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Original Article

Matrine induces cell apoptosis and necroptosis dependent on RIPKs of lung cancer cells

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Abstract: Background: Lung cancer is a malignant tumor with high incidence and morality. Matrine is an alkaloid extracted from the dried roots of traditional Chinese medicine Sophora flavescens. In the present study, we aim to explore the antitumor efficiency of matrine in lung cancer cells, as well as its molecular mechanism in lung cancer cell lines. Material & method: The lung cancer cells were treated with increasing concentrations of matrine and cell viability was determined by MTT assay. Cell apoptosis rate and necrosis rate was determined by FACS assay. The levels of RIP-1 and RIP-3 were determined by western blotting analysis. Results: The MTT assay results demonstrated that matrine inhibited the proliferation of A549 and H460 in a time- and dose-dependent manner. Moreover, FACS assay results demonstrated that matrine could enhance cell apoptosis rate and necrosis rate as the increasing concentrations of matrine at 24 hours. Importantly, the matrine-induced cell death could be partly reversed by the pancasepase z-VAD-fmk suggesting that matrine-induced cell apoptosis contributed to the cell death of lung cancer cells. Additionally, western blotting analysis results demonstrated that RIP-1 and RIP-3 levels significantly increased as the increasing concentrations of matrine suggesting matrine promoted the expression of RIP-1 and RIP-3. Conclusion: In conclusion, matrine induced cell apoptosis and necroptosis of lung cancer cells. It has a potential to be used as an effective drug to treat human lung cancers.

Keywords: Matrine, apotosis, necroptosis, RIP-1, RIP-3

Introduction

Traditional Chinese herbal medicine originated in ancient China, which has evolved over thousands of years [1-3]. It is still used as a major part of healthcare provision in China, which is also used in the clinical therapy for various cancers in China [3-5]. Recently, looking for a safe and effective anticancer drug with little toxic side effects has become the hotspot, which is also helpful in the therapy of cancers. As the development of analysis methods in biochemistry and molecular biology, a large amount of the active compounds was extracted from Chinese Herbal Medicine [6-8]. Matrine is an alkaloid found in plants of the *Sophora genus*, with the molecular formula of $C_{15}H_{24}N_{20}$, which is one of the active components and extracted from the dried roots of traditional Chinese medicine *Sophora flavescens* [9, 10]. A large number of pharmacological and clinical studies have found that matrine has the anti-fibrosis, anti-arrhythmic, immunosuppressive, anti-inflammatory, anti-hypertensive, vascular remodeling, anti-tumor and other pharmacological effects etc [11-15].

Recently, muchstudies have found matrine induced cell death *in vitro* and *in vivo* on a variety of tumor cells. However, the exact molecular mechanism is not clearly clarified till now. Li, H. has found that matrine suppressed cell proliferation of human breast cancer cells by promoting cell apoptosis *via* upregulation of Bax and downregulation of Bcl-2 [16]. It also reported that matrine inhibited the invasion and metastasis of lung cancer cell by regulating the expression levels of miRNAs, such as elevating expression of miR-133a which further suppressed the activation of EGFR/Akt/MMP-9 pathway [17]. The combination therapy with matrine and platinum-based doublet chemotherapy (PBDC) versus PBDC alone was reported to treat advanced non-small cell lung cancers and the results revealed that matrine combined with PBDC decreased the incidence of

