

Original Research

The Effect of Liraglutide on Lung Cancer and Its Potential Protective Effect on High Glucose-Induced Lung Senescence and Oxidative Damage

Zhiyan Pu¹, Yanxia Yang¹, Shuanghong Qin¹, Xiaojuan Li¹, Can Cui², Weiyu Chen^{1,*}

¹Department of Endocrinology, The Second People's Hospital of Gansu Province, Northwest Minzu University, 730000 Lanzhou, Gansu, China

²Department of Endocrinology, Second Affiliated Hospital of Harbin Medical University, 150086 Harbin, Heilongjiang, China

*Correspondence: weiyuchen8109@163.com (Weiyu Chen)

Academic Editor: Peter Brenneisen

Submitted: 3 September 2022 Revised: 27 March 2023 Accepted: 10 April 2023 Published: 24 October 2023

Abstract

Background: Lung cancer is a malignant disease with high morbidity and mortality. Lung cancer and diabetes are closely related, and diabetic patients with lung tumors are common in clinical practice. Liraglutide, a glucagon-like peptide-1 receptor (GLP-1R) agonist, is commonly used in the treatment of type 2 diabetes. In this study, we examined the effect of liraglutide on lung cancer and its potential protective effect on high glucose-induced lung aging. Methods: Indirect mmunofluorescence was done to assess the expression levels of p-AKT, ki67, Caspase3, Bax and PI3K. Western blotting was conducted to determine the expression levels of BAX, BCL2, Caspase9, Ecadherin, N-cadherin, PI3K, AKT and vimentin. Cell viability, cell cycle and cell apoptosis were evaluated by colony formation, CCK-8 assay and flow cytometry. Immunohistochemistry was performed to evaluate the expression of Nf-kb, p15, p16, p21 and SMA in vivo. Besides, a high glucose-induced lung cell injury model was established to evaluate the effect of liraglutide on lung aging and oxidative damage. Sa-\beta-gal staining was used to assess cellular/ tissue senescence. Cell senescence-related markers (p16, p21 and p53) were determined by Western-blot analysis. Results: The proliferation, cell cycle, migration of lung cancer cells were significantly inhibited after treatment with liraglutide compared to control group (p < 0.05). Furthermore, Liraglutide inhibited the epithelial-mesenchymal transition process of lung cancer cell compared to control group (p < 0.05). Liraglutide also suppressed the proliferation of lung cancer in vivo. Besides, the BEAS-2B cell senescence induced by high glucose was significantly alleviated after treatment with liraglutide compared with control group (p < 0.05). The lung aging and endoplasmic reticulum stress was significantly suppressed after liraglutide treatment. Conclusions: This work indicates that liraglutide could inhibit lung cancer cell proliferation in vitro and in vivo. In addition, liraglutide exhibited anti-aging effects in vivo and in vivo. The current work has important implications for the treatment of patients with diabetes and lung cancer.

Keywords: liraglutide; lung cancer; proliferation; aging; lung cells

1. Introduction

In China, lung cancer is a malignant disease with high morbidity and mortality, which is exacerbated by the fact that making an early diagnosis is difficult [1], with about 70% of patients having advanced disease at the time of diagnosis. Lung cancer is mainly divided into two types: non-small cell lung cancer (NSCLC) and small cell lung cancer [1]. About 80% of all lung cancer patients have NSCLC, which is currently the focus of lung cancer prevention and treatment [2]. Radiotherapy and chemotherapy do not significantly improve the survival of patients with advanced lung cancer, and the lung cancer mortality rate is still very high [2]. Although platinum-based single or combined chemotherapy drugs are widely used in the treatment of lung cancer, their side effects include nephrotoxicity, bone marrow suppression, and gastrointestinal reactions [3,4]. Therefore, the search for new lung cancer drugs is crucial.

Glucagon-like peptide-1 receptor (GLP-1R) agonist is a new type of widely used hypoglycemic drug [5]. There are two types of GLP-1R agonists commonly used in clinical practice. One is a short-acting GLP-1 receptor agonist such as exenatide and another is a long-acting GLP-1R agonist such as liraglutide [6], which is highly stable. Liraglutide is an effective hypoglycemic agent, and its safe and convenient use is increasingly favored by diabetic patients. GLP-1 is an incretin hormone secreted by the gut; the continuous use of GLP-1R agonists can induce the occurrence and development of tumors such as colorectal cancer. Koehler et al. [7] have found that the GLP-1R agonist exenatide can inhibit tumor cell proliferation and induce increased apoptosis in mouse colon cancer cells. It was also shown by Ligumsky et al. [8] to inhibit the proliferation of breast cancer cells, but it was not dependent on the expression of GLP-1R. Zhao et al. [9] found that the GLP-1 receptor agonist liraglutide could inhibit the proliferation of pancreatic cancer cells and promote cancer cell apoptosis. Similarly, Müller et al. [10] found that for human prostate

Copyright: © 2023 The Author(s). Published by IMR Press. This is an open access article under the CC BY 4.0 license.

Publisher's Note: IMR Press stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

cancer cells with GLP-1R expression, the GLP-1R agonist exenatide could inhibit the proliferation of human prostate cancer cells by L cells in the small intestine.

