Lycorine inhibits the proliferation of neuroblastoma neuro-2a cells by inducing G2/M phase cell cycle arrest and apoptosis

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Abstract: Lycorine, a benzylphenanthridine-type alkaloid extracted form Amarillidaceae genera, exhibits an efficacy against various types of cancer. Nonetheless, the impact of lycorine treatment on neuroblastoma has not yet been investigated. Here we utilized a combinatorial strategy to explore and to understand the effect of lycorine on neuroblastoma Neuro-2a cells. Our results indicated that lycorine inhibits the Neuro-2a cells proliferation by promoting cell apoptosis. In addition, wound healing assay revealed that lycorine inhibits the Neuo-2a cells migration. Comparative transcriptome analysis showed that lycorine has the potential to affect cycle pathway. Flow cytometry analysis confirmed that lycorine arrested the Neuro-2a cell cycle at G2/M phase. Furthermore, we detected that the protein expression of Cyclin A, Cyclin B1 and Cyclin E were decreased, whereas protein of p53, Tgfβ3, Gadd45β, Gadd45γ, p21 and p27 were increased after treatment with lycorine. Collectively, we propose that lycorine might be a valuable candidate therapeutic agent in combating neuroblastoma.

Keywords: Lycorine; neuroblastoma; neuro-2a cells; cell cycle arrest; apoptosis.

INTRODUCTION

Neuroblastoma is one of the most common childhood solid tumor (Davidoff, 2021). Among all the childhood tumors, neuroblastoma is listed the second leading cause of death in children after lymphoblastic leukemia (Steiner *et al*., 2016). Neuroblastoma might affect certain lethal malignancies because of its heterogeneity (Elizabeth *et al*., 2019). Nevertheless, half of the cases caused by neuroblastoma are approximately classified as high risk group, in which overall survival rates around 40% despite aggressive multimodal therapy (Olecki and Grant 2019). Therefore, there is clearly a need for novel drugs against it. Numerous studies have reported that natural plant compounds have shown extensive and effective anti-tumor activity, have become a very valuable source of new chemotherapeutic agents. For example, camptothecin, vinblastine, paclitaxe and their derivatives proved to possess anti-tumour activity against different types of human cancer (Gordaliza, 2007; Liu *et al*., 2017).

Lycorine, an natural alkaloid isolated from different *Amaryllidaceae genera* and have various biological activities, such as anti-viral (Guo *et al*., 2015), anti-malarial (Toriizuka *et al*., 2008) and anti-inflammation (Yui *et al*., 2001). Moreover, numerous studies reported that lycorine have excellent anti-cancer activity in various cancer, including leukemia (Jian *et al*., 2013), prostate cancer (Hu *et al*., 2015), multiple myeloma (Roy *et al*.,

2016) and breast cancer (Yu *et al*., 2016). Nevertheless, the effect and the molecular mechanisms of lycorine on neuroblastoma remain poorly understood. Here, the mouse neuroblastoma Neuro-2a cell line, which is widely used for studying neuroblastoma, to investigate the effects of lycorine against neuroblastoma (Dickey *et al*., 2011; Paramasivam *et al*., 2014). We aimed to explore the effect and possible molecular mechanisms of lycorine on Neuro-2a cells and expected that lycorine can be used as an anti-neuroblastoma reagent.

MATERIALS AND METHODS

Materials
Lycorine (Yuanye, China) and prepared in DMSO (Sigama, USA) and stored at -20°C until use. Antibodies for western blot including p21, Akt, p-Akt(S473), p-Akt(S308), Cyclin B1, cleaved-Caspase 3, Caspase 3, Cyclin D1, Bax, Bcl2, β-actin, p53, Cyclin E1, p27 and Secondary antibodies (Beyotime, China); Tgfβ3, Gadd45 β and Gadd45 γ (Santa Cruz, USA).

Cell culture
Neuro-2a cells (ATCC, Manassas, USA) were seeded in MEM (Hyclone, USA) containing 10% FBS (Hyclone, USA) and 1% penicillin/streptomycin (Beyotime, China) maintained at 37 °C in culture incubator with 5% CO_2.

MTT assay
Cell viability was measured using the MTT assay (Jiang *et al*., 2020). Neuro-2a cells (5×10^3 cells/well) were seeded

into 96-well plate and then treated by lycorine (0-10μM) for 24 hours. Followed, 10μl of MTT solution (5 mg/ml) was added and incubated for 4 hours. The medium removed and the formazan blue was solubilized with DMSO (100μL), followed by reading the absorbance at 490nm using microplate reader.