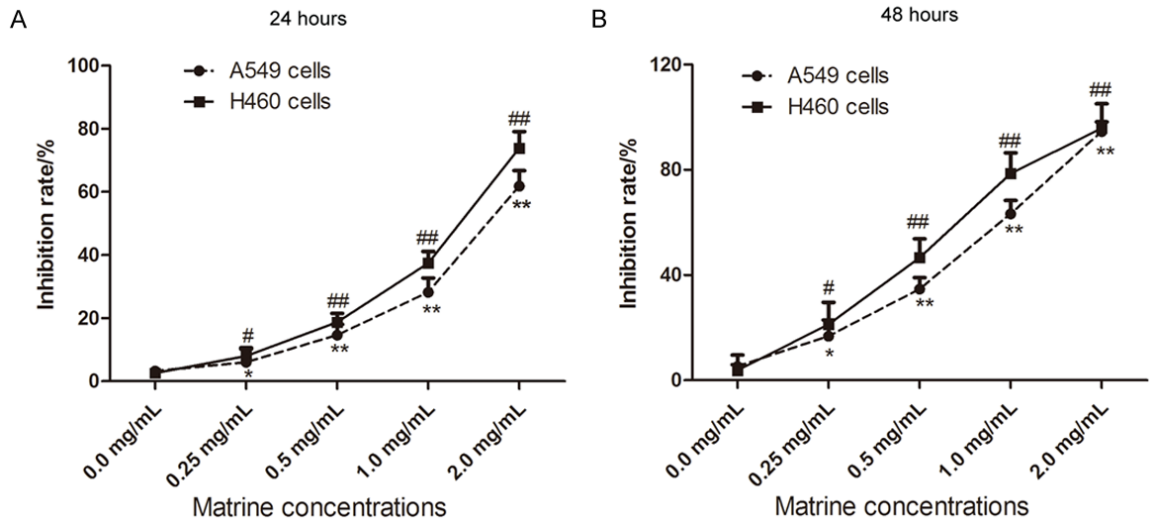


Figure 1. Matrine inhibits the proliferation of lung cancer cells in a dose-dependent manner. A549 cells and H460 cells were treated with increasing concentrations of matrine for 24 hours (A) and 48 hours (B). MTT assay was used to determine the cell survival rate of lung cancer cells. ##P<0.01, compared with negative H460 cells. **P<0.01, compared with negative A549 cells.

adverse reactions compared with PBDC alone [18]. Moreover, an involvement of the NF-kappa B signaling pathway was reported in matrine-treated cancer cells, in which, matrine inhibited the proliferation, invasion and migration of castration-resistant prostate cancer cells by decreasing the levels of P65, p-P65, IKKalpha/beta, p-IKKalpha/beta, IKBalpha and p-IKBalpha [19].

In cancer therapy, one idea is to effectively induce cell death of tumor cells. As we known, programmed cell death, such as apoptosis, autophagy, and necroptosis, played an important role in the development and progression of cancers [20-22]. In the present study, we used human lung cancer cells A549 and H460 as the cell model and explored whether programmed cell death was induced in matrine-treated lung cancer cells. It is helpful to find the new targets in the therapy of lung cancers.

Material and methods

Cell lines and agents

The lung cancer cell lines (A549 and H460) were purchased from American Type Culture Collection (Manassas, VA). The cells were cultured in Dulbecco's Modified Eagles Medium (DMEM) with 10% fetal bovine serum at 37°C in an atmosphere of 95% air and 5% CO_2. DMEM was purchased from Hyclone corporation (Logan, Utah, USA) and FBS was obtained from Gibco Life Technologies. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (Cat. No. M2128) was purchased from Sigma-Aldrich Corporation (Sigma). Mouse Anti-Human/Mouse/Rat RIPK1/RIP1 monoclonal antibody (Catalog # MAB3585) was purchased from R&D Corporation. RIP-3 Antibody (B-2) is a mouse monoclonal IgG1 provided at 200 µg/ml (Cat. No. sc-374639) and obtained from Santa Cruz corporation. HRP-conjugated anti-mouse IgG secondary antibody (Cat. No. A3682) was also purchased from Santa Cruz Corporation.

MTT assay

Cell viability was determined by MTT assay. The A549 cells and H460 cells were treated with matrine for 24 hours and 48 hours. The concentration of matrine was 0.25 mg/ml, 0.5 mg/ml, 1.0 mg/ml and 2.0 mg/ml. In the other experiment, the lung cancer cells were treated with different concentration of matrine (0.25 mg/ml, 0.5 mg/ml, 1.0 mg/ml and 2.0 mg/ml) in combination with 10 µM of z-VAD-fmk or 0.1% DMSO for 24 hours. Four hours before test, 10 µL of MTT solution (5 mg/mL) was added into the cultured medium and incubated at 37°C in 5% CO_2. The absorbance of each well was read at 490 nm.

