Latcripin-13 domain induces apoptosis and cell cycle arrest at the G1 phase in human lung carcinoma A549 cells

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Abstract. Latcripin-13 domain, isolated from the transcriptome of *Lentinula edodes* C_{91-3}, contains a regulator of chromosome condensation (RCC1) domain/β-lactamase-inhibitor protein II (BLIP-II) and a plant homeodomain (PHD). Latcripin-13 domain has been shown to have antitumor effects. However, the underlying molecular pharmacology is largely unknown. We report here that Latcripin-13 domain induced cell cycle arrest in the G1 phase and caused the apoptosis of human lung carcinoma A549 cells via the GSK3β-cyclin D1 and caspase-8/NF-κB signaling pathways. Western blot analysis showed that Latcripin-13 domain decreased cyclin D1 and cyclin-dependent kinase 4 (CDK4), while it increased the ratio of GSK3β/phosphorylated GSK3β. Importantly, Latcripin-13 domain induced nuclear fragmentation and chromatin condensation in the A549 cells. In addition, treatment of the A549 cells with Latcripin-13 domain resulted in the loss of mitochondrial membrane potential, accompanied by an increase in the Bax/Bcl-2 ratio and activation of caspase-3, -8, and -9. Intriguingly, western blot analysis revealed that NF-κB was significantly downregulated by Latcripin-13 domain. These results demonstrated that Latcripin-13 domain induced apoptosis and cell cycle arrest at G1 phase in the A549 cells, providing a mechanism for the antitumor effects of Latcripin-13 domain.

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Abbreviations: RCC1, regulator of chromosome condensation; PHD, plant homeodomain; *L. edodes*, *Lentinula edodes*; CD, circular dichroism; BLIP-II, β-lactamase-inhibitor protein II; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide

Key words: Latcripin-13 domain, RCC1, PHD, apoptosis, G0/G1 arrest, GSK3β

Introduction

Lung cancer is one of the leading causes of cancer-related deaths worldwide. During the past decades, great advances have been made in the management of lung cancer; however, the 5-year survival rate is still less than 15% (1). Most lung cancer patients eventually develop drug resistance following systematic surgery, chemotherapy and radiotherapy (2). Recently developed molecularly targeted therapies, such as epidermal growth factor receptor-tyrosine kinase inhibitors, have improved the survival time of some patients. Nevertheless, drug resistance is still a current challenge for molecularly targeted therapies (3). Recent studies have clearly demonstrated that natural bioactive compounds can effectively inhibit growth and proliferation, and induce apoptosis in various cancer cells, with marginal side effects. This suggests that the use of natural products is a promising alternative strategy for the treatment of lung cancer (4).

Fungal-derived chemical compounds with novel structure and biological activity are potential agents for anticancer drug development (5,6). *Lentinula edodes* is the second most popular edible mushroom in the world market (7). It has not only been widely used as a health food for thousands of years in China, Japan and Korea, but has also become popular in nutritional and medicinal products throughout Europe and North America (8). Dried *L. edodes* contains carbohydrates, proteins, fiber, lipids and ash (9). As a medicinal material, *L. edodes* has many pharmacological activities, including antibacterial, antiviral, immunomodulatory and antitumor activities (10), but the molecular pharmacology of *L. edodes* remains to be determined.

In our previous studies, we found that Latcripin-13 domain, a novel protein from *L. edodes* C_{91-3}, demonstrated tumor-suppressive activity via inducing the apoptosis of tumor cells without toxicity in normal cells (11). However the underlying molecular mechanism is largely unknown. Latcripin-13 domain belongs to the secretion-regulating guanine nucleotide exchange factor (also known as DelGEF) family, which may be involved in the secretion process. Latcripin-13 domain contains a regulator of chromosome condensation (RCC1) domain/β-lactamase-inhibitor protein II (BLIP-II) and a plant homeodomain (PHD). RCC1 and PHD domains are involved in several key cellular processes, including nucleocytoplasmic

