Matrine suppresses invasion of castration-resistant prostate cancer cells by downregulating MMP-2/9 via NF-κB signaling pathway

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Abstract. Matrine is an alkaloid from *Sophora flavescens* that exhibits multiple protective effects on cancers. However, the molecular mechanisms of anti-metastatic effects of matrine on castration-resistant prostate cancer (CRPC) remain unknown. This study investigated the anti-metastatic effects of matrine on CRPC to identify the underlying mechanisms. The effects of matrine on the cell viability of DU145 and PC-3 cells were measured using MTS assay. The impact of matrine on expression levels of matrix metalloproteinase (MMP)-9, MMP-2, nuclear factor-κB (NF-κB) subunit p65 and phosphorylated p65 in cells untreated or treated with matrine were analyzed by western blotting. The inhibitory effects of matrine on cell migration and invasion were examined by Transwell assay. The impact of matrine on tumorigenesis in male Balb/c nude mice inoculated subcutaneously with cells were investigated *in vivo*. We found that matrine inhibited the growth of DU145 and PC3 cells time- and dose-dependently both *in vitro* and *in vivo*. Migration and invasion capabilities of cells were also suppressed by matrine. At the same time, matrine markedly reduced the expression levels of MMP-9, MMP-2 and p-p65 in both cell lines. Further experiments revealed that matrine exhibited inhibitory effects of migration and invasion of CRPC by downregulating MMP-2/9 through NF-κB pathway. Matrine inhibits invasion of CRPC by reducing levels of MMP-9 and MMP-2 through NF-κB pathway. Therefore, it may be a potential anti-metastatic therapeutic agent for CRPC.

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Introduction

Prostate cancer (PCa) is one of the most common malignancies for men (1). Most PCa depends on androgens for growth and survival, and androgen ablation therapy represents the most effective initial treatment (2). Unfortunately, most PCa will progress to castration-resistant prostate cancer (CRPC) with higher potential of invasion and metastasis in 2-3 years (3). Though efforts have been made for treating CRPC for years, an efficient therapeutic treatment is still not available (4). Therefore, identification of mechanisms involved in CRPC carcinogenesis and metastasis may be the key to novel treatments.

Tumor metastasis is a crucial hallmark of cancer progression. It involves numerous factors including defects in programmed cell death, degradation of the extracellular matrix (ECM) and tumor angiogenesis (5). ECM provides structural support to cells and tissues by heterogeneous macromolecules

and is a rich source of angiogenesis promoters (6). Matrix metalloproteases 9 and 2 (MMP-9 and MMP-2) catalyze the degradation of essentially the majority of ECM and components of the basement membrane and proteolytically cleave and activate precursors of angiogenesis promoters, which play an important role for tumor invasion and metastasis (7-10). The degradation activities of MMP-9 and MMP-2 are regulated by NF-κB activity (11,12).

Matrine ($C_{15}H_{24}N_2O$), an alkaloid derived from *Sophora flavescens*, a traditional Chinese herb medicine, has been identified to exhibit bioactive and pharmacological effects such as anti-inflammatory (13) and antiviral activities (14). It also exhibits an anticancer effect on malignancies such as lung cancer, breast cancer and castration-sensitive prostate cancer (15-17). In our previous study, we have identified the inhibitory abilities of matrine for growth of CRPC cells through NF-κB signaling pathway *in vitro* (18). However, the anti-metastatic effects of matrine on CRPC *in vivo* and the underlying mechanisms regulating NF-κB by matrine are still unknown.

In this study, we proved that matrine reduced the growth and invasion abilities of CRPC cell lines both *in vitro* and *in vivo* by reducing the expression levels of MMP-2 and MMP-9 via NF-κB signaling pathway. Our results imply that matrine may be developed as a novel anti-metastasis therapeutic drug for CRPC in future.

Materials and methods

Reagents. Matrine [chemical formula: $C_{15}H_{24}N_2O$; molecular weight: 248.36] was purchased from Dalian Mellon (Dalian, China). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, phosphate-buffered saline, and RPMI-1640 media were purchased from Hyclone (Logan, UT, USA).

Cell lines and culture. Human castration-resistant prostate cancer cell lines DU145 and PC-3 were obtained from the cell bank of the Center for Experiment Animals of Sun Yat-sen University (Guangzhou, China). Cells were maintained in RPMI-1640 supplemented with 10% (vol/vol) FBS, 1% penicillin and streptomycin at 37˚C in an incubator containing 5% CO_2. Different concentrations of matrine were added into culture medium to treat cells for different times.

Cell viability assay. DU145 and PC-3 cells were seeded in 96-well plates (100 μl/well) at a density of $1x10^4$ cells/well for 24 h. Then the cells were treated with various concentrations of matrine (0, 0.025, 0.05, 0.1, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 3.0, 4.0 and 6.0 g/l). After incubation for 0, 24, 48 and 72 h, the viability of the cells was evaluated using the MTS kit (cellTiter96AQ, Promega Corp., Madison, WI, USA) according to the manufacturer's instructions, and the absorption was read at 490 nm.