Under physiological conditions, GLP-1 is rapidly digested and degraded by DPP4 (dipeptidyl peptidase-4) within about 1-2 minutes (half-life), which results in low plasma concentrations. GLP-1 inhibits gastric emptying, increases anorexia, reduces body weight, and increases the sensitivity of surrounding tissues to insulin [10]. The physiological role of GLP-1 is mainly mediated through activation of GLP-1R. Studies have shown that GLP-1Rs are widely distributed in a variety of cells and tissues, including islet beta cells, brain, heart, smooth muscle, as well as others [5]. Binding of GLP-1 to GLP-1R can activate the protein kinase A signaling pathway as well as the calmodulin pathway and the mitogen-activated protein kinase and phosphatidylinositol 3 kinase signaling pathways, which are also involved in Wsnt signaling. Although most clinical research data show that GLP-1 depends on the expression of GLP-1R for its physiological role, it has been reported that GLP-1 can induce hepatic glycogen production when it acts on hepatocytes and skeletal muscle cells, even though there is no GLP-1R expression on the surface of these cells [11,12]. Furthermore, Montrose-Rafizadeh et al. [13] found that GLP-1 can induce the physiological effects of insulinoma when it acts on 3T3-L1 adipocytes, even in the absence of GLP-1R expression on these cells. These experimental results show that GLP-1 can still exert physiological effects when it acts on GLP-1R-negative cells. One possible reason is the existence of GLP-1 alternate receptors on the surface of these cells. Therefore, for GLP-1Rnegative cells, in the presence of GLP-1 alternate receptor expression, GLP-1 will still have a physiological effect on these cells. Previous studies have found that in patients with diabetes, the incidence of tumors is higher, indicating that the two are related. Perfetti et al. [14] showed that GLP-1R agonists can activate the Wnt signaling pathway in islet β cells, thereby promoting the proliferation of pancreatic β cells, which may lead to an increased risk of pancreatitis and even pancreatic cancer. The Wnt signaling pathway has also been shown to be closely related to colorectal cancer [14]. Therefore, Sun et al. [15] hypothesized that long-term use of GLP-1R agonists in diabetic patients with high risk factors for colorectal cancer may lead to the development of colorectal cancer. However, in contrast, Kakkar et al. [16] suggested that GLP-1R agonists can selectively activate the Wnt signaling pathway in pancreatic β cells but not in colorectal cancer cells; hence, GLP-1R agonists may not lead to proliferation of these cells. Therefore, further studies are needed to confirm that this biological behavior occurs and that it depends on the expression of the GLP-1R [17]. He et al. [18] found that the GLP-1R agonist exenatide could inhibit the proliferation, migration, and invasion of human ovarian cancer cells by acting on the PI3K/Akt signaling pathway and promote their apoptosis. However, thus far

it is unclear whether liraglutide has an inhibitory effect on lung cancer. Until recently, a series of new GLPIR agonists have been developed and showed promising application prospects [19,20].

In the current study, we evaluated the effect of liraglutide on lung cancer. First, we found that liraglutide could inhibit lung cancer cell proliferation and induce lung cancer cell apoptosis. This has important significance for the treatment of patients with diabetes and lung cancer. On this basis, we further evaluated the effect of liraglutide on high glucose-induced lung aging and oxidative damage and found that liraglutide could protect the lung from aging and endoplasmic reticulum stress (ERS).

2. Materials and Methods

2.1 Antibodies and Reagents

Liraglutide was from Novo Nordisk A/S (China). The Annexin V-FITC apoptosis detection kit and RIPA lysate were purchased from Beyotime Biotechnology Co., Ltd (Shanghai, China). The BCA protein concentration assay kit was purchased from Shanghai Biyuntian Biotechnology Co., Ltd (Shanghai, China). Lung normal epithelial cells (BEAS-2B, CRL-9609) were purchased from ATCC. DMEM high glucose was purchased from Gibco (California USA). PVDF membranes were purchased from Bio-Rad (Shanghai, China). The SDS-PAGE gel preparation kit was purchased from China Salorbio Biotechnology Co., Ltd (Beijing, China). The hypersensitive ECL chemiluminescence kit was purchased from Salorbio Biotechnology Co., Ltd (Beijing, China). The penicillin-streptomycin solution (100 \times) was purchased from Amresco (Shanghai, China). The rabbit anti-human caspase-3 monoclonal antibody and the rabbit anti-human caspase-9 monoclonal antibody were purchased from Abcam (Cambridge, UK). Cell culture dishes were purchased from Corning Company (New York, USA). Fetal bovine serum (FBS) was purchased from Thermo Fisher Scientific (Shanghai, China). Details of the antibodies used in the current study are shown in Supplementary Table 1. Diabetic mice were purchased from Cyagen company (Guanzhou, China). The experimental protocol was approved by the Institutional Animal Care and Use Committee of Northwest Minzu University (IACUC-20210215).

2.2 Cell Culture

A549 (SCSP-503), H1299 (Catalogue number TCHu160) and BEAS-2B were purchased from National Collection of Autheticated cell cultures (Shanghai, China). H1299 and A549 cells were cultured in DMEM+10% FBS and placed in a 37 °C, 5% CO₂ incubator. In addition, mycoplasma testing has been done for the A549, H1299 and BEAS-2B. A549, H1299 and BEAS-2B have been authenticated by STR and sequencing.

2.3 Western Blot Analysis

Lung cancer cells were collected after liraglutide treatment. Proteins in cell samples were measured using the BCA protein kit (Beyotime). The protein samples were separated using SDS-PAGE and transferred to PVDF membranes. PVDF membranes were incubated in 5% skimmed milk solution for 1 h. Membranes were washed 3 times with TBST (5 min each time) and then incubated with diluted primary antibodies at 4 °C overnight. The membrane was then washed 3 times with TBST (5 min each time) and incubated with secondary antibodies for 2 h at room temperature. After washing three times with TBST (10 min each time), ECL was added to detect the immunoprotein bands.

2.4 Flow Cytometry

Cellular apoptosis was detected using an apoptosis detection kit (BD Bioscience, Bedford, MA, USA) according to the manufacturer's instructions. Briefly, the cells were digested with trypsin, then washed twice with PBS, and subsequently mixed in the binding buffer. Then 5 μ L Annexin and 5 μ L propidium iodide were added to the cell suspension and incubated at room temperature for 15 min (in the dark). Cell samples were analyzed using flow cytometry.

2.5 Cell Viability Assay

Cells were seeded in 96-well plates at a density of 6000 cells per well. After 24 h, cells were treated with different concentrations of liraglutide for 72 h. Cell viability was determined using the MTT kit (Abcam, Shanghai, China) according to the manufacturer's instructions.

2.6 Xenograft Tumor Experiment in Nude Mice

 5×10^6 A549 cells in 0.2 mL PBS were suspended in Matrigel and injected subcutaneously into BALB/c nude mice (female nu/nu athymic nude, 4–6 weeks, n = 6 mice/group), which were purchased from Huafukang Laboratory Animals Ltd (Beijing, China). One week later, mice were treated with liraglutide, and tumor volume was measured for 4 weeks. The tumor volume was measured every 3 days, and the tumor volume was calculated using the following formula: volume = (length × width²) × 0.5. Mice were then sacrificed by cervical dislocation, and tumors were harvested and analyzed accordingly.

