Original Article

Lycorine induces apoptosis in human pancreatic cancer cell line PANC-1 via ROS-mediated inactivation of the PI3K/Akt/mTOR signaling pathway

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Abstract: Lycorine, a main phenanthridine Amaryllidaceae alkaloid, has shown wide ranging pharmacological activities. This study aimed to investigate the antitumor activity of lycorine against pancreatic cancer and to elucidate the underlying mechanisms. Human pancreatic cancer cell line PANC-1 was treated with lycorine. Cell viability was monitored by MTT assay. The expression of related proteins was identified by western blot analysis. Antitumor activity of lycorine in vivo was assessed in BALB/c athymic nude mice. The results demonstrated that lycorine induced apoptosis in PANC-1 cells, which was supported by PARP cleavage, activation of caspase-3 and caspase-9 as well as down-regulation of Bcl-2, upregulation of Bax and Bax/Bcl-2 ratio. Meanwhile, lycorine treatment decreased the phosphorylation of Akt and mTOR, while LY294002 pretreatment enhanced lycorine-triggered dephosphorylation of Akt, cell apoptosis and cleavage of PARP. In addition, lycorine induced ROS generation, pretreatment of cells with ROS scavenger NAC reduced ROS generation, rescued cell from apoptosis, reversed Akt suppression and PARP cleavage. These results demonstrated that lycorine induced apoptosis in PANC-1 cells via ROS-mediated PI3K/Akt/mTOR signaling pathway. In vivo, lycorine effectively inhibited tumor growth in xenografts model in BALB/c mice without obviously additional toxicity. Lycorine may be a promising option in the treatment of pancreatic cancer.

Keywords: Lycorine, pancreatic cancer, ROS, PI3K/Akt/mTOR, apoptosis

Introduction

Pancreatic cancer is one of the most aggressive tumors of the digestive system with poor response to current therapeutics [1, 2]. Due to a largely asymptomatic progress of the disease, only 10-15% of the patients have a tumor localized to the pancreas at the time of diagnosis allowing of a potentially curative resection [3]. Besides surgical therapy, the major therapies for pancreatic cancer treatment include chemotherapy, radiotherapy, immunotherapy, and vaccine therapy [4, 5]. However, the therapeutic outcome of pancreatic cancer is disappointing in clinic owing to the resistance to cytotoxic chemotherapeutic agents and/or radiotherapy, the treatment of pancreatic cancer remains a major challenge [6]. Therefore, there is an urgent need of therapeutic strategies for pancreatic cancer treatment.

Emerging evidence has shown that naturally occurring products from medicinal plants or diets is a fertile source of potential cancer chemotherapeutic and chemoprevention agents, and they have received great attention because they are considered to be safe and to reduce the risk of mutagenicity in normal cells [7]. Lycorine, an alkaloid extracted from amaryllis plants, has shown to exhibit various pharmacological effects, including anti-inflammatory activities, anti-malarial properties, emetic actions, anti-virus activities [8, 9]. Recent studies have focused on the potential antitumor activity of lycorine. Numerous evidence have demonstrated that lycorine could suppress the growth of various cancer cells, including poliovirus-infected Hela cells [10], leukemia cell [11-13], mammary cancer MM46 cells [14], human anaplastic oligodendroglioma Hs683 cells and mouse melanoma B16F10 cells [15]. Interestingly,
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Figure 1. Chemical structures of lycorine.

Figure 2. Effects of lycorine on the cell viability of PANC-1 cells. Cells were treated with 0 μM, 0.5 μM, 1 μM, 3 μM, 5 μM and 10 μM lycorine for 24 h and the cell viability was monitored by MTT assay. Data were represented as means ± SD. **P < 0.01 vs. the control.

Lycorine displays significantly higher anti-proliferative activities in tumor cells than in normal cells [16]. Up to now, whether lycorine can inhibit the proliferation of human pancreatic cancer cell line PANC-1 is still unknown. In this study, we investigated the anticancer activity of lycorine in PANC-1 cells and explored the potential mechanisms.

Materials and methods

Chemicals and antibodies

Lycorine (purity > 98%) was purchased from Sigma Chemical Co. (St. Louis, MO, USA) and its chemical structure is shown in Figure 1. Lycorine was dissolved in dimethyl sulfoxide (DMSO; Sigma) as a stock solution and diluted in serum-free medium until needed. LY294002 and N-acetyl-cysteine (NAC) were purchased from Sigma (St. Louis, MO, USA). Z-VAD-FMK (Z-VAD), anti-cytochrome c, anti-Bax and anti-Bcl-2 were obtained from Abcam (Cambridge, MA, USA). Anti-Akt, anti-phosphorylated Akt, anti-mTOR, anti-phosphorylated-mTOR, anti-caspase-3, anti-caspase-8, anti-caspase-9, anti-poly (ADP-ribose) polymerase (PARP), anti-β-actin, goat anti-rabbit IgG (H&L)-HRP secondary antibody were purchased from Cell Signaling Technology (Danvers, MA, USA). All reagents were prepared and used as recommended by their suppliers.

