Lycorine Promotes Autophagy and Apoptosis via TCRP1/Akt/mTOR Axis Inactivation in Human Hepatocellular Carcinoma

Haiyang Yu¹, Yuling Qiu², Xu Pang¹, Jian Li¹, Song Wu¹, Shuangshuang Yin¹, Lifeng Han¹, Yi Zhang¹, Chengyun Jin³, Xiumei Gao¹, Wenwei Hu⁴, and Tao Wang¹

Abstract

Lycorine is a multifunctional bioactive compound, and it possesses potential anticancer activities. However, little is known about the underlying mechanism. In this research, we have found that lycorine significantly induces the apoptotic and autophagic capacities of hepatocellular carcinoma (HCC) cells in vitro and in vivo. Treatment with specific autophagy inhibitor (3-methyladenine/Bafilomycin A1) or knockdown of LC3B/Atg5 by siRNA drastically enhances the apoptotic cell death effect by facilitating the switch from autophagy to apoptosis. Molecular validation mechanistically demonstrates that lycorine-induced apoptosis and autophagy in HCC cells is associated with decreased protein levels of tongue cancer resistance–associated protein 1 (TCRP1), and we further find that inhibition of TCRP1 decreases phosphorylation level of Akt and represses Akt/mTOR signaling. Finally, lycorine-induced apoptosis and autophagy suppress the growth of xenograft hepatocellular tumors without remarkable toxicity. Our results elucidate a novel molecular mechanism whereby lycorine promotes apoptosis and autophagy through the TCRP1/Akt/mTOR pathway in HCC. Our results reveal that lycorine might be a potential therapeutic agent for the treatment of HCC. Mol Cancer Ther. 16(12): 2711–23. ©2017 AACR.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common aggressive types of tumor worldwide. HCC is a highly mortal tumor and its median survival rate remains under 1 year after diagnosis (1, 2). HCC is a heterogeneous disease originated from chronic liver diseases, such as chronic inflammation and cirrhosis. To date, the therapeutic strategy against HCC is mainly composed of resection, transplantation, local ablation, and chemotherapy (3, 4). Because early symptoms of HCC are not obvious, most patients with HCC are diagnosed at advanced stages and are unsuitable for potentially curative therapies such as resection, transplantation, and local ablation (5–7). Among the chemotherapeutic agents, sorafenib, a small-molecule multikinase inhibitor, is considered as an active treatment and is the only approved systemic agent to prolong survival period for advanced HCC (8). However, life expectancy of advanced HCC patients on sorafenib is only about 8 to 11 months (9, 10). In the past decades, although remarkable progress has been made in conventional treatment options for HCC patients, it remains one of the most lethal malignancies worldwide due to its limited therapeutics, poor prognosis and high recurrence rate (11–13). Novel effective and promising treatment strategies still need to be explored urgently across the globe.

Natural products possessing diverse bioactivities and mechanisms usually serve as excellent drug leads for cancer prevention and anticancer drug discovery. Lycorine, an active alkaloid from a common folk medicine, Lycoris radiata (Amaryllidaceae), has been explored for its various biological effects including anticancer, antiviral, antimalarial, antibacterial, and anti-inflammatory activities (14–18). Although the targets or mechanisms of lycorine are still undefined, the dominant biological effects and low cytotoxicity of lycorine render it as a potential clinical drug or lead. For instance, it has received much attention as a promising anticancer agent for bladder cancer, cervical cancer, leukemia, prostate cancer, and multiple myeloma (14, 19–23). However, the defined molecular mechanisms underlying lycorine-regulated suppression of tumorigenesis remain elusive.

In this study, we have demonstrated that lycorine suppresses the growth of HCC by inducing the apoptotic and autophagic capacities in vitro and in vivo. Lycorine decreases the levels of tongue cancer resistance–associated protein 1 (TCRP1) protein via promoting the TCRP1 protein degradation pathway, which results in the repressing of Akt/mTOR signaling, and thereby activates apoptotic and autophagic capacities. Furthermore, lycorine exhibited potential antitumor activities in HepG2 liver cancer.
xenografts without remarkable toxicity. Finally, we evaluated the clinical relevance between Akt and TCRP1 in HCC patient tissues. As expected, TCRP1 expression was positively associated with p-Akt. Therefore, lycorine that inhibit TCRP1-dependent signaling is a novel and promising therapeutic strategy in the treatment of HCC.

Materials and Methods

Cell culture and cell treatments

Human HCC cell lines HepG2, SMMC-7721, HuH-7, and Chang liver cell were obtained from the ATCC in April 2015. The used cells were resuscitated within 1 month. The cell lines were identified by PCR-amplified short tandem repeat analysis. Mycoplasma contamination was excluded in these cell lines. Cells were maintained at 37°C in DMEM or RPMI1640 supplemented with 10% FBS and 1% penicillin/streptomycin in a humidified atmosphere with 5% CO2. Expression vectors of human TCRP1 and Akt were designed and purchased from Servicebio Technologies (Wuhan, China). For siRNA knockdown, siRNA oligos against LC-3B and Atg5 were purchased, respectively, from GenePharma. Lycorine (purity > 98%) was purchased from Shanghai YuanYe Bio-Technology. A 20 mmol/L stock solution was prepared in dimethyl sulfoxide (DMSO), and stored in aliquots at ~20°C. 3-MA (3-methyladenine), BA (Bafilomycin A1), rapamycin, MG-132, and LY294002 were purchased from Selleck Chemicals. Thapsigargin and MTT (3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2-H-tetrazolium bromide) reagent were purchased from Sigma-Aldrich.

Cell viability assay

Cell viability was determined by MTT assay. In brief, human HCC cells or Chang liver cells (5 × 103 cells/well) were treated with lycorine at various concentrations for 48 hours, and MTT solution was added and incubated for 4 hours at 37°C. Then, medium was removed. One-hundred microliter DMSO was added and absorbance at 570 nm was determined by microplate reader. For blocking study, cells were pretreated with 20 μmol/L LY294002 or 1 mmol/L 3-MA for 1 hours, and then treated with lycorine (40 μmol/L) for 24 hours.

