RESEARCH ARTICLE

Matrine Reduces Proliferation of Human Lung Cancer Cells by Inducing Apoptosis and Changing miRNA Expression Profiles

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Abstract

Matrine, a main active component extracted from dry roots of *Sophora flavecens* , has been reported to exert antitumor effects on A549 human non-small lung cancer cells, but its mechanisms of action remain unclear. To determine effects of matrine on proliferation of A549 cells and assess possible mechanisms, MTT assays were employed to detect cytotoxicity, along with o flow cytometric analysis of DNA content of nuclei of cells following staining with propidium iodide to analyze cell cycle distribution. Western blotting was performed to determined expression levels of Bax, Bcl-2, VEGF and HDAC1, while a microarray was used to assessed changes of miRNA profiles. In the MTT assay, matrine suppressed growth of human lung cancer cell A549 in a dose- and time-dependent manner at doses of 0.25-2.5 mg/ml for 24h, 48h or 72h. Matrine induced cell cycle arrest in G0/G1 phase and decreased the G2/M phase, while down-regulating the expression of Bcl2 protein, leading to a reduction in the Bcl-2/Bax ratio. In addition, matrine down regulated the expression level of VEGF and HDAC1 of A549 cells. Microarray analysis demonstrated that matrine altered the expression level of miRNAs compared with untreated control A549 cells. In conclusion, matrine could inhibit proliferation of A549 cells, providing useful information for understanding anticancer mechanisms.

Keywords: Matrine - A549 lung cancer cells - miRNA profile - apoptosis - proliferation

Asian Pac J Cancer Prev, **15 (5)**, 2169-2177

Introduction

Lung cancer is still leading cause of cancer- related death in worldwide. And in them, 80% of lung cancers are non-small cell lung cancer (NSCLC). The mortality rates with an overall 5-year survival of adenocarcinoma are only 15%.To date, chemotherapy been the most frequently used for NSCLC. However, as a subgroup of NSCLC, adenocarcinoma shows resistance to exiting treatments, such as chemotherapy, radiotherapy, and platinum-based doublet chemotherapy (Hu et al., 1996; Zhang et al., 2001a; Zhang et al., 2001b; Liu et al., 2003; Long et al., 2004; Cheng et al., 2006). Fortunately, there are many extracts from Traditional Chinese Medicines showing better therapies to human NSCLC, leading us a new way to treat lung cancer in the future (Tan et al., 2013; Ulasli et al., 2013).

Matrine, with the molecular formula C15H24N20, is a naturally monomer compound of Traditional Chinese Medicine *Sophora flavecens* Ait with various biological activities such as anti-inflammatory (Hu et al., 1996; Cheng et al., 2006), antiviral (Liu et al., 2003; Long et al., 2004), immunoinhibitory, antifibrotic (Zhang et al., 2001a; Zhang et al., 2001b), analgesic, antiarrhythmic (Zhang et al., 1990; Ai et al., 2001; Xu et al., 2004) and anti-diarrhea effects (Zhang et al., 2001c). Recently, some studies displayed that matrine had potent anti-tumor bioeffect against various cancer cell lines by inhibiting and inducing apoptosis of gastric and cervical cancer cells, prostate cancer cells, leukemia and glioma cells (Hu et al., 2005; Liu et al., 2006; Jiang et al., 2007; Luo et al., 2007; Zhang et al., 2007; Liu et al., 2008; Zhang et al., 2009; Zhang et al., 2012a; Tan et al., 2013;), and also suppressed invasiveness and malignant of melanoma cell line A375 in vitro (Croce and Calin, 2005).

However, there is few report about precise mechanism underlying the against A549 cell line activity of matrine. Hence, we designed the present study to investigate the antitumor effects of matrine in human lung adenocarcinoma A549 cells, and further elucidate the molecular mechanisms involved in its antineoplastic activities.

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MicroRNA (miRNA), also called small non-coding RNA, has been reported to negatively control expression of target genes in animal and plants. These RNAs play a significant role in regulating cell proliferation and apoptosis in different cancer cells. However, unusual expressions of miRNAs contribute to carcinogenesis and tumor progression (Gregory et al., 2005; Blower et al., 2008). Moreover, it has been reported that miRNAs could be as a biomarker for detection of lung cancer at early stages (Yao et al., 2014; Zhu et al., 2014). Recent study showed that suppression of miRNAs by chemotherapeutic agents induced apoptosis in some cancer cells (Zhang et al., 2009). On the basis of the early results, in this research we investigated the underlying mechanism by which matrine may suppress cell proliferation in NSCLC cells. Eventually, the role of matrine-treated miRNAexpression profiles using miRNA microarray analysis was also employed.

Recent data suggested a correlation between miRNAs expression and human lung cancer (Eder et al., 2005; Esquela-Kerscher et al., 2006; Nana-Sinkam et al., 2006; Yang et al., 2014). Since changes in miRNAs expression may play an important role in the anti-tumor progress of matrine, it is necessary to study the expression changes of miRNA profiles in matrine-treated A549 cells. In this study, we firstly evaluated the growth inhibition and apoptosis ratio of A549 cells treated by matrine in different concentration. Furthermore, we examined expression of apoptosis-related genes: Bcl-2 and Bax. Finally, by using microarray, we investigated the effect of matrine on changes of miRNAs expression in A549 cells. Our results show that matrine could inhibit growth and induce apoptosis of A549 cells and these whole processes may be controlled through expression changing of relative miRNAs by matrine treatment.

