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Original Research Article

Matrine induces cell cycle arrest and apoptosis in hepatocellular carcinoma cells via miR-122 mediated CG1/livin/survivin signal axis

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Abstract

Purpose: *To study the impact of matrine on cell cycle and apoptotic changes in hepatoma cells, and the mechanism involved.*
Methods: *Human hepatoma cell line HepG2 was treated with different concentrations of matrine. The blank control cells were maintained in 1640 medium only. The influence of matrine on proliferative ability was determined with 3-(4,5)-dimethylthiahiazo (-z-y1)-3,5-di- phenytetrazoliumromide (MTT) method. Flow cytometry was used to determine its effect on cell cycle and apoptosis; RT-PCR (reverse transcription-polymerase chain reaction) was applied to assay the mRNA expressions of miR-122, cyclin G1 (CG1), livin and survivin mRNA, while the protein expressions of CG1, livin and surviving were assayed by Western blotting.*
Results: *Matrine time- and dose-dependently suppressed the proliferative capacity of the cells. At a concentration of 0.5 mg/mL, matrine had no significant effect on the cell cycle. However, 1.0 mg/mL matrine blocked the cell cycle in G1 phase, while 1.5 mg/mL matrine blocked HepG2 cells in G2/M phase ($p < 0.05$). Moreover, matrine induced apoptosis in HepG2 cells, and markedly downregulated the expressions of miR-122 concentration- time-reliantly ($p < 0.05$). In addition, matrine markedly and concentration-dependently reduced mRNA and protein expression levels of CG1, livin and survivin, with the strongest inhibitory effect at a level of 1.5 mg/mL.*
Conclusion: *Matrine induces cell cycle block and apoptotic changes in hepatoma cells through a mechanism related to regulation of the CG1/livin/survivin signal axis mediated by miR-122. Matrine may be a potential treatment for liver cancer. However, clinical trials are needed to confirm this potential.*

Keywords: *Matrine, miR-122, CG1/livin/survivin signal axis, Hepatoma cells, Stagnation, Apoptosis*

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INTRODUCTION

Liver cancer is a frequently diagnosed primary carcinoma, accounting for about 90 % of all cases. China accounts for more than half of the 1 million new cases of HCC worldwide, and in recent years, cases of HCC have been increasing markedly. In addition, liver cancer is not easily diagnosed at the early stages, and it progresses rapidly. Thus, at the point of diagnosis, most patients are already in the middle and late stages of the disease, thereby limiting the clinical effectiveness of treatment, shortening the survival time and increasing mortality rate [1]. The pathogenesis of liver cancer is associated with multiple factors and multiple gene interactions. Some of the factors associated with incidence of HCC are hepatitis B virus infection, aflatoxin, alcohol and nitrosamines [2].

At present, the clinical treatment of hepatocellular carcinoma focuses mainly on surgery in combination with radiotherapy and chemotherapy. However, due to the adverse side effects of radiotherapy and chemotherapy, drug resistance and other problems may arise. Traditional Chinese medicine has unique advantages in enhancing the survival of patients with advanced hepatocellular carcinoma. Matrine is a bioactive substance extracted from dried roots of *Sophora flavescens*. It has antiviral, anti-inflammatory, sedative, antihypertensive and immunomodulatory properties [3]. Investigations have shown that matrine effectively inhibited the proliferative and metastasis potential of tumors, and induced apoptosis and differentiation [4]. MicroRNA (miRNA) plays an important role in tumorigenesis. It inhibits the translation of target gene by binding to its 3'-terminal non-coding region. It has been reported that miR-122 is one of the unique miRNAs which regulate the proliferation liver cells and lipid metabolism [5,6].

The aim of study was to investigate the effect of matrine on hepatoma cells, as well as the underlying mechanism.

EXPERIMENTAL

Reagents and instruments

Human hepatocellular carcinoma cell line HepG2 was obtained from Shanghai Yiyan Biotechnology Co. Ltd. The reagents and instruments used, and their suppliers (in brackets) were: matrine (Aimeijie Technology Co. Ltd), calf serum (ChuzhouShinoda Biotechnology Co. Ltd), PCR Amplification Kit (Beijing See Microgene Technology Co. Ltd), miRNA Reverse Transcription Kit (GeneCopoeia, USA); protein pre-staining marker (Beijing Beyosentai Biotechnology Co. Ltd), Trizol extract and RIPA protein lysate (Shanghai Yanjing Biotechnology Co. Ltd), and primary antibodies against cyclin G1 (CG1), livin and survivin (American Cell Signaling Company).