transport, regulation of spindle formation, nuclear envelope reassembly at mitosis, gene transcription, cell cycle and apoptosis (12,13). However, BLIP-II does share significant sequence identity with the regulator of chromosome condensation (RCC1) family of proteins. These two families are clearly related, both having a seven-bladed β-propeller structure, although they differ in the number of strands per blade; BLIP-II having three anti-parallel β-strands per blade, while RCC1 has four-stranded blades (14). BLIP-II is a secreted protein produced by the soil bacteria *Streptomyces exfoliates* SMF19. BLIP-II acts as a potent inhibitor of β-lactamases such as TEM-1, which is the most widespread resistance enzyme to penicillin antibiotics (15). Based on the previous study, we hypothesized that Latcripin-13 domain may induce cancer cell apoptosis by disrupting pathways important for cell cycle progression. Whether or not Latcripin-13 domain has the activity of inhibiting the β-lactamases, will be investigated in the future.

In this study, we treated human lung carcinoma A549 cells with different concentrations of Latcripin-13 domain for varying times, analyzed cell apoptosis and cell cycle progression, and explored the potential molecular mechanisms.

Materials and methods

Preparation and analysis of Latcripin-13 domain. The expression, purification and analysis of the amino acid sequence and protein structure of Latcripin-13 domain were performed as previously described (11). In brief, Latcripin-13 domain, cloned from the transcriptome of *L. edodes*, was expressed in *E. coli* Rosetta-gami (DE3) in the form of inclusion bodies. The Latcripin-13 domain was purified by Ni-His affinity chromatography with high purity and refolded by urea gradient dialysis. The amino acid sequence of Latcripin-13 protein was analyzed using the online tool ExPASy ProtParam. The protein structure of Latcripin-13 was analyzed with the circular dichroism (CD) spectra, Swiss-Model, Pfam and InterPro databases.

Cell line and cell culture. Human lung carcinoma A549 cells were obtained from the Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). The cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 ng/ml streptomycin and 100 U/ml penicillin at 37˚C in a humidified atmosphere with 5% CO_2.

Cell viability assay. The effects of Latcripin-13 domain on A549 cell viability were measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. Cells were treated with Latcripin-13 domain at various concentrations. After treatment for 24 or 48 h, medium was replaced and 20 μl MTT [5 mg/ml in phosphate-buffered saline (PBS) solution] was added into each well. After incubation for 4 h, culture supernatants were aspirated, and purple insoluble MTT product was redissolved in 150 μl of dimethyl sulfoxide (DMSO). Absorbance at 562 nm was measured via an ELISA reader (Thermon, USA).

Nuclear staining with Hoechst 33258. The nuclear morphology of cells was evaluated using the cell-permeable DNA dye, Hoechst 33258. A549 cells ($1x10^5$ cells/ml) were placed in 6-well plates containing 1,500 μl culture medium and permitted to adhere for 24 h. Different concentrations of Latcripin-13 domain were added. After 48 h, the supernatant was aspirated and 100 μl of Hoechst 33258 was added to each well, followed by incubation at 37˚C for 10 min. The stained cells were observed under a fluorescence microscope at the wavelength of 340 nm.

Cell cycle analysis. Cell cycle distribution was assayed using the Cell Apoptosis PI detection kit (KeyGen Biotech, Nanjing, China) according to the manufacturer's protocol. Briefly, $1x10^6$ cells were harvested and washed with 1X buffer A after Latcripin-13 domain treatment. The cells were fixed with cold 70% ethanol for 24 h at -20˚C, and then resuspended in 500 μl 1X buffer A, followed by incubation with 5 μl propidium iodide (PI) for 30 min in the dark. After 30 min, fluorescence-activated cells were sorted in the FACSCalibur™ flow cytometer (BD Biosciences, San Jose, CA, USA) with excitation at 488 nm and detection at 620 nm. Data were gated to exclude cellular debris. The proportions of G1, S and G2-M phase cells were calculated from the DNA content histograms.

Assay of mitochondrial membrane potential. Mitochondrial membrane potential ($\Delta\psi m$) was measured using the mitochondrial membrane sensor kit containing the dye JC-1 (KeyGen Biotech) according to the manufacturer's instruction. Cells were treated with Latcripin-13 domain for 48 h, and then harvested for flow cytometric analysis.