Materials and Methods

Cell culture

Human NSCLC cell line A549 was obtained from Shanghai Institute of Cell Biology, Chinese Academic of Science (Shanghai, China). A549 cells were cultured in DMEM medium (Hyclone, Logan, UT, USA) containing 10% heat-inactivated fetal bovine serum (GIBCO, Grand Island, NY, USA), and incubated at 37°C in a humidified atmosphere with 95%air/5% CO_2.

MTT assay

Matrine was purchased from sigma (Sigma, USA). A549 cells (4×10^3 per well) were seeded into 96 well plate (corning Inc, NY, USA) and treated with different concentrations of matrine: 0.25 mg/ml, 0.5 mg/ml, 1.0 mg/ml, 1.5 mg/ml, and 2.0 mg/ml. The control group contained A549 cells only. After treatment, the medium were replaced with 20μl of MTT reagent (5 mg/ml) and incubated in 5% CO_2 at 37°C for 4 h. Then, 150μl DMSO was added to each well to solubilise the MTT tetrazolium crystal. Absorbance was measured at 570nm using a microplate reader (Bio-Rad, Hercules, CA, USA). All MTT assay were repeated three times. The inhibitory rate of A549 proliferation was calculated as [1-A570 (test) / A570 (control)] × 100%

Cell cycle analysis

To analyze cell cycle distribution, A549 cells were seeded into 60-mm culture (corning Inc, NY, USA) dishes with different concentration matrine. The cells were then fixed in ice-cold 70% ethanol for overnight. After an additional wash with PBS, the cell pellets were stained with 1ml of propidium iodide (PI) staining solution containing 200μg of PI in 1 ml of PBS containing 2mg of DNase free RNase for 30 min. Acquisition and analysis were performed by Cytomics FC500 flow cytometry (Beckman Coulter, Fullerton, CA, USA) with excitation at 488 nm.

Morphological evaluation of apoptosis cells

After treatment with different concentration matrine for 72 h, A549 cells fixed with 1% glutaraldehyde in PBS for 30 min at room temperature, then, washed in PBS, and stain with 1mM Hoechst 33342 for 20 min at 37°C. The cells were observed under a fluorescent microscope, ix-81 (Olympus, Tokyo, Japan).

Western blot analysis

The treated cells were collected and washed twice in 1× PBS at 72 h, then lysed in ice-cold lysis buffer (50mMTris–HCl, pH 7.4, 150mMNaCl, 5mMEDTA, 50mMNaF, 1%Triton X-100, 1mM sodium orthovanadate, 1mM phenylmethanesulfonyl fluoride, 1 mg/ml aprotinin, 2g/ml pepstatin A, and 2 g/ml leupeptin) for 5min. Proteins were separated using sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred to PVDF (Millipore, Bedford, MA, USA) membrane. The blots were blocked with 5% non-fat milk in TBST saline (20 mM Tris–HCl, pH 7.4, 137mMNaCl, and 0. 1% Tween-20) at RT for 1 h and incubated with the appropriate primary antibody at room temperature. After wash, the blots were incubated with peroxidaseconjugated secondary antibody for 1 h. Bands were monitored using Western blot chemiluminescence reagent (Bio-Rad, Hercules, CA, USA).

Microarray analysis of miRNA profiles

Total RNA was extracted from cells by using mirVana miRNA Isolation Kit (Invitrogen, CA, USA) 72 h after matrine treatment. For microarray studies, both the RNA samples were determined with Agilent 2100 Bioanalyzer (Agilent Technologies, Cam USA) and an Ultrospec 3300 Pro UV/Visible Spectrophotometer (Amersham Biosciences, USA). The RNA quality parameters for microarray analysis were as follows: UV spectroscopy A260/A280 ratio of 1.8-2.0 and A260/A230 radio greater than 1.8, 18S/28S rRNA radio of 1.8-2.1, and RNA integrity number greater than 8.0. TaqMan Array Human MicroRNA A+B Cards Set V3.0 was used for analyzing miRNA profiles. 350 ng RNA was converted to cDNA by using Megaplex™ RT Primers, Human Pool Set V3.0 (Applied Biosystems, California, USA) containing looped primers of 770 known human miRNAs. The expression of miRNAs was profiled by using 7900HT real-time PCR instrument (Applied Biosystems, Foster City, California).