Migration and invasion assay. The migration and invasion assays were performed using Transwell® Inserts (BD Biosciences, Bedford, MA, USA). Approximately $1x10^5$ cells in 100 μl serum-free RMPI-1640 medium were placed in the upper chamber, and 600 μl RMPI-1640 medium containing 10% (vol/vol) FBS were placed in the lower chamber. For the invasion assay, Transwell® membranes were pre-coated with 25% Matrigel (BD Biosciences). The cells were then incubated for 48 h at 37˚C in 5% CO_2 with 0.5 g/l matrine, 10 μM Bay11-7082 (Bay), 0.5 g/l matrine plus 10 μM Bay or fresh median (the untreated control). Cells were fixed in 4% paraformaldehyde for 20 min and stained with 0.05% crystal violet in PBS for 10 min. The cells on the upper side of the filter were removed with cotton-tipped swabs, and the filter was washed with 1X PBS. The cells on the underside of the filter were examined and counted under a microscope with a power of x200 magnification.

Western blotting. Cells treated with different concentrations of matrine, Bay and TNF-α (T6674 Sigma, USA) were lysed in a RIPA lysis buffer system (50 mM Tris-HCl, 1 mM EDTA, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS) and 1 mmol/l PMSF, pH 7.4). Supernatants were then collected via centrifugation at 14,000 x g for 15 min at 4˚C. Proteins were separated by electrophoresis in 10% SDS-polyacrylamide gel and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore Corp., Billerica, MA, USA). PVDF membranes were incubated in blocking buffer (1X Tris-buffered saline, 0.1% Tween-20 with 5% non-fat dry milk) for 1 h and probed overnight at 4˚C with specific primary antibodies against p-P65, P65, MMP-2, MMP-9 (1:1,000; Cell Signaling Technology, Beverly, MA, USA) or GAPDH (1:1,000; Kangcheng Biology, Shanghai, China). After being washed, membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (Dako) for 1 h at room temperature. Membranes were subsequently visualized with the Western Blotting Luminal Reagent (Millipore Corp.) according to the manufacturer's protocol.

Animal xenograft model assays. Male Balb/c nude mice (5-weeks old) were purchased from Experimental Animal Center of Sun Yat-sen University (Certificate of compliance: 44008500010058; Guangzhou, China) and raised under specific pathogen-free (SPF) conditions. All experimental procedures were performed according to the institutional ethical guidelines approved by the Institutional Animal Care and Use Committee of Sun Yat-sen Memorial Hospital, Sun Yat-sen University. Suspensions of DU145 and PC-3 cells (0.1 ml $5x10^7$ viable cells/ml) were subcutaneously injected into the right flank of mice. Nine days later, the tumor volumes were ~100 mm^3 in all mice. The treatment and control groups were subjected to intraperitoneal injection of 50 or 200 mg/kg matrine and an equal volume of saline three times per week, respectively (6 mice in each group). Tumor sizes were measured twice weekly, and the volumes (cm^3) were calculated according to the formula: $V = (\text{length} \times \text{width}^2) / 2$. Animals of both groups were sacrificed to measure tumor weights 3 weeks later.

Statistical analysis. All the experiments were repeated successfully at least three times. Two-tailed Student's t-test was used to determine the difference between groups by GraphPad Prism (San Diego, CA, USA). A P-value ≤0.05 was considered statistically significant.