2.7 CCK8 Assay

Cells in logarithmic growth were trypsinized and harvested using centrifugation. Cells were then resuspended and added to 96-well plates (2×10^4 cells/well), which were then placed in a constant temperature cell incubator at 37 °C for 12 h. After the cells adhered, liraglutide was added (final drug concentrations were 10, 100, and 1000 nmol/L), and then the 96-well plates were placed in a 37 °C constant temperature cell incubator for 24 h, 48 h, 72 h, and 96 h. After the cell culture plates were washed, 90 µL



of cell culture medium and 10 μ L of CCK-8 reagent were added to each well, and after culturing in a constant temperature cell incubator at 37 °C for 2 h, the absorbance value (OD value) was measured and recorded using a microplate reader at a wavelength of 450 nm. The cell proliferation rate was then calculated as follows: cell proliferation rate = (drug group-blank)/(control group-blank).

2.8 Colony Formation Experiments

Lung cancer cells were inoculated into 6-well culture plates, and control plates and drug-added plates were established. Then liraglutide was added (150 nmol/L), and the 6-well plates were placed in a 37 °C cell incubator for 14 days, while the culture medium was changed regularly. The culture was terminated when cell clones could be observed, and then the number of cell clones were counted from images taken after crystal violet staining.

2.9 High Glucose-Induced Lung Cell Senescence Injury Model

For the long-term continuous exposure to glucose, BEAS-2B cells were cultured in 6-well plates and stimulated with 5 mM glucose (normal glucose) or 25 mM glucose (high glucose). All BEAS-2B cells were propagated and maintained in the same treatment conditions. During cell passage, the cells were collected for senescenceassociated β -galactosidase (SA- β -gal) staining and other experimental analyses. All experiments were performed with six biological replicates.

2.10 Immunohistochemistry

We detected the expression of lung tissue-related proteins using immunohistochemistry. Mouse lung tissues were cut into 0.5 cm \times 0.5 cm pieces, fixed in 4% paraformaldehyde for 24 h, dehydrated with conventional gradient alcohol, embedded in paraffin, and sectioned (4 µm). Sections were attached to polylysine-treated slides, incubated overnight at 60 °C, and then stored in a 4 °C freezer. After the sections were removed and treated with alcohol, a drop (~50 µL) of 3% hydrogen peroxide was added to each section and incubated at room temperature for 15-20 min to block endogenous peroxidase. The sections were rinsed with distilled water 3 times (5 min each time). Then non-immunized animal serum was added to the sections and incubated at room temperature for 60 min to block non-specific antigens. After washing, primary and secondary antibodies were added to the sections sequentially. The sections were then observed and imaged under the microscope. Photos were analyzed using image J software v1.8.0 (developed by the National Institutes of Health (NIH), Bethesda, MD, USA). In addition, the same method was used to detect xenograft tumor samples and lung tissues of diabetic mice.

2.11 HE Staining

The isolated tissues were fixed into 4% formalin for 48 h, and then the tissues were immersed in alcohol after dehydration through a graded ethanol series. After paraffin embedding and dewaxing, H&E staining was performed.

2.12 β -gal Staining

SA- β -gal activity is one of the biomarkers for identifying cellular senescence, which was used to test whether the cell senescence model was successfully established. First, cells were seeded into 6-well plates and the cells were cultured to 80% confluence. After three washes with PBS, paraformaldehyde and glutaraldehyde were added to fix the cells at room temperature for 5–15 min. The fixative was then discarded, the cells were washed three times with PBS, and then β -galactosidase staining solution was added to each well and incubated at 37 °C for different time points. When some cells turned blue as seen under the microscope, cell culture dishes were examined, and cells were counted (500–1000 cells were counted).

2.13 Indirect Immunofluorescence

Tissue sections were blocked with 5% BSA for 30 min. The blocking solution was discarded, the primary antibody was then added and placed in a wet box overnight at 4 °C. Sections were washed three times (5 min each), secondary antibodies were added to cover the tissue and incubated for 60 min at room temperature. After washing, DAPI solution was added and incubated for 5 min at room temperature. Sections were sealed with resin. Then laser confocal microscopy was performed to observe samples using an Olympus FV-3000 confocal laser-scanning microscope (DAPI: excitation wavelength: 330–380 nm, emission wavelength 420 nm, blue; Alexa Fluor 488: excitation wavelength 495 nm, emission wavelength 519 nm, green; DyLight 594: excitation wavelength 593 nm, emission wavelength 618 nm, red).

2.14 Detection of the Mitochondrial Membrane Potential

After liraglutide treatment of the cells, 1×10^5 cells were extracted and re-suspended in 0.5 mL of cell culture medium. 0.5 mL of JC-1 staining working solution was added and mixed. The cells were incubated for 20 min at 37 °C in a cell incubator. Then JC-1 staining buffer was added and incubated at 37 °C according to the kit's protocol. After cell incubation, cells were centrifuged at 600 ×g for 3–4 min at 4 °C to precipitate cells. The samples were assayed by Flow cytometry (BD FACSCanto II).

2.15 Statistical Analysis

Statistical analysis was performed on the experimental data using SPSS21.0 software (IBM Corp., Chicago, IL, USA). The data were represented by the mean \pm standard deviation. Multiple samples were compared using one-way ANOVA, and the two groups were compared using the t-test. A p-value less than 0.05 was considered statistically significant.



Fig. 1. Liraglutide inhibited the proliferation of lung cancer cells in A549 and H1299 cell. (A) Effect of different concentrations of liraglutide (10 nmol/L–1000 nmol/L) on the proliferation of lung cancer cells (A549 and H1299). Lung cancer cells were treated with liraglutide for 48 h. (B) Effect of Liraglutide (150 nmol/L of liraglutide) on the proliferation of lung cancer cells. The cells were treated with Liraglutide (150 nmol/L) at 24–96 h. The proliferation of lung cancer cells was detected by CCK8 Kit according to the manufacturer's instructions. The absorbance value (OD value) was measured and recorded using a microplate reader at a wavelength of 450 nm. Data were presented as mean \pm SD. n = 3, **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

3. Results and Discussion

3.1 Analysis of the Inhibitory Effect of Liraglutide on Human Lung Cancer Cells

A CCK-8 kit was used to assess the effect of liraglutide on the proliferation of human lung tumor cells (H1299 and A549). Liraglutide, at increasing concentrations (10 nmol/L, 50 nmol/L, 100 nmol/L, 200 nmol/L, 500 nmol/L, and 1000 nmol/L) inhibited the proliferation of human lung cancer cells (A549 and H1299) (Fig. 1A). The IC50s of liraglutide were 146.4 nmol/L for A549 cells and 122.9 nmol/L for H1299 cells. Given these results, we selected 150 nmol/L of liraglutide for subsequent experiments. We also assessed the inhibitory effect of liraglutide on the proliferation of lung cancer cells at different time points (24 h, 48 h, 72 h, and 96 h). The results showed that compared with the controls, liraglutide was able to inhibit the proliferation of lung cancer cells at different time points (p < 0.05), indicating that liraglutide had an inhibitory effect on human lung cancer cells (Fig. 1B).



