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IL-37 inhibits glycolysis of lung adenocarcinoma by inhibiting the expression of PFKFB3

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ABSTRACT

Aerobic glycolysis is one of the hallmarks of cancer. The metabolic phenotype of tumor cells is characterized by preferential dependence on glycolysis under aerobic conditions. Recent researchers have provided a piece of information on the effectiveness of targeting glycolysis. Thus, targeted glucose metabolism therapy is still a research hotspot. Interleukin 37 (IL-37) plays an important role in tumor development. Previous studies have found that IL-37 can inhibit the progression of lung adenocarcinoma in a variety of ways. For example, IL-37 can inhibit the migration and invasion of lung adenocarcinoma by inhibiting the interleukin 6(IL-6)/ Signal transducing activator of transcription 3(STAT3) pathway. IL-37 inhibits tumor growth by regulating RNA methylation at the M6A site of lung adenocarcinoma. It has been found that overexpression of IL-37 in macrophages can reverse the Warburg effect. The mechanism of IL-37 on glucose metabolism of tumor cells has not been studied. In research, glucose uptake and lactic acid production were inhibited in A549 cells with recombinant human IL-37(rhIL-37). Also, rhIL-37 inhibited the expression level of PFKFB3 in A549 cells. To verify whether the two aspects of rhIL-37's effects on A549 cells are related, we applied PFK15, a specific inhibitor of PFKFB3, to prove that rhIL-37 inhibits the glucose uptake and lactate production of A549 cells by inhibiting the expression of PFKFB3, and further inhibits the progression of lung adenocarcinoma.

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Introduction

Metabolic reprogramming is one of the main characteristics of malignant tumors, including aerobic glycolysis, lipid metabolism, and glutamine metabolic reprogramming, of which aerobic glycolysis is the most important (1). The concept of oxyglycolysis was first introduced in the 1920s when Warburg (A German physiologist) found that liver cancer cells consume less oxygen than normal liver tissue but can consume more glucose and produce more lactic acid (2). Aerobic glycolysis of tumor cells is a process of compensating for metabolic changes to maintain tumor cell proliferation. This process often involves the high expression of a variety of metabolism-related proteins (3). PFKFB3 is a member of the PFKFB family. It can activate fructose-2,6-diphosphate, an allosteric activator of fructose 1,6 diphosphate, and increase aerobic glycolysis (4). Studies have shown that PFKFB3 is overexpressed in a variety of tumors, and PFKFB3 is overexpressed in lung cancer, which is an independent risk factor for lung cancer (5).

IL-37 is expressed in a variety of immune cells, such as macrophages, dendritic cells (DCs), ton-sillar B cells, and plasma cells (6). The expression of IL-37 in the peripheral blood of healthy people is low, but IL-37 can be activated by proinflammatory stimuli such as LPS, the expression of IL-37 will increase to achieve the purpose of suppressing excessive inflammation(7).

IL-37 also plays a key role in tumor therapy, which is related to tumor proliferation, metastasis, transformation, and angiogenesis (8). It has been found that IL-37 can inhibit the progression of a variety of tumors, such as lung cancer (9), liver cancer (10), breast cancer (11), and colon cancer (12). Our previous study found that IL-37 can promote Wnt5B mRNA methylation by inhibiting the expression of a-ketoglutarate-dependent dioxygenase ALKB homolog 5 (ALKBH5), thereby inhibiting the expression of Wnt5B and affecting the progression of lung adenocarcinoma (13). The effect of IL-37 on tumors is very complicated, and the specific mechanism of IL-37 on tumor suppression is not yet fully cleared.

Studies have found that overexpression of IL-37 in macrophages can convert effective aerobic glycolysis to ineffective aerobic glycolysis, indicating that IL-37 has the ability to reverse the Warburg effect (14). Whether IL-37 can affect the aerobic glycolysis of tumors is still unclear, and we need to find out. We treated A549 cells with Recombinant Human IL-37 (rhIL-37) to detect changes in glucose uptake and lactic acid production. In order to explore the mechanism of the effect of rhIL-37 on glucose consumption and lactic acid production in A549 cells, we then used RT-PCR and WB to detect the changes in the expression of glucose metabolism-related enzymes HK2, PFKFB3, and PKM after rhIL-37 treatment in A549 cells. Furthermore, specific inhibitors were used to inhibit the target protein, which proved that rhIL-37 inhibited glucose uptake and lactic acid production by inhibiting the expression of related glucose metabolism enzymes. This study

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suggests that rhIL-37 may inhibit glucose uptake and lactic acid production by inhibiting the expression of glucose metabolism-related enzymes in A549 cells, which may influence the progression of lung adenocarcinoma.

