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Original Article

ERK1/2 mediates lung adenocarcinoma cell proliferation and autophagy induced by apelin-13

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The aim of this study was to investigate the role of apelin in the cell proliferation and autophagy of lung adenocarcinoma. The over-expression of APJ in lung adenocarcinoma was detected by immunohistochemistry, while plasma apelin level in lung cancer patients was measured by enzyme-linked immunosorbent assay. Our findings revealed that apelin-13 significantly increased the phosphorylation of ERK1/2, the expression of cyclin D1, microtubule-associated protein 1 light chain 3A/B (LC3A/B), and beclin1, and confirmed that apelin-13 promoted A549 cell proliferation and induced A549 cell autophagy via ERK1/2 signaling. Moreover, there are pores on the surface of human lung adenocarcinoma cell line A549 and apelin-13 causes cell surface smooth and glossy as observed under atomic force microscopy. These results suggested that ERK1/2 signaling pathway mediates apelin-13-induced lung adenocarcinoma cell proliferation and autophagy. Under our experimental condition, autophagy associated with 3-methyladenine was not involved in cell proliferation.

Keywords apelin; APJ; lung adenocarcinoma; ERK1/2; proliferation; autophagy

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Introduction

APJ receptor, described as a member of G-protein-coupled receptor by O'Dowd *et al*. [1], is similar to angiotensin II (Ang II) receptor type 1 (AT1). Because its endogenous ligand was not found until 1998, it was called as orphan G-protein-coupled receptor. Apelin consisting of 377 amino acid residues was first identified as an endogenous ligand of APJ [2].

Apelin peptides (the length range from 13 to 36 residues) are produced from the C-terminal portion of the pre-proprotein consisting of 77 amino acid residues and both apelin-13 and apelin-36 are the most important members among these peptides [3]. The amino acid sequences of mature apelin peptides (e.g. apelin-36) are highly conserved among humans, bovine, rat, and mouse. Despite apelin has high sequence homology with Ang II, apelin does not bind to AT1 and Ang II does not bind to APJ receptor, indicating that APJ is not functionally related to the AT1. It also suggested that apelin/APJ and Ang II/AT1 maybe have similar effect but different functions. Apelins and APJ are widely expressed in animal and human heart, lung, kidney, brain, liver, breast, and skeletal muscle and abundantly found in cardiovascular, brain, lung, and breast cancers. Previous studies have shown that apelin/APJ system has effects on the function of cardiovascular system [4], has the functional and histopathological protective effects against renal ischemia/reperfusion injury [5], and has the effect of analgesia [6]. In addition, apelin/APJ system is involved in many processes such as regulation of the release of hormone and metabolism [7], regulation of cell proliferation, migration, and apoptosis [8], and reduction of pulmonary inflammation and fibrin deposition [9]. It is also related to angiogenesis and atherosclerosis [10], insulin sensitivity [11], obstructive sleep apnea [12], and so on.

Recent studies demonstrated that high level of *apelin* mRNA is expressed in cultured Hs578T cells, a human breast carcinoma cell line [13]. Apelin is expressed in malignant tumor cells of tubulolobular carcinoma. Previous studies showed that apelin over-expression clearly increased tumor growth in malignant gliomas [14], suggesting that apelin over-expression might be associated with tumor development. Apelin expression was up-regulated under

hypoxic conditions, and exogenous apelin enhanced the proliferation and migration of oral cancer cells [15]. Taken together, these data suggested that apelin over-expression is closely related to the occurrence and development of tumor. However, detailed molecular and immunohistochemical analysis of apelin and its receptor APJ expression in human lung adenocarcinoma tissue is lacking. Also, the status of plasma apelin level in lung cancer is unknown.

Interestingly, specific over-expression of AT1 receptor was observed in the breast cancer cells [16]. It has been reported that Ang II/AT1 could promote cancer cell proliferation and tumor angiogenesis but inhibit the differentiation of tumor cells. It suggested that apelin/APJ is related to tumorigenesis. It had been assumed that apelin may promote proliferation and aggregation of endothelial cells, and regulate the caliber size of blood vessels [17]. Apelin is a novel angiogenic factor in human non-small cell lung cancer (NSCLC) [18]. Our recent studies demonstrated that apelin-13-induced adhesion of monocytes to human umbilical vein endothelial cells via PI3K/14-3-3 [19] and apelin-13 also promoted vascular smooth muscle cell (VSMC) proliferation through PI3K-Akt-ERK1/2-cyclin D1 pathway [20] and Jagged-1/Notch3 signaling pathway [21]. We also confirmed that apelin-13 stimulates VSMC proliferation by promoting the G1/S phase transition, and this effect is mediated in part by an apelin-pERK1/2-cyclin D1 signal cascade [22]. The over-expression and the phosphorylation of ERK may play an important role in the generation and development of human breast cancer [23] and ERK1/2 is an important signal protein of the apelin/APJ system. In addition, the over-expression of cyclin D1 was found in lung cancer and breast cancer. Furthermore, cyclin D1 and cyclin-dependent kinase 4 formed complexes and then promoted G1/S transformation.

The present study was designed to determine the expression and distribution of APJ in human lung cancer cell line A549 and plasma apelin level in lung cancer patients. It also aimed to study whether apelin promotes lung adenocarcinoma cell proliferation and whether ERK1/2 mediates the process of lung adenocarcinoma cell proliferation.

It has been reported that autophagy is involved in cell proliferation and the proliferation of cervical cancer CaSki cells is significantly inhibited when autophagy-related gene *beclin1* was over-expressed [24]. A recent study indicated that tephrosin induces A549 cancer cell death via the autophagy pathway [25]. In addition, a previous study revealed that arsenite can induce the ERK1/2 signaling pathway to stimulate autophagy and death-associated protein kinase promoter hypermethylation in human uroepithelial SV-HUC-1 cells [26]. Some data suggested that the activity of autophagy fluctuates in lung cancer, and it is closely related to the occurrence and development of lung cancer [27]. However, whether apelin could induce autophagy of lung cancer cells and the underlying mechanism are still unclear. It is reported that apelin stimulates proliferation and suppresses apoptosis of mouse osteoblastic cell line MC3T3-E1 [28], and apelin suppresses apoptosis of human VSMCs via APJ/PI3K/Akt signaling pathways [29]. Hence, we predicted that the mechanism may be related to the over-expression of APJ.

In the present study, we determined the expression of APJ and the plasma apelin level in lung cancer. It was found that apelin promotes proliferation and autophagy of lung cancer cells.

Materials and Methods

Ethics statement

The protocol of this study followed the principles of the Declaration of Helsinki. The sections were the archived microsection by tumor resection, that is, from Pathology Department of the first Affiliated Hospital of University of South China in 2005–2007. Furthermore, all human plasma samples were collected from peripheral blood of fasting human, that is, from Clinical Laboratory of the First Affiliated Hospital of University of South China in 2009–2010. All experiments were approved by the Ethics Committee of the First Affiliated Hospital of University of South China. The written informed consent was received from all participating patients.

Reagents and materials

Human lung adenocarcinoma cell line A549 was from Xiangya School of Medicine, Central South University (Changsha, China). Apelin-13 was purchased from Phoenix Pharmaceuticals, Inc. (Belmont, USA). Bicinchoninic acid (BCA) protein assay kit was purchased from Hyclone (Logan, USA). Polyvinylidene difluoride (PVDF) membranes were purchased from Millipore Biosciences (Billerica, USA). Monoclonal anti-human APJ antibody was purchased from R&D Systems, Inc. (Minneapolis, USA). Human apelin-12 enzyme-linked immunosorbent assay (ELISA) kit was from Phoenix Pharmaceuticals, Inc. (catalog No. EK-057-23). Anti-ERK1/2 antibody [ERK1/2 (K-23)], anti-phospho-ERK1/2 [pERK1/2 (E-4) antibody] or [phospho-PI3K P85 (Tyr458)/p55 (Tyr199) antibody], and PD98059 were purchased from Cell Signaling Technology (Beverly, USA). Beclin1 (H-300): sc-11427 was purchased from Santa Cruz Biotechnology (Santa Cruz, USA). Anti-LC3A/B antibody was purchased from Epitomics, Inc. (Burlingame, USA). Mouse anti-cyclin D1 antibody was purchased from Boster Biological Technology (Wuhan, China). Anti-β-actin antibody, anti-β-tubulin antibody, and horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG were purchased from Beijing CoWin Bioscience Co., Ltd (Beijing, China). HRP-conjugated goat anti-mouse IgG(h+1) was purchased from Immunology Consultants Laboratory, Inc. (Portland, USA). Cell counting kit-8 and Hoechst staining kit

were purchased from Beyotime Institute of Biotechnology (Haimen, China).