3.2 The Effect of Liraglutide on the Clone Formation Ability of Lung Cancer Cell

The results of the colony formation assay showed that after liraglutide was added to A549 and H1299 cells for 14 days, the number of lung cancer tumor cell colonies in the liraglutide treatment group was significantly reduced compared with the control group (p < 0.05) (Fig. 2A).

In addition, we examined the effect of liraglutide on the migration ability of human lung tumor cells (A549 and H1299). The results showed that after liraglutide (150 nmol/L) was added to the cells for 48 h, the migration of liraglutide-treated tumor cells was significantly reduced compared with that of the non-treated control cells (p <0.05) (Fig. 2B). These findings showed that liraglutide inhibited the migration ability of lung cancer cells.



Fig. 2. Liraglutide inhibited the proliferation and migration of lung cancer cells. (A) Effect of Liraglutide (150 nmol/L) on the colony formation of lung cancer cells. Lung cancer cells were inoculated into 6-well culture plates. Liraglutide was then added (150 nmol/L) and placed in a 37 °C cell incubator for 14 days. The number of cell clones were counted from images taken after crystal violet staining. (B) Liraglutide inhibited the migration of lung cancer cells (A549 and H1299). Data were presented as mean \pm SD. n = 3, *p < 0.05.

IMR Press

3.3 The Effect of Liraglutide on Apoptosis of Human Lung Cancer Cells Determined by Flow Cytometry

The effect of liraglutide on lung cancer cells was assessed using flow cytometry. The results showed that liraglutide induced apoptosis in H1299 and A549 cells (p < 0.05) (Fig. 3A). Furthermore, mitochondrial membrane potentials were also significantly downregulated after liraglutide treatment (Fig. 3B). Flow analysis showed that liraglutide induced apoptosis in lung cancer cells. We further analyzed the effect of liraglutide on apoptosis-related signaling proteins. Western blot results showed that BCL2 levels decreased after liraglutide treatment, while those of Bax, caspase-3, caspase-9 and and Cytochrome c (Cyt-C) increased (Fig. 3C).

3.4 The Effect of Liraglutide on Cell Cycle of Lung Cancer Cell

We analyzed the effect of liraglutide on lung cancer cells to determine whether the inhibitory effect of liraglutide on lung cancer cell growth was associated with cell cycle arrest. We performed cell cycle assays on liraglutidetreated cells using flow cytometry. Cells were stained with propidium iodide after treatment, and cell cycle distribution was detected using flow cytometry. The results are shown in Fig. 4. The cell cycle distribution in the A549 control cells was: G1: 64 \pm 2.5%; S phase: 25 \pm 1.3%; and G2 phase: $11 \pm 1.7\%$. The cell cycle distribution in the liraglutide-treated A549 cells was: G1: 83 \pm 3.5%; S phase: $9 \pm 1.5\%$; and G2 phase: $8 \pm 0.9\%$. The cell cycle distribution in the H1299 control cells was G1: 66 \pm 3.2%; S phase: 22 \pm 1.9%; and G2 phase: 12 \pm 0.8%. The cell cycle distribution in the liraglutide-treated H1299 cells was: $81 \pm 3.3\%$; S phase: $13 \pm 1.6\%$; and G2 phase: $7 \pm 1.2\%$. These results showed that the number of cells entering S phase was significantly reduced in the liraglutide-treated groups, indicating that liraglutide inhibited lung cancer cells from going from the G0/G1 phase to the S phase and hence blocked the cell cycle of lung cancer cells within the G0/G1 phase, inhibiting lung cancer cell proliferation.

3.5 Liraglutide Inhibited the EMT Process of Lung Cancer Cells

EMT is generally considered to be an important factor leading to the invasion and migration of cancer cells during tumorigenesis and development [19]. Therefore, we evaluated the effect of liraglutide on the EMT process in lung cancer cells. To this end, 150 nmol/L of liraglutide was added to the medium of A549 cell cultures for 24 h, and western blots were used to detect the cellular expression of EMT-related markers. It was found that liraglutide could significantly reduce the expression of mesenchymal cell markers N-cadherin and vimentin and increase the expression of the epithelial marker E-cadherin (Fig. 5A), Similar results were obtained with the small cell lung cancer



Fig. 3. Liraglutide (150 nmol/L) treatment increased the apoptosis rate of lung cancer cells. (A) Effect of liraglutide on the apoptosis of lung cancer cells. The cells were digested with trypsin, then washed twice with PBS. Then 5 μ L Annexin and 5 μ L propidium iodide were added to the cell suspension and incubated at room temperature for 15 min (in the dark). Cell samples were analyzed using Flow cytometry. (B) Effect of liraglutide treatment on Mitochondrial membrane potential. (C) Effect of liraglutide on the expression of apoptosis-related molecules (Bax, Caspase-3, Caspase-9 and Cytochrome c). Lung cancer cells were collected after liraglutide treatment. The protein samples were separated using SDS-PAGE and transferred to PVDF membranes. PVDF membranes were incubated in 5% skimmed milk solution for 1 h. After washing, the membranes were incubated with diluted primary antibodies at 4 °C overnight. The membrane was then washed 3 times with TBST and incubated with secondary antibodies for 2 h at room temperature. After washing, ECL was added to detect the immunoprotein bands. Data were presented as mean \pm SD. n = 3, *p < 0.05.

line H1299 (Fig. 5B). These results indicated that liraglutide can inhibit the EMT process of lung cancer cells. Further study found that liraglutide treatment decreased the level of PI3K/AKT phosphorylation (Fig. 5C). These results suggested that liraglutide could inhibit the PI3K/AKT signaling pathway. To further clarify whether the inhibitory effect of liraglutide on EMT was related to the PI3K/AKT signaling pathway, lung cancer cells were treated with liraglutide and 20 μ M 1,3-dicaffeoylquinic acid, a PI3K agonist. Western blotting showed that the PI3K agonist could reverse the liraglutide-induced increase in E-cadherin levels and decrease N-cadherin and vimentin levels (Fig. 5D), indicating that activation of the PI3K/AKT signaling pathway is the key to the EMT process and that liraglutide suppressed the EMT process through the PI3K/AKT pathway.

