Abstract. Lycorine, a natural alkaloid extracted from the Amaryllidaceae plant family, has been reported to exhibit anti-cancer effects in various types of cancer cells. However, the molecular mechanisms through which lycorine exhibits anti-hepatoblastoma activity are unclear. In the present study, the inhibitory effects of lycorine on the proliferation and migration of HepG2 hepatoblastoma cells were investigated. Lycorine inhibited the proliferation of HepG2 cells in a dose-dependent manner by inducing cell cycle arrest at the G2/M phase, via downregulation of cyclin A, cyclin B1 and cyclin dependent kinase 1. Additionally, wound healing and Transwell assays revealed that treatment with lycorine resulted in a decrease in the migratory ability of HepG2 cells. Also, treatment with lycorine decreased the expression levels of matrix metalloproteinase (MMP)-9 and MMP-2. Furthermore, lycorine induced the cleavage/activation of Rho associated coiled-coil containing protein kinase 1 (ROCK1) and the downregulation of cofilin, accompanied by an increase in polymerized filamentous actin and a loss of depolymerized globular actin. Furthermore, pre-incubation of cells with Y-27632, a specific ROCK1 inhibitor, markedly attenuated lycorine-induced anti-proliferative and anti-migration effects. Taken together, the results demonstrated that lycorine inhibited the proliferation and migration of HepG2 cells by suppressing ROCK1/cofilin-induced actin dynamics, which suggests that lycorine has the potential to be developed into a novel drug for hepatoblastoma treatment.

Introduction

Hepatoblastoma, the most commonly diagnosed malignant pediatric liver tumor, is frequently diagnosed in the first 3 years of life. In recent years, the combination of surgery and chemotherapy has improved the prognosis of patients with hepatoblastoma (1). Furthermore, chemotherapeutic agents, including cisplatin, have been applied in therapeutic strategies for hepatoblastoma (2,3). However, conventional chemotherapy agents frequently have limited clinical applications due to the adverse side effects and drug resistance acquired following long-term use. Consequently, it is vital to develop safe and affordable alternative therapeutic agents for the treatment of hepatoblastoma.

Recently, naturally occurring compounds have been valued as potential anticancer therapies due to their safety and efficacy. Additionally, the majority of clinical chemotherapeutic drugs have an alkaloid structure, suggesting that alkaloids are important antitumor agent candidates. Lycorine, a crude alkaloid extracted from Amaryllidaceae genera, is reported to have antimalarial, antiviral and anti-inflammatory properties (4-7). Notably, lycorine is at least 15-fold more effective against cancer cells compared with normal cells, suggesting that lycorine is a selective anti-tumor compound (8). Multiple molecular mechanisms have been reported to be involved in the anticancer effects of lycorine. Inducing apoptosis of cancer cells has a pivotal role among these mechanisms. For example, lycorine induces apoptosis of A549 cells via the adenosine monophosphate-activated protein kinase/mTOR/S6 kinase signaling pathway (9), and lycorine induces apoptosis of bladder cancer T24 cells by inhibiting protein kinase B phosphorylation and activating the intrinsic apoptotic cascade (10). However, increasing evidence has emphasized that lycorine also inhibits cancer cell proliferation and migration (11). Thus, it is important to examine the molecular mechanisms underlying the anti-proliferative and anti-migration effects.

In the present study, the HepG2 hepatoblastoma cell line was used to investigate the inhibitory effects of lycorine on cell proliferation and migration. Lycorine inhibited the proliferation of HepG2 cells and induced cell cycle arrest at the G2/M phase. Additionally, lycorine inhibited the migration...
Materials and methods