The instruments used and their sources were: ultra-pure water meter (Beijing Zeping Science and Technology Co. Ltd), micro-high-speed low-temperature centrifuge (Sichuan Shuke Instrument Co. Ltd), pressure steam disinfector (Nanjing Beden Medical Co. Ltd), porous plate enzyme marker (Meigu Molecular Instrument (Shanghai) Co. Ltd), constant-temperature water bath (Shanghai Fuze Commercial Co. Ltd), flow cytometer (Shanghai Ranzhe Instrument Equipment Co. Ltd), electrophoresis instrument (Beijing Yiaobai Co. Ltd.), gel imaging system (Shanghai Jinpeng Analytical Instrument Co. Ltd.), and UV spectrophotometer (Beijing Anmage Trade Co. Ltd.).

Cell culture and grouping

Frozen HepG2 cells were taken out and thawed in a 37 ºC water bath. They were maintained in IMDM spiked with FBS (10 %) at 37 ºC and 5% CO_2. The cells were permitted to attain 80 -90 % confluence prior to digestion and passaging. Three groups of cells at logarithmic phase were set up. They were exposed to matrine at doses of 0.5, 1.0, 1.5 mg/mL. Cells in the blank control received 1640 medium only.

Determination of influence of matrine on proliferative potential

This was determined with MTT assay. Logarithmic growth-phase cells were seeded in 96-well plates after removal of culture medium and rinsing with PBS. They received either matrine at doses of 0.5, 1.0, 1.5 mg/mL, or 1640 medium only, followed by culturing at 37 ℃ and 5 % CO_2 for 48 h. After 44 h of culture, 10 µl of MTT (5 mg/mL) was put into every well, followed additional 4 h of culture. Thereafter, the supernatant was replaced with 150 µl DMSO to dissolve the formazan crystals formed. The absorbance of the solution in each well was read at 490 nm. Inhibition of cell proliferation (H) was calculated as shown in Eq 1.

$$H (\%) = \{(1 - Ae)/Ae\}100 \quad \text{..... (1)}$$

where *Ae* is absorbance of experimental group and *Ac* is absorbance of control group.

Determination of influence of matrine on HepG2 cell cycle

This was determined using flow cytometry. Cells at logarithmic phase were inoculated in 6-well plates (5 x 10^4 cells/well), and were treated separately with matrine at doses of 0.5, 1.0 and 1.5 mg/mL. A blank control group was set up. After 48 h of continuous culture, the cells were subjected to digestion and counted. Then, the cells (1 x 10^6 cells) were re-suspended in cooled absolute ethanol and centrifuged for 12 h, and re-suspended once more. again. This was followed by addition of 50 µl RNase solution and 400 mL of propidium bromide staining solution. After a 10-min incubation in darkness, the cells were analyzed using a flow cytometer.

Determination of effect of matrine on mRNA expressions

The mRNA expressions levels of miR-122, CG1, livin and survivin in HepG2 cells were determined using RT-PCR. TRIzol was used to extract total RNA from HepG2 cells in logarithmic growth phase. The content and integrity of the RNA extract were determined. Diluted RNA samples were subjected to reverse-transcription. The enzyme RNase was inactivated at 37 ℃ for 1 h and at 85 ℃ for 5 min. The resultant cDNA was subjected to PCR, with GAPDH as internal reference gene, under the conditions: pre-denaturation at 95 ℃ for 10 min, 95 ℃ for 10 sec, 57 ℃ for 20 sec, and 72 ℃ for 10 sec. The Ct values of each target gene were obtained, and the relative gene expressions were calculated using the $2^{-\triangle\triangle CT}$ method.

Determination of effect of matrine on protein expressions

Extraction of total from cells in various groups was done with RIPA buffer, and protein concentration was measured with BCA method. Following SDS-PAGE, the proteins were transferred to PVDF membrane, and the membrane was incubated with PBS solution without fat milk powder for 1 h, shaken slowly for 2 h, and washed thrice with PBST. Thereafter, the membrane was incubated overnight at 4 ℃ with primary antibodies against CG1, livin and survivin, each diluted 1:100. Thereafter, the membrane was rinsed thrice with TBST and incubated with HRP-linked 2º antibody for 1 h at laboratory temperature, followed by rinsing with PBST. The protein bands were developed using electro-chemiluminescence.

Statistical analysis

All measurement data are presented as mean ± standard deviation. Independent sample *t*-test was used for two-group comparison, while comparison amongst many groups was performed with ANOVA. Values of $p < 0.05$ were taken as indicative of statistical significance. All statistical analyses were carried out with SPSS20.0 software package.