3.6 Liraglutide Exhibited Antitumor Effect in Vivo

To explore whether liraglutide could also inhibit the growth of NSCLC *in vivo*, a lung cancer xenograft model in nude mice was established. BALB/c nude mice were divided into two groups: a control group and a liraglutide-treated group (six nude mice in each group). The mice were subcutaneously injected with lung cancer A549 cells (1 \times 10⁷ A549 cells per mouse) to establish the xenograft model. Tumor size was measured every 48 h. The mice were treated with Liraglutide (200 µg/kg s.c.) once a day, and



H1299



Fig. 4. The effect of liraglutide (150 nmol/L of liraglutide) on cell cycle in the lung cancer. The cells were digested by using trypsin, the lung cancer cells were collected by centrifugation. After washing, the cells were treated with RNaseA solution for 20 min. The propidium iodide (PI) staining solution was assdded, and incubated at 37 °C for 30 min. The cell samples were analyzed by using Flow cytometry. Histogram represents the percentage of cell cycle (G1, S and G2). Asterisks indicate significant differences compared with control group. Data were presented as mean \pm SD. n = 3, *p < 0.05. Three independent experiments were conducted.

nude mice were sacrificed on day 30. The results showed that compared with the control group, the liraglutide-treated group had slower tumor growth and smaller tumor volume (p < 0.05) (Fig. 6A). In addition, the expression of apoptosis-related signaling proteins was significantly downregulated in the liraglutide-treated group (Fig. 6B–D).

3.7 Protective Effect of Liraglutide on High Glucose-Induced Lung Cell Damage

In the above studies, we found that liraglutide exhibited a certain potential anti-lung cancer effect. In this part of the study, we evaluated the protective effects of liraglutide on the lung. We established a high glucose-induced lung cell injury model using BEAS-2B cells. We estab-

i IMR Press

lished three different glucose cell groups: a control cell group, a high glucose cell group, and a high glucose + liraglutide cell group. The results showed that in the highglucose group, BEAS-2B cells were senescent using SA- β gal staining (Fig. 7A). Furthermore, the expression of P53, P21, and P16 were also significantly upregulated in the high glucose group (Fig. 7B). Flow cytometry showed that high glucose did not induce significant cell apoptosis (Fig. 7C). However, in the liraglutide group, SA- β -gal staining was significantly downregulated. Furthermore, P53, P21, and P16 were significantly downregulated. Additionally, the cell cycle showed that the cell ratio of S phase was significantly elevated compared with controls (Fig. 7D).



Fig. 5. Liraglutide inhibited the EMT process of lung cancer cells. (A) Effect of liraglutide on N-cadherin, vimentin and E-cadherin in the A549 cell. Data were presented as mean \pm SD. n = 3. *p < 0.05. (B) Effect of Liraglutide on N-cadherin, vimentin and E-cadherin in the H1299 cell. Lung cancer cells were collected after liraglutide treatment. The protein samples were separated using SDS-PAGE and transferred to PVDF membranes. PVDF membranes were incubated in 5% skimmed milk solution for 1 h. After washing, the membranes were incubated with diluted primary antibodies at 4 °C overnight. The membrane was then washed 3 times with TBST and incubated with secondary antibodies for 2 h at room temperature. After washing, ECL was added to detect the immunoprotein bands. Data were presented as mean \pm SD. n = 3. *p < 0.05. (C) Liraglutide treatment decreased the phosphorylation level of PI3K/AKT. *p < 0.05. (D) Liraglutide suppressed EMT process through the PI3 and AKT pathway. Data were presented as mean \pm SD. n = 3. Different letters indicate significant differences among group.



Fig. 6. The effect of liraglutide on tumor growth *in vivo*. (A) Liraglutide treatment inhibited the proliferation of lung cancer *in vivo*. Mice were treated with liraglutide, and tumor volume was measured for 4 weeks. (B) The effect of Liraglutide treatment on the expression of Bax and Ki67. Tissue sections were blocked with 5% BSA for 30 min. The primary antibody was then added and placed in a wet box overnight at 4 °C. After washing, secondary antibodies were added to cover the tissue and incubated for 60 min at room temperature. Then laser confocal microscopy was performed to observe samples using an Olympus FV-3000 confocal laser-scanning microscope (DAPI: excitation wavelength: 330–380 nm, emission wavelength 420 nm, blue; Alexa Fluor 488: excitation wavelength 495 nm, emission wavelength 519 nm, green; DyLight 594: excitation wavelength 593 nm, emission wavelength 618 nm, red). Mean fluorescence intensity of Bax and Ki67 was measured using Image J software (right penal). (C) Caspase3 was down-regulated in the liraglutide-treated group. Mean fluorescence intensity of Caspase3 was determined using Image J software (right penal). (D) Liraglutide treatment down-regulated p-PI3K and p-AKT expression. Mean fluorescence intensity of P-PI3K was determined using Image J software. Data were presented as mean \pm SD. n = 6 mice. ns, Not significant. *p < 0.05, ***p < 0.001.

Additionally, high glucose also caused ERS in BEAS-2B cells. As shown in Fig. 8A, the level of reactive oxygen species (ROS) was significantly increased in the high glucose group. The expression of ERS-related markers GRP78 and CHOP was significantly increased in the high glucose group, while the expression of ERS-related markers was significantly downregulated in the liraglutide treatment group (Fig. 8B). In addition, the expression of unfolded protein response-related molecules (XPB1, ATF6, and IRE1) were also significantly increased in the BEAS-2B group. However, the ubiquitin–proteasome system (UPS)-related signaling molecules (including XPB1, ATF6 and IRE1) were significantly decreased in the liraglutide treatment group (Fig. 8C). These findings showed that liraglutide can significantly alleviate the ERS caused by high glucose. The above studies evaluated the effect of liraglutide on lung cell damage *in vitro*. Therefore, in a diabetes mouse model, we further evaluated the effect of liraglutide on the lungs of diabetic mice. The mice were treated with Liraglutide (200 µg/kg s.c.) once a day (4 weeks). SA- β -gal staining of lungs showed that diabetes-induced lung aging was significantly alleviated by liraglutide (Fig. 9A). In addition, the aging-related markers P15, P16, and P21 were significantly downregulated in the liraglutide-treated group (Fig. 9B). Furthermore, the expression of the inflammationrelated signaling molecule NF- κ b was also significantly downregulated (Fig. 9C). Moreover, α -SMA (a fibrosisrelated marker) was also reduced in the liraglutide group (Fig. 9D). Lung injury was significantly mitigated by liraglutide treatment (Fig. 9E). To sum up, the current work