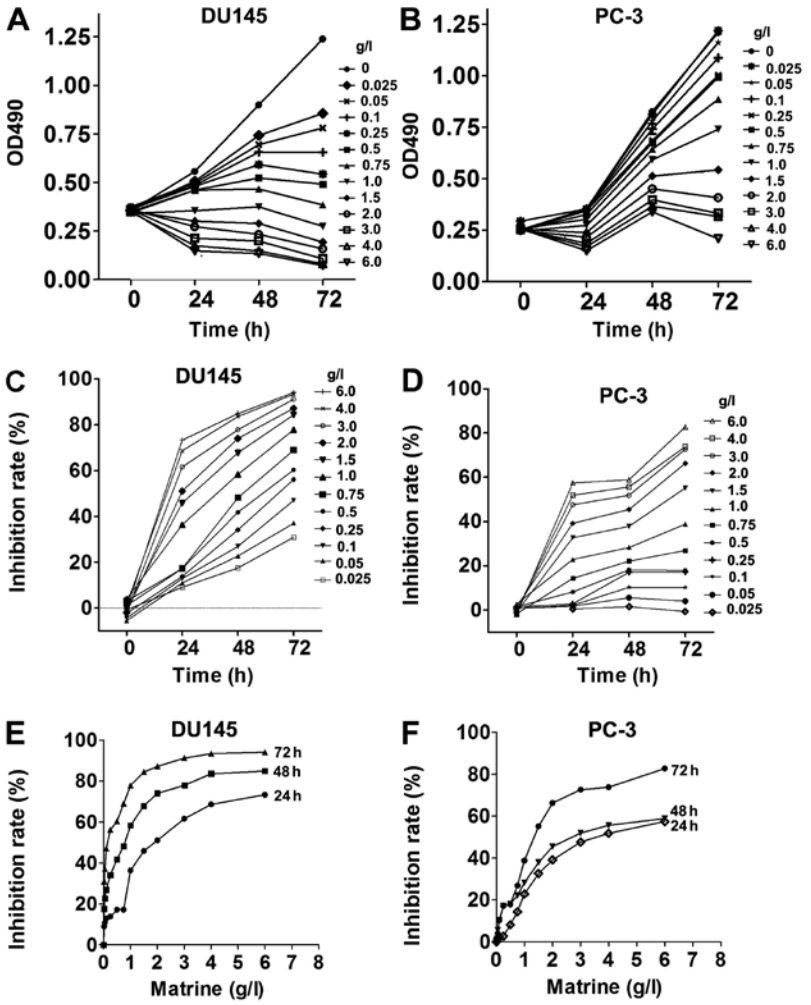

Figure 1. Matrine reduces the proliferation of CRPC cells *in vitro*. (A and B) Plots of the proliferation rates of prostate DU145 and PC-3 cells in the presence of increasing concentrations of matrine for different times. Relative OD490 values represent the number of viable cells to the number of cells before treatment. (C and D) Plots of the rates of growth inhibition in the presence of increasing concentrations of matrine for different times in DU145 and PC-3 cells. (E and F) Plots of IC_{50} values in the presence of increasing concentrations of matrine for different times in DU145 and PC-3 cells.

Results

Matrine reduces the viability of DU145 and PC-3 cells in vitro. DU145 and PC-3 cells were exposed to increasing concentrations of matrine, and their viabilities were evaluated by MTS assays at different time-points. Matrine inhibited the proliferation of both CRPC cell lines in a dose- and time-dependent manner (Fig. 1A and B). The inhibition rates of both CRPC cell lines reached almost 90% when cells were treated with 6.0 g/l matrine for 72 h (Fig. 1C and D). The potency of matrine to suppress the growth of CRPC cells was increased with increasing time (Fig. 1E and F). Thus, matrine suppresses the viabilities of both CRPC cell lines DU145 and PC-3.

Matrine suppresses tumor growth in nude mice inoculated with CRPC cells. DU145 and PC-3 cells were inoculated into male Balb/c nude mice. The time courses of CRPC xenograft growth with and without matrine treatment are shown in Fig. 2. Inhibitory impacts of matrine on tumor volume and weight were observed in DU145-inoculated mice treated with 50 mg/kg/d matrine (Fig. 2A-C). Such impact of matrine on tumor growth was only observed on the 19th and 22nd days in PC-3-inoculated mice treated with 50 mg/kg/d matrine (Fig. 2D-F). Interestingly, matrine at dosage of 200 mg/kg/d did not show any impact on the tumor size and weight of either DU145- or PC-3-inoculated mice, we did notice that tumors in the sacrificed mice treated with high dosage of matrine started ulcerating. The results suggest that matrine may suppress the tumor growth of CRPC at low dose but exhibit no antitumor effect at high dose due to interruption of the homeostasis of mice.

Matrine reduces the ability of migration and invasion of CRPC cells. We further tested whether matrine has any effect on the migration and invasion of CRPC cells. Cells were untreated or treated with 0.5 g/l matrine to measure cell migration and invasion. The migration rates of both DU145 and PC-3 cells were significantly reduced when cells were treated with matrine for 48 h (Fig. 3A-D). Invasion assay with Transwell chambers coated with Matrigel indicated that the number of either DU145 or PC-3 cells that invaded to the lower chamber significantly decreased after cells were treated with matrine for 48 h (Fig. 3E-H). Therefore, matrine effectively impairs the ability of migration and invasion of CRPC cells.

