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Short communication

Expression and functional analysis of novel molecule — Latcripin-13 domain from *Lentinula edodes* $C_{91\text{-}3}$ produced in prokaryotic expression system

Jia Wang, Mintao Zhong, Ben Liu, Li Sha, Yongzhi Lun, Wei Zhang, Xingyun Li, Xiaoli Wang, Jing Cao, Anhong Ning, Min Huang *

Department of Microbiology, Dalian Medical University, Dalian 116044, China

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ABSTRACT

The shiitake mushroom *Lentinula edodes* has health benefits and is used to treat various diseases due to its immunomodulatory and antineoplastic properties. In the present study, the Latcripin-13 domain, isolated from *L. edodes*, was expressed in *Escherichia 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 product showed biological activity in A549 cells, a human lung cancer cell line, by flow cytometry and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) method. The MTT assay and the flow cytometry results revealed that there was a great difference between the Latcripin-13 domain-treated group and the control group ($p < 0.05$). Similarly, cell apoptosis observed by transmission electron microscopy (TEM) supported the flow cytometry results. This work demonstrated that the Latcripin-13 domain can induce apoptosis of A549 cells, which will bring new insights into the development of new antitumor drugs in the future.

1. Introduction

Lung cancer remains the most common cancer diagnosed worldwide and has one of the lowest survival rates of all cancers (Jahangeer et al., 2013). The high mortality rate is related to the low cure rate, which in turn is associated with the lack of adequate screening and early detection measures (Jahangeer et al., 2013; Bremnes et al., 2006). Thus, there is a need for new screening and treatment modalities within this field.

Numerous effective anticancer drugs have been developed from natural sources. Most anticancer drugs are secondary metabolites of plants and animals and other small molecule compounds. However, the activity of biological macromolecules has attracted great attention of researchers in recent years. The earthworm fibrinolytic enzyme (EFE) has shown significant antitumor activity in hepatoma cells both *in vitro* and *in vivo* (Chen et al., 2007). In addition, protein-bound polysaccharide (PSK) derived from the CM-101 strain of the fungus *Coriolus versicolor* has shown anticancer activity *in vitro*, in *in vivo* experimental models, and in human cancers (Jiménez-Medina et al., 2008). Moreover, trichosanthin, a type I ribosome-inactivating protein, induces cell death in various cell types, including several tumor cell lines, and induces apoptosis in K562 cells (Li et al., 2007). *Lentinula edodes* (Berk.) Sing, the shiitake mushroom, has been used for traditional foods and medicine in Asia for over 2000 years (Choi et al., 2006). Many studies have shown that Lentinan can inhibit tumor cell growth, improve patient symptoms, and reduce adverse reactions. However, the antitumor activity of *L. edodes* rarely has been reported at the protein level (Zhong et al., 2013). *L. edodes* $C_{91\text{-}3}$ is an edible mushroom isolated from Basidiomycetes Umbelliferae fungi. Since 1991, according to the international study of pharmaceutical research for *L. edodes*, six strains of mycels of *L. edodes* have been fermented with the fermentation technology of bioengineering. One of the strain's fermentation broths was found to have a direct anti-tumor effect (Takehara et al., 1981; Wu et al., 2007). The extracts contain a variety of proteins in addition to polysaccharides and amino acids (Chen and Chang, 2004; Moradali et al., 2007; Wasser, 2002). In our previous studies, the protein components of *L. edodes* $C_{91\text{-}3}$ mycelia had significant antitumor effects on inducing apoptosis both *in vivo* and *in vitro* (Liu et al., 2012).

In this study, thousands of genes from *L. edodes* $C_{91\text{-}3}$ were extracted in order to further analyze the antitumor effects. According to the transcriptome sequence of *L. edodes* $C_{91\text{-}3}$, we designed primers and used the 3′-Full RACE and 5′-Full RACE methods to produce the *Latcripin-13* full-length gene. Based on the preliminary results of the gene functional annotation, the domains were analyzed using the online tool Sanger Pfam. Then, we designed primers of the functional area to get truncated

Abbreviations: RCC1, regulator of chromosome condensation; PHD, plant homeodomain; IPTG, isopropyl β-D-thiogalactoside; LB, Luria Bertani; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide.