Assay for Annexin V staining

Apoptosis was measured by staining cells using the Muse Annexin V and Dead Cell Assay Kit (Millipore), and cells were analyzed in a bench Muse Cell Analyzer (Millipore) according to the manufacturer’s instructions.

Quantitative real-time PCR

Total RNA was purified as described previously (24). All primers were purchased from Sangon Biotech. Real-time PCR was done in triplicate with SYBR Green PCR mixture (Applied Biosystems). The expression of genes was normalized to the Actin gene. The primers used for quantitative real-time PCR were as follows: For TCRP1, 5’-CTCACGCCCTTCTGGCGATGATG-3’ and 5’-AAGCTGAGGGGTTCCGACAG-3’; For Actin, 5’-GGACATGGAGCACAGACAGATGG-3’ and 5’-ACAGCTGTGGTGGCGTACAC-3’.

Colony formation assay

Colony formation assay was performed as described previously (25). Tumor cells were seeded into 6-well plates and cultured overnight. Cells were then treated with lycorine at the indicated concentrations for 48 hours. After being rinsed with fresh medium, the colonies were formed, fixed with 4% paraformaldehyde, stained with 0.1% crystal violet, and then counted in indicated time periods.

Xenograft tumorigenicity assays

All animal experiments were conducted under protocols approved by the Animal Care and Use Committee of Tianjin University of Traditional Chinese Medicine. No specific exclusion or inclusion used for animal experiments. HepG2 Cells (5 × 106 in 0.2-ml PBS) were inoculated (via subcutaneous injection) into 7-week-old BALB/c female athymic nude mice (Taconic). When tumor volumes reached 100 mm3, mice were randomized into 3 groups (n = 8/group) and received intraperitoneal injection with lycorine (10 or 20 mg/kg/d) or vehicle every other day for 33 days. Tumor volume and body weight were monitored every 3 days. After mice were sacrificed, their tumors were weighed, photographed, and fixed in 4% paraformaldehyde for IHC assay.

Immunofluorescence assay

Immunofluorescence (IF) analysis was performed as described above (24). In brief, cells were fixed with 4% paraformaldehyde for 30 minutes and incubated with 0.5% Triton X-100 in PBS, blocked with 5% BSA for 30 minutes. The slides were stained with anti-LC-3B antibody overnight at 4°C followed by Alexa-Fluor 488-conjugated goat anti-rabbit IgG antibody for 1 hour at room temperature. Nuclei were stained with 2.5 μg/mL of DAPI (Invitrogen), and visualized by an inverted fluorescent microscope (Carl Zeiss).

Tissue samples

Forty-five pairs of human liver cancer and their matched adjacent non-tumor tissue arrays were purchased from Shanghai Biochip Company Ltd. These samples were collected from August 2006 to December 2008. All patients were followed up until September 2013. The study was approved by Taizhou Hospital of Zhejiang Province for Biomedical Research Ethics Committee, and all of the patients provided informed consent. All these samples are deidentified, and the cohort has information of clinical outcome.

IHC assay

IHC analysis was performed as described previously (24). Tissue sections were excised, formalin-fixed, paraffin-embedded, and then incubated with anti-Ki67, anti-cleaved caspase 3, anti-PARP, anti-LC-3B, and anti-TCRP1 antibodies overnight at 4°C, followed by biotinylated secondary antibody. Immunoreactivity was visualized by using the Vectastain Elite ABC Kit (Vector Laboratories). Known positive controls were included in each experiment, and negative controls were obtained by omitting the primary antibody.

Caspase colorimetric assay

The enzymatic activity of the caspases was assayed using a colorimetric assay kit according to the manufacturer’s protocol (Calbiochem). The cells were incubated in the absence and presence of lycorine at indicated concentrations, respectively, for 48 hours, and then the cells were incubated with 50 μL reaction buffer and 5 μL specific colorimetric peptide substrates, Ac-Asp-Glu-Val-Asp (DEVD)-pNA for caspase-3, at 37°C for 1
hour in the dark. The absorbance at 405 nm was determined by measuring the changes with an ELISA reader.

**Analysis of autophagic flux**

To determine autophagic flux, the cells were transfected with a tandem mRFP-GFP–tagged LC-3 according to the manufacturer’s instructions (GeneChem). The transfected cells were then treated with lycorine at 40 μmol/L for 48 hours. Subsequently, these cells were fixed with 4% paraformaldehyde for 10 minutes and washed with PBS. Finally, the GFP/mRFP images were visualized with a laser scanning confocal microscope (Olympus FV1000).

**Western blot assays**

Standard Western blot assays were analyzed as described above (24). Antibodies against SQSTM1/p62 (D5E2), Atg5 (D5F5U), Atg7 (D12B11), Atg12 (D88H11), anti-LC-3B (D11), phospho-p70S6 kinase (Thr-389), total p70S6K, phospho-Akt (Ser-473), total Akt, phospho-4-Erk1/2 (Thr-37/46), total 4-EBP1, and cleaved caspase-3 (D175) were purchased from Cell Signaling Technology. Anti-PARP1/2 (H250) and TCRP1 (E-13) antibodies were purchased from Santa Cruz Biotechnology. Anti-β-actin (A5441) antibody was purchased from Sigma-Aldrich. Full scans of Western blot data were shown in Supplementary Figs. S8–S12.

**Statistical analysis**

Data were presented as mean ± SD of at least three independent experiments. The statistical differences in xenograft tumor growth in response to lycorine treatment were analyzed by one- or two-way ANOVA, followed by the Student t test with Welch correction (assume unequal variance). The correlation between two factors was evaluated by correlation analysis, and Spearman correlation coefficients were calculated to estimate the correlations. The statistically variation (P < 0.05) was considered to be significant.

