Matrine reduces the proliferation and invasion of colorectal cancer cells via reducing the activity of p38 signaling pathway

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Matrine has been used in anti-inflammatory and anti-cancer therapies for a long time. However, the anti-metastatic effect and related mechanism(s) in colorectal cancer (CRC) are still unclear. In this study, we investigated whether the administration of matrine could inhibit the proliferation, motility, and invasion of human CRC cells via regulating p38 signaling pathway. Results showed that matrine inhibited migration and invasion of CRC cells in vitro and in vivo. Additionally, after being treated with matrine for 24 h, the expression levels of matrix metalloproteinase-2 (MMP-2) and MMP-9 as well as proteinase activity in CRC cells were reduced in a dose-dependent manner. Moreover, matrine reduced the phosphorylation level of p38 obviously. Combined treatment with p38 inhibitor (SB203580) and matrine resulted in a synergistic reduction of invasion as well as MMP-2/-9 expression in CRC cells. It was also found that matrine inhibited the proliferation and metastasis of CRC tumor in vivo. In conclusion, p38 signaling pathway may involve in matrine’s inhibitory effects on migration and invasion of CRC cells by reducing the expression of MMP-2/-9, suggesting that matrine may be a potential therapeutic agent for CRC.

Keywords colorectal cancer; invasion; matrine; p38

Introduction

Colorectal cancer (CRC) is one of the most common cancers and the leading causes of cancer-related death worldwide [1]. CRC is often characterized by aggressive local invasion and metastasis which makes it be recalcitrant to treatment [2]. Most of the drugs were considered incompetent in curing CRC, thus finding new therapeutic targets for developing novel drugs is urgent and of significance. Further understanding of mechanism of CRC could provide clues in discovering the targets.

Various proteases participate in the process of cancer metastasis [3]. These proteases facilitate cancer cell metastasis by degrading the basement membranes. Matrix metalloproteinase-2 (MMP-2) and MMP-9 play important roles in CRC and are considered to correlate with the prognosis of patients [4,5]. Decreased MMP-9 expression level was reported to associate with longer survival time and lower risk of cancer recurrence [6]. Low pro-matrix MMP-2 levels and high tissue inhibitor of MMP-1 levels correlate with parameters of CRC disease [7].

Matrine, also known as C_{14}H_{24}N_{2}O, is one of the effective monomers found in Saphora flavescens, which has been found to be effective in inhibiting the proliferation and invasion of different kinds of cancer cells [8,9]. But up to now, there are still few reports concerning the anti-metastatic effect and possible mechanism(s) of matrine in CRC. In the study, we investigated the effect of matrine on the migration and invasion of CRC cells.

Materials and Methods

Cell culture

Human colon carcinoma HT29 and DLD1 cell lines were obtained from the American Type Culture Collection (Manassas, USA) and cultured in RPMI-1640 (Hyclone, Logan, USA) medium containing 10% fetal bovine serum (FBS; Hyclone). All cells were incubated at 37°C with 5% CO₂.
previously described [10]. Briefly, CRC cells were plated at a density of $1 \times 10^5$ cells per well into 6-well plates for 24 h. The cells were treated with various concentrations of matrine (0, 0.5, 1, and 1.5 mg/ml) and incubated at 37°C for 48 h. The cells were washed with cold PBS and resuspended in Annexin V binding buffer. The cells were stained with Annexin V-FITC for 15 min, washed, and then stained with PI. The samples were analyzed by flow cytometer with CellQuest software.

**Colony formation assay**

Cells of each type were cultured in 10-cm culture dishes and exposed to fresh media every 4 days [11]. Colonies were fixed at day 16 with 10% formaldehyde for 10 min and then stained with 1% crystal violet for 30 s. The colonies with diameters $>0.2$ mm were counted.

**Migration and invasion assay**

The migration and invasion potential of the cancer cells was assessed in vitro with Transwell chambers (Corning, Tewksbury, USA). For migration assays, $2 \times 10^5$ cells in serum-free medium were added into the upper chambers, and FBS (10%) was added into bottom chambers. For invasion assay, the upper chambers were coated with Matrigel (BD Biosciences, San Jose, USA).

**Western blot analysis**

After treatment with different concentrations of matrine or SB203580 (Cell Signaling Technology, Beverly, USA), $2 \times 10^6$ cells were suspended in 200 μl of lysis buffer (1 mM EDTA, 40 mM Tris–HCl, 150 mM KCl, 1% Triton X-100,100 mM NaVO₃, and 1 mM PMSF, pH 7.5). The proteins (60 μg) were separated by 10% or 12% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) electrophoresis, and then transferred onto polyvinylidene difluoride membranes. After being blocked in defatted milk (5% in Tris-buffered saline with Tween-20 buffer) at 37°C, the membranes were incubated with various antibodies against MMP-2, MMP-9, p38, p-p38, p-JNK, JNK, p-ERK, ERK, or β-actin overnight at 4°C (all the antibodies were purchased form Cell Signaling). The membranes were then incubated with appropriate secondary antibodies for 1 h at room temperature. The bands were detected and expressed as arbitrary units (a.u.).