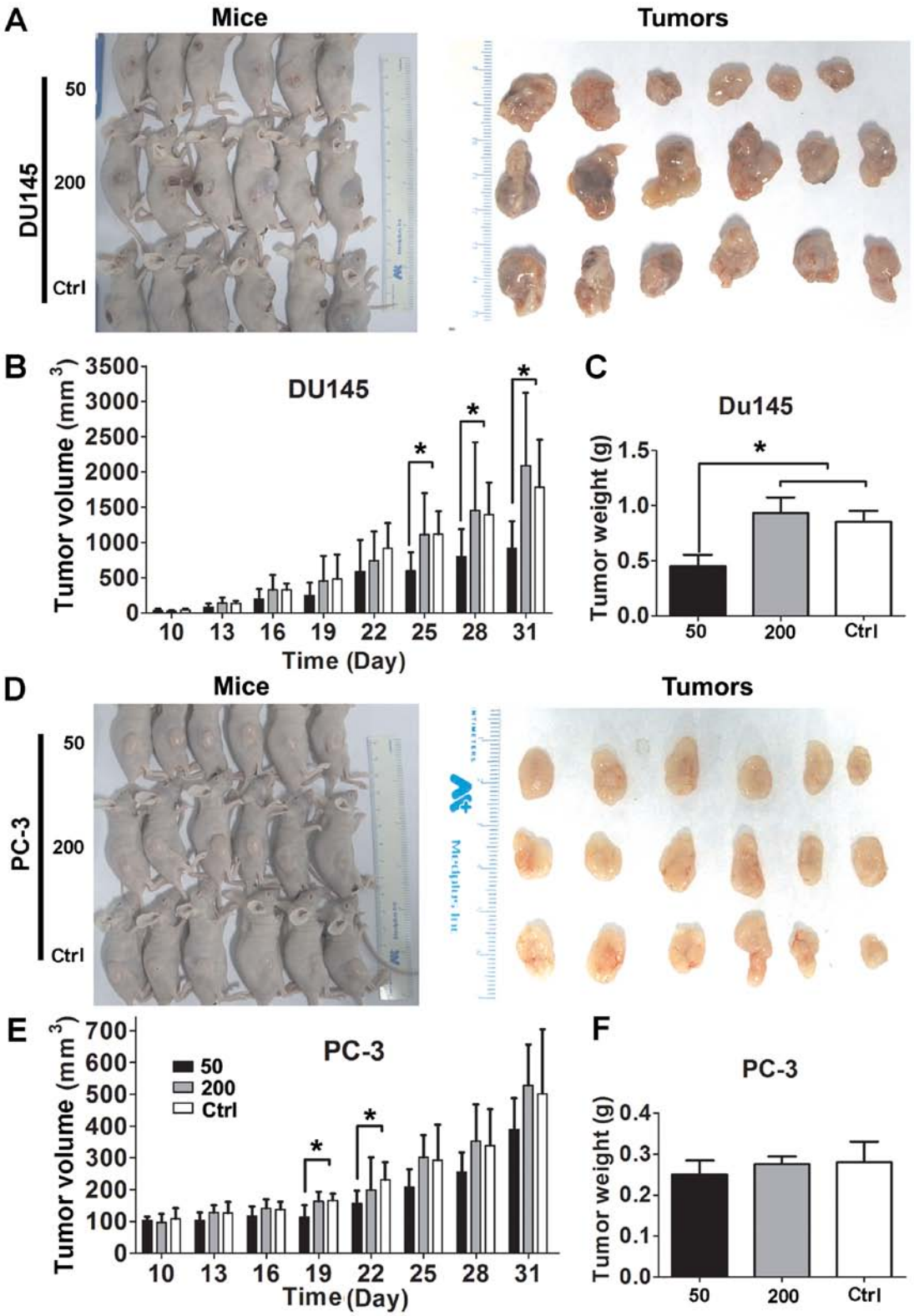

Figure 2. Matrine inhibits growth of tumors in nude mice inoculated with CRPC cells. (A-F) DU145 (A-C) and PC-3 cells (D-F) were implanted subcutaneously into BALB/c nude mice. Mice were treated without or with matrine by intraperitoneal injection (50 or 200 mg/kg, three times a week) after the tenth day. Representative images of animals and tumors after the mice were sacrificed (A and D), plots of tumor volumes in the presence of increasing time periods (B and E) and plots of final tumor weights, after the mice were sacrificed, are shown. Data shown here and later are the mean ± standard deviation of at least three repeats. Statistical significance was determined by the Student's t-test. $^{*}P \leq 0.05$.

Matrine reduces the expression levels of MMP-9 and MMP-2 and impairs NF-κB pathway in CRPC cells. Since MMPs play important roles in the invasion of cancer cells. We examined the expression levels of MMP-9 and MMP-2 in CRPC cells exposed to different concentrations of matrine by immunoblotting. Similar to NF-κB inhibitor Bay, matrine reduced