Immunohistochemistry

The sections were fixed in 10% neutral buffered formalin, paraffin-embedded, rehydrated routinely, and cut into 4 μm serial section. Then, these sections were dried overnight at 60°C and fixed. After being treated overnight with dimethylbenzene, the sections were rehydrated through graded ethanol (100%–70%), rinsed in distilled water, and soaked in 0.1 M sodium phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4, pH 7.4) for 5 min. These sections were subject to antigen retrieval at 98°C for 25 min in citric acid salt buffer in a microwave oven and washed in PBS, then incubated with 3% H_2O_2 for 15 min, and washed three times in PBS (each 3 min) to block endogenous peroxidase activity. The sections were then incubated for 30 min with the monoclonal anti-human APJ antibody under ambient temperature and washed three times with PBS (each 10 min). Here, PBS instead of the monoclonal anti-human APJ antibody was used as a negative control. The sections were then incubated with secondary antibodies for 30 min under room temperature and washed three times with PBS (each 10 min). Finally, the sections were stained by hematoxylin and observed under a microscope and the images were taken by a digital camera.

Apelin level in plasma of lung cancer patients

All blood samples were collected from peripheral blood of fasting human and were centrifuged for 15 min at 4°C at 400 *g*. Plasma was taken and immediately frozen at −80°C for analysis. Apelin level in plasma of cancer patients were measured by ELISA and the absorbance was detected at 450 nm.

Cell culture

A549cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) in a humidified incubator with 5% CO_2. When the cells reached 70%–80% confluent, they were treated with 0.25% trypsin and passaged to a fresh culture. A549 cells were serum starved by changing the culture media to RPMI 1640 containing 0.1% FBS 24 h before experiments.

Cell proliferation assay

Cells at the exponential phase were chosen to measure the extent of cell proliferation by cell counting kit-8 (CCK-8) assay. After being digested with trypsin and suspended in RPMI 1640 containing 10% FBS, the cells were seeded into 96-well plate (100 μl/well) with 6 parallel wells in one group. Cells were synchronized in RPMI 1640 containing 0.1% FBS for 24 h. After treatment with different concentrations of apelin-13 for different time periods, 10 μl of CCK-8 was added to each well of 96-well plates and incubated for 4 h. Then the absorbance at 450 nm was measured [30].

Cell-cycle analysis using flow cytometry

A549 cells were seeded in six-well plastic culture dishes at a density of 5×10^5 cells/ml, and apelin-13 was added in CCK-8 assay after cell synchronization and cultured for 12 and 24 h, respectively. Then, A549 cells were harvested by trypsinization, collected from the six-well plate, placed in Eppendorf tubes, washed with PBS at 4°C twice, and fixed with 2 ml of 70% ice-ethanol overnight. After centrifugation, A549 cells were washed with PBS twice again. Finally, the DNA contents of A549 cells were determined by iodinated pyridine (PI) staining and flow cytometry (FCM) analysis.

Western blot analysis

A549 cells were treated with the indicated concentrations of apelin-13 for 24 h or 30 min in the presence or the absence of 10 μM PD98059 (ERK1/2 inhibitor). Whole cell lysates were prepared with lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate and 0.1% sodium dodecyl sulphate) containing 1 mM phenylmethanesulfonyl fluoride and protease inhibitor. Protein concentrations were determined using BCA kit. Total protein samples from cell lysates (50 μg) were separated on 12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis and then transferred onto a PVDF membrane. The membranes were blocked with 5% non-fat milk dissolved in Tris-buffered saline containing 0.05% Tween-20 (TBST) at room temperature for 1 h and then incubated with a primary antibody [phospho(p)-ERK1/2, 1 : 2000, Cell Signaling; ERK1/2, 1 : 1000; cyclinD1, 1 : 200; BECN1, 1 : 600; LC3A/B: 1 : 3000; β-tubulin, 1 : 10000; β-actin, 1 : 1000] at 4°C overnight. After three times wash (each for 10 min) in TBST, membranes were incubated with either HRP-conjugated goat anti-rabbit (1 : 2000–1 : 5000) or HRP-conjugated goat anti-mouse IgG (1 : 5000) for 45 min at 37°C. After three additional washes (each for 15 min) with TBST, the membranes were visualized using an enhanced chemiluminescence reagent (Millipore) and exposed onto X-films for data analysis. β-Actin and β-tubulin were used as loading controls.

Scanning cell morphology and ultrastructure by atomic force microscopy

In order to visualize surface morphology of cells, atomic force microscopy (AFM) was used. For this purpose, cell suspension was placed on freshly cleaved mica at room temperature. A549 cells were cultured on the cover glass in a six-well plate and treated for different time, and then the cover glass was put out, cells were washed with PBS, and

fixed with 4% paraformaldehyde. After 30 min, cells were washed with distilled water for 2 min and then dried in the air. Then, the prepared samples were fixed in AFM XY scanning platform and the location area of samples was scanned. The experiment was carried out with tapping mode and with the software in AFM for image data collection.

Statistics

Data were analyzed by one-way analysis of variance followed by the Student–Newman–Keuls test for multiple comparisons or by the unpaired Student's *t*-test for pairwise comparison. Data were expressed as the mean ± SEM. Statistical significance was defined as $P < 0.05$.

Results

Expression of APJ in lung adenocarcinoma tissue and plasma of lung cancer patients

We examined 20 patients' pathological tissue section and each section included the control and the experimental groups, respectively. ELISA was used to determine the plasma of 10 healthy people and 10 patients, respectively. The expression of APJ in human lung adenocarcinoma tissue was detected by immunohistochemistry. The results in **Fig. 1** showed that the expression of APJ is positive in cells of cancer nest in lung adenocarcinoma tissue, with brown granules clearly distributed in the cell membrane and the cytoplasm as shown with red arrow (**Fig. 1A**). Interestingly, the expression of APJ in interstitial inflammatory cells in adjacent tissues was negative as indicated with blue arrow (**Fig. 1A**). However, the expression of APJ in cells of normal bronchial submucous glands was negative (**Fig. 1B**). This result revealed that the expression of APJ was higher in lung adenocarcinoma tissue than that in neighboring bronchial submucous tissue.

To examine the plasma level of apelin in tumor patients, we collected blood samples and detected the apelin level by ELISA. The results in **Fig. 1C** demonstrated that the plasma apelin level in lung cancer patients was remarkably higher than that in normal healthy persons.

Apelin-13 promotes A549 cell proliferation

A549 cells were treated with different concentrations of apelin-13 (0, 0.0001, 0.001, 0.01, 0.1, and 1 μM) and 10% FBS for 24 h, and the results shown in **Fig. 2A** demonstrated

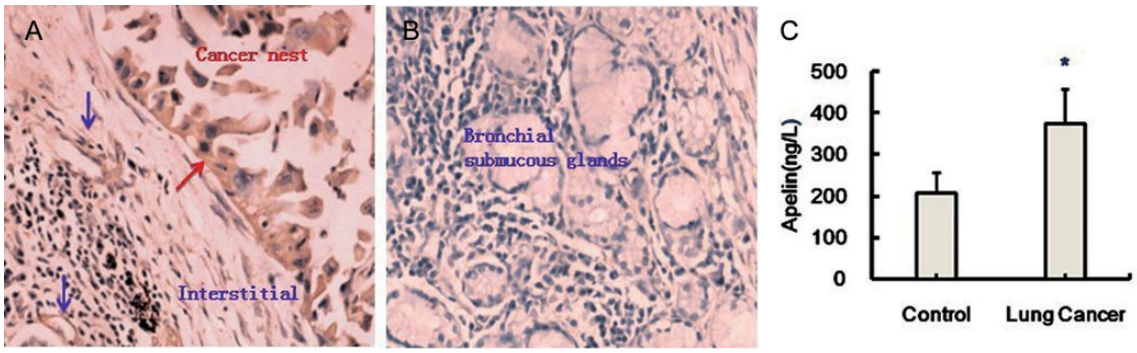

Figure 1. Expression of APJ in lung adenocarcinoma tissue and plasma of lung cancer patients (A) The expression of APJ in human lung adenocarcinoma tissue and (B) adjacent bronchial submucous tissue ($n = 3$). (C) The plasma apelin level of the lung cancer patients. *$P < 0.05$ vs. control ($n = 10$).