Clonogenic assay

Clonogenic assay was performed as described by Dickey *et al.* (Dickey *et al.*, 2011). Neuro-2a cells (2×10^3 cells/well) were seeded in 6-well plates for 24 hours. Thereafter cells were treated by lycorine (0-10μM) for 7 days. Following incubation, cells were fixed in 4% paraformaldehyde and stained with crystal violet dye. The plates washed with tap water, then photographed and the cell colonies were counted.

Cell migration assay

Neuro-2a cells allowed to grow at least 90% confluence then scratched with a pipette tip (100μL) causing 650μm gap. After washing twice with PBS, cells were treated by lycorine (0-10μM). Cell migration was visualized and measured using inverted microscope (Olympus, Japan) after 48 hours.

Cell-cycle and apoptosis analysis

Flow cytometry analysis according to the protocol described previously ((Paramasivam *et al.*, 2014)). Neuro-2a cells (at least 1×10^6 cells) were collected by trypsinization. For cell cycle analysis, cell washed twice with ice-cold PBS then fixed in ice-cold 70% ethanol. Fixed cells were washed with PBS and stained with propidium iodide (PI)/RNase buffer (BD, USA) for 30 min. For cell apoptosis analysis, cells washed with ice-cold PBS, mixed with binding buffer and stained with Annexin V-FITC/PI (Biolegend, USA). Finally, Data acquisition and analysis were performed by the samples were analyzed by the CytoFLEX cytometer (Beckman-Coulter, USA).

Differential expression analysis Transcriptome sequencing

Neuro-2a cells were harvested after treated by 10μM lycorine or solvent control for 12 h and each treatment had three duplicates. Total RNA isolation from Neuro-2a cells by TRIzol (Invitrogen, USA). Transcriptome sequencing was performed by Illumina Hiseq platform (Novogene, China).

Protein extraction and western blot

Cell proteins were collected using RIPA buffer (CWBIO, China) and protein concentration was quantified by BCA protein kit (TIANGEN, China). Protein samples were subjected to SDS-PAGE, followed by electrophoretic transferred onto PVDF membranes. Membrane strips were incubated with primary antibody, then with secondary antibody. The immunostaining was visualized by electrochemiluminescence (ECL) detection.

STATISTICAL ANALYSIS

GraphPad Prism (version 5.0) was used for statistical analysis and graphical display. Values presented as mean±SEM. One-way ANOVA was used for multiple comparisons and statistically significant difference considered as $*P<0.05$, $**P<0.01$ and $**P<0.001$.

RESULTS

The effect of lycorine on proliferation and migration in Neuro-2a cells

Lycorine is a natural benzylphenethylamine-type alkaloid (fig. 1A). Numerous earlier studies have demonstrated that lycorine inhibited the proliferation of various malignant tumors. To investigate whether lycorine inhibited the proliferation of neuroblastoma, a typical neuroblastoma cell lines, Neuro-2a cells were exposure to different concentrations of lycorine (0.1 to 10μM) for 24 hours. fig. 2B showed that lycorine dramatically suppressed the viability of Neuro-2a cells in a dose-dependent manner (IC50 value was 3.274μM). Next, the effect of lycorine on clone formation was evaluated by clonogenic assay, which is closer to physiological state of tumor ((fig. 1C)). As shown in fig. 1D, a low concentration of lycorine (0.1μM) possess significant inhibition of clone formation (fig. D). The scratch wound healing assay was used to assess lycorine-treated Neuro-2a cells migration (fig.1E). The results showed that Neuro-2a cells migration was significantly inhibited by lycorine (fig.1F). These results indicated that lycorine inhibits neuroblastoma cell proliferation and migration.

Effect of Lycorine on Neuro-2a cell apoptosis

Exposure of Neuro-2a cells to lycorine exhibited characteristic morphologic changes of apoptotic, such as cellular shrinkage and reduction of the cytoplasmic volume (fig. 2A). The apoptotic cells were detected by Hochest33342 staining (fig. 2B). As show in fig. 2B, we noticed that condensation of chromatin in lycorine-treated Neuro-2a cells, indicating the occurrence of cell death by apoptosis. Furthermore, apoptotic cell populations induced by lycorine were evaluated using flow cytometry assay (fig. 2C). After treatment with 0, 1, 5and 10μM lycorine for 24 hours, the apoptotic rates were 5.07%, 10.41%, 13.75% and 19.27%, respectively.

