Original Article

Matrine inhibits proliferation and migration of HepG2 cells by downregulating ERK1/2 signaling pathways

Haipeng Yu[1,2,3#], Bolan Zhu[4#], Wan Yang[5#], Qian Zhang[6], Wei Wang[4], Weichun Zhai[4], Lixia Lu[4], Ying Zheng[4], Zheng Dang[7], Bin Li[4], Chuyi Li[4], Bin Liang[8*], Jiucong Zhang[4*], Xiaohui Yu[4*]

[1]Department of Interventional Therapy, Tianjin Medical University Cancer Institute and Hospital, [2]National Clinical Research Center for Cancer, Tianjin's Clinical Research Center for Cancer, [3]Key Laboratory of Cancer Prevention and Therapy, Tianjin, Departments of [4]Gastroenterology, [6]Hematology and [7]Hepatobiliary Surgery, The 940 Hospital of Joint Logistic Support Force of People's Liberation Army, Lanzhou, [5]Department of Gastroenterology, Daxing Teaching Hospital Affiliated to Capital Medical University, Beijing, [8]Department of Gastroenterology and Liver Diseases, Ganzhou Fifth People's Hospital, Jiangxi, China

ABSTRACT

Objective: To research the effect of matrine on the proliferation and migration of HepG2 cells through extracellular signal-regulated kinase 1/2 (ERK1/2) signaling pathway.

Methods: HepG2 cell was selected and divided into blank control group, experimental group (matrine 1, 2, and 4 mg/mL), and positive control group (PD98059, ERK1/2 inhibitor). MTT measure was used to detect the effective time and concentration which matrine inhibits HepG2 cells. After 24 h, the effect of effective concentration of matrine on the of morphological changing HepG2 cells was observed. The invasion ability was assayed by transwell method, the expression of ERK1/2 and pERK1/2 were detected through Western blot, and reverse transcription polymerase chain reaction was used to test the expression level of ERK1/2 mRNA.

Results: With the increase of matrine concentration, the number of adherent HepG2 cells gradually decreased, the morphologic changes gradually became spherical, some cell morphology was incomplete, and even cell fragments appeared. The proliferation and invasion ability of HepG2 cells decreased. The expression of ERK1/2, pERK1/2, and ERK1/2 mRNA downregulated with the increase of matrine concentration ($P < 0.05$).

Conclusion: Matrine inhibits the proliferation and migration of HepG2 cells by downregulating the ERK1/2 signaling pathway.

KEY WORDS: Extracellular signal regulated kinase 1/2 pathway, hepatocellular carcinoma, matrine, migration, proliferation

INTRODUCTION

Hepatocellular carcinoma (HCC) represents as the sixth leading cause of cancer-related deaths worldwide and its occurrence is the result of a variety of factors, for instance, hepatitis virus infection, food contaminated by aflatoxin and cirrhosis.[1,2] One of the pathogenesis is that it has malignant proliferation and invasion identify,[3] which is also an important bottleneck restricting the clinical efficacy of existing anti-HCC drugs. The mechanism of malignant proliferation and invasion of HCC is extremely complex, and the activation of related signaling pathways is one of its important pathogenesis.

Extracellular signal-regulated kinase (ERK), one of the mitogen-activated protein kinase (MAPK) family of signal transduction, is widely distributed in various tissues, and plays a role in cell proliferation and differentiation and regulates growth factor receptors. Studies have shown that the activation of Studies have shown that the activation of ERK1/2 is not only involved in inflammation but also frequently associated with the occurrence and development of malignant tumors, which involved proliferation and migration of HCC has been reported literature;[4] however, inhibiting the proliferation and migration of HCC cells to prevent ERK1/2 signaling pathway activation becomes the current research focus of anti-HCC drugs.

