Curcumin-targeting Pericellular Serine Protease Matriptase Role in Suppression of Prostate Cancer Cell Invasion, Tumor Growth and Metastasis

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Abstract

Curcumin has been shown to possess potent chemopreventive and antitumor effects on prostate cancer. However, the molecular mechanism involved in curcumin’s ability to suppress prostate cancer cell invasion, tumor growth and metastasis is not yet well understood. In this study, we showed that curcumin can suppress EGF- and heregulin-stimulated PC-3 cell invasion, as well as androgen-induced LNCaP cell invasion. Curcumin treatment significantly resulted in reduced MMP-9 activity and down-regulation of cellular matriptase, a membrane-anchored serine protease with oncogenic roles in tumor formation and invasion. Our data further show that curcumin is able to inhibit the induction effects of androgens and EGF on matriptase activation, as well as to reduce the activated levels of matriptase after its overexpression, thus suggesting that curcumin may interrupt diverse signal pathways to block the protease. Furthermore, the reduction of activated matriptase in cells by curcumin was also partly due to curcumin’s effect on promoting the shedding of matriptase into an extracellular environment, but not via altering matriptase gene expression. Additionally, curcumin significantly suppressed the invasive ability of PCa cells induced by matriptase overexpression. In xenograft

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model, curcumin not only inhibits PCa tumor growth and metastasis but also
down-regulates matriptase activity \textit{in vivo}. Overall, the data indicate that curcumin
exhibits a suppressive effect on prostate cancer cell invasion, tumor growth and
metastasis, at least in part \textit{via} down-regulating matriptase function.
Introduction

Prostate cancer (PCa) is currently the second leading cause of cancer-related death in men in the western world (1). Localized PCa is highly curable by surgery or chemotherapy to remove or destroy the cancerous lesions. Yet, there currently exist no efficacious therapies for advanced PCa with hormone refractory or metastatic phenotypes (2). Therefore, it is a critical issue to discover a chemical or compound valuable for developing new therapies that inhibit the progression or invasive abilities of PCa.

It has been shown that the expression levels of matriptase, a membrane-anchored serine protease, are increased in PCa and correlated with tumor grades and poor prognosis (3). Several agents including androgens, EGF, sphingosine 1-phosphate and suramin exhibit stimulatory effects on the matriptase activation in mammalian epithelial, prostate and breast cancer cells (4-6). In matriptase transgenic mice, matriptase overexpression promoted tumorigenicity and carcinogen-induced tumor formation (7). Matriptase is also involved in ErbB-2-induced PCa cell invasion (6). This suggests that dysregulation of matriptase exhibits oncogenic effects and can also promote the progression of human cancer including PCa. Inhibition of matriptase may
be an opportunity to reduce prostate tumor growth and progression (8). Thus, matriptase may present a new potential target for PCa therapies.

Curcumin has emerged as a compound with multiple biological properties for health maintenance and cancer prevention. The anti-inflammatory and anti-oxidant activities of curcumin have been proposed via inhibiting NF-κB, COX-2, iNOS and cytokine production (9, 10). In rodents, curcumin can prevent carcinogenesis induced by various carcinogens (11). A more recent phase II clinical study has pointed out that allotting low concentrations of curcumin in patients has a similar biological impact on NF-κB, COX-2 and phospho-STAT-3 in peripheral blood mononuclear cells, as those observed in the in vitro studies with 5-50 μM of curcumin treatments (12, 13). In hepatocellular cancer cells, curcumin can inhibit cancer cell invasion and MMP-9 secretion (14). In PCa cells, curcumin can down-regulate androgen receptors (10, 15), decrease cell proliferation (16), and inhibit DU145 cell invasion via decreasing MMP-2/-9 activity (17). However, the more detailed molecular mechanisms how curcumin inhibits PCa cell invasion, tumor growth and metastasis have not been yet clearly elucidated. In this study, we show that curcumin can significantly suppress PCa cell invasion, tumor growth and metastasis. Curcumin not only inhibits MMP-9
activity but also decreases the cellular levels of matriptase. Furthermore, the invasive
ability of PCa cells induced by matriptase overexpression was also significantly
suppressed by curcumin. In summary, the data indicate that curcumin exhibits a
suppressive effect on PCa cell invasion, tumor growth and metastasis, at least in part
by down-regulating matriptase function. Thus, the data suggest that curcumin may
exhibit a therapeutic potential for invasive prostate cancer.
Materials and Methods

Materials - DMEM, FBS, and RPMI1640 media were obtained from Hyclone (Logan, UT). Protein Assay kits were from Bio-Rad (Hercules, CA). Anti-V5 Ab and Lipofectamine™ 2000 reagent were purchased from Invitrogen (Carlsbad, CA). Curcumin (Cat.# C7727, purity 94% curcuminoid, ≥80% curcumin) and all other reagents were purchased from Sigma-Aldrich, MO, unless otherwise noted. M24, M69 and M19 antibodies were gifts from Dr. Chen-Yong Lin at the Georgetown University, DC.