Western blot analysis. Protein extracts of A549 cells treated with or without Latcripin-13 domain were prepared by lysing cells in RIPA buffer on ice for 30 min. Samples were centrifuged at 15,000 x g for 10 min. Protein concentration was determined with the BCA protein assay (KeyGen Biotech). For each sample, 30 μg/lane of protein was loaded onto 12% polyacrylamide gels and transferred to Total Blot NC membranes (Pall, USA), followed by blocking with 5% fat-free milk and incubation with the appropriate specific primary and secondary antibodies. The antibodies used were as follows: NF-κB (p65; ZSGB-BIO, Beijing, China); phosphorylated-GSK3β (S9; Bioworld Technology, Nanjing, China); GSK3β, Bcl-2, Bax, cyclin D1, CDK4, β-actin (Proteintech, Wuhan, China); GAPDH (TransGen Biotech, Beijing, China); secondary antibody (ZSGB-BIO). Signals were detected using a chemiluminescent gel imaging system according to the manufacturer's instructions (Bio-Rad).

Caspase activation assay. Caspase-3, caspase-8 and caspase-9 activation was determined using Caspase Colorimetric assay kits (KeyGen Biotech) following the manufacturer's instructions. In brief, $2x10^6$ cells were collected following treatment with various concentrations of Latcripin-13 domain (50, 100 μg/ml) for 48 h. After washing with PBS, cells were lysed in 50 ml of cold lysis buffer and incubated on ice for 20 min. The cell lysate was centrifuged at 15,000 rpm for 10 min at 4˚C. Reaction buffer/dithiothreitol (DTT) was then added to the supernatant. After incubation at 37˚C for 2 h with substrate DEVD-pNA, the absorbance was measured at the wavelength

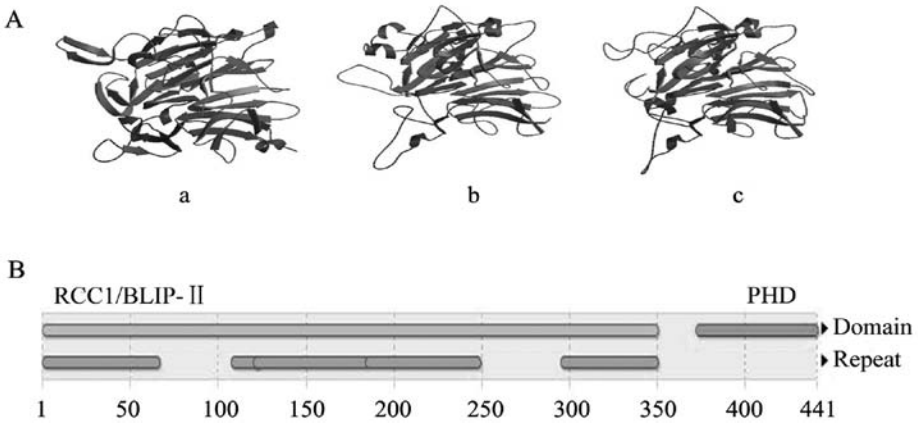

Figure 1. The model building results and InterPro databases of Latcripin-13 domain. (A) The SWISS-MODEL template library was searched with Blast and HHBlits for evolutionary related structures matching the target sequence which show the three possible model building results (a-c). (B) The InterPro databases show that Latcripin-13 domain may belong to the secretion-regulating guanine nucleotide exchange factor (also known as DelGEF) family and contain a regulator of chromosome condensation (RCC1) domain/β-lactamase-inhibitor protein II (BLIP-II) and a plant homeodomain (PHD).

