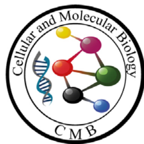

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GLP-1 receptor agonist liraglutide inhibits the proliferation and migration of thyroid cancer cells

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ABSTRACT

This research was done to find out how liraglutide affected the growth and movement of two human thyroid cancer cell lines overexpressing GLP-1 receptor (migration of medullary thyroid cancer TT/GLP-1R and papillary thyroid carcinoma TCP-1/GLP-1R). Flow cytometer and cAMP assays were used to identify the expression and activation of GLP-1R in two stable cell lines. Counting Kit-8 for Cells was applied to examine the proliferative ability of cells in two stable cell lines after treatment with different concentrations of 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 phosphorylatios 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.

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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). However, in the preclinical stage of drug development, medullary thyroid cancer had been observed from laboratory rodent studies following treatment with GLP-1R activators. With the clinical use of liraglutide, Kannan S. *et al* reported that GLP-1R agonists can be safely applied to all Thyroid follicular epithelial cells (papillary and follicular) in patients with thyroid tumors (7). Although GLP-1R agonists have not been confirmed the occurrence of medullary thyroid cancer cases in clinical research and post-marketing surveillance all GLP-1R agonists except exenatide are still contraindicated for use in those who have MEN2 (Multiple Endocrine Neoplasia Type II), or medullary thyroid carcinoma (7). Therefore, as GLP-1R agonist, the safety of liraglutide still needs to be further investigated in clinical trials. Elucidation of the underlying mechanism of liraglutide in tumor biological characteristics is expected to offer a theoretical foundation for clinical treatment.

In this study, two thyroid cancer cell lines with different drug safety risks during liraglutide therapy were selected: medullary thyroid cancer TT cells and papillary thyroid carcinoma TCP-1 cells. We constructed TCP-1/GLP-1R and TT/GLP-1R cell lines stably expressing GLP-1R. The aim of this study was to further demonstrate the safety of liraglutide by investigating its effect on the proliferation and migration of thyroid tumor cells with different expressions of GLP-1R, and to explor the possible risks of liraglutide in thyroid tumors.

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,

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MN, USA). Liraglutide was purchased from Novo Nordisk Pharmaceutical Co., Ltd. (Tianjin, China).

Cells and cell culture

Human medullary thyroid carcinoma (TT) TCP1 (human particle cell) (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 ug/mL streptomycin and 100 ug/mL, 37°C. 10% fetal bovine serum, 100 U/mL penicillin and 100 ug/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 96-6 well plates and implanted into 96-pore plates at the logarithmic growth stage according to the concentration of 2×10^4/ pores, and cultured overnight. After starving serum for 12 hours, Lilaru peptide was added, and the final concentration was 10 nmol/L,100nmo1/L,500nmo1/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 by 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/ holes. Each cell line was divided into 7 groups with 5 wells in each group. Cells were treated with liraglutide 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 liraglutide; the Blank group contains only medium but not cells and liraglutide. 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 absorptivity (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 liraglutide 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 integrity of the overall RNA is evaluated using the RNA- Nano 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 (TT/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