Cell culture - PC-3, CWR22Rv1 and WPMY-1 cells were obtained from ATCC. PNT-2 cells were purchased from Sigma-Aldrich. PC-3 and WPMY-1 cells were maintained in DMEM containing 10% FBS and 2 mM glutamine in a 5% CO₂, 37°C incubator. DU145, C-33 and C-81 LNCaP cells were gifts from Dr. Ming-Fong Lin at the University of Nebraska Medical Center, NE. CWR22Rv1, C-33 and C-81 LNCaP, DU145 and PNT-2 cells were maintained in 5% FBS, 2 mM glutamine RPMI1640 medium. No authentication for all cell lines used was done by the authors.

Cell growth and cytotoxicity assays - Cells were seeded at a density of 1.5x10⁴ or 3x10⁵ cells/cm² for cell growth and cytotoxicity assays, respectively. For growth assay,
one day after seeding, cell amounts were daily analyzed by MTT assays according to the manufacturer’s protocol and other cells were accordingly refreshed with media. For cell cytotoxicity assay, one day after seeding, cells were treated with indicated concentrations of curcumin for 16 hours and the viable cell numbers were counted by a hemacytometer with a trypan blue exclusion method.

Cell invasion and migration assays- Transwell assays were performed according to the previously described procedures (18). In brief, transwells were coated with or without 20 μg of matrigel (BD biosciences, NJ) for cell invasion or migration assay. Serum-starved cells were then seeded at a density of 3x10^5 cells/cm² in the upper chambers of transwells with serum-free medium. The lower chambers were filled with the medium containing 10% FBS or growth factors as chemoattractants. After 16-hour incubation, cells were fixed and stained with 1% GIEMSA dye. The penetrating cells were photographed (100X) and counted using a light microscope. All experiments were performed in triplicate.

RNA extraction and quantitative real-time PCR- Both assays were performed according to the previously described procedures (19). Briefly, 16-hour, curcumin-treated cells were used for total RNA extraction using Trizol reagent...
(Invitrogen) and RNA was reversely transcribed using SuperScript One-step RT-PCR System (Invitrogen) according to the manufacturer’s instructions. Quantitative real-time PCR (Q-PCR) was performed using the StepOne Real-time PCR system (Applied Biosystems). The primers for the experiments were listed as follows:

Matriptase: forward, 5’-CACCTCAGTGGTGCTTTCC-3’ and reverse, 5’-GCGTGCAGGCCAAAGCT-3’; GAPDH: forward, 5’-AAAGGATCCACTGGCTCTTCACCACC-3’ and reverse, 5’-GAATTGTCATGGATGACCTTGGCCAG-3’.

HAI-1 primers were obtained from ABI (Cat.#Hs00173678). All Q-PCR reactions were performed three times.

Gelatin zymography- For gelatin zymography, cells were treated with different concentrations of curcumin for 16 hours. The supernatants of the conditioned media were collected and concentrated using Amicon Ultra-4 centrifuge filter devices (Millipore) at 3,000 r.p.m. at 4°C for 30 minutes. Without heating or reducing, samples underwent 0.1% (w/v) gelatin SDS-PAGE. After electrophoresis, the gel was incubated in a renaturation buffer (50 mM Tris-HCl, pH7.5, 10 mM NaCl, 2.5% v/v Triton X-100) at 37°C for 1.5 hours, and following with a developing buffer (50 mM Tris-HCl, pH7.5, 5 mM CaCl2) at 37°C for 18 hours with gentle agitation. Gels were
then stained and clear bands shown on blue background. The bands were detected by a luminescent image analyzer (LAS-4000; Fujifilm, Japan).

**Western blot analysis**- For western blotting by M24, M69 and M19 mAbs, equal amounts of cell lysates were mixed with protein loading dye in a non-reducing and non-boiling condition (20). For the other samples, equal amounts of cell lysates were mixed with regular protein loading dye and boiled for 10 minutes. Samples were separated by SDS-PAGE and transferred to nitrocellular membranes (Whatman, Piscataway, NJ). The membranes were blocked with 5% skim milk in TBST at RT for 1 hour and then incubated with primary antibodies at 4°C overnight, followed by secondary antibody incubation. The protein images were visualized using an Enhanced Luminol Reagent Plus (Perkin Elmer, PA) and detected by a luminescent image analyzer (LAS-4000; Fujifilm, Japan).

