Matrine induces mitochondrial apoptosis in cisplatin-resistant non-small cell lung cancer cells via suppression of β-catenin/survivin signaling

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Abstract. Matrine is an alkaloid isolated from *Sophora flavescens* and shows anticancer activities. The present study was carried out to determine the cytotoxic effects of matrine on cisplatin-resistant non-small cell lung cancer (NSCLC) cells and the associated molecular mechanisms. Parental and cisplatin-resistant A549 and H460 NSCLC cells were treated with 1 or 2 g/l of matrine for 48 h, and cell viability and apoptosis were assessed. β-catenin-mediated transcriptional activity, mitochondrial membrane potential (ΔΨm) changes, activation of caspases, and survivin expression were examined. The effect of overexpression of survivin on the anticancer activity of matrine was investigated. Compared to the parental cells, cisplatin-resistant NSCLC cells showed increased β-catenin transcriptional activity. Matrine treatment resulted in a significant reduction in β-catenin activation and survivin expression in the cisplatin-resistant cells. Matrine caused apoptotic death in the cisplatin-resistant NSCLC cells, coupled with loss of ΔΨm and activation of caspase-9 and -3. Matrine-induced apoptosis of the cisplatin-resistant NSCLC cells was significantly reversed by overexpression of survivin. In conclusion, matrine exposure induces mitochondrial apoptosis in cisplatin-resistant NSCLC cells, which is largely mediated through inactivation of β-catenin/survivin signaling. Further investigation of the therapeutic benefit of matrine in overcoming cisplatin resistance in NSCLC is warranted.

Introduction

Non-small cell lung cancer (NSCLC) accounts for ~85% of all lung cancers and is the leading cause of cancer-related mortality worldwide (1). Surgery is currently the most effective treatment for early NSCLC (2). However, most NSCLC patients are inoperable due to advanced disease, and are managed with systemic therapies (3,4). Cisplatin-based palliative chemotherapy has been commonly used in patients with advanced NSCLC, yielding a slight survival benefit (5,6). Development of drug resistance is regarded as a major cause for chemotherapy failure in the treatment of cancer (7). Therefore, establishment of effective approaches to overcome cisplatin chemoresistance is of paramount importance in the management of unresectable NSCLC.

The anticancer activity of cisplatin involves the generation of DNA lesions followed by the activation of the DNA damage response and the induction of mitochondrial apoptosis. Numerous mechanisms are responsible for the development of cisplatin resistance, including reduced drug uptake, accelerated drug inactivation, increased DNA damage repair and inhibition of transmission of DNA damage recognition signals to the apoptotic pathway (8). It has been documented that oncogenic β-catenin signaling plays a critical role the acquisition of cisplatin resistance in NSCLC cells (9). Glycogen synthase kinase 3β (GSK3β) is a multifunctional serine/threonine protein kinase that acts as a negative regulator of β-catenin signaling (10). GSK3β is activated upon phosphorylation at Tyr216, which leads to phosphorylation and degradation of β-catenin (11). In contrast, phosphorylation of GSK3β at Ser9 inhibits its ability to promote the degradation of β-catenin (12). Inactivation of GSK3β results in the translocation of active β-catenin to the nucleus, where it interacts with the transcription factor Tcf/Lef to activate multiple pro-proliferative and survival genes such as c-Myc, cyclin D1 and survivin (13). The β-catenin pathway has been suggested as an attractive target pathway for improving the susceptibility of cancer cells to cisplatin (14,15).

Matrine is an alkaloid isolated from *Sophora flavescens* and possesses multiple biological activities including anti-inflammatory (16), antiviral (17) and antitumor (18) activities. Different molecular pathways mediate the cytotoxic effects of matrine on tumor cells (19,20). For instance, Niu *et al* (19) reported that matrine induces apoptosis of lung cancer cells through inhibition of Akt signaling. Downregulation of the ERK-NF-κB pathway is causally linked to the inhibition of

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human osteosarcoma cell invasion by matrine (20). In Hep3B hepatoma cells, matrine has been shown to decrease β-catenin-dependent transcriptional activity (21). Given the involvement of the β-catenin pathway in cisplatin resistance in NSCLC cells, we hypothesized that matrine could sensitizes NSCLC cells to cisplatin through inactivation of β-catenin signaling.