**Zymography**

The assay was carried out as previously described [3]. After being treated with different concentrations of matrine at 37°C for 24 h, conditioned media of cells were collected and separated by 0.1% gelatin-8% SDS-PAGE electrophoresis. Then, the gels were washed twice in 2.5% Triton X-100 for 45 min at room temperature and then incubated in reaction buffer (40 mM Tris–HCl, 10 mM CaCl₂ and 0.01% NaN₃, pH 8.0) at 37°C for 14 h. The gels were stained with coomassie brilliant blue R-250 gel stain. The intensities of bands were calculated using an image analysis system (Bio-Rad Laboratories, Richmond, USA). The last volumes of samples were adjusted by vital cell number.

**Animal and tumor xenograft assays**

In vivo tumorigenicity was done as previously described [12]. In brief suspensions of tumor cells HT29 (1 $\times 10^6$ viable cells/mouse) were implanted into the right flank region of BALB/c nude mice. Forty-eight hours after the injection (day 1), all mice were randomly divide into two groups ($n = 3$). The animals were pair matched to make sure the median tumor volume for each group was similar: treatment group received matrine (60 mg/kg per day) via intra-gastric administration; control group were treated with saline. Tumor volumes were measured twice a week with calipers and volumes (cm³) were calculated according to the standard formula: length $\times$ width$^2$/2. After 3 weeks of drug administration, mice were sacrificed and tumors were dissected out and weighed. The experimental protocols used were evaluated and approved by the Animal Care and Use Committee of the Medical School of Xi’an Jiaotong University.

**Xenograft animal model of lung metastasis**

The effect of matrine on CRC metastasis in xenograft mouse models of lung metastasis was determined as previously described [13]. In the lung metastasis model, single-cell suspensions of CRC cells ($2 \times 10^6$) in 100 μl HBSS were injected into the lateral tail vein. Five mice were included in the...
control and treatment group (60 mg/kg per day), respectively. Mice were sacrificed 6 weeks after injection and lungs were surgically excised. The number and size of metastatic foci in the lung were documented. Specimens were fixed in 10% formalin or stored at −80°C for future analysis.

**Statistical analysis**

Experiments were repeated three times, and the results of the studies were expressed as the mean ± SD. Statistical differences were analyzed by one-way or two-way analysis of variance and further by *post hoc* tests using the statistical software of GraphPad Prism 5. All statistical tests and corresponding *P* values were two sided. *P* < 0.05 was considered as statistically significant. Correlation analysis was performed by the *Z*-test.

**Results**

**Matrine inhibits the proliferation of HT29 and DLD1 cells**

The anti-proliferation effects of matrine at various concentrations (0 to 2 mg/ml) on CRC cells are shown in Fig. 1. At 1 mg/ml, matrine significantly inhibited the proliferation of HT29 and DLD1 cells. At concentrations < 1 mg/ml, the inhibition effect was not obvious; thus, we chose a concentration range of matrine lower than this for all subsequent experiments. The effect of 5-Fu on the CRC cells was also detected. The IC$_{50}$ of 5-Fu is 10.78 μM ($R^2 = 0.9203$) and the IC$_{50}$ of matrine is 1.569 mg/ml ($R^2 = 0.8914$). Matrine also induced the apoptosis of HT29 cells (Supplementary Fig. S1). Results showed that matrine could inhibit the

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**Figure 2. Effect of matrine on in vitro migration and invasion of HT29 cells**

(A) For the migration assay, after incubating with different concentrations of matrine (0, 0.25, 0.50, and 0.75 mg/ml), cells were seeded into the upper wells of the chamber, and FBS (10%) was added into the lower wells for 24 h to induce cell migration. Then, cells on the bottom side of the filter were fixed, stained, and measured. Cell migration spontaneous migration in DMSO was designated as control. (B) The percent migration rate was expressed as a percentage of the control (0 mg/ml). (C) For the invasion assay, the filter was coated with Matrigel, and other steps were the same as the migration assay. (D) The percent invasion rate was expressed as a percentage of the control (0 mg/ml). Values were represented as the mean ± SD of three independent experiments ($n = 3$). *$P < 0.05$ and **$P < 0.01$ compared with the control group.
cloning formation ability and induce the apoptosis of HT29 cells (Supplementary Fig. S1).

**Matrine inhibits the migration and invasion of HT29 cells**

Figure 2 shows the effect of matrine on cell migration and invasion in HT29 cells treated with 0, 0.25, 0.50, and 0.75 mg/ml of matrine for 16 h (cell migration) and 24 h (cell invasion), respectively. Results showed that matrine reduced the invasion and migration of HT29 cells substantially in a concentration-dependent manner. Similar anti-metastatic effect of matrine was observed in DLD1 cells (Supplementary Fig. S1).