**Tumor xenografts and bioluminescence analysis**- All procedures for animal experimental protocols were approved by Institutional Animal Care and Use Committee (IACUC) of Academia Sinica and NTU. For xenograft study, six-week-old male nude mice were inoculated subcutaneously into the dorsal flank with $2 \times 10^6$ luciferase-expressed PC3 (PC3-Luc) cells. After 2 weeks, mice were
randomly assigned into two groups (6 mice/group): one group receiving 100 mg/kg of curcumin and the other receiving vehicle (corn oil) by daily i.p. injection. The tumor volume and body weight of each mouse were monitored weekly. After 3-week treatment, mice were sacrificed and individual tumors were taken, weighted and snap frozen in liquid nitrogen for western blot analysis. For *ex vivo* bioluminescence images of lymph nodes, the images were acquired with Xenogen IVIS50 Imaging System and measurements of bioluminescent signals were performed with Living Image 2.50 software. 150 mg/kg D-luciferin was injected into the mice 10 minutes prior to imaging. Mice were humanely sacrificed and both brachial lymph nodes were taken and imaged for 10 seconds.

**Statistical analysis**- A mean±S.E. was calculated from three repeated groups in all experiments. A statistical significance between groups was determined by Student’s *t*-test. A *p*-value below 0.05 was considered as a significant difference between the two groups.
Results

Curcumin Inhibition of Prostate Cancer Cell Migration and Invasion

To examine the effects of curcumin on the growth of PC-3 cells, and ensure that the cell growth kept in the log phase after 5-day culture, cells were seeded at a density of 1.5x10^4 cells/cm^2 and treated with 5, 25 and 50 μM of curcumin. With MTT assays, Figure 1A showed that curcumin ably inhibited PC-3 cell growth in a time- and dose-dependent manner. We then tested the cytotoxicity of curcumin on PC-3 cells in a cell density of 3x10^5 cells/cm^2, because this cell density would be used in following transwell assays. Using trypan blue exclusion assays, the results displayed that there was no significant cytotoxicity on PC-3 cells after the curcumin treatment (Fig. 1B). That 25 or 50 μM of curcumin dramatically decreased PC3 cell growth without any significant effect on cell cytotoxicity may be partly due to the difference of cell-seeding density and/or the inhibitory effect of curcumin on dehydrogenases (21). To further investigate the effect of curcumin on PCa cell migration and invasion, we performed transwell assays and found that the migration and invasion of PC-3 cells were significantly decreased by approximately 50% after 5 μM-curcumin treatment and suppressed up to 80% upon 25 and 50 μM curcumin treatments (Fig. 1C and 1D).
Taken together, these data indicate that curcumin can significantly suppress PC-3 cell migration and invasion.

Since recent studies have shown that oncogenic signaling of EGFR/ErbB-2 participates in metastatic PCa (22), the effect of curcumin on ErbB ligands (EGF or heregulin)-induced PCa cell invasion was further analyzed. As shown in Fig. 1E, EGF or heregulin was able to increase PC-3 cell invasion and this induced cancer cell invasion was ably antagonized by curcumin. Since androgen signaling has been another important factor for PCa progression, to further explore the effect of curcumin on androgen-induced PCa cell invasion, we treated androgen-sensitive LNCaP cells with DHT in the presence or absence of curcumin. The result (Fig. 1F) showed that DHT significantly enhanced the invasion of LNCaP cells and curcumin suppressed DHT-induced PCa cell invasion. Thus, the data indicate that curcumin can block ErbB ligands (EGF and heregulin)- and DHT-induced PCa cell invasion.