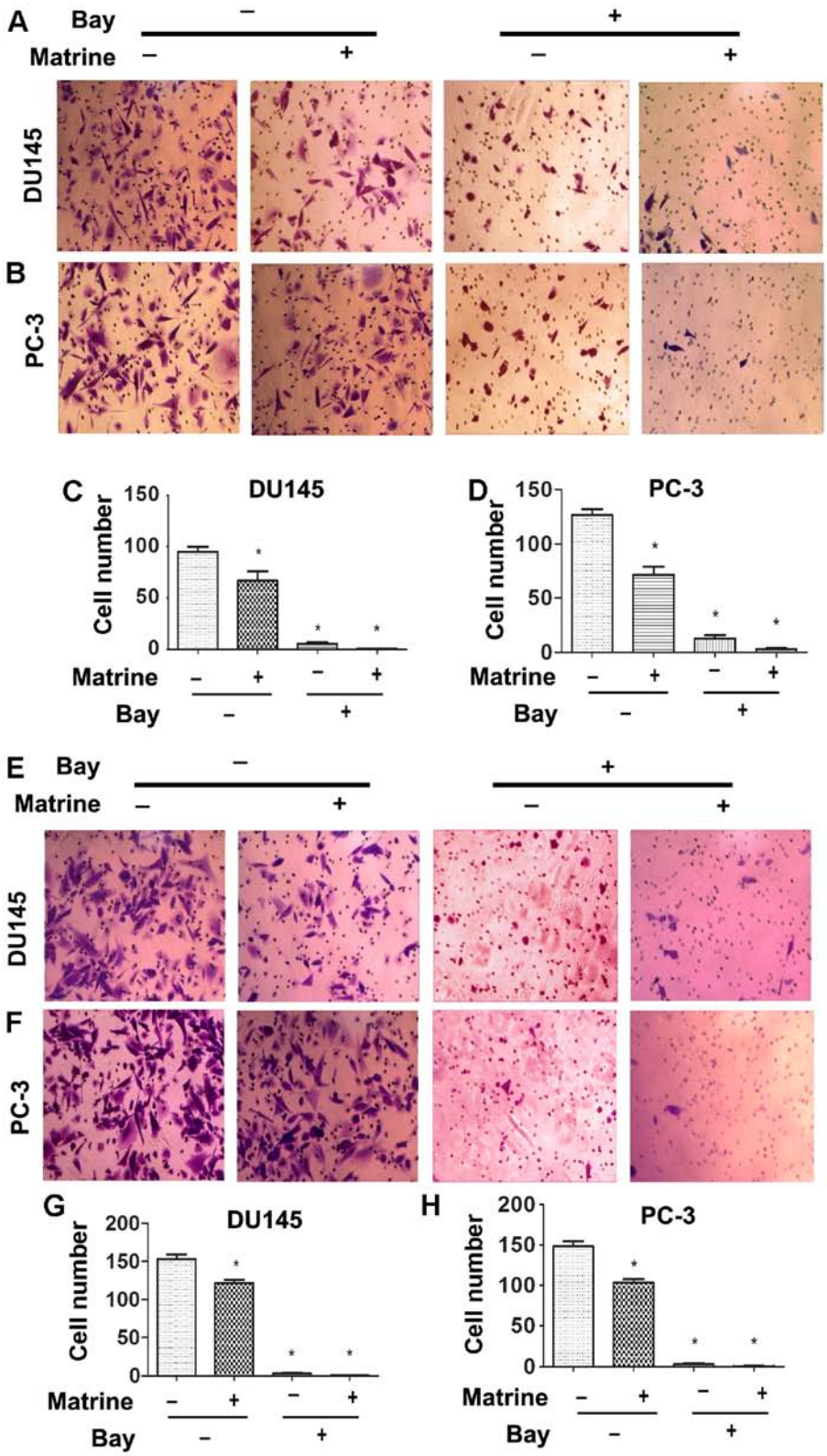

Figure 3. Matrine impairs the migration and invasion of CRPC cells *in vitro*. (A-D) Representative images (A and B) and plots of the number (C and D) of migrated DU145 (A and C) and PC-3 cells (B and D) per 10,000 seeded cells in the absence or presence of 0.5 g/l matrine and/or 10 μM Bay for 48 h. (E-H) Representative images (E and F) and plots of the number (G and H) of invaded DU145 (E and G) or PC-3 cells (F and H) per 10,000 seeded cells in the absence or presence of 0.5 g/l matrine and/or 10 μM Bay for 48 h.

the expression levels of MMP-9 and MMP-2 in DU145 cells in a concentration-dependent manner (Fig. 4A-C). NF-κB is reported as a key signaling molecule that mediates the expression of MMPs (11). We hypothesized that NF-κB signaling pathway might also be affected by matrine. As shown in Fig. 4A, D and E, the expression levels of p-P65 and P65 in DU145 cells were markedly downregulated upon treatment with matrine for 48 h. The same trends were observed in PC-3

