Lycorine induces apoptosis by enhancing protein degradation of survivin in human oral cancer cell lines

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Lycorine, a natural alkaloid extracted from the Amaryllidaceae plant family, was reported to various physiological and pharmacological effects including anti-cancer activity. Nevertheless, there is no report of the anticancer effect of lycorine in oral cancer cells. The effects of lycorine on cell proliferation and apoptosis were examined through trypan blue exclusion assay, 4′-6-diamidino-2-phenylindole (DAPI) stain, Live/Dead assay, Western blot analysis and RT-PCR. Lycorine suppressed cell viability and induced apoptosis in MC3 and HSC-3 cell lines. Lycorine decreased survivin protein but did not affect its mRNA. It regulated survivin through accelerating protein degradation in a time-dependent manner although neither proteasome nor lysosome was not associated with lycorine-mediated protein degradation. Collectively, our results suggest that lycorine may be a potential therapeutic anti-cancer drug candidate for the treatment of human oral cancer.

Key words: Lycorine, Apoptosis, Survivin, Human oral cancer

I. INTRODUCTION

Lycorine is a natural alkaloid extracted from Amaryllidaceae plant family and possesses various biological activities such as anti-inflammatory properties, anti-malarial activities, anti-virus effects and so forth. Recently, many studies have focused on its anti-cancer properties through cytostatic effects, apoptosis-inducing potentials, anti-metastatic activities and cell cycle regulations in many cancer cell lines.
However, there was no such a report describing anti-cancer activity of lycorine in human oral cancer cell lines.

Inhibitor of apoptosis proteins (IAPs) are recognized by the presence of Baculovirus IAP Repeat (BIR), a zinc finger fold at least once located in each family member. Survivin, one of IAP family members plays an important role in anti-apoptotic process and tumor progression. Survivin can suppress mitochondria-driven apoptosis by inhibiting caspase-9 activity in concert with the caspase inhibitor, XIAP10-12). Our group reported that YM155 known as a survivin inhibitor strongly induced apoptosis in human oral cancer cell lines implying that survivin can be good molecular target for the treatment of oral cancer.

Our aim was to determine the anti-cancer effect of lycorine in MC3 and HSC-3 human oral cancer cell lines and identify the molecular target underlying its apoptotic activity. In the present study, our results demonstrated that inactivation of survivin protein by lycorine can induce apoptosis of human oral cancer cells.

II. Materials and methods

1. Cell culture and chemical treatment

MC3 cells and HSC-3 cells were provided by Prof. Wu Junzheng (Fourth Military Medical University, Xi’an, China) and Prof. Shindo (Hokkaido University, Sapporo, Japan), respectively. Both cell lines were cultured in Dulbecco modified eagle medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) and 100 U/mL each of penicillin and streptomycin in a humid atmosphere of 5 % CO₂ at 37 °C in incubator. Cycloheximide (CHX) was purchased from Sigma-Aldrich chemical Co. (Sigma-Aldrich, Louis, MO, USA). Lycorine and MG132 were supplied by Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Lycorine was dissolved in dimethyl sulfoxide (DMSO), aliquoted, and stored at -20 °C. Final concentration of DMSO did not exceed 0.1 %.

2. Trypan blue exclusion assay

The growth inhibitory effect of lycorine was determined using trypan blue solution (Gibco, Paisley, UK). Cells stained with trypan blue (0.4 %) were counted using a hemocytometer. Each experiment was carried out in triplicate and the results were expressed as the mean ± SD.

3. Western blot analysis

Whole-cell lysates were prepared with lysis buffer and protein concentration in each sample was measured using a DC Protein Assay Kit (BIO-RAD Laboratories, Madison, WI, USA). After normalization, equal amount of protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to Immun-Blot™ PVDF membranes. The membranes were blocked with 5 % skim milk in tris buffered saline with Tween20 (TBST) at RT for 1 hr 30 min, incubated overnight at 4 °C with primary antibodies and followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies at RT for 1 hr 30 min. Antibodies against cleaved PARP, cleaved caspase-3 and survivin were purchased from Cell Signaling Technology, Inc., (Charlottesville, VA, USA) and actin antibody was obtained from Santa Cruz Biotechnology, Inc., (Santa Cruz, CA, USA). The immunoreactive bands were visualized by ImageQuant™ LAS 500 (GE Healthcare Life Sciences, Piscataway, NJ, USA).