**Results**

**Lycorine inhibits growth in HCC cells**

The chemical structure of lycorine is shown in Fig. 1A. The MTT assay was used to assess the growth-inhibitory activity of lycorine against three typical HCC cell lines (HepG2, SMMC-7721, and HuH-7) and the nontumorigenic human liver cell line (Chang liver). The result in Fig. 1B showed that following exposure to lycorine (0–50 μmol/L) for 48 hours, cell viability of HCC cell lines is remarkably decreased dose-dependently with IC₅₀ values of 34.1, 36.60, and 38.34 μmol/L respectively (Fig. 1B). Although lycorine did not affect the cell viability of Chang liver cells in the same dosage comparably with an IC₅₀ value of 254.84 μmol/L (Fig. 1B). Consistently, as evidenced by decreased clonogenicity (Fig. 1C), lycorine dramatically suppressed cell proliferation in HCC cells (HepG2, SMMC-7721, and HuH-7). Next, we established a xenograft tumor model by injecting HepG2 cells subcutaneously into the mammary of nude mice. As shown in Fig. 1D, tumor size was markedly decreased after being treated with lycorine compared with that of the control group. Consistently, the weight of control tumors was much larger than that of lycorine-treated tumors (Fig. 1E). Xenografts being treated with lycorine had a markedly decreased growth rate as compared with those treated with control (Fig. 1F). To further assess the relative change of proliferation in tumors growth, we determined xenografts by Ki67 staining, a proliferation marker antigen. There was a dramatic decrease in the percentage of Ki67-positive staining in lycorine-treated tumors, as compared with that of control tumors (Fig. 1G). However, there was no obvious difference in cell morphology of major target organs (Fig. 1H) and in body weight (Supplementary Fig. S1A) between the control and lycorine-treated groups. Collectively, these results suggest that lycorine inhibits the proliferation of HCC in vitro and in vivo.

**Lycorine promotes HCC apoptosis via the caspase-dependent pathway**

To explore a possible mechanism of apoptosis induction effect of lycorine on HCC cells (HepG2, SMMC-7721, and HuH-7), we performed Western blot assays and caspase activity detection. In all three cell lines, along with activation of cleaved caspase-3 and the subsequent proteolytic cleavage of PARPs by lycorine dose dependently (Fig. 2A; Supplementary Fig. S2A), a marked increase in caspase activity was observed (Fig. 2C, Supplementary Fig. S2C). The apoptotic effects were further confirmed by employing Annexin V staining in HCC cells treated with lycorine (Fig. 2B, Supplementary Fig. S2B). Likewise, treating HCC cells with lycorine (Fig. 2D, Supplementary Fig. S2D) led to nuclear fragmentation and cytoplasmic shrinkage as shown by DAPI staining.

To further evaluate the effect of lycorine on HCC apoptosis in vivo, Western blot assays and IHC staining detection was performed in HCC xenograft tumors. As shown in Fig. 2E, lycorine treatment clearly increased the levels of cleaved caspase-3 and PARP in HepG2 tumors compared with that of the control group. Consistently, a similar tendency was observed by IHC staining of cleaved caspase-3 and PARP. The number of apoptotic cells with positive staining of cleaved caspase-3 and PARP was much higher in lycorine-treated tumors compared with that of control group (Fig. 2F). Furthermore, to investigate the significance of caspases activation in lycorine-induced apoptosis, HCC cells (HepG2, SMMC-7721, and HuH-7) were pretreated with Z-VAD-FMK, a Pan-caspase inhibitor, which was employed to block caspase. As shown in Fig. 2G and H and Supplementary Fig. S2E and S2F, Z-VAD-FMK greatly attenuated the effect of lycorine-induced cell death. Importantly, Z-VAD-FMK treatment largely abolished the effect of lycorine-induced activation of cleaved caspase-3 and PARP proteins. Z-VAD-FMK treatment also largely abolished the effect of lycorine-induced caspase-3 activities (Fig. 2I, Supplementary Fig. S2G). However, Z-VAD-FMK treatment did not affect lycorine-induced autophagy activation by Western blot assays (Supplementary Fig. S2H). Taken together, these results suggest that lycorine-promoted apoptosis is mainly dependent on caspase activation in HCC cells.