Therefore, in the present study, we attempted to explore the cytotoxic effects of matrine on cisplatin-resistant NSCLC cells and to ascertain whether the anticancer activity of matrine is mediated through modulation of β-catenin signaling.

Materials and methods

Cell lines. Two human NSCLC cell lines A549 and H460 were purchased from the American Type Culture Collection (ATCC; Rockville, MD, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA, USA).

In accordance with previously described methods (23), cisplatin (CDDP)-resistant sublines A549/CDDP and H460/CDDP were established by continuous exposure of the parental cells to increasing concentrations of cisplatin, ranging from 2 nM to 4 μM for >6 months. The drug-resistant cell lines were maintained in DMEM containing 4 μM cisplatin.

Matrine treatment. A549/CDDP and H460/CDDP and their parental cells were seeded at 4-6x10^4 cells/well onto 12-well plates and cultured overnight to allow attachment. The cells were exposed to 1 or 2 g/l of matrine (Sigma, St. Louis, MO, USA) for 48 h. After treatment, the cells were subjected to gene expression and apoptosis analysis.

Plasmid transfection. Human survivin-expressing plasmid (pcDNA3.1-survivin) was kindly provided by Dr Altieri (University of Massachusetts, Worcester, MA, USA). Cells were seeded at a density of 3x10^5 cells/well onto 6-well plates and pre-transfected with empty vector or pcDNA3.1-survivin using Lipofectamine 2000. The transfection efficiency was ~70%, which was determined by transfection of a green fluorescent protein-expressing plasmid (pGFP-N1; Clontech, Mountain View, CA, USA). After incubation for 24 h, the transfected cells were exposed to matrine for an additional 48 h before apoptosis analysis.

Cell viability assay. Cells were seeded onto 96-well plates at 3x10^3 cells/well and cultured overnight to allow adherence. Different concentrations of cisplatin (i.e., 0.5, 1, 2, 4, 8, 16, 32 and 64 μM) were added to the cell culture. After incubation for 48 h, cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. MTT solution (5 mg/ml) (Sigma) was added to each well and incubated for 4 h. After removal of the MTT solution, formazan crystals were dissolved in dimethyl sulfoxide. The absorbance was measured at a wavelength of 570 nm. The 50% inhibitory concentration (IC_{50}) was calculated from the survival curve.

Western blot analysis. Primary antibodies used in the present study are as follows: anti-β-catenin (#9562), anti-phospho-β-catenin (Ser33/37/Thr41) (#9561), anti-non-phospho (active) β-catenin (Ser33/37/Thr41) (#4270), anti-GSK-3β (#9315), anti-phospho-GSK-3β (Ser9) (#9323) (Cell Signaling Technology, Danvers, MA, USA), anti-survivin (sc-10811) and anti-β-actin (sc-130301) (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Following treatment, the cells were lysed in radioimmunoprecipitation assay (RIPA) buffer [150 mM NaCl, 1% NP40, 0.5% deoxycholic acid, 0.1% sodium dodecylsulfate (SDS), 50 mM Tris-HCl (pH 8.0)] supplemented with protease and phosphatase inhibitors. The protein samples were separated on polyacrylamide gels and then transferred to a nitrocellulose membrane. After blocking for 1 h in a Tris-buffered solution (TBS) containing 5% fat-free dried milk and 0.5% Tween-20, the membrane was incubated with individual primary antibodies overnight at 4˚C. The membrane was washed three times and incubated for 1 h with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology). The signals were visualized with an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Densitometric analysis of the protein bands was performed using the Quantity One software (Bio-Rad, Hercules, CA, USA).

Luciferase reporter gene assay. A β-catenin/TCF firefly luciferase reporter construct (pTopFlash) was purchased from Upstate Biotechnology (Waltham, MA, USA). A control pRL-TK reporter plasmid encoding *Renilla* luciferase was purchased from Promega (Madison, WI, USA). Cells were seeded onto 12-well plates at 6x10^4 cells/well and transiently transfected with 0.2 μg of pTopFlash along with 0.02 μg pRL-TK using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. Cells were collected 24 h post-transfection or treated with matrine for 48 h before collection. The cells were then lysed and centrifuged and the supernatant was obtained for measurement of luciferase activities using the Dual-Luciferase Assay System (Promega). The firefly luciferase activity was normalized to *Renilla* luciferase activity and expressed as relative luciferase activity.