FACS assay

Annexin V–fluorescein isothiocyanate (FITC)/PI dural staining was used to detect the cell apop-

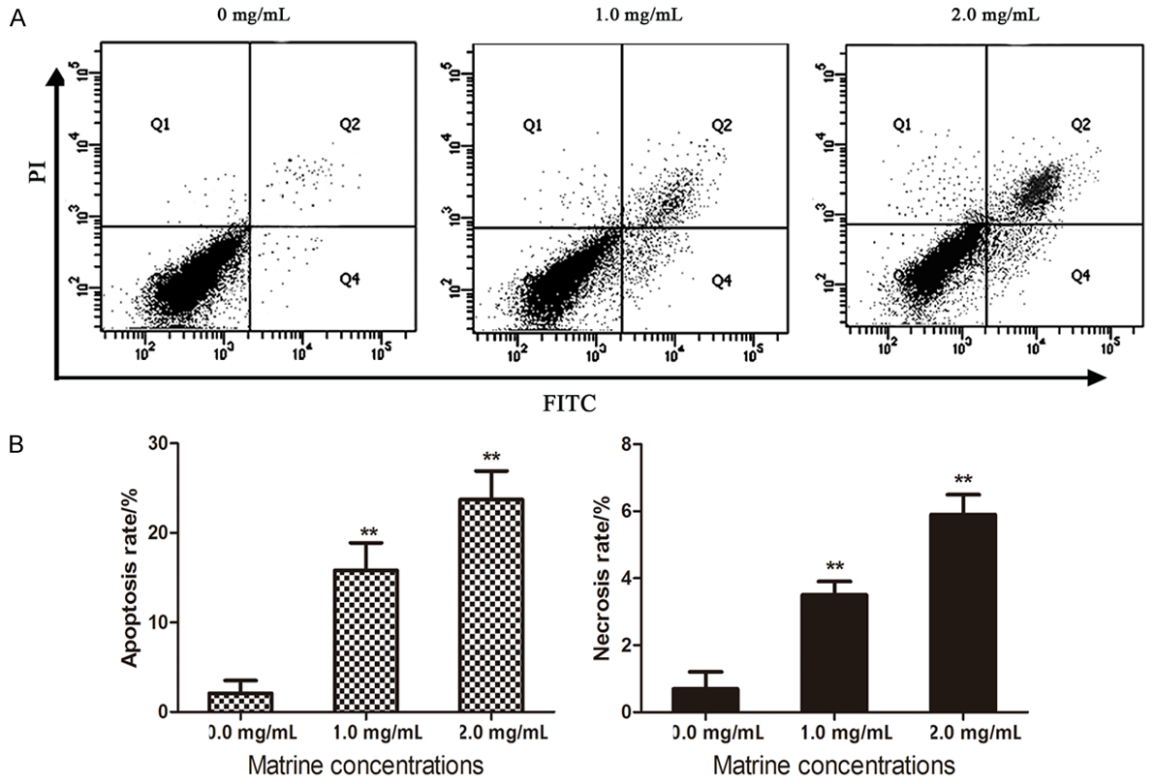


Figure 2. FACS assay was performed to determine the cell apoptosis rate and necrosis rate in matrine-treated H460 cells. (A) H460 cells were treated with different concentrations of matrine (1.0 mg/ml and 2.0 mg/ml) for 24 hours. 2×10^6 cells were washed and collected for Annexin V-FITC/PI staining. (B) Cell apoptosis rate and necrosis rate was shown in histogram. The apoptosis rate (A) and necrosis rate (B) from FACS assay was shown in histogram. The data is shown as mean value ± SD. **P<0.01, compared with untreated H460 cells.

tosis rate and necrosis rate according to the protocols [23]. Briefly, the lung cancer cells were treated with increasing concentrations of matrine for 24 hours. Then, 2×10^6 lung cancer cells were washed twice in cold PBS buffer and fixed in 4% paraformaldehyde for 30 min. The cells were washed with PBS again, FITC and propidium iodide were added at a final concentration 100 ng/ml in each sample. After incubation for 15 min on the ice in dark atmosphere, the cells were analyzed by flow cytometry (FACScan; Belgium).