**Lycorine induces autophagy in HCC cells**

Besides apoptosis, autophagy is an alternate mode of programmed cell death in response to cellular stress (27, 28). To
identify whether the cytotoxic effect of lycorine could result from autophagy activation, we first investigated the effect of lycorine on the formation of autophagosome by evaluating LC-3B and p62, two classical markers of autophagy. As shown in Fig. 3A and Supplementary Fig. S3A, the result demonstrated that the expression of LC-3B-II was markedly increased and the expression of p62 was significantly decreased in response to indicated concentrations of lycorine. Expression levels of autophagy related proteins, such as Atg5, Atg7, and Atg12, were also investigated by Western blot assays. As shown in Fig. 3B and Supplementary Fig. S3B, an obvious upregulation of Atg5, Atg7, and Atg12 was observed as a result of lycorine exposure. To further verify lycorine-induced autophagy, we investigated the intracellular morphologic change of HCC cells by transmission electronic microscopy. As shown in Fig. 3C and Supplementary Fig. S3C, we found in lycorine-treated cells a significant accumulation of double membrane vesicles, which contain subcellular materials, as compared with control cells. Using a tandem mRFP-GFP–tagged LC-3, we found weak green fluorescence scattered LC-3, and red puncta autolysosomes and yellow puncta autophagosomes being generated. Lycorine treatment led to increased LC-3-II conversion, promoted LC-3 lipidation and puncta, and accumulated yellow puncta autophagosomes (Fig. 3D and E, Supplementary Fig. S3D and S3E). Furthermore, we evaluated the effect of lycorine on HCC autophagy in vivo by Western blot assays and IHC staining. As shown in Fig. 3F, lycorine treatment clearly increased LC-3-II levels and significantly decreased p62 levels in HepG2 tumors compared with that of the control group. Consistently, a similar tendency was observed by IHC staining of multiple organ sections was determined, scale bar, 100 μm. For D-H, Data are shown as mean ± SD; (n = 8); *P < 0.05; **P < 0.01 compared with control (Student t test).
Figure 2.
Lycorine promotes HCC apoptosis via the caspase-dependent pathway. A, HepG2 and SMMC-7721 cells were treated with indicated concentrations of lycorine for 48 hours. The protein levels of cleaved caspase-3 and PARP were detected by Western blot assays. B, The percentage of apoptotic cells was determined by Annexin V staining. C, Activity of caspase-3 was evaluated by caspase colorimetric assays. For B and C, data are shown as mean ± SD; (n = 3); *, P < 0.05; **, P < 0.01 compared with control (Student t test). D, The cells were stained with DAPI solution and then photographed with a fluorescent microscope (magnification, ×400). E and F, Cleaved caspase-3 and PARP levels and expressions in xenograft tumors were examined by Western blot assays and IHC staining; scale bar, 50 μm. For E and F, data are shown as mean ± SD; (n = 8). G-I, HepG2 and SMMC-7721 cells were treated with lycorine with and without Z-VAD-FMK, respectively. G, Cell viability was measured by MTT assay. H, The levels of cleaved caspase-3 and PARP proteins were determined by Western blot assays. I, Activity of caspase-3 was evaluated by caspase colorimetric assays. All the Western blot data are representative of three independent experiments. For G and I, Data are shown as mean ± SD; (n = 3); **, P < 0.01 compared with control; ###, P < 0.01 compared with control cells treated with lycorine (Student t test).
Figure 3.
Lycorine induces autophagy in HCC cells. A and B, Western blotting analysis shows that the protein expression of LC-3B, p62, Atg5, Atg7, and Atg12 was measured in HepG2 and SMMC-7721 cells treated with indicated concentrations of lycorine for 48 hours. C, Autophagy was measured in cells treated with DMSO or 40 μmol/L lycorine for 48 hours by transmission electron microscopy. D, Cells were transiently transfected with tandem fluorescent mRFP-GFP-tagged LC-3 plasmid (RFP-GFP-LC-3). In addition, cells were treated as in C, followed by confocal fluorescence microscopy; scale bar, 10 μm. E, The formation of endogenous LC-3 puncta was determined by immunofluorescence analysis in cells treated as in C; scale bar, 10 μm. F, LC-3B and p62 levels in xenograft tumors were examined by western blot assays. (G) LC-3B expressions in xenograft tumors were examined by IHC staining. Scale bar, 50 μm. For F and G, data are shown as mean±S.D. (n = 8). All the western data are representative of three independent experiments.
Inhibition of autophagy promotes lycorine-induced apoptosis

Although apoptosis and autophagy are two distinct modes of programmed cell death, they have complex interconnections to maintain cellular homeostasis (29). To investigate the cross talks between autophagy and apoptosis induced by lycorine on HCC cells, 3-Methyladenine (3-MA), an autophagy inhibitor, was employed to block autophagy, and then the effect of lycorine on autophagy and apoptosis was determined. HCC cells were treated with lycorine with and without 3-MA treatment, respectively. As shown in Fig. 4A, 3-MA dramatically attenuated lycorine-induced autophagy in HCC cells (HepG2 and SMMC-7721). Interestingly, 3-MA further increased lycorine-induced apoptosis by activating cleaved caspase-3 and PARP (Fig. 4A). Here, we used Bafilomycin A1 (BA), an autophagy–lysosomal inhibitor, Annexin V staining, and MTT assay showed that the apoptotic effect of lycorine was enhanced when lycorine was combined with 3-MA or BA in comparison with treatment with lycorine alone (Fig. 4B; Supplementary Fig. S4A). Consistent results were obtained when the
autophagy function was blocked by using siRNA targeting LC-3B or Atg5. Knockdown of endogenous LC-3B or Atg5 by siRNA markedly abolished lycorine-induced autophagy and further promoted lycorine-induced apoptosis in HCC cells (Fig. 4C and D; Supplementary Fig. S4B–S4D). However, induction of autophagy by thapsigargin did not affect apoptosis induction by lycorine (Supplementary Fig. S4E). Taken together, these results strongly suggest that inhibition of autophagy could promote lycorine-induced apoptosis.

**Akt/mTOR pathway plays an important role in lycorine-induced apoptosis and autophagy**

It has been elucidated that Akt/mTOR, and the subsequent phosphorylation and activation of the downstream targets (p70S6K and 4-EBP1), are composed of a series of pivotal regulators regulating cell survival and proliferation, which play a critical role during the entire life of a cell (30, 31). Recent studies have also demonstrated that the Akt signaling pathway plays a key role in lycorine-induced apoptosis of bladder cancer T24 cells (14). In this study, we found that phosphorylation levels of Akt (Ser-473), 4-EBP1 (Thr-37/46), and p70S6K (Thr-389) were downregulated in response to lycorine dose-dependently, but there was no noticeable change in their total expression in HCC cells (Fig. 5A, Supplementary Fig. S5A). Combination of lycorine with LY294002, an Akt inhibitor, further decreased phosphorylation levels of 4-EBP1 and p70S6K compared with lycorine treatment alone (Fig. 5B, Supplementary Fig. S5B). Furthermore, pretreatment of LY294002 enhanced the conversion of LC-3B, cleavage of caspase-3 and cleavage of PARP, and blocked the expression of p62 induced by lycorine (Fig. 5C, Supplementary Fig. S5C). In addition, combination of lycorine with LY294002 further promoted cell death (Fig. 5D; Supplementary Fig. S5D) and increased autophagic flux in HCC cells compared with lycorine treatment alone (Fig. 5E; Supplementary Fig. S5E). Consistently, ectopic Akt expression in HCC cells clearly suppressed lycorine-induced conversion of LC-3B, cleavage of caspase-3, and cleavage of PARP, as well as blocked lycorine-inhibited p62 protein expression (Fig. 5F; Supplementary Fig. S5F). Furthermore, Akt overexpression also suppressed lycorine-induced cell death (Fig. 5G; Supplementary Fig. S5G) and autophagic flux (Fig. 5H; Supplementary Fig. S5H) in HCC cells. Consistently, a similar tendency was observed by employing Western blot and MTT assay. Combination of lycorine with rapamycin, an mTOR inhibitor, further enhanced cleavage of PARP and promoted cell death (Supplementary Fig. S5I and S5J). Taken together, these results suggest that the Akt/mTOR pathway plays an important role in lycorine-induced apoptosis and autophagy.

