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Lycorine inhibited the cell growth of non-small cell lung cancer by modulating the miR-186/CDK1 axis

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ABSTRACT

Aims: Lycorine is a kind of natural alkaloid with anti-cancer potential. It has been demonstrated that lycorine processes high activity and specificity against the progression of cancers. However, the underlying molecular mechanisms by which lycorine regulates the formation and development of non-small cell lung cancer (NSCLC) remain largely unknown.

Main methods: The effects of lycorine on the growth of NSCLC cells were determined by the cell counting kit-8 (CCK-8) assay, colony formation and flow cytometry analysis. RT-qPCR was performed to detect the expression of microRNA with lycorine treatment. The binding of miRNA and target genes was confirmed by luciferase reporter assay.

Key findings: Lycorine significantly inhibited the proliferation and induced apoptosis of NSCLC cells. Mechanistically, lycorine up-regulated the expression of microRNA-186 in NSCLC cells. Depletion of miR-186 significantly reversed the suppressive effect of lycorine on the proliferation of NSCLC cells. Furthermore, the cyclin dependent kinase 1 (CDK1) was identified as one of the binding candidates of miR-186. Experimental analysis showed that miR-186 bound the 3′-untranslated region (3′-UTR) of CDK1 and suppressed the level of CDK1 in NSCLC cells. Consistently, exposure of lycorine significantly decreased the expression of CDK1. Restoration of CDK1 remarkably attenuated the inhibition of lycorine on the proliferation of NSCLC cells.

Significance: Our results uncovered the novel molecular mechanism of lycorine in suppressing the progression of NSCLC partially via regulating the miR-186/CDK1 axis.

1. Introduction

Lung cancer has been considered as one of the most common malignancies with high morbidity and mortality around the world, which contributes heavily to cancer-associated death [1,2]. Non-small cell lung cancer (NSCLC) accounts for approximately 85% of all the lung cancer cases [3–6]. The research about NSCLC has attracted increasing attention in the past decades and huge progress including radiotherapy, chemotherapy has been made in the diagnosis and treatment of NSCLC. Nevertheless, the five-year overall survival of NSCLC patients still remains very poor [7]. Therefore, exploring innovative strategies for the treatment of NSCLC is imperatively required.

Nature products processing multiple biological activities display remarkable effects on inhibiting the progression of cancers, which have become promising candidates in anticancer drug discovery [8–12]. Lycorine is a natural isoquinoline alkaloid. The anti-cancer feature of lycorine has been characterized in human cancers and has the potential to be used in the treatment of cancers [13–17]. Previous studies showed that lycorine inhibited the growth of NSCLC cells [18,19], however, the underlying mechanisms by which lycorine regulates the progression of NSCLC remain largely unknown.

MircroRNAs (miRNAs) are a class of small, single-stranded transcripts with the length of approximate 20–24 nucleotides (nt) lacking protein-coding capacity [20–22]. MiRNAs act as negative regulators of gene expression via binding to the 3′-untranslated region (3′-UTR) of targeted mRNAs, consequently leading to the instability or translation inhibition of the mRNAs [21]. Increasing evidence suggested that miRNAs play important roles in a wide range of physiological processes including cell proliferation, differentiation and apoptosis [20,23]. Accordingly, the involvement of miRNAs in the progression of cancer is also well-established by recent studies [24–29]. Aberrant expression of miRNAs has been found in cancers, which is tightly associated with the proliferation, epithelial-mesenchymal transition and drug resistance in tumorigenesis [26,28,29]. An increasing body of evidence has