* Corresponding author at: Department of Medical Microbiology, Dalian Medical University, 9 Western Section, Lvshun South Road, Lvshunkou District, Dalian 116044, China.
E-mail address: huangminchao@163.com (M. Huang).

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Latcripin-13, encoding the protein with the regulator of chromosome condensation (RCC1) and the plant homeodomain (PHD). We induced and expressed the protein with the *Escherichia coli* Rosetta-gami(DE3) expression system and incubated the human lung cancer cell line A549 with the identified protein. The biological function of the Latcripin-13 domain on A549 cells was studied by flow cytometry, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), and transmission electron microscopy (TEM) methods. Finally, the role of the protein in apoptosis was analyzed.

2. Materials and methods

2.1. Strains, plasmids, and reagents

The pET-32a(+) vector and *E. coli* Rosetta-gami(DE3), used for Latcripin-13 domain expression, were purchased from Invitrogen Co. (Beijing, China). The In-Fusion™ Advantage PCR Cloning Kit, 3′-Full RACE Core Set Ver2.0, 5′-Full RACE Kit, BigDye Terminater V3.1 Cycle Sequencing Kit, plasmid purification kit, Lysis Buffer for Microorganisms to Direct PCR, restriction polymerases, and primers were from Takara (Dalian, China). Trizol, Penta His Antibody, and HRP-Rabbit Anti-Mouse IgG (H + L) were from Invitrogen Co. (Beijing, China). The Bicinchoninic Acid Kit for Protein Determination and the Annexin V-FITC/PI kit were from keyGEN BioTECH (Nanjing, China).

2.2. Method of obtaining the functional region of Latcripin-13

Total RNA was extracted from the mycelium of *L. edodes* $C_{91\text{-}3}$ with Trizol reagent. Primers for the 3′-RACE and 5′-RACE experiments were designed with oligo6.0 software according to the transcriptome sequence results. The cDNA was synthesized with Takara 3′-Full Race Core Ver.2.0. The M-MLV (−) control was established at the same time. The cDNA was amplified by polymerase chain reaction (PCR) using the sense primer 5′-GGCTGATATCGGATCCGATGGAAACGCGTG GATTT-3′ and the antisense primer 5′-GTGGTGGTGCTCGAGGTCTTTC ACGCAGCTTGGAC-3′. Both primers contained BamH I and Xho I restriction enzyme sites, respectively. The PCR program included 30 cycles of 98 °C for 10 s, 55 °C for 10 s, and 72 °C for 1.5 min; and then a final extension of 72 °C for 5 min. The sequence of the amplified gene was analyzed and confirmed by an ABI PRISM™ 3730XL DNA Sequencer (Applied Biosystems). The general amino acid analysis of Latcripin-13 protein was done using the Swiss-Model database. The protein structure of Latcripin-13 was analyzed with the Swiss-Model and Pfam databases.

2.3. Construction of the expression vector

The prokaryotic expression vector, plasmid pET-32a(+), was digested by BamH I/Xho I in a 50 μL volume at 37 °C for 1 h. The products were purified with a TaKaRa MiniBEST Agarose Gel DNA Extraction Kit Ver.3.0. In order to identify, separate, and purify the protein, a 6× His-tag was added at the end of the truncated *Latcripin-13* gene. In order to ligate the gene and expression vector, an In-Fusion® HD Cloning Kit was used for In-Fusion cloning. The constructed plasmid was transformed into JM109 cells, an *E. coli* competent cell line, which was spread on Luria Bertani (LB) agar plates (pH 7.0) containing 100 μg/ml ampicillin and incubated overnight. The expression plasmid was obtained from the screened positive transformants and verified by both BamHI and XhoI digestion. Sequencing was conducted at Takara Dalian Co. The pET-32a(+)-truncated *Latcripin-13* was transformed into *E. coli* Rosetta-gami(DE3). A colony of positive cells was selected on LB agar plates containing tetracycline (12.5 μg/ml), kanamycin (15 μg/ml), and chloromycetin (34 μg/ml).