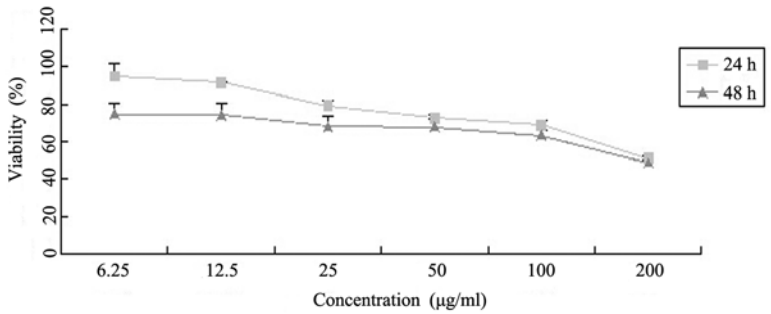

Figure 2. Latcripin-13 domain inhibits the proliferation of A549 cells. A549 cells were exposed to the indicated concentrations of Latcripin-13 domain for 24 and 48 h. Cell viability was determined using the MTT assay. Results represent the mean ± SD of three independent experiments.

of 405 nm using an ELISA microreader. Caspase-3, -8 and -9 activity was denoted as U and calculated from the standard curve generated with pNA solutions of different concentrations.

Statistical analysis. All the data are presented as mean ± standard deviations of three independent experiments. Statistical analyses were performed by one-way ANOVA using SPSS 11.0 software. Significant difference was set at $P<0.05$.

Results

Preparation and properties of Latcripin-13 domain. The amino acid sequence of the Latcripin-13 domain was obtained according to the gene sequence (GenBank accession number: KF682439, the sites from 235 to 1557). The number of amino acids of Latcripin-13 domain was 441 with molecular weight of 46705.2 Da and theoretical isoelectric point of 5.07. The CD spectra of the Latcripin-13 domain exhibited a high content of α-helix (4.7%), β-sheets (38.7%), turns (21.6%) and unordered (35%), as predicted using the Swiss-Model database. The InterPro database showed that Latcripin-13 domain belongs to the secretion-regulating guanine nucleotide exchange factor (also known as DelGEF) family containing the RCC1/BLIP-II and PHD domain. The model building results of Latcripin-13 domain by Swiss-Model database are showed in Fig. 1.

Latcripin-13 domain inhibits the proliferation of A549 cells. To assay the effects of Latcripin-13 domain on the proliferation of A549 cells, we treated A549 cells with varying concentrations of Latcripin-13 domain for 24 and 48 h, and determined cell viability with the MTT assay. As shown in Fig. 2, Latcripin-13 domain inhibited the viability of the A549 cells in dose- and time-dependent manners.

Latcripin-13 domain induces cell cycle arrest at G1 phase in A549 cells. To investigate the underlying mechanism by which Latcripin-13 domain inhibits cell proliferation of A549 cells, we analyzed the cell cycle progression following Latcripin-13 domain treatment. A549 cells were incubated with 50 or 100 μg/ml of Latcripin-13 domain for 24 and 48 h. The distribution of the cell cycle was analyzed using PI staining. Treatment with 50 or 100 μg/ml of Latcripin-13 domain for 24 h led to 79.6 and 82.01%, respectively, of cells in the G0/G1 phase compared with 64.53% in the control group. Moreover, 85.15 and 86.68% of cells were in G0/G1 phase following treatment for 48 h with 50 and 100 μg/ml, respectively (Fig. 3A). Thus, Latcripin-13 domain arrested A549 cells in the cell cycle at the G0/G1 phase.

To determine the molecular mechanism underlying cell cycle arrest in the G1 phase by Latcripin-13 domain, the expression of G1 phase regulatory proteins, CDK4, cyclin D1,

