) primer (27,28). Quantitative real-time PCR (qPCR) was carried out using a TaqMan One-step PCR Master Mix (Applied Biosystems, CA). Total complementary DNA (100 ng) was added to each 25 μl reaction with sequence-specific primers and TaqMan probes. All target gene primers and probes were purchased commercially, including those for glyceraldehyde-3-phosphate dehydrogenase as an internal control (Applied Biosystems). qPCR was carried out in triplicate with a StepOnePlus (Applied Biosystems) sequence detection system. The cycling conditions were 10 min at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. To calculate the cycle number at which the transcript was detected (C_T), the threshold was set above the non-template control background and within the linear phase of target gene amplification.

Reporter gene assay

The prostate cancer cells were transfected with reporter plasmid using Lipofectamine 2000 according to the manufacturer’s recommendations. Twenty-four hours after transfection, the cells were treated with inhibitors for 30 min and then CCN3 or vehicle was added for 24 h. Cell extracts were then prepared, and luciferase and β-galactosidase activities were measured.

Chromatin immunoprecipitation

Chromatin immunoprecipitation was performed as described (29). DNA was immunoprecipitated using an anti-p65 antibody, then extracted, purified and resuspended in H_2O. Immunoprecipitated DNA was amplified by PCR using the following primers: 5′-AGACCTTAGCGCGGTGTAGA-3′ and 5′-GGCA CTCGAGGAGGAGCTGGA-3′ (30). PCR products were resolved by 1.5% agarose gel electrophoresis and visualized by UV light.

Electrophoretic mobility shift assay

Electrophoretic mobility shift assay was performed using an EMSA ‘gel shift’ kit (Panomics, Redwood City, CA) and an oligonucleotide corresponding to the NF-xB binding sequence [5′-AGCTTGGAATTCGGAG-3′; (31)]. Nuclear extracts of PC-3 cells (3 μg) were incubated with poly (dI-dC) at room temperature for 5 min. The nuclear extract was incubated with biotin-labeled probes at room temperature for 30 min. After electrophoresis on a 6% polyacrylamide gel, the samples were transferred onto a polyvinylidene fluoride membrane (Millipore, Billerica, MA). The membrane was baked at 80°C for 1 h, cross-linked in an oven for 3 min, incubated with blocking buffer followed by streptavidin-horseradish peroxidase conjugate and then subjected to western blot analysis.

Intratibial injection of PC-3 cells in nude mice

CCN3 shRNA-expressing PC-3 cells were cultured with fresh culture medium for 24 h before intratibial injection. Controls included untransfected PC-3 cells and cells transfected with vector control plasmid. Cells were harvested with trypsin-ethylenediaminetetraacetic acid (Invitrogen), resuspended in PBS and kept at 4°C before injection. Male CB17–severe combined immunodeficiency mice (4 weeks old) were deeply anesthetized using trichloroacetaldehyde monohydrate (0.4 mg/g body wt; KANTO Chemical Co., Tokyo, Japan). Intratibial injection of cells was performed using polyethylene tubing (Recorder No. 427401; Becton Dickinson) fit around a 30-G needle. This design was used to ensure the depth (1.5 mm) of the needle as it was inserted into the proximal tibia, thereby preventing the cells from spilling out of the injection site. The cell suspension (150 μl containing 2 × 10^6 cells) was slowly injected into the bone marrow cavity of the tibia. A tumor mass was visualized around the proximal tibia 28 days after injection. To make sure of bone osteolysis, radiographs were taken using a soft X-ray-generating unit. Animals were deeply anesthetized with trichloroacetaldehyde monohydrate, placed in a prone position on a Kodak Scientific Imaging film (13 × 18 cm) and exposed to X-rays (45 kV) for 5 s.

Statistical analysis

Data are presented as mean ± standard error of the mean. Statistical analysis between two samples was performed using the Student’s t-test. Statistical comparisons of more than two groups were performed using one-way analysis of variance with Bonferroni’s post hoc test. In all cases, P <0.05 was considered significant.

Results

CCN3 expression is positively correlated with prostate cancer cell motility, and this effect requires αvβ3 integrin receptor

CCN3 has been shown to increase cell migration and metastasis in a variety of human cancer cells (8,22). We first examined the levels of CCN3 in three human prostate cancer cell lines: PC-3, DU145 and LNCaP. It has been reported that CCN3 antibody will recognize two major forms of CCN3 (glycosylation and non-glycosylation forms) (32). The non-glycosylation form of CCN3 influenced the cell motility of human cancer cells (32). However, both glycosylation and non-glycosylation forms of CCN3 were significantly elevated in PC-3 cell line compared with DU145 and LNCaP cell lines (Figure 1A). The CCN3 messenger RNA (mRNA) in PC-3 cells was also higher than in DU145 and LNCaP (Figure 1B). In addition, PC-3 cells were more migratory than DU145 and LNCaP (Figure 1C). Moreover, treatment with CCN3 (10–100 ng/ml) dramatically increased migration in all the three cell lines (Figure 1D). Therefore, the expression of CCN3 is positively correlated with motility of prostate cancer cells.