Materials and Methods

Cell culture

The human lung adenocarcinoma A549 cell line was stored in the central laboratory of Qingdao Central Hospital and derived from the Shanghai Institute of Biology, Chinese Academy of Sciences. A549 cells were cultured in F-12 (DMEM/F-12) medium containing 10%FBS and 1% penicillin/streptomycin at 37℃ and 5% CO_2.

Cell treatments

A549 cells were treated with different concentrations (0 ng/ml, 10 ng/ml, 50 ng/ml, 100 ng/ml, 200 ng/ml) of rhIL-37 (R&D Systems, Minneapolis, MN, USA)for different times (24 h, 48 h, 72 h) to explore the optimal concentration and time; 1-(4-pyridinyl)-3-(2-quinolinyl)-2-propen-1-one(PFK15) (Sigma-Aldrich, St. Louis, MO, USA) treat A549 cells with different concentrations (0 μM, 2 μM, 4 μM, 6 μM, 8 μM, 10 μM) for 24 h to find the optimal concentration.

Measurement of glucose uptake and lactic acid production

After treating the A549 cells with the drugs for a suitable period of time, the culture medium was collected, and the glucose uptake and lactic acid production were detected with a Glucose assay kit and lactate acid assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

RNA extraction and real-time polymerase chain reaction

Total RNA was used to extract from A549 cells by using the TRIzol method. The primer sequences are shown in Table 1. PrimeScriptTMRT Reagent Kit with gDNA Eraser (TaKaRa, Dalian, China) was used for reverse transcription. Real-time PCR amplification was performed by using TB Green Premix Ex TaqTM II (Tli RNaseH Plus) (TaKaRa, Dalian, China). 7500 fast Real-time PCR system (Applied Biosystems, Foster City, CA, USA) was used to real-time fluorescence quantitative PCR instrument for amplification, use PCR instrument with 7500 software to analyze gene amplification, use 2-ΔΔc(t) method to calculate genes Differential expression.

Table 1. The information on primers used in this study.

Primers	Sequence (5'-3')
ACTB-F	GGGAAATCGTGCGTGACATT
ACTB-R	GGAACCGCTCATTGCCAAT
IL-37-F	CAGCTGAAGAAGGAGAAACTGATG
IL-37-R	ACAATTGCAGGAGGTGCAGAT
HK2-F	TCACCAAGCGTGGACTACTCTTC
HK2-R	CAGGTGCTCTCAAGCCCTAAGT
PFKFB3-F	GGGAGGCTGTGAAGCAGTACA
PFKFB3-R	CATCGAAAACCGCAATTTGTC
PKM-F	GAAGCCTGTCATCTGTGCTACTCA
PKM-R	GGATAGTCCCCTTTGGCTGTT

Western blotting

RIPA and PMSF (SolarBio Life Sciences, Beijing, China) were prepared into cell lysate at a ratio of 100:1. Cells and tissues were lysed to obtain protein extract, which was electrophoresis separated by SDS-PAGE and transferred to PVDG membrane. Membranes were blocked with 5% skimmed milk in tris-buffered saline containing 0.1% Tween 20 (TBST buffer) and incubated with anti-PFKFB3 antibody (Abcam, Cambridge, UK) and anti-IL-37 antibody (Abcam, Cambridge, UK) and incubated with goat anti-rabbit immunoglobulin G horse-radish peroxidase (ABclonal Technology, Wuhan, China). The presentation of the results depends on a suitable chemiluminescent substrate.

Statistical analysis

All data were analyzed by GraphPad Prism software (La Jolla, California, USA), gray value analysis was performed by Image J software (NIH), and pictures were sorted by Adobe Photoshop CS6 (SanJose, California, USA). The data was analyzed using t t-test to compare the two groups. A P-value of <0.05 was considered statistically significant.

