Liraglutide suppresses the metastasis of PANC-1 co-cultured with pancreatic stellate cells through modulating intracellular calcium content

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Abstract. In this study, we aim to explore the anti-tumor effect of liraglutide (Lira), an anti-diabetic medicine, on pancreatic cancer cell PANC-1 co-cultured with or without pancreatic stellate cells (PSCs). The chemical count kit-8 and Annexin V-FITC apoptosis detection were conducted to investigate the effect of Lira on cell viability and proliferation of PANC-1 with or without PSCs co-culture. Then, the wound healing and transwell experiments were performed to explore the influence of Lira on PANC-1 cells’ migration and invasion capabilities. To identify the potential action mechanism of Lira on PANC-1, the expression of E-cadherin and N-cadherin and the intracellular calcium content in PANC-1, after Lira administration, were detected. The results indicated that Lira in 100 and 1,000 nmol/L, effectively decreased the cell viability and dose-dependently promoted cell apoptosis of PANC-1 co-cultured with or without PSCs. Lira significantly reduced the migration and invasion of PANC-1 and also reduced the inducing effect of PSCs to PANC-1. Lira effectively induced the expression of E-cadherin and suppressed the expression of N-cadherin with a dose-dependent manner. Otherwise, Lira significantly reduced the abnormal high content of calcium in PANC-1 and also weakened the elevation of calcium in PANC-1 induced by cell-cell interaction. The current study firstly indicated that Lira suppressed the cell proliferation, migration and invasion of PANC-1 with or without PSCs co-culture. This effect was partially due to the calcium modulation of Lira and its influence on Ca²⁺-binding proteins, such as E-cadherin and N-cadherin.

Key words: Liraglutide, Pancreatic cancer, Pancreatic stellate cell, Calcium modulation
population suggested that there is no association between Lira treatment and the increased risk of acute pancreatitis and pancreatic cancer [12]. Otherwise, in human pancreatic cancer cell MiaPanca-2, Lira combined with metformin exerted anti-tumor effect through upregulating the expression of Bax and cleaving caspase-3 [13]. Therefore, study on therapeutic effect of Lira on pancreatic cancer cells can provide some Enlightenments to solve the complex diabetic pancreatic cancers. The excessive desmoplasia with plentiful extracellular matrix protein (ECM) which is produced by activated pancreatic stellate cells (PSCs), is the most common phenotype of pancreatic cancers [14, 15]. PSCs promote the proliferation, invasion and migration of pancreatic cancer cells, through secreting paracrine stimulants and growth factors to tumor microenvironment [16-18]. Therefore, studying drug influence on pancreatic cancer cells cocultured with PSCs can provide Enlightenments to comprehensively evaluating drug effects. By far, the effect of Lira on pancreatic cancer cells coexisting with PSCs, the study of which is more credible than study only on pancreatic cancer cells, hasn’t been discussed.

In this study, we explored the effect of Lira on pancreatic cancer cell line PANC-1, under co-culture with PSCs. The PANC-1 viability inhibition and apoptosis promotion effect of Lira were determined under conditions with or without PSCs. Then, the PANC-1 migration and invasion capabilities were also investigated at the same conditions with above. The expression of epithelial (E)-cadherin and neuronal (N)-cadherin in PANC-1 was detected. To evaluate the influence of Lira on calcium homeostasis in PANC-1, the intracellular calcium content ([Ca²⁺]) was also quantified by Ca²⁺ indicator Fluo-4/AM.

Materials and Methods

Materials

Antibodies against E-cadherin and β-actin were purchased from Cell Signaling Technology (MA, USA). Antibody against N-cadherin was obtained from Abcam (Cambridge, UK). Goat anti-mouse IgG H&L (Alexa Fluor™ 594) secondary antibody and HRP-conjugated secondary antibody were acquired from Abcam. Annexin V-FITC apoptosis kit was obtained from BD Biosciences (NJ, USA). Cell counting kit-8 (CCK-8) and Fluo-4/AM were purchased from Dojindo Molecular Technologies Inc. (Tokyo, Japan). All cell culture reagents were bought from Gibco BRL Life Technologies (MD, USA). Pluronic F-127 was acquired from Genecopoeia (MA, USA). All other chemicals were bought from Sigma-Aldrich (CA, USA).

Cell culture

Pancreatic cancer cell lines (PANC-1) were purchased from RIKEN Bio-Resource Center (Tsukuba, Japan) and were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin (0.1 mg/mL) at 37°C in a humidified atmosphere with 5% CO₂. Human pancreatic stellate cells (HPaSteCs, PSCs) were bought from American Type Culture Collection (ATCC, MD, USA) and its culture medium was the same as PANC-1 cells.