Matrine is an alkaloid extracted from matrine, and studies have shown that it has a certain the anticancer activity, including HCC;[5] however, the exact mechanisms of matrine-induced malignant HCC cell death still remain unclear. In this study, we researched whether matrine inhibits the

Cite this article as: Yu H, Zhu B, Yang W, Zhang Q, Wang W, Zhai W, *et al*. Matrine inhibits proliferation and migration of HepG2 cells by downregulating ERK1/2 signaling pathways. J Can Res Ther 2020;16:209-14.

Submitted: 03-May-2019
Revised: 23-Sep-2019
Accepted: 14-Nov-2019
Published: 16-Apr-2020

Access this article online
Website: www.cancerjournal.net
DOI: 10.4103/jcrt.JCRT_331_19
Quick Response Code:

[#]Haipeng Yu, Bolan Zhu, Wan Yang contributed equally to this work

For correspondence:
Dr. Xiaohui Yu, Department of Gastroenterology, The 940 Hospital of Joint Logistic Support Force of People's Liberation Army, Lanzhou, 730050, China. E-mail: yuxiaohui528@126.com
Dr. Jiucong Zhang, Department of Gastroenterology, The 940 Hospital of Joint Logistic Support Force of People's Liberation Army, Lanzhou, 730050, China. E-mail: zhangjiucong@163.com
Dr. Bin Liang, Department of Gastroenterology and Liver Diseases, Ganzhou Fifth People's Hospital, Jiangxi 341000, China. E-mail: liangbin8128@163.com

proliferation and migration of HCC through ERK1/2 signaling pathway.

METHODS

Reagents

The following reagents were purchased for this study: Matrine from Shanghai Aladdin Company, MTT solution from Amresco (USA), ERK1/2 monoclonal antibody from Cell Signaling Technology, polymerase chain reaction (PCR) kit from Takara, western blot kit from Abcam, transwell (pore size 0.8 μm) from Corning. The HepG2 cell line was purchased from the Shanghai Institute of Biology at the Chinese Academy of Sciences.

Assessment of effective time and matrine concentration for inhibition of HepG2 cell proliferation using the MTT assay

HepG2 cells in the logarithmic growth phase were harvested and were seeded in a 96-well plate at a concentration of 1 × 105 cells/mL. The zeroing and control wells were set, and matrine was added at concentrations of 0.25, 0.5, 1, 2, and 4 mg/mL. Six replicate wells were used for each concentration. After culturing cells for 24, 48, and 72 h, 20 μL MTT solution and 150 μL DMSO solution were added to each well, and the absorbance values were measured in a microplate reader. The rate of inhibition was calculated according to the following equation:

Inhibition rate = 1−(OD of the experimental sample−OD blank)/(OD of the control sample-OD blank) ×100

The experiment was repeated three times; the effective time of 24 h was selected and the effective matrine concentrations were 1, 2, and 4 mg/mL.

Experimental grouping

There are blank control, experimental, and positive control groups including in the experiment on the basis of removed culture medium of HepG2 cells when cultured to logarithmic growth phase. While the blank control group was not treated with matrine, the experimental group was treated with 1, 2, or 4 mg/mL matrine and the positive control group was treated with 20 μmol/L PD98059.

Effect of matrine treatment on HepG2 cell morphology

The morphological characteristics of HepG2 cells in all three groups were assessed, and images were acquired using an inverted phase contrast microscope.

Assessment of the migration ability of matrine using transwell assay

Three groups of HepG2 cells suspension were taken 2 μL vaccination in the room, at a concentration of 1 × 105 cells/mL, and 500 μL complete medium containing RPMI-1640 and serum was added to the lower chamber. After 24 h of crystal violet staining, five horizons were selected at random. The average number of HepG2 cells that migrated from the upper to the lower transwell chamber was calculated. The experiment was repeated three times.