**Lycorine promotes apoptosis and autophagy via TCRP1/Akt/mTOR axis inactivation**

TCRP1, a novel candidate chemotherapeutic resistance-associated human gene, previously has been reported to be able to promote tumorigenesis through selective activation of PI3K/Akt and NFκB pathways in oral squamous cell carcinoma (OSCC) radioresistance (32, 33). As shown in Fig. 6A and Supplementary Fig. S6A, we found that lycorine treatment dramatically decreased the levels of TCRP1 protein in HCC cells. However, lycorine treatment did not markedly change the mRNA level of TCRP1 by real-time PCR (Fig. 6B; Supplementary Fig. S6B), suggesting that TCRP1 protein is mostly turned over through proteasomal degradation. Subsequently, we found that treatment with MG-132, a proteasome inhibitor, clearly abolished the inhibitory effect of lycorine on the TCRP1 protein level in HCC cells (Fig. 6B; Supplementary Fig. S6B). Taken together, these results suggest that lycorine may decrease TCRP1 protein levels through promoting the TCRP1 protein degradation pathway.

Next, we found that ectopic TCRP1 expression in HCC cells dramatically blocked the inhibitory effect of lycorine on the Akt/mTOR pathway (Fig. 6C; Supplementary Fig. S6C). Furthermore, TCRP1 overexpression also blocked lycorine-induced apoptosis and autophagy related protein expressions, cell death, and autophagic flux (Fig. 6D–F; Supplementary Fig. S6D–S6F). In addition, TCRP1 overexpression strongly blocked the inhibitory effect of lycorine on colony formation (Fig. 6G and Supplementary Fig. S6G). Consistently, lycorine treatment attenuated protein levels of TCRP1, Akt, 4-EBP1, and p70S6K in HepG2 xenograft tumors (Fig. 6H). Accordingly, IHC staining demonstrated that positive staining of phosphorylated Akt and TCRP1 was much stronger in control xenografts tumors compared with lycorine-treated xenografts tumors (Fig. 6I and J). Next, we evaluated the clinical relevance between Akt and TCRP1 in HCC patient tissues. As expected, TCRP1 expression was positively associated with p-Akt (Supplementary Fig. S7A, S7B, S7K, and S7L), which supports our results in both cultured cells and mouse tumor models. Collectively, our results suggest that the TCRP1/Akt/mTOR axis plays a key role in lycorine-induced apoptosis and autophagy in HCC.

**Discussion**

Lycorine, an active alkaloid compound, has been reported to possess multiple biological effects. For instance, several studies have demonstrated that lycorine displays remarkable efficacy in the suppression of tumorigenesis (14, 19–23). However, the underlying mechanism involved remains poorly understood. In this study, we have demonstrated that lycorine represses Akt/mTOR signaling via decreasing the levels of TCRP1 protein, which results in activating apoptotic and autophagic processes and leading to suppressing the growth of HCC in vitro and in vivo.

Programmed cell death is a commonly seen cellular self-destruction process for the cell to remove harmful cells and maintain an inner homeostasis under emergency, including neoplastic disease (34). On the basis of this fact, programmed cell death inhibits tumor growth and constitutes potential target for cancer therapy. Of the three common types of programmed cell death, including apoptosis, autophagy and necrosis, apoptosis is the most extensively studied and well-defined one (35–38). We have found that lycorine initiates apoptotic cell death in HCC cells, which is supported by the results of DAPI staining and V-FITC/PI double staining. Caspases that belong to the family of cysteine proteases are integral components of the apoptotic pathway. Recently, many studies have shown that a variety of chemotherapeutic agents promote apoptosis through the activation of caspases (38–40). In this study, we have found that lycorine treatment markedly augments caspase activity, and upregulates cleaved caspase-3 and PARP. Furthermore, blocking the caspase pathway largely abolishes the apoptotic effect of lycorine. Together, these results suggest that lycorine induces apoptosis via the caspase-dependent pathway in HCC cells.

Autophagy is a cellular process characterized by formation of autophagosome which is a double membrane structure enveloping cytoplasmic material (41). Autolysosome is the result of the fusion of autophagosomes and lysosomes, and then executes...
Akt/mTOR pathway plays an important role in lycorine-induced apoptosis and autophagy. **A,** Western blotting analysis shows that the levels of p-Akt, p-4-EBP1, and p-p70S6K were measured in cells treated with indicated concentrations of lycorine for 48 hours. Total Akt, 4-EBP1, and p70S6K expressions were used as the internal control, respectively. **B–E,** Cells were treated with lycorine with and without LY294002, respectively. **B** and **C,** The levels of p-Akt, p-4-EBP1, p-p70S6K, LC-3B, p62, cleaved caspase-3, and PARP proteins were determined by Western blot assays. **D,** The cell viability was measured by MTT assay. Data are shown as mean ± SD; (**n** = 3); **##,** *P* < 0.01 compared with control; **##,** *P* < 0.01 compared with control cells treated with lycorine (Student’s *t* test).

**E,** Cells were transiently transfected with tandem fluorescent mRFP-GFP-tagged LC-3 plasmid (RFP-GFP-LC-3), followed by confocal fluorescence microscopy; scale bar, 10 µm. **F–H,** Cells transfected with Akt (Akt Vec) or empty vector (Control Vec) followed by lycorine treatment. **F,** The levels of p-Akt, LC-3B, p62, cleaved caspase-3, and PARP proteins were determined by Western blot assays. **G,** The cell viability was measured by MTT assay. Data are shown as mean ± SD; (**n** = 3); **##,** *P* < 0.01 compared with control; **##,** *P* < 0.01 compared with Vector control–transfected cells treated with lycorine (Student’s *t* test). **H,** Cells were transiently transfected with tandem fluorescent mRFP-GFP-tagged LC-3 plasmid (RFP-GFP-LC-3), followed by confocal fluorescence microscopy; scale bar, 10 µm. All the Western blot data are representative of three independent experiments.