Identification of Matriptase as a Curcumin-targeting Serine Protease in PCa Cells

It has been shown that both matrix metalloproteinases (MMPs) and serine proteases play important roles in cancer cell invasion (23, 24). To further explore the
involvement of MMPs or serine proteases in curcumin-inhibited PCa cell invasion, we analyzed the effects of a broad MMP inhibitor GM6001, a serine protease inhibitor AEBSF, or curcumin on PC-3 cell invasion. As shown in Fig. 2A, curcumin, GM6001 and AEBSF suppressed the invasion of PC-3 cells approximately by 76%, 38% and 70%, respectively. With the combination treatment of GM6001 and curcumin, curcumin further reduced the invasion of PC-3 cells approximately by 35% in comparison with GM6001 alone. Moreover, curcumin only had a marginal effect to further reduce the invasion of AEBSF-treated cells, and the degree of curcumin to inhibit PCa cell invasion was quite similar to the combined effect of GM6001 and AEBSF on this event. The data suggest that curcumin-inhibited PCa cell invasion is mainly via suppressing both MMPs and serine proteases. Indeed, the activity of secreted MMP-9 in PCa cells was significantly suppressed by curcumin, with a less effect on MMP-2 (Fig. 2B).

Since the expression of matriptase has been shown to be correlated with the PCa progression (3) and also that matriptase is involved in ErbB-2-induced PCa cell invasion (6), we then proposed that matriptase was a curcumin-targeting serine protease in PCa cells, by examining the effect of curcumin on matriptase using...
western blotting with anti-total matriptase (M24), anti-activated matriptase (M69) and anti-HAI-1 (M19) mAbs. As shown in Fig. 2C, curcumin proficiently decreased the total levels of matriptase including latent matriptase (70 kDa) and activated matriptase (a 120 kDa complex of activated matriptase and its cognate inhibitor HAI-1) in a dose-dependent manner. The decrease in matriptase/HAI-1 complexes by curcumin was further validated by the western blotting with M69 Ab. Additionally, the protein levels of HAI-1 or in a complex with activated matriptase were also reduced by curcumin. To further assess the effect of curcumin on the transcription levels of matriptase and HAI-1, we performed Q-PCR and showed that curcumin had no significant effect on the expression of both genes (Fig. 2D). Thus, the data indicate that the reduction of matriptase and HAI-1 protein levels by curcumin is not via transcription regulation, but may utilize a post-translational mechanism for decreasing cellular matriptase.

Since epithelial-mesenchymal transitions (EMT) have been shown to be implicated in the PCa progression (25), we then investigated if curcumin functioned as an antagonist for EMT to reduce PCa cell invasion, by using the immunoblotting analyses of several EMT markers including E-cadherin, β-catenin, vimentin and snail.
As shown in Fig. 2E, 25 μM curcumin had no significant effect on those biomarkers in PC-3 cells.

Next, we examined the time-kinetic effect of curcumin on matriptase in PC-3 cells. The data showed that the levels of activated matriptase dramatically decreased at 30 minutes after curcumin treatment, and up to 16 hours, whereas the level of latent matriptase began to increase after 8-hour treatment, lasting to 24 hours (Fig. 2F). Moreover, to determine whether this decrease in matriptase by curcumin was a ubiquitous phenomenon in PCa and prostate cells, we analyzed the effect of curcumin on matriptase in various human PCa cells, prostatic stromal myofibroblast WPMY-1 cells and immortalized prostatic epithelial PNT2 cells. The result (Fig. 2G) showed that curcumin also suppress the activated levels of matriptase in androgen-sensitive C-33 LNCaP cells and androgen-independent PCa cells including C-81 LNCaP, DU145 and CWR22Rv1 cells. Moreover, matriptase protein in WPMY-1 cells and the activated level of matriptase in PNT2 cells were negligibly detectable. Apparently, curcumin can reduce the level of latent matriptase in PNT2 cells (Fig. 2G). Thus, the data indicate that curcumin exhibits an inhibitory role in matriptase in PCa cells, with a less effect on prostatic epithelial PNT2 cells.
In addition, we examined the effects of two curcumin analogues, dimethoxycurcumin (DMC) and EF24, on matriptase in PC3 cells and found that both analogues also can reduce the activated levels of matriptase (Fig. 2H). Taken together, these data suggest that curcumin-decreased PCa cell invasion is at least partly due to decreasing both MMP-9 activity and cellular matriptase, rather than altering the EMT process.

Curcumin-reduced Matriptase Function in PC-3 Cells

Next we examined the pretreatment effect of curcumin on the FBS-induced matriptase activation in PCa cells. The data showed that the pretreatment of curcumin dramatically depressed the stimulatory effects of FBS on matriptase activation (Fig 3A). To further evaluate the duration of curcumin on reducing cellular matriptase in PCa cells, the effect of 1-hour acute curcumin treatment on matriptase was examined. The data showed that curcumin could decrease the activated matriptase up to 8 hours after the acute treatment (Fig. 3B). Four hours after the acute treatment, the decreased levels of latent matriptase began to rebound. We then analyzed the effect of the acute curcumin treatment on PCa cell invasion and found that the acute treatments of 25 and 50 μM curcumin suppressed PC3 cell invasion by ~50 % and ~64 % (Fig. 3C).
This result indicates that an acute exposure of curcumin remains effective in reducing both cellular matriptase and PCa cell invasion.