Western blot analysis of extracellular signal-regulated kinase 1/2 and pERK1/2 expression in HepG2 cells treated with matrine

Proteins were extracted from the HepG2 cells of all three groups for quantitative analysis. The sample protein concentration was determined from the standard curve. The samples were subjected to SDS-PAGE in stacking and separating gels. The resolved proteins were transferred to a polyvinylidene fluoride (PVDF) membrane, which was blocked overnight at 4°C with the primary antibody and subsequently with the secondary antibody. Chemiluminescence was performed according to the ECL A/ECL B ratio of 1. The glass plate with the PVDF membrane was placed in a gel imaging analyzer, and the molecular weight and net optical density of the target protein were analyzed using a gel imaging processing system. The procedure was repeated three times.

Reverse transcription polymerase chain reaction quantification of extracellular signal-regulated kinase 1/2 mRNA expression in HepG2 cells treated with matrine

Total RNA was extracted from HepG2 cells. The lysate was placed on ice for cell lysis. RNA was separated by centrifugation at 4°C, and dissolved in 10 μL RNase-free water. For cDNA synthesis, total RNA was mixed with ×5 PrimeScript RT Master Mix (Perfect Real Time) and RNase-free dH20 and subjected to reverse transcription PCR (RT-PCR) at 37, 85, and 94°C. The following ERK1/2 primer sequences were used for RT-PCR amplification [Table 1]:

The RT-PCR results were subjected to $2^{-\Delta\Delta Ct}$ (Livak) statistical analysis.

Statistical analysis

Data were analyzed using the SPSS for Windows software, version 17.0 (Chicago, IL, USA). The correlation test was used to evaluate the statistical strength of independent associations among covariates. Two-tailed $P < 0.05$ was considered as statistically significant.

RESULTS

Assessment of the effective time and matrine concentration for inhibition of HepG2 cell proliferation using MTT assays

HepG2 cells were treated with various concentrations of matrine for 24 h. We observed that the rate of inhibition of matrine on HepG2 cell proliferation at a specific concentration increased with time. Moreover, the rate of inhibition on HepG2 cell growth gradually increased with increasing matrine concentration [Table 2 and Figure 1]. As shown in Figure 1, the 24-, 48-, and 72-h growth curves of HepG2 cells were compared. A difference between the 24- and 48-h growth curves and the 72-h growth curve was observed ($P < 0.05$). However, no difference was observed between the 48-h and 72-h growth curves ($P > 0.05$). Moreover, the slope of the 24-h growth curve of HepG2 cells treated with 1, 2, or 4 mg/mL matrine was the highest, indicating that these concentrations of matrine inhibited HepG2 cell growth over 24 h with better sensitivity. The low sensitivity of HepG2 cells to toxic drug effects may affect experimental results. Therefore, 1, 2, and 4 mg/mL matrine were selected as the effective concentrations for the inhibition of HepG2 cell growth within a 24-h period for subsequent experiments.

Effect of matrine treatment on HepG2 cell morphology

HepG2 cells in the blank control group resembled fusiform, rhomboid, and polygonal epithelial-like cells. The cells and cells were connected to each other, closely arranged, and the morphology was full, and the adherence was well, and the growth density was large, as shown in Figure 2a. As shown in Figure 2b, the number of adherent HepG2 cells decreased and the cells became spherical 24 h after treatment with 1 mg/mL matrine. As shown in Figure 2c, HepG2 cells were poorly attached, showed reduced growth density, and appeared morphologically incomplete with transparent particles when treated with 2 mg/mL matrine for 24 h. The number of cells was significantly reduced and the morphology was extremely irregular 24 h after treatment with 4 mg/mL matrine. Most cells appeared spindle shaped and extended tentacles to contact the neighboring scattered cells. The cell density was significantly reduced, and necrotic cell debris was observed [Figure 2d].

Effect of matrine treatment on HepG2 cell migration

In the experimental group, the number of HepG2 cells penetrating the basement membrane of the small chamber was reduced, and their invasion and penetration abilities weakened at high concentrations of matrine. As shown in Figure 3a-c, the density of tumor cells decreased gradually. The positive control and experimental (4 mg/mL matrine) group showed similar results [Figure 3d]. In the blank control group, HepG2 cell migration remained unaffected. As shown in Figure 3e, a large number of cells passed through the basement membrane of the compartment after 24 h. Dense clusters of the cells were formed as the HepG2 cell nucleus was not severely damaged by drug treatment.