Previous study has shown that CCN family affects cell migration by binding to integrin receptors on the cell surface (5). We hypothesized, therefore, that αvβ3 integrin signaling pathway may be involved in CCN3-induced prostate cancer cell migration. Pretreatment of cells with an anti-αvβ3 integrin monoclonal antibody (5 μg/ml) for 30 min markedly inhibited CCN3-induced cancer cell migration (Figure 1E). Similarly, CCN3-induced migration was essentially abolished by pretreatment with 100 nM cyclic RGD peptide [which is known to block αvβ3 function (33)] but not by pretreatment with cyclic RAD peptide (a negative control). To confirm the positive correlation between integrin αvβ3 and CCN3-induced cell migration, we measured integrin αv and β3 mRNA expression by qPCR following CCN3 treatment.
As expected, CCN3 upregulated both $\alpha_v$ and $\beta_3$ integrin expression in a dose-dependent manner (Figure 1F). Collectively, these data indicated that CCN3 expression was positively correlated with a higher metastatic potential (i.e. rate of migration) in prostate cancer cells and that this effect may have been mediated by integrin $\alpha_v\beta_3$ receptor.

ICAM-1 is involved in CCN3-induced cell migration

Recently, it has been reported that ICAM-1 plays a key role in cancer cell migration and invasion (17). We examined whether ICAM-1 was involved in CCN3-induced migration of prostate cancer cells. Western blot and qPCR analyses indicated that CCN3 expression was positively correlated with a higher metastatic potential (i.e. rate of migration) in prostate cancer cells and that this effect may have been mediated by integrin $\alpha_v\beta_3$ receptor.

CCN3 promotes cell migration and ICAM-1 expression via an ILK- and Akt-dependent pathway

ILK is a downstream regulator of integrin signaling (34). To determine whether ILK participates in CCN3-dependent effects in prostate cancer cells, we first treated PC-3 cells with CCN3 and measured ILK activity. As shown in Figure 3A, GSK3$\beta$ was used as substrate to measure ILK activity. Following CCN3 stimulation, ILK activity increased in a time-dependent manner. Treatment with an ILK-specific inhibitor (KP-392, 1 nM) or transfection with an ILK siRNA decreased CCN3-induced migration and ICAM-1 expression (Figure 3B and C). In addition, CCN3-dependent increases in ICAM-1 expression were reduced by KP-392 treatment or ILK siRNA transfection (Figure 3D and E). Therefore, ILK is involved in CCN3-mediated migration and ICAM-1 expression.
It is well established that Akt is regulated by ILK signaling (26). We asked, therefore, whether CCN3 stimulation could activate the Akt signaling pathway. CCN3 treatment increased the level of phosphorylated Akt (Figure 4A). Transfection of cells with CCN3 shRNA reduced CCN3-mediated Akt phosphorylation (Figure 4B). CCN3-induced migration and ICAM-1 expression of prostate cancer cells was greatly reduced by addition of Akt inhibitor (Akti, 1 μM) (Figure 4C and D) or expression of a dominant-negative mutant of Akt (Figure 4E and F). Therefore, CCN3 induced cell migration and ICAM-1 expression through Akt signaling pathway.

NF-κB is involved in CCN3-induced cell migration and ICAM-1 expression

NF-κB is a primarily cytosolic transcription factor whose activity is often correlated with cancer cell migration and invasion (23). Therefore, we used western blot analysis to investigate whether components of the NF-κB signaling pathway are involved in the CCN3-dependent effects on prostate cancer cells. Levels of phosphorylated IκKα/β, IκBα and NF-κB subunit p65 were higher after 15 min of CCN3 treatment and diminished after ~120 min (Figure 5A). Moreover, cell migration in response to CCN3 was decreased by pretreatment with the NF-κB inhibitor PDTC (10 μM) or the IκB protease inhibitor TPCK (1 μM; Figure 5B). Furthermore, expression of dominant-negative mutant of IκKα or IκKβ markedly inhibited CCN3-induced cell migration (Figure 5B). Similarly, each of these treatments reduced ICAM-1 expression (Figure 5C). It is clear, therefore, that CCN3-mediated effects on cell migration and ICAM-1 expression are mediated by the NF-κB signaling pathway.