Co-culture procedures were as follows: PANC-1 cells were seeded into a 6-well plate (BD Biosciences, NJ, USA) with a density of 1 × 10⁵ cells/well and the PSCs were plated into a culture insert (BD Biosciences) with 1.0 μm pores at a density of 5 × 10⁵ cells/insert. The culture insert plated with PSCs were placed into the 6-well plate seeded with PANC-1 cells at 37°C humidified incubator for 24 h. The PANC-1 cells co-cultured with PSC cells were regarded as PANC-Co cells.

CCK-8 assay

To explore the effect of Lira on cell viability of PANC-1 and PANC-Co cells, the CCK-8 assay was conducted. The cells were plated in 96-well plate at a density of 1 × 10⁴ cells/well with complete medium for 24 h. Then, cells were incubated with medium containing 0, 10, 100 or 1,000 nmol/L Lira for 12 h. After 12 h incubation, cells were cultured in CCK-8 solutions for another 3 h. Then, the absorbance was observed at optical density (OD) 450 nm by a Thermolabsystem Multiskan Spectrum spectrophotometer (Franklin, MA, USA).

Cell apoptosis assay

The Annexin V-FITC apoptosis detection kit was adopted to conduct the cell apoptosis assay. Briefly, PANC-1 cells and PANC-Co cells were incubated in complete medium for 24 h. Culture medium containing 0, 10, 100 or 1,000 nmol/L Lira were used to treat cells for 8 h. After the samples treatment, cells were washed three times with ice-cold PBS and then resuspended by 1 × binding buffer at a density of 1 × 10⁶ cells/mL. 100 μL suspension containing 1 × 10⁵ cells was transferred into a 5 mL culture tube and next, 5 μL Annexin V-FITC were added to stain cell in dark for 15 min. Then, the cells were washed by PBS and stained with deliquated binding buffer containing 10 μL propidium iodide (PI) (20 μg/mL). The apoptosis assay was performed by flow cytometry (Beckman Coulter, CA, USA) at the wavelength of 488 nm.

Wound healing assay

PANC-1 cells and PANC-Co cells were incubated in
complete medium for 24 h. A pipette tip scratched line wounds on the completely confluent monolayer cells. Then, cells were incubated in serum-free medium containing 0, 10, 100 and 1,000 nmol/L Lira, and photographed at 0, 24 and 48 h by an inverted microscope. The images were analyzed by the Scion image software (version 4.0.3.2, Scion Corporation, MD, USA). The migration capability of PANC-1 and PANC-Co cells was expressed as migration % = (the distance of two sides at 0 h (D0)–the distance at 24 or 48 h (D24 or D48))/D0.

Transwell assay
For invasion assay, PANC-1 cells were plated at 1 × 10⁵ cells/well on the top chambers of 24-well transwell plates (8.0 μm-pore, BD Biosciences) with serum-free medium containing 0, 10, 100 and 1,000 nmol/L Lira while the bottom chambers were added 10% FBS medium. For PANC-Co cells, the PSC cells were seeded into the culture insert (8.0 μm pores) and placed on the 24-well plate containing PANC-1 cells. After 24 h incubation, PANC-1 cells that migrated to the bottom surface of the top chamber membranes were stained with 0.1% crystal violet and photographed by a microscope. The values of invasion were expressed with the mean absorbance of OD 570 nm per assay.

Immunofluorescence assay
PANC-1 cells were cultured in 6-well plates with or without culture inserts seeded with PSC cells and incubated in complete medium for 24 h. Then the cells were pretreated with culture medium containing 0, 10, 100 and 1,000 nmol/L Lira for 8 h and then the following experiments were conducted.

Western blot
The total protein extractions of PANC-1 cells were performed by lysing cells in ice-cold RIPA buffer for 30 min, then centrifugated at 15,000 rpm at 4°C for 15 min and heated to 100°C for 5 min. The protein concentration was detected by a bicinchoninic acid protein assay kit (Beyotime Biotechnology, Shanghai, China). The equivalent protein of each sample was separated by 10% SDS-PAGE and next, transferred to PVDF membranes. The membranes were blocked by 10% goat serum and then incubated with primary antibodies diluted in TBST: mouse anti-human E-cadherin, N-cadherin and β-actin, overnight at 4°C. The next day, PVDF membranes were incubated with HRP-conjugated goat anti-mouse secondary antibodies at room temperature for 1 h. The protein bands were visualised with immobilon western chemiluminescent HRP substrate detection reagent (Millipore, MA, USA).

Fluorescence imaging of Fluo-4-Ca²⁺ complex
When cells reached 60% confluency, it were pretreated with culture medium containing 0, 10, 100 and 1,000 nmol/L Lira for 8 h and then the following experiments were conducted.