HepG2 cells migrated into the lower chamber. The average migration rates of HepG2 cells in the experimental group were

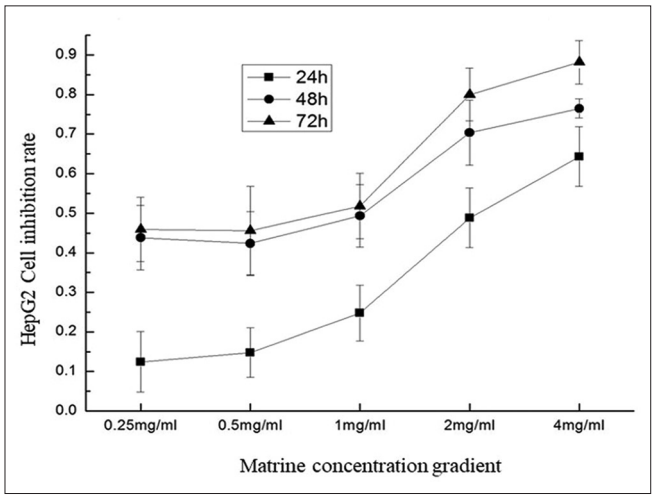

Figure 1: Inhibition rate of matrine at different concentrations on HepG2 cells

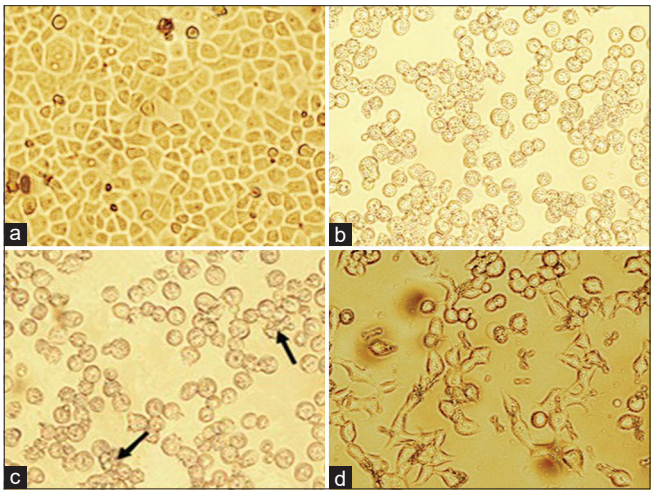

Figure 2: Morphological changes of HepG2 cells after matrine intervention. (a) Blank control group, (b) 1 mg/mL matrine, (c) 2 mg/mL matrine, incomplete cells can be seen at the arrow, (d) 4 mg/mL matrine. All above were observed under 100 times microscope

Table 1: Primer sequence of ERK1/2 and ERK1/2

Gene	Upstream sequence	Downstream sequence
ERK1/2	5'-TATGCTAATCCTGCTGTGTTGTCAT-3'	5'-AAATGTTGTGCAGGCGAATG-3'
B-actin	5'-GCACCGTCAAGGCTGAGAAC-3'	5'-TGGTGAAGACGCCAGTGGA-3'