RESULTS

Matrine suppressed cell proliferative capacity

Matrine suppressed proliferative capacity of HepG2 cells. These results are shown in Table 1

Table 1: Influence of matrine on HepG2 cell proliferation

Matrine concentration (mg/mL)	24h	48h	72h
0.025	5.58±3.72	10.11±7.74	9.31±8.26
0.05	8.84±3.62	12.11±7.52	12.46±10.13
0.1	10.47±5.26	16.25±7.85	16.13±12.88
0.5	18.85±11.42	29.58±6.62	37.45±12.41
1.0	24.42±8.74	34.85±3.62	45.21±11.22
1.5	35.78±16.52	57.62±14.03	67.05±15.47

Values are mean ± SD

Influence on cell cycle

Based on the results of MTT assay, matrine was used at concentrations of 0.5, 1.0 and 1.5 mg/mL in the determination of its effect on cell cycle. At a concentration of 0.5 mg/mL, matrine produced no significant influence on the cell cycle (Table 2). However, 1.0 mg/mL dose blocked the cells in G1 stage, while at the higher dose of 1.5 mg/mL, it blocked the cycle in G2/M stage.

Table 2: Influence of matrine on cell cycle

Group	G1 stage	G2/M stage	S stage
Control	64.12±6.23	11.52±2.13	24.26±4.45
Matrine (0.5mg/mL)	62.85±7.54	11.44±2.60	25.61±9.32
Matrine (1.0mg/mL)	75.23±6.48*	7.21±1.87	17.56±5.52
Matrine (1.5mg/mL)	51.12±7.03	29.11±9.05*	19.78±3.15

Values are mean ± SD. *$P < 0.05$, vs the control

Influence of matrine on apoptosis of HepG2 cells

As shown in Table 3, matrine time- and dose-dependently induced apoptosis in HepG2 cells (*p*

< 0.05).

Table 3: Apoptotic effect of matrine

Group	24h	48h	72h
Control	5.82±0.62	7.78±1.52	7.77±1.84
Matrine (0.5mg/mL)	6.74±0.86	12.12±1.26	18.84±2.32
Matrine (1.0mg/mL)	10.62±0.48	28.26±6.13	36.42±2.33
Matrine (1.5mg/mL)	17.47±6.23	54.82±13.52	67.68±8.45

Values are presented as mean ± SD

Effect of matrine on miR-122 in HepG2 cells

Relative to control group, matrine markedly downregulated the expression of miR-122 in HepG2 cells, while the expression of miR-122 was markedly upregulated (p < 0.05). These results are presented in Figure 1.

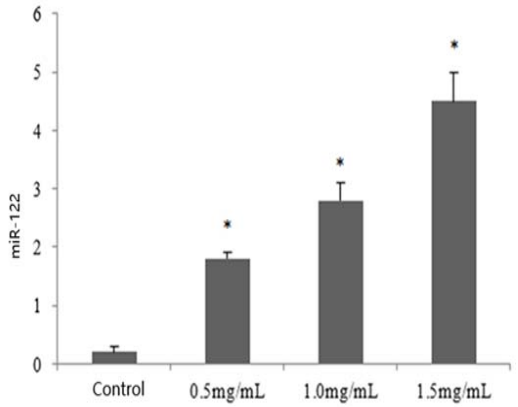

Figure 1:: Effect of matrine on the expression of miR-122 in HepG2 cells. $^{*}P$ < 0.05, vs control

Effect of matrine on CG1/livin/survivin signaling pathway in HepG2 Cells

As shown in Table 4 and Figure 2, matrine dose-dependently downregulated the mRNA and protein expressions of CG1, livin and survivin, relative to control.