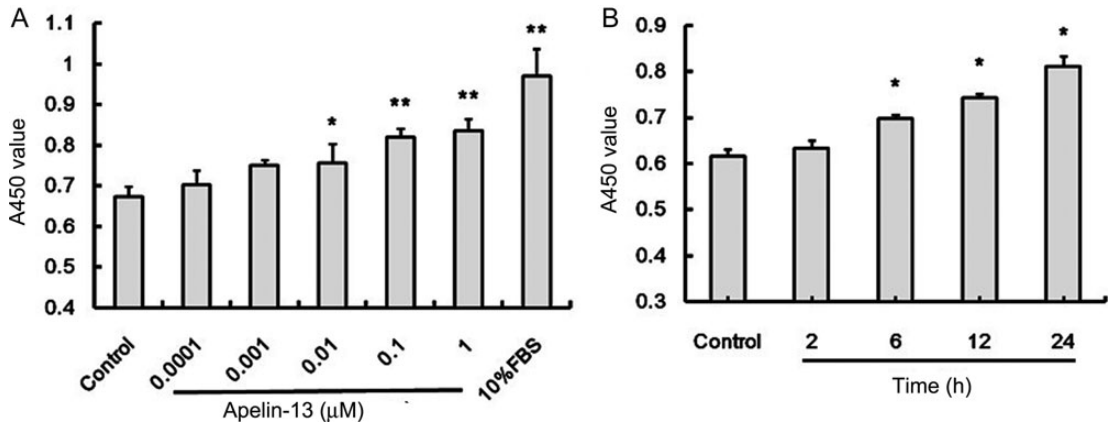

Figure 2. The dose effect and time effect of apelin-13 on the A549 cell proliferation (A) The dose effect of apelin-13 on the proliferation of A549 (incubated for 24 h). Data are expressed as the mean ± SD of six independent experiments. *$P < 0.05$ vs. control. **$P < 0.01$ vs. control. (B) The time effect of apelin-13 (0.1 μM) on the proliferation of A549 cells. Data are expressed as the mean ± SD of six independent experiments. *$P < 0.01$ vs. control.

Table 1. The cell cycle distribution of A549 induced by apelin-13 detected by FCM

Group	G0/G1 (%)	S (%)	G2/M (%)
Control	72.5 ± 4.4	19.2 ± 5.0	8.3 ± 0.9
Apelin-13 (0.1 μM) for 12 h	75.8 ± 4.1	18.4 ± 4.6	8.12 ± 0.6
Apelin-13 (0.1 μM) for 24 h	51.0 ± 1.3*	42.2 ± 2.0*	6.75 ± 1.4

Data are expressed as the mean ± SD of three independent experiments. *$P < 0.01$ vs. control.

that apelin-13 increase the absorbance in CCK-8 assay. When A549 cells were treated with apelin-13 (0.1 μM) for 0, 2, 6, 12, and 24 h, apelin-13 could also significantly increase the proliferation rate in concentration-dependent and time-dependent manners, with significant proliferation at 0.1 μM and 24 h (**Fig. 2B**). The absorbance of A549 cell lines was 0.819 ± 0.023, while the absorbance of the control was 0.672 ± 0.027, and 10% FBS (used as positive control) significantly increased the absorbance value. Compared with the control, 0.1 μM of apelin-13 increased the cell proliferation by 22% in A549 cells.

Apelin-13 accelerates the conversion of G0/G1 to S phase in lung adenocarcinoma cells

The results shown in **Table 1** demonstrated that apelin-13 (0.1 μM) has no apparent effect on cell cycle after 12 h of incubation, but significantly increases the percent of S phase cells and decreases the percent of G0/G1 phase cells in the cell cycle after 24 h of incubation ($P < 0.01$). These data suggested that apelin-13 induces A549 cell proliferation by accelerating the G0/G1 phase to S phase progression in the cell cycle.

PD98059 inhibits apelin-13-induced A549 cell proliferation

To understand the mechanism of apelin-13-induced A549 cell proliferation and whether ERK1/2 has any effect on apelin-13-induced A549 cell proliferation, CCK-8 assay was carried out. PD98059 (10 μM) was used to block ERK1/2 activity. The results shown in **Fig. 3** demonstrated that apelin-13 significantly reduces A549 cell proliferation after pre-incubated with PD98059 for 1 h, suggesting that apelin-13 influences A549 cell proliferation most probably via ERK1/2 signaling cascades.

Apelin-13 promotes the phosphorylation of ERK1/2 in A549 cells

In order to examine the role of ERK1/2 in the process of apelin-13-induced A549 cell proliferation, western blot analysis was carried out. After A549 cells were treated with apelin-13 (0.1 μM) for 0, 5, 15, 30, 45, and 60 min respectively, the results showed that apelin-13 promotes the expression of phospho-ERK1/2, and its expression level peaked at 30 min, while returns to normal at 60 min (**Fig. 4**). These data suggested that apelin-13 promotes the phosphorylation of ERK1/2.

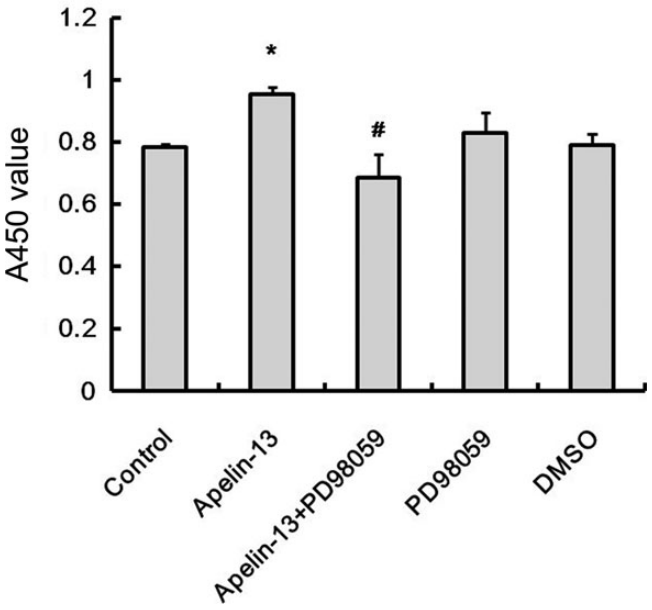

Figure 3. Effects of specific ERK1/2 inhibitor PD98059 on the proliferation of A549 cells induced by apelin-13 Data are expressed as the mean ± SD of six independent experiments. *$P < 0.01$ vs. control; #$P < 0.01$ vs. apelin-13.

PD98059 inhibits the phosphorylation of ERK1/2 induced by apelin-13 in A549 cells

As reported previously, apelin-13 could promote the phosphorylation of ERK1/2. Then, we explored the effect of ERK1/2 by western blot analysis. The results demonstrated that pre-incubation by PD98059 significantly inhibits the phosphorylation of ERK1/2 (**Fig. 5**). It revealed that apelin-13 promotes A549 cell proliferation via the increased phosphorylation of ERK1/2.