The primer pairs used were as follows in order to reverse transcribed RNA

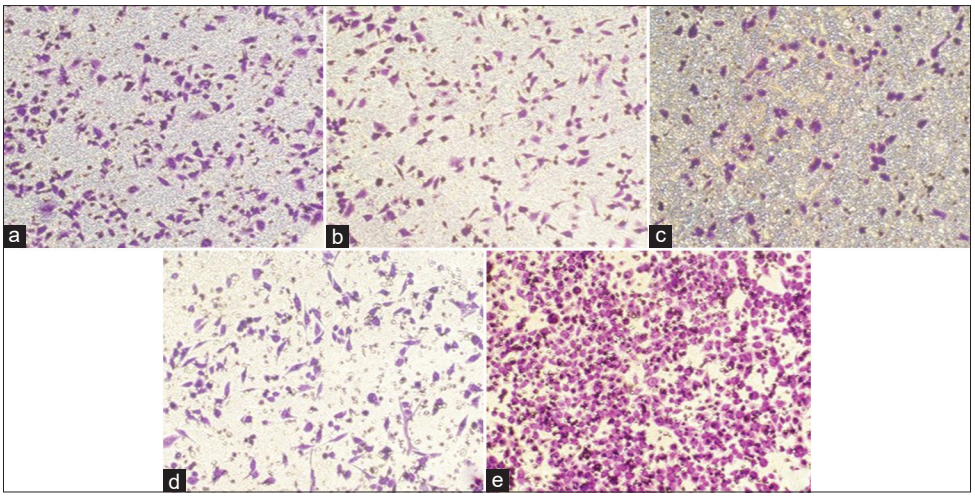

Figure 3: Effect of each group on the migration ability of HepG2 cells. (a) Migration status of HepG2 cells after 1 mg/mL matrine treatment. (b) Migration status of HepG2 cells after 2 mg/mL matrine treatment. (c) Migration status of HepG2 cells after 4 mg/mL matrine treatment. (d) Migration status of HepG2 cells after 20 μmol/mL PD98059 treatment. (e) Blank control group HepG2 cell migration status. All above were observed under 100 times microscope

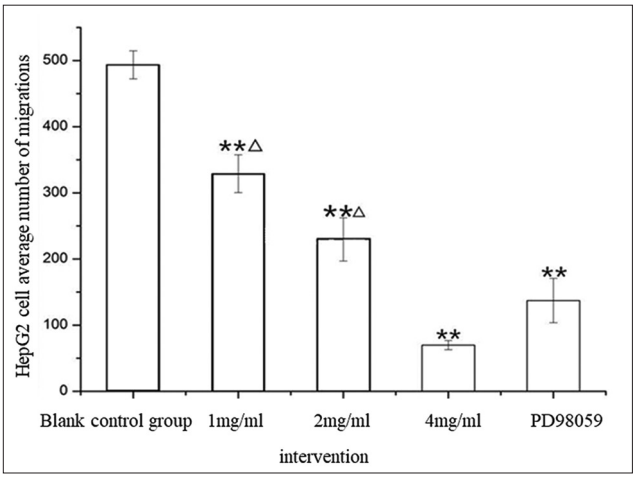

Figure 4: Number of HepG2 cell migration in each group. Note: $^{**}P < 0.01$ compared with the blank control; ΔIs compared with PD98059 positive control group, $P < 0.05$

365.80 ± 58.53, 236.00 ± 42.644, and 69.80 ± 6.94, while that in the blank control group was 501.6 ± 21.26. The number of migrated cells was significantly lower in the experimental group than that in the blank control group ($P < 0.01$). Moreover, the number of migrated HepG2 cells in the 1 mg/mL and 2 mg/mL matrine treatment groups was statistically different from that in the positive control group ($P < 0.05$). However, there was no difference in the 4 mg/mL matrine treatment group ($P > 0.05$). The number of migrated HepG2 cells in the experimental groups treated with 1, 2, and 4 mg/mL was reduced by 27.0%, 53.0%, and 86.1%, respectively, compared with that in the blank group [Figure 4].