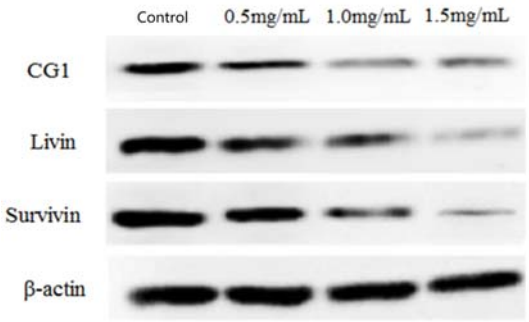

Figure 2: Effect of matrine on protein expressions of CG1, livin and surviving

DISCUSSION

Extracts from Chinese Traditional Medicine (TCM) have been widely used in clinical practice to induce apoptosis in tumor cells. The involvement of inhibition of telomerase activity in the anti-cancer effects of drugs has continued to attract the attention of researchers. Studies have shown that TCMs induce apoptosis by arresting the cell cycle at some phases, and studies have demonstrated that telomerase plays an important role in cell immortalization and malignancy [7,8]. Matrine, an effective anticancer compound isolated from *Sophora flavescens*, is a tetracyclic quinazine molecule with numerous pharmacological properties. Studies have demonstrated that matrine suppresses tumor proliferation, and induces apoptosis and tumor cell adhesion [9]. In addition, matrine reverses drug resistance of tumors, mitigates the toxic and adverse side effects of chemotherapeutic agents, and enhances the survival of cancer patients.

Some investigators have reported that oxymatrine blocked the G0/G1 stage of hepatoma cell lines, decreased fraction of cells in S phase, and inhibited cell proliferation [10]. Moreover, matrine inhibited the proliferation of hepatoma cell line SMMC-7721 in a dose-dependent manner. At moderate concentrations, oxymatrine inhibited tumor cell growth, but it had

Table 4: Effect of matrine on the mRNA and protein expressions of CG1, livin and survivin in HepG2 cells

Group	CG1		Livin		Survivin	
	mRNA	Protein	mRNA	Protein	mRNA	protein
Control	0.46±0.05	0.54±0.02	0.48±0.02	0.70±0.03	0.44±0.03	0.67±0.04
Matrine (0.5mg/mL)	0.37±0.04*	0.47±0.03*	0.41±0.01*	0.63±0.04*	0.35±0.04*	0.59±0.04*
Matrine (1.0mg/mL)	0.25±0.03*	0.33±0.21*	0.28±0.04*	0.55±0.05*	0.30±0.02*	0.42±0.03*
Matrine (1.5mg/mL)	0.19±0.02$^{*\#}$	0.20±0.02$^{*\#}$	0.21±0.05$^{*\#}$	0.36±0.09$^{*\#}$	0.18±0.04$^{*\#}$	0.30±0.04$^{*\#}$

Values are mean ± SD. $^{*}P$ < 0.05, vs control; $^{\#}p$ < 0.05, vs matrine groups

very minimal effect on cell growth at low concentrations [11].

MicroRNAs (miRNAs) are a non-coding single-stranded RNAs with lengths of 19-25 nucleotides. They participate in the regulation of cell proliferation, differentiation and apoptosis. These miRNAs regulate the expressions of several target genes at the translation level through generation of one or more complementary base pairs of partial sequences of mRNA [12]. In tumorigenesis, different miRNAs may act on the same target gene to form a complex regulatory network. High or low expressions of miRNAs are akin to the effects of oncogenes or tumor suppressor genes, and are involved in the formation, survival and migration of tumors. Similar variations in the expressions of specific miRNAs are implicated in the pathogenesis and growth of hepatocellular carcinoma [13]. About 70 % of the total liver miRNAs is MiR-122 which is one of the important regulators of liver differentiation [14]. Some researchers have found that miR-122 regulates cell proliferation and DNA repair by downregulating *CG1* [15]. Livin and survivin are anti-apoptosis proteins closely related to apoptosis regulatory genes.

The results of this investigation indicate that matrine blocked HepG2 cell proliferative capacity in a time- and dose-based fashion: 1.0 mg/mL blocked the cells in G1 stage, but 1.5 mg/mL blocked the cells in G2/M phase. Moreover, it was found that matrine triggered apoptotic changes in these cells. These results suggest that matrine suppresses cell proliferation and promotes apoptosis by prolonging cell cycle through inhibition of the growth of HepG2 cells. In addition, matrine upregulated miR-122 and downregulated the expressions of CG1, livin and survivin. Thus, matrine enhanced apoptosis of hepatoma cells through a mechanism involving the CG1/livin/survivin signaling pathway.

CONCLUSION

Matrine induces apoptotic changes and cell cycle block in HCC cells through a mechanism involving regulation of the miR-22-mediated CG1/livin/survivin signaling pathway. Matrine may be a potential treatment for liver cancer. However, clinical trials are needed to confirm this potential.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this work.

Authors' contribution

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Haiqing Hua was involved in the conception and design of the study, manuscript revision, funding support and study supervision. Zhongjian Pu contributed to design of the study as well as the experiments, data analysis and manuscript writing. Others contributed to the design of the study and interpretation of the data. All authors read and approved the final manuscript.

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