**Inhibitory Effects of Curcumin on DHT- and EGF-induced Matriptase Activation**

Since androgens can proficiently induce matriptase activation in PCa cells (26), we then examined the effect of curcumin on androgen-induced matriptase activation in LNCaP cells. As shown in Fig. 3D, curcumin had an inhibitory effect on DHT-induced matriptase activation in a dose-responsive manner. Moreover, we examined if curcumin also exhibited an antagonized effect on EGF-induced matriptase activation in PCa cells. As shown in Fig. 3E, EGF could dramatically induce matriptase activation in LNCaP cells and curcumin ably reduced EGF-induced matriptase activation. Likewise, we further analyzed the acute effect of curcumin on EGF-induced matriptase activation. After the treatment with EGF for two hours and followed by curcumin treatment for 30 minutes, the results showed that curcumin could quickly decrease the stimulatory effect of EGF on matriptase in LNCaP cells (Fig. 3F). These results display that curcumin can potently inhibit the stimulatory effects of androgens and EGF on matriptase in PCa cells.
Curcumin Promotion of Matriptase Shedding and Suppression of Matriptase-induced PCa Cell Invasion

The shedding of matriptase-HAI-1 complexes to extracellular environments is thought of as a mechanism to remove matriptase from cells (26). Since Figure 2 shows that curcumin can reduce the cellular content of matriptase but has no effect on the gene expression, we accordingly examined whether curcumin could promote the shedding of matriptase, leading to decreasing cellular matriptase in PCa cells. The effect of curcumin on matriptase shedding was examined by using western blotting to analyze the shed matriptase in the conditioned media. The data revealed that curcumin promoted the shedding of the matriptase-HAI-1 complex in a dose-dependent manner, with less effect on the latent form of matriptase (Fig. 4A). We then further analyzed whether the shed matriptase in the conditioned media retains its function for cancer cell invasion. Following the curcumin treatment, the conditioned media were collected, washed out of curcumin and used to treat PC-3 cells. Through cell invasion assays, the data showed that the shed matriptase had no significant effect on PCa cell invasion (Fig. 4B).

Moreover, to recapitulate the increased levels of matriptase in advanced PCa,
PC3 cells were transiently transfected with V5-tagged matriptase plasmids. We then analyzed whether curcumin could also affect the activated levels of matriptase caused by overexpression. As shown in Fig. 4C, the overexpression of matriptase increased the activated levels of matriptase, and curcumin effectively reduced the cellular levels of matriptase, at least in part by promoting the shedding of the matriptase-HAI-1 complex into the conditioned media. Thus, the data indicate that curcumin-induced matriptase shedding may play an important role in reducing the cellular level of activated matriptase.

Since matriptase overexpression is observed in a variety of human carcinomas including PCa (3, 27), we further examined the role of matriptase in PCa cell invasion and the effect of curcumin on matriptase-induced cancer malignancy by establishing stable pools of matriptase-overexpressing CWR22Rv1 cells. The data displayed that the total and activated levels of matriptase increased in matriptase-overexpressing CWR22Rv1 cells (Fig. 4D), which were concurrent with increased invasion capabilities (Fig. 4E). We then examined the effect of curcumin and AEBSF on the invasion of matriptase-overexpressing CWR22Rv1 cells. As shown in Fig. 4F, similar to AEBSF, curcumin significantly inhibited the matriptase-induced PCa cell invasion,
up to 80%, down to the levels of curcumin-treated control cells. Thus, the data denote that curcumin exhibits a strong inhibitory potential for PCa cell invasion, especially that caused by matriptase overexpression or dysregulation.