To further evaluate whether the effect of lycorine on apoptosis of Neuro-2a cells was on cell apoptosis regulation, cell apoptosis-related proteins were investigated (fig. 2D). Western blot revealed that the Bcl-2 (anti-apoptotic protein) expression was suppressed while the Bax (pro-apoptotic protein) expression was promoted in lycorine-treated Neuro-2a cells. In addition, lycorine decreased the level of precursor caspase 3 while the cleaved caspase 3 protein expression was increased. The anti-apoptotic Akt pathway plays a pivotal role in maintaining cell survival. Interestingly, we observed that

the Akt phosphorylation level was significantly decreased following lycorine treatment. These observations demonstrated that lycorine-induced Neuro-2a cell apoptosis mediated by reducing the activation of Akt and activating the caspase 3 signal pathway.

The impact of lycorine on the transcriptional level in Neuro-2a cells

To examine the early responses to lycorine, transcriptome analysis was performed using high-throughput sequencing platform of Illimina Hiseq2500. We identified 8891 genes as differentially expressed genes (DEGs) after treated with lycorine. Among them, 4441 up-regulated genes while 4450 were down-regulated (fig. 3A). Furthermore, all the DEGs were selected for GO function and KEGG pathway enrichment analysis. The significantly enriched GO and KEGG signaling pathways are listed in fig. 3B, C.

Effect of Lycorine on cell cycle procession in Neuro-2a cells

To assess whether the anti-proliferative and apoptogenic activities of lycorine were associated to cell cycle regulation, the flow cytometry was applied (fig. 4A). Results are presented in fig. 4A, the percentage of cells at G2/M phase increased from 0.81% to 15.45% in lycorine-treated Neuro-2a. The results demonstrated that lycorine can arrest Neuro-2a cells at the G2/M cell phase. Furthermore, to confirm whether cell cycle-related protein involved in lycorine-induced cell cycle arrest, three key cell cycle regulators (cyclin B1, cyclin D1 and cyclin E1) were detected by western blot. Interestingly, we found that lycorine does-dependently decreased the expression of cyclin B1, cyclin D1 and cyclin E1 (fig. 4C). Therefore, we concluded that the lycorine arrested Neuro-2a cell cycle at G2/M phase by regulating the cell cycle-related proteins, which may alter cell proliferation.

Effect of Lycorine on cell cycle-related genes in Neuro-2a cells

To understand the mechanism by which lycorine arrested Neuro-2a cell at G2/M phase, the expression of cell cycle-related genes was listed in fig 5.A. There are 80 DEGs found in the cell cycle pathway. Among them, 31 genes expression levels were increased, while 49 genes were decreased after 12 h lycorine treatment. We mapped the expression status of DEGs on the cell cycle as an example (fig. 5B). Six of the assayed cell cycle genes were significantly (fold change≧1.5, lycorine/DMSO) up-regulated in Neuro-2a cells treated with lycorine, including some that relevant to the observed G2/M arrest (e.g. Tgfβ3, Gadd45β and Gadd45γ) (fig. 5C). The western blot results of selected 6 genes were consistent with the results of transcriptomic analyses, indicating that the RNA-sequencing results are credible (fig. 5D).

DISCUSSION

Neuroblastoma is the commonest extracranial malignant tumors occurring in childhood (Maris *et al*., 2007). Although development a variety of treatment strategies, the treatment of high-risk neuroblastoma remains challenging. Therefore, it will be necessary to explore more potential candidate drugs for the treatment of neuroblastoma.

Natural compounds extracted from Chinese herbs are valuable resource for cancer prevention and anticancer drug discovery (Gordaliza, 2007). It has been revealed that lycorine has an inhibitory activity on various malignant tumors. Here, we found that lycorine inhibits Neuro-2a cells proliferation and migration in a dose-dependent manner. Moreover, we confirmed that lycorine induced apoptosis of Neuro-2a cells as measured using Hoechest33342 staining and Annexin V-FITC/PI apoptosis assay. Furthermore, our data described the mechanisms of lycorine related to cell apoptosis.