Cells and antibodies. Lycorine (cat. no. A0415) was purchased from Chengdu Must Biotechnology Co., Ltd. (Chengdu, China) and dissolved in PBS as a stock solution. Y-27632 (cat. no. S1049) was obtained from Selleck Chemicals (Houston, TX, USA). Antibodies against cyclin A (cat. no. sc-751; 1:500), cyclin B1 (cat. no. sc-752; 1:500), cyclin-dependent kinase 1 (cdk2; cat. no. sc-8395; 1:1,000) and GAPDH (cat. no. sc-51905; 1:10,000) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA); antibodies against cofilin (cat. no. ab42824; 1:2,000) and ROCK1 (cat. no. ab45171; 1:1,000) were from Abcam (Cambridge, MA, USA); and antibodies against matrix metalloproteinase (MMP)-9 (cat. no. 13667; 1:1,000) and MMP-2 (cat. no. 40994; 1:1,000) were from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Cell culture. The human HepG2 hepatoblastoma cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; cat. no. PM150212; Procell Life Science & Technology Co., Ltd., Wuhan, China) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and cultured in a 37°C incubator with a humidified atmosphere of 5% CO2. Once cells were adhering to the flask, the medium was changed every 2 days and the cells were digested using 0.25% trypsin (Gibco; Thermo Fisher Scientific, Inc.).

MTT assay. Cells were seeded in a 96-well culture plate at a density of 1x10^4 cells/well overnight, and treated with various concentrations of lycorine (0.2, 0.5, 1, 2, 10, 20, and 100 µM) the following day. Following incubation in a 5% CO2 incubator at 37°C for 24 or 48 h, the medium was removed and 20 µl MTT solution (5 mg/ml) was added to each well. Following incubation at 37°C for an additional 4 h, 150 µl dimethyl sulfoxide (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was used to dissolve the dark blue crystals. The optical density value was determined at 570 nm and measured on a microplate reader (Varioskan Flash; Thermo Fisher Scientific, Inc.). All these results are expressed as a percentage of the control, which was set at 100%. Each experiment was repeated three times individually.

Clone formation assay. Cells (200 cells/well) were seeded in a 6-well plate. Cells were allowed to attach overnight and exposed to different concentrations of lycorine (10 and 20 µM) for 48 h, following which the culture medium was replaced with fresh DMEM and cultured for 2 weeks; cells were subsequently fixed with 4% paraformaldehyde for 15 min and stained with 0.1% crystal violet for 10 min at room temperature. The number of colonies was counted using Photoshop CS6 software (Adobe Systems, Inc., San Jose, CA, USA). Each group had three repeat wells and this experiment was repeated three times.

Flow cytometry. The cell cycle distribution was determined by flow cytometry. Cells were seeded in a 6-well culture plate at a density of 1x10^6 cells/well. Lycorine (10 and 20 µM) were added the subsequent day. Following incubation for 48 h, cells were harvested and washed twice with PBS. Cells were fixed in cold 75% ethanol overnight in 4°C. Cells were washed twice with cold PBS and suspended in PBS with 200 µg/ml RNase and 50 µg/ml propidium iodide (cat. no. 556547; BD Biosciences, San Jose, CA, USA) in the dark for 30 min. The results were measured by flow cytometry (FACScan; BD Biosciences) and analyzed using ModFit LT 3.2 software (Verity Software House, Inc., Topsham, ME, USA).

Wound healing assay. A wound healing assay was used to assess the migration ability of HepG2 cells. Briefly, cells were seeded in a 6-well culture plate. When cells reached 90% confluence, a wound was scratched with a 200 µl pipette tip. Cells were washed with PBS three times to remove the scratched cells, and lycorine (10 and 20 µM) was added and incubated for 24 or 48 h. The cells were imaged following replacement of the medium. The wound width was measured using ImageJ software (version 1.48; National Institutes of Health, Bethesda, MD, USA): Wound healing rate (%) = 100 x (0 h width - 24/48 h width)/2/0 h width.

Transwell assay. HepG2 cells were adjusted to a density of 2x10^5 cells/well, resuspended and seeded in the upper chamber of Transwell chambers with 200 µl serum-free medium, and 600 µl complete medium containing 30% FBS was added in the lower chamber. Lycorine (10 and 20 µM) was added to the two chambers. Following incubation for 48 h, non-migrated cells on the top surface of the upper chamber were gently scraped away with a cotton swab. The lower membrane containing migrated cells was fixed in 4% paraformaldehyde for 10 min and stained with 0.1% crystal violet for 15 min at room temperature. A microscope (x4 magnification; Olympus IX51; Olympus Corporation, Tokyo, Japan) was used to image the migrated cells. The total cell numbers were calculated from three different fields and three independent experiments.