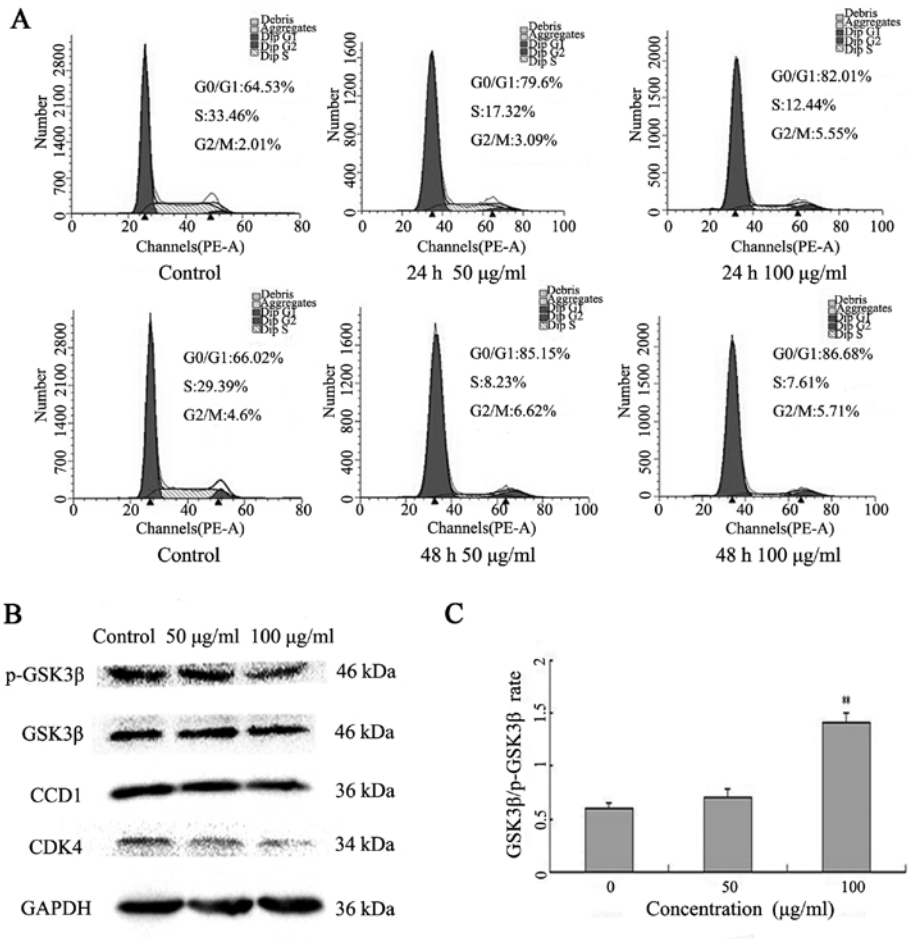

Figure 3. Cell cycle arrest by Latcripin-13 domain. (A) Changes in the cell cycle distribution in A549 cells treated with Latcripin-13 domain. Accumulation of G0/G1 phase cells was observed after treatment with Latcripin-13 domain (50 or 100 μg/ml for 24 and 48 h). (B) The effects of Latcripin-13 domain on G1 cell-cycle regulatory proteins. G1 cell-cycle regulatory proteins, including cyclin D1, CDK4 and GSK3β/phosphorylated GSK3β were examined using western blot analyses. (C) The GSK3β/phosphorylated GSK3β ratio in cells exposed to Latcripin-13 domain (50 or 100 μg/ml for 48 h) or unexposed. *P<0.05, **P<0.01 compared to the controls. Data from optical density measurements were tested using one-way ANOVA.

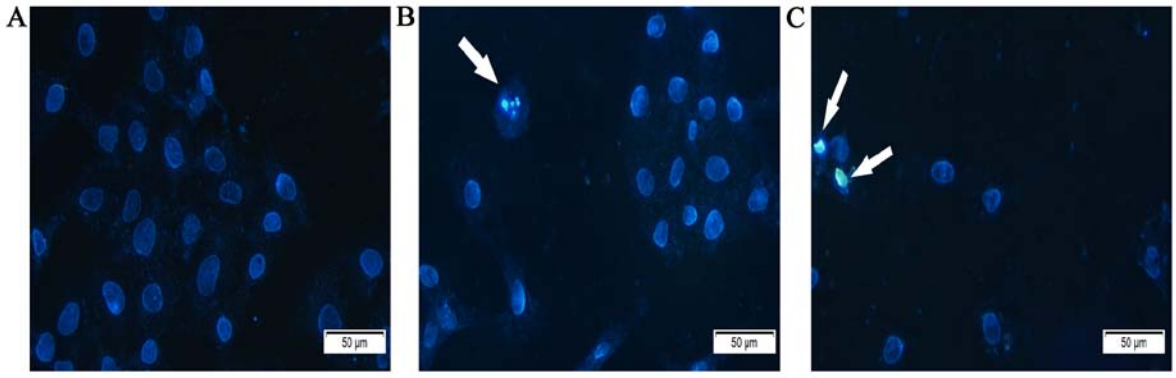

Figure 4. Fluorescence micrographs of A549 cells stained with Hoechst 33258 (x100 magnification). Cells were treated with 0 μg/ml (A), 50 μg/ml (B) or 100 μg/ml (C) Latcripin-13 domain for 48 h. Latcripin-13 domain induced nuclear condensation and fragmentation.