Table 1. Changes of Cell Cycle Progression by Matrine in A549 Cancer Cells

Mtrine does (mg)	NO.of miRNAs	miRNA	
		upregulated	downregulated
0.5	48	hsa-let-7f, hsa-let-7g, hsa-miR-10a, hsa-miR-17, hsa-miR-19a, hsa-miR-19b, hsa-miR-20a, hsa-miR-20b, hsa-miR-24, hsa-miR-25, hsa-miR-27a, hsa-miR-27b, hsa-miR-28-3p, hsa-miR-28, hsa-miR-29a, hsa-miR-29c, hsa-miR-30b, hsa-miR-30c, hsa-miR-31, hsa-miR-34a, hsa-miR-99b, hsa-miR-101, hsa-miR-106b, hsa-miR-125a-5p, hsa-miR-130a, hsa-miR-132, hsa-miR-135b, hsa-miR-148a, hsa-miR-148b, hsa-miR-152	hsa-miR-199a, hsa-miR-202, hsa-miR-205, hsa-miR-208b, hsa-miR-216a, hsa-miR-219, hsa-miR-302a, hsa-miR-302b, hsa-miR-331-5p, hsa-miR-381, hsa-miR-382, hsa-miR-409-5p, hsa-miR-672, hsa-miR-885-5p, hsa-miR-147, hsa-miR-520f, hsa-miR-516-3p,
0.5 & 1.0	48	hsa-let-7a, hsa-let-7b, hsa-let-7c, hsa-let-7e, hsa-miR-100, hsa-miR-103a, hsa-miR-128, hsa-miR-130b, hsa-miR-139-5p, hsa-miR-18a, hsa-miR-192, hsa-miR-193b, hsa-miR-194, hsa-miR-200c, hsa-miR-212, hsa-miR-222, hsa-miR-223, hsa-miR-26a, hsa-miR-29b, hsa-miR-30a, hsa-miR-339-5p, hsa-miR-365, hsa-miR-424, hsa-miR-425, hsa-miR-486-3p, hsa-miR-520c-3p, hsa-miR-660, hsa-miR-96, hsa-miR-98, hsa-miR-30d, hsa-miR-328, hsa-miR-362-3p, hsa-miR-342-3p, hsa-miR-95, hsa-miR-500, hsa-miR-502-3p, hsa-miR-519a	hsa-let-7i-star, hsa-miR-10b, hsa-miR-140-5p, hsa-miR-29c-star, hsa-miR-301a, hsa-miR-455-5p, hsa-miR-532-5p, hsa-miR-193-5p, hsa-miR-324-3p, hsa-miR-362-5p, hsa-miR-450-5p
1	70	hsa-miR-1, hsa-miR-23b, hsa-miR-33b, hsa-miR-34c, hsa-miR-122, hsa-miR-127hsa-miR-127-5p, hsa-miR-129hsa-miR-133b, hsa-miR-136, hsa-miR-138, hsa-miR-139-3p, hsa-miR-141, hsa-miR-142-5p, hsa-miR-145, hsa-miR-146b-3p, hsa-miR-147b, hsa-miR-149, hsa-miR-150, hsa-miR-154, hsa-miR-188-3p, hsa-miR-197, hsa-miR-198, hsa-miR-199b, hsa-miR-200a, hsa-miR-204, hsa-miR-214, hsa-miR-215, hsa-miR-216b, hsa-miR-217, hsa-miR-361, hsa-miR-369-3p, hsa-miR-369-5p, hsa-miR-370, hsa-miR-372, hsa-miR-373, hsa-miR-507, hsa-miR-510, hsa-miR-520a, hsa-miR-520a#, hsa-miR-520d-5p, hsa-miR-539, hsa-miR-541, hsa-miR-551b, hsa-miR-556-3p, hsa-miR-556-5p, hsa-miR-561, hsa-miR-570, hsa-miR-655, hsa-miR-674, hsa-miR-920, hsa-miR-921 , hsa-miR-337-3p , hsa-miR-936, hsa-miR-938 , hsa-miR-939 , hsa-miR-18b#, hsa-miR-543	hsa-miR-9, hsa-miR-371-3p, hsa-miR-508, hsa-miR-512-3p, hsa-miR-542-3p, hsa-miR-654, hsa-miR-33a, hsa-miR-922, hsa-miR-924, hsa-let-7a#, hsa-miR-20b#

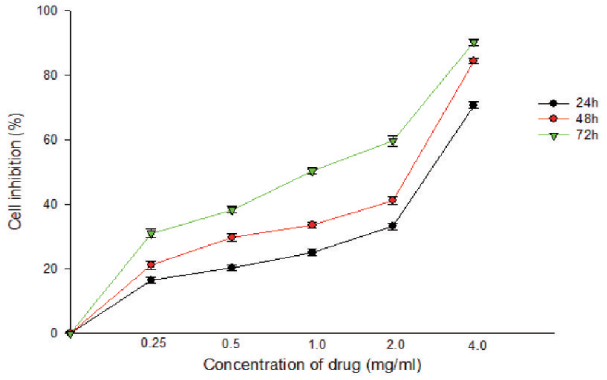

Figure 1. The Anti-proliferative Effect of Matrinel on A549 Cells in a Dose Dependent Manner. The cells were treated with martine at concentration of 0.25, 0.50, 1.0, 1.5, 2.0, 4.0 mg/ml. The deadcells were determined and presented as a percentage of the untreated cells as a control. The shown data are the mean from three independent experiments. all results $P < 0.05$ vs control group

Real-time PCR was performed by using standard conditions. Statistical tests for variant expressions were conducted between matrine-treated and control groups. Data were presented as fold differences based on calculations of 2 (-△△Ct). Of the total miRNAs probed on the microarray, 770 human miRNAs were selected for further analysis. The miRNAs whose flags were present in at least one sample were filtered and applied to the fold-change analysis. The fold-change analysis was conducted by a factor of 2-fold among 3 groups: A549 control and 0.5 mg and 1.0 mg of matrine-treated A549. Candidate miRNAs that were either increased or decreased in miRNAs expression more than 2-fold following matrine treatment were chosen for target prediction and analyzed with miRBase Targets Version 5 (http://microrna.sanger.ac.uk/targets/v5/). Human genes that have been experimentally verified functions correlated to cell cycle regulation, apoptosis, cell proliferation, differentiation and chromatin modification were selected by Gene Ontology (http://www.geneontology.org/).

Statistical analysis

Data were expressed as means±SD. Statistical comparisons of the results were made using analysis of variance (ANOVA). $P<0.05$ was considered to be statistically significant.