Matrine inhibits the expression of extracellular signal-regulated kinase 1/2 and pERK1/2 in HepG2 cells

In the experimental group, the expression of ERK1/2 and pERK1/2 was downregulated with the increase of matrine concentration. ERK1/2 expression in HepG2 cells treated with 1 mg/mL matrine was not significantly different from that in the blank control group ($P > 0.05$). However, ERK1/2 expression in HepG2 cells treated with 2 mg/mL and 4 mg/mL matrine was significantly different ($P < 0.01$). pERK1/2 expression was significantly different among HepG2 cells treated with 1, 2, and 4 mg/mL matrine ($P < 0.01$). ERK1/2 expression in HepG2 cells treated with 1 m, 2, and 4 mg/mL matrine was significantly different from that in the positive control group ($P < 0.01$). Moreover, pERK1/2 expression in HepG2 cells treated with 1 mg/mL and 2 mg/mL matrine was significantly different ($P < 0.01$). However, pERK1/2 expression in HepG2 cells treated with 4 mg/mL matrine was not significantly different from that in the positive control group [Table 3 and Figures 5, 6].

Table 2: Inhibitory rates of matrine at different concentrations on HepG2 cells in different time periods

Matrine concentration (mg/mL)	Time group		
	24 h	48 h	72 h
0.25	0.12 0.03	0.44 0.08	0.46 0.08
0.5	0.15 0.05	0.46 0.06	0.48 0.11
1	0.25 0.07	0.49 0.08	0.52 0.08*
2	0.49 0.08	0.70 0.08**	0.79 0.07**
4	0.64 0.08*	0.76 0.02**	0.88 0.06**

*Compared with the concentrations of 0.25, 0.5, 1 and 2 mg/mL matrine in the same period, $P<0.05$; **Compared with the concentrations of 0.25, 0.5, 1 and 2 mg/mL matrine in the same period, $P<0.01$

Matrine downregulated extracellular signal-regulated kinase 1/2 mRNA expression in HepG2 cells

The treatment of HepG2 cells with matrine significantly decreased ERK1/2 mRNA expression. Compared with the blank control and positive control groups, there were significant differences between HepG2 cells treated with 2 mg/mL and 4 mg/mL matrine ($P < 0.05$). There was no difference in the HepG2 cells treated with 1 mg/mL matrine ($P > 0.05$), indicating that 2 mg/mL and 4 mg/mL matrine treatment

can significantly downregulate ERK1/2 mRNA expression in HepG2 cells [Table 4 and Figure 7].

DISCUSSION

HCC presents the poor prognosis and high recurrence rate which is closely related to the malignant biological behavior of proliferation and migration.[3] The ERK1/2 MAPK signaling pathway regulates the growth, development, division, and death of tumor cells, and its abnormal activation promotes proliferation and migration of tumor cells.[6] Therefore, inhibiting the activation of critical signaling pathways is extensively studied. In addition, inhibiting the proliferation and migration of hepatocarcinoma cells becomes a hot research for the research and development of anti-HCC drugs.

Table 3: ERK1/2 and pERK1/2 protein

Group	ERK1/2	pERK1/2
Blank control group	1.47±0.55	1.76±0.05
1 mg/mL	1.47±0.43$^{\Delta\Delta}$	1.34±0.18$^{\Delta\Delta}$
2 mg/mL	1.18±0.67*$^{\Delta}$	0.83±0.42**$^{\Delta\Delta}$
4 mg/mL	0.97±0.78**	0.47±0.01**
Positive control group	0.14±0.60	0.10±0.00

The statistical method of gray value is: Measuring integral optical density, integral optical density=Strip area × average density. *Is the comparison between the experimental group and the blank control group, P<0.05, **<0.01. $^{\Delta}$Is the comparison between the experimental group and positive control group, P<0.05, $^{\Delta\Delta}$<0.01

Table 4: Expression of ERK1/2 mRNA in HepG2 cells

Group	ERK1/2 mRNA ($2^{\Delta\Delta}$)
Blank control group	2.53±0.31
1 mg/mL	1.65±0.59
2 mg/mL	0.40±0.86*$^{\Delta}$
4 mg/mL	0.14±0.20*$^{\Delta}$
Positive control group	1.44±0.39

