GLP-1 receptor agonist liraglutide inhibits the proliferation and migration of thyroid cancer cells

Xuelian Zhang¹, Liqiang Zhang², Bo Wang¹, Xuane Zhang¹, Lei Gu¹, Kai Guo¹, Xiaoyan Zhang¹, Zunhai Zhou¹*¹

¹Department of Endocrinology, Yangpu District Central Hospital, Tongji University School of Medicine, Shanghai 200090, China
²Shanghai Jemincare Pharmaceutical Co., Ltd, Shanghai 201315, China

ARTICLE INFO

Original paper

Article history:
Received: September 30, 2023
Accepted: December 07, 2023
Published: December 20, 2023

Keywords:
liraglutide; Thyroid cancer cells in the medulla; Papillary thyroid carcinoma cells; GLP-1R; Proliferation; Migration

Introduction

At the beginning of the last century, Maynard discovered that there is a statistical correlation between diabetes and tumorigenesis. Multiple clinical and epidemiological studies, such as cohort studies and observational studies, have shown that diabetes can increase the incidence of multiple tumors (1-3). Diabetes and tumors share a common biological pathogenesis such as hyperinsulinemia, inflammatory cytokines, IGF-1, etc. (4). Furthermore, hypoglycemic agents may affect the occurrence and development of tumors by reducing cyclin expression, activating P53 or through other mechanisms (5). Therefore, it is necessary to select the hypoglycemic agents carefully for diabetes mellitus patients with tumors during clinical treatment, considering the side effects of the treatment.

Liraglutide, an agonist of the glucagon-like peptide-1 receptor (GLP-1R), was launched in China in October 2011. As a new GLP-1R agonist, liraglutide can control blood sugar level effectively, reduce body weight and improve systolic blood pressure, which suggest that liraglutide has a broad clinical application prospect (6). As a new GLP-1R agonist, liraglutide at indicated time points. To track the capacity for cell migration, the Transwell test was utilized. We found that liralutide-activated GLP-1R could significantly reduce the growth and metastasis of two kinds of thyroid tumor cells, and the inhibitory effect was dose- and time- dependent. The phosphorylations of Akt, S6K1, and 70SK declined after receiving liraglutide therapy. In our previous studies, we found that the GLP-1 receptor agonist Liraglutide inhibited the proliferation and migration of thyroid cancer cells through the PI3K/Akt/mTOR pathway. This finding provides a theoretical basis for the treatment of diabetes mellitus complicated with medullary thyroid cancer, and is relatively safe.

Copyright: © 2023 by the C.M.B. Association. All rights reserved.

Doi: http://dx.doi.org/10.14715/cmb/2023.69.14.37

Materials and Methods

Plasmids and reagents

GLP1R-Tango plasmid was purchased from Addgene. Liposome transfection reagent Lipofectamine 3000 was purchased from Life Technologies (Gaithersburg, MD, USA). G418 was purchased from Sigma (St. Louis, MO, USA). Human GLP-1R Alexa Fluor® 488-conjugated antibody and mouse IgG2B Alexa Fluor® 488-conjugated Isotype Control was purchased from R&D (Minneapolis, USA). 

* Corresponding author. Email: zunhaizhou@163.com

Cellular and Molecular Biology, 2023, 69(14): 221-225
Liraglutide was purchased from Novo Nordisk Pharmaceutical Co., Ltd. (Tianjin, China).

Cells and cell culture

Human medullary thyroid carcinoma (TT) TCP1 (human cell line) (TCP1) was purchased from the Chinese Academy of Sciences (China Science Library) (Shanghai, China). TT cells of human medullary carcinoma were cultured by DMEM/F12 K method under the condition of 37°C, 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin and 100 ug/mL, 37°C. 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin were cultured in vitro under the condition of 37°C. According to the convention, under the condition of 75-85%, it is hydrolyzed with 0.25% trypsin and subcultured. This experiment uses cells in the logarithmic growth stage.