**Curcumin-inhibited Tumor Growth and Metastasis in a PCa Xenograft Model**

To further assess if curcumin can inhibit prostate tumor growth and metastasis, we examined the effects of curcumin on the tumor growth of PC3-Luc cells using a xenograft model. As shown in Fig. 5A, there was a significant regression of tumor volume in the group of mice receiving the curcumin treatment. No animal body weight was significantly altered during the treatment, indicating that the curcumin treatment had no toxicity in mice (Fig. 5B). After scarification, the tumor mass was measured and significantly reduced in the group of curcumin-treated mice (Fig. 5C). Moreover, we checked if there was any metastatic nodule in the lymph nodes nearby the xenografted tumors, and found that curcumin treatment significantly reduced the metastatic lesions at brachial lymph nodes in the xenografted mice, indicating that curcumin exhibited an inhibition effect on PCa metastasis (Fig. 5D). We then further analyzed the total and activated levels of matriptase in the xenografted tumors with or without curcumin treatment. As shown in Fig. 5E, the level of activated matriptase
was remarkably deceased in the xenografted tumors treated with curcumin, compared with vehicle control. Taken together, the data indicate that curcumin can inhibit PCa tumor growth and metastasis, at least in part due to curcumin’s effect on down-regulating the activated matriptase in tumors.
Discussion

Recent findings have recommended curcumin as a beneficial chemotherapeutic and chemopreventive agent for PCa (28). However, its functions in PCa cell invasion and metastasis are still lacking. In this study, we found that curcumin exhibits an inhibitory effect on PCa cell invasion, tumor growth and metastasis, not only in terms of inhibiting MMP9 activity, but also on the down-regulation of cellular matriptase, achieved through inhibiting the stimulatory effects of androgens or EGFR ligands on the protease and promoting the protease shedding.

For cancer cell invasion or metastasis, the epithelial–mesenchymal transition (EMT) and the degradation of ECM have been proposed as two important processes. ECM degradation has been mainly attributed to dysregulation of pericellular proteolysis. In curcumin-inhibited PCa cell invasion, our results suggest that some proteases from the families of MMPs and serine proteases are critically inhibited by curcumin while the EMT is apparently not affected by this treatment. Moreover, our data indicate that in PCa cells, MMP-9 activity is significantly inhibited by curcumin with less effect on MMP-2. Unexpectedly, we further observed that the combination treatment of GM6001 and AEBSF could reach to the similar inhibitory effect of
curcumin on PCa cell invasion, implying that some serine protease(s) was curcumin-targeting and involved in promoting PCa cell invasion. In PCa, ErbB-2 is overexpressed in approximately 80% of metastasized PCa lesions (29) and matriptase is involved in ErbB-2-induced PCa cell invasion (6). These previous findings inspired us to explore whether matriptase can promote PCa cell invasion and if curcumin can inhibit matriptase-induced PCa cell invasion. Interestingly, our data revealed that matriptase overexpression can induce PCa cell invasion, suggesting that the dysregulation of matriptase is a factor in allowing cancer cells to obtain high invasion capabilities during the PCa progression. Comparatively, our data further suggest that curcumin can inhibit matriptase-induced PCa cell invasion through reducing the cellular matriptase. In addition, curcumin also can inhibit prostate tumor growth and metastasis, at least partly via down-regulating the level of activated matriptase in PCa xenografted tumors (Fig. 5). Thus, curcumin exhibits a chemopreventive and therapeutic potential in matriptase-involved PCa malignancy.

As shown in Fig. 6, matriptase function is mainly regulated by three steps: activation, inhibition, and ectodomain shedding. Matriptase is synthesized as a single-chain zymogen, through two sequential endoproteolytic cleavages for
activation: The first cleavage at G149 for matriptase maturation (30) and the second cleavage at Arg614 for activation (4). Matriptase overexpression, HAI-1 knockdown, S1P, EGF, Heregulin or DHT can induce the protease activation (4-6, 26, 31). After activation, two mechanisms are proposed for inhibiting matriptase activity: 1) its inhibitor HAI-1-mediated inhibition by forming 120-kDa complexes and 2) the ectodomain shedding of the activated matriptase-HAI-1 complex from cells into the extracellular environments with a molecular mass of 95 or 110 kDa (30, 32). Based on this model, we propose two possible mechanisms in which curcumin can down-regulate cellular matriptase. First, perhaps curcumin promotes the shedding process to reduce the cellular levels of matriptase, leading to decreased PCa cell invasion. This suggests that curcumin’s ability to decrease the cellular content of matriptase provides a way to reduce the protease’s oncogenic effect on PCa. However, how curcumin enhances the matriptase shedding is still elusive and remains under further investigation. Second, curcumin can interrupt the stimulatory effects of EGF, heregulin and androgens in the process of matriptase activation. This may be explained by curcumin’s multiple inhibitory effects on several signal molecules including androgen receptor, HER-2, EGFR, PI3K/Akt, etc. (9, 10, 33). Thus,
Curcumin may block the androgen- and ErbB ligand-induced signal pathways for matriptase activation, leading to reducing PCa cell invasion. In conclusion, the data suggest that curcumin may be a potent phytocomponent able to efficiently inhibit matriptase-mediated PCa malignancy.