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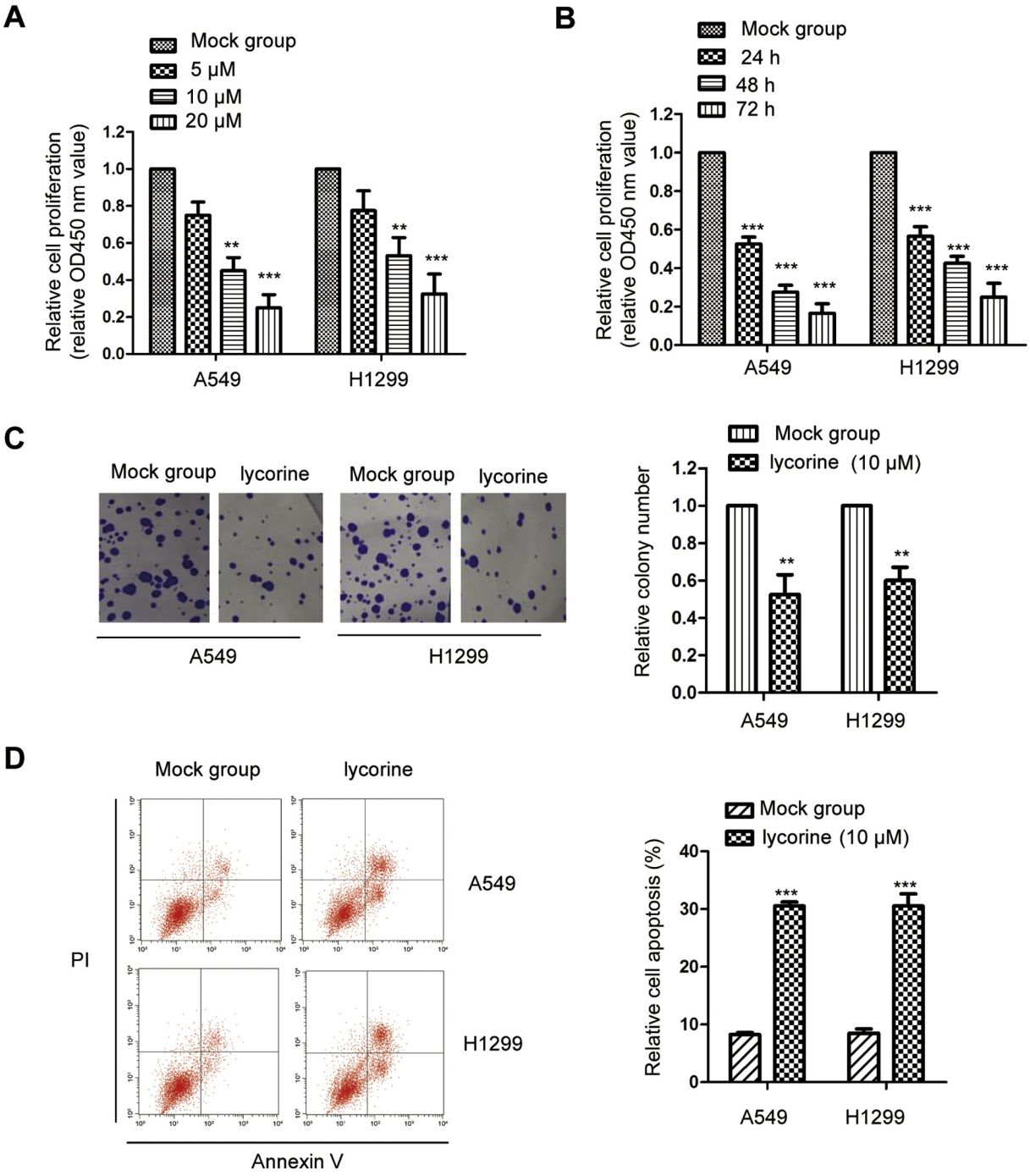

Fig. 1. Lycorine inhibited the growth of NSCLC cells. (A) A549 and H1299 cells were exposed to different concentration of lycorine for 24 h and the cell viability was tested by CCK-8 assay. (B) A549 and H1299 cells were treated with 10 μM lycorine for the indicated time and then the cell proliferation was determined. (C) Treatment of both A549 and H1299 cells with lycorine decreased the colony formation. (D) Lycorine exposure promoted the apoptosis of NSCLC cells.

uncovered the critical roles of miRNAs in the development of NSCLC. For example, recent study showed that miR-150-5p suppressed the metastasis and recurrence of NSCLC [30]. MiR-889 acted as a tumor suppressor in NSCLC that inhibited the proliferation and invasion via targeting TAB1 [31]. The oncogenic miR-421 promoted the progression of NSCLC by modulating the Wnt/β-catenin pathway [32]. Considering the important roles in NSCLC, miRNAs are promising targets of anti-tumor strategies.

MiR-186 has been identified as a tumor suppressive miRNA involved in the development of human malignancies including bladder cancer, gastric cancer and prostate cancer [33–35]. MiR-186 targeted Twist to inhibit the proliferation, migration and epithelial-mesenchymal transition in breast cancer [36]. MiR-186 was found to attenuate the cisplatin resistance of glioma cells via degrading Yin Yang 1 [37]. The tumor suppressive role of miR-186 was also established in cutaneous malignant melanoma, which inhibited the proliferation and invasion of cancer cells [38]. Consistent with these findings, the anticancer effect of miR-186 in NSCLC was also reported. MiR-186 suppressed the malignant behaviors of NSCLC cells via down-regulating diverse targets [39–41].

Aberrant cell cycle progression plays an important role in the tumorigenesis. Cyclin-dependent kinases are serine/threoinie kinases that