GSK3β and phosphorylated GSK3β were examined after treatment with Latcripin-13 domain for 48 h by western blotting. We found that 100 μg/ml of Latcripin-13 domain significantly decreased cyclin D1 and CDK4, and increased the ratio of GSK3β/phosphorylated GSK3β in the A549 cells (Fig. 3B and C). These results indicated that Latcripin-13 domain arrested cell cycle at the G1 phase, at least in part by decreasing cyclin D1/CDK4 and phosphorylation of GSK3β.

Latcripin-13 domain induces apoptosis in A549 cells. To assess whether induction of apoptosis contributed to the inhibition of cell proliferation of A549 cells by Latcripin-13 domain,

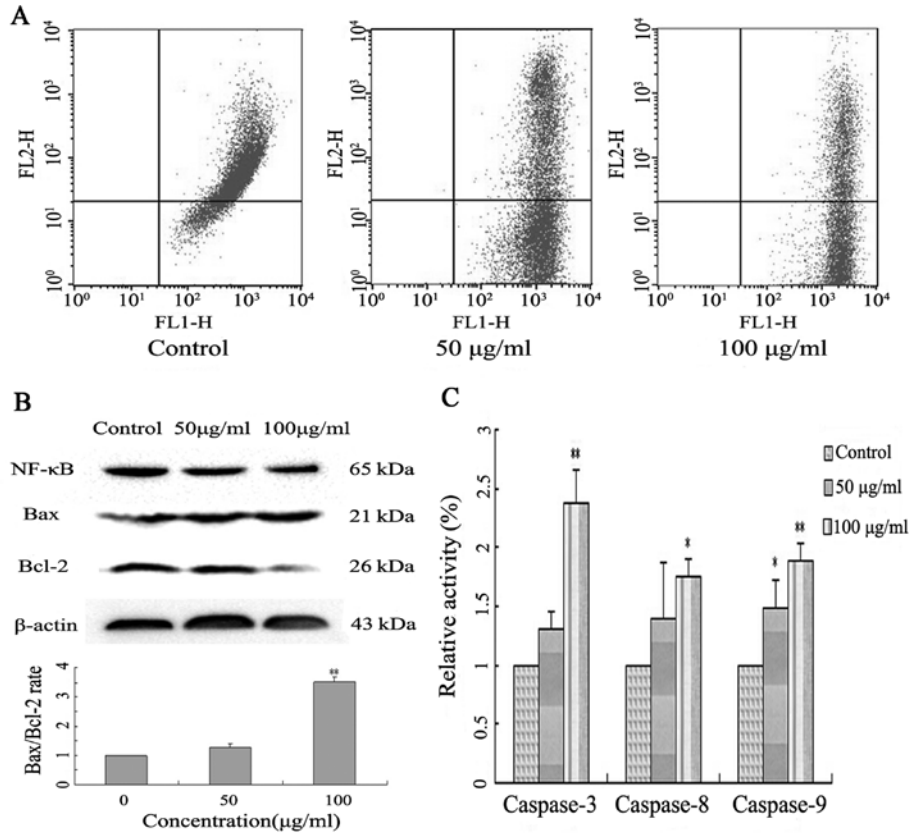

Figure 5. Latcripin-13 domain induces apoptosis by the mitochondrial-mediated pathway in A549 cells. (A) Depolarization of mitochondrial membrane potential (MMP, Δψm) was examined in control A549 cells and A549 cells exposed to Latcripin-13 domain (50 or 100 μg/ml for 48 h). Cells were stained with JC-1 and analyzed by flow cytometry. (B) Western blot analysis of NF-κB, Bcl-2 and Bax expression, and the Bax/Bcl-2 ratio in the cells exposed to Latcripin-13 domain (50 or 100 μg/ml for 48 h) or unexposed. *P<0.05, **P<0.01 compared to the controls. Data from optical density measurements were tested using one-way ANOVA. (C) The relative activities of caspase-3, caspase-8 and caspase-9 in A549 cells treated with Latcripin-13 domain for 48 h were higher than the relative activities in the untreated control cells.