The transcriptome sequencing technology has been extensively used to investigate the molecular mechanisms of drug action (Ying *et al*., 2016; Chauhan, *et al*., 2019). Therefore, we used RNA-seq combined with comparative transcriptome analysis to explain the molecular mechanism of lycorine action. We identified 8891 genes (4441 up-regulated and 4450 down-regulated) were differentially expressed in lycorine-treated Neuro-2a cells. In our observation, these DEGs are associated with a variety of signaling transduction pathways, including cell apoptosis, cell cycle, neurotrophin, glycolysis/gluconeogenesis and HIF-1 pathways in neuro-2a cells. Notably, there are multiple DEGs are associated with cell cycle regulation, suggesting that the cell cycle procession may be disturbed by lycorine in Neuro-2a cells. Moreover, some studies have confirmed that lycorine inhibited cell proliferation by arresting cell cycle progression (Liu *et al*., 2004; Li *et al*., 2012). Our observations showed clearly that the lycorine treatment arrested Neuro-2a cell cycle at the G2/M phase (fig.4). The G2/M checkpoint is crucial for repairing DNA damage before cells entering mitosis and is a prime strategic target for many anticancer drugs, which would lead to cell death by inducing apoptosis (Halike *et al*., 2021). Based on these findings, it is possible to explain that lycorine induced Neuro-2a cells apoptosis could be though the G2/M cell cycle arrest.