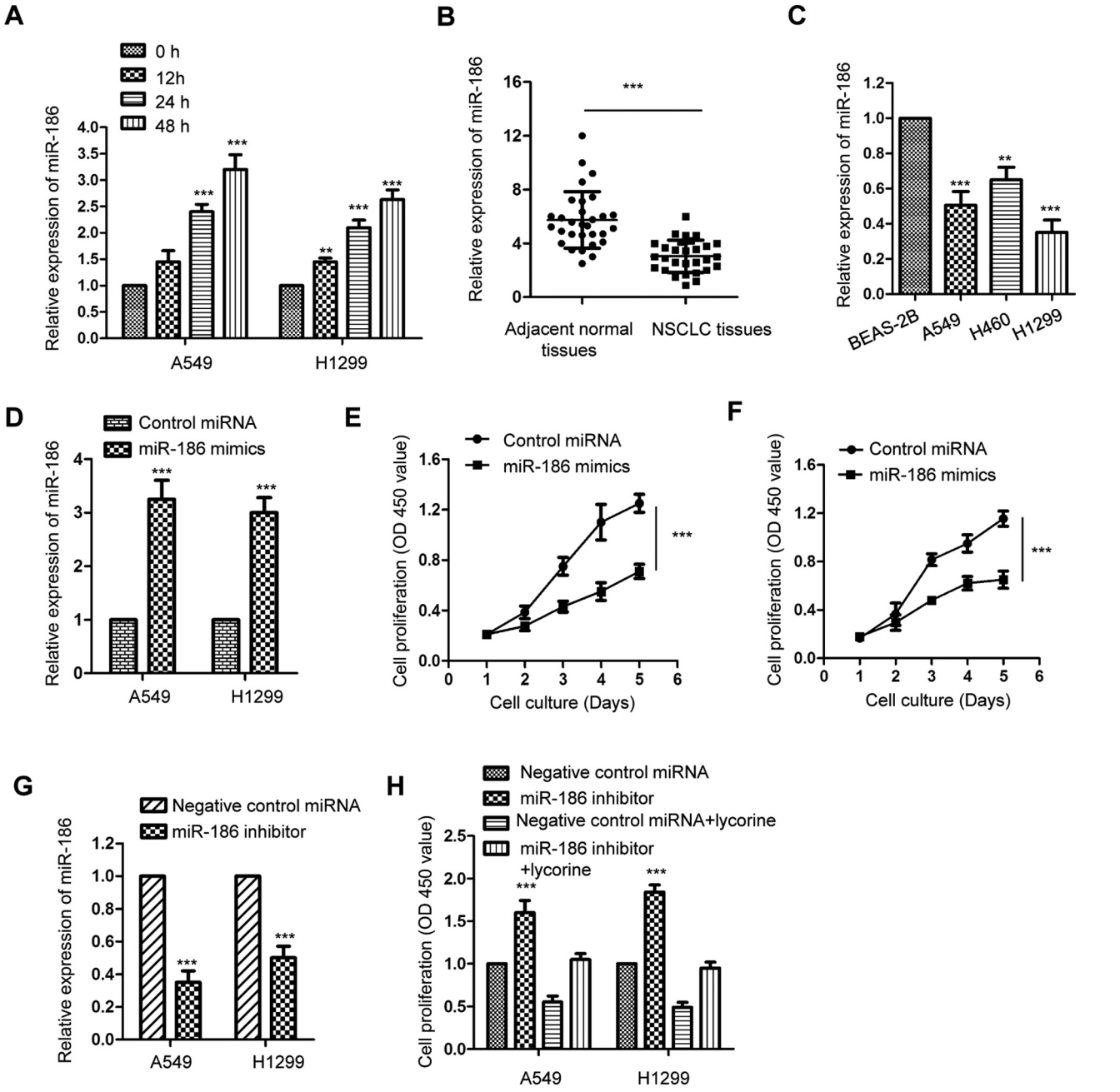

Fig. 2. Lycorine up-regulated the expression of miR-186 in NSCLC. (A) Both A549 and H1299 cells were treated with 10 μM lycorine for the indicated time and then the expression of miR-186 was determined with the RT-qPCR assay. (B) Thirty paired NSCLC tissues and adjacent normal tissues were collected and the level of miR-186 was detected by RT-qPCR with the U6 RNA as the internal control. (C) The level of miR-186 in NSCLC cell lines (A549, H1299 and H460) and normal cell BEAS-2B was detected by RT-qPCR. (D) Both A549 and H1299 cells were transfected with control miRNA or miR-186 mimics, and the expression of miR-186 was verified by RT-qPCR (E, F) Both A549 (E) and H1299 (F) cells were transfected with control miRNA or miR-186 mimics, and the cell proliferation was detected by CCK-8 assay at indicated time. (G) Both A549 and H1299 cells were transfected with negative control miRNA or miR-186 inhibitor, and the knockdown efficiency of miR-186 was detected by RT-qPCR. (H) NSCLC cells transfected with control miRNA or miR-186 inhibitor were treated with or without lycorine. The cell proliferation was determined by the CCK-8 assay.

regulated the cell cycle by interacting with specific cell-cycle-regulatory cyclins [42]. Cyclin dependent kinase 1 (CDK1, Gene ID: 983) acts together with cyclin B, which forms an active maturation-promoting factor to make cell enter the mitosis phase [43]. Due to the leading function of CDK1 in cell cycle control, increasing studies have showed that the up-regulated CDK1 in cancer promoted the progression of cancer [43]. Therefore, targeting CDK1 with selective CDK1 inhibitor to disrupt cancer development is undergoing evaluation in clinical trials. Notably, CDK1 has been identified as the target of miRNAs in NSCLC. Recent study reported that miR-181a inhibited the proliferation of NSCLC cells via targeting CDK1 [44]. The regulation of CDK1 by more miRNAs remains to be explored.

