

DDIT4 is involved in PPAR- γ mediated Fatty Acid intake to promote the Proliferation of Pancreatic Cancer

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Abstract

Background: DDIT4 is a tumor metabolism-related protein which is involved in the proliferation and survival of various tumors. However, the function of DDIT4 in the pancreatic cancer is still unknown.

Methods: We collected clinical samples of pancreatic cancer and established animal models of pancreatic cancer and DDIT4-HET animal models. We examined the expression of DDIT4 in pancreatic cancer tissues by IHC and WB and the degree of fat infiltration in the pancreas by HE staining and oil-red O staining. What's more, the Western blot and immunohistochemical methods were applied to test the expression levels of PPAR- γ and CD36. Meanwhile, the apoptosis and proliferation levels were also examined in DDIT4-HET pancreatic cancer model.

Results: The expression of DDIT4 is decreased in pancreatic cancer and DDIT4 regulates the development of pancreatic cancer by participating in fat infiltration. The degree of fat infiltration in DDIT4-HET pancreatic cancer tumors is higher than that in wild-type pancreatic cancer tumors. Through transgenic animal models, we found that DDIT4 regulates lipid metabolism in pancreatic cancer by regulating PPAR- γ and its downstream proteins CD36 and ATGL, and DDIT4-mediated lipid metabolism abnormality is involved in the proliferation and apoptosis of pancreatic cancer.

Conclusion: The low expression of DDIT4 promotes the proliferation and survival by regulating lipid metabolism in pancreatic cancer cells.

Background

The American Cancer Society speculated approximately 56,000 new cases of pancreatic cancer in the USA in 2019 with an estimated 45,000 deaths, and the 5-year survival rate of patients is 10% in the USA [1]. The metabolism reprogrammed was one of the most important characteristics in the cancer cells in order to meeting energy requirements [2]. Indeed, A large number of lipids supported the survival and proliferation of cancer cells via the expression level of several lipogenic enzymes [3]. *Increased fatty acid synthesis* exhibits important roles in the growth [4], survival [5], drug resistance [6] and invasion [5] of *pancreatic cancer*.

DDIT4 played an important role in lung cancer [7], bladder cancer [8], ovarian cancer [9] and other malignant tumors, and it was involved in the prognosis assessment of breast cancer [10], lymphoma [11] and other diseases, and it was further confirmed to be involved in tumor autophagy [8], proliferation [12] and invasion [9], etc. DDIT4 may influence malignant behavior of cancer cells by affecting lipid metabolism. DDIT4 participated in the activation of insulin signaling pathway through mTORC1-dependent inhibition [13]. DDIT4 mediated tumor suppressor whose survival is defined by endoplasmic reticulum stress is mediated by DDIT4 [14]. DDIT4 mediated reprogramming of lipid metabolism involved in malignant behavior of a tumor such as invasive and metastatic progression, and poor prognosis [15].

We speculate that DDIT4 plays an important role in pancreatic cancer, but its specifics are still unknown. Then, we investigated the role of DDIT4 in pancreatic cancer through clinical samples, animal models and transgenic animals.

Methods

Clinical samples

A total of 20 fresh primary pancreatic cancer samples and matched adjacent non-cancerous tissues were obtained from patients who completed the surgery at the First Affiliated Hospital of Fujian Medical University. All samples were confirmed by the Department of Pathology at the First Affiliated Hospital of Fujian Medical University, and all stored in -80 °C refrigerator and the 10% formalin. All enrolled patients signed the informed consent for excess specimens to be used for subject research and all protocols in this study were approved by the Ethics Committee of the First Affiliated Hospital of Fujian Medical University. The study have been performed in accordance with the Declaration of Helsinki.