To unravel how lycorine cause Neuro-2a cell cycle arrest, the key regulators mediating cell cycle progress were further investigated. Results described here show that the expression of cyclin B1, cyclin D1 and cyclin E1 were down-regulated, while levels of the negative regulator p53, p21, p27, Gadd45β, Gadd45γ and Tgfβ3 were up-regulated by lycorine treatment. The p53 as a tumor suppressor protein, is capable of regulating cell cycle and apoptosis in carcinoma cells (Silveira *et al*., 2020). Several studies have reported p53 arrests cell cycle at the G1 or G2/M phases by up-regulating the cell cycle inhibitor p21 and the pro-apoptotic Bax (Taylor and stark, 2001; Choi *et al*., 2020). In addition, p27 as a tumor suppressor modulates G2/M checkpoint transition in cell cycle (Lee *et al*., 2008; Hsu *et al*., 2011).
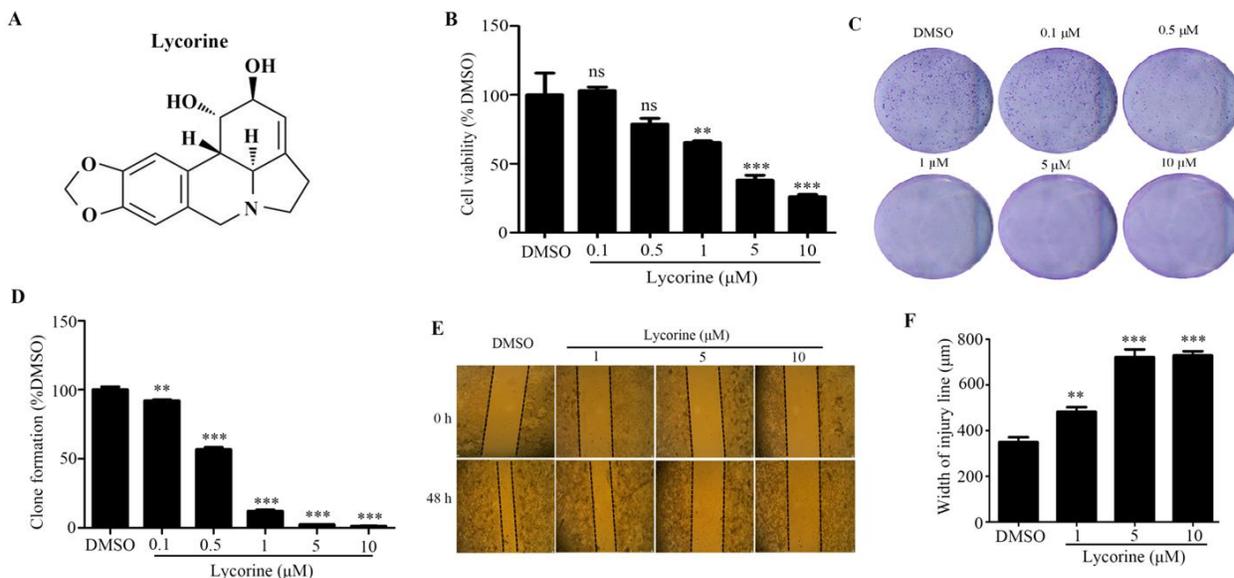
Fig. 1: Lycorine inhibits the proliferation, colony formation and migration of Neuro-2a cells. (A). The chemical structure of lycorine. (B). Inhibitory effect of lycorine on the cell viability of Neuro-2a cells measured by MTT assay. (C). Inhibition of Neuro-2a cell clone formation after treatment with Lycorine. The number of colonies are presented in (D). (E). Lycorine suppressed the Neuro-2a cells migration. The statistical results of cell migration are presented in (F). Values represent the mean±SEM. **, $p < 0.01$, *** $p < 0.001$, ns. not significant.
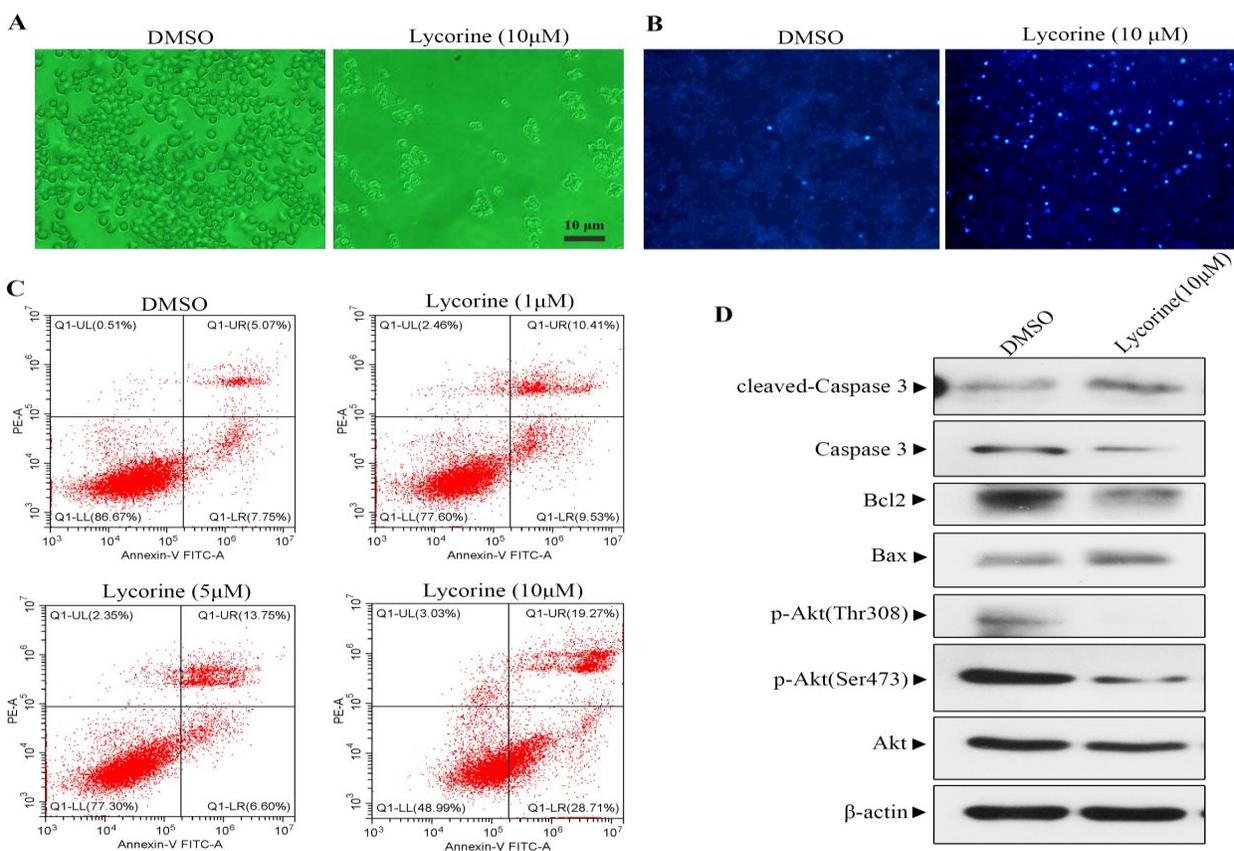
Fig. 2: Lycorine induces Neuro-2a cell apoptosis. (A). Apoptotic morphological changes in Lycorine-treated Neuro-2a cells. (B). Cell apoptosis was assessed by nuclear morphologic staining (Hoechst 33342). (C). A flow cytometric method using Annexin V/PI for cell apoptosis assay (D). Western blot analysis of Akt, p-Akt (Ser308), p-Akt (Ser473), Bcl2, Bax, Caspase 3 and cleaved-Caspase 3.
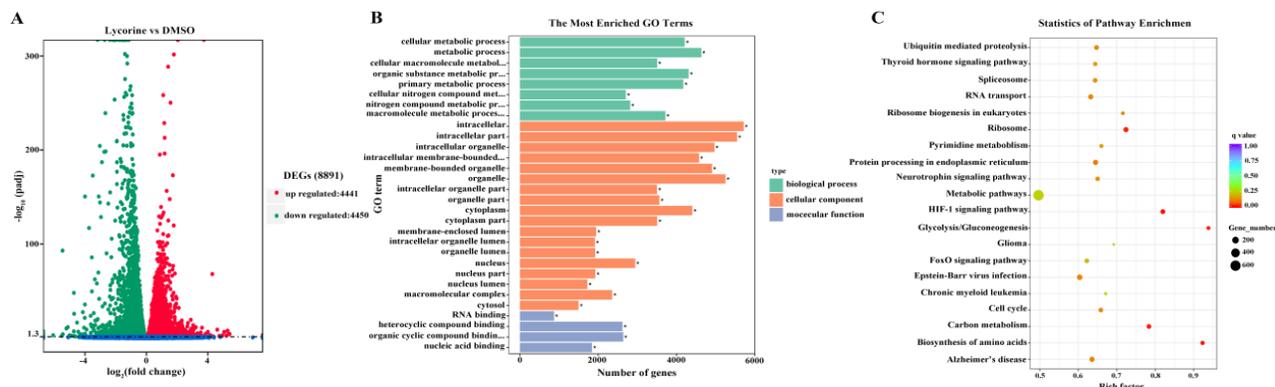
Fig. 3: Differential gene expression analysis after treated with lycorine. (A). DEGs were clustered via volcano plots analysis. GO (B) and KEGG (C) enrichment analysis of DEGs in Neuro-2a cells treated by lycorine.
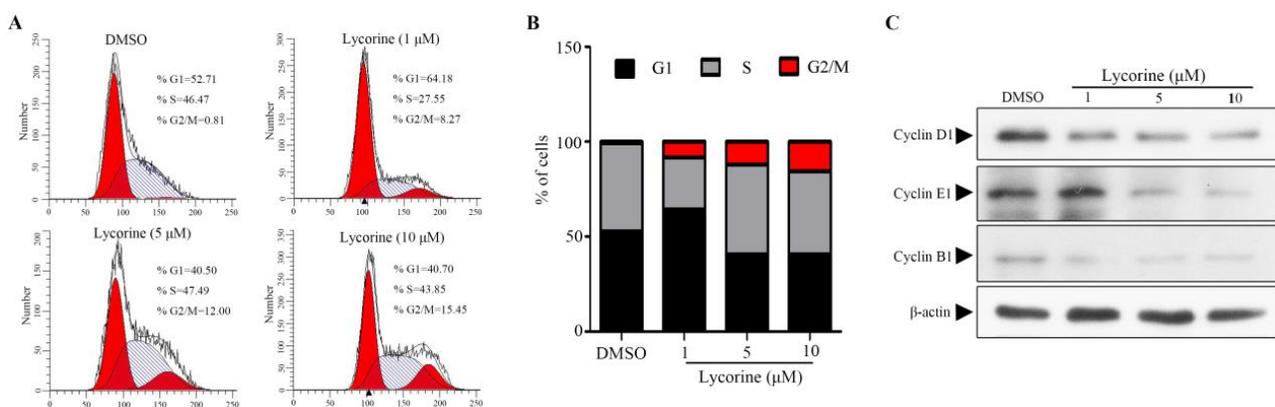