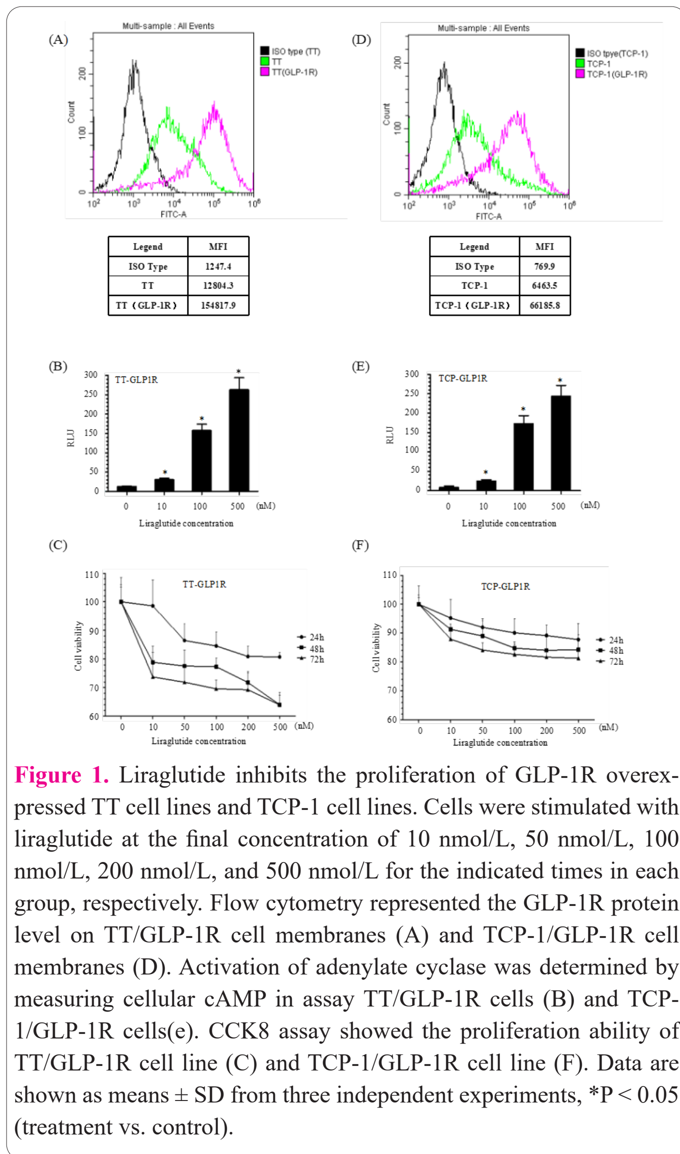

Figure 1. Liraglutide inhibits the proliferation of GLP-1R overexpressed TT cell lines and TCP-1 cell lines. Cells were stimulated with liraglutide at the final concentration of 10 nmol/L, 50 nmol/L, 100 nmol/L, 200 nmol/L, and 500 nmol/L for the indicated times in each group, respectively. Flow cytometry represented the GLP-1R protein level on TT/GLP-1R cell membranes (A) and TCP-1/GLP-1R cell membranes (D). Activation of adenylate cyclase was determined by measuring cellular cAMP in assay TT/GLP-1R cells (B) and TCP-1/GLP-1R cells(e). CCK8 assay showed the proliferation ability of TT/GLP-1R cell line (C) and TCP-1/GLP-1R cell line (F). Data are shown as means ± SD from three independent experiments, *P < 0.05 (treatment vs. control).

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 liralutide 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).

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 100nm 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

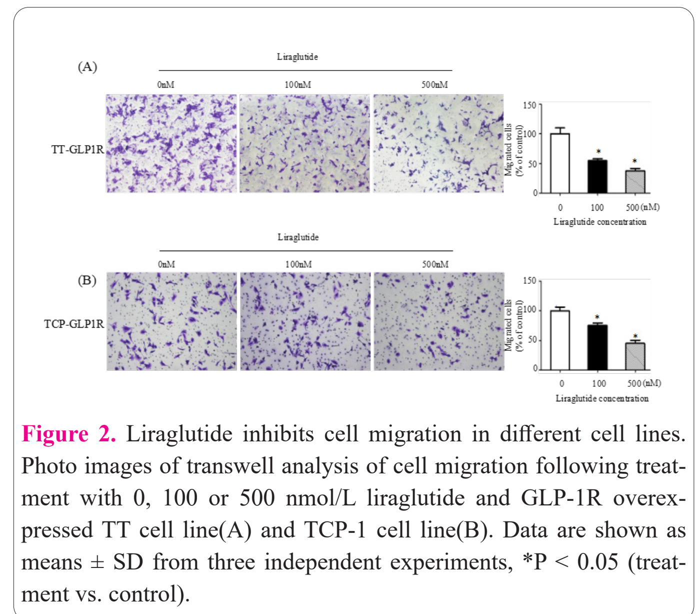

Figure 2. Liraglutide inhibits cell migration in different cell lines. Photo images of transwell analysis of cell migration following treatment with 0, 100 or 500 nmol/L liraglutide and GLP-1R overexpressed TT cell line(A) and TCP-1 cell line(B). Data are shown as means ± SD from three independent experiments, *P < 0.05 (treatment vs. control).