Apoptosis detection by Annexin V/PI staining. After drug treatment, the cells were trypsinized and centrifuged. The cell pellet was resuspended and incubated with 1 μl of fluorescein isothiocyanate (FITC)-conjugated Annexin V and 5 μl of propidium iodide (PI) (Becton-Dickinson Biosciences, San Diego, CA, USA) for 15 min at 4˚C in the dark. Apoptotic cells were analyzed on a FACSCalibur flow cytometer using CellQuest software (Becton-Dickinson Biosciences).

Mitochondrial membrane potential (ΔΨm) assay. ΔΨm was measured using the JC-1 mitochondrial membrane potential assay kit (Biotium, Hayward, CA, USA). When ΔΨm is relatively low, the cyanine dye JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimi-dazolylcarbocyanine iodide) localizes in the cytoplasm in a green fluorescent monomeric form. At a high ΔΨm, the JC-1 dye aggregates and yields red fluorescence. A decrease in the ratio of the red/green fluorescence indicates loss of ΔΨm. In brief, cells were harvested after drug treatment and stained with 10 mM JC-1 at 37˚C for 15 min in the dark. Cells were washed and green/red fluorescence was analyzed by flow cytometry.

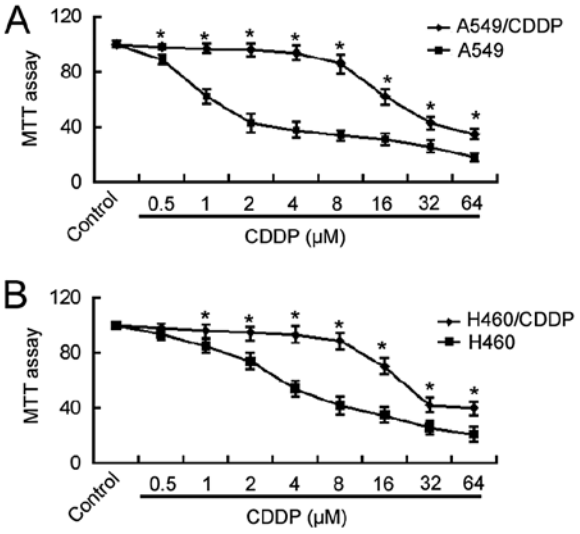

Figure 1. Assessment of the susceptibility to cisplatin (CDDP) in CDDP-resistant and parental cells. CDDP-resistant (A) A549 and (B) H460 cells and their parental cells were treated with a series of concentrations of CDDP for 48 h, and cell viability was assessed using the MTT method. *P<0.05 vs. the parental cells.