Results

Antiproliferation effect of matrine in A549 cell

By employing MTT assay, we examined the anti-proliferation effect of matrine in A549 cell. As displayed in Figure 1, matrine suppressed growth of human lung

Table 2. miRNA Target Genes Displayed Greaterly than 2-fold Dependent on Concentration of Matrine from Table 1

miRNA name	Functions of target genes			
	Cell cycle	Apoptosis	Cell proliferation & differentiation	chromatin modification
hsa-let-7a	MYC, E2F1, PTEN, NCAPG, CDC34, CDK6, CCNG1, CDKN1C, E2F3, CCNA2, CDKN1B, EPHB2, CDKN2A, CDKN1A, CDC25A, NF2, TGFB1, CDKN2D, CDC25C, DDIT3, UBE2I, CCND1, EGFR, CHAF1A, RASSF1, RBL2, MAPK3, APC, HDAC4, TP73, WEE1, CYLD, E2F2, CCND2, UHRF1, TUSC2	E2F1, PTEN, CDKN1B, CDKN2A, CDKN1A, CDKN2D, KRAS, BCL2, AKT1, TP53, DYRK2, PAX7, NPM1, NFKB1, IL2RA, IL1B, BCL2L1, IL6, RTN4, BIRC3, PDCD6, BRCA1, TNF, IL17A, BRCA2, TP63, CYFIP2, TIMP3, FAS, DDX20, JAK2, HMOX1, BRAF, NOL3, CYCS, IFNG, E2F2, NRAS, CASP3	CCND2, UHRF1, NRAS, TUSC2, CCND1, MCL1, CEBPA, TWIST1, FOXA1, BCL6, SHH, FXR1, CEBPB, SYNE1, STMN1, SOCS1, HAND2, GLI1, NRAS	DNMT1, DNMT3B
hsa-let-7b	HSPA1B, TNFRSF10B, PTEN, SAC3D1, NAT6, EPHB2, SMC1A, CHAF1A, NCAPG, CUL4B, CCNT2, GAK, SH3BP4, AURKB, CDC25A, CCND2, CCNF, CDC34, E2F6, UHRF1	HSPA1B, TNFRSF10B, TNFRSF10A, TNFSF9, DDX20, STK3, NOTCH2, HBXIP, CEBPB, SQSTM1, ARHGDIA, JAK2, HMOX1, VEGFA, BCL2, PTEN, IFNG, TP53, RTN4, F2, NRAS, PLAGL2	PTEN, RHOG, TACSTD2, IFRD2, KLK8, VTI1B, TXNRD1, LAMC1, KLF5, PLK1, PTGS2, MYC, LRP1, FGF2, EGFR, PURA, CDC25A, CCND2, NRAS, UHRF1TNFAIP2, GNA13, NARG1, SYNE1, RHOB, DOCK7, SERPINE2, MTPN, IFRD1, PDLIM7, WNT1, ANPEP, CAND1, CCND1, NRP1, JUN, DZIP1, NRAS	N/A
hsa-let-7c	MCL1, SSSCA1, E2F3, CDK4, MYC	BBC3, CD40LG, TNFSF10, CDKN2A, DDIT4, BNIP3L, BCL2, MCL1, SIRT1, IGF1R, IGFBP3, BCL2L1, NRAS	ODZ1, NRAS, TGFBR1, BMP5, NRAS	N/A
hsa-let-7e	SMC1A	AKT1	JAG1, SOCS1, CSF1, WNT1	N/A
hsa-let-7i-star	STAG2, NKX2-2, CUL3	CUL3	CUL3, NKX2-2	N/A
hsa-miR-100	CDC25C	TP53	PLK1, SIRT1	N/A
hsa-miR-103a	WWOX, CCNE1, CDK2	IL1B	IL1B, FGF23, BMP7	N/A
hsa-miR-10b	CDK6, E2F3, E2F7, HDAC4, MAPRE1, PAPD5, STARD13, UBE2I, XRN1, ZMYND11	BCL2L11, BCL2L2, DPF2, GCLM, TBX5	BCL6, CDK6, CHL1, DAZAP1, EPHA2, HDAC4, INHBB, MDGA2, MED1, MYT1L, NAV1, PURB, TFAP2C	GATAD2A
			CDK6, CTNNBIP1, HDAC4, KLF11, MAPRE1, ZMYND11	
hsa-miR-128	E2F3	N/A	BMI1, TGFBR1, DCX, NTRK3, RELN	N/A
hsa-miR-130b	RUNX3	RUNX3, TP53INP1	RUNX3, PPARG	NAT13
hsa-miR-139-5p	SSSCA1, CDKN1B	CDKN1B	CDKN1B	N/A
hsa-miR-140-5p	ALS2CR2, CTCF, CUL3, EIF4G2, ERBB2IP, HDAC4, KLK10, MAPK1, PARD6B, PIN1, PPP1CC, RBBP4, SIAH1, SLC44A2, HDAC4	VEGFA, BAG2, BCL2L1, BCL2L2, BIRC6, CUL3, FXR1, IGF1R, MAPK1, NCKAP1, RFFL, SIAH1, STK3, SULF1	VEGFA, BIRC6, CAPN1, CUL3, FGF9, HDAC4, LAMC1, LIFR, NUMBL, PDGFRA, RBBP4, TGFBR1, HDAC4, BMP2, DPYSL2, FGF9, FXR1, HAND2, HDAC4, JAG1, PPP1R9A, PURB, SIAH1, SMURF1, TLR4, TSSK2, UBE2V1, HDAC4	CTCF, NDST1
hsa-miR-18a	ATM, BTG3, CCND2, CDK2, ESCO2, HMG20B, MAPK4, NEDD9, NOTCH2, PARD6B, RABGAP1, SH3BP4, ZAK	FAM130A2, GCLC, NOTCH2, STK4, TNFAIP3, UBE2Z	BTG3, CCND2, CDK2, IGF1, IRF2, KIT, CTGF, CDC42, MBNL1, MDGA1, NAV1, NOTCH2, PAPPA, ROBO2, RUNX1, SIM2	EHMT1, SATB1, ZCCHC4
hsa-miR-192	N/A	DLG5	DLG5	N/A
hsa-miR-193b	CCND1	MCL1	ETS1, CCND1, ETS1, MCL1	N/A
hsa-miR-194	N/A	IGF1R, SOCS2	HBEGF, IGF1R	DNMT3A
hsa-miR-200c	FZR1, CCND1, SSSCA1, CDC25C, JAG2, SUFU, CCND2, TGFB1, CDKN1B, EGFR, CCPG1, IRF1, EP300, UBE2I	SFRP1, SIRT1, IHH, MAP3K10, FOXC2, BCL2, TP53, FAS, GULP1, KRAS, IL2RA, EP300	BAP1, BMI1, ZEB1, CCND1, JAG2, TWIST1, TWIST2, SFRP1, SIRT1, QKI, IHH, NTRK2, TUBB3	EP300
hsa-miR-212	BRCA1	BRCA1, PEA15	BRCA1	N/A
hsa-miR-222	CDKN1B, CDKN1C	BBC3, CDKN1B, FOXO3	CDKN1B, CDKN1C, KIT, FOXO3	FOS
hsa-miR-223	RHOB	RHOB	MEF2C, RHOB	N/A
hsa-miR-26a	CCND2, CDC6, PTEN, RB1	PTEN	CCND2, CDC6, LIF, PTEN, RB1, SMAD1, SMAD4, CTGF, RB1	N/A
hsa-miR-29b	TGFB1, HELLS, E2F3, RASSF1, WWOX, CDKN2B, FHIT, PLK2, CDK4, SMAD3, FH, PTEN, CCND1, MYC, CDK6	BCL2, TNFSF10, PAX7, PAK1, MYBL2, IL1B, AKT1, MMP9, MCL1, VEGFA	SMAD3, CDK6, VEGFA, FGA, CCND1, ZFP36, GLI1, ZAP70, CEBPA, PDLIM7, ID3, BMP1, IL4, SHH, DNMT3B, DNMT3A, DNMT1, HDAC2, CDK6, MCL1	DNMT3A, DNMT3B
hsa-miR-29c-star	TGFB1, TGFB2, EPHB2, TP53	TGFB1, TGFB2, TP53, PRKCA	TGFB1, ZEB1, TGFB2, TP53, ZIC1, COL15A1, TP53	CREBZF, DNMT3B, DNMT3A