Animal models and animal samples

The DDIT4-HET mice required for the experiment which were completed by Shanghai Southern Model Biotechnology Co., Ltd, with a genetic background of C57BL/6J. 20 wild type mice(6-8week old 18-22g, male:female=13:7)were provided by the Shanghai Slack Laboratory Animal Co. Ltd and were housed in pathogen-free conditions(22-25°C with adequate food and water). All animal related studies complied with the National Institutes of Health guidelines for the care and used of laboratory animals, and the protocol was approved. The study experiments and relevant details were approved by the Ethics Committee of the First Affiliated Hospital of Fujian Medical University. The study was carried out in compliance with the ARRIVE guidelines for the reporting of animal experiments. The model of pancreatic cancer induced by DMBA(Sigma) was accomplished according to previously paper[16]. Briefly, mice were anesthetized with isoflurane inhalation, fixation and midline laparotomy. A pocket was blunt dissected in the parenchyma of the pancreatic tail, and 1 mg of DMBA were placed into it. Then, pocket was stitched with a 6-0 purse-string suture. mice were sacrificed at 1 months following DMBA implantation. We euthanized the mice by using 50% CO₂ *chamberreplacement* rate[17]. Tissue near the implantation site and adjacent normal pancreatic tissue were collected.

Western blot

Tissue proteins were prepared from fresh frozen tumor tissue in RIPA lysis extraction buffer with protease inhibitors. Tissue proteins were separated by SDS-PAGE and transferred to PVDF membranes. The primary antibodies were used as the manufacturer's instructions: anti-TAGL (Dilution 1:1000) and anti-β-actin (Dilution 1:1000), anti-CD36 (Dilution 1:1000), anti-PPAR-γ (Dilution 1:1000), anti-DDIT4 (Dilution 1:1000) All bands was analyzed by Image J 1.80 software (National Institutes of Health, Bethesda, MD, USA), and the grey value were normalized against the β-actin.

Oil-red O staining and HE staining

Frozen tissue sections were used in oil-red O stain and the paraffin section were used in HE staining. Frozen sections were fixed in 10% formalin, and an oil-red O staining solution and 60% isopropyl alcohol was used as the manufacturer's recommended methods. The paraffin section were dipped into Hematoxylin solution for 5-10 min and placed in eosin solution for 0.5~2 min. The sections were placed in 95% ethanol for re-dewatering and dehydrated tissue is placed in xylene and sealed with neutral gum.

Immunohistochemistry and Immunofluorescence

The paraffin sections were deparaffinized and rehydrated. The tissue sections are placed in a repair box filled with citric acid antigen retrieval buffer for antigen retrieval in a microwave oven. the sections are placed in 3% hydrogen peroxide and incubated at room temperature in darkness for 25 minutes. After being soaked in PBS 3 times. Part of the paraffin section were incubated with primary antibody for 2 h, The HRP-conjugated secondary antibody was diluted with antibody diluent to an proper concentration and incubated with sections at room temperature for 30 min. Costain with hematoxylin. Visualize staining of tissue under a microscope, acquisitive and analysis image. Part of the paraffin section were incubated by FITC-conjugated goat anti-mouse or Cy3-conjugated goat anti-rabbit secondary antibodies at room temperature for 2 h. Spontaneous fluorescence quenching reagent was incubated for 5 min. Immunofluorescence and DAPI-stained nuclei signals were visualized at room temperature using a fluorescence microscope. The primary antibodies were used as the manufacturer's instructions: DDIT4 (Dilution 1:200), ATGL (Dilution 1:200), CD36 (Dilution 1:200), ki-67 (Dilution 1:200), PPAR- γ (Dilution 1:200), Caspase-3 (Dilution 1:200).

Statistical analysis

SPSS software (SPSS Inc., Chicago, IL, USA) was used for relevant quantitative data statistical analyses. $P < 0.05$ was considered statistically significant. P-values are depicted in the figures as follows: P-value < 0.05 (*) and not significant (ns).