PD98059 inhibits the expression of cyclin D1 induced by apelin-13 in A549 cells

In order to explore the effect of apelin-13 on cyclins, the expression level of cyclin D1 was detected by western blot analysis. As shown in **Fig. 6**, after A549 cells were incubated with apelin-13 (0.1 μM) for 24 h, the expression level of cyclin D1 was significantly increased, suggesting that apelin-13 affects cell cycle through increasing cyclin D1 expression, and then inducing lung adenocarcinoma cell line A549 proliferation.

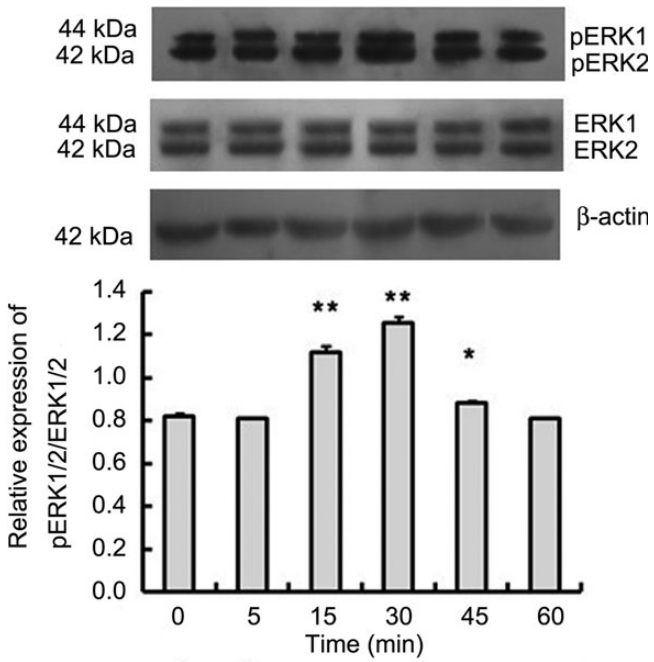

Figure 4. The phosphorylation of ERK1/2 was detected when apelin stimulated A549 cells for different time Data are expressed as the mean ± SD of three independent experiments. *$P < 0.05$ vs. control; **$P < 0.01$ vs. control.

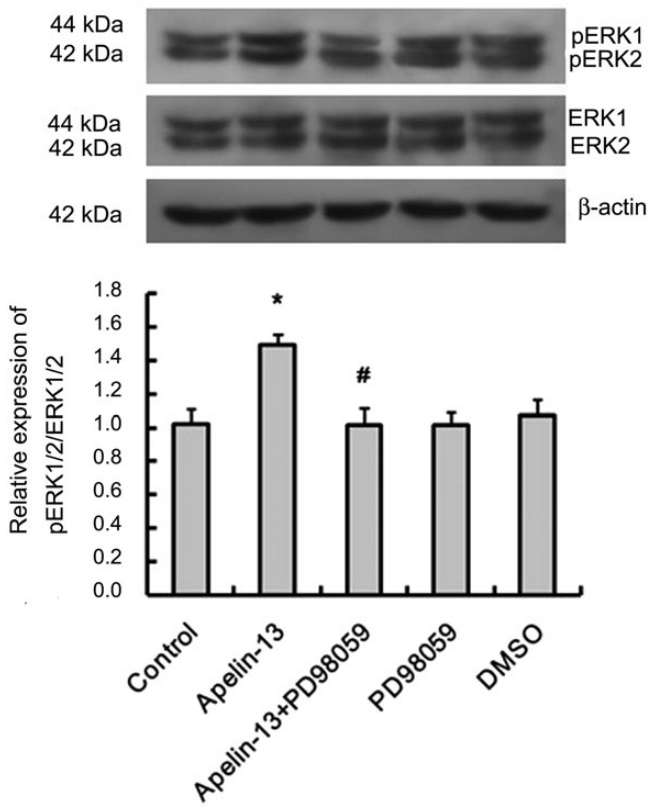

Figure 5. Effects of ERK1/2 inhibitor PD98059 on the expression of pERK1/2 in A549 induced by apelin-13 Data are expressed as the mean ± SD of three independent experiments.*$P < 0.01$ vs. control; #$P < 0.01$ vs. apelin-13.

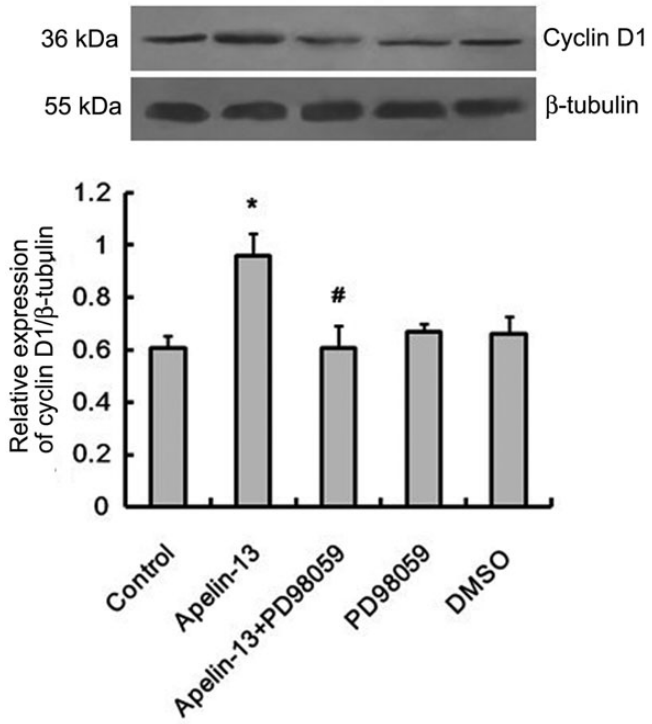

Figure 6. Effects of specific ERK1/2 inhibitor PD98059 on the expression of cyclin D1 in A549 cells induced by apelin-13 Data are expressed as the mean ± SD of three independent experiments. *$P < 0.01$ vs. control; #$P < 0.01$ vs. apelin-13.

We further explored the effect of PD98059 on the expression of cyclin D1 induced by apelin-13. Cyclin D1 expression levels were dramatically decreased by PD98059 (10 μM). These results showed that the PD98059 can suppress the expression of cyclin D1 induced by apelin-13, suggesting that apelin-13 can promote the expression of cyclin D1 via phosphorylation of ERK1/2 and subsequently induce A549 cell proliferation.

Apelin-13 induces the expression of LC3A/B and beclin1 and PD98059 inhibits the expression of LC3A/B and beclin1 induced by apelin-13 in A549 cells

LC3 has been used as a marker of autophagy. Beclin1 is one of the important factors for regulating autophagy. The expression of LC3 and beclin1 can reflect the autophagy activity. To study whether or not apelin-13 could induce the autophagy in A549 cells, we detected the expression of LC3A/B and beclin1 after treating with apelin-13 for 24 h in A549 cells by western blot analysis. The results indicated that apelin-13 increases the expression of LC3A/B and beclin1 (**Fig. 7A,B**), suggesting that apelin-13 could induce autophagy in A549 cells. As expected, pre-incubation for 1 h with PD98059 (10 μM) reduced the increased expression of LC3A/B and beclin1. These results suggested that apelin-13 induces autophagy of A549 cells and ERK1/2 is involved in this signaling pathway.