The inhibitory effects on the activated matriptase in PCa cells after an acute curcumin treatment only last up to 16 hours. This may be due to curcumin consumption by the cells or the instability of curcumin in the culture medium, since curcumin has been shown to be unstable at neutral and basic pH and decomposed approximately by 50% in the culture medium after 8-hour incubation (34). Therefore, two curcumin analogs (DMC and EF24) with an enhanced metabolic stability and a higher potency than curcumin (35, 36) were used in this study and show both analogs also can reduce the cellular levels of activated matriptase in PCa cells. Thus, this study points out that enhancing the stability and potency of curcumin will improve the efficiency of curcumin in the chemoprevention or cancer therapy, especially in cancers with matriptase dysfunction.

Curcumin has been shown to down-regulate urokinase-type plasminogen activator (uPA) in PCa cells (37). Pro-uPA has been shown to be one of matriptase
substrates (38, 39). Consequently, curcumin’s ability to inhibit the activities of uPA may be partly attributed to its inhibitory effects on matriptase.

In conclusion, our data reveal that matriptase can be activated by multiple factors, including its overexpression, growth factors or androgens, and ultimately lead to PCa cell invasion. Curcumin can significantly decrease the cellular levels of matriptase in PCa cells, by promoting protease shedding. Therefore, this study provides a novel mechanism for establishing how curcumin can inhibit PCa cell invasion, tumor growth and metastasis by the down regulation of matriptase function.
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Figure legends

**Fig.1. Effect of curcumin on prostate cancer cell migration and invasion.**

(A) Analysis of curcumin effect on PC-3 cell growth using MTT methods. (B) Examination of curcumin effect on the cytotoxicity of PC3 cells with a trypan blue exclusion method. Curcumin effects on PC3 cell migration (C) and invasion (D) were analyzed using cell migration or invasion assays. Examination of curcumin effects on EGF- and HRG-induced PC3 cell invasion (E), and on androgen-induced LNCaP cell invasion (F). Each assay was performed in triplicate and statistically calculated (mean±S.E., **: P<0.001).

**Fig.2. Identification of matriptase as a curcumin-targeting serine protease in PCa cells.**

(A) Effects of curcumin, GM6001, and AEBSF on PC-3 cell invasion were analyzed and statistically calculated (mean±S.E., **: P<0.001). (B) Curcumin’s effect on MMP activity using gelatin zymography was analyzed in triplicate and statistically calculated (mean±S.E., **: P<0.001). (C) Analysis of curcumin’s effects on matriptase and HAI-1 in PC3 cells using immunoblotting assays with M24, M69 and M19 mAbs. (D) Effects of curcumin on the gene expression of matriptase and HAI-1 in PC-3 cells were analyzed by Q-PCR. (E) Analysis of curcumin effects on the
expression of E-cadherin, β-catenin, vimentin and snail in PC3 cells by immunoblot assays. (F) Time-kinetic effect of curcumin on matriptase in PC-3 cells. Cells were treated with 25 μM curcumin for indicated times for western blot analysis with M24 and M69 Abs. (G) Effect of curcumin on matriptase in different PCa and epithelial cells. LNCaP (LN, C-33 and C-81), DU145, CWR22Rv1 (22Rv1) and prostate epithelial PNT2 cells were treated with or without curcumin for 16 hours and used for the immunoblot analysis. (H) Effects of 10 μM dimethoxycurcumin (DMC) and 5 μM EF24 on matriptase in PC-3 cells were analyzed by immunoblot assay. β-actin was used as control.

**Fig.3. Curcumin reduced matriptase activation in PCa cells.** (A) Analysis of curcumin pretreatment on FBS-induced matriptase activation in PC-3 cells. Cells were pre-treated with the indicated concentrations of curcumin for 24 hours and recultured with the medium containing 10% FBS for indicated times. (B) Analysis of acute curcumin treatment on matriptase in PC-3 cells. Cells received 1-hour curcumin treatment and regrew in a regular culture medium for indicated times. Cell lysates were used for immunoblot analysis. (C) Examination of acute curcumin treatment on PC3 cell invasion. One-hour, curcumin-treated PC-3 cells were used for cell invasion
assay with statistical calculation. **, P<0.001. (D) Analysis of curcumin effect on DHT-induced matriptase activation in C-33 LNCaP cells by immunoblot analysis. (E) Effects of curcumin on EGF-induced matriptase activation in C-33 LNCaP cells. Cells were treated with or without curcumin in the presence or absence of DHT (D) or EGF (E) for 16 hours, and used for immunoblot analysis. (F) Analysis of acute curcumin treatment on EGF-induced matriptase activation in C-33 LNCaP cells. Cells were pre-treated with EGF for 2 hours and then recived an acute treatment of curcumin for 30 minutes. Cell lysates were used for immunoblot analysis.