Fig. 4: Lycorine arrested the Neuro-2a cell cycle at the G2/M phase. (A, B). The effect of lycorine-treated (1, 5 and 10μM) Neuro-2a cells in different phases (G1/G0, S and G2/M) were measured by Flow cytometric analysis. (C). Levels of Cyclin D1, Cyclin E1 and Cyclin B1 were detected by western blot.
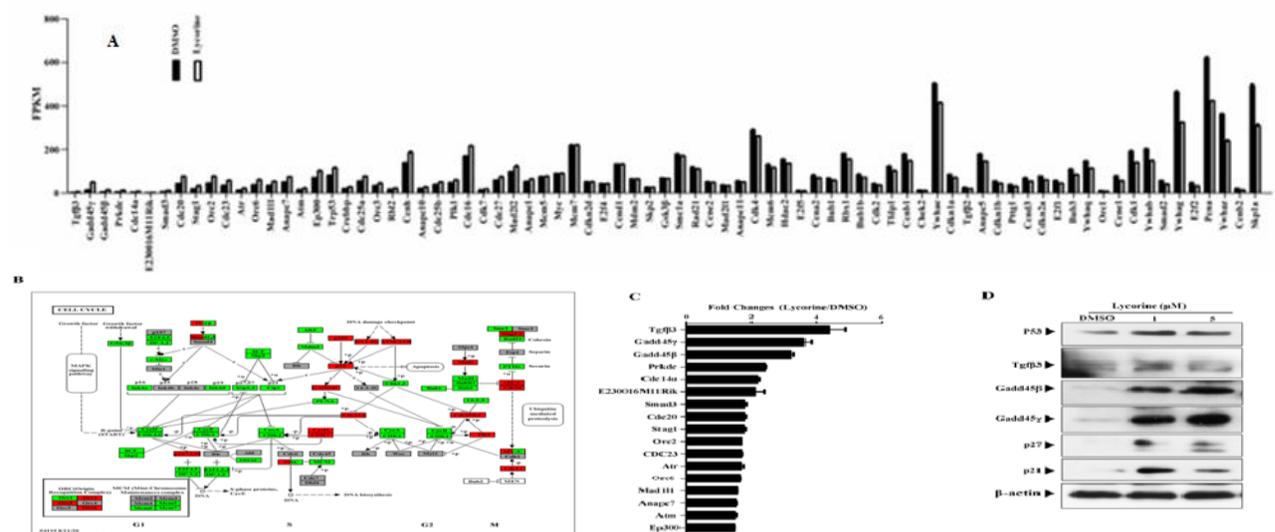