Results

The expression level of DDIT4 is decreased in pancreatic cancer tissues

In order to further clarify the expression level of DDIT4 in pancreatic ductal adenocarcinoma tissues, we detected the expression level of DDIT4 in tumor tissues of clinical patients, animal models and control groups respectively. Immunohistochemical showed that the expression level of DDIT4 in pancreatic cancer tissues of clinical patients samples (Fig.1A) and animal model samples (Fig.1B) was lower than that in control group. So did the result of Western blot(Fig.1C, D). The DMBA induced animal model of pancreatic cancer was successfully constructed and the tumor is located in the tail of the pancreas(Fig.1E).

DDIT4 is involved in fatty infiltration in pancreatic cancer tissues

In order to further explore the fatty infiltration in pancreatic cancer tissues, HE staining and oil-red O staining were performed on the clinical tumor samples and animal tumor samples. HE staining showed that the degree of fatty infiltration in clinical samples of pancreatic cancer tissues was significantly higher than that in control group (Fig. 2A). So did the result of oil-red O staining (Fig. 2B). As the HE staining (Fig. 2C) and oil-red O staining (Fig. 2D) show, the degree of fatty infiltration in DDIT4-HET pancreatic cancer animal model was much higher than that in wild-type animal model .

DDIT4 is involved in lipid metabolism of pancreatic cancer cells through PPAR- γ /CD36 and PPAR- γ /ATGL

In order to further clarify the downstream pathway proteins in DDIT4 signaling pathways that regulate lipid metabolism, Western blot and immunohistochemistry were applied to the clinical samples and animal models tissues. In the clinical samples, the results of Western blot suggest that the expressions of PPAR- γ , CD36 and ATGL in pancreatic cancer tissue were higher than those in control group (Fig. 3A). And the immunohistochemical or immunofluorescence results suggest CD36, ATGL and PPAR- γ expressions in pancreatic cancer tissue were higher than those in the control group (Fig. 3B). In the animal models tissues, Western blot results showed that the expressions of PPAR- γ , CD36 and ATGL in the tumor tissues of DDIT4-HET were higher than those in the wild type (Fig. 3C), and immunohistochemical or immunofluorescence results suggest CD36, ATGL and PPAR- γ in the tumor tissues of DDIT4-HET were higher than those in the wild type (Fig. 3D).

The DDIT4 is involved in apoptosis and proliferation of DDIT4-HET pancreatic cancer cell

To further clarify the role of DDIT4- mediated lipid metabolism in pancreatic cancer cells, we examined the levels of cell proliferation and apoptosis in the animal model of DDIT4-HET pancreatic cancer induced by DMBA. Immunohistochemical results showed that the expression level of ki-67 in DDIT4-HET pancreatic cancer animal model was significantly higher than that in the control group (Fig. 4A), and immunofluorescence results showed that the expression level of caspase-3 in DDIT4-HET pancreatic cancer animal model was lower than the control group (Fig. 4B). And we found that the volume of tumor tissue in the DDIT4-HET pancreatic cancer animal model was larger than that in the control group (Fig. 4C).

Discussion

DDIT4 can be involved in tumor regulation through a variety of pathways. Our work show that DDIT4 mediated fatty acid intake promote the proliferation of pancreatic cancer as the previous literature [15]. DDIT4/mTORC1 induced autophagy and promoted the survival of lung cancer cells [7] and glioblastoma [18], while it inhibited the growth of breast cancer cells [19]. DDIT4 promoted the proliferation and tumorigenesis of gastric cancer through the p53 and MAPK pathways [12], and it could promote the proliferation of non-small cell lung cancer cells regulated by the PI3K signaling pathway [20]. DDIT4 deficiency in tumor-associated macrophages lead to the formation of functional blood vessels and metastasis [21].