Western blotting assay

The lung cancer cells were treated with different concentration of matrine (0.25 mg/ml, 0.5 mg/ml, 1.0 mg/ml and 2.0 mg/ml) for 24 hours. The cells were washed with PBS buffer and lysed in RIPA buffer (Cat. No. P0013B, Beyotime, Nanjing, China). 10% SDS-PAGE was used to separate the proteins. Then, the proteins were transferred to PVDF at 400 mA for 1 hour, which was blocked with 5% non-fat milk in TBST buffer. The membrane was incubated with specific primary antibody and followed by HRP-conjugated secondary antibody. Finally, chemiluminescent ECL reagent kit (Millipore) was used for development.

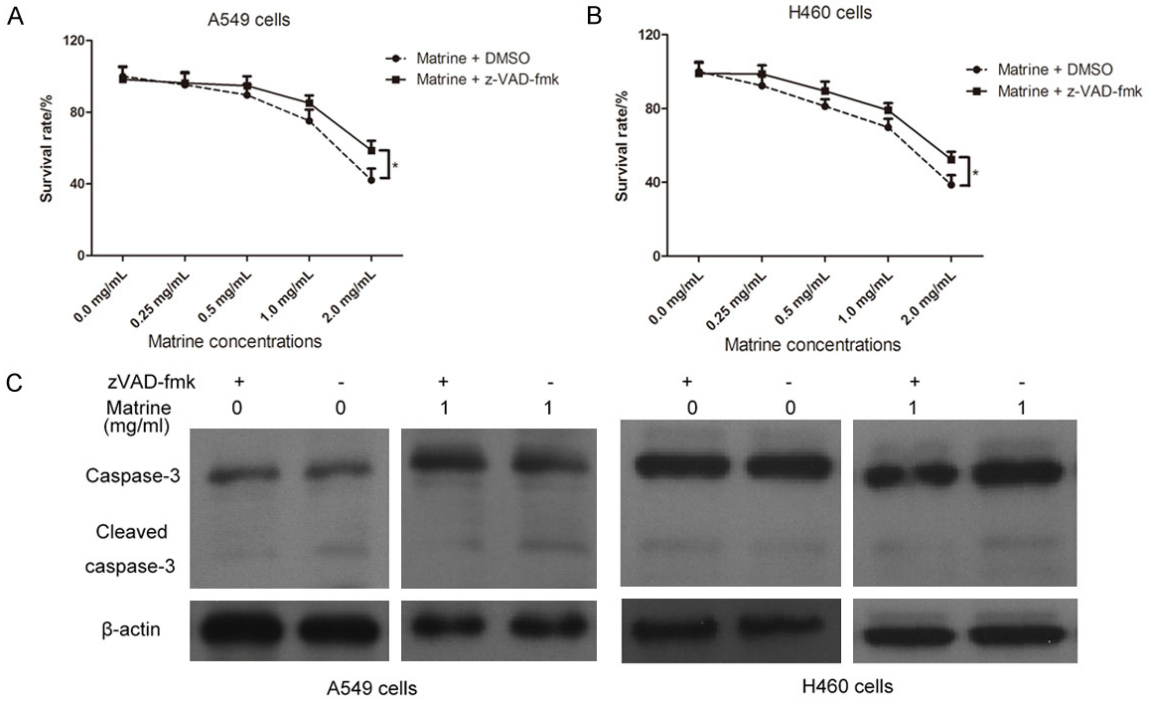
Statistical analysis

SPSS 20.0 software was used to analyze the data (SPSS Inc., Illinois, USA). The multiple comparisons were analyzed by using Two-way ANOVA followed by Tukey test. The others were used two sets of independent samples *t*-test. All the data was shown as mean values ± S.D. The $p<0.05$ was considered as statistically different.