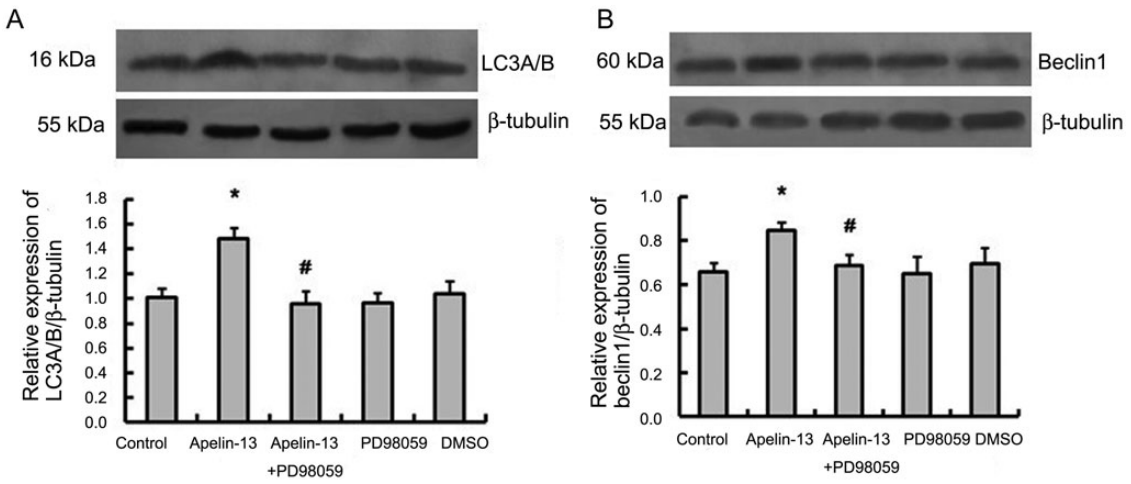

Figure 7. The influence of apelin-13 on the expression of LC3A/B and beclin1 in A549 cells (A) The influence of apelin-13 (0.1 μM) on the expression of LC3A/B in A549 cells. Data are expressed as the mean ± SD of three independent experiments.*$P < 0.01$ vs. control; #$P < 0.01$ vs. apelin-13. (B) The influence of apelin-13 on expression of beclin1 in A549 cells. Data are expressed as the mean ± SD of three independent experiments. *$P < 0.01$ vs. control. #$P < 0.01$ vs. apelin-13.

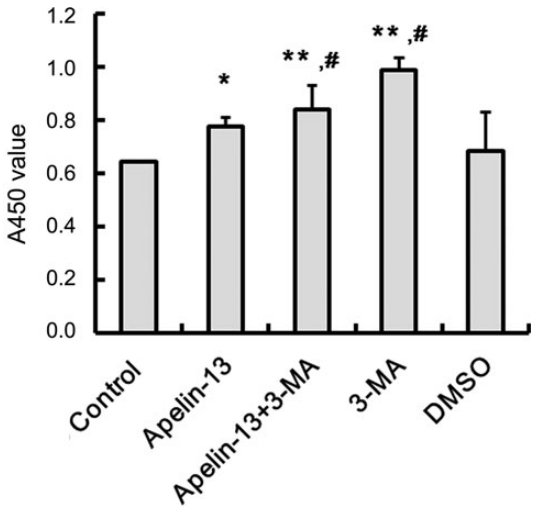

Figure 8. Effects of autophagy inhibitor on the proliferation of A549 cells induced by apelin-13 Data are expressed as the mean ± SD of six independent experiments.*$P < 0.05$ vs. control; **$P < 0.01$ vs. control; #$P < 0.01$ vs. apelin-13.

Interference to autophagy has no effect on A549 cell proliferation induced by apelin-13

Apelin can promote A549 cell proliferation and autophagy. We next investigated the relationship between autophagy and proliferation using autophagy inhibitor 3-methyladenine (3-MA) (1 μM). The results of CCK-8 assay showed that pre-treatment with 3-MA significantly increases cell proliferation when compared with only apelin-13-stimulated A549 cells (**Fig. 8**). These results suggested that apelin-13 and inhibition of autophagy both promote A549 cell proliferation. Moreover, apelin-13 could not reduce A549 cell proliferation after pre-incubated with 3-MA for 1 h, inferring that the autophagy associated with 3-MA was not involved in cell proliferation.

Apelin-13 can make A549 cell surface smooth and form glossy

The cell membrane is an important structure to maintain the stability of the intracellular environment. AFM was utilized to study the surface morphology of fixed cells. Hence, we used AFM to scan the cell surface stimulated by apelin-13 at 12 and 24 h. The surface topography (**Fig. 9A–C**) and the corresponding ultrastructure (**Fig. 9D–F**) showed that the individual cell had no significant change (**Fig. 9A–C**) when treated with apelin-13. However, cell surface had pores, became smooth, and some formed ravine (**Fig. 9D–F**).

Discussion

Apelin/APJ system was widely distributed in lung, heart, skeletal muscle, kidney, brain, and liver and involved in the fluid homeostasis, decompression, reinforcement of myocardium contractility, inhibition of human immunodeficiency virus infection, and so on. However, Maguire *et al.* [31] and our lab both found that apelin could influence vascular blood pressure, suggesting that apelin may play dual roles: relaxes blood vessels and makes vein contraction. The over-expression of *apelin* gene in the rostral ventrolateral medulla of spontaneously hypertensive rat results in chronic blood pressure elevation [32]. It is different from the regulation of peripheral blood pressure.

We found that APJ is expressed in human lung adenocarcinoma tissues and the expression of APJ in lung adenocarcinoma is higher compared with the adjacent tissues of lung cancer. Furthermore, we also found that the plasma apelin level of lung cancer patients were higher than the normal persons. These results suggested that the over-expression of

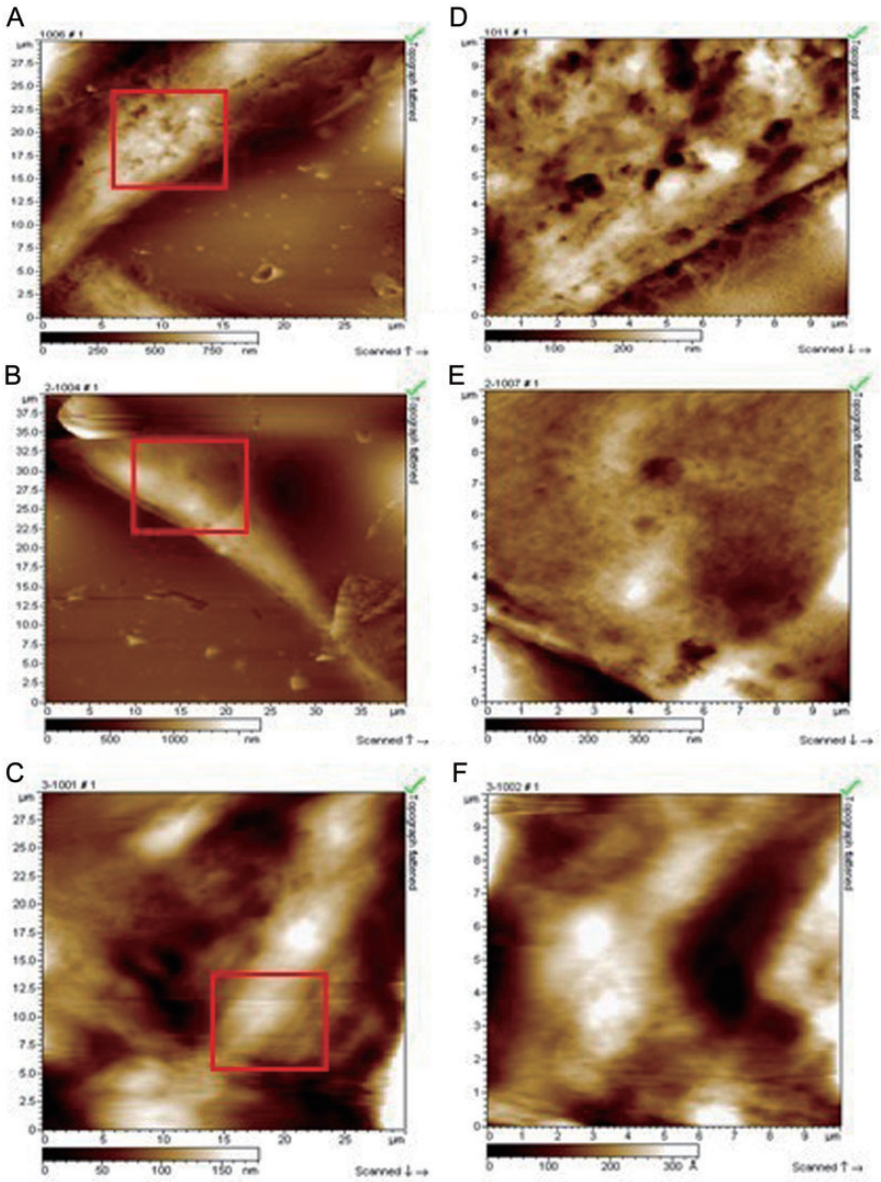

Figure 9. AFM images of A549 morphology and ultrastructure induced by apelin-13 for different time Data are expressed as the mean ± SD of three independent experiments. (A–C) The topographies of the three cells; (D–F) Cell membrane ultrastructures corresponding to the positions of red boxes in (A–C). (A,D) The control group; (B,E) Treatment with apelin-13 for 12 h; (C,F) Treatment with apelin-13 for 24 h.