In this study, the effect of lycorine on the growth of NSCLC cells was explored, which showed that lycorine inhibited the proliferation and induced apoptosis of NSCLC cells. Mechanistically, we found that lycorine significantly up-regulated the expression of miR-186. MiR-186 targeted the cyclin dependent kinase 1 (CDK1) and caused defects in cell cycle progression. Our results uncovered the anti-cancer effect of lycorine via targeting the miR-186/CDK1 axis.

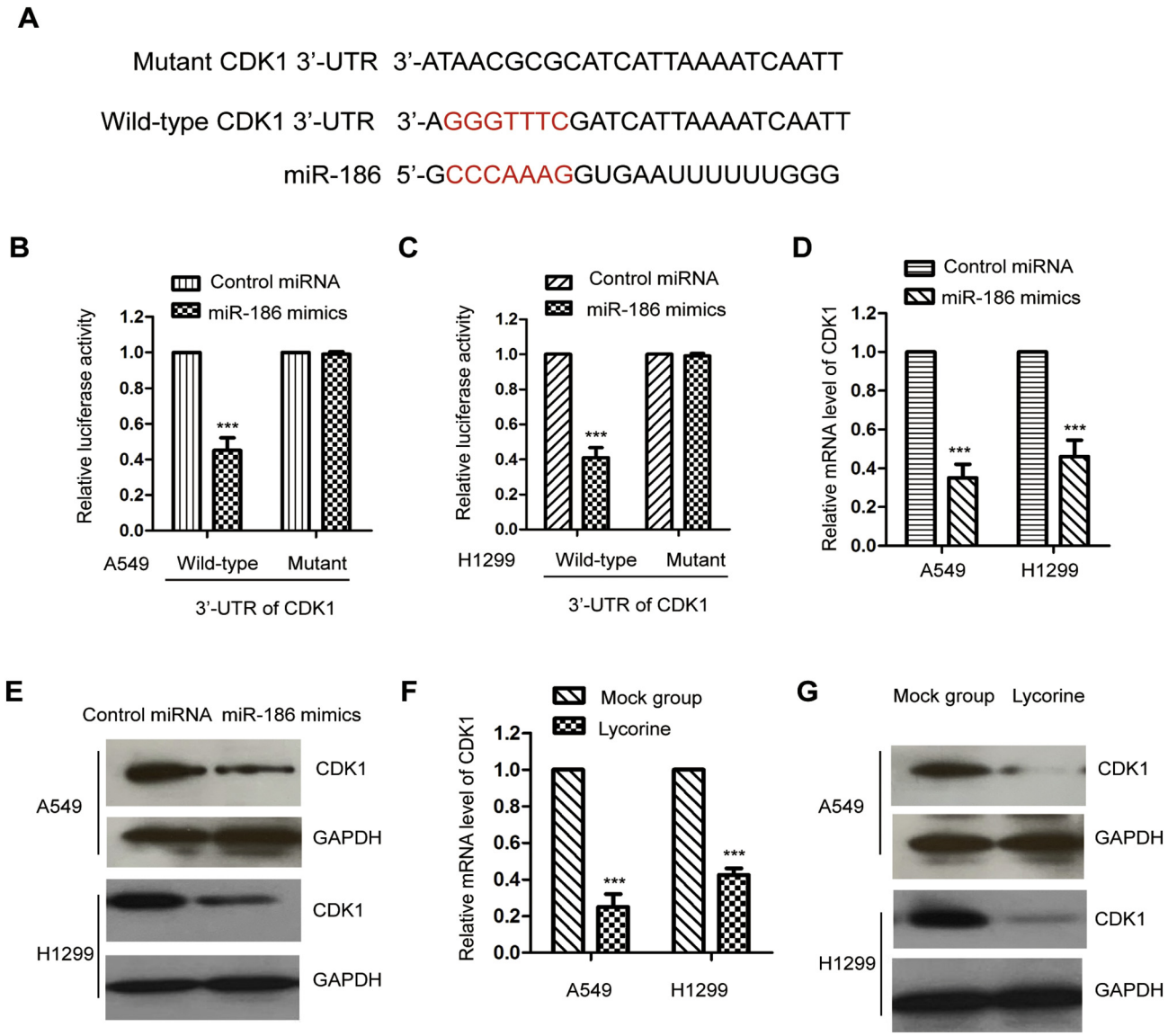

Fig. 3. CDK1 was a target of miR-186 in NSCLC cells. (A) The predicted binding sites of miR-186 at the 3′-UTR of CDK1. (B, C) Overexpression of miR-186 decreased the luciferase of wild-type 3′-UTR of CDK1. (D) Transfection of miR-186 in both A549 and H1299 cells inhibited the mRNA level of CDK1. (E) Overexpression of miR-186 decreased the protein expression of CDK1 in NSCLC cells. (F, G) Treatment of NSCLC cells with lycorine reduced both the mRNA and protein levels of CDK1.