Fig. 7. Protective effect of liraglutide on high glucose-induced lung cell damage. (A) SA-β-gal staining of BEAS-2B cells. Cells were seeded into 6-well plates and the cells were cultured to 80% confluence. After three washes with PBS, paraformaldehyde and glutaraldehyde were added to fix the cells at room temperature for 5 min. The fixative was then discarded, the cells were washed three times with PBS, and then Sa-β-gal staining solution was added to each well and incubated at 37 °C for different time points. Bar = 50 µM. Data were presented as mean ± SD, n = 3, *p < 0.05. (B) Western blotting analysis of P53, P21 and P16 in the liraglutide treatment group. The protein samples were separated using SDS-PAGE and transferred to PVDF membranes. PVDF membranes were incubated in 5% skimmed milk solution for 1 h. After washing, the membranes were incubated with diluted primary antibodies at 4 °C overnight. The membrane was then washed 3 times with TBST and incubated with secondary antibodies for 2 h at room temperature. After washing, ECL was added to detect the immunoprotein bands. β-actin was used as the loading control. (C) Effect of high-glucose on cell apoptosis. (D) Effect of liraglutide on the cells were treated with RNaseA solution for 20 min. The propidium iodide (PI) staining solution was added, and incubated at 37 °C for 30 min. The cell samples were analyzed by using Flow cytometry. Histogram represents the percentage of cell cycle (G1, S and G2). Asterisks indicate significant differences compared with control group. Data were presented as mean ± SD. n = 3. ns, Not significant. *p < 0.05.



Fig. 8. High glucose caused ERS in BEAS-2B cells. (A) Effect of high glucose on ROS level. (B) Western blotting analysis of GRP78 and CHOP in the liraglutide treatment group. The protein samples were separated using SDS-PAGE and transferred to PVDF membranes. PVDF membranes were incubated in 5% skimmed milk solution for 1 h. After washing, the membranes were incubated with diluted primary antibodies at 4 °C overnight. The membrane was then washed 3 times with TBST and incubated with secondary antibodies for 2 h at room temperature. After washing, ECL was added to detect the immunoprotein bands. β -actin was used as the loading control. (C) Western blotting analysis of AFT6, IRE1 and XPB1 in the liraglutide treatment group. Data were presented as mean \pm SD, n = 3, **p* < 0.05. ROS, reactive oxygen species; PVDF, polyvinylidene fluoride; ECL, enhanced chemiluminescence; TBST, Tris Buffered Saline+Tween-20.

shows that liraglutide can significantly alleviate the lungaging damage caused by diabetes.

Studies have shown that diabetes is closely related to the incidence of lung cancer [21] and that there is a link between diabetes and lung cancer [22]. There are many diabetic patients who present with malignant tumors in clinical practice [23]. Therefore, we examined the effect of liraglutide on lung cancer and found that it has a potential antilung cancer effect. Moreover, we found that liraglutide had a protective effect on lung damage. In lung cancer patients with diabetes mellitus, an important scientific question to address is whether glucagon analogs or agonists (liraglutide) used in the treatment of diabetes have effects on tumors. In the current study, we found that liraglutide exhibited partial anti-lung cancer potential. However, previous studies on the effects of glucagon analogs or agonists on tumors have been conflicting. Studies have shown that GLP-1R agonists can activate the Wnt signaling pathway in islet β cells, thereby promoting their proliferation, which may lead to an increased risk of pancre-



Fig. 9. Effect of liraglutide on the lung tissues of diabetic mice. (A) SA- β -gal staining of the lung tissues from diabetes mice in liraglutide treatment group. Sa- β -galactosidase staining solution was added to lung tissues and incubated for 12 h. Bar = 50 μ M. Data were presented as mean \pm SD, n = 6, *p < 0.05. (B) Analysis of P15, P16 and P21 by IHC. The sections were rinsed with distilled water 3 times. Then non-immunized animal serum was added to the sections and incubated at room temperature for 60 min to block non-specific antigens. After washing, primary and secondary antibodies were added to the sections sequentially. The sections were then observed and imaged under the microscope. Photos were analyzed using image J software. (C) Analysis of Nf- κ b expression by IHC. (D) The α -SMA (Fibrosis-related marker) was reduced in the liraglutide treatment group. (E) Effect of liraglutide treatment on Lung injury. Data were presented as mean \pm SD, n = 6, *p < 0.05.

atitis and even pancreatic cancer [14]. The Wnt signaling pathway is also closely related to the occurrence and development of rectal cancer and other tumors. Therefore, Sun et al. [15] hypothesized that long-term use of GLP-1R agonists in diabetic patients may lead to the occurrence and development of colorectal cancer. In contrast, Kakkar et al. [16] surmised that GLP-1R agonists may selectively activate the Wnt signaling pathway in pancreatic β cells but not in colorectal cells, so GLP-1R agonists may not be involved in colorectal cancer. These findings suggest that further studies are needed to confirm whether continued use of GLP-1R agonists can induce the development of tumors such as colorectal cancer. Koehler et al. [7] found in in vitro studies that when the GLP-1R agonist exenatide was applied to CT26 mouse colon cancer cells, tumor cell proliferation was inhibited and cell apoptosis was increased. Similarly, Ligumsky et al. [8] showed that exenatide could inhibit the proliferation of breast cancer cells, but it did not depend on the expression of GLP-1R. This suggests that when GLP-1R agonists act on breast cancer cells, they may act through the alternate receptor of GLP-1 [8]. In addition, it has been reported that liraglutide could inhibit the proliferation of pancreatic cancer cells and promote cancer cell apoptosis [9]. Furthermore, exenatide could inhibit the proliferation, migration, and invasion of human ovarian cancer cells by acting on the PI3K/Akt signaling pathway. Exenatide exhibits its anti-tumor effects through interaction with GLP-1R. The study by Nomiyama *et al.* [17] also found that exenatide inhibited the proliferation of human prostate cancer cells, which depended on the expression of GLP-1R. However, Ligumsky *et al.* [8] found that exenatide suppressed the proliferation of breast cancer cells, which was not dependent on the expression of GLP-1R. In the current study, through a series of experiments, we found that liraglutide has anti-tumor activity, which could effectively inhibit the development of lung cancer.