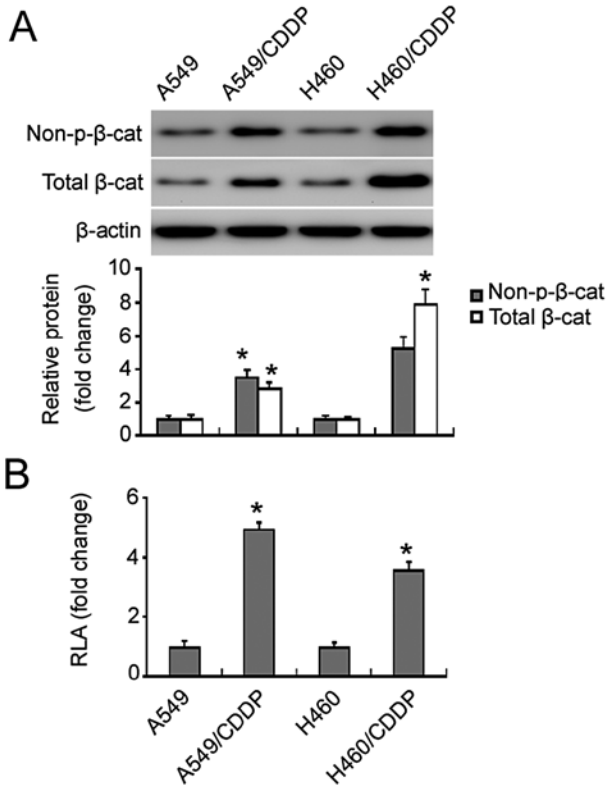

Figure 2. Increased β-catenin activity in the CDDP-resistant NSCLC cells. (A) Western blot analysis of total and non-phospho (active) β-catenin (non-p-β-cat). Representative blots are shown in the top panels, and densitometric quantification of the blots is shown in the bottom graph. (B) CDDP-resistant A549 and H460 cells and their parental cells were transfected with pTop-Flash and pRL-TK. After incubation for 24 h, the cells were treated with matrine for an additional 48 h. The cells were collected and tested for luciferase activities. The results are expressed as relative luciferase activity (RLA) compared to the parental cells arbitrarily assigned as 1. *P<0.05 vs. the untreated cells. CDDP, cisplatin; NSCLC, non-small cell lung cancer.

Measurement of caspase-9 and -3 activities. Measurement of cellular caspase-9 and -3 activities was carried out using the caspase-3 and -9 activity kits (Beyotime, Haimen, Jiangsu, China) according to the manufacturer's instructions. Briefly, cells were collected after drug treatment, washed and lysed in lysis buffer on ice for 10 min. Cell lysates were incubated at 37˚C for 4 h with 1X reaction buffer containing a caspase-3 substrate (acetyl-Asp-Glu-Val-Asp-p-nitroanilide) or caspase-9 substrate (acetyl-Leu-Glu-His-Asp-p-nitroanilide). Caspase activities were measured by spectrofluorometry.

Statistical analysis. All data are expressed as mean ± standard deviation (SD). Statistical significance was analyzed using the Student's t-test or one-way analysis of variance with Tukey's *post-hoc* test. A difference was defined as significant at P<0.05.

Results

Activation of β-catenin signaling is associated with acquisition of cisplatin resistance. Cisplatin-resistant A549 cells were generated by culturing cells in gradually increasing concentrations of cisplatin. The MTT assay revealed that the IC_{50} value of A549/CDDP cells for cisplatin was ~15-fold higher than that of the parental A549 cells (24.7±1.5 vs. 1.6±0.2 μM; Fig. 1A). Similarly, H460/CDDP cells showed an ~6-fold increase in the IC_{50} value for cisplatin compared to the parental cells (28.3±1.2 vs. 4.6±0.4 μM; Fig. 1B). These results indicate the acquisition of cisplatin resistance in NSCLC cells following long-term exposure to cisplatin.

Western blot analysis identified a marked increase in non-phospho (active) β-catenin (Ser33/37/Thr41) and total β-catenin protein in the cisplatin-resistant NSCLC cells relative to the parental cells (Fig. 2A). To evaluate the changes in β-catenin transcriptional activity, cells were transiently transfected with the β-catenin luciferase reporter plasmid. We found that there was a 3.5- and 5-fold increase in the β-catenin reporter activity in the cisplatin-resistant H460 and A549 cells relative to the parental cells, respectively (Fig. 2B).

Matrine suppresses β-catenin signaling in the cisplatin-resistant NSCLC cells. Next, we explored the effect of matrine on β-catenin signaling in the cisplatin-resistant cells. As shown in Fig. 3A and B, matrine treatment reduced the Ser33/37/Thr41-unphosphorylated active β-catenin protein level in the cisplatin-resistant cells. Moreover, matrine exposure resulted in a marked decrease in the phosphorylated level of GSK3β (Ser9) and increase in the total level of GSK3β protein (Fig. 3A and B). The β-catenin luciferase reporter assay confirmed a significant decrease in the β-catenin-mediated transcriptional activity in matrine-treated cisplatin-resistant cells (Fig. 3C). Additionally, survivin, a target gene of β-catenin, was downregulated by matrine exposure (Fig. 3D).