**Matrine suppresses the expression and activity of MMP-2/-9**

The expression and activity of MMP-2/-9 in HT29 cells that were exposed to different concentrations of matrine were examined. Cells were treated with 0, 0.25, 0.50, and 0.75 mg/ml matrine for 24 h and then subject to western blot analysis. Figure 3A,B showed that matrine significantly reduced the protein levels of MMP-2/-9 in a concentration-dependent manner compared with the control group. Figure 3C,D showed the results of gelatin zymography. Matrine could reduce the activity of MMP-2/-9. Matrine could also reduce the expression of MMP-2/-9 in DLD1 cells (data not shown).

**The p38 signaling pathway is involved in the anti-metastatic mechanism of matrine**

p38 signaling pathway plays important roles in the invasion of cancer cells via regulating MMP-2/-9 [14]; thus, we investigated the effect of matrine on the p38 signaling pathway in HT29 cells. Western blot analysis showed that matrine could reduce the phosphorylation of p38 in a concentration-dependent manner (Fig. 4A,B). The results also showed that treatment with SB203580 (a p38 inhibitor) and matrine significantly reduced both cell invasion (Fig. 5A,B) and MMP-2/-9 protein expression (Fig. 5C,D). Matrine had no effect on the activity of JNK and ERK signaling pathway (Supplementary Fig. S2).

**Matrine inhibits the CRC tumor growth in vivo**

The time course of HT29 xenograft growth with and without matrine treatment is shown in Fig. 6A. A significant inhibition of tumor growth by matrine was observed. Twenty-one days after cell implantation, the xenograft tumors were removed and weighed. Compared with the control group, matrine decreased the solid tumor mass significantly (Fig. 6B).

**Matrine inhibits CRC metastasis in vivo**

Overgrowth and metastasis are two major characteristics of malignant tumors [15]. Nude mice were treated with diluent vehicle or matrine. The average number of lung metastases in the treated and control groups were 14.6 and 8.4, respectively. Lung metastases in the treated group decreased by 42.47% compared with those in the control group (Fig. 6C,D).

**Discussion**

The anti-tumor effect of matrine has been shown in various cancers [6,8,9,16,17]. However, the anti-metastatic effect and the related mechanism(s) in CRC cells are still unclear.
This study revealed that matrine significantly suppressed the invasive and metastatic ability of CRC cells. Concurrently, MMP-2 and MMP-9 in CRC cells were down-regulated via inhibition of the p38 signaling pathway after matrine treatment. To the best of our knowledge, this is the first scientific report on the anti-metastatic effect of matrine on CRC.

Transwell assay was used to detect the anti-metastatic effect of matrine on CRC cells. The results showed that matrine inhibited the migration and invasion of CRC cells. These results are similar to previous studies in other malignant tumors such as breast cancer [18,19].

Metastasis is one of the leading causes of cancer-related death among CRC patients [16]. Degradation of the ECM of blood or lymph vessels is critical to metastasis because loss of the ECM allows cancer cells to invade the blood or lymphatic system and spread to distal tissues and organs [20]. MMPs, especially MMP-2 and MMP-9, are believed to be potent in degrading ECM [7,17,21]. Matrine has been proved to be able to reduce the expression of MMPs in many cancers [22,23]. In the study, we found that matrine reduced the expression and activity of MMP-2 and MMP-9 in CRC cells. The results suggested that the anti-metastatic effect of matrine is related to its inhibitory effect on MMP-2 and MMP-9 in CRC cells.

Previous studies showed that in both normal and cancerous tissues, p38 signaling pathway is correlated with MMPs [24–26]. Increased phosphorylated p38 has been deemed to be a negative independent prognostic factor for CRC [27]. Matrine has also been reported to regulate the activity of p38 signaling pathway [28]. In lung cancer and leukemia cells, it
was shown that matrine inhibited the proliferation and invasion by regulating p38 signaling pathway [28,29]. Furthermore, MMP-2 and MMP-9 were proved to be the down-stream effecters of p38 signaling pathway [30,31]. To further explore the possible mechanism of anti-metastatic effect of matrine, the effect of matrine on the phosphorylation level of p38 in CRC cells was detected. The results showed that matrine reduced the phosphorylation level of p38 in a concentration-dependent manner. With the combined treatment of p38 inhibitor (SB203580), matrine reduced CRC cell invasion and expression and activities of MMP-2 and MMP-9 more dramatically. In addition, results from in vivo analyses demonstrated that matrine decreases the growth of CRC xenografts and metastasis in nude mice.

In conclusion, this study demonstrated the inhibitory effects of matrine on the proliferation, invasion, and metastatic capabilities of CRC cells. Furthermore, the decreased expression and activity of MMP-2 and MMP-9 induced by matrine was found to contribute to p38 signaling pathway inhibition. These findings not only improved our understanding of the mechanisms of matrine’s anti-invasion effects on CRC, but also revealed a new potential therapeutic application of matrine in anti-metastatic therapy for CRC.

Supplementary Data

Supplementary Data is available at ABBS online.

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