Figure 5.
Figure 6.
Lycorine promotes apoptosis and autophagy via TCRP1/Akt/mTOR axis inactivation. A and B, HepG2 and SMMC-7721 cells were treated with indicated concentrations of lycorine for 48 hours. The mRNA and protein levels of TCRP1 were detected by real-time PCR and Western blot assays. B, Cells were treated with lycorine with and without MG-132, respectively. The level of TCRP1 protein was determined by Western blot assays. C–G, Cells were transfected with TCRP1 (TCRP1 Vec) or empty vector (Control Vec) and followed by lycorine treatment. C and D, The levels of TCRP1, p-Akt, p-4-EBP1, p70S6K, LC-3B, p62, cleaved caspase-3, and PARP proteins were determined by Western blot assays. E, The cell viability was measured by MTT assay. F, Cells were transiently transfected with tandem fluorescent mRFP-GFP-tagged LC-3 plasmid (RFP-GFP-LC-3), followed by confocal fluorescence microscopy; scale bar, 10 μm. G, The colony formation capability was detected by colonogenic assay. H, TCRP1, p-Akt, Akt, p-4-EBP1, 4-EBP1, p70S6K, and p70S6K levels in xenograft tumors were examined by Western blot assays. All the Western blot data are representative of three independent experiments.

I-L, The relevance between Akt and TCRP1 in xenograft tumor models and HCC patient tissues. I and J, p-Akt and TCRP1 expressions in xenograft tumors were examined by IHC staining. Representative images were provided as indicated: **, P < 0.01; scale bars, 50 μm. K and L, IHC analysis of TCRP1 and p-Akt expression in Human HCC specimens; scale bars, 20 μm. For B and E, Data are shown as mean ± SD; (n = 3); **, P < 0.01 compared with control; ###, P < 0.01 compared with Vector control-transfected cells treated with lycorine (Student t test).
Lycorine Promotes Autophagy and Apoptosis

autophagy through degrading the enveloped cytosol. LC-3B-II, forming from lipidated LC-3B-I, participates in autolysosome formation, and it is so far a crucial marker of autophagy (41, 42). In this study, we have demonstrated that lycorine accumulates the formation of autophagosomes by IF staining and transmission electron microscopy. Lycorine also promotes LC-3 lipidation and puncta, increases conversion of LC-3B-I to LC-3B-II, and induces autophagic flux formation. P62, a negative marker of autophagy, promotes transferring ubiquitinated substrates to the autophagosomes (43, 44). We have found that lycorine markedly decreases the protein expression level of p62. The autophagy occurs through two ubiquitin-like reactions which involve a series of regulators, such as Atg5, Atg7, and Atg12. Recent studies have demonstrated that the expression of Atg5, Atg7, and Atg12 proteins plays a role in autophagic/apoptotic cell death and gastrointestinal cancer pathogenesis (45). In this study, we have found by Western blot assays that lycorine increases the expression levels of Atg5, Atg7 and Atg12 in HCC cells. Together, these results strongly suggest that lycorine induces autophagy in HCC cells.

Although autophagy plays an important role in cancer progression, it is not clear whether autophagy suppresses tumorigenesis or promotes cell adaptation and survival with a rescue mechanism under unfavorable circumstances (46, 47). Generally, autophagy is crucial for cancer cell survival strategy by degrading intracellular macromolecules and eliminating altered cytosolic constituents. However, under some conditions, it may play a suppressive role and lead to cancer cell death. Cell death is most commonly associated with apoptosis, but it can also occur via autophagic cell death (47, 48). Many studies have demonstrated that these two types of cell death are predominantly distinctive but that cross-talk also occurs between them.

Previous studies have shown that autophagy occurs through a temporary survival mechanism after chemotherapies, and the disruption of autophagy induces apoptosis, thus augmenting antitumor effects; however, some chemotherapies also have shed light on the induction of autophagic cell death or both apoptosis and autophagy (28, 36). It seems necessary to sort out the complexity of the interplay between autophagy and apoptosis following treatment with different substances. In this study, we have demonstrated that lycorine-induced autophagy and apoptosis inhibit the growth of HCC cells in vitro and in vivo. Treatment with specific autophagy inhibitor (3-MA) or knockdown of LC-3B by siRNA drastically enhances the apoptotic cell death effect by speeding up the switch from autophagy to apoptosis. Therefore, lycorine-induced autophagy may serve as a temporary protective mechanism against lycorine-induced apoptosis.

The Akt/mTOR pathway that is very frequently dysregulated in the pathogenesis of human cancers is a major pathway accounting for autophagy, proliferation, and apoptosis of cancer cells (26, 49). Therefore, it is a potential pathway and target for intervention in cancer prevention and treatment. In this study, we have found that lycorine significantly inhibits the Akt/mTOR signaling pathway in HCC cells. Moreover, pretreatment of Akt inhibitor (LY294002) promotes not only the apoptosis-inducing activity of lycorine, but also probably autophagy induction. Consistently, ectopic Akt expression in HCC cells clearly suppresses lycorine-induced apoptotic cell death and autophagic flux in HCC cells. Together, these findings suggest that the Akt/mTOR pathway plays an important role in lycorine-induced apoptosis and autophagy.