we treated A549 cells with 0, 5 or 100 μg/ml of Latcripin-13 domain for 48 h and assessed the cell morphology under a microscope following Hoechst 33258 staining. In comparison to the control, Latcripin-13 domain induced apparent nuclear fragmentation and chromatin condensation, the typical morphological characteristics of apoptotic cells (Fig. 4). These results showed that Latcripin-13 domain induced apoptosis of the A549 cells.

Latcripin-13 domain induces apoptosis by mitochondria-mediated pathway in A549 cells. To support the above observation, we evaluated the mitochondrial function of Latcripin-13 domain-treated A549 cells by measuring mitochondrial membrane potential (MMP, Δψm) using the fluorochrome JC-1 and flow cytometry. A549 cells treated with Latcripin-13 domain demonstrated significant dissipation of the MMP in a concentration-dependent manner (Fig. 5A), indicating that treatment with Latcripin-13 domain led to loss of mitochondrial membrane potential in the A549 cells.

We next evaluated the anti-apoptotic proteins NF-κB, Bcl-2 and the pro-apoptotic protein Bax in the Latcripin-13 domain-treated A549 cells by western blotting. Latcripin-13 domain downregulated the expression of NF-κB, Bcl-2, and moderately increased the expression of Bax (Fig. 5B). Quantification analysis showed that Latcripin-13 domain induced a concentration-dependent increase in the Bax/Bcl-2 ratio in the A549 cells, an indication of cell apoptosis (Fig. 5B).

To assess whether the Latcripin-13-induced apoptosis in A549 cells is caspase-dependent, caspase-3, -8 and -9 enzyme activity was analyzed using a Caspase Colorimetric assay. In comparison to the control, 100 μg/ml of Latcripin-13 domain significantly induced the activation of caspase-3 (P<0.01), caspase-8 and caspase-9 (P<0.05) (Fig. 5C).

Discussion

In the present study, we demonstrated that Latcripin-13 domain inhibited proliferation and induced G1 phase cell cycle arrest of the A549 cells. The cell cycle arrest was accompanied by decreased cyclin D1 and CDK4, and an increased ratio of GSK3β/phosphorylated GSK3β. Importantly, Latcripin-13 domain induced the apoptosis of the A549 cells, as demonstrated by loss of mitochondrial membrane potential, increase in the Bax/Bcl-2 ratio and activation of the caspases. Our results indicated that Latcripin-13 domain arrested cell cycle progression and induced apoptosis in lung carcinoma A549 cells, suggesting that Latcripin-13 domain is a potential agent for the treatment of lung cancer.

Uncontrolled regulation of the cell cycle, leading to unrestrained cell proliferation, is a hallmark of cancer. Normal cell cycle progression requires the sequential expression of cyclins,

resulting in the activation of the cyclin-dependent kinases (CDKs) to phosphorylate target proteins (16). Cyclin D1 is a positive cell-cycle regulator necessary for the transition of cells from G1 to S phase. Cyclin D1 is expressed relatively early in the G1 phase as a crucial control of the G1 checkpoint of the cell cycle, after which cyclin D1/CDK4/6 forms a complex that is important in cell cycle regulation by retinoblastoma (Rb) phosphorylation (17-19). Overexpression of cyclin D1 is among the most commonly observed alterations in human malignant disorders. In this study, we found that Latcripin-13 domain inhibited proliferation and arrested A549 cells in G1 phase, accompanied by a decrease in cyclin D1 expression. These results suggest that Latcripin-13 domain may inhibit the growth of cancer cells by decreasing cyclin D1.