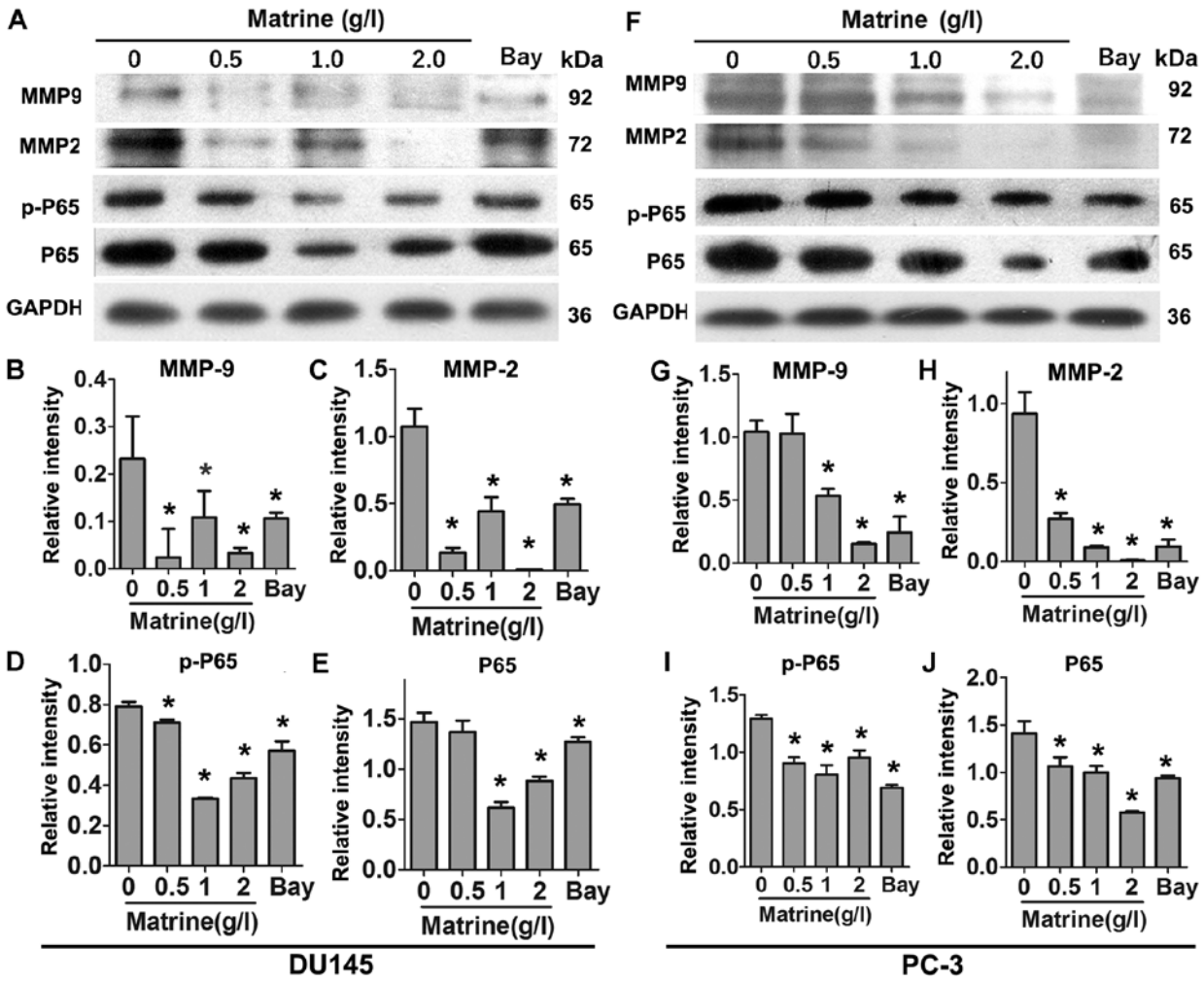

Figure 4. Matrine reduces the expression levels of p-p65, p65, MMP-9 and MMP-2 in CRPC cells. (A-J) Representative immunoblot results (A and F) and plots of relative intensity of MMP-9 (B and G), MMP-2 (C and H), p-P65 (D and I) and P65 (E and J) in DU145 (A-E) and PC-3 cells (F-J) treated with different concentrations of matrine or 10 μM Bay. Relative intensity of proteins are their ratios to levels of GAPDH.

cells (Fig. 4F-J). Collectively, these results demonstrate that matrine suppresses the expression of MMP-9 and MMP-2 and inactivates NF-κB signaling pathway in CRPC cells.

Matrine inhibits migration and invasion of CRPC cells by suppressing MMP-9 and MMP-2 through NF-κB pathway. In addition, NF-κB is involved in the control of migration and invasion of tumor cells. We hypothesized that the suppression of migration and invasion of CRPC cells may be caused by downregulation of NF-κB. Combined treatment of NF-κB inhibitor Bay and matrine caused a synergistic reduction in migration and invasion of both in DU145 and PC-3 cells (Fig. 3). We further investigated whether the inhibitory effects of matrine on cell invasion and expression of MMP-9 and MMP-2 were correlated with the inhibition of NF-κB pathway. Either DU145 or PC-3 cells were cultured in the absence or presence of 0.5 g/l matrine, 10 μM Bay and/or 10 ng/ml TNF-α for 48 h. Consistently, the levels of p-P65 and P65 proteins were significantly reduced when DU145 cells were treated with matrine and/or Bay (Fig. 5A-C). Consequently, the expression levels of MMP-9 and MMP-2 in DU145 cells were significantly reduced upon exposure to matrine and/or Bay (Fig. 5A, D and E). When NF-κB pathway was activated by TNF-α, matrine significantly reduced expression levels of p-P65 and MMP-9 in DU145 cells (Fig. 5F-J). However, expression levels of MMP-2 were not significantly changed when DU145 cells were cultured in the presence of both matrine and TNF-α, suggesting different regulatory mechanisms for MMP-9 and MMP-2. Similar results were obtained when the tested cell line was changed to PC-3 (Fig. 5K-T). Therefore, inhibitory effects of matrine on migration and invasion of CRPC cells act by reducing levels of MMP-9 and MMP-2 through the NF-κB pathway.