The degree of pancreatic *fatty infiltration*(FI) in *pancreatic* ductal adenocarcinoma patients was significantly higher than that in non-cancer patients, and pancreatic FI was involved in the development of animal and human pancreatic cancer [22]. Our results show that fat infiltration was significant in pancreatic cancer tissues in human and animal models, and oil-red O staining revealed intracellular and extracellular fat accumulation in pancreatic tumors. pancreatic cancer with increased expression of 5-lipoxygenase (an enzyme that converts omega-6 fatty acids into eicosanes) were characterized by increased mast cell infiltration and pancreatic progression [23]. Lipolysis of peripancreatic adipose tissue enhanced local invasion and metastasis through fatty acids released by adipocytes [24]. Fatty acid-mediated interstitial reprogramming of pancreatic stellate cells induced inflammation and fibrosis, thereby contributing to pancreatic cancer [25]. CD36 and ATGL are involved in fat infiltration of pancreatic cancer, while CD36 and ATGL expression is higher in DDIT4-HET animal models. CD36 promoted tumor metastasis and treatment of drug resistance by promoting lipid uptake and fatty acid oxidation [26],

CD36 can act as a direct target molecule of hydrogen sulfide and directly activate long-chain fatty acids into the cytoplasm [27]. CD36-mediated metabolic reprogramming was defined as a basic survival mechanism for HER2-positive breast cancer [28]. Omentum majus adipose cells reprogramed tumor metabolism and promoted tumor metastasis by upregulating CD36 expression in ovarian cancer cells [26]. ATGL expression was often reduced in various human cancers, such as lung, muscle, and pancreatic cancer [29]. *ATGL promoted the proliferation of hepatocellular carcinoma cells via the p-AKT signaling pathway* [30].

Conclusion

The low expression of DDIT4 was observed in the clinical samples of pancreatic cancer. We further demonstrated that DDIT4 promotes the proliferation and apoptosis of tumor cells through lipid metabolism, And it regulates lipid metabolism mainly through activation of PPAR- γ /CD36 or PPAR- γ /ATGL signaling pathways.

Declarations

Ethical approval and consent to participate

The study has been approved by the ethics committee of the First Affiliated Hospital of Fujian Medical University. All enrolled patients signed the informed consent for excess specimens to be used for subject research and the study have been performed in accordance with the Declaration of Helsinki.

The study experiments and relevant details on animal experiments were approved by the Ethics Committee of the First Affiliated Hospital of Fujian Medical University. The study was carried out in compliance with the ARRIVE guidelines for the reporting of animal experiments.

Consent for publication.

Not applicable.

Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Competing interests

The authors declare not having competing interests.

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Authors' contributions

CYT contributed to the project design and experiment design. DFD contributed to the writing and critical revision of the manuscript; GF contributed to the design and has been involved in the critical revision of the manuscript; HSR and HHJ contributed to experimental operation and results analysis of the manuscript; LW and LXT contributed to Discussion of experimental result and article translation of the manuscript. LQC and ZSY contributed to the analysis and interpretation as well as critical revision of the manuscript. All authors read and approved the final manuscript.