CAMP was measured on 96-well plates at the logarithmic growth stage according to the concentration of 2×10^4/ pores, and cultured overnight. After starving serum for 12 hours, Liluar peptide was added, and the final concentration was 10 nmol/L, 100 nmol/L, 500 nmol/L. At 37 degrees Celsius, these cells were cultured for another 30-60 min. The content of cAMP was determined by the cAMP method and their fluorescence was measured using fluorometer.

**Assay for cell proliferation**

CCK-8 (Japanese colleague Chemical Institute) measured cell proliferation according to the manufacturer's operating procedures. In short, cells at the logarithmic growth stage were planted on 96-well plates at the concentration of 1×10^4/ wells. Each cell line was divided into 7 groups with 5 wells in each group. Cells were treated with liralutide at the final concentration of in each group 10 nmol/L, 50 nmol/L, 100 nmol/L, 200 nmol/L, and 500 nmol/L, respectively. The control group was seeded with cells but wasn’t treated with liralutide; the Blank group contains only medium but not cells and liralutide. After being cultured for 72 h, 10 μg 8-acetylline L solution was added respectively. The cells were cultured in CCK-8 solution for 1 to 2 h, and then the weekly absorbitivity (A) was determined at 450 nm. The absorbance value of the experimental groups was referred as As; the absorbance value of the control group was referred to as Ac and the absorbance value of the blank group was referred as Ab. The relative cell proliferation rate was calculated using the following formula: Cell relative proliferation rate (%) = ((As-Ab)/(Ac-Ab))×100%.

**Transwell assay**

The Transwell method was used to observe the effect of TT/GLP-1 receptor and TCP-1/GLP-1 receptor on tumor metastasis. 10% fetal bovine serum was added to the culture medium. 105 cells with 0.1% bovine serum albumin were added to 100 microliter serum-free medium. Subsequently, 0, 100 or 500 nmol/L liralutide was added to the culture medium in the upper chamber. After 24 hours of culture, methanol was used as the carrier and 0.1% crystal violet as dye, and counted. The experiment was repeated three times.

High-throughput sequencing and differential expression analysis

Using MagzolTM reagent (Chinese Magen), the total RNA was extracted according to the manufacturer's instructions. Use ThermoFisher Science, Inc. (Waltham, MA, USA). The content and purity of RNA samples were determined by NanoDrop 2000 spectrophotometer. The integrality of the overall RNA is evaluated using the RNA-6000 parsing kit on Agilent Biological Analyzer 2100™ (Agilent Technologies, Santa Clara, CA, USA). Taking RIN 8 as an example, RIN 8 RNA samples are made. The sequence is completed on IlluminaNovaseq6000 software, and the reading at both ends is 150 bp. Before studying different gene expressions, the readings of each sequence were corrected by Edger software package. Edger R (3.18.1) was used to compare the gene expression. The value of P < 0.05 and the change of magnification > 1.5, indicating the difference between the two groups. The function and pathway of Genetics (GO) between Kyoto gene and genome were studied by ClusterProfiler software. Tiangen Biotechnology Co., Ltd. (Beijing, China) has carried out library construction, sequence determination and data analysis.

**Western blot analysis**

As described above, the cells are collected and dissolved in a RIPA buffer containing protease and phosphatase inhibitors. Using 10% Mel 12% SDS-PAGE as a solvent, the dissolved protein was trans-imprinted and transferred to the PVDF film (Millipore, Billerica, MA, USA). The film was sealed for 1 hour with a skim milk of 5% (Wamp V) and cultured for one night at 4°C with antibodies against phosphorylated-70S6K (Thr389/Thr412, TA3228), phosphorylated-S6K1 (T421Universe S424 camera T55261), GAPDH (# m20006), Akt (# 9272), cell signaling, Danvers, MA, USA), and p-Akt (Ser473, #9271, cell signaling, Danvers, MA, USA). This project intends to use double antibody technology to characterize and characterize it in Shanghai Taineng Technology Co., Ltd. (General Chemical Organization, GFP).