Discussion

Naturally-occurring phytochemicals play important roles in the prevention and treatment of cancers (19,20). As a naturally-occurring phytochemical, matrine has been reported to exhibit anticancer effects on different malignancies such as lung cancer, breast cancer, and castration-sensitive prostate cancer (15-17). In our previous study, we identified the inhibitory ability of matrine in CRPC cells *in vitro*. This study investigated the mechanisms of anti-metastatic effects of matrine against CRPC cells. Our data showed that matrine inhibited the viability of CRPC cells time- and dose-dependently.

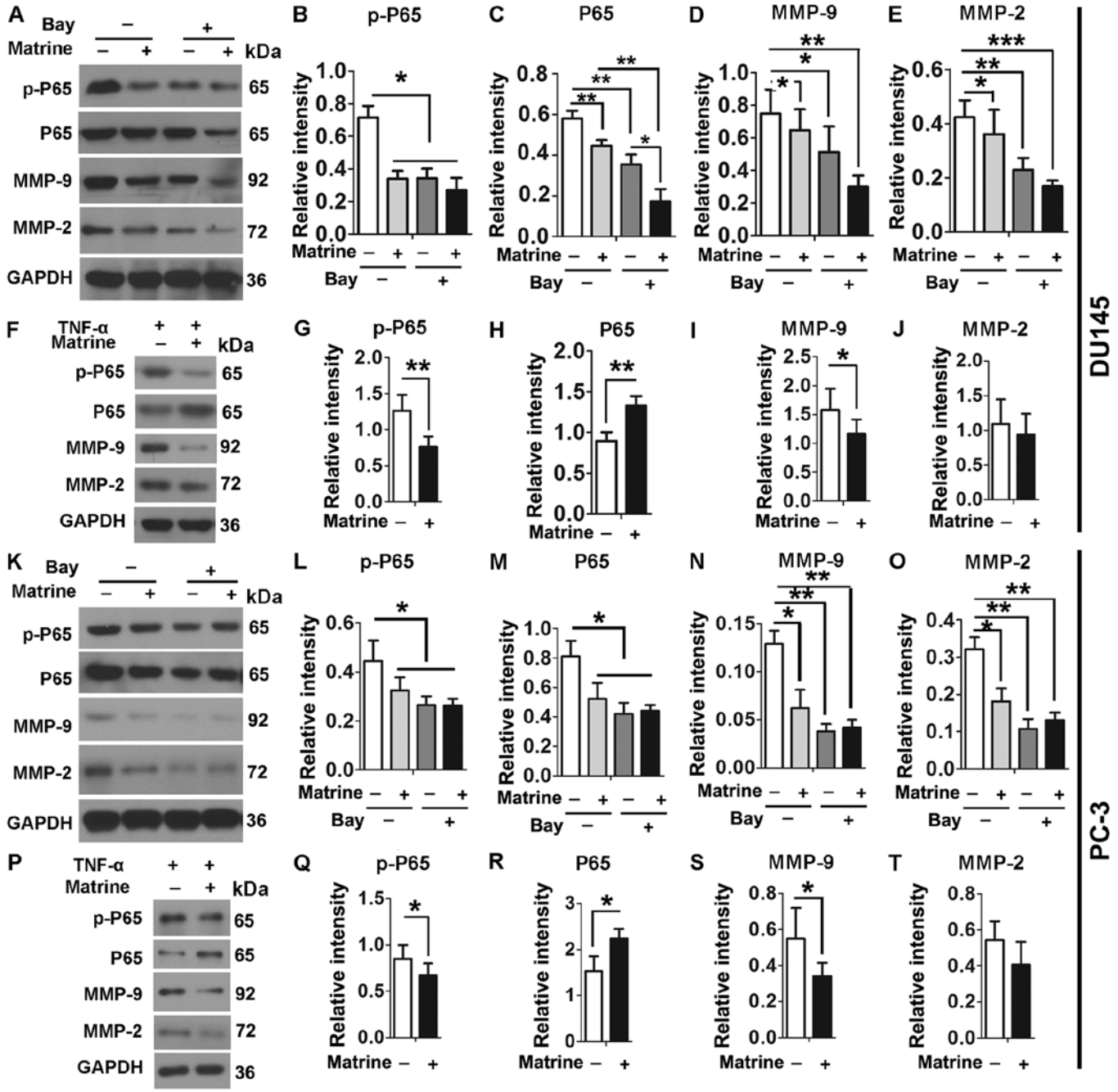

Figure 5. Matrine reduces the levels of MMP-2 and MMP-9 via NF-κB signaling pathway in CRPC cells. (A-T) Representative immunoblot results (A, F, K and P) and plots of relative intensity of p-P65 (B, G, L and Q), P65 (C, H, M and R), MMP-9 (D, I, N and S) and MMP-2 (E, J, O and T) in DU145 (A-J) or PC-3 cells (K-T) in the absence or presence of 0.5 g/l matrine in the absence or presence of 10 μM Bay (A-E and K-O), or in the absence or presence of 0.5 g/l matrine in the presence of 10 ng/ml TNF-α for 48 h (F-J and P-T). Relative intensity of proteins are their ratios to levels of GAPDH. *P≤0.05; **P≤0.01; and ***P≤0.001.