Results

Matrine inhibits the proliferation of lung cancer cells in a dose-dependent manner

In order to detect the anti-tumor effects of matrine in human lung cancer cells, we used



Matrine concentration	A549	NCI-H460
0.0 mg/mL	98.4	99.1
0.25 mg/mL	96.3	98.7
0.5 mg/mL	94.8	89.5
1.0 mg/mL	85.2	79.1
2.0 mg/mL	58.7	52.4

Figure 3. Caspase inhibitor z-VAD-fmk suppresses matrine-induced cell viability. The lung cancer cells A549 (A) and H460 (B) were treated with different concentrations of matrine (0.25 mg/ml, 0.5 mg/ml, 1.0 mg/ml and 2.0 mg/ml) in combination with 10 μM of z-VAD-fmk or 0.1% DMSO for 24 hours. Cell viability was determined by MTT assay. The data is shown as mean value ± S.D. *p<0.05 between matrine-DMSO group and matrine-z-VAD-fmk group. (C) The levels of caspase-3 and cleaved caspase-3 were detected by western blotting analysis. Beta-actin was used as internal reference gene.

increasing concentration of matrine to treat A549 cells and H460 cells. As shown in **Figure 1**, the concentrations of matrine were 0.25 mg/ml, 0.5 mg/ml, 1.0 mg/ml and 2.0 mg/ml. A549 cells and H460 cells were treated with different concentration of matrine for 24 h and 48 h, respectively. The results demonstrated that the inhibition rate of matrine was gradually increased as the increasing concentration of matrine in both 24 hours group and 48 hours group (*p<0.05, **p<0.01, compared with negative control A549 cells; ##p<0.01, compared with negative control H460 cells).

Matrine promotes apoptosis and necrosis

To further identify the molecular mechanism of matrine in lung cancer cells, FACS assay was performed to determine the cell apoptosis rate and necrosis rate in matrine-treated H460 cells. As shown in **Figure 2A**, H460 cells were treated with increasing concentrations of matrine for 24 h, and the concentrations of matrine were 0.5 mg/ml, 1.0 mg/ml and 2.0 mg/ml. Moreover, the results of FACS assay demonstrated that the apoptosis and necrosis of H460 was significantly increased compared with the negative control cells (**p<0.01) (**Figure 2B**). All the results revealed that matrine-induced lung cancer cell death was partly due to the cell apoptosis and cell necrosis.

Caspase inhibitor z-VAD-fmk suppresses matrine-induced cell apoptosis

Z-VAD-fmk is an irreversible general caspase inhibitor to inhibit the activity of caspases. In order to identify whether cell death could be reversed by z-VAD-fmk, the lung cancer cells were treated with increasing concentration of matrine in combination with DMSO or z-VAD-fmk. As shown in **Figure 3**, the survival rate of A549 and H460 in matrine plus z-VAD-fmk-treated group was higher than that in matrine-treated group, suggesting inhibition cell apop-