The promoter region of human ICAM-1 contains an NF-κB-binding site (30); therefore, we asked whether CCN3 stimulation caused NF-κB to bind to the ICAM-1 promoter in prostate cancer cells. To this purpose, the electrophoretic mobility shift assay was used. As shown in Figure 5D, the p65 subunit of NF-κB bound to the NF-κB element was increased by CCN3 treatment. We confirmed that NF-κB activity was increased by κB-luciferase activity assay (Figure 5E). In addition, CCN3-mediated binding of p65 to the NF-κB element was repressed by KP-392, Akti, PDTC and TPCK (Figure 5F). Based on these results, activation of the ILK and Akt signaling pathways was required for the
CCN3-induced increase in NF-κB activity in human prostate cancer cells.

Knockdown of CCN3 expression inhibits cell migration and osteolytic metastasis in a mouse model of prostate cancer

To confirm the role of CCN3 in prostate tumor metastasis, we took advantage of PC-3 cells that stably express CCN3 shRNAs. Following puromycin (1 μg/ml) selection, individual stable clones (CCN3 sh1, CCN3 sh2 and CCN3 sh3) were collected for analysis. Empty vector plasmid was used as a negative control. Levels of both CCN3 and ICAM-1 were decreased in all three CCN3 shRNA stable clones, and CCN3 sh3 showed most decreased (Figure 6A). CCN3 knockdown did not alter the rate of cell proliferation (Figure 6B) but significantly reduced migration (Figure 6C).

The PC-3 cell line is an androgen-independent human prostate cancer line that frequently metastasizes to bone (35). To examine the effect of CCN3 on prostate tumor growth in vivo, PC-3 cells (2 × 105) were locally injected into the tibial bone marrow cavity of 4-week-old severe combined immunodeficiency mice. In each mouse, one tibia was injected with negative PC-3 cells and the contralateral tibia was injected with CCN3 sh3 stable clone. Mice were sacrificed after 28 days later. To analyze bone osteolysis in these mice, radiographs were taken using a soft X-ray-generating unit. Radiographs, which were taken 28 days post-injection revealed osteolytic lesions in tibia injected with negative PC-3 cells. In contrast, osteolytic lesions were dramatically reduced in mice injected with CCN3 sh3 stable clone (Figure 6D). Tumor weight was recorded after the animals were sacrificed. Quantitative assessment of tumor weight and osteolytic lesions confirmed that knockdown of CCN3 inhibited both tumor growth and formation of osteolytic lesions (Figure 6E and F).

Discussion

In recent years, correlations between CCN3 and tumorigenesis have been identified in a variety of cancers, but the mechanism of CCN3 activity in these contexts remains unclear (8). In both osteosarcoma and Ewing sarcoma, high levels of CCN3 expression indicate a poor prognosis for patients (9,36). In contrast, reduced levels of CCN3 expression are thought to promote cancer progression in childhood adrenocortical tumors and melanomas (10). A correlation between prostate cancer and CCN3 has been reported previously (11), but
the mechanism by which CCN3 affects prostate cancer remains unclear. In this study, we have demonstrated for the first time that CCN3 (an extracellular matrix-associated protein) increased migration and metastasis of prostate cancer cells by transcriptionally upregulating ICAM-1 expression. CCN3 increased ICAM-1 expression by activating the \( \alpha_v \beta_3 \) integrin, ILK, Akt and NF-\( \kappa \)B signaling pathways.

Prostate cancer is prevalent in developed countries worldwide. Most prostate tumors remain confined to the prostate gland and adjacent soft tissue and cause little to no harm. However, nearly one in eight cases leads to metastasis, typically to bone (37). The role of androgenic hormones in prostate cancer progression and survival has been reported previously and is supported by the ability of androgen ablation therapy to cause regression of both primary and metastatic disease (38). Here, we found a correlation between CCN3 expression and the rate of prostate cancer cell migration. The highest level of CCN3 expression and the greatest ability to migrate were seen in the most malignant prostate cancer cell line (PC-3; Figure 1A–C).

Although the PC-3 expressed highly CCN3 level than DU145 and LNCaP, however, we did not find any significant differences on migration activity after response to CCN3. Therefore, the maximum increase in migration activity after CCN3 stimulation of prostate cancer cells is 2- to 3-fold.