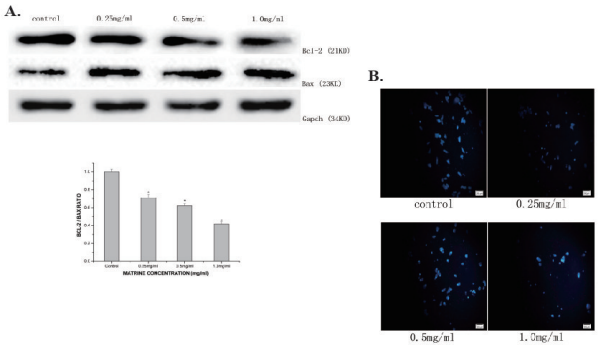

Figure 2. Effect of Matrine on Inducing Apoptosis of A549. (A). A549 cells was treated with matrine (0.25 mg/ml, 0.5 mg/ml, and 1.0 mg/ml). After treatment for 72 h, the cells were harvested and the total cell lysates were prepared. The expression of Bax and Bcl-2 were determined by immunoblotting analysis. GAPDH was used as the loading control in this study. Experiments are repeated for three times and similar results were obtained.(*P<0.05,**P<0.01); (B). A549 cells were treated in the presence (0.25mg/ml, 0.5 mg/ml, and 1.0 mg/ml) for 72 h. The morphological features were observed with optical microscope with a 100-fold magnification

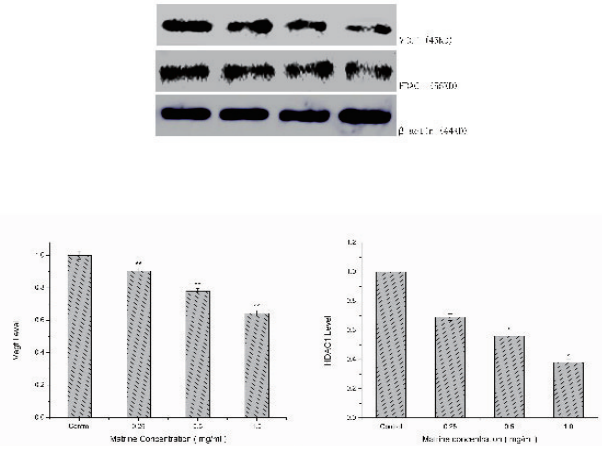

Figure 3. Effect of Matrine on VEGF and HDAC-1 of A549. A549 cells were treated with matrine (0.25 mg/ml, 0.5 mg/ml, and 1.0 mg/ml). After treatment for 72 h, the cells were harvested and the total cell lysates were prepared. The expression of VEGF and HDAC-1 were determined by immunoblotting analysis. GAPDH was used as the loading control in this study. Experiments are repeated for three times and similar results were obtained (*P<0.05,**P<0.01)