4. 4’, 6-diamidino-2-phenylindole (DAPI) staining

Morphological changes of apoptotic cells were determined by DAPI staining. MC3 and HSC-3 cells were seeded in 60 mm² dishes and treated with lycorine for 24 hr. Cells were harvested by trypsinization and fixed in 100% ethanol overnight at -20 °C. The next day, the cells were washed with ice-cold PBS three times and fixed with 1 mL 100 % methanol at room temperature (RT) for 10 min. They were deposited on slides, and then stained with DAPI solution (2 µg/ml). DAPI stained cells were observed using
a fluorescence microscope (x 400) to detect apoptotic characteristics (Nuclear condensation and fragmentation).

5. Live/Dead assay

Cell viability was further investigated with LIVE/DEAD viability/cytotoxicity kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Briefly, MC3 and HSC-3 cells seeded in 60 mm² dishes were treated with various concentrations of lycorine (1, 3, 10, 30 µM). After treatment for 24 hr, cells were harvested by trypsinization and then pelleted by centrifugation for 3 min at 3500 rpm. Cell pellets were then resuspended with calcein acetoxymethyl ester (2 µM) and ethidium homodimer (4 µM) at RT for 30 min, followed by washing with ice-cold PBS. Cells were finally observed with a fluorescence microscope (x 200).

6. Reverse transcription–polymerase chain reaction (RT–PCR)

Total RNA was extracted by easy-BLUE Total RNA Extraction Kit (INTRON, Daejeon, Korea), one microgram of RNA was reversely transcribed by TOPscript RT DryMIX (Elpis Biotech, Daejeon, Korea), and the resultant cDNA was subjected to PCR using HiPi PCR PreMix (Elpis Biotech, Daejeon, Korea). The PCR condition of survivin was as follows: (28 cycles: 1 min at 95 °C, 1 min at 60 °C and 1 min 30 sec at 72 °C), and the PCR condition of β-actin was as follows: (30 cycles: 1 min at 95 °C, 1 min at 62 °C and 1 min 30 sec at 72 °C). The primer sequences were used: survivin sense 5’-ATG GCC GAG GCT GGC TTC ATC-3’, survivin anti-sense 5’-ACG GCG CAC TTT CTT CGC AGT T-3’, β-actin sense 5’-GTG GGG CGC CCC AGG CAC CA-3’, β-actin anti-sense 5’-CTT CCT AAT GTC ACG CAC GAT TTC-3’. The intensities of each band were normalized to β-actin. The amplified products were analyzed by 1.2 % agarose gel electrophoresis and stained with ethidium bromide.

7. Statistical Analysis

The data were analyzed for statistical significance using a Student’s t-test, and P value compared with vehicle control was considered statistically significant.

III. Results

1. Lycorine suppress the proliferation of MC3 and HSC-3 oral cancer cell lines.

To determine the effect of Lycorine on the growth of MC3 and HSC-3 cells, cells treated with Lycorine at various concentrations for 24 hr were measured using a trypan blue exclusion assay. Figure 1A showed that the viability of both

![Fig. 1](image_url)

**Fig. 1.** The effect of lycorine on cell viability and apoptosis in MC3 and HSC-3 cells

(A) MC3 and HSC-3 cells were treated with DMSO or various concentrations of lycorine (1, 3, 10 and 30 µM) for 24 hr. The effects of lycorine on cell viability were analyzed by Trypan blue exclusion assay. The graph represents mean ± SD of three independent experiments and significance (p<0.01 and 0.001) compared with the DMSO-treated group was indicated (** and ***). (B) Western blot analysis was performed to detect cleaved PARP and caspase-3. Actin was used to normalize the protein loading from each treatment.
cells exposed to various concentrations of lycorine (1, 3, 10 and 30 μM) was decreased from 77 % to 37 % (MC3 cells) and from 86 % to 31 % (HSC-3 cells). Its IC50 values for MC3 and HSC-3 cells were approximately 15.8 and 13.1 μM, respectively.

2. Lycorine induces apoptosis in oral cancer cell lines.

To demonstrate whether the growth-inhibitory effects of lycorine may be due to its apoptotic potential, we performed Western blot analysis using antibodies against cleaved PARP and caspase 3, DAPI staining and Live/Dead assay. As shown in figure 1B, the treatment of lycorine remarkably activated caspase 3 and accompanied by PARP cleavage in both cell lines. MC3 and HSC-3 cell lines treated with lycorine displayed nuclear condensation and fragmentation which are typical apoptotic features while DMSO-treated cells had an intact nucleus (Figure 2A). In addition, lycorine clearly increased red fluorescence-positive cells implying the increasing number of dead cells in MC3 and HSC-3 cells (Figure 2A).