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Figures

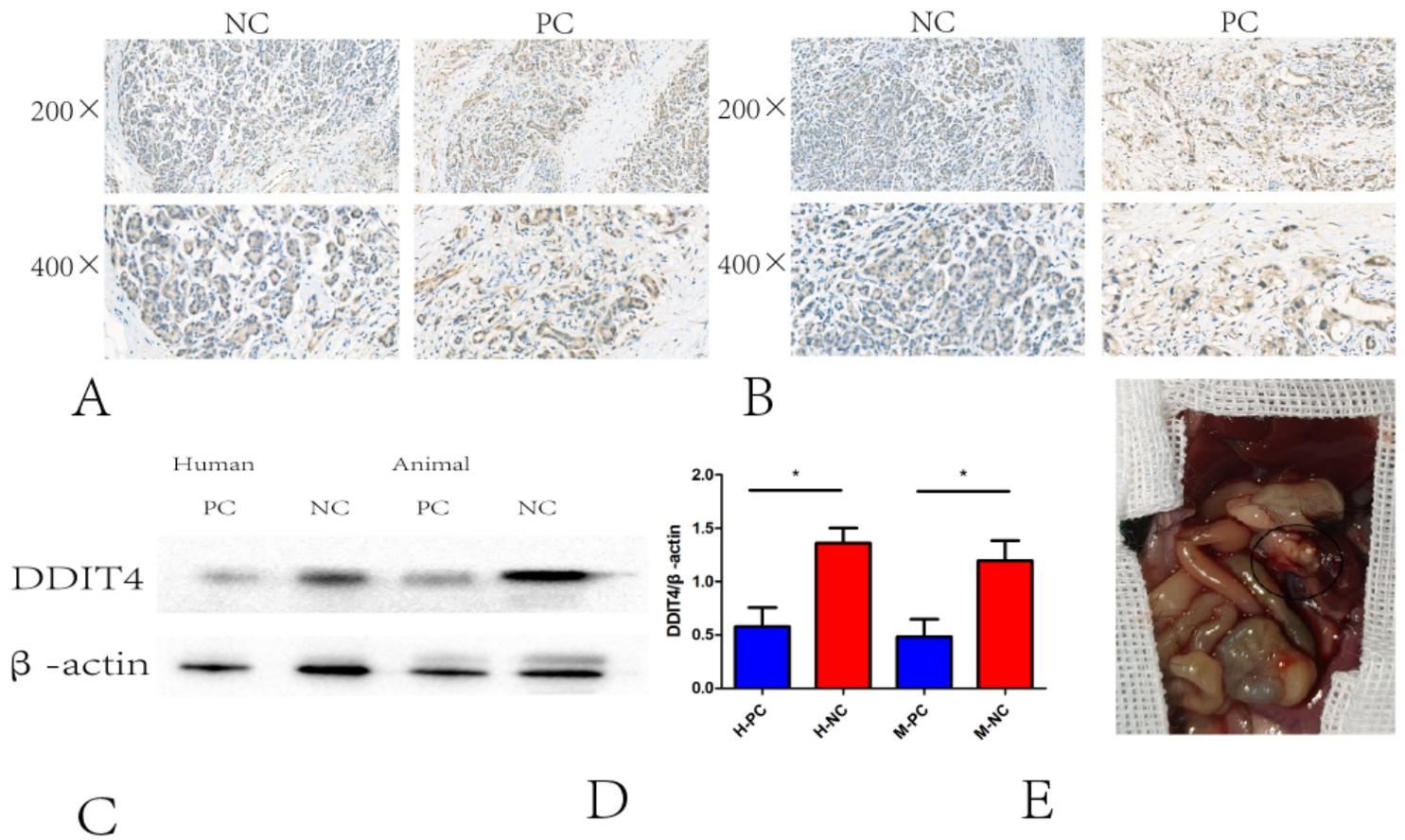


Figure 1

The expression level of DDIT4 in pancreatic cancer. (A) Immunohistochemical results of clinical samples; (B) Immunohistochemical results of animal models; (C) Western blot of tumor tissues; (D) Analysis of Western blot; (E) Anatomy and tumor in animal models.