Statistical analysis
Statistical analysis was carried out by SPSS v.18.0 statistical analysis software (SPSS Inc., Chicago, USA) and all data were presented as the mean ± standard deviation (SD). Statistical comparisons between two groups were conducted with an unpaired, two tailed Student’s t-test and statistical comparisons between multiple groups were conducted with one-way ANOVA. P values ≤0.05 were considered significant.

Results
Lira inhibited cell viability of PANC-1 and PANC-Co cells
Cell viability was determined by CCK-8 assay. The absorbance of CCK-8 in PANC-1 was dose-dependently reduced by Lira treatment (Fig. 1A). After co-culture, the CCK-8 absorbance of PANC-Co cells was also decreased by 100 and 1,000 nmol/L Lira administration. Furthermore, the co-culture with PSC cells improved the cell viability of PANC-1 cells.

Lira induced cell apoptosis in PANC-1 and PANC-Co cells
The early apoptotic cells were labeled with Annexin V
(+), PI (–) and located in the Q3 area in Fig. 1B. The late apoptotic cells were labeled with Annexin V(+), PI (+) and located in the Q2 area in Fig. 1B. When the PANC-1 and PANC-Co cells were stained by Annexin V-FITC and PI, the dose-dependent induction of cell apoptosis was observed after Lira treatment. From Fig. 1B, 10, 100 and 1,000 nmol/L Lira significantly increased the percentage of apoptotic cells in PANC-1 and PANC-Co cells. Moreover, the proportion of apoptotic PANC-Co cells was obviously lower than the proportion of apoptotic PANC-1 cells, which indicated that the co-culture with PSC cells significantly inhibited apoptosis of PANC-1 cells in Lira treatment.

Fig. 1 Lira suppressed cell viability and induced cell apoptosis in PANC-1 co-cultured with or without PSCs. (A) The absorbance value (λ = 450 nm) of PANC-1 and PANC-Co cells after Lira treatment, detected by CCK-8 kit (n = 6). The lower absorbance value represents the weaker cell viability. (B) The cell apoptosis analysis of PANC-1 and PANC-Co cells after 8 h treatment by 0, 10, 100 and 1,000 nmol/L Lira (n = 3). Each bar represents the mean ± S.D.; * p < 0.05, ** p < 0.01, *** p < 0.001 compared to the PANC-1 samples without Lira treatment; # p < 0.05, ## p < 0.01, ### p < 0.001 compared to the PANC-Co samples without Lira treatment; Δ p < 0.05, ΔΔ p < 0.01, ΔΔΔ p < 0.001.
Lira inhibited migration of PANC-1 and PANC-Co cells

The results in Fig. 2A showed that Lira inhibited the migration of PANC-1 and PANC-Co cells with a dose-dependent manner. The migrations of PANC-1 and PANC-Co cells were both suppressed by 1,000 nmol/L Lira in 24 h. In 24 h, 1,000 nmol/L Lira also suppressed the migration of PANC-1 cells induced by PSCs. In 48 h, Lira exerted the dose-dependent suppression of PANC-1 and PANC-Co cells’ migration and the migrations of PANC-1 and PANC-Co cells showed no significant difference after Lira treatment.

Lira inhibited invasion of PANC-1 and PANC-Co cells

A modified Matrigel invasion assay was performed to
investigate the impact of Lira on the invasion capability of PANC-1 and PANC-Co cells. PANC-Co cells exerted stronger invasion capability than PANC-1 cells, which indicated that the presence of PSC cells induced the invasion of PANC-1 (Fig. 2B). After Lira administration, the invasion ability was effectively inhibited in PANC-1 and PANC-Co cells. Furthermore, after 100 and 1,000 nmol/L Lira administration, the invasion abilities of PANC-1 and PANC-Co cells had no significant difference, which indicated that Lira inhibited the induction effect of PSC on PANC-1 cells’ invasion.

Lira induced the expression of E-cadherin

As shown in immunofluorescence experiment, Lira significantly increased the expression of E-cadherin in PANC-1 and PANC-Co cells (Fig. 3A). Furthermore, the
result of immunofluorescence was validated by the western blot assay. From the result of western blot, the expression of E-cadherin in PSC, PANC-1 and PANC-Co cells was dose-dependently induced by Lira and this induction exhibited no significant difference between PANC-1 and PANC-Co cells (Fig. 3B).

**Lira suppressed the expression of N-cadherin**

The result of immunofluorescence showed that Lira suppressed the expression of N-cadherin in PANC-1 and PANC-Co cells (Fig. 4A). The western blot indicated that 100 and 1,000 nmol/L Lira significantly suppressed the expression of N-cadherin in PSC, PANC-1 and PANC-Co cells (Fig. 4B). Moreover, the expression of
N-cadherin in PANC-Co cells was obviously lower than that in PANC-1 cells after 100 and 1,000 nmol/L Lira administration.