**Statistical analysis**

Three groups of separate tests were compared, and the data were processed by statistics. Data processing uses Statistic Package for Social Science (SPSS) 17.0 (IBM, Armonk, NY, USA) as the main processing tool, and the data between groups are tested by t-test. There was a significant difference in (P<0.05).

**Results**

Expression of GLP-1R in TT/GLP-1R and TCP/GLP-1R cell lines

TT and TCP-1 cell lines that stably express GLP-1R were constructed and selected by G418. The membrane protein level of GLP-1R in TT/GLP-1R and TCP-1/GLP-1R stable cell lines were examined using flow cytometry. The average fluorescence intensity of GLP-1R on cell membranes of the GLP-1R stable cells line was 12.1 times higher (TCP-1/GLP-1R cells versus TT cells) (Figure 1A) and 10.2 times higher (TCP-1/GLP-1R cells versus TCP-1 cells), respectively (Figure 1D). Activation of GLP-1R can lead to a rapid elevation of cyclic adenosine monophosphate (cAMP) in cells. So we detected the cAMP level using the HitHunter® cAMP Assay kit in GLP-1R overexpressed cells. After treatment with liralutide, the content of cAMP increased gradually in a concentration-dependent
untreated control group (Figure 2A). Likewise, liraglutide also inhibited the cell migration of TCP-1/GLP-1R cells (Figure 2B).

Liraglutide prevented the PI3K/Akt/mTOR pathway from activating in thyroid cancer cell lines

On this basis, this project intends to use RNA-seq technology to study the transcriptome of TT/GLP-1R cells in TT/GLP-1R. The results of our previous study showed that under the condition of 100 nm and 24 h, Lilarutide could significantly increase the expression level of FC > 1.5 by 616s and decrease it by 322s. Compared with the control (Figure 3 An on the right), the expression of 522 genes increased significantly and the expression of 254 genes decreased significantly in FC or 1.5 treated with lialutide (100 nm, 72 h) compared with the control (Figure 3 An manner, indicating that GLP-1R was activated in such cells (Figure 1B and 1e).

Effect of liraglutide on the proliferation of thyroid cancer cells with different GLP-1R expression levels

Objective: to study the inhibitory effect of liraglutide on the growth of tumor cells with high expression of GLP-1 receptor. The survival of tumor cells was determined by CCK-8 colorimetric assay, the widely used liralutide (10, 50, 100, 200, 500 nm/L) within the specified time (24, 48, 72 h). The inhibition of liraglutide on cell proliferation of two thyroid cancer cell lines with stable expression of GLP-1R are shown in Figure 1. We found that 10 nM liraglutide showed significant inhibition on cell proliferation of GLP-1R overexpressed TT or TCP-1 cells 48 hours after the treatment. A high concentration of liraglutide induced significant inhibition in GLP-1R overexpressed TT and TCP-1 cells 24 hours after the treatment. The inhibition effect was in a time- and dose-dependent manner (Figures 1C and 1F).

Effect of liraglutide on the migration of thyroid cancer cells with different GLP-1R protein expression levels

Study on the effect of GLP-1R activation on thyroid tumor cells by Transwell method. Transwell assay showed liraglutide significantly inhibited cell migration in a dose-dependent manner in TT/GLP-1R cells, compared with the untreated control group (Figure 2A). Likewise, liraglutide also inhibited the cell migration of in TCP-1/GLP-1R cells (Figure 2B).
occurrence of medullary thyroid carcinoma (MTC) (10-13). Therefore, in the United States, labels of GLP-1RAs product contain a black box warning. Medullary cell carcinoma of the thyroid accounts for 1% of primary thyroid tumors and is differentiated from adjacent cells (14). From 1983 to 2012, the average annual incidence of age-adjusted MTC increased notably, from 0.14 to 0.21 cases per 100,000 persons (15). MTC cases are mostly sporadic (80%) (15). In rats, the activation of GLP-1R can increase the amount of cyclic adenosine monophosphate in C-cells, which leads to the secretion of intracellular calcitonin, which promotes the proliferation and carcinogenesis of C-cells (16, 17). Although calcitonin is a critical biomarker for MTC existence (13, 18, 19), it was not found that GLP-1RAs mediates elevation of calcitonin in non-human primates (17, 18) or patients with type 2 diabetes (20, 21). In view of these findings, we need to comprehensively and scientifically balance the benefits and possible risks of GLP-1RAs treatment in diabetes. To do this, we must find a consensus to understand the molecular mechanism of the hypothetical pathology of GLP1 analogue-induced thyroid disease, and to what extent experimental animal data can be inferred to clinical application.