Although existing clinical studies have shown that exenatide can inhibit the proliferation and migration of human pancreatic cancer, prostate cancer, breast cancer, and other tumor cells, these studies were mostly limited to investigating the effect of short-acting GLP-1R agonists on the biological behavior of tumor cells, while study of the long-acting GLP-1R agonist liraglutide has been limited. In our study, we explored the biological effect of liraglutide on human lung cancer cells. The results showed that liraglutide could significantly inhibit the migration of lung cancer cells. EMT is generally considered to be an important factor leading to the invasion and migration of cancer cells during tumorigenesis and development [24]. Therefore, we evaluated the effect of liraglutide on the EMT process of lung cancer cells, and results showed that it could inhibit the EMT process.

Diabetes can cause lung damage [25]. Diabetes is a chronic metabolic disease with complications that can affect various organs throughout the body. However, most of the attention has been given to the damage that diabetes causes to the heart, kidney, and eye, while the lung has been neglected, even though it is one of the target organs [26]. Therefore, we investigated whether liraglutide had a protective effect on the lung damage caused by hyperglycemia. We established a model of high glucose-induced lung cell injury and showed that high glucose induced the senescence of BEAS-2B cells and that liraglutide could significantly inhibit high glucose-induced lung cell damage. High glucose also caused ERS in BEAS-2B cells. The expression of ERS markers was significantly increased in the control cells, while in liraglutide-treated cells, the ERS-related protein expression was significantly downregulated. In addition, the expression of UPS-related molecules was also significantly increased in BEAS-2B control cells, while in the liraglutide-treated cells, the expression of UPS-related signaling molecules was significantly decreased. These results showed that liraglutide could significantly alleviate the ERS caused by high glucose.

The dose of liraglutide used in the current study was based on the following two considerations: (1) One is based on the findings of previous studies [27-30]; (2) The other one is based on our pre-experimental results. The concentration of liraglutide used in in vitro experiments varies depending on the cell type. There is no absolute standard dose. Another interesting phenomenon is that liraglutide induces apoptosis in tumor cells, but liraglutide protects normal somatic cells from senescence damage. In this work, Liraglutide indicated an anti-ageing effect on normal lung cells. In contrast, liraglutide exhibited a pro-apoptotic effect on the tumour model. They are not contradictory. Previous study has showed that Liraglutide can induce apoptosis in other types of tumours [31-33]. However, in normal somatic cells, liraglutide exhibits a protective effect [27–30]. In addition, the anti-ageing effect displayed by liraglutide in lung tissue can also be explained, our finding showed that liraglutide is able to reduce oxidative stress which is considered to be one of the important factors that induce aging. Of course, the deep molecular mechanism of anti-aging of liraglutide needs to be further revealed in future research.

4. Conclusions

We evaluated the effects of liraglutide on lung cell damage *in vitro* and on the lungs of diabetic mice. The results showed that aging of the lungs was alleviated by liraglutide. Therefore, we have shown that liraglutide can not only inhibit lung cancer but can also significantly alleviate lung damage.



Abbreviations

ERS, endoplasmic reticulum stress; FBS, Fetal Bovine Serum; NSCLC, non-small cell lung cancer; CLSM, Confocal Laser scanning microscopy; GLP-1R, Glucagon-like peptide-1 receptor; EMT, Epithelial-Mesenchymal Transition; PI3K, phosphatidylinositol 3 kinase; AKT, protein kinase B.

Availability of Data and Materials

Data are available from the corresponding author on reasonable request.

Author Contributions

Conceptualization, ZP, YY and WC; formal analysis, ZP and SQ; funding acquisition, WC; investigation, ZP and XL and CC; writing-original draft, ZP, WC and CC; writing-review and editing, ZP and WC. All authors contributed to editorial changes in the manuscript. All authors have read and agreed to the published version of the manuscript.

Ethics Approval and Consent to Participate

The experimental protocol was approved by the Institutional Animal Care and Use Committee of Northwest Minzu University (IACUC-20210215).

Acknowledgment

We thank Dr. Zhang Wei for technical assistance.

Funding

This work was supported by Gansu Province Science and Technology Program Project (2022) - Natural Science Foundation (22JR5RA738), and by Central Universities Basic Research Business Fund (2021) - Young Teachers Innovation Project (31920210047).

Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10. 31083/j.fbl2810259.

References

- Luo YH, Chiu CH, Scott Kuo CH, Chou TY, Yeh YC, Hsu HS, et al. Lung Cancer in Republic of China. Journal of Thoracic Oncology: Official Publication of the International Association for the Study of Lung Cancer. 2021; 16: 519–527.
- [2] Wu J, Gou F, Tan Y. A Staging Auxiliary Diagnosis Model for Nonsmall Cell Lung Cancer Based on the Intelligent Medical System. Computational and Mathematical Methods in Medicine. 2021; 2021: 6654946.
- [3] Yuan C, Huang M, Wang H, Jiang W, Su C, Zhou S. Pretreatment Fibrinogen-Albumin Ratio (FAR) Associated with Treat-

ment Response and Survival in Advanced Non-Small Cell Lung Cancer Patients Treated with First-Line Anti-PD-1 Therapy Plus Platinum-Based Combination Chemotherapy. Cancer Management and Research. 2022; 14: 377–386.