Results

Expression of IL-37 and glucose metabolism-related proteins in normal lung epithelial cell line BEAS-2B cells and lung adenocarcinoma cell line A549 cells

First, we detected the expression of IL-37 and glucose metabolism-related proteins HK2, PFKFB3, and PKM in normal lung epithelial cell lineBEAS-2B cells and lung adenocarcinoma cell line A549 cells by RT-PCR. As shown in Figure 1, compared with BEAS-2B cells, the expression of IL-37 and HK2 in A549 cells was much lower, while the expression of PFKFB3 and PKM was much higher. These results indicate that the expressions of IL-37, HK2, PFKFB3 and PKM in normal lung epithe-

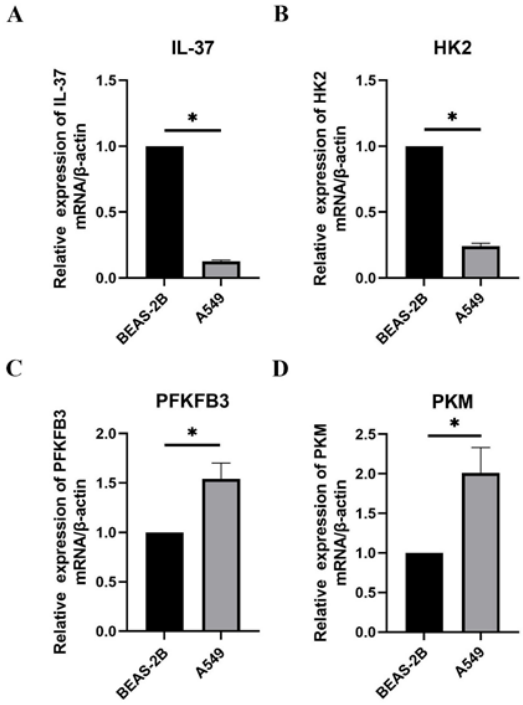

Figure 1. Expression of IL-37, HK2, PFKFB3 and PKM in A549 cells and BEAS-2B. *P < 0.05

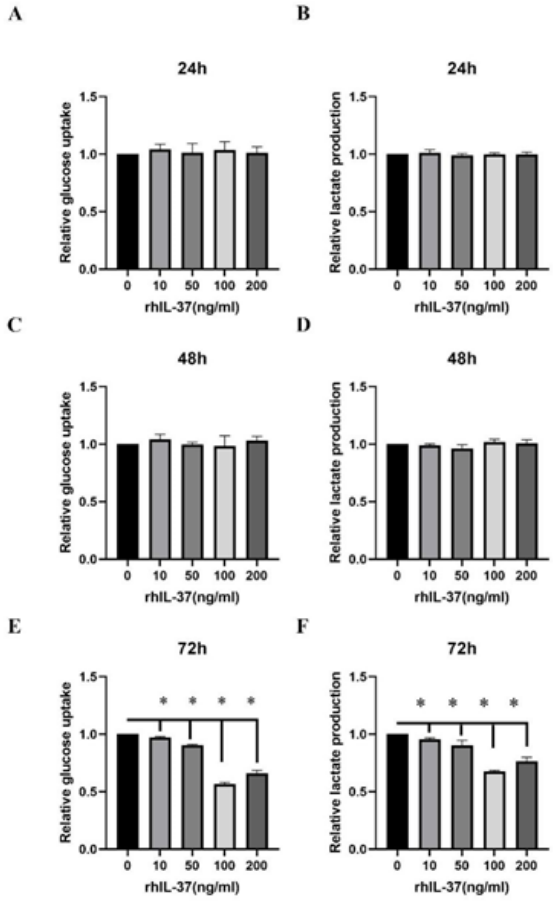

Figure 2. Glucose uptake and Lactate acid production of A549 cells with different concentrations of rhIL-37 protein (0, 10, 50, 100, 200 ng/mL). *P < 0.05.

lial BEAS-2B cells and lung adenocarcinoma A549 cells are different, and Whether this difference is related to the malignant phenotype of lung adenocarcinoma remains to be further studied.