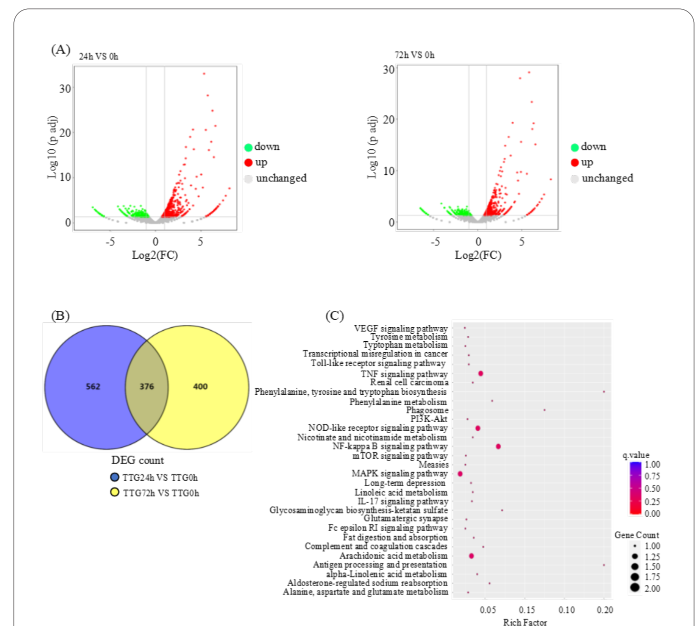

Figure 3. Differentially expressed genes (DEGs) KEGG enrichment analysis in TT/GLP-1R cell lines. (A) the volcano map showed that the expression of TT/GLP-1R increased or decreased after liralutide treatment, while that in the control was as follows: (B) Nonproportional Venn diagram of differentially expressed genes following treatment with 100 nmol/L liraglutide for 24 h and 72 h. (C) The KEGG annotation results of the DEGs.

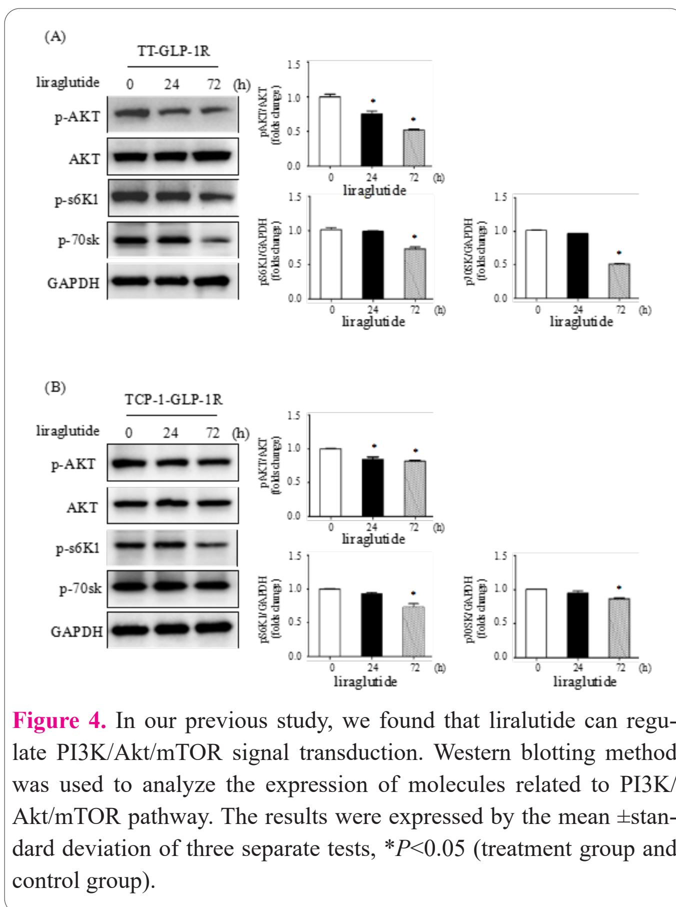

Figure 4. In our previous study, we found that liralutide can regulate PI3K/Akt/mTOR signal transduction. Western blotting method was used to analyze the expression of molecules related to PI3K/Akt/mTOR pathway. The results were expressed by the mean ±standard deviation of three separate tests, *$P<0.05$ (treatment group and control group).

on the right). Secondly, the functional annotation of candidate genes was carried out by using the bioinformatic method, and the preliminary functional study of candidate genes was carried out. Lilarutide can regulate PI3K/Akt/mTOR signal pathway, MAPK signal pathway, TNF signal pathway, nuclear transcription factor-KB (Nuclear factor kb) signal pathway and metabolic pathway (shown in Figure 3C). PI3K/Akt/mTOR is an important way to regulate cell proliferation, metabolism and differentiation. Survival, invasion, protein synthesis, etc. Taking PI3K/Akt/mTOR as the starting point, this project intends to phosphorylate several important molecules in the PI3K/Akt/mTOR pathway by Western blot technology. We found that the expressions of p-Akt, phosphorylated S6K1 and phosphorylated S6K genesIn cells were significantly lower than those in the normal group after 72 hours of liralutide. Therefore, we speculate that Lilarutide has a anti-tumor effect on tumors with high expression of GLP-1R, and its mechanism is related to the activation of Akt/mTor/S6K and related signal transduction (Figure 4).

Discussion

Glucagon-like peptide-1 receptor agonists (GLP-1RAs), such as liraglutide, exenatide, and dulaglutide, It was approved to improve the blood sugar level of patients with type 2 diabetes. Starting with the approval of GLP-1RAS, there have been many discussions about the potential safety of GLP-1RAs for the treatment of diabetes, since it may induce thyroid disease. Although GLP-1RAs can effectively control blood sugar and reduce body weight, based on research on rodents during the drug development stage, people have raised concerns about the 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 cancera 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 y 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

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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.

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