TCRP1 has been reported as a novel candidate for chemotherapeutic resistance–associated human gene, which is expressed extensively in multiple types of cancer cells, such as lung cancer, breast cancer, and tongue cancer (32, 33, 50). Previous studies have shown that TCRP1 plays an essential role in mediating DDP-resistant lung cancer cells through inhibition of Pol b degradation (50). Recently, TCRP1 has been reported to promote tumorigenesis in OSCC radioreistance through selective activation of PI3K/Akt and NFκB pathways (32, 33). Clinical studies have suggested that high TCRP1 levels are associated with a poor prognosis of OSCC radioreistance patients (33). Thus, it is possible that further study targeting TCRP1 can provide us an optimal therapeutic strategy for cancers with TCRP1. In this study, we have found that lycorine markedly decreases the levels of TCRP1 and inhibits the Akt/mTOR signaling pathway. Clinical studies have showed that TCRP1 expression is positively correlated with the phosphorylation of Akt. We also have found that lycorine downregulates TCRP1 protein levels by promoting its degradation. However, the detailed mechanism underlying lycorine-regulated TCRP1 still needs further studies. Taken together, these results suggest that the TCRP1/Akt/mTOR axis as a novel pathway may play an important role in lycorine-induced apoptosis and autophagy.

In summary, this study demonstrates that lycorine promotes apoptosis and autophagy through the TCRP1/Akt/mTOR pathway, which in turn inhibits the tumor growth of HCC. These findings have provided a preclinical rationale for exploring and broadening the clinical evaluation of lycorine for HCC treatment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: H. Yu, T. Wang
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Li, Y. Zhang
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H. Yu, Y. Qiu, S. Wu, S. Yin, C. Jin, T. Wang
Writing, review, and/or revision of the manuscript: H. Yu, Y. Qiu, W. Hu, T. Wang
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H. Yu, Y. Qiu, X. Fang, J. Li, S. Wu, S. Yin, L. Han, Y. Zhang
Study supervision: X. Gao, T. Wang

Acknowledgments

We thank Dr. Yuhong Hu (Hebei Normal University) for her technical assistance in electron microscope analysis. This work was supported by grants from National Natural Science Foundation of China (81603253, to H. Yu; 81673703, to T. Wang; and 81602614, to Y. Qiu) and Natural Science Foundation of Tianjin City (15CYBJC54900, to H. Yu; 15PTCSY00030, to Z. Li).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received May 30, 2017; revised July 27, 2017; accepted September 5, 2017; published OnlineFirst September 28, 2017.
References

1. Choo SP, Tan WL, Goh BK, Tai WM, Zhu AX. Comparison of hepatocellular carcinoma in Eastern versus Western populations. Cancer. 2016 Sep 13. [Epub ahead of print].

2. Nault JC. The end of almost 10 years of negative RCTs in advanced hepatocellular carcinoma. Lancet 2017;6:4–6.

3. Takahashi A, Sawaki N, Tsuqarui Y, Iwabuchi S, Matsunaga K, Ebinuma H, et al. Phase 2 study of stereotactic body radiotherapy and optional transarterial chemoembolization for solitary hepatocellular carcinoma not amenable to resection and radiofrequency ablation. Cancer 2016;122:2041–9.

4. Molinad AA, Zhu H, Marrero JA, Masoumier JC, Singal AG, Yopp AC. Hospital volume and survival after hepatocellular carcinoma diagnosis. Am J Gastroenterol 2016;111:967–75.

5. Villanueva A, Hernandez-Gea V, Llovet JM. Medical therapies for hepatocellular carcinoma: a critical view of the evidence. Nat Rev Gastroenterol Hepatol 2013;10:34–42.

6. Bruix J, Roaul JL, Sherman M, Mazzaferro V, Bolondi L, Craxi A, et al. Efficacy and safety of sorafenib in patients with advanced hepatocellular carcinoma: subanalyses of a phase III trial. J Hepatology 2012;57:821–9.

7. El-Serag HB, Marrero JA, Rudolph L, Reddy KR. Diagnosis and treatment of hepatocellular carcinoma. Gastroenterology 2008;134:1752–63.

8. Wilhelm S, Carter C, Lynch M, Lowinger T, Dumas J, Smith RA, et al. Decade in review—hepatocellular carcinoma: HCC-subtypes, biomarkers and new therapeutic strategies. Lancet Oncol 2015;16:599–707.

9. Bruix J, Qin S, Merle P, Granito A, Bodoky G, et al. Regorafenib for patients with hepatocellular carcinoma who progressed on sorafenib treatment (RESORCE): a randomised, double-blind, placebo-controlled, phase 3 trial. Lancet Oncol 2014;15:81–91.

10. Bruix J, Qin S, Merle P, Granito A, Huang YH, Bodoky G, et al. Regorafenib for patients with hepatocellular carcinoma who progressed on sorafenib treatment (REACH): a randomised, double-blind, multicentre, phase 3 trial. J Hepatol 2015;63:244–55.

11. Toriizuka Y, Kinoshita E, Kogure N, Kitajima M, Ishiyama A, Otoguro K, et al. Lycorine reduces mortality of bladder cancer T24 cells by inhibiting phospho-Akt and activating the intrinsic apoptotic cascade. Biochem Biophys Res Commun 2013;430:197–202.

12. Liu Y, Levine B. Autosis and autophagic cell death: the dark side of apoptosis. Cell Death Differ 2015;22:367–76.

13. Liu PH, Hsu CY, Hsia CY, Lee YH, Su CW, Huang YH, et al. Prognosis of hepatocellular carcinoma: subanalyses of a phase III trial. J Hepatol 2012;57:81–90.

14. Wang C, Wang Q, Li X, Jin Z, Xu P, Xu N, et al. Lycorine induces apoptosis of bladder cancer T24 cells by inhibiting inhibiting phospho-Akt and activating the intrinsic apoptotic cascade. Biochem Biophys Res Commun 2013;430:197–202.

15. Liu J, Yang Y, Xu Y, Ma C, Qin C, Zhang L. Lycorine reduces mortality of human enterocinoma 71-infected mice by inhibiting virus replication. Virol J 2011;8:183.

16. Loncak M, Novakova J, Kloucek P, Hostalkova A, Kokoska L, Lucie M, et al. Anti-influenza and antibacterial activity of extracts and alkaloids of selected amaryllidaceae species. Nat Prod Commun 2015;10:1537–40.