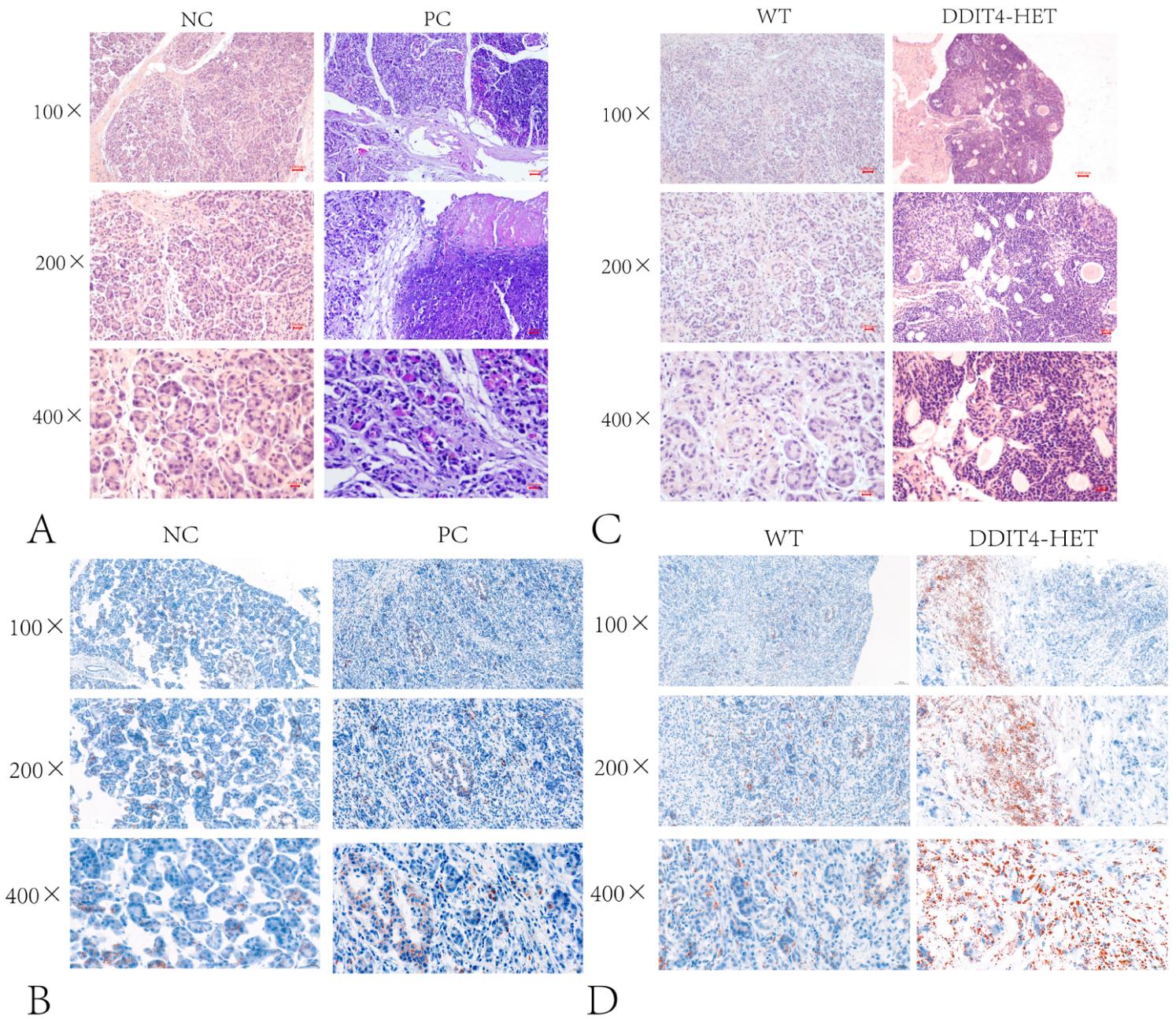


Figure 2

The degree of fat infiltration in pancreatic cancer tissues. (A) The HE staining of patients tissues; (B) The HE staining of DDIT4-HET mice tissues; (C) The oil-red O staining of patients tissues; (D) The oil-red O of DDIT4-HET mice tissues.