Matrine induces apoptosis in cisplatin-resistant cells via the mitochondrial death pathway. Flow cytometric analysis revealed that matrine at 2 g/l significantly induced apoptosis in the A549/CDDP and H460/CDDP cells, with a 5-8-fold increase in the apoptosis rates relative to the untreated cells (Fig. 4A). Matrine-induced apoptosis was accompanied by a marked loss

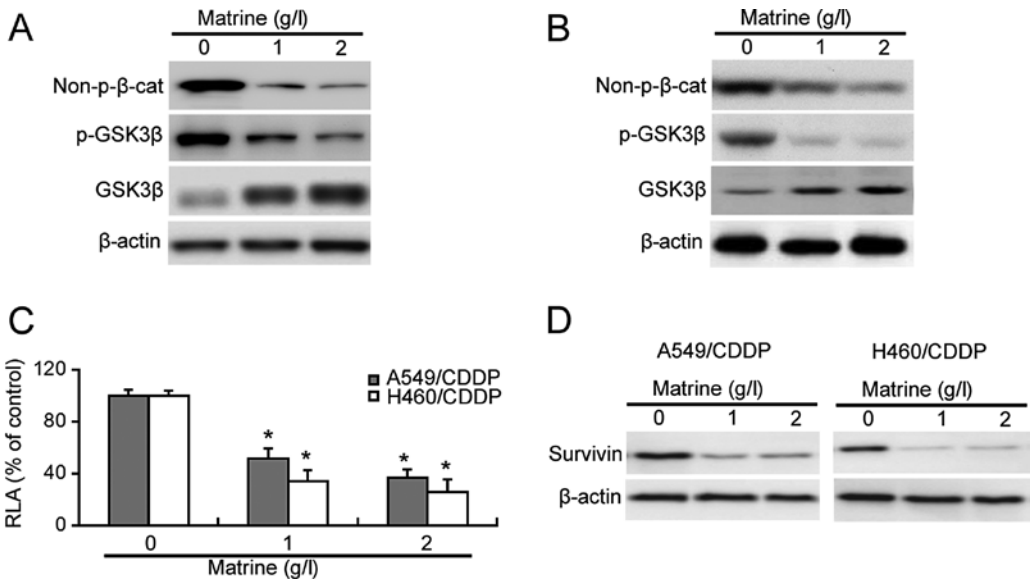

Figure 3. Effect of matrine treatment on β-catenin signaling in CDDP-resistant NSCLC cells. CDDP-resistant (A) A549 and (B) H460 cells were untreated or treated with 1 or 2 g/l of matrine for 48 h, and western blot analysis was carried out to analyze changes in β-catenin signaling. Representative blots are shown. non-p-β-Cat, non-phospho β-catenin. (C) CDDP-resistant A549 and H460 cells were pre-transfected with pTopFlash and pRL-TK and 24 h later, treated with or without matrine for an additional 48 h. The results are expressed as relative luciferase activity (RLA) compared to the control cells (without matrine treatment) arbitrarily assigned as 100%. *P<0.05 vs. the control cells. (D) Western blot analysis of survivin expression in the CDDP-resistant NSCLC cells with or without matrine treatment. CDDP, cisplatin; NSCLC, non-small cell lung cancer.