17. Park JB. Synthesis and characterization of norbelladine, a precursor of amaryllidaceae alkaloid, as an anti-inflammatory/anti-COX compound. Bioorg Med Chem Lett 2014;24:5381–4.

18. Chen S, Jin G, Huang KM, Ma JJ, Wang Q, Ma Y, et al. Lycorine suppresses RANKL-induced osteoclastogenesis in vitro and prevents ovariectomy-induced osteoporosis and titanium particle-induced osteolysis in vivo. Sci Rep 2015;5:12853.

19. Hu M, Peng S, He Y, Qin M, Cong X, Xing Y, et al. Lycorine is a novel inhibitor of the growth and metastasis of hormone-refractory prostate cancer. Oncotarget 2015;6:15348–61.

20. Ghavre M, Freese J, Pour M, Hudilicky T. Synthesis of amaryllidaceae constituents and unnatural derivatives. Angew Chem Int Ed Engl 2016;55:5642–91.

21. Lamoral-Theyss D, Daecasteecker C, Mathieu V, Dubois J, Kornienko A, Kiss R, et al. Lycorine and its derivatives for anticancer drug design. Mini Rev Med Chem 2010;10:41–50.

22. Roy M, Liang L, Xiao X, Peng Y, Luo Y, Zhou W, et al. Lycorine downregulates HMGB1 to inhibit autophagy and enhances bortezomib activity in multiple myeloma. Theranostics 2016;6:2209–21.

23. Yu H, Yue X, Zhao Y, Li X, Wu L, Zhang C, et al. LiF negatively regulates tumour-suppressor p53 through Stat3/ID1/MDM2 in colorectal cancers. Nat Commun 2014;5:5218.

24. Chen K, Xie J, Wang H, Zhang H, Yu M, Fu L, et al. Cambogin induces caspase-independent apoptosis through the ROS/NK pathway and epigenetic regulation in breast cancer cells. Mol Cancer Ther 2015;14:1738–49.

25. Dou Q, Chen HN, Wang K, Yuan K, Lei Y, Li K, et al. Ivermectin induces cytoplastic autophagy by blocking the PAK1/Akt axis in breast cancer. Cancer Res 2016;76:4457–69.

26. Liu Y, Levine B. Autosis and autophagic cell death: the dark side of apoptosis. Cell Death Differ 2015;22:367–76.

27. Marino G, Niso-Santano M, Bachrecke EH, Kroemer G. Self-consumption: the interplay of autophagy and apoptosis. Nat Rev Mol Cell Biol 2014;15:81–94.

28. Fuchs Y, Steller H. Live to die another way: modes of programmed cell death and the signals emanating from dying cells. Nat Rev Mol Cell Biol 2015;16:329–44.

29. Polivka J Jr, Janku F. Molecular targets for cancer therapy in the PI3K/AKT/mTOR pathway. Pharmacol Ther 2014;142:164–75.

30. Matter MS, DeCaaens T, Andersen JB, Thorgerisson SS. Targeting the mTOR pathway in hepatocellular carcinoma: current state and future trends. J Hepatol 2014;60:855–65.

31. Peng B, Gu Y, Xiong Y, Zheng G, He Z. Microarray-assisted pathway analysis identifies MT1X & NEKappal as mediators of TCRP1-associated resistance to cisplatin in oral squamous cell carcinoma. PLoS ONE 2012;7:e51413.

32. Yu H, Fan S, Liu B, Zheng G, Yu Y, Ouyang Y, et al. TCRP1 promotes radiosensitivity of oral squamous cell carcinoma cells via Akt signal pathway. Mol Cell Biochem 2011;357:107–13.

33. Bidle KD. The molecular ecophysiology of programmed cell death in marine phytoplankton. Annu Rev Mar Sci 2015;7:341–75.

34. Roos WP, Thomas AD, Kaina B. DNA damage and the balance between survival and death in cancer biology. Nat Rev Cancer 2016;16:20–33.

35. Lalouei N, Lindquist LM, Sandow JI, Ekert PG. The molecular relationships between apoptosis, autophagy and necroptosis. Semin Cell Dev Biol 2015;39:63–9.

36. Gibson CJ, Davids MS. BCL-2 antagonism to target the intrinsic mitochondrial pathway of apoptosis. Clin Cancer Res 2015;21:5021–9.

37. Roy MJ, Yoon A, Cazbatar PE, Lessene G. Cell death and the mitochondria: therapeutic targeting of the BCL-2 family-driven pathway. Br J Pharmacol 2014;171:1973–87.

38. Bai L, Wang S. Targeting apoptosis pathways for new cancer therapeutics. Annu Rev Med 2014;65:139–55.

39. Creagh EM. Caspase crosstalk: integration of apoptotic and innate immune signalling pathways. Trends Immunol 2014;35:631–40.

40. Boya P, Reggiori F, Codogno P. Emerging regulation and functions of mammalian 26S proteasome. Proc Natl Acad Sci U S A 2016;113:20202–7.
on transcriptional upregulation and autophagy-derived amino acids. Autophagy 2014;10:431–41.
45. Feng Y, He D, Yao Z, Klionsky DJ. The machinery of macroautophagy. Cell Res 2014;24:24–41.
46. Lorin S, Hamai A, Mehrpour M, Codogno P. Autophagy regulation and its role in cancer. Semin Cancer Biol 2013;23:361–79.
47. White E. The role for autophagy in cancer. J Clin Invest 2015;125:42–6.
48. Amaravadi R, Kimmelman AC. White E. Recent insights into the function of autophagy in cancer. Genes Dev 2016;30:1913–30.
49. Kim YC, Guan KL. mTOR: a pharmacologic target for autophagy regulation. J Clin Invest 2015;125:25–32.
50. Liu X, Wang C, Gu Y, Zhang Z, Zheng G, He Z. TCRP1 contributes to cisplatin resistance by preventing Pol beta degradation in lung cancer cells. Mol Cell Biochem 2015;398:175–83.