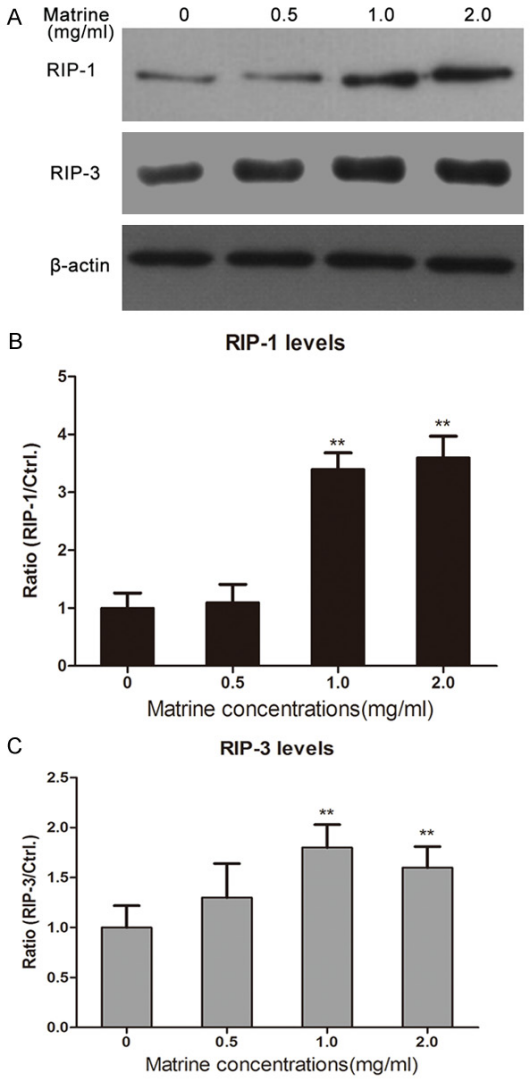


Figure 4. The levels of RIP-1 and RIP-3 are up-regulated in matrine treated H460 cells. (A) The lung cancer cells were treated with increasing concentrations of matrine (0.5 mg/ml, 1.0 mg/ml, 2.0 mg/ml) for 24 hours. The levels of RIP-1 and RIP-3 were detected by western blotting analysis. The band of RIP-1 was analyzed by Image J software and the levels of RIP-1 (B) and RIP-3 (C) was shown in histogram. **p<0.01, compared with untreated H460 cells.

tosis would partly block matrine-induced cell death.

The level of RIP-1 and RIP-3 is up-regulated in matrine treated H460 cells

In order to indentify whether necroptosis was involved in matrine-induced cell death, western blotting analysis was used to determine the levels of RIP-1 and RIP-3 in matrine-treated H460 cells. As shown in **Figure 4**, H460 cells were treated with various concentration of matrine (0.5 mg/ml, 1.0 mg/ml, 2.0 mg/ml) for 24 hours, and the expression level of RIP-1 and RIP-3 significantly increased in matrine-treated cells. Both RIP-1 and RIP-3 were important kinases in the process of necroptosis and the results demonstrated that matrine could induce the necroptosis in the lung cancer cells.

Discussion

Lung cancer, also known as lung carcinoma, is one of the most common cancers in the world. It is estimated that 174000 Americans are diagnosed as lung cancers each year [24-26]. While in China in 2010, 605900 patients were diagnosed and 486600 patients died of lung cancer [26]. It is an important way to effectively induce cell death of lung cancer cells in cancer therapy. In the present study, we used matrine, the active component in Chinese herbal medicine *Sophora* to treat lung cancer cells A549 and H460 and explored the molecular mechanism of matrine, which would give some useful clue for the therapy of lung cancers.

Firstly, the lung cancer cells were treated with different concentration of matrine for 24 hours and 48 hours. MTT assay results demonstrated that the inhibition rate significantly decreased as the increasing concentration of matrine. The inhibitory effects in 48 hours were much more obvious than that of 24 hours, which demonstrated that matrine induced the cell death of lung cancer cells in a dose-dependent manner, as well as in a time-dependent manner. All the results revealed that matrine had anti-tumor effects in human lung cancer cells, which was consistent with the results from other researchers [27, 28].

Programmed cell death has three major different ways of cell death, including cell apoptosis, autophagy and necroptosis. Cell apoptosis is a normally seen form of programmed cell death, which is a naturally occurring process in the body and attracted considerable attention in the study of cancerigenesis. Cell autophagy is a self-degradation process which sometimes mediates cell death while sometimes contributes to cell survival [29, 30]. Till now, few studies have focused on cell necroptosis. In the present study, we have identified that matrine promoted cell death of the lung cancer cells. In order to identify whether the cell death is

induced by cell apoptosis or necroptosis, we used FACS assay to determine the cell apoptosis rate and necrosis rate. Our results demonstrated that matrine-induced cell apoptosis and necroptosis contributed to the cell death of lung cancer cells, which would be a new drug candidate with little side effects for human lung cancers.