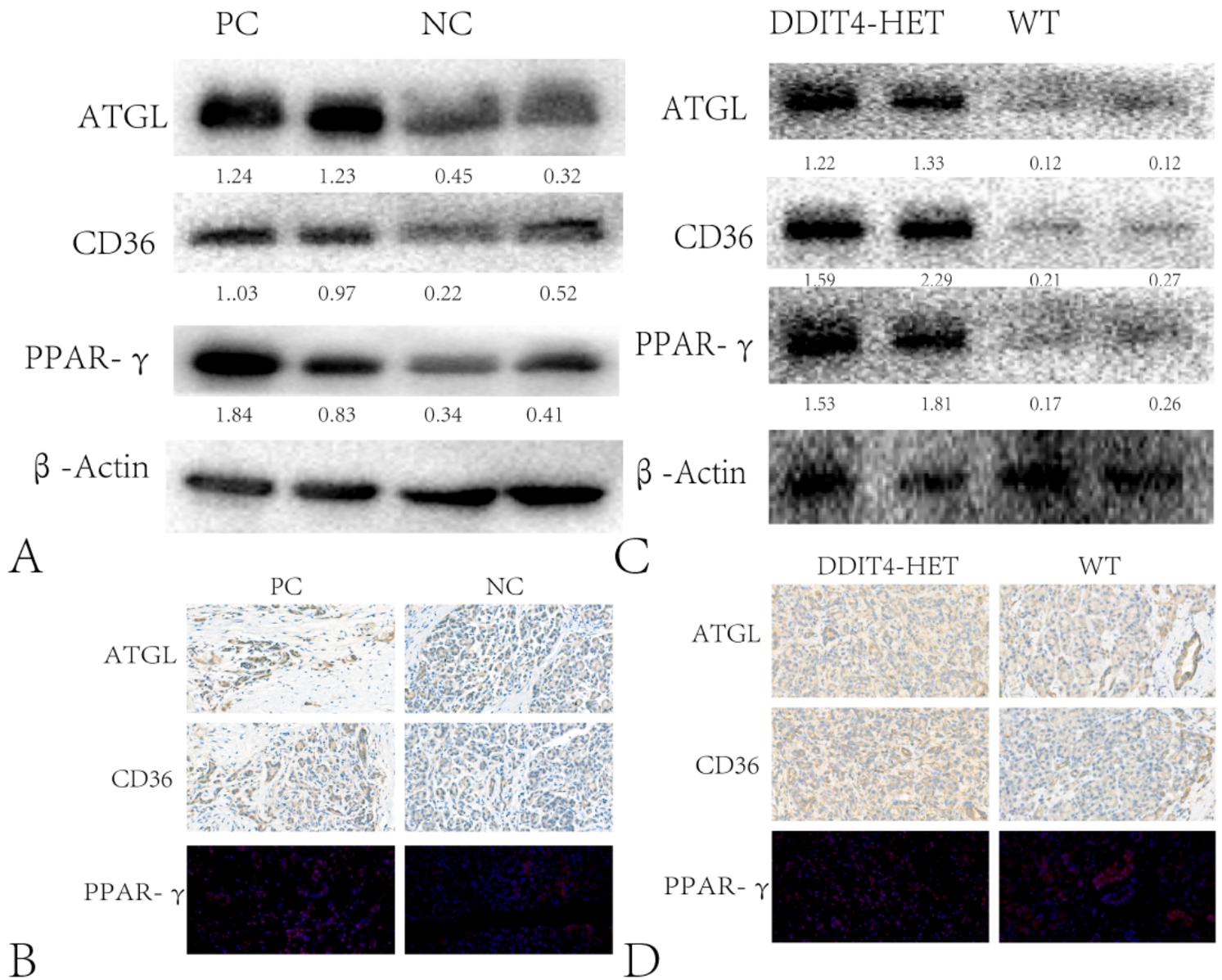


Figure 3

DDIT4 is involved in lipid metabolism of pancreatic cancer cells through PPAR- γ and CD36/ATGL. (A) The western blot in patients tissues; (B) The western blot in mice tissues; (C) The immunohistochemistry in patients tissues; (D) The immunohistochemistry in mice tissues.