*Is compared with the blank control group (P<0.05). $^{\Delta}$Is compared with positive control group (P<0.05). Matrine 1mg/mL was significantly different from 4 mg/mL and 2 mg/mL with P<0.05 and no difference between 4 mg/mL and 2 mg/mL

ERK1/2 signaling pathways controls the growth, development, division, and death of tumor cells, and its abnormal activation promotes the malignant proliferation and migration of tumor cells.[7] At present, several studies have demonstrated that the activation of the ERK1/2 signaling pathway is closely related to HCC pathogenesis. It has been found that upregulation of MKP-4 can dephosphorylate ERK1/2 and inhibit the proliferation of HCC cells. As one of the MAPK signaling pathways, ERK1/2 controls the growth, development, division, and death of tumor cells, and its abnormal activation promotes the malignant proliferation.[8] In addition, Liang *et al*.[9] found that through targeted adjustment the GRB2-ERK1/2-AKT axis can inhibit the proliferation and invasion of SMCC-7721 cells. Therefore, the intervention of ERK1/2 signaling pathway is a fatal issue to inhibit the occurrence and development of HCC.

There are abundant Chinese herbal medicines in China. Studies have shown that some extracts of Chinese herbal medicine have significant anti-HCC effects, such as curcumin[10] and salvianolic acid B.[11] Matrine, as an alkaloid extracted from Sophora flavescens, also has certain antitumor effects. Many literatures reported that the mechanism is mainly to interfere with the growth cycle of cancer cells, so as matrine can blocked G2/M stage.[12,13] It can break ATK/mTOR signaling pathway to promote apoptosis of cancer cells and inhibit proliferation.[14]

In this study, we assessed the ability of matrine to inhibit the proliferation and migration of HCC cells by interfering with the ERK1/2 signaling pathway. We found that different concentrations of matrine affect the morphology of HepG2 cells. The number of adherent HepG2 cells decreased with the increase in matrine concentration. The morphology of HepG2 cells appeared spherical, incomplete, and even as cell fragments [Figure 2]. We also found that matrine inhibits the proliferation and migration of HepG2 cells in a concentration-dependent manner [Table 1 and Figures 1-4].

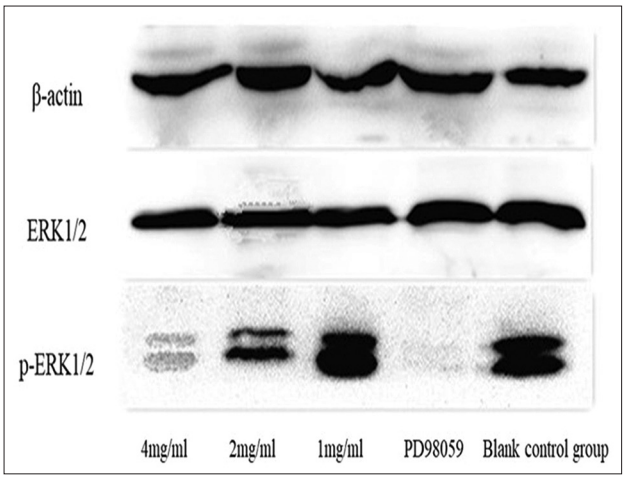

Figure 5: Extracellular signal-regulated kinase 1/2 and pERK1/2 expression bands by Western blot. Note: the statistical method of gray value is: measuring integral optical density, integral optical density = strip area × average density