Western blot analysis. Cells were harvested and lysed in lysis buffer containing 1 mM phenylmethylsulfonly fluoride (Beyotime Institute of Biotechnology, Haimen, China). Bicinchoninic acid protein quantification kits (Beyotime Institute of Biotechnology) were used to measure protein concentrations. A total of 15 µg protein was separated via 12% SDS-PAGE and transferred to polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). Following blocking with 5% skim milk in Tris-buffered saline (TBS) containing 0.1% Tween-20 for 2 h at room temperature, specific primary antibodies were added to the membranes.
and incubated at 4˚C overnight on a shaker. Membranes were washed in TBS with Tween-20 three times and incubated with anti-rabbit or anti-mouse horseradish peroxidase secondary antibodies (cat. nos. 074-1516 and 074-1802, respectively; 1:100,000; Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD, USA) for a further 2 h at room temperature. Enhanced chemiluminescence reagent (EMD Millipore) was used to visualize the bands. Densitometric analysis was performed using Quantity One software version 4.6.2 (Bio-Rad Laboratories, Inc., Hercules, CA, USA). GAPDH was used as an internal control.

Immunofluorescence assay. Following treatment with lycorine (10 and 20 µM) for 48 h, cells were washed twice with PBS and fixed with ice-cold 75% ethanol for 15 min at room temperature. Cells were permeabilized with 0.1% Triton X-100 for 5 min, fluorescent staining of filamentous and globular actin was performed by staining with fluorescent deoxyribonuclease I conjugates and fluorescent phallotoxins (Molecular Probes; Thermo Fisher Scientific, Inc.) for 30 min in the dark, and slides were washed and stained with DAPI for 5 min at room temperature (cat. no. C1002; Beyotime Institute of Biotechnology). Images were captured using a Leica scanning confocal microscope (x40 magnification; TCS SP2 AOB; Leica Microsystems GmbH, Wetzlar, Germany).

Statistical analysis. All data were analyzed using GraphPad Prism 6.0 software (GraphPad Software, Inc., San Diego, CA, USA). Data presented are expressed as the mean ± standard deviation at least three independent experiments. Differences between groups were analyzed by one-way analysis of variance (ANOVA) with Dunnett’s or Tukey’s test. P<0.05 was considered to indicate a statistically significant difference.

Results

Lycorine inhibits the proliferation of HepG2 cells. The chemical structure and molecular weight of lycorine is presented in Fig. 1A. The cytotoxicity of lycorine in HepG2 cells was determined by MTT assay. Cells were treated with various concentrations of lycorine (0.2, 0.5, 1, 2, 10, 20 and 100 µM) for 24 or 48 h, which resulted in significant decreases in cell viability in a dose-dependent manner (P<0.01, P<0.001; Fig. 1B). Exposure of cells to 2 µM lycorine resulted in a modest decrease in cell viability, and these events became significant following exposure of cells to ≥10 µM lycorine (Fig. 1B). To further confirm the anti-proliferative effect of lycorine, a clone formation assay was performed. In accordance with the results of the MTT assay, lycorine significantly inhibited the clone formation of HepG2 cells compared with the control cells (P<0.01, 10 µM; P<0.001, 20 µM; Fig. 1C). Taken together, these results demonstrated that lycorine inhibited the proliferation of HepG2 cells.