cancer cell A549 in does-and time-dependent manners after cells were treated with matrine at 0.25-2.5 mg/ml for 24h, 48h, and 72h, respectively. Proliferation of A549 cells treated with 0.025 mg/ml, 0.5 mg/ml, 1.0, mg/ml 1.5 mg/ml, 2.0 mg/ml, and 4.0 mg/ml of matrine was inhibited significantly.

Effect of matrine on distribution of the cell cycle

We performed flowcytometric analysis for determining the mechanism responsible for matrine-mediated cell growth inhibition and cell cycle. Matrine treatment for 72 h induced sub-G0/G1 accumulation in a dose dependent manner as compared with (Table 1). After treating with increasing concentration of matrine for 72 h, the distribution of A549 cells at each phase of cell cycle was analyzed by flow cytometry as described. The shown data are the mean from three independent experiments.

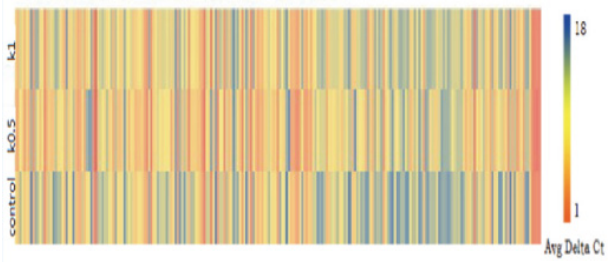

Figure 4. Microarray Analysis of miRNA Expression Profile. After flag-filtration, a total of 166 human miRNA expression average delta Ct are exhibited by color bar from 1(red) to 18 (blue). The data were separated into three groups: control, 0.5 mg matrine (k 0.5) and 1.0 mg matrine. (k 1.0)

Matrine induced apoptosis by reducing the Bcl-2/Bax in A549 cells

To invesgitate the effects of matrine on apoptosis, we employing western blot to detect the expression of Bcl-2 and Bax proteins in A549 cells. By analysis, we confirmed that matrine does-dependently down-regulated the expression of antiapoptosis protein Bcl-2, affected the level of proapoptosis protein Bax (Figure 2A). Matrine-induced apoptotic effect was further validated by using Hoechst 33342 staining assay in A549 cells (Figure 2B). The staining showed that the typical changes, such as formation of apoptical bodies, appeared in cells with martine treatment (0.25 mg/ml, 0.5 mg/ml, and 1.0 mg/ml) for 72 h.

Reduction of expression level of VEGF and HDAC1 in A549 cells by matrine

Vascular endothelial growth factor and histone deacetylases plays a vital role of tumorigenesis, and their expression are upregulated in many cancer cell lines including A549. Hence, we investigated the effects of matrine on the expression of VEGF and HDAC1in A549 cells. The data from western blotting showed that matrine dose-dependently reduced the expression of VEGF and HDAC1 in A549 cells after the cells were treated for 72h with matrine at the concentrations of 0.25-1.0 mg/ml (Figure 3).

Changes of miRNA expression of A549 cells after matrine treatment and target prediction of miRNAs

TaqMan Array Human MicroRNA A+B Cards Set v3.0 was employed for analysis of miRNA profiles. miRNA expression profiles were compared between matrine (1.0 mg/ml) and matrine (0.5 mg/ml) treated A549 cells for 72h. A total of 770 human miRNAs were chosen for detection of miRNA expression profiles. Among them, 206 miRNAs are differentially expressed between the untreated and treated A549 cells (Figure4). The color closet to blue reflects high average Ct value, conversely red color displays low average △Ct value. This result showed that matrine treatment induced changes in certain miRNAs expression levels of A549 cells.206 miRNAs was identified that it differentially regulated by 2-fold change in 0.5 mg and 1.0 mg treated cells compared to control. 48 of 770 in 0.5 mg matrine-treated and 70 of 770 in 1.0 mg matrine-treated displayed more than 2-fold alterations in expression (Table 2). Interestingly, 48 miRNAs displaying greater than 2-fold expression changes in 0.5 mg treated