2. Materials and methods

2.1. Reagents and cell culture

The lycorine was purchased from Shanghai Yuanye Biological Technology Company (Shanghai, China). Lycorine was dissolved in dimethyl solfoxide (DMSO) to the stock solution of 20 mM and filtered through a 0.22 μm filter. Lycorine was maintained at −20 °C before use.

NSCLC cell lines including A549, H1299 and H460, normal cell BEAS-2B were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured with the Dulbecco's modified Eagle's medium (Invitrogen, Thermo Fisher Inc., Carlsbad, CA USA) supplemented with 10% fetal bovine serum (Gibco, Thermo Fisher Inc., Carlsbad, CA USA). Cells were maintained at 37 °C with 5% CO_2.

2.2. Tissue samples

The NSCLC tissues and paired adjacent normal tissues were obtained from patients who were diagnosed as NSCLC at the Cangzhou Central Hospital between April 2011 to September 2013. Patients were not received any treatment before the tissue collection via surgical resection. Samples were maintained in the liquid nitrogen prior to the experiment. Informed consents were received from all the participants. This study was approved by the Ethics Committee of CangZhou Central Hospital.

2.3. Cell proliferation analysis

The proliferation of NSCLC cells was measured with the Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies, Inc., Kumamoto, Japan) according to the manufacturer's instruction. Briefly, both A549 and H1299 cells were cultured in 96-well plate and treated with lycorine at the indicated concentration. 10 μl of CCK-8 solution was added into the medium and then incubated for 3 h at 37 °C. The absorbance of each well at 450 nm was detected with the SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA, USA).