APJ is closely related to the occurrence and development of tumor.

It was reported that apelin inhibited apoptosis in human VSMCs through APJ/PI3K/Akt signaling pathways [28], suggesting apelin/APJ system is closely related to cell proliferation and apoptosis, and the over-expression of APJ might promote the proliferation of lung adenocarcinoma cell, then affect the occurrence and development of lung adenocarcinoma.

Our experiment was designed to investigate the apelin-13-mediated cell proliferation and autophagy in lung adenocarcinoma cell line A549. The results showed that apelin-13 promotes A549 cell proliferation in concentration- and time-dependent manners with the significant effects when cells were incubated with 0.1 μM of apelin-13 for 24 h. Hence, we choose 0.1 μM of apelin-13 treating with A549 cells for 24 h in the subsequent experiments.

We also investigated the role of apelin-13 in cell cycle and cyclins in A549 cells. In our experiments, we found that apelin-13 promotes the transformation of G0/G1 phase to S phase by FCM and apelin-13 significantly increases the expression of cyclin D1 as revealed by western blot analysis.

In addition, to reveal the mechanism of apelin-13-induced A549 cell proliferation, we blocked the ERK1/2 activity using PD98059. The results from the western blot analysis suggested that apelin-13 could increase the phosphorylation of ERK1/2, facilitate the expression of cyclin D1, and further promote A549 cell proliferation. Several studies

showed that autophagy was closely related to cell proliferation and played a key role in cell proliferation. However, whether apelin is related to autophagy and what is the mechanism is currently unknown. In this study, we showed that apelin-13 could increase the expression of LC3A/B and beclin1 and induce A549 autophagy via ERK1/2 signaling pathway. However, it is reported that beclin1 had gene deletion in breast carcinoma and ovarian cancer and acted as a tumor-suppressor gene [33,34]. The expression of beclin1 and LC3 were higher in the lung carcinoma cells than those in the adjacent tissues. This discrepancy is related to lung carcinoma cell autophagy, which is caused by nutrient deficiency. Autophagy is a compensatory mechanism providing nutrients to cells.

What is the relationship between autophagy and proliferation in A549 cells? In this study, we found that autophagy associated with 3-MA was not involved in proliferation. Whether autophagy caused by other signaling pathways is involved in proliferation is still need to be investigated. However, it was reported that fibrin could activate autophagy and autophagy is involved in cell proliferation and angiogenesis. It was demonstrated that interferon-γ (IFN-γ) induces autophagy and induction of autophagy contributes to the growth-inhibiting effect of IFN-γ in human liver cancer cells [35]. In addition, induced autophagy could suppress cell proliferation of glioblastoma cells, MCF-7, HepG2, and normal hepatocytes [36,37]. These data suggested that inducing or suppressing autophagy may both involve in cell proliferation.

Other findings suggested that autophagy suppression leads to inhibition of NSCLC cell proliferation and sensitizes them to cisplatin-induced caspase-dependent and -independent apoptosis by stimulation of reactive oxygen species (ROS) formation [38]. However, induction of autophagy leads to a down-regulation of human immunodeficiency virus type 1 Vpr-binding protein (VPRBP) and promotes VPRBP-LC3/p62 interaction. Moreover, patients with low expression of both p62 and VPRBP showed the best prognosis in lung adenocarcinoma [39]. These data suggested that autophagy had dual roles. Autophagy is involved in cell proliferation and autophagy is related to prognosis of lung adenocarcinoma.

These above-mentioned results could be summarized in **Fig. 10** and these findings showed that ERK1/2 signaling pathway mediates apelin-13-induced lung A549 cell proliferation and autophagy.

Cell membrane is an important structure of the cells and regulates cell survival, material transportation, metabolism, cell surface recognition, and other physiological process. Therefore, the normal function is closely related to the structure of cell membrane. The surface topography of fixed cells and the corresponding ultrastructure by AFM showed that individual cell had no significant change, but cell surface

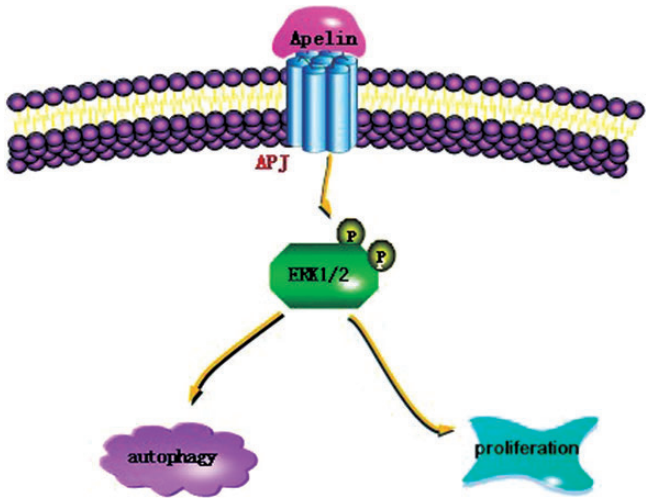

Figure 10. Molecular mechanism of A549 cell proliferation, autophagy induced by apelin-13

had pores, became smooth, and some formed ravine in the ultrastructure (**Fig. 9D–F**). This change of cell membrane structure was probably attributed to the treatment with apelin-13, which caused lower cell membrane permeability, thus preventing A549 cells from harmful stimulation and facilitating cell proliferation.

Drugs that could induce autophagy can also change the level of ROS. It was reported that ROS can make mitochondrial inner membrane lipid peroxidation [40]. It is concluded that inhibition of autophagy blocks ROS accumulation and cell death, and the cause of abnormal ROS accumulation is the selective autophagic degradation of the major enzymatic ROS scavenger, catalase [41]. These data suggested that the ROS involved in the regulation of autophagy may exist in mitochondria.

Recent studies demonstrated that dihydrocapsaicin down-regulated catalase, led to ROS accumulation, and blocked LC3 conversion in A549 cells [42]. H460 cells over-expressing catalase were able to induce autophagy, suggesting that catalase regulates autophagy, which protects cells against apoptotic and necrotic cell death. Our recent study reported that NOX4-derived ROS drive apelin-13-induced VSMC proliferation via the ERK pathway [43]. These results concluded that apelin-13 can induce autophagy of A549 and ERK1/2 is involved in this process. However, whether apelin-13 induces A549 autophagy through the change of ROS or not still needs to be explored.

Some data concluded that autophagy is involved in heart disease [44]. Many studies demonstrated that the effect of autophagy on the vascular function and vascular diseases such as atherosclerosis, abdominal aortic aneurysm, pulmonary hypertension, diabetes mellitus, and some drugs influenced cell autophagy of blood vessel wall [45]. As previously demonstrated, apelin is closely related to blood pressure and heart disease (e.g. myocardial hypertrophy). It

was recently reported that autophagy is a new target for disease treatment, particularly in some cardiovascular, neuronal, lung and metabolism diseases, and in tumor development and infection [46].

It has been confirmed that apelin has minimal effect on sprouting retinal angiogenesis, but contributes significantly to pathogenic non-neovascular remodeling [47], suggesting that apelin plays an important role in angiogenesis. Apelin/APJ system and vascular endothelial growth factor play a role in contributing retina vascular formation. Thus, we inferred that apelin may have dual functions on angiogenesis through correlation factor.