- [4] van den Boogaard WMC, Komninos DSJ, Vermeij WP. Chemotherapy Side-Effects: Not All DNA Damage Is Equal. Cancers. 2022; 14: 627.
- [5] Doggrell SA. Do glucagon-like peptide-1 receptor (GLP-1R) agonists have potential as adjuncts in the treatment of type 1 diabetes? Expert Opinion on Pharmacotherapy. 2018; 19: 1655– 1661.
- [6] Holst JJ. Long-acting glucagon-like peptide-1 receptor agoniststatus December 2018. Annals of Translational Medicine. 2019; 7: 83.
- [7] Koehler JA, Kain T, Drucker DJ. Glucagon-like peptide-1 receptor activation inhibits growth and augments apoptosis in murine CT26 colon cancer cells. Endocrinology. 2011; 152: 3362– 3372.
- [8] Ligumsky H, Wolf I, Israeli S, Haimsohn M, Ferber S, Karasik A, *et al.* The peptide-hormone glucagon-like peptide-1 activates cAMP and inhibits growth of breast cancer cells. Breast Cancer Research and Treatment. 2012; 132: 449–461.
- [9] Zhao H, Wei R, Wang L. Activation of glucagon-like peptid-1 receptor inhibits growth and promotes apoptosis of human pancreatic cancer cells in a cAMP-dependent manner. American Journal of Physiology-Endocrinology and Metabolism. 2014; 306: E1431–E1441.
- [10] Müller TD, Finan B, Bloom SR, D'Alessio D, Drucker DJ, Flatt PR, *et al.* Glucagon-like peptide 1 (GLP-1). Molecular Metabolism. 2019; 30: 72–130.
- [11] Villanueva-Peñacarrillo ML, Delgado E, Trapote MA, Alcántara A, Clemente F, Luque MA, *et al.* Glucagon-like peptide-1 binding to rat hepatic membranes. The Journal of Endocrinology. 1995; 146: 183–189.
- [12] Yang H, Egan JM, Wang Y, Moyes CD, Roth J, Montrose MH, et al. GLP-1 action in L6 myotubes is via a receptor different from the pancreatic GLP-1 receptor. The American Journal of Physiology. 1998; 275: C675–C683.
- [13] Montrose-Rafizadeh C, Yang H, Wang Y, Roth J, Montrose MH, Adams LG. Novel signal transduction and peptide specificity of glucagon-like peptide receptor in 3T3-L1 adipocytes. Journal of Cellular Physiology. 1997; 172: 275–283.
- [14] Perfetti R, Zhou J, Doyle ME, Egan JM. Glucagon-like peptide-1 induces cell proliferation and pancreatic-duodenum homeobox-1 expression and increases endocrine cell mass in the pancreas of old, glucose-intolerant rats. Endocrinology. 2000; 141: 4600– 4605.
- [15] Sun Y, Fan L, Meng J, Zhang F, Zhang D, Mei Q. Should GLP-1 receptor agonists be used with caution in high risk population for colorectal cancer? Medical Hypotheses. 2014; 82: 255–256.
- [16] Kakkar AK. GLP-1 receptor agonists and the risk of colorectal cancer. Medical Hypotheses. 2014; 82: 796.
- [17] Nomiyama T, Kawanami T, Irie S, Hamaguchi Y, Terawaki Y, Murase K, *et al*. Exendin-4, a GLP-1 receptor agonist, attenuates prostate cancer growth. Diabetes. 2014; 63: 3891–3905.
- [18] He W, Yu S, Wang L, He M, Cao X, Li Y, et al. Exendin-4 in-

hibits growth and augments apoptosis of ovarian cancer cells. Molecular and Cellular Endocrinology. 2016; 436: 240–249.

- [19] Girdhar K, Thakur S, Gaur P, Choubey A, Dogra S, Dehury B, et al. Design, synthesis, and biological evaluation of a small molecule oral agonist of the glucagon-like-peptide-1 receptor. The Journal of Biological Chemistry. 2022; 298: 101889.
- [20] Kim TH, Jung IH, Kim K, Lee B, Kim MK, Chae Y. 1403-P: DA-1726, a Balanced GLP1R/GCGR Dual Agonist, Effectively Controls Both Body Weight and Blood Glucose. Diabetes. 2022; 71: 1403-P.
- [21] Brabletz T, Kalluri R, Nieto MA, Weinberg RA. EMT in cancer. Nature Reviews. Cancer. 2018; 18: 128–134.
- [22] Leiter A, Charokopos A, Bailey S, Gallagher EJ, Hirsch FR, LeRoith D, *et al.* Assessing the association of diabetes with lung cancer risk. Translational Lung Cancer Research. 2021; 10: 4200–4208.
- [23] Yao L, Liu M, Huang Y, Wu K, Huang X, Zhao Y, et al. Metformin Use and Lung Cancer Risk in Diabetic Patients: A Systematic Review and Meta-Analysis. Disease Markers. 2019; 2019: 6230162.
- [24] Davis FM, Stewart TA, Thompson EW, Monteith GR. Targeting EMT in cancer: opportunities for pharmacological intervention. Trends in Pharmacological Sciences. 2014; 35: 479–488.
- [25] Khateeb J, Fuchs E, Khamaisi M. Diabetes and Lung Disease: A Neglected Relationship. The Review of Diabetic Studies: RDS. 2019; 15: 1–15.
- [26] Pitocco D, Fuso L, Conte EG, Zaccardi F, Condoluci C, Scavone G, et al. The diabetic lung–a new target organ? The Review of Diabetic Studies: RDS. 2012; 9: 23–35.
- [27] Kornelius E, Tsou SH, Chang CC, Ho YJ, Lin SC, Chen WL, et al. Liraglutide Attenuates Glucolipotoxicity-Induced RSC96 Schwann Cells' Inflammation and Dysfunction. Biomolecules. 2022; 12: 1338.
- [28] Song S, Guo R, Mehmood A, Zhang L, Yin B, Yuan C, et al. Liraglutide attenuate central nervous inflammation and demyelination through AMPK and pyroptosis-related NLRP3 pathway. CNS Neuroscience & Therapeutics. 2022; 28: 422–434.
- [29] Li K, Sun J, Huang N, Ma Y, Han F, Liu Y, et al. Liraglutide improves obesity-induced renal injury by alleviating uncoupling of the glomerular VEGF-NO axis in obese mice. Clinical and Experimental Pharmacology & Physiology. 2020; 47: 1978–1984.
- [30] Shao S, Nie M, Chen C, Chen X, Zhang M, Yuan G, et al. Protective action of liraglutide in beta cells under lipotoxic stress via PI3K/Akt/FoxO1 pathway. Journal of Cellular Biochemistry. 2014; 115: 1166–1175.
- [31] Lu R, Yang J, Wei R, Ke J, Tian Q, Yu F, *et al.* Synergistic antitumor effects of liraglutide with metformin on pancreatic cancer cells. PloS one. 2018; 13: e0198938.
- [32] Chequin A, Costa LE, de Campos FF, Moncada ADB, de Lima LTF, Sledz LR, *et al.* Antitumoral activity of liraglutide, a new DNMT inhibitor in breast cancer cells *in vitro* and *in vivo*. Chemico-biological Interactions. 2021; 349: 109641.
- [33] Zhu XX, Feng ZH, Liu LZ, Zhang Y. Liraglutide suppresses the proliferation of endometrial cancer cells through the adenosine 5'-monophosphate (AMP)-activated protein kinase signaling pathway. Chinese Medical Journal. 2021; 134: 576–578.