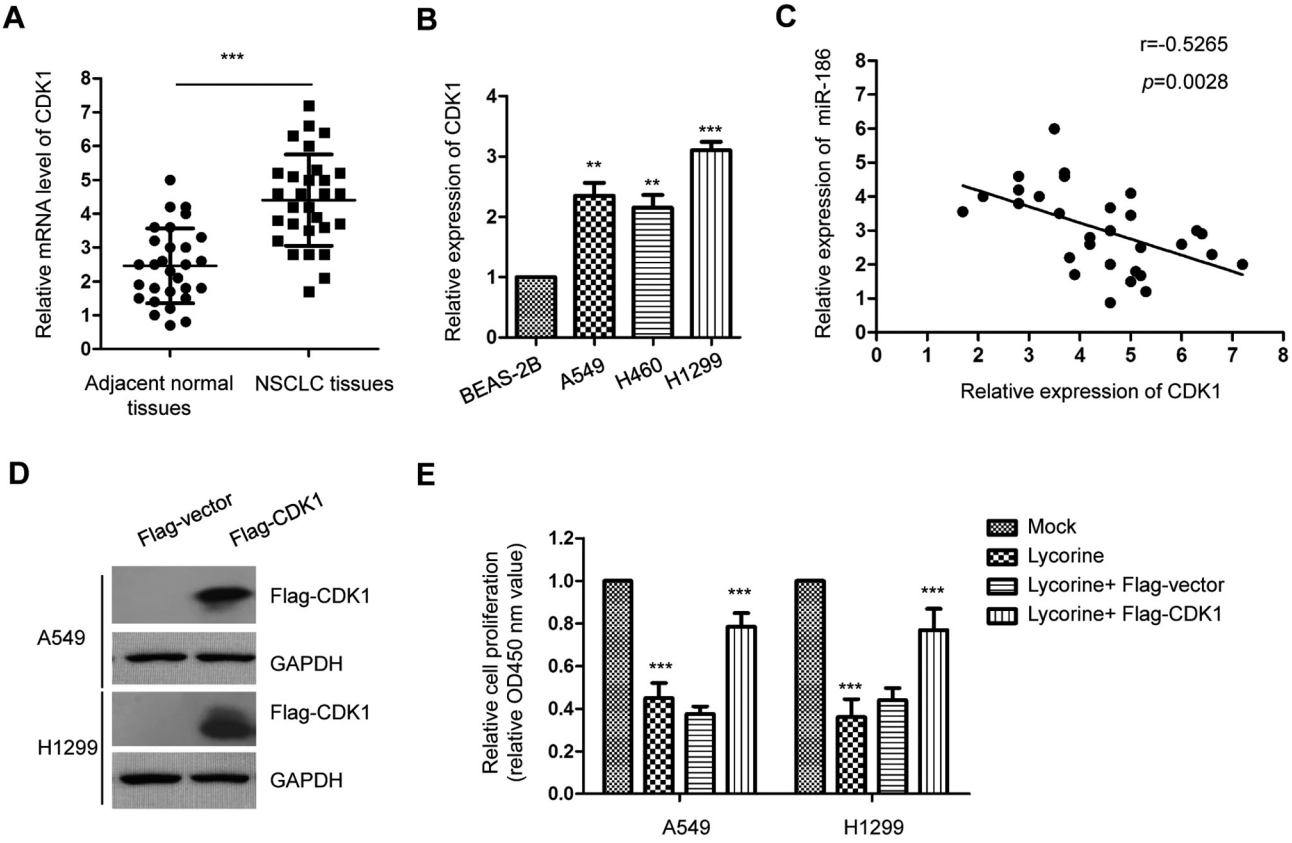

Fig. 4. Restoration of CDK1 reversed the inhibition of lycorine on the growth of NSCLC cells. (A) The mRNA level of CDK1 in paired NSCLC tissues and corresponding normal tissues was detected by RT-qPCR analysis. (B) The expression of CDK1 in NSCLC cell lines and normal cell BEAS-2B. (C) The expression of miR-186 in NSCLC tissues was significantly inversed correlated with that of CDK1. (D) Both A549 and H1299 cells were transfected with Flag-vector or Flag-CDK1. (E) Restoration of CDK1 reversed the inhibition of lycorine on the proliferation of both A549 and H1299 cells.

2.4. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was isolated from cells using TRIzol regent (Invitrogen, Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's instruction. Reverse transcription of miRNA was performed using the TaqMan microRNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). To detect the expression of CDK1 mRNA level, cDNA was synthesized using the Moloney-murine leukemia virus reverse transcriptase (BioTeke Corporation, Beijing, China). Real-time quantitative PCR was performed with the SYBR Green PCR Mixture (Takara, Dalian, China) on the ABI Prism 7900 real-time PCR detection system (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The expression of U6 or GAPDH was determined for the normalization of miR-186 and CDK1, respectively. The relative expression of miR-186 and CDK1 was calculated with the equation $2^{-\Delta\Delta CT}$. The PCR conditions were as follows: 95 °C for 5 min followed by 40 cycles of 95 °C for 10 s and 58 °C for 60 s. The primers used for RT-qPCR were as follow: miR-186 forward: 5′-CCCGATAAAGCTAGATAACC and reverse, 5′-CAGTGC GTCTCGTGGAGT; U6 forward, 5′-CGCTTCGGCAGCACATATACTAA and reverse, 5′-TATGGAACGCTTCACGAATTTGC; CDK1 (Gene ID: 983) forward, 5′-GGATGTGCTTATGCAGGATTCC and reverse, 5′-CATGTAC TGACCAGGAGGGATAG; GAPDH forward, 5′-CATCTTCTTTTGCGTCG CCA and forward, 5′-TTAAAAGCAGCCCTGGTGACC.

2.5. Western blot

Cells were lysed with the NP-40 lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China) containing protease/phosphatase inhibitor (Sigma, CA, USA). Equal amount of protein was loaded and separated with 15% SDS-PAGE. Proteins were transferred onto the polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). Membranes were firstly blocked with 5% non-fat milk prior to overnight incubation with the primary antibody at 4 °C. Membranes were washed twice with TBST buffer and then incubated with HRP-conjugated secondary antibody at room temperature (RT) for 1 h. The signals were visualized with the ECL solution (Pierce, Thermo Fisher Scientific, Inc. CA, USA) according to the manufacturer's instruction.

2.6. Dual-luciferase reporter assay

The wild-type or mutant 3′-UTR of CDK1 was cloned into the psiCHECK-2 vector (Promega, Madison, WI, USA). Both A549 and H1299 cells were co-transfected with miR-186 or control miRNA and wild-type or mutant 3′-UTR of CDK1 using Lipofectamine 2000 (Invitrogen, Thermo Fisher Scientific, Inc. Waltham, MA, USA) according to the manufacturer's instruction. 48 h after transfection, cells were harvested and the luciferase activity was qualified using the Dual-Luciferase Reporter assay system (Promega, Madison, WI, USA). The activity of *Renilla* was also determined for normalization.

2.7. Flow cytometry

Both A549 and H1299 cells were treated with the indicated concentration of lycorine. The apoptosis of cells was determined with the Annexin V-FITC Apoptosis Detection Kit (eBioscience, San Diego, CA, USA) according to the manufacturer's instruction. Cells were harvested and washed twice with PBS. 1×10^6 cells were resuspended in 200 μl of 1× binding buffer. 5 μl of Annexin V-FITC was added for 15 min at RT and then 10 μl of Propidium Iodide (100 μg/mL) was added into the cell

suspension. Cells were diluted with 400 μl of 1× binding buffer and immediately analyzed with the flow cytometry (Beckman Coulter, Brea, CA, USA). The data analysis was performed with the FlowJ software (BD Biosciences, San Jose, CA, USA).