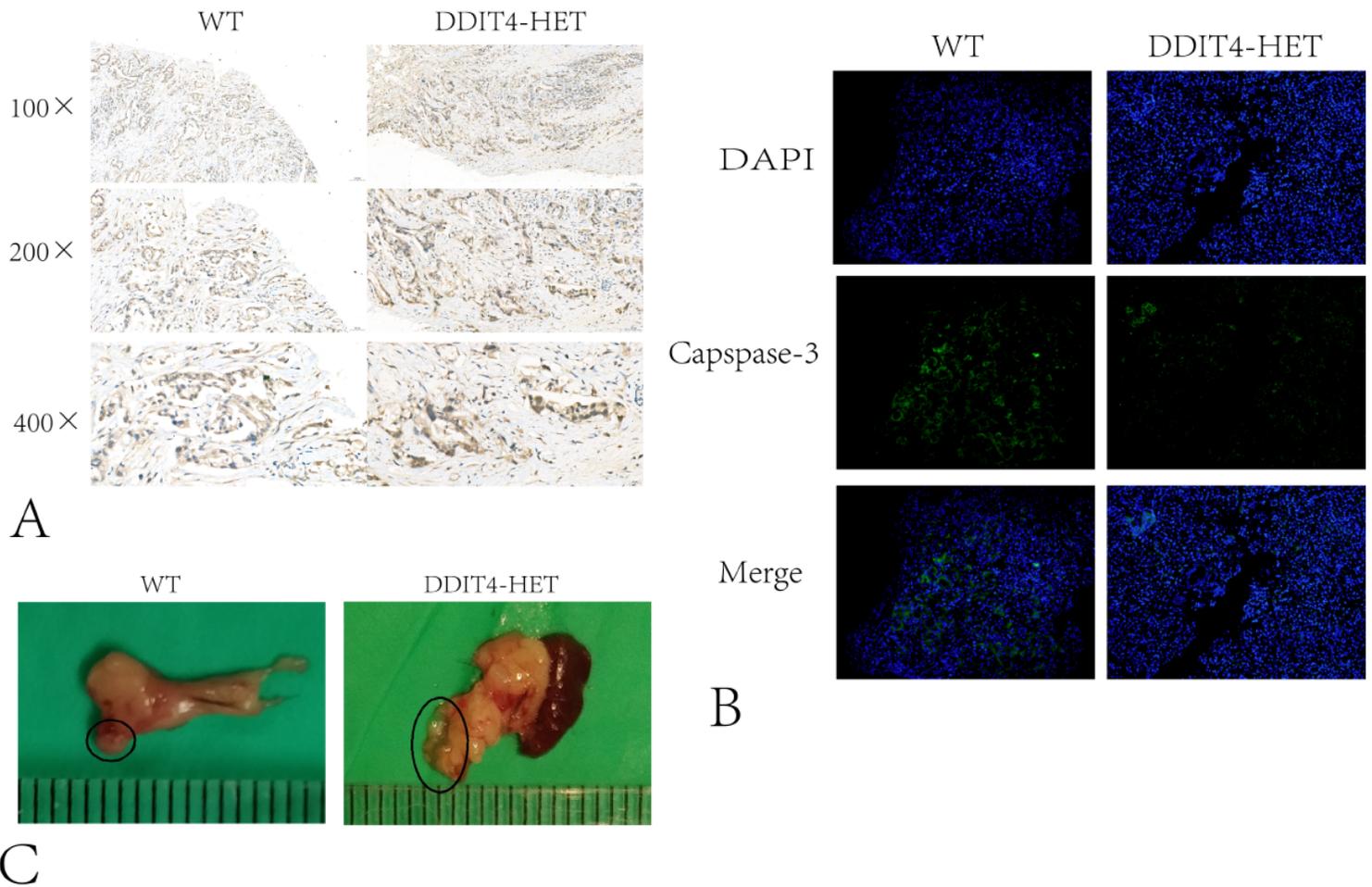


Figure 4

The DDIT4 is involved in apoptosis and proliferation of DDIT4-HET pancreatic cancer cell. (A) Immunohistochemical results of ki-67; (B) Immunofluorescence results of caspase-3; (C) Tumor specimens from animal model.