3. Lycorine downregulates survivin protein in oral cancer cell lines through translational and posttranslational modifications.

Recently, our laboratory reported that down-regulation of survivin might be a good molecular target to induce apoptosis in oral cancer13, 14. Based on these studies, we evaluated the effects of lycorine on the expression of survivin protein and mRNA in both cell lines. Figure 3A

![Figure 2](image_url)

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**Fig. 2.** The apoptotic effects of lycorine in MC3 and HSC-3 cells

(A) Fluorescence microscopy images of the DAPI-stained MC3 and HSC-3 cells (Magnification, ×400). The apoptotic cells which have nuclear condensation and DNA fragmentation were quantified. (B) Live/dead cells were observed by fluorescence microscope (Magnification, ×200), and then dead cells were counted. The graph represents mean ± SD of three independent experiments and significance (p<0.05, 0.01 and 0.001) compared with the DMSO-treated group was indicated (*, ** and ***).
showed concentration-dependently reduction of survivin protein level in MC3 and HSC-3 cells incubated with various concentrations of lycorine whereas the survivin mRNA expression levels were not altered by the treatment of lycorine (Figure 3B).

4. Lycorine accelerates survivin protein degradation in a time-dependent manner regardless of proteasome and lysosome.

To demonstrate the post-transcriptional modification of lycorine on survivin protein, we treated both cell lines with/without 10 μM of lycorine in the presence of a protein...
synthesis inhibitor, cycloheximide (CHX). As shown in figure 4, co-treatment of lycorine and CHX significantly decreased survivin protein levels quickly within 3 hr. These findings suggest that lycorine reduces the survivin protein level through the post-transcriptional modification rather than transcriptional regulation.

IV. Discussion

Lycorine, a natural alkaloid extracted from the Amaryllidaceae plant family, were performed detailed in vitro and in vivo on the anti-cancer effects of lycorine via possesses various biological effects including inducing apoptosis. In previous studies, chromatin condensation and nuclear fragmentation were known for hallmarks of apoptotic characterization. Several studies recently reported that lycorine inhibited cancer cell proliferation and metastasis and induced cell cycle arrest and apoptosis in various cancer cell lines. In the present study, we explored the anti-cancer effects of lycorine on the cell growth and apoptosis of human oral cancer cell lines (MC3 and HSC-3 cell lines). Our results strongly suggest that lycorine suppressed oral cancer cell growth through its apoptotic activity (Figure 1 and 2). These results suggest that lycorine might be a potentially growth suppressor of human oral cancer cell lines.

According to a recent study, lycorine induces apoptosis in human leukemia cells via intrinsic apoptotic pathways through a rapid-turnover of protein level of myeloid cell leukemia-1 (Mcl-1). Tumor necrosis factor (TNF)-alpha and p21 may be involved in lycorine-induced apoptosis in HL-60 cells. Even though many studies demonstrated the relationship between multiple signaling pathway and lycorine-induced apoptosis, the involvement of survivin protein in lycorine-induced apoptosis in human cancer cell lines was not elucidated yet. Our data findings suggest that lycorine reduces the survivin protein level through the post-transcriptional modification rather than transcriptional regulation (Figure 3). On the contrary to this, Zhang and Cui revealed that lycorine notably changed the gene expressions of survivin as well as Bcl-2, Bax and p53 to induce apoptosis of A549 pulmonary carcinoma cells meaning that it regulates the survivin molecule at a transcriptional level. It means that lycorine can modulate survivin at either transcriptional or post-transcriptional level. Previously, several studies showed that lycorine reduced the survival and induced apoptosis in human cancer cells through Bcl-2 family proteins. Thus, we confirmed whether Bcl-2 family proteins may be involved in lycorine-induced apoptosis in human oral cancer cells. Unfortunately, we did not find any common target for lycorine-related apoptosis in both cell lines (data not shown) meaning that Bcl-2 proteins may not be a molecular targets for apoptotic activity of lycorine against oral cancer. As a result, we conclude that lycorine can induce apoptosis by down-regulating survivin at a post-transcriptional modification. To the best of our knowledge, this is the first demonstration of the anticancer effect of lycorine on human oral cancer. Consequently, our results suggest that lycorine inhibits of oral cancer cell proliferation potential drug candidate for anti-cancer therapy.

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