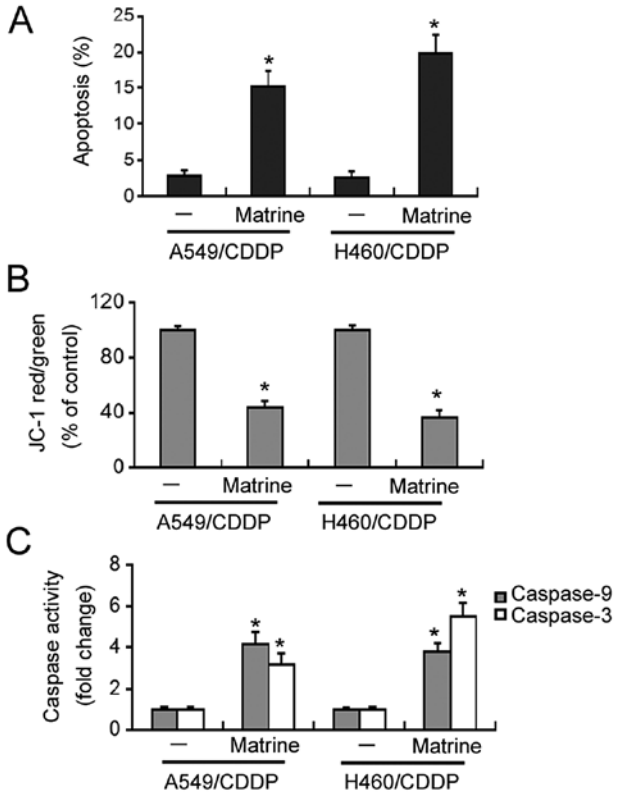

Figure 4. Matrine induces mitochondrial apoptosis in CDDP-resistant NSCLC cells. A549/CDDP and H460/CDDP cells were untreated or treated with 2 g/l matrine for 48 h and apoptotic changes were assessed. (A) Flow cytometric analysis of apoptotic cells stained with Annexin V and PI. Annexin V-positive cells were considered apoptotic. (B) Loss of ΔΨm was determined by flow cytometry using JC-1 staining. The ratio of the red/green fluorescence was calculated. The results are expressed as a percentage of the control values (assigned 100%). (C) Measurement of activities of caspase-3 and -9. The relative caspase activity is expressed as fold-change compared to the control cells (assigned 1). *P<0.05 vs. untreated cells. ΔΨm, mitochondrial membrane potential. CDDP, cisplatin; NSCLC, non-small cell lung cancer.

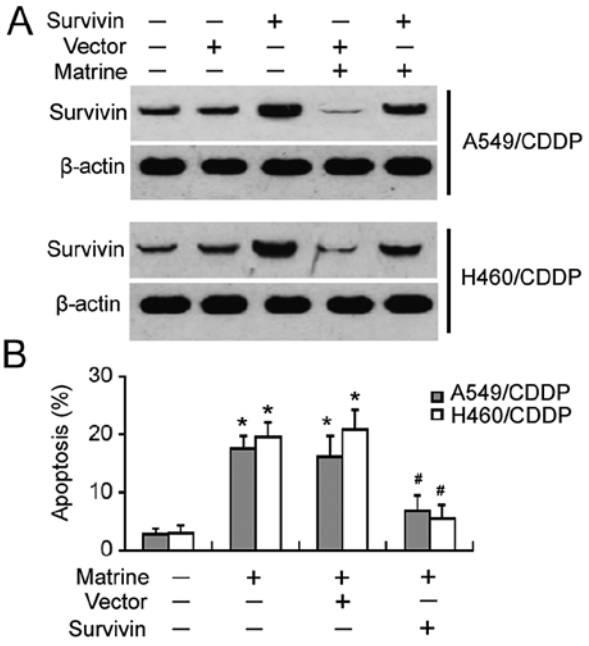

Figure 5. Effect of enforced expression of survivin on matrine-induced apoptosis. (A) Western blot analysis of survivin expression in the A549/CDDP and H460/CDDP cells transfected with the control vector or survivin-expressing plasmid after exposure to 2 g/l matrine for 48 h. Representative blots of three independent experiments are shown. (B) A549/CDDP and H460/CDDP cells were treated with 2 g/l matrine or pre-transfected with the control vector or survivin-expressing plasmid before matrine treatment. After incubation for 48 h, the cells were subjected to apoptosis detection using Annexin V and PI staining. *P<0.05 vs. the untreated cells; #P<0.05 vs. matrine alone. CDDP, cisplatin.

in ΔΨm (Fig. 4B). Moreover, both caspase-9 and -3 activities were significantly increased in the matrine-treated cisplatin-resistant cells, compared to the untreated cells (Fig. 4C).

Ectopic expression of survivin protects against matrine-induced apoptosis. To determine the role of survivin in matrine-induced apoptosis of cisplatin-resistant NSCLC cells, the cells were pre-transfected with the survivin-expressing plasmid before exposure to matrine. Western blot analysis confirmed that the expression level of survivin remained high after matrine treatment in survivin-transfected cells, yet was markedly reduced in vector-transfected cells (Fig. 5A). After a 48-h incubation with matrine, apoptotic death was significantly observed in the vector-transfected cells, yet not in the survivin-transfected cells (Fig. 5B). These results indicate that survivin can rescue cisplatin-resistant cells from matrine-induced apoptosis.