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Disclosure of conflict of interest

None.

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References

[1] Chen Y and Zhu J. Anti-HBV effect of individual traditional Chinese herbal medicine in vitro and in vivo: an analytic review. J Viral Hepat 2013; 20: 445-452.

[2] Li N, Ma Z, Li M, Xing Y and Hou Y. Natural potential therapeutic agents of neurodegenerative diseases from the traditional herbal medicine Chinese dragon's blood. J Ethnopharmacol 2014; 152: 508-521.

[3] Zhao L, Zhao AG, Zhao G, Xu Y, Zhu XH, Cao ND, Zheng J, Yang JK and Xu JH. Survival benefit of traditional chinese herbal medicine (a herbal formula for invigorating spleen) in gastric cancer patients with peritoneal metastasis. Evid Based Complement Alternat Med 2014; 2014: 625493.

[4] Zhang L, Wu C, Zhang Y, Liu F, Wang X, Zhao M and Hoffman RM. Comparison of efficacy and toxicity of traditional Chinese medicine (TCM) herbal mixture LQ and conventional chemotherapy on lung cancer metastasis and survival in mouse models. PLoS One 2014; 9: e109814.

[5] Xu Y, Zhao AG, Li ZY, Zhao G, Cai Y, Zhu XH, Cao ND, Yang JK, Zheng J, Gu Y, Han YY, Zhu YJ, Yang JZ, Gao F and Wang Q. Survival benefit of traditional Chinese herbal medicine (a herbal formula for invigorating spleen) for patients with advanced gastric cancer. Integr Cancer Ther 2013; 12: 414-422.

[6] Teschke R, Wolff A, Frenzel C, Eickhoff A and Schulze J. Herbal traditional Chinese medicine and its evidence base in gastrointestinal disorders. World J Gastroenterol 2015; 21: 4466-4490.

[7] Zhang Y, Ma XJ and Shi DZ. Antiplatelet effect of active components derived from Chinese herbal medicine. Chin J Integr Med 2015.

[8] Zhang FY, Hu Y, Que ZY, Wang P, Liu YH, Wang ZH and Xue YX. Shikonin inhibits the migration and invasion of human glioblastoma cells by targeting phosphorylated beta-catenin and phosphorylated PI3K/Akt: a potential mechanism for the anti-glioma efficacy of a traditional Chinese herbal medicine. Int J Mol Sci 2015; 16: 23823-23848.

[9] Wang HQ, Jin JJ and Wang J. Matrine induces mitochondrial apoptosis in cisplatin-resistant non-small cell lung cancer cells via suppression of beta-catenin/survivin signaling. Oncol Rep 2015; 33: 2561-2566.

[10] Yong J, Wu X and Lu C. Anticancer advances of matrine and its derivatives. Curr Pharm Des 2015; 21: 3673-3680.

[11] Zhang L, Zhang H, Zhu Z, Jiang L, Lu X, Zhou M, Sun X, He L, Bai Y and Ma L. Matrine regulates immune functions to inhibit the proliferation of leukemic cells. Int J Clin Exp Med 2015; 8: 5591-5600.

[12] Liu J, Zhang L, Ren Y, Gao Y, Kang L and Lu S. Matrine inhibits the expression of adhesion molecules in activated vascular smooth muscle cells. Mol Med Rep 2016; 13: 2313-2319.

[13] Sun N, Sun P, Lv H, Sun Y, Guo J, Wang Z, Luo T, Wang S and Li H. Matrine displayed antiviral activity in porcine alveolar macrophages co-infected by porcine reproductive and respiratory syndrome virus and porcine circovirus type 2. Sci Rep 2016; 6: 24401.