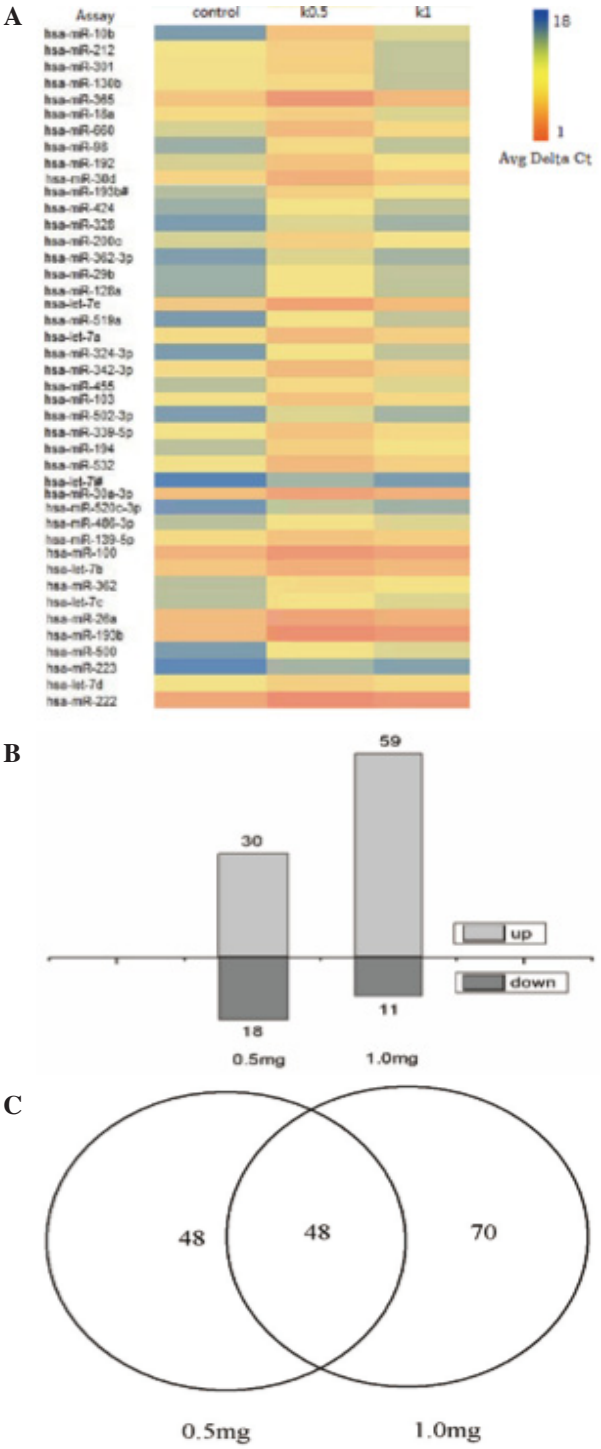

Figure 5. Changes of miRNA Expression of A549 Cells after Matrine Treatment and Target Prediction of miRNAs. (A) miRNA expression profiles that showed >2-fold change in comparison with each group after matrine treatment. (B) 48 miRNAs exhibited than 2-fold changes in expression after matrine treatment indicated by color. The Venn diagram depicts the expression patterns of several miRNAs that are influenced by matrine dose. Among 70 miRNAs showing 2-fold change, the miRNAs demonstrate up- or down-regulation in each matrine treatment. (C) 48 miRNAs are changed in both conditions

cells were also confirmed in 1.0 mg treated cells (Figure 5A, B). It could be found 30 up-regulated and 18 down-regulated miRNAs in 0.5 mg treated cells. We also found 59 up-regulated and 11 down-regulated miRNAs in 1.0 mg treated cells (Figure 5C).

Discussion

Matrine has various pharmacological effects including anti-tumor activities on different cancer cell lines (Cress et al., 2000; Zhang et al., 2014). In this study, our data demonstrated the anti-proliferative effect of matrine in human NSCLC A549 cells, this is consistent with previous study (Chang et al., 2014; Zhang et al., 2014). MTT assay showed that matrine suppressed the viability of human lung cancer line A549 in dose- and time- dependent manners after cells were treated with matrine at 0.25-4.0 mg/ml for 24, 48, 72 h, respectively. Cell cycle plays a critical role in regulating cell proliferation, growth as well as cell division (Wang et al., 2012; Hernandez-Hernandez et al., 2013; Jiang et al., 2013) . The cell cycle analysis showed that at duration matrine can induce cell cycle arrest at G0/G1 phase and simultaneously decreasing the G2/M phase. This result suggests that retardation of cell cycle progression may be one of the mechanisms underlying the antiproliferative effect of matrine. Thus we assume that matrine may prove to be a valuable tool for inducing cell cycle arrest at G0/G1 phase. In the repression of gene transcription expression, histone deacetylases (HDACs) play a significant role by changing chromatin structure (Thiagalingam et al., 2003) and affecting proliferation, cell cycling, apoptosis, differentiation, DNA replication, DNA damage response and migration of cancer cells (Langer et al., 2010; Li et al., 2013a). It has been reported that over-expression of HDACs has been detected in lung cancer tissues (Sasaki et al., 2004; Krusche et al., 2005; Nakagawa et al., 2007; Fritzsche et al., 2008; Weichert et al., 2008a; Weichert et al., 2008b; Kallsen et al., 2012) and that the expression of HDAC promotes the progress of lung cancer (Zhang et al., 2012b). Specifically, the expression of HDAC1 is related to advanced non small cell lung cancers (NSCLCs) (Minamiya et al., 2011), and an independent predictor of poor prognosis in patients with NSCLC (Bremnes et al., 2006). As well as HDAC1, VEGF also is a poor prognostic indicators for tumor aggressiveness and survival in human NSCLC (Thorburn., 2004). Vascular endothelial growth factor plays a vital role of angiogenesis, and its expression are upregulated in many cancer cell lines including A549. Recent study reported that VEGF might be a marker for non-small cell lung cancer (Liang et al., 2013). By employing western blot, the collective data displayed that matrine matrine dose-dependently reduced the expression of VEGF and HDAC1 in A549 cells after the cells were treated for 72h with matrine at the concentrations of 0.25-1.0 mg/ml, and suggested that the reduction of VEGF and HDAC1 expression in A549 cells by matrine may be one of important mechanisms of matrine against growth in A549 cells.