Lycorine induces HepG2 cell cycle arrest at the G2/M phase via downregulation of cyclin A, cyclin B1 and cdc2. Cell cycle arrest is an important mechanism involved in the inhibition of cell growth (12). To examine the effect of lycorine on the cell cycle of HepG2 cells, cell cycle dynamics were assessed by flow cytometry. Following exposure of cells to lycorine (10 and 20 µM) for 48 h, the proportion of cells in the G2/M phase increased from 10.81% (without lycorine) to 14.64% (in the presence of 10 µM lycorine) and 20.15% (in the presence of 20 µM lycorine; P<0.01 vs. control; Fig. 2A). These results indicated that lycorine induced HepG2 cell cycle arrest at the G2/M phase. To further examine the molecular mechanisms under lycorine-induced G2/M phase arrest, western blot
Figure 2. Lycorine induces cell cycle arrest at the G2/M phase in HepG2 cells. (A) The cell cycle was analyzed by flow cytometry following treatment with lycorine (10 and 20 µM) for 48 h. The relative ratios of the G0/G1, S and G2/M phases were analyzed using flow cytometry by ModFit LT 3.2 software. (B) Western blot analysis of the cellular G2/M phase regulators cyclin A, cyclin B1 and cdc2 under treatment with lycorine (1, 2.5, 5, 10 and 20 µM) for 48 h. GAPDH served as the loading control and the relative quantification of the detected proteins was analyzed using Quantity One software. Data are presented as the mean ± standard deviation. **P<0.01 and ***P<0.001 vs. control. cdc2, cyclin-dependent kinase 1.

Figure 3. Lycorine inhibits the migration of HepG2 cells. (A) Cells were exposed to serial concentrations of lycorine (10 and 20 µM) for 24 or 48 h. The effects of lycorine on HepG2 cell migration were evaluated by wound healing assay. Scale bar, 200 µm. (B) The effect of lycorine on the migration of HepG2 cells was assessed in a Transwell assay. HepG2 cells were treated with lycorine at 10 or 20 µM for 48 h. Scale bar, 200 µm. (C) Following treatment of HepG2 cells with lycorine (1, 2.5, 5, 10 and 20 µM) for 48 h, western blotting was performed to assess the expression of MMP-9 and MMP-2. The relative quantification of proteins was analyzed using Quantity One software. Data are presented the mean ± standard deviation (n=3). *P<0.05, **P<0.01 and ***P<0.001 vs. control. MMP, matrix metalloproteinase.
analysis was performed to assess the expression of cyclin A, cyclin B1 and cdc2. The results revealed that exposure of cells to lycorine resulted in a marked decrease in the expression of cyclin A, cyclin B1 and cdc2 in HepG2 cells (P<0.001; Fig. 2B). These data suggested that lycorine has an inhibitory effect on HepG2 cell cycle progression, which may cause the inhibition of HepG2 cell proliferation.

Lycorine inhibits the migration of HepG2 cells. The wound healing assay indicated that lycorine induced a marked decrease in cell migration in HepG2 cells in a dose and time-dependent manner (P<0.01 and P<0.001; Fig. 3A). Furthermore, the Transwell assay revealed that lycorine significantly inhibited the migratory ability of HepG2 cells (P<0.001; Fig. 3B). Evidence has confirmed that MMPs, particularly MMP-9 and MMP-2, are important regulators in the process of cancer migration (13,14). To investigate whether lycorine alters the expression of MMP-9 and MMP-2, western blot analysis was performed. The results demonstrated that compared with the control group, lycorine decreased the expression levels of MMP-9 and MMP-2 in a concentration-dependent manner (P<0.05, P<0.01 and P<0.001; Fig. 3C). These data indicated that lycorine inhibits HepG2 cell migration.

Lycorine alters actin cytoskeletal dynamics by suppressing cofilin expression. Actin, an essential component of the cytoskeleton, has a critical role in a wide range of cellular processes, including cell migration and cell division (15). In the current study, exposure of cells to lycorine (10 and 20 µM) resulted in an increase in polymerized filamentous actin (F-actin) and a decrease in depolymerized globular actin (G-actin; Fig. 4A). It has been reported that cofilin regulates actin dynamics by severing actin filaments (16). Therefore, western blot analysis was performed to determine whether cofilin is involved in the lycorine-induced altering of actin cytoskeletal dynamics. As presented in Fig. 4B, HepG2 cells were treated with lycorine (1, 2.5, 5, 10 and 20 µM) for 48 h, which resulted in a decrease in the expression of cofilin (P<0.01 and P<0.001). Collectively, these results suggested that lycorine suppresses the expression of cofilin and alters actin cytoskeletal dynamics.