[14] Zhang X, Jin L, Cui Z, Zhang C, Wu X, Park H, Quan H and Jin C. Antiparasitic effects of oxymatrine and matrine against toxoplasma gondii in vitro and in vivo. Exp Parasitol 2016; 165: 95-102.

[15] Kan QC, Lv P, Zhang XJ, Xu YM, Zhang GX and Zhu L. Matrine protects neuro-axon from CNS inflammation-induced injury. Exp Mol Pathol 2015; 98: 124-130.

[16] Li H, Li X, Bai M, Suo Y, Zhang G and Cao X. Matrine inhibited proliferation and increased apoptosis in human breast cancer MCF-7 cells via upregulation of Bax and downregulation of Bcl-2. Int J Clin Exp Pathol 2015; 8: 14793-14799.

[17] Liao H, Zhao X, Qu J, Zhang J and Cai H. Matrine suppresses invasion and metastasis of NCI-H1299 cells by enhancing microRNA-133a expression. Int J Clin Exp Med 2015; 8: 10714-10722.

[18] Rong B, Zhao C, Gao W and Yang S. Matrine promotes the efficacy and safety of platinum-based doublet chemotherapy for advanced non-small cell lung cancer. Int J Clin Exp Med 2015; 8: 14701-14717.
[19] Li Q, Lai Y, Wang C, Xu G, He Z, Shang X, Sun Y, Zhang F, Liu L and Huang H. Matrine inhibits the proliferation, invasion and migration of castration-resistant prostate cancer cells through regulation of the NF-kappaB signaling pathway. Oncol Rep 2016; 35: 375-381.
[20] Su Z, Yang Z, Xu Y, Chen Y and Yu Q. MicroRNAs in apoptosis, autophagy and necroptosis. Oncotarget 2015; 6: 8474-8490.
[21] Davidovich P, Kearney CJ and Martin SJ. Inflammatory outcomes of apoptosis, necrosis and necroptosis. Biol Chem 2014; 395: 1163-1171.
[22] Feoktistova M, Wallberg F, Tenev T, Geserick P, Leverkus M and Meier P. Techniques to distinguish apoptosis from necroptosis. Cold Spring Harb Protoc 2016; 2016: pdb.top070375.
[23] Hussain AR, Khan AS, Ahmed SO, Ahmed M, Platanias LC, Al-Kuraya KS and Uddin S. Apigenin induces apoptosis via downregulation of S-phase kinase-associated protein 2-mediated induction of p27Kip1 in primary effusion lymphoma cells. Cell Prolif 2010; 43: 170-183.
[24] Baker H. Overtreatment in stage IV lung cancer in the USA. Lancet Oncol 2015; 16: e532.
[25] Matsuda A and Katanoda K. Five-year relative survival rate of lung cancer in the USA, Europe and Japan. Jpn J Clin Oncol 2013; 43: 1287-1288.
[26] Chen W, Zheng R, Zeng H and Zhang S. Epidemiology of lung cancer in China. Thorac Cancer 2015; 6: 209-215.
[27] Zhang Y, Zhang H, Yu P, Liu Q, Liu K, Duan H, Luan G, Yagasaki K and Zhang G. Effects of matrine against the growth of human lung cancer and hepatoma cells as well as lung cancer cell migration. Cytotechnology 2009; 59: 191-200.
[28] Tan C, Qian X, Jia R, Wu M and Liang Z. Matrine induction of reactive oxygen species activates p38 leading to caspase-dependent cell apoptosis in non-small cell lung cancer cells. Oncol Rep 2013; 30: 2529-2535.
[29] Su Z, Yang Z, Xu Y, Chen Y and Yu Q. Apoptosis, autophagy, necroptosis, and cancer metastasis. Mol Cancer 2015; 14: 48.
[30] Lalaoui N, Lindqvist LM, Sandow JJ and Ekert PG. The molecular relationships between apoptosis, autophagy and necroptosis. Semin Cell Dev Biol 2015; 39: 63-69.