2.8. Statistical analysis

Data was presented as mean ± standard deviation. Statistical analysis was performed using the GraphPad Prism 6.0 (San Diego, CA, USA). The difference between two groups was analyzed with the Student's *t*-test. Comparison between more than two groups was performed with the One-way ANOVA test. $P < 0.05$ was considered as statistical difference. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

3. Results

3.1. Lycorine suppressed NSCLC cell growth

To detect the effect of lycorine on the growth of NSCLC cells, both A549 and H1299 cells were treated with increasing dose of lycorine for 24 h and the cell proliferation was detected. The CCK-8 assay indicated that treatment of lycorine reduced the proliferation of NSCLC cells in a dose-dependent manner (Fig. 1A). Moreover, both A549 and H1299 cells were exposed to 10 μM lycorine for the indicated time. The results showed that treatment of lycorine significantly inhibited the proliferation of NSCLC cells with the extension of incubation (Fig. 1B). To further confirm the suppressive role of lycorine in regulating the growth of NSCLC cells, in vitro colony formation assay was performed. As presented in Fig. 1C, the number of colonies formed with A549 and H1299 cells was significantly decreased with the treatment of lycorine. Additionally, the effect of lycorine on the apoptosis of both A549 and H1299 cells was also evaluated with the FACS analysis. The data indicated that exposure of lycorine remarkably promoted the apoptosis of NSCLC cells compared with that of the control cells (Fig. 1D). These results demonstrated the negative regulation of lycorine on the growth of NSCLC cells.

3.2. Lycorine up-regulated the expression of miR-186 in NSCLC cells

Increasing evidence has illustrated the critical roles of miRNAs in the progression of human cancers. To investigate the involvement of miRNAs in lycorine mediated growth inhibition of NSCLC cells, A549 cells were treated with 10 μM lycorine for 48 h and then microarray was performed to evaluate the regulation of lycorine on the miRNA expression. These miRNAs with significantly changed expression in the exposure of lycorine was summarized in Supplementary Table 1. Among these miRNAs, the expression of miR-186 was dramatically increased with the treatment of lycorine. To confirm this data, both A549 and H1299 cells were treated with lycorine and the level of miR-186 was detected by RT-qPCR analysis. As shown in Fig. 2A, the abundance of miR-186 was significantly up-regulated with the exposure of lycorine in NSCLC cells. To validate the involvement of miR-186 in the growth of NSCLC cells, the expression of miR-186 was examined in 30 paired NSCLC tissues and corresponding adjacent normal tissues. The result showed that the expression of miR-186 was significantly decreased in NSCLC tissues compared with that of the normal tissues (Fig. 2B). Consistently, the level of miR-186 was also down-regulated in NSCLC cell lines in comparison with that of the normal cells (Fig. 2C). To check the influence of miR-186 on the growth of NSCLC cells, both A549 and H1299 cells were transfected with control miRNA or miR-186 mimics (Fig. 2D). The cell proliferation was detected with the CCK-8 assay. As presented in Fig. 2E and F, overexpression of miR-186 significantly suppressed the proliferation of both A549 and H1299 cells.

To investigate whether the up-regulation of miR-186 mediated the suppressive effect of lycorine on the proliferation of NSCLC cells, both A549 and H1299 cells were transfected with negative control miRNA or miR-186 inhibitor. The down-regulation of miR-186 was confirmed by RT-qPCR (Fig. 2G). Cells transfected with negative control miRNA or miR-186 inhibitor were treated with lycorine. CCK-8 assay showed that down-regulation of miR-186 significantly attenuated the suppressive function of lycorine in regulating the proliferation of both A549 and H1299 cells (Fig. 2H). These results indicated the involvement of miR-186 in lycorine induced growth defects of NSCLC cells.

3.3. CDK1 was a target of miR-186 in NSCLC cells

To further characterize the underlying molecular mechanism by which miR-186 regulated the growth of NSCLC cells, the possible targets of miR-186 were predicted with the microRNA.org database. The prediction uncovered that the 3′-UTR of CDK1 contained the potential binding sites of miR-186 (Fig. 3A). To confirm the prediction, luciferase reporter assay was performed by transfecting luciferase plasmid expressing wild-type or mutant 3′-UTR into NSCLC cells. The data showed that overexpression of miR-186 significantly down-regulated the luciferase activity of wild-type but not the mutant 3′-UTR of CDK1 (Fig. 3B and C). To detect whether the binding of miR-186 with the 3'-UTR of CDK1 affected the mRNA stability of CDK1, the mRNA level of CDK1 in both A549 and H1299 cells with the transfection of miR-186 was checked with RT-qPCR. The data demonstrated that overexpression of miR-186 significantly decreased the mRNA level of CDK1 (Fig. 3D). Consistently, the protein abundance of CDK1 was also examined by western blot, which indicated the down-regulation of CDK1 with the transfection of miR-186 in both A549 and H1299 cells (Fig. 3E).

As lycorine up-regulated the expression of miR-186, to investigate the role of lycorine in modulating the expression of CDK1, A549 and H1299 were treated with lycorine and the mRNA and protein levels of CDK1 were detected. The data indicated that lycorine treatment significantly suppressed both the mRNA and protein abundance of CDK1 in NSCLC cells (Fig. 3F and G). These results suggested that lycorine suppressed the expression of CDK1 via modulating miR-186.