Cell proliferation and apoptosis are very important for normal cell growth and metabolism. Interestingly, Ang II stimulates cell proliferation and apoptosis, while p53 was not involved [48]. Both Ang II and transforming growth factor beta-1 have been reported to modulate the balance between cell proliferation and apoptosis. Ang II induced apoptosis in the absence of DNA synthesis [49]. The data as mentioned above suggested that apelin was also related to A549 apoptosis. But the mechanism needs further study.

It was recently reported that sodium selenite treatment of A549 cells appeared to trigger both apoptosis and cytoprotective autophagy, which were both mediated by ROS [50]. Thus, whether apelin could lead to both apoptosis and autophagy of A549 cells and whether ROS are involved in this process also need to be studied.

In addition, there are data suggesting that cytochrome C has a close relationship with cell apoptosis. Cytochrome C activates procaspase-9 and procaspase-3 successively and induces apoptosis through increasing the level of ROS. Recent studies indicated that apelin possibly affected A549 cells apoptosis through the production of caspase-3 induced by cytochrome C.

It has been reported that 14-3-3 protein levels are higher in human lung cancers as compared with normal tissues. Kawamoto *et al*. [51] obtained 14-3-3 protein-inducible A549 by transfection with 14-3-3 expression vector and found that 14-3-3 promoted cell proliferation. Our group has confirmed that 14-3-3 and ERK1/2 were involved in the signaling pathway of rat VSMCs proliferation induced by apelin-13 [52], suggesting that apelin probably promotes A549 cell proliferation via 14-3-3/ERK1/2 signaling pathway.

Some recent studies have discovered the new roles of apelin in regulating miRNA generation [53], which opens a new field for further research.

Taken together, our findings showed that the expression of APJ in lung adenocarcinoma tissue was higher than adjacent bronchial submucous tissue and the plasma apelin level of the lung cancer patients was higher than that in normal persons. To our knowledge, this is the first report on the plasma apelin level in lung cancer patients. Furthermore, we confirmed that apelin promotes cell proliferation and autophagy in A549 cells and ERK1/2 signaling pathway mediates apelin-13-induced A549 cell proliferation and autophagy. We then explored the relationship between apelin-induced proliferation and autophagy. In addition, we presented several images for A549 surface topography and the corresponding ultrastructure. We preliminarily explored the apelin's biological effect and related signal transduction mechanism. Apelin/APJ system is expected to be therapeutic targets of lung cancer. In future, more detailed studies need to be performed including detailed mechanism of apelin-13-induced A549 cell autophagy, whether 14-3-3-ERK1/2 signal cascade were involved in A549 cell proliferation, as well as the relationship between cell proliferation and angiogenesis of lung cancer. It provides more evidence to understand the mechanism of occurrence and development of lung cancer. Moreover, we further resolve the problem more directly through observation of living cells with respect to exploring cell surface morphology and ultrastructure. These results provided the basis for lung cancer treatment as well as the drug screening and development. Furthermore, apelin/APJ system will probably become therapeutic targets for treating lung cancer.

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References

1. O'Dowd BF, Heiber M, Chan A, Heng HH, Tsui LC, Kennedy JL and Shi X, *et al*. A human gene that shows identity with the gene encoding the angiotensin receptor is located on chromosome 11. Gene 1993, 136: 355–360.
2. Tatemoto K, Hosoya M, Habata Y, Fujii R, Kakegawa T, Zou MX and Kawamata Y, *et al*. Isolation and characterization of a novel endogenous peptide ligand for the human APJ receptor. Biochem Biophys Res Commun 1998, 251: 471–476.