2.4. Induction and expression of the Latcripin-13 domain in E. coli Rosetta-gami(DE3)

The selected recombinant strains that were integrated with truncated *Latcripin-13* and pET-32a(+) were cultured in LB medium containing tetracycline (12.5 μg/ml), kanamycin (15 μg/ml), chloromycetin (34 μg/ml), and carbenicillin (50 μg/ml) at 37 °C with shaking until the optical density of the culture at 600 nm reached 0.5. Isopropyl β-D-thiogalactoside (IPTG) was then added to a final concentration of 0.5 mM or 1 mM to induce the expression at 37 °C for 2 h. The empty pET-32a(+) vector-transformed culture was used as a control. The bacteria were centrifuged at 6000 rpm at 4 °C for 5 min. The pellets were resuspended in phosphate-buffered saline (PBS) and lysed with liquid nitrogen four times, the lysate was centrifuged at 6000 rpm for 5 min, and the supernatant and the precipitate were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

2.5. Characterization of the Latcripin-13 domain with western blot

For each sample, the protein was loaded onto 12% polyacrylamide gels and transferred to TotalBlot nitrocellulose membranes (Pall, USA), which were blocked with 5% fat-free milk and immunostained with anti-His antibody (diluted 1:300). Thereafter, membranes were incubated for 1 h with horseradish peroxidase-labeled secondary antibodies, sheep anti-rabbit (diluted 1:5000), and then developed by a chemiluminescent gel imaging system according to the manufacturer's instructions (Bio-Rad, USA).

2.6. Affinity purification of the Latcripin-13 domain

For every 100 mg of bacteria (wet weight), 1–2 ml of bacterial lysis solution was added, which included phenylmethanesulfonyl fluoride (100 mM), DNaseI (1000 U/ml), and lysozyme (50 mg/ml); the bacteria were lysed with liquid nitrogen four times. The bacteria were centrifuged at 10,000 × *g* at 4 °C for 20 min. The pellets were washed in buffer I (100 mM phosphate buffer, 10 mM Tris–HCl, pH 8.0, and 2 M urea) at 4 °C overnight. After washing, the lysate was centrifuged at 10,000 × *g* at 4 °C for 20 min, and the supernatant was discarded and the pellet was gently resuspended in buffer II (100 mM phosphate buffer, 10 mM Tris–HCl, pH 8.0, and 8 M urea) at 4 °C overnight.

The nickel-chelated column was uncapped, and the alcohol was allowed to drain from the gel bed. The gel was equilibrated with 8 ml of binding buffer. Next, 5 ml of the resuspended protein was added to the column. The flow rate was controlled at 10 ml/h. After that, the column was washed with 15 ml of binding buffer. The fractions were collected. Finally, the 6× His-tagged protein was eluted with 5 ml of elution buffer and the fractions were collected. The eluted fractions were analyzed by 12% SDS-PAGE. Then, the Bicinchoninic Acid Kit for Protein Determination was used to measure the concentration of the purified protein (Smith et al., 1985).

2.7. Renaturation of the fusion protein by dialysis

The dialysis bag was boiled in 2% $NaHCO_3$ for 10 min, washed with distilled water, boiled in 1 mM EDTA for 10 min, and then kept in 50% ethanol at 4 °C prior to renaturation. The purified protein-containing dialysis bag was placed into renaturation solution I (PBS, 4 M urea, 0.2 mM oxidized glutathione, and 2 mM reduced glutathione) and kept at 4 °C for 12 h, and the fluid was changed once every 4 h. Next, the dialysis bag was placed into renaturation solution II (PBS, 2 M urea, 0.2 mM oxidized glutathione, and 2 mM reduced glutathione) and kept at 4 °C for another 12 h, and the fluid was changed once every 4 h. After that, the dialysis bag was placed into new renaturation solution III (PBS, 0.2 mM oxidized glutathione, and 2 mM reduced glutathione) at 4 °C for 12 h, and the fluid was changed once every 4 h. Finally, the dialysis