ROCK1 activation has an important role in lycorine-induced anti-proliferative and anti-migration effects. ROCK1 has been confirmed to have an important role in regulating cell polarity and migration (17,18). In the present study, it was investigated whether ROCK1 activation is involved in lycorine-induced anticancer effects. Treatment of HepG2 cells with lycorine (1, 2.5, 5, 10 and 20 µM) for 48 h
induced cleavage/activation of ROCK1 in a dose-dependent manner (Fig. 5A). To further confirm the role of ROCK1 in lycorine-induced cell proliferation and migration inhibition, Y-27632, a ROCK1 specific inhibitor, was used. Western blot analysis indicated that pre-incubation of cells with Y-27632 inhibited lycorine-induced ROCK1 cleavage/activation (Fig. 5B). Pre-incubation of cells with Y-27632 attenuated the lycorine-induced cofilin decrease (Fig. 5C). Furthermore, pre-incubation with Y-27632 also attenuated the decreases in cyclin A, cyclin B1, cdc2, MMP-9 and MMP-2, which indicated...
that Y-27632 attenuated lycorine-induced G2/M cell cycle arrest and migration ability (Fig. 5D and E). A clone formation assay demonstrated that combined treatment with Y-27632 and lycorine significantly increased the colony number compared with treatment with lycorine alone (P<0.01; Fig. 5F). The wound healing and Transwell assays revealed that co-administration of Y-27632 and lycorine markedly attenuated the lycorine-induced inhibitory effect on cell migration (P<0.01, P<0.001; Fig. 5G and H). Collectively, these results demonstrated that ROCK1 activation has a critical role in the lycorine-induced effects on actin cytoskeletal dynamics, and anti-proliferative and anti-migration activity in HepG2 cells.

**Discussion**

In recent years, naturally occurring compounds have received increasing attention in cancer research. Lycorine, a natural compound obtained from the Amaryllidaceae plant family, possesses anti-cancer activity in breast cancer, bladder cancer and multiple myeloma (10,19,20). However, the function and associated mechanisms of lycorine have not been examined in hepatoblastoma. In the current study, the inhibitory effects of lycorine on cell proliferation and migration were investigated in HepG2 cells. The HepG2 cell line was originally established in 1979 by Aden et al (21) and mistakenly reported as hepatocellular carcinoma. In recent years, the HepG2 cell line has frequently been used for hepatoblastoma research (3). The results of the present study indicated that lycorine inhibited HepG2 cell proliferation by inducing cell cycle arrest at the G2/M phase, and decreasing the expression of cyclin A, cyclin B1 and cdc2. In addition, lycorine decreased the migration ability of HepG2 cells. Furthermore, lycorine altered actin cytoskeletal dynamics by suppressing the expression of cofilin, and ROCK1 activation was demonstrated to have an important role in the anti-proliferative and anti-migration effects of lycorine in HepG2 hepatoblastoma cells.

Agents that possess the ability to inhibit the proliferation and migration of tumor cells may be used to inhibit cancer progression and increase survival rates (22). In the present study, treatment with lycorine effectively inhibited the cell proliferation and colony formation of HepG2 cells. It has been previously reported that lycorine induces cell cycle arrest at the G0/G1 phase in K562 cells (23) and KM3 cells (24). However, in the current study, lycorine induced HepG2 cell cycle arrest at the G2/M phase in a dose-dependent manner. The cell cycle is a physiological process, including the G0/G1, S and G2/M phases. A series of proteins, including cyclins and cyclin-dependent kinases, regulate the cell cycle (25). In the present study, cyclin A, cyclin B1 and cdc2 were significantly downregulated in HepG2 cells following treatment with lycorine. These findings suggested that lycorine induced cell cycle arrest at the G2/M phase via inhibition of cyclin A, cyclin B1 and cdc2 expression in HepG2 cells.