3. Charo DN, Ho M, Fajardo G, Kawana M, Kundu RK, Sheikh AY and Finsterbach TP, *et al*. Endogenous regulation of cardiovascular function by apelin-APJ. Am J Physiol Heart Circ Physiol 2009, 297: H1904–H1913.
4. Liu C, Li X, Chen F, Pan W, Feng F, Qin X and Li L, *et al*. ERK1/2 mediated the vasodilatation of apelin-13 on vascular rings of spontaneously hypertensive rat *in vitro*. Prog Biochem Biophys 2009, 36: 1578–1588.
5. Sagiroglu T, Torun N, Yagci M, Yalta T, Sagiroglu G and Oguz S. Effects of apelin and leptin on renal functions following renal ischemia/reperfusion: an experimental study. Exp Ther Med 2012, 3: 908–914.
6. Xu N, Wang H, Fan L and Chen Q. Supraspinal administration of apelin-13 induces antinociception via the opioid receptor in mice. Peptides 2009, 30: 1153–1157.
7. Heinonen MV, Laaksonen DE, Karhu T, Karhunen L, Laitinen T, Kainulainen S and Rissanen A, *et al*. Effect of diet-induced weight loss on plasma apelin and cytokine levels in individuals with the metabolic syndrome. Nutr Metab Cardiovasc Dis 2009, 19: 626–633.
8. Zhang Z, Yu B and Tao GZ. Apelin protects against cardiomyocyte apoptosis induced by glucose deprivation. Chin Med J 2009, 122: 2360–2365.
9. Visser YP, Walther FJ, Laghmani el H, Laarse Av and Wagenaar GT. Apelin attenuates hyperoxic lung and heart injury in neonatal rats. Am J Respir Crit Care Med 2010, 182: 1239–1250.
10. Lv D, Li H and Chen L. Apelin and APJ, a novel critical factor and therapeutic target for atherosclerosis. Acta Biochim Biophys Sin 2013, 45: 527–533
11. Attané C, Foussal C, Le Gonidec S, Benani A, Daviaud D, Wanecq E and Guzmán-Ruiz R, *et al*. Apelin treatment increases complete fatty acid oxidation, mitochondrial oxidative capacity, and biogenesis in muscle of insulin-resistant mice. Diabetes 2012, 61: 310–320.
12. Zirlik S, Hauck T, Fuchs FS, Neurath MF, Konturek PC and Harsch IA. Leptin, obestatin and apelin levels in patients with obstructive sleep apnoea syndrome. Med Sci Monit 2011, 17: CR159–CR164.
13. Wang Z, Greeley GH, Jr and Qiu S. Immunohistochemical localization of apelin in human normal breast and breast carcinoma. J Mol Histol 2008, 39: 121–124.
14. Sorli SC, Le Gonidec S, Knibiehler B and Audigier Y. Apelin is a potent activator of tumor neoangiogenesis. Oncogene 2007, 26: 7692–7699.
15. Heo K, Kim YH, Sung HJ, Li HY, Yoo CW, Kim JY and Park JY, *et al*. Hypoxia-induced up-regulation of apelin is associated with a poor prognosis in oral squamous cell carcinoma patients. Oral Oncol 2012, 48: 500–506.
16. De Paepe B, Verstraeten VL, De Potter CR, Vakaet LA and Bullock GR. Growth stimulatory angiotensin II type-1 receptor is upregulated in breast hyperplasia and *in situ* carcinoma but not in invasive carcinoma. Histochem Cell Biol 2001, 116: 247–254.
17. Takakura N and Kidoya H. Maturation of blood vessels by haematopoietic stem cells and progenitor cells: involvement of apelin/APJ and angiopoietin/Tie2 interactions in vessel caliber size regulation. Thromb Haemost 2009, 101: 999–1005.
18. Berta J, Kenessey I, Dobos J, Tovari J, Klepetko W, Ankersmit HJ and Hegedus B, *et al*. Apelin expression in human non-small cell lung cancer: role in angiogenesis and prognosis. J Thorac Oncol 2010, 5: 1120–1129.
19. Li X, Zhang X, Li F, Chen L, Li L, Qin X and Gao J, *et al*. 14-3-3 mediates apelin-13-induced enhancement of adhesion of monocytes to human umbilical vein endothelial cells. Acta Biochim Biophys Sin 2010, 42: 403–409.
20. Liu C, Su T, Li F, Li L, Qin X, Pan W and Feng F, *et al*. PI3K/Akt signaling transduction pathway is involved in rat vascular smooth muscle cell proliferation induced by apelin-13. Acta Biochim Biophys Sin 2010, 42: 396–402.
21. Li L, Li L, Xie F, Zhang Z, Guo Y, Tang G and Lv D, *et al*. Jagged-1/Notch3 signaling transduction pathway is involved in apelin-13-induced vascular smooth muscle cells proliferation. Acta Biochim Biophys Sin 2013, 45: 875–881.
22. Li F, Li L, Qin X, Pan W, Feng F, Chen F and Zhu B, *et al*. Apelin-induced vascular smooth muscle cell proliferation: the regulation of cyclin D1. Front Biosci 2008, 13: 3786–3792.
23. Yao Q, Luo JR, Chen JH, Zhang JL, Yuan SF, Ling R and Wang L. Expression and activation of MAPK pathway signaling molecules in human breast cancer cell lines. Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi 2004, 20: 328–330.
24. Sun Y, Liu JH, Sui YX, Jin L, Yang Y, Lin SM and Shi H. Beclin1 overexpression inhibits proliferation, invasion and migration of CaSki cervical cancer cells. Asian Pac J Cancer Prev 2011, 12: 1269–1273.
25. Li J, Wang XL, Fang YC and Wang CY. Tephrosin-induced autophagic cell death in A549 non-small cell lung cancer cells. J Asian Nat Prod Res 2010, 12: 992–1000.
26. Huang YC, Hung WC, Chen WT, Yu HS and Chai CY. Sodium arsenite-induced DAPK promoter hypermethylation and autophagy via ERK1/2 phosphorylation in human uroepithelial cells. Chem Biol Interact 2009, 181: 254–262.
27. Yang L, Xiao L and Chen L. Research progress of autophagy and pulmonary diseases. Prog Biochem Biophys 2012, 39: 861–868.
28. Tang SY, Xie H, Yuan LQ, Luo XH, Huang J, Cui RR and Zhou HD, *et al*. Apelin stimulates proliferation and suppresses apoptosis of mouse osteoblastic cell line MC3T3-E1 via JNK and PI3K/Akt signaling pathways. Peptides 2007, 28: 708–718.
29. Cui RR, Mao DA, Yi L, Wang C, Zhang XX, Xie H and Wu XP, *et al*. Apelin suppresses apoptosis of human vascular smooth muscle cells via APJ/PI3-K/Akt signaling pathways. Amino Acids 2010, 39: 1193–1200.
30. Zhang HY and Sun H. Up-regulation of Foxp3 inhibits cell proliferation, migration and invasion in epithelial ovarian cancer. Cancer Lett 2010, 287: 91–97.
31. Maguire JJ, Kleinz MJ, Pitkin SL and Davenport AP. [Pyr1]Apelin-13 identified as the predominant apelin isoform in the human heart: vasoactive mechanisms and inotropic action in disease. Hypertension 2009, 54: 598–604.
32. Zhang Q, Yao F, Raizada MK, O'Rourke ST and Sun C. Apelin gene transfer into the rostral ventrolateral medulla induces chronic blood pressure elevation in normotensive rats. Circ Res 2009, 104: 1421–1428.
33. Liang XH, Jackson S, Seaman M, Brown K, Kempkes B, Hibshoosh H and Levine B. Induction of autophagy and inhibition of tumorigenesis by beclin1. Nature 1999, 402: 672–676.
34. Qu X, Yu J, Bhagat G, Furuya N, Hibshoosh H, Troxel A and Rosen J, *et al*. Promotion of tumorigenesis by heterozygous disruption of the beclin1 autophagy gene. J Clin Invest 2003, 112: 1809–1820.
35. Li P, Du Q, Cao Z, Guo Z, Evankovich J, Yan W and Chang Y, *et al*. Interferon-γ induces autophagy with growth inhibition and cell death in human hepatocellular carcinoma (HCC) cells through interferon-regulatory factor-1 (IRF-1). Cancer Lett 2012, 314: 213–222.
36. Yao F, Wang G, Wei W, Tu Y, Tong H and Sun S. An autophagy inhibitor enhances the inhibition of cell proliferation induced by a proteasome inhibitor in MCF-7 cells. Mol Med Report 2012, 5: 84–88.
37. Filippi-Chiela EC, Villodre ES, Zamin LL and Lenz G. Autophagy interplay with apoptosis and cell cycle regulation in the growth inhibiting effect of resveratrol in glioma cells. PLoS ONE 2011, 6: e20849.
38. Kaminskyy VO, Piskunova T, Zborovskaya IB, Tchevkina EM and Zhivotovsky B. Suppression of basal autophagy reduces lung cancer cell proliferation and enhances caspase-dependent and -independent apoptosis by stimulating ROS formation. Autophagy 2012, 8: 1032–1044.
39. Wang BS, Liu YZ, Yang Y, Zhang Y, Hao JJ, Yang H and Wang XM, *et al*. Autophagy negatively regulates cancer cell proliferation via selectively targeting VPRBP. Clin Sci 2013, 124: 203–214.
40. Kirkland RA, Adibhatla RM, Hatcher JF and Franklin JL. Loss of cardiolipin and mitochondria during programmed neuronal death: evidence of a

role for lipid peroxidation and autophagy. Neuroscience 2002, 115: 587–602.
41. Yu L, Wan F, Dutta S, Welsh S, Liu Z, Freundt E and Baehrecke EH, *et al*. Autophagic programmed cell death by selective catalase degradation. Proc Natl Acad Sci USA 2006, 103: 4952–4957.
42. Choi CH, Jung YK and Oh SH. Selective induction of catalase-mediated autophagy by dihydrocapsaicin in lung cell lines. Free Radic Biol Med 2010, 49: 245–257.
43. Li L, Li F, Li F, Mao X, Yang L, Huang H and Guo Y, *et al*. NOX4-derived reactive oxygen species drive apelin-13-induced vascular smooth muscle cell proliferation via the ERK pathway. Int J Pept Res Ther 2011, 17: 307–315.
44. Xie F, Liu W and Chen L. The progress of autophagy involved in heart disease. Prog Biochem Biophys 2012, 39: 224–233.
45. Xiao L, Li L and Chen L. Research of the autophagy in vessel function and related diseases. Prog Biochem Biophys 2013, 40: 1177–1185.
46. Xie F, Li L and Chen L. Autophagy, a new target for disease treatment. Sci China Life Sci 2013, 56: 856–860.
47. McKenzie JA, Fruttiger M, Abraham S, Lange CA, Stone J, Gandhi P and Wang X, *et al*. Apelin is required for non-neovascular remodeling in the retina. Am J Pathol 2012, 180: 399–409.
48. Efrati S, Berman S, Goldfinger N, Erez N, Averbukh Z, Golik A and Rotter V, *et al*. Enhanced angiotensin II production by renal mesangium is responsible for apoptosis/proliferation of endothelial and epithelial cells in a model of malignant hypertension. J Hypertens 2007, 25: 1041–1052.
49. Redondo S, Ruiz E, Padilla E, Gordillo-Moscoso A and Tejerina T. Role of TGF-beta1 in vascular smooth muscle cell apoptosis induced by angiotensin II. Eur J Pharmacol 2007, 556: 36–44.
50. Park SH, Kim JH, Chi GY, Kim GY, Chang YC, Moon SK and Nam SW, *et al*. Induction of apoptosis and autophagy by sodium selenite in A549 human lung carcinoma cells through generation of reactive oxygen species. Toxicol Lett 2012, 212: 252–261.
51. Kawamoto S, Iemura N, Inoue Y, Katakura Y and Shirahata S. Effect of 14-3-3 protein induction on cell proliferation of A549 human lung adenocarcinoma. Cytotechnology 2000, 33: 253–257.
52. Pan W, Li F, Mao X, Qin X, Deng Sh, Feng F and Chen F, *et al*. 14-3-3 protein is involved in ERK1/2 signaling transduction pathway of rat vascular smooth muscle cells proliferation induced by apelin-13. Prog Biochem Biophys 2011, 38: 1153–1161.
53. Lv D, Lu Q, Cao J and Chen L. Unanticipated role of apelin: regulation of miRNA generation. Acta Biochim Biophys Sin 2013, 45: 896–898.