**Lira decreased the intracellular Ca\(^{2+}\) content**

To explore the effect of Lira on [Ca\(^{2+}\)]\(_i\) modulation, the Fluo-4/AM as a [Ca\(^{2+}\)] indicator, was applied. As shown in Fig. 5, the fluorescence intensity of Fluo-4-Ca\(^{2+}\) complex was increased in PANC-Co, which suggested that co-culture with PSC induced the increase of [Ca\(^{2+}\)]\(_i\) in PANC-1 cells. In PANC-Co cells, Lira treatment dose-dependently reduced the [Ca\(^{2+}\)]\(_i\) (Fig. 5).

**Discussion**

E-cadherin, a paradigmatic cadherin, plays a key role in adherens junctions which is pivotal in modulating contact inhibition of proliferation and keeping epithelial phenotype of cells [19]. Therefore, loss of E-cadherin leads to lack of contact inhibition and increase of cell migration [20]. Furthermore, loss of E-cadherin promotes the epithelial-to-mesenchymal transition (EMT) that is extremely active to accelerate tumor metastasis [21]. Therefore, E-cadherin is a well-known tumor invasion suppressor. Although N-cadherin sometimes exerts similar biological function of E-cadherin during EMT, it isn’t always a beneficial cadherin. N-cadherin can activate fibroblast growth factor receptor 1 (FGFR1) through binding to this membrane protein and then, disturbs the FGFR1 relevant signaling pathway and facilitate tumor malignancy [22, 23]. Moreover, high expression of N-cadherin in tumors of epithelial origin, such as pancreatic cancer, promotes the tumors acquiring a heterotypic cell adhesion mode, which contributes to metastatic dissemination of cancer cells [24]. In the current study, Lira effectively induced the expression of E-cadherin and suppressed the expression of N-cadherin in PANC-1 cells co-cultured with or without PSCs, which suggested Lira has the potential of suppressing PANC-1 metastasis and the results of migration and invasion assay also proved that Lira exerted the suppression of migration and invasion of PANC-1 cells with or without PSCs co-culture.

Ca\(^{2+}\), as a secondary messenger, plays various roles in regulating cell fate. In cancer cells, the intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) is far higher than normal cells and high [Ca\(^{2+}\)]\(_i\) promotes proliferation and migration of cancer cells by activating PLC, PKC, Ras and other oncoproteins [25, 26]. The activity of pancreatic cancer cells enormously rely on the [Ca\(^{2+}\)]\(_i\), and aberrant [Ca\(^{2+}\)]\(_i\) is always observed in active pancreatic cancer cells [27, 28]. Therefore, maintaining calcium homeostasis and modulating [Ca\(^{2+}\)]\(_i\) plays important role in the metastasis and malignancy of pancreatic cancer. In this study, cell-cell interaction of PANC-1 and PSC cells elevated the [Ca\(^{2+}\)]\(_i\) of PANC-1 and Lira effectively decreased [Ca\(^{2+}\)]\(_i\), in PANC-1 co-cultured with PSCs, which may be partly responsible for Lira inhibiting PANC-1 cells proliferation and migration. The ectodomain of E-cadherin binds Ca\(^{2+}\) at junctions, constructs a rigid conformation between cadherin domains [29]. Ca\(^{2+}\) homeostasis, espe-
Lira facilitating the expression of E-cadherin. Therefore, Lira potentially affects the folding of Ca\(^{2+}\)-binding proteins directly and restoring proteostasis of transmembrane proteins, such as cadherins [30, 31]. Some research reported that, in melanoma cells, downregulating [Ca\(^{2+}\)], effectively suppressed the expression of N-cadherin [32], which is coincident with our result that Lira decreased the expression of N-cadherin by inhibiting [Ca\(^{2+}\)], Suppression the increase of [Ca\(^{2+}\)], which maintains the function of ER as a calcium store, may promote the conformation of E-cadherin, which may be one of causes for Lira facilitating the expression of E-cadherin. Therefore, Lira may suppress the development of PANC-1 without or with PSCs co-culture through inhibiting extortionate [Ca\(^{2+}\)], and then regulating the expression of E-cadherin and N-cadherin. Due to the anti-tumor effect of Lira on PANC-1, Lira may be a promising option for improving pancreatic cancer associated with diabetes.

**Conclusion**

In the current study, we explored the role of Lira in human pancreatic cancer cell line PANC-1 with or without PSCs co-culture. Our result indicated that Lira inhibited cell viability, migration and invasion, and promoted cell apoptosis. We firstly revealed that Lira promoted the expression of E-cadherin and suppressed the expression of N-cadherin, which was partially, if not in all, due to its modulation in calcium homeostasis.

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**Disclosure**

The present study has not been published in any other journal, in whole or in part, and not being considered for publication elsewhere in any language. There is no conflict of interest. The final manuscript was reviewed and approved by all of the co-authors.

**References**