Discussion

Aberrant activation of β-catenin signaling is causally linked to the development of resistance to anticancer drugs (24). It has been reported that miR-29a-induced resistance to gemcitabine in pancreatic cancer cells is mediated through activation of the Wnt/β-catenin signaling pathway (25). In contrast, inhibition of Wnt/β-catenin signaling reverses multidrug resistance in cholangiocarcinoma cells (26). A previous study showed that inhibition of cytoplasmic GSK3β in A549/CDDP cells leads to activation of Wnt/β-catenin signaling, consequently increasing cisplatin resistance (9). In line with these studies, our data demonstrated that the acquisition of cisplatin resistance in NSCLC cells after chronic exposure to cisplatin was associated with elevated β-catenin activity. Downregulation of β-catenin via RNA interference technology reversed drug resistance in A549/CDDP cells (27), confirming the essential role for β-catenin activity in cisplatin resistance in NSCLC cells.

Matrine has been shown to inhibit β-catenin signaling in hepatoma cells (22). However, in WB-F344 rat liver epithelial stem-like cells, matrine has been found to induce β-catenin activation (21). Our data provide initial evidence that matrine treatment led to impaired β-catenin activation in cisplatin-resistant NSCLC cells. These findings suggest that the regulatory effects of matrine on β-catenin signaling are cellular context-dependent. GSK3β is a pivotal negative regulator of β-catenin signaling and its phosphorylation at Ser9 decreases its ability to promote β-catenin degradation (12). We found that matrine-treated cells showed increased total GSK3β protein and reduced Ser9-phosphorylated GSK3β protein, coupled with reduced β-catenin-dependent transcriptional activity. These results indicate that upregulation of GSK3β accounts for matrine-mediated inactivation of β-catenin in cisplatin-resistant NSCLC cells.

Apoptosis induction is an important mechanism for the action of anticancer agents. The proapoptotic activity of matrine has been described in a variety of cancer cells, such as lung cancer (19) and hepatocellular carcinoma cells (28). Our data confirmed that matrine was also able to induce apoptotic death in cisplatin-resistant NSCLC cells. The mitochondrial pathway is an important pathway of apoptosis (29), which involves loss of ΔΨm and release of several proapoptotic proteins including cytochrome *c* from the mitochondrial intermembrane space to the cytosol, leading to activation of procaspase-9 and -3. Notably, matrine treatment of cisplatin-resistant NSCLC cells resulted in dramatic loss of ΔΨm and increased caspase-9 and -3 activities, indicating activation of the mitochondrial death pathway. In agreement with our findings, matrine also induced mitochondrial apoptosis in human acute myeloid leukemia cells (30).

Having identified that matrine suppressed β-catenin signaling and induced apoptosis in cisplatin-resistant NSCLC cells, we next checked whether inactivation of β-catenin signaling is causally linked to the proapoptotic activity of matrine. It has been documented that activated β-catenin regulates the transcription of several oncogenic target genes (13). Survivin is an important target gene of β-catenin and its overexpression induces anticancer drug resistance (31). In NSCLC, survivin expression affects the susceptibility to drug-induced cell apoptosis (32,33). Okamoto *et al* (32) reported that stable overexpression of survivin attenuated apoptotic death induced by gefitinib, an epidermal growth factor receptor-tyrosine kinase inhibitor. Targeting survivin has been shown to enhance cisplatin sensitivity in lung cancer xenografts (33). Notably, we found that matrine treatment resulted in a significant reduction in survivin expression. Moreover, restoration of survivin counteracted matrine-induced apoptosis in cisplatin-resistant NSCLC cells. These findings highlight an important role for survivin in the regulation of NSCLC cell susceptibility to matrine. Matrine-induced downregulation of survivin has also been described in multiple myeloma cell lines (34). Despite the importance of survivin, we cannot exclude the possibility that other target genes of β-catenin may have an impact on the anticancer activity of matrine in NSCLC cells.

In conclusion, matrine has the capacity to induce mitochondrial apoptosis in cisplatin-resistant NSCLC cells, which is associated with inactivation of β-catenin/survivin signaling. Therefore, matrine represents a novel anticancer agent for overcoming cisplatin resistance in NSCLC.

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