Boess et al. reported that GLP-1R expression is present in the thyroid of rodents is much higher than that of non-primate and human thyroids (22), and they also found that GLP-1 was not observed in human thyroid culture. The role of GLP-1R agonists in humans is different from that in mice. However, its mechanism is not clear.

In this study, the papillary thyroid carcinomaTCP-1/GLP-1R cell line and the medullary thyroid cancer TT/GLP-1R cell line that stably overexpressed GLP-1R were constructed for the first time. This laid a solid foundation for studying whether liraglutide affects the proliferation and migration of cell lines expressing GLP-1R.

Proliferation and metastasis is the major cause during cancer progression. Our results showed that GLP-1R activation hindered the proliferation and migration of GLP-1R overexpressed human TT or TCP-1 cells, and the inhibitory effect is dose- and time-dependent, suggesting that high expression of GLP-1R maybe not the root cause of medullary thyroid disease in rodents. Also in humans, liraglutide may not accelerate the proliferation of cells expressing GLP-1R. Liraglutide can inhibit the activation of Akt and mTOR signaling pathways.

The same is true of Liang and them. The literature suggests that Lilarutide has no obvious inhibitory effect on the growth of PTC (23). h. Zhao, and other people. Activation of GLP-1 receptor (GLP-1) can down-regulate PI3K/Akt signal and exert anti-cancer effect. Study on Meta method of Liu Yufang et al. GLP-1RA is safe in cancer treatment (25). They conducted a study in a 24-week GLP-1RAs-based study of patients with type 2 diabetes. No tumorigenesis was found when GLP-1 receptor agonists were used. Liu Yufang et al found that GLP-1RAs has good controllability and can be used as an effective drug for the treatment of type 2 diabetes. These studies further support our finding that GLP-1R suppresses the progression of medullary thyroid cancer. Our findings demonstrate that GLP-1RA-based treatment may be beneficial to comorbid diabetes and thyroid cancer and that it is not harmful. Therefore, more extensive research is required to further evaluate whether the safety concerns about the occurrence of human medullary thyroid carcinoma (MTC) based on rodent experiments are scientifically adequate.
Conclusions
Our results showed that liraglutide reduces proliferation and migration by inhibiting the activation of Akt/mTOR/S6K signaling in medullary thyroid cancer TT/GLP-1R and papillary thyroid carcinoma TCP-1/GLP-1R cell lines and that the inhibitory effect was dose- and time-dependent. Our data indicate that liraglutide is a promising drug for the treatment of comorbid diabetes and thyroid cancer.

Acknowledgements
This work was supported by the Natural Science Foundation of Shanghai (18ZR1435800).

Conflict of interest
The authors declare no conflict of interest.

Authors’ contributions
Xuelian Zhang performed most of the experiments. Liqiang Zhang, Bo Wang and Xiaoyan Zhang performed the bioinformatics analysis. Xuane Zhang, Lei Gu and Kai Guo collected the data. Zunhai Zhou supervised the entire project and wrote the manuscript.

References
2. Stier-Jarmer M, Frisch D, Neuy S, Schuh A. A 3-Week Naturopathic Intervention Improves HbA1c, Weight, and Quality of Life Among Overweight and Obese Adults With Type 2 Diabetes: 6-Month Results From a Randomized Trial. Altern Ther Health M 2021; 27(61-71).