Fig. 5: Expression variability of cell cycle-related genes in Lycorine-treated Neuro-2a cells. (A). RNA-seq reveals Lycorine-induced gene expression changes in cell cycle pathway. (B) KEGG analysis of cell cycle network. Boxes colored Red or green indicate genes which were down-regulated or up-regulated, respectively. Two-part coloured box indicate this gene family some are up-regulated and some are down-regulated by lycorine. (C) A total of 17 up-regulated DEGs in the cell cycle pathway (fold change≥1.5, lycorine/DMSO). (D) The 7 genes related with cell cycle regulation confirmed by western blotting.

Interestingly, here we discovered that the p53, p21, p27 and Bax were up-regulated after treatment with lycorine in Neuro-2a cells. Therefore, it is noteworthy that lycorine arresting cell cycle arrest and inducing cell apoptosis may be correlated with up-regulation of p53, p21 and p27 expression. Gadd45 (growth arrest and DNA damage), a p53-regulated stress protein, that regulate a variety cellular processes, such as cell cycle checkpoint, DNA repair process, signal transduction and maintenance of genomic integrity (Mo *et al.*, 2021). Previous studies revealed that over-expression of Gadd45 caused a G2/M arrest (Zhu *et al.*, 2009). In our experiment, lycorine significantly increased both the mRNA and protein levels of p53, Gadd45β and Gadd45γ in Neuro-2a cells. The evidence of our findings indicates that lycorine inducing G2/M phase cell cycle arrest was related to the up-regulated expression of p21, p27, p53, Gadd45β, Gadd45γ and Tgfβ3, but down-regulated cyclin B1, cyclin D1 and cyclin E1.

CONCLUSION

This work demonstrates that lycorine inhibited neuroblastoma Neuro-2a cell proliferation through arresting cell cycle at G2/M phase. This effect was involved in the regulation of cell cycle-related protein expression and activating intrinsic apoptotic pathway, leading to apoptosis of Neuro-2a cells. Collectively, our results provide insight for lycorine as a potential natural drug for neuroblastoma cancer.

CONFLICTS OF INTEREST

The authors declare that they have no conflict interests.

ACKNOWLEDGMENTS

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