Metastasis is considered to be the primary cause of mortality in the majority of patients with cancer. The migration and invasion ability of tumor cells are key factors in tumor metastasis. Previous studies have demonstrated that lycorine inhibits the growth and metastasis of breast cancer through inhibition of signal transducer and activator of transcription 3 signaling (19). Similarly, in the current study, lycorine inhibited the migration of HepG2 cells. The MMP family, generally considered to be biomarkers for cancers, are reportedly upregulated in the majority of types of cancer (26). Furthermore, MMPs have also been considered to be potential therapeutic targets in cancer (27-29). In particular, MMP-9 and MMP-2, exhibiting enzymatic collagenase activity, are typical members among them (30). ROCK1, which serves an important role in cell polarity and migration, regulates the expression of MMP-9 and MMP-2 (31,32). Jeong et al (33) demonstrated that lysophosphatidic acid increases ovarian cancer cell invasion via a Ras/Rho/ROCK signaling pathway and subsequent production of the proteolytic enzyme MMP-9. Cofilin is an important downstream mediator of ROCK1, and the present study revealed that inhibition of the ROCK1/cofilin pathway decreased the expression of MMP-9 and MMP-2. These results indicated that the anti-migration effects of lycorine on HepG2 cells may be associated with the lycorine-induced downregulation of MMP-9 and MMP-2.

Previous studies have indicated that the reorganization of the actin cytoskeleton is the basis of cancer cell migration, adhesion and invasion (34-36). The current findings demonstrated that lycorine blocked the normal dynamic turnover of the actin cytoskeleton, with an increase in polymerized F-actin and a loss of depolymerized G-actin. A number of actin-binding proteins have been reported to be involved in the regulation of actin dynamics. For example, cofilin, a member of the actin depolymerizing factor/cofilin family, exerts its effects on actin filament dynamics by binding to F-actin and severing actin filaments. Cofilin has been reported to be overexpressed in pancreatic cancer cells, A549 lung cancer cells and the rat C6 glioblastoma cell line (14,37,38). Furthermore, Yap et al (39) reported that overexpression of cofilin enhanced cell motility in U373 astrocytoma cells. In the present study, treatment with lycorine decreased the expression of cofilin in a dose-dependent manner. ROCK1, an upstream regulator of cofilin, has an important role in regulating cell polarity and migration (34,40-42). Furthermore, Y-27632, a ROCK1 specific inhibitor, stimulates proliferation in various cell lines (43-45), indicating that the activation of ROCK1 inhibits cell proliferation. In the present study, ROCK1 activation was associated with lycorine-induced anti-proliferative and anti-migration effects in HepG2 cells. Treatment with lycorine resulted in the cleavage/activation of ROCK1, and pre-incubation of cells with Y-27632 blocked lycorine-induced decreases in cofilin, cyclin A, cyclin B1, cdc2, MMP-9 and MMP-2. Furthermore, combined treatment with Y-27632 and lycorine markedly attenuated lycorine-induced clone formation inhibition and its inhibitory effects on migratory ability. Taken together, these results demonstrated that lycorine alters actin cytoskeletal dynamics by suppressing the expression of cofilin and activating ROCK1.

In conclusion, the data demonstrated that lycorine inhibited HepG2 hepatoblastoma cell proliferation and migration through inhibition of ROCK1/cofilin-induced actin dynamics. All of the findings provide support for the development of lycorine as a potential drug candidate for anti-hepatoblastoma therapy.

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Availability of data and materials
The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors’ contributions
WL, RZ and GL conceived and designed the study. WL, QZ, QT and CH performed the experiments. JH, YLi, YLu and QW analyzed the data. WL and RZ wrote the manuscript. GL and QZ reviewed and edited the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the report work are appropriately investigated and resolved.

Ethics approval and consent to participate
Not applicable.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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