Cyclin D1 is overexpressed in most cancers (20). Genetic mutations or aberrant control of cyclin D1 expression contribute to cyclin D1 upregulation and accelerated G1 to S phase transition (21). GSK-3 is a serine-threonine kinase involved in a variety of physiologic functions such as glycogen metabolism, gene expression and apoptosis (22-24). Moreover, GSK-3β plays a key role in the regulation of cyclin D1 by both regulating cyclin D1 mRNA transcription and ubiquitin-dependent proteolysis of cyclin D1 protein (25). Accordingly, GSK-3β is implicated in the pathogenesis of a number of diseases, including diabetes, bipolar disorder, Alzheimer's disease, heart failure and cancer (26-29). Proteasome-dependent degradation of cyclin D1 is triggered by GSK-3β-mediated phosphorylation at threonine 286 (Thr286), which targets cyclin D1 for ubiquitination and proteolytic destruction (30,31). Our study showed that with an increasing concentration of Latcripin-13 domain, the GSK3β/phosphorylated GSK3β ratio increased. Our results suggest that Latcripin-13 domain may inhibit the G1 to S phases transition of A549 cells via a GSK3β-cyclin D1 signaling pathway.

Cell apoptosis is characterized by cytoplasmic shrinkage, chromatin condensation and DNA fragmentation (32,33). As shown in Fig. 2, the growth of A549 cells was inhibited by Latcripin-13 domain in a dose- and time-dependent manner. Fluorescence microscopy of cells stained with the DNA-binding dye Hoechst 33258 revealed that Latcripin-13 peptide treatment resulted in significant cell shrinkage and obvious chromatin condensation (Fig. 4).

Cell apoptosis is controlled by the mitochondrial Fas signaling pathways (34). Dysfunction of mitochondria results in a permeabilization of the outer membrane and concomitant release of mitochondrial apoptotic components into the cytosol, leading to caspase activation (35). Mitochondrial membrane permeability is controlled by a balance of pro- and anti-apoptotic proteins, including Bax and Bcl-2. In addition, apoptosis in cancer cells is mediated by cell surface death receptors, activation of which leads to activated caspase-8 and downstream caspases, such as caspase-3 (36). Activated caspase-8 can also cleave and stimulate Bid protein, which further promotes caspase-9 activation (37,38). Caspase-3, a downstream effector of both apoptosis pathways, is activated to regulate the caspase signaling cascade, eventually inducing apoptosis (39,40). Thus, these three caspases play important roles in the induction, transduction and amplification of intracellular apoptotic signals (41). Our results showed that Latcripin-13 domain decreased MMP, accompanied by an increase in Bax, but a decrease in Bcl-2. In addition, Latcripin-13 domain at 100 μg/ml significantly activated caspase-3, -8 and -9 in A549 cells. Importantly, NF-κB was also downregulated by Latcripin-13 domain (Fig. 5B). RCC1 is a eukaryotic nuclear protein that acts as a guanine nucleotide exchange factor for Ran, a member of the Ras GTPase family. RCC1 mediates a Ran-GTP gradient necessary for the regulation of spindle formation and nuclear assembly during mitosis, as well as for the transport of macromolecules across the nuclear membrane during interphase (42). The nuclear RanGTP level is diminished during the early stages of apoptosis, which correlates with immobilization of RCC1 on the chromosomes. Nuclear localization signal (NLS)-containing proteins that transport to the nucleus have been limited, including NF-κB-p65, which has important roles in rescuing cells from apoptosis. Wong *et al* has reported that RCC1 reads the histone code created by caspase-activated Mst1 to initiate apoptosis by reducing the level of RanGTP in the nucleus (43). Therefore, we propose that Latcripin-13 domain may reduce the concentration of nuclears RanGTP and recruit the Fas-associated via death domain (FADD) to bind to death receptors, which in turn activates caspase-8 to inhibit NF-κB signaling.

In conclusion, our data indicate that Latcripin-13 domain inhibits the proliferation of A549 cells via G1 cell cycle arrest and also inhibits the mitochondrial-mediated pathway. In addition, Latcripin-13 domain induces apoptosis through caspase-8 and NF-κB signaling. These findings provide further mechanistic evidence of the anticancer activity of Latcripin-13 domain.

Acknowledgements

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