Apoptosis resistance is a biology characteristic of cancers by developing resistance to chemotherapy and radiotherapy (Bremnes et al., 2006). At present, platinum-based chemotherapy demonstrates placebo effect to the NSCLC patients. Previous study reported that matrine induced apoptosis autophagy in rat C6 glioma cells. Activation of apoptosis pathways is a significant mechanism by which cytotoxic drugs suppress

proliferation of cancer cells. There are two main pathways that activate apoptosis. One is mediated by the activation of so-called death receptors (Thorburn., 2004), the other is mediated by loss of mitochondria (Green et al., 2004). Mitochondria play a significant role in inducing apoptosis. Bcl-2 is one of the major genes that inhibit apoptosis by protecting cells from causing cell death, whereas Bax members (e.g., Bax, Bad) induce apoptosis, indicating that the Bcl-2 family regulates a common cell death pathway and functions at a point where various signals unite (Hengartner., 2000; Jiang et al., 2004; Antignani et al., 2006). Bax oligomerization and translocation to mitochondria is an essential step in inducing apoptosis through mitochondrial mediated pathway (Cai et al., 2009). The ratio between expression level of Bax and the expression level of Bcl-2 determines whether a cell responds to an apoptotic signal. These apoptosis pathway is tightly synchronized under the control of several signalling pathways. Recent study has demonstrated that Curcumin, one of extracts from Traditional Chinese Medicines, can inhibit proliferation of lung cancer cells by regulating the balance between Bcl2 and Bax (Li et al., 2013b). In present study, we observed that martine treatment increased expression of Bax and decreased expression of Bcl2, suggesting that matrine induced remarkable changes in the Bax/Bcl2 ratio. Apoptosis induced by matrine treatment was confirmed using morphological evaluation which gave a detailed understanding of the drug interaction with cells. We observed lots of apoptotic bodies in matrine- treated A549 cells. These results suggested that matrine can induce apoptosis in A549 cells.

miRNAs are a group of non-coding RNA molecules, whose functions are regulating gene expression by inhibiting translation and/or triggering degradation of their target mRNAs and thus could be used as a biomarker. Moreover, it has been reported that miRNA could interact with other factor to co-regulate the proliferation of lung cancer cells (Liu et al., 2011). Suberoylanilide hydroxamic acid (SAHA) treatment in A549 cells shows changes in the expression profiles of miRNA that has several targets related to angiogenesis, apoptosis, chromatin modification, cell proliferation and differentiation (Lee et al., 2009). The let-7 family has been reported to express at lower level in lung cancer (Takamizawa et al., 2004). Suppressed let-7 expression was significantly associated with shortened postoperative survival and over-expression of let-7 caused the inhibition of lung cancer cell growth in vitro, indicating a vital role of let-7 in proliferation pathways (Johnson et al., 2005). It has been reported that Ras is directly downstream target of let-7 and reduced expression of let-7 enhanced expression of Ras, finally contributing to lung carcinogenesis (Shivapurkar et al., 2003). These studies collectively suggest that down-regulation of let-7 is a key event in lung cancer (Erridge et al., 2007). Our study shows that matrine treatment in A549 cells shows alteration in the expression of miRNAs and that let-7a, miR-18a and hsa-miR-29b may act as tumor suppressors, suggesting that matrine could inhibit growth of lung cancer cells through affecting miRNAs expression profiles.

MiR-18a may play a potential role in suppressing tumor by targeting K-Ras (Tsang et al., 2009). It has been reported that hsa-miR-18a can suppress the K-Ras expression, cell proliferation and anchorage-independent growth in A431 cell line (Cordes et al., 2004). Hsa-miR-29b has been predicted to regulate CDK6 and Bcl-2 (http://www.targetscan.org/index.html). The up-regulated CDK6 can function towards cell cycle arrest and apoptosis through several regulatory axes of the signaling pathway. In this study, we also find hsa-miR-532-5p and 486-3p show higher expression level than other miRNAs. The present data clearly demonstrate that all the miRNAs were up or down-regulated after matrine treatment, indicating that miRNAs play a key role in regulating apoptosis pathway in A549 cells. The present results show that some certain miRNAs undergo alteration in response to matrine and may differentially regulate their target genes, which may provide a useful approach to clarify the role of matrine in cellular response. We could draw a conclusion that matrine could suppress proliferation, growth and induce apoptosis of A549 cells in vitro by changing miRNA expression profiles. Furthermore, it can be predicted that mechanisms of matrine in different concentrations displaying cytotoxicity in A549 are different. In summary, our study indicated that inhibition of cell cycle progression and induction of apoptosis contributes to the anti-proliferative effects of matrine in human lung adenocarcinoma A549 cells. We further found that matrine induces cell death by altering the miRNA expression profiles. Nevertheless, the role of miRNAs in inducing the apoptosis pathway after matrine treatment is still not fully understood. However, additional studies are in progress to identify the possible gene targets of miR-15a, 18a, 486, 2861 and 486-5p and general cellular changes influenced by these miRNAs after matrine treatment. With this basic understanding and puzzle, we would like to further evaluate the sensitising effect of matrine in low concentration on A549 cells. Taken all these data together, we infer that matrine could be developed as an adjuvant chemotherapeutic application for non-small cell lung cancer.

Acknowledgements

We are grateful to all other members in our research group for their support and advice in regards to this study. The study was supported by The National Natural Science Foundation of China (81060351/H2810, 81272454).

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