Integrins, which link the extracellular matrix to intracellular signaling molecules, regulate a number of cellular processes, including adhesion, signaling, motility, survival, gene expression, growth and differentiation. Integrins are known to serve as receptors for CCN3 (5). CCN3 has been shown to bind \( \alpha_v \beta_3 \) integrin and increase cell migration (8). In this report, \( \alpha_v \beta_3 \) integrin mRNA expression was elevated following CCN3 treatment. Moreover, CCN3-induced cell migration and ICAM-1 expression was inhibited by pretreatment with a neutralizing antibody against integrin \( \alpha_v \beta_3 \) or with an RGD peptide. This indicates that integrin \( \alpha_v \beta_3 \) receptor played an important role in CCN3-induced migration and ICAM-1 expression in prostate cancer cells.

ILK is activated by integrins, growth factors and chemokines (34). In bone, ILK promotes chondrocyte proliferation, adhesion and spreading (39), but its role in prostate cancer differentiation is still unclear. In this study, we presented the first evidence to indicate that ILK plays a critical role in prostate cancer progression. Treatment of prostate cancer cells with CCN3 increased the activity of ILK, and CCN3-induced ICAM-1 expression and cell migration were both inhibited by the ILK inhibitor KP-392 and by ILK-specific siRNA. These data suggest that ILK activation is an obligatory step in CCN3-induced prostate cancer cell migration. ILK may regulate these cellular functions by promoting phosphorylation of Akt on Ser473.
thereby activating this important downstream pathway (26). Akt has been shown to regulate cancer progression (40). We found that CCN3 treatment increased the level of Akt phosphorylation in a time-dependent manner and that inhibition of Akt reduced the effects of CCN3 on cell migration and ICAM-1 expression (Figure 4). Taken together, our results provide evidence that CCN3 upregulated ICAM-1 expression and promoted cell migration via the integrin/ILK/Akt signaling pathway in prostate cancer cells.

Previous reports have indicated that NF-κB is involved in tumor metastasis (23,41). Here, we demonstrated that the NF-κB inhibitors PDTC and TPCK reduced CCN3-induced cell migration, indicating that NF-κB acts downstream of CCN3. In its inactivated state, NF-κB is held in the cytoplasm by the inhibitory protein IκB. Upon stimulation (for example, by tumor necrosis factor α), IκB proteins become phosphorylated by the multisubunit IKK complex. This targets IκB for ubiquitination and subsequent degradation by the 26S proteasome. Free NF-κB can then translocate to the nucleus, where it regulates the transcription of target genes. The NF-κB pathway has also been linked to CCN3 signaling (42). Treatment of PC-3 cells with CCN3 led to increased levels of phosphorylated IKK, IκBα and p65 (Figure 5A). Using transient transfection of a κB-luciferase reporter construct that indicates NF-κB activity, we found that CCN3
increased NF-κB activity. Moreover, chromatin immunoprecipitation and electrophoretic mobility shift assays clearly demonstrated that p65 binds to the NF-κB element in the ICAM-1 promoter in response to CCN3 stimulation and that p65 binding to the NF-κB element was attenuated by KP-392, Akti, PDTC and TPCK (Figure 5D–F). Taken together, these results indicate that CCN3 acted through the αvβ3 integrin, ILK, Akt and NF-κB pathways to induce ICAM-1 expression in human prostate cancer cells.

Finally, to directly determine the effect of CCN3 on prostate cancer progression, we knocked down CCN3 expression in PC-3 cells using shRNA (Figure 6). CCN3 knockdown significantly reduced ICAM-1 expression and inhibited migration in PC-3 cells. We then injected negative PC-3 cells and PC-3 cells-expressing CCN3 shRNA into the contralateral tibia of severe combined immunodeficiency mice to investigate the in vivo effects of CCN3 on tumor growth and bone resorption (i.e. osteolysis). Tumor growth in the bone was clearly decreased in PC-3 cells that lacked CCN3, as was bone metastasis. These data indicated that CCN3 play an important role in prostate cancer metastasis to bone in vivo.

The rate-limiting step in metastasis and a critical stage in cancer progression is the acquisition of motility by a tumor cell. Bone metastasis is the major cause of mortality for patients with prostate cancer. Here, we have revealed critical new insights into CCN3 function and its role in prostate cancer progression. CCN3 expression is upregulated in prostate cancer cells, promotes cell migration in vitro and promotes tumor growth in vivo. Although the mechanisms involved in CCN3-induced bone metastasis are not yet complete, we have provided evidence that CCN3 works via the integrin αvβ3, ILK, Akt and NF-κB signaling pathways. Our data demonstrate the importance of CCN3 in the growth and metastasis of prostate cancer and identify CCN3 as a novel therapeutic target for the clinical treatment of prostate cancer.

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References


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