Fig. 4. Curcumin promoted matriptase shedding and reduced matriptase-induced PCa cell invasion.

(A) Analysis of curcumin effect on matriptase shedding in PC-3 cells. The conditioned media of 16-hour, curcumin-treated PC-3 cells were collected and concentrated. Cell lysates and the conditioned media were analyzed by immunoblot analysis. (B) Examination of shed matriptase on PCa cell invasion. The conditioned media of 16-hour, 50 μM curcumin-treated PC3 cells were collected, washed out of curcumin and used for cell invasion assay. (C) Effects of curcumin on the cellular and shed matriptase in matriptase-overexpressing PC3 cells. PC-3 cells were transiently
transfected with V5-tagged matriptase plasmids (MTX), and their cell lysates and conditioned media were collected for immunoblot analyses. (D) Immunoblotting analysis of matriptase in V5-tagged MTX-overexpressed CWR22Rv1 cells. Stable pools of MTX-V5 plasmids (MTX)-transfected CWR22Rv1 cells were selected by 400 μg/ml G418. Cell lysates were prepared for immunoblot analyses. (E) Examination of matriptase role in PCa cell invasion. CWR22Rv1 (C), vector (Vec)- and matriptase (MTX)-transfected CWR22Rv1 cells were used for cell invasion assays with statistical calculation of mean±S.E.. (F) Effects of curcumin and AEBSF on matriptase-induced PCa cell invasion with statistical calculation of mean±S.E. **, P<0.001.

Fig.5. Curcumin suppression of tumor growth and metastasis in a PC3 xenograft model.

(A) Effect of curcumin on the tumor volumes of PC3-Luc cell xenograft. The tumor volumes in vehicle-treated (control; n=6) and curcumin-treated (100mg/kg/day; n=6) mice were plotted and calculated as mean±S.E. ** P<0.01. (B) The mouse body weight after curcumin treatment was weekly monitored. (C) After 3-week treatment, tumor weights were measured, plotted and statistically calculated as mean±S.E.. (D)
Curcumin effect on tumor metastasis to brachial lymph nodes was examined by bioluminescent images as photon flux per second. (E) Whole cell homogenates from xenografted tumor tissues with vehicle and curcumin treatment were immunoblotted. β-actin was used as loading control.

**Fig.6. Model of curcumin’s effects on matriptase in PCa cells.**

Matriptase is synthesized as a single-chain polypeptide and its activation requires two sequential endoproteolytic cleavages: the first cleavage at G149 for matriptase maturation (30) and the second cleavage at R614 for activation (4). Active matriptase is quickly inhibited by HAI-1 with formation of 120 kDa complexes. Then, the matriptase-HAI-1 complex is shed with a molecular mass of 95 or 110 kDa into extracellular environments. The mechanism for curcumin to affect matriptase activity is proposed as follows: First, curcumin induces matriptase shedding, leading to decreasing cellular matriptase and PCa cell invasion. Second, curcumin may inhibit matriptase activation by targeting multiple signaling pathways, such as androgens, EGF and heregulin signaling, even by its overexpression.
References

11. Lopez-Lazaro M. Anticancer and carcinogenic properties of curcumin: considerations for its clinical development as a cancer chemopreventive and...


Figure 1

A

Cell growth (Ratio to Day 0)

1.5x10^4 cells/cm^2

Curcumin (µM)

0 5 25 50

Day(s)

B

Cell Viability (Ratio to control)

3x10^5 cells/cm^2

Curcumin (µM)

0 5 25 50

C

Cell Migration (Ratio to control)

Curcumin (µM)

0 5 25 50

D

Cell Invasion (Ratio to control)

Curcumin (µM)

0 5 25 50

E

Cell Invasion (Ratio to control)

Cur (µM) 0 25 0 25 0 25

EGF (ng/ml) 0 0 50 50 0 0

HRG (ng/ml) 0 0 0 40 40

F

Cell Invasion (Ratio to control)

Cur (µM) 0 25 0 25

DHT (nM) 0 0 10 10

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Curcumin-targeting Pericellular Serine Protease Matriptase Role in Suppression of Prostate Cancer Cell Invasion, Tumor Growth and Metastasis

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