Cell line and cell culture

Human pancreatic cancer cell line PANC-1 was obtained from American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml of penicillin and 100 μg/ml of streptomycin. The cells were maintained in a humidified incubator containing 5% CO₂ in air at 37°C.

Cell viability assay

The viability of cell treated with lycorine was determined by MTT assay. Exponentially growing PANC-1 cells were suspended at a final concentration of 1×10⁴ cell/well and cultured in a 96-well flat-bottomed microplate. After 12 hour’s incubation, the cells were treated with 0 μM, 0.5 μM, 1 μM, 3 μM, 5 μM and 10 μM lycorine for 24 h. Subsequently, the drug containing medium was removed and replaced by fresh medium. MTT solution was added to each well and cells were then incubated for 4 h at 37°C. The culture media were then replaced with 150 μL DMSO. After gentle mixing, the absorbance was measured at 570 nm on a Bio-Rad Model 680 microplated reader (Bio-Rad Laboratories, Hercules, CA, USA).

Western blot analysis

Collected cells were lysed immediately in RIPA buffer (20 mm Tris, 2.5 mm EDTA, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 40 mm NaF, 10 mm Na₂HPO₄, and 1 mm phenylmethylsulphonyl fluoride) supplemented with a protease inhibitor cocktail (Calbiochem, San Diego, CA, USA). Cytosolic and mitochondrial protein extracts were isolated using the cytochrome c
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Releasing Apoptosis Assay kit (Millipore, Germany) according to the manufacturer’s instruction. The nuclear protein extracts were isolated using the Nuclear Extraction kit from Activate Motif (Carlsbad, USA). Protein concentration was determined using Micro BCA kit (Beyotime Biotechnology, Haimen, China). Proteins were separated by SDS-PAGE, and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). After being blocked with 5% non-fat milk, blots were incubated with primary antibodies overnight at 4°C, and then exerted to incubation with the corresponding secondary antibody at room temperature for 2 h. Immunoreactive bands were then developed using an enhanced chemiluminescence reagent (Pierce, Rockford, IL, USA) according to the manufacturer’s instruction. The bands were quantitatively analyzed using the public domain NIH Image software (open source Image J software available at http://rsb.info.nih.gov/ij/).

ROS assay

Intracellular ROS generation was measured by using the fluorescent dye 2', 7'-dichlorofluorescein diacetate (DCFH-DA; Sigma, USA) as a substrate. After being pretreated with 0 μM, 1 μM, 3 μM and 5 μM lycorine for 24 h, PANC-1 cells were incubated with DCFH-DA for 30 min at 37°C in dark. After loading, cells were rinsed with PBS and DCF fluorescence was measured using the FlexStation II 384 fluorometric imaging plate reader (Molecular Devices, Sunnyvale, CA, USA) with excitation wavelength of 488 nm and an emission wavelength of 525 nm.

**Figure 3.** Lycorine induced apoptosis in PANC-1 cells. (A) PANC-1 cells were treated with 0 μM, 1 μM and 3 μM lycorine for 24 h and the expression of apoptosis related proteins was determined by western blot assay. (B and C) Cells were pretreated with 10 μM Z-VAD for 1 h, then treated PANC-1 cells with 3 μM lycorine for 24 h. The cell viability (B) and cleavage of caspase-3 and PARP (C) were detected. Data were represented as means ± SD. **P < 0.01 vs. cells treated with lycorine alone.
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Antitumor activity in vivo

Animal experiments were conducted in accordance with the Bioethics Committee guidelines in Yantaishan Hospital. Six-week-old BALB/c athymic nude mice obtained from Shanghai SLAC Laboratory Animal Co. Ltd (Shanghai, China) were established as a xenograft tumor model of PANC-1. PANC-1 cells (5 × 10^6 cells) were inoculated subcutaneously into the dorsal area of the mice. When the tumors grew to approximately 100 mm², the mice were randomly assigned into 3 groups (n = 8, saline control group, low dose lycorine group and high dose lycorine group) and injected intraperitoneally with the following treatments: the same volume saline, 10 mg/kg/2 day lycorine and 20 mg/kg/2 day lycorine, respectively. Tumor growth was measured with a microcaliper every 3 days throughout the experiment. Animals were sacrificed at 21 days after cancer cells inoculation. The implanted sarcomas were separated and weighed. Tumor volume was calculated as the following: volume = d² × D/2, where d and D were the shortest and the longest diameters, respectively.