3.4. Overexpression of CDK1 attenuated the inhibitory effect of lycorine on the proliferation of NSCLC cells

As miR-186 targeted CDK1 and negatively regulated the expression of CDK1, to support this conclusion, the level of CDK1 in paired NSCLC tissues and corresponding normal tissues was detected. As shown in Fig. 4A, CDK1 was significantly up-regulated in NSCLC tissues than that of the adjacent normal tissues. Additionally, the expression of CDK1 in NSCLC cell lines and normal cells was also compared. The data showed that the level of CDK1 was increased in NSCLC cell lines in comparison with that of the normal control (Fig. 4B). The correlation between the expression of miR-186 and CDK1 was evaluated with the Spearman test. Significantly inversed correlation between the expression of miR-186 and CDK1 in NSCLC was observed (Fig. 4C). These findings collectively supported the conclusion that CDK1 was a target of miR-186 in NSCLC.

Lycorine treatment down-regulated the expression of CDK1 in NSCLC cells. To confirm whether the inhibition of lycorine on the proliferation of NSCLC cells was through regulating CDK1, the full-length cDNA of CDK1 was inserted into the pcDNA-3.0Flag vector. The overexpression of CDK1 was detected by western blot (Fig. 4D). The result showed that the level of CDK1 was significantly increased with the transfection of Flag tagged CDK1. Both A549 and H1299 cells were transfected with Flag vector or Flag-CDK1 and treated with lycorine. The CCK-8 assay showed that restoration of CDK1 significantly attenuated the suppressive role of lycorine in regulating the proliferation of both A549 and H1299 cells (Fig. 4E). These results indicated the critical involvement of CDK1 in the tumor-suppressive function of lycorine in NSCLC.

4. Discussion

Emerging evidence showed that the small molecules derived from natural products are potential therapeutic agents in treatment of cancer [45–49]. As a natural alkaloid, lycorine has been found to suppress the malignant behaviors of human cancers [13–17,50,51]. Here, our results provided novel insights into the functional mechanism of lycorine in NSCLC via regulating the expression of miR-186 and CDK1.

An increasing number of studies have reported the critical roles of miRNAs in tumorigenesis [24,25,28]. Aberrant expression of miRNAs function as oncogenes or tumor suppressors and regulate the proliferation, migration and metastasis of cancer cells. Therefore, miRNAs have been considered as the targets of anti-cancer drugs to inhibit the progression of malignancy. Previous studies showed that lycorine suppressed the growth of cancer cells including NSCLC [19]. However, whether miRNAs are also involved in lycorine-mediated growth defect of lung cancer cells remains to be investigated. To test this idea, we performed microarray analysis to screen the aberrant expression of miRNAs with the treatment of lycorine. The results revealed that miR-186 was significantly up-regulated with the treatment of lycorine. Notably, recent studies demonstrated that miR-186 acted as a tumor suppressor, which suppressed the proliferation, invasion and migration of human cancers [33,34,52–54]. The down-regulation of miR-186 in NSCLC tissues was also confirmed in this study. Consistent with the suppressive role of miR-186 in NSCLC, depletion of miR-186 significantly reversed the inhibitory effect of lycorine on the proliferation of lung cancer cells. Further study might be necessary to investigate the underlying mechanism by which lycorine up-regulated the expression of miR-186. Additionally, the involvement of other miRNAs in lycorine-induced growth defects of NSCLC remains to be evaluated.

CDK1 is a member of the serine/threonine kinase family and controls the cell cycle progression [55]. CDK1 has been reported to be up-regulated in human cancers and overexpression of CDK1 was associated with the poor prognosis of cancer patients [56]. Aberrant expression of CDK1 was suggested as an independent prognostic factor for predicting the outcome of patients with colon cancer [57]. Given the oncogenic function of CDK1, CDK1 was identified as the targets of miRNAs to modulate the malignant behaviors of cancer cells. For example, miR-769 inhibited the progression of colorectal cancer via directly targeting CDK1 [58]. In NSCLC, miR-181a was found to inhibit the proliferation of cancer cells by down-regulating CDK1 [44]. In the present study, our results showed that miR-186 bound the 3'-UTR of CDK1 and decreased the expression of CDK1 in NSCLC cells. Up-regulation of CDK1 was observed in NSCLC tissues and negatively correlated with that of the miR-186. Overexpression of CDK1 reversed the inhibitory effect of lycorine on the growth of NSCLC cells. These results demonstrated that CDK1 mediated the suppressive function of lycorine in lung cancer.

5. Conclusion

Our results uncovered that lycorine inhibited the growth of lung cancer cells via modulating miR-186/CDK1 axis. Understanding the functional mechanism would benefit the application of lyroine in cancer treatment. Our findings demonstrated that molecular therapy against CDK1 and the treatment of lycorine might be an attracting therapeutic strategy for the treatment of lung cancer.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.06.003.

Declaration of Competing Interest

The authors declare that they have no conflicts of interests.

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