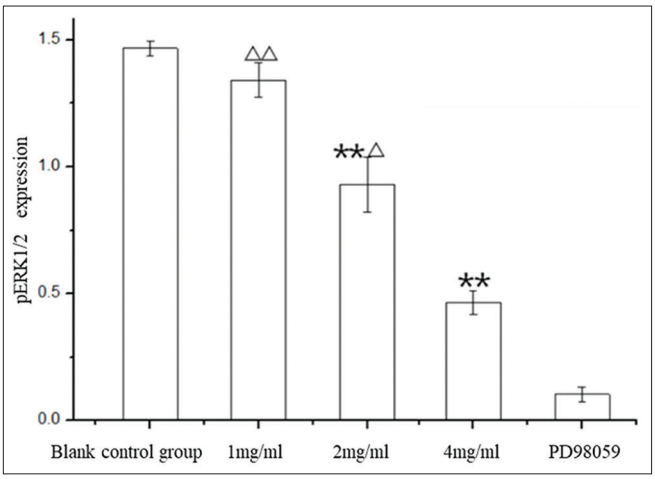

Figure 6: pERK1/2 expression Note: *Is the comparison between experimental group and blank control, P < 0.05, **<0.01. Δ is the comparison between experimental group and positive control group, P < 0.05, ΔΔ <0.01

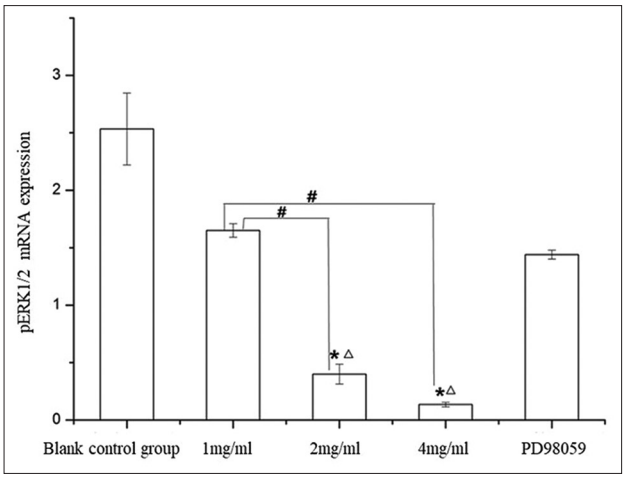

Figure 7: Extracellular signal-regulated kinase 1/2 mRNA expression in each group after matrine intervention Note: *Is compared with blank control group ($P < 0.05$). Δ is compared with positive control group ($P < 0.05$). #For experimental comparison is 4 mg/mL that is different from 1 and 2 mg/mL, and there is no difference between 1 and 2 mg/mL ($P < 0.05$)

The mRNA expression of ERK1/2, pERK1/2, and ERK1/2 in HepG2 cells was examined at transcriptional and translation levels after matrine treatment. The mRNA expression of ERK1/2, pERK1/2, and ERK1/2 decreased with increasing matrine concentrations, indicating that the treatment of HepG2 cells with matrine reduces the activity of the ERK1/2 signaling pathway. Proliferation and migration signals were transmitted to inhibit HCC [Tables 2, 3 and Figures 5-7]. A previous study has reported that WM130, a matrine derivative, can inhibit proliferation, invasion, migration, and induce apoptosis of HCC cells through the EGFR/ERK/MMP-2 signaling pathway.[15] This result is consistent with the results of this study. Similarly, it has been reported that matrine can inhibit autophagy and induce apoptosis of HCC cells by mediating the interaction between the JNK-Bcl-2/Bcl-xL-Bax/Bak MAPK signaling pathway and Beclin1.[16] However, there has been no report demonstrating the ability of matrine to inhibit the proliferation and migration of HCC cells through the ERK1/2 signaling pathway. According to the results of this and previous studies, matrine downregulates the ERK1/2 signaling pathway and inhibits the proliferation and migration of HCC. These results suggest that the ERK1/2 signaling pathway can serve as a specific target for matrine for the treatment of HCC. This study provides a theoretical basis for the molecular targeting therapy of HCC.

Financial support and sponsorship

Scientific Research Project of Science and Technology Bureau, Lanzhou, Fund number: 2015-2-68.

Key Project of Tianjin Science and Technology Support Program (15ZCZDSY00890), Oncology Intervention Research Fund of China Health Promotion Foundation (XM-2018-011-0006-01).

Conflicts of interest

There are no conflicts of interest.

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