Statistical analysis

Data were presented as means ± SD. The SPSS19.0 software package was used to perform all statistical analysis. Comparisons between two group were using the Student’s test and between multiple group using ANOVA analysis. The level of statistical significance was set at P < 0.05.

Results

Lycorine inhibited proliferation of PANC-1 cells

To determine the appropriate inhibitory concentrations of lycorine, PANC-1 cells were treated with different concentrations (0–10 μM) of
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As illustrated in Figure 3A, lycorine induced clearly down-regulations of anti-apoptotic proteins Bcl-2, and upregulation of proapoptotic protein Bax, indicating the apoptosis-promoting effect of lycorine was partly attributed to up-regulating Bax/Bcl-2 protein ratio. In addition, exposure of cells to different concentrations of lycorine induced cleavage of caspase-3, caspase-9 and PARP. Lycorine treatment also triggered increasing release of cytochrome c from the mitochondria of PANC-1 cells without affecting caspase-8, suggesting that the intrinsic apoptosis (mitochondrial apoptosis) instead of extrinsic apoptosis was induced. To ascertain if caspase-dependent apoptosis was induced, the pan-caspase inhibitor z-VAD was used. As demonstrated in Figure 3B and 3C, pre-treatment with z-VAD significantly attenuated cell apoptosis induced by lycorine treatment and suppressed the cleavage of caspase-3 and PARP, implying lycorine induced caspase-dependent apoptosis in PANC-1 cells.
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As demonstrated in Figure 4A, lycorine administration decreased phosphorylation of Akt, mTOR in a dose-dependent manner. To further confirm the role of PI3K/Akt/mTOR pathway in lycorine treatment-induced apoptosis, cells were pretreated with PI3K kinase inhibitor LY294002. As manifested in Figure 4B and 4C, pretreatment of LY294002 further promoted lycorine-induced the dephosphorylation of Akt, cell death and cleavage of PARP, suggesting that lycorine-induced apoptosis was mediated through the PI3K/Akt/mTOR pathway.

Lycorine elevated the level of ROS in PANC-1 cells

ROS involved in the regulation of cell apoptosis if generated in excessive amounts [17]. Intracellular ROS generation plays crucial role in the apoptosis of cancer cells triggered by various anticancer agents [18]. Recently, ROS was demonstrated to be related to apoptosis induced by antineoplastic drugs and may be upstream of PI3K/Akt/mTOR signaling pathways [19]. In this study, we investigated whether excessive generation of ROS was involved in lycorine induced apoptosis. As demonstrated in Figure 5A, lycorine treatment elevated the generation of ROS in a dose-dependent manner. Furthermore, pretreatment of cells with the ROS scavenger NAC not only abrogated lycorine induced ROS generation but also rescued cell from lycorine-induced apoptosis. In addition, pretreatment with NAC resulted in the reversal of Akt inhibition and PARP cleavage induced by lycorine (Figure 5B and 5C). Collectively, lycorine-induced apoptosis and inactivation of PI3K/Akt/mTOR signaling pathway was partly modulated by ROS.

Lycorine demonstrated antitumor activity in vivo

In order to translate the results reported above in vivo, a xenograft model with subcutaneously implanted PANC-1 cells was established. As demonstrated in Figure 6, lycorine significantly reduced tumor volume in mice compared with...
the control group. Meanwhile, mean tumor weight in mice treated with lycorine was lower than that in control group, implying that lycorine possesses potent antitumor activity. Meanwhile, the body weight of mice treated with lycorine has not significantly different from those in the control group, which was consistent with the notion that lycorine preferably targets tumor cells and thus exhibited little toxicity (Supplemental Table 1).

**Discussion**

Pancreatic cancer is a common cancer of the digestive system and is a leading cause of morbidity and mortality worldwide. Since toxicity and limited efficacy are common disadvantages of the antitumor drugs, identification of the new agents with low toxicity is very useful for treatment cancer. Lycorine is a natural anti-tumor alkaloid extracted from Amaryllidaceae and has various biological effects on malignant cells. In the present study, we found that lycorine demonstrated the antitumor effects in vivo and in vitro by inducing apoptosis in pancreatic cancer cell line PANC-1.