In vivo study further demonstrated that matrine inhibited the growth of CRPC tumors in nude mice. Transwell analysis showed that matrine also inhibited the migration and invasion of CRPC cells dose-dependently through NF-κB pathway. The underlying mechanism is potentially that suppressing NF-κB activities causes reduction in levels of MMP-9 and MMP-2. These data imply that matrine is a potent candidate for CRPC treatment.

PCa mainly depends on the presence of androgens for growth and survival, and blocking androgen secretion or activity represents the most effective initial treatment (2). However, after the initial surgical or medical ablation, most PCa will progress to CRPC stage with increased malignancy in 2-3 years and need for second-line therapy (3,21). Although cytotoxic drugs and recently approved drugs for more efficient blockade of androgen signaling are available, there is still a need for new and more efficient treatment strategies in CRPC (22). Matrine has been reported to inhibit the proliferation and invasion of different malignancies (23-25). This study initially investigated whether matrine had similar effects on CRPC. Results proved that matrine did reduce the viability of DU145 and PC-3 cells time- and dose-dependently. Moreover,

matrine inhibited migration and invasion of the CRPC cells. These results may be translated into the development of novel treatment approach with matrine. To further confirm the hypothesis of such a novel treatment modality, we conducted *in vivo* analyses to demonstrate that matrine decreased the growth of tumor xenografts in nude mice inoculated with CRPC cells by inhibiting cell proliferation.

Tumor metastasis involves a complex process and various cellular physiological changes. Cancer cell invasion is the first step for metastasis, which is characterized by increasing cell motility caused by alterations in cell-cell and cell-ECM interactions (26). Apoptotic defects may cause cells to be resistant to cell death induced by such alterations, which may promote cancer cell invasion (27). Apoptosis is a type of programmed cell death that is characterized by cell membrane blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation (28). After initiating invasion, cancer cells undergo various stresses that may trigger apoptosis by the extrinsic and the intrinsic pathways (29). However, even with the successful micro-metastases, there is only ~0.01% of tumor cells that ultimately develop into macro-metastases in their destined organs. Such inefficiency may be closely related to the stress-induced apoptosis because of changed cell environment (30). Many small molecules targeting apoptotic pathways, such as ABT-263, and GX15-070, have been developed for cancer therapy (31). Other studies indicated that matrine exerts anticancer effects by inducing apoptosis in different malignancies such as cholangiocarcinoma, medulloblastoma and lung cancer (23-25). Studies also showed that matrine exhibits anti-metastatic effects by inducing apoptosis in lung cancer and breast cancer (16,32). Matrine induces apoptosis by decreasing the expression of Bcl-2 and increasing expression of Bax. Apoptosis may block metastatic dissemination by killing misplaced cells. Apoptotic resistance induced by the loss of cell-cell and cell-ECM contacts may promote metastatic progression (27).

The ECM is composed of heterogeneous macromolecules and provides structural support to cells. It stores rich angiogenesis promoters and inhibitors (6). The degradation or breakdown of the ECM is a critical step in tumor invasion, leading to the separation of the intercellular matrix to promote metastasis. MMPs, which are a family of structurally related zinc-dependent endo-peptidases, are collectively capable of degrading essentially all components of ECM including collagens, gelatin and proteoglycan (8,33). Among MMPs, MMP-2 and MMP-9 were reported to play the most important roles for cancer invasion and metastasis (34,35). Previous studies have reported that matrine can reduce the expression of MMP-2 and MMP-9 in tumor cells (32,36,37). NF-κB, promoting expression of MMP-2 and MMP-9, has previously been reported as a downstream target of matrine in cancer cells (32,36,37). Our study indicated that matrine significantly reduces the expression levels of MMP-2 and MMP-9, implying that matrine may inhibit the invasion of DU145 and PC-3 cells by down-regulating the expression and activity of MMP-2 and MMP-9. Further analysis on the signaling pathway regulating MMP-2 and MMP-9 suggested that matrine significantly decreased the expression levels of NF-κB subunit p65 and p-p65 that are located within the nuclei of DU145 and PC-3 cells. All results indicated that NF-κB-MMP-2/9 is a key signaling pathway by which matrine regulates the invasive and metastatic abilities of CRPC cells.

In conclusion, this study provides evidence that matrine is capable of inhibiting the proliferation, migration and invasion of CRPC cells. The invasion is most likely inhibited by reducing expression levels of MMP-2 and MMP-9 through inactivation of NF-κB pathway. A novel potential therapeutic application of matrine for anti-metastatic therapy of CRPC is revealed.

Acknowledgements

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