Apoptosis is a programmed cell death mechanism that can be driven by two major apoptotic pathways: the cell death receptor-mediated extrinsic pathway and the mitochondrial-mediated intrinsic pathway [20]. Caspases, a family of cysteine proteases, play an important role in the induction of apoptosis through cleavage of different substrates [21, 22]. The mitochondrial mediated apoptotic pathway causes cytochrome c release from mitochondria, and the cleavage of executioner caspase-3, ultimately resulting in chromatin condensation, DNA fragmentation and the formation of apoptotic bodies [23]. Our results demonstrated that lycorine treatment released cytochrome c from mitochondria into cytoplasm as well as activated and induced cleavage of caspase-3, caspase-9 and PARP, but not caspase-8, indicating an activation of the mitochondrial-mediated intrinsic pathway. Bcl-2 family members located on the mitochondrial membrane are involved in the regulation of intrinsic apoptosis pathway by altering the permeability of the mitochondrial membrane and triggering the release of cytochrome c [24]. To further confirm the involvement of the mitochondrial pathway in lycorine induced apoptotic death, the expression of Bax and Bcl-2 were evaluated. These results indicated that upregulation of Bax and Bax/Bcl-2 ratio as well as downregulation Bcl-2 and may be involved in the release of cytochrome c from mitochondria into cytosol after lycorine treatment.

The PI3K/Akt/mTOR signaling pathway is the major pathway that regulates cell proliferation, differentiation, apoptosis, and chemotherapy resistance of tumor cells and is one of the most commonly deregulated pathways in cancer [25]. The constitutive activation of this pathway is considered to be corrected with aggravated clinical chemoresistance and poor prognosis for pancreatic cancer patients [26]. Suppression of the PI3K/Akt/mTOR signaling pathway has been extensively investigated for anticancer therapy [27]. The key molecules involved in PI3K/Akt/mTOR pathway include PI3K, PTEN, Akt and mTOR [28]. Akt is an evolutionarily conserved serine/threonine kinase, located mainly in the cytoplasm in resting status. The activated protein p-Akt is translocated into cytoplasm or nucleus, where it will further phosphorylate a series of substrates that may modulate mechanisms including protein synthesis and gene transcription and thus regulate the survival and apoptosis of cells [25, 29]. mTOR is a serine/threonine kinase of the PI3K kinase family and is considered to be a key effector of PI3K/AKT/mTOR pathway, which involves in regulating cell proliferation, survival and angiogenesis [28, 30]. Akt and mTOR are two key kinases in the PI3K downstream of PI3K/Akt/mTOR signaling pathway and often abnormally activated in many tumors [31, 32]. In the present study, we found that lycorine treatment decreased the phosphorylation of Akt and mTOR. However, pretreatment cells with LY294002 enhanced lycorine-triggered the dephosphorylation of Akt, cell apoptosis and cleavage of PARP, indicating that lycorine-induced apoptosis was regulated via the PI3K/Akt/mTOR pathway.

ROS is an important secondary messenger and normally exists in all cells in balance with biochemical antioxidants. Appropriate ROS levels are pivotal for cell survival, while excessive ROS could oxidize the mitochondrial pores, disrupt the mitochondrial membrane potential leading to cytochrome c release, and finally lead to cell death [33, 34]. The mitochondrial apoptotic signaling pathway has been described as an
important downstream event mediated by ROS in apoptotic cell death [35]. In the present study, lycorine administration increased ROS generation, while pretreatment of cells with ROS scavenger NAC reduced ROS generation, rescued cell from apoptosis, reversed Akt suppression and PARP cleavage induced by lycorine. Taken together, these results indicated that lycorine treatment-induced apoptosis and inhibition of PI3K/Akt/mTOR signaling pathway were at least partly mediated by generation of ROS.

Conclusion

In summary, the current study demonstrated that lycorine exerts potent antitumor activity both in vitro and in vivo against pancreatic cancer and the underlying mechanisms of lycorine induced apoptosis may be through ROS generation to modulate the PI3K/Akt/mTOR signaling pathway. These results support the notion that lycorine could facilitate development of an effective strategy for pancreatic cancer.

Disclosure of conflict of interest

None.

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References

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### Supplemental Table 1. Body weight of mice treated with or without lycorine

<table>
<thead>
<tr>
<th>Group</th>
<th>Body weight (g)</th>
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<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>Final</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>18.08 ± 0.79</td>
<td>24.89 ± 2.46</td>
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<tr>
<td>Lycorine (10 mg/kg)</td>
<td>18.19 ± 0.62</td>
<td>23.78 ± 3.12</td>
<td></td>
</tr>
<tr>
<td>Lycorine (20 mg/kg)</td>
<td>18.91 ± 0.83</td>
<td>21.49 ± 3.56</td>
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</tbody>
</table>

Data are mean ± SD for 8 mice.