rhIL-37 inhibits glucose uptake and lactate production in A549 cells

In order to prove the role of IL-37 in the glucose metabolism of A549 cells. A glucose assay kit and lactate acid assay kit were used to detect the changes in glucose uptake and lactic acid production of A549 cells treated with rhIL-37 in different concentrations (0ng/ml, 10ng/ml, 50ng/ml, 100ng/ml, 200ng/ml) for different period (24 h, 48 h, 72 h), and the appropriate stimulation concentration and action time of rhIL-37 were selected. As shown in Figure 2, there was no significant difference in glucose uptake and lactate production after A549 cells were treated with rhIL-37 for 24 and 48 hours. Glucose uptake and lactate production of A549 cells were significantly reduced after 72 hours of treatment with rhIL-37, and the most significant changes were observed when the concentration of rhIL-37 was 100 ng/ml. Therefore, rhIL-37 at a concentration of 100 ng/ml was used for further research.

rhIL-37 inhibits the expression of PFKFB3 in A549 cells

We used RT-PCR technology to detect the expression of HK2, PFKFB3 and PKM in A549 cells treated with rhIL-37. As shown in Figure 3(A-C), after A549 cells were treated with rhIL-37, there was no significant difference in the expression of HK2 and PKM, while the expression of PFKFB3 was significantly reduced. We further applied WB technology to detect the expression of the protein level of PFKFB3. As shown in Figure 3(D), the protein level of PFKFB3 was significantly reduced after A549 cells were treated with rhIL-37. These results indicate that rhIL-37 can inhibit the expression of PFKFB3 in A549 cells.

PFK15 can inhibit glucose uptake and lactate production in A549 cells

Our above experimental results found that rhIL-37 can inhibit the glucose uptake and lactate production in A549 cells and rhIL-37 can inhibit the expression of PFKFB3 in A549 cells. However, it is still unclear whether rhIL-37 can inhibit glucose uptake and lactic acid production by inhibiting the expression of PFKFB3 in A549 cells. Therefore, we applied PFK15, which is a specific inhibitor of PFKFB3 and can specifically inhibit the expression of PFKFB3.

First, we explored the optimal concentration of PFK15. As shown in Figure 4, PFK15 inhibited the expression of PFKFB3 in protein level of A549 cells in a dose-dependent manner. When the concentration of PFK15 is 10 μM, it can significantly inhibit PFKFB3 in A549 cells. Therefore, the concentration PFK15 of 10 μM was used for further research.

We used PFK15 at a concentration of 10 μM to treat A549 cells for 24 hours and then collected the culture medium to detect glucose uptake and lactate production. As shown in Figure 5, PFK15 can significantly inhibit the glucose uptake and lactate production of A549 cells. These results indicate that rhIL-37 can inhibit glucose uptake and

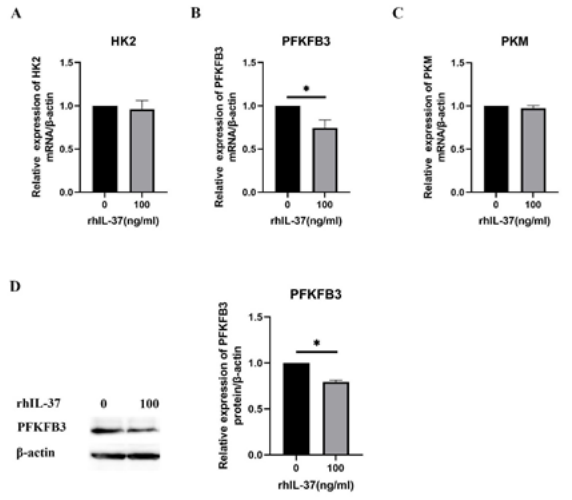

Figure 3. (A-C) Expression of HK2 mRNA, PFKFB3 mRNA and PKM mRNA in rhIL-37 treated A549 cells. *P < 0.05. (D) Expression of PFKFB3 protein in rhIL-37 treated A549 cells. *P < 0.05.

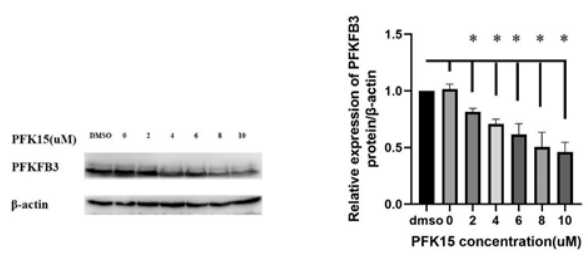

Figure 4. PFK15 suppresses the expression of PFKFB3 in a dose-dependent manner. *P < 0.05.

lactate production by inhibiting the expression of PFKFB3.

Expression of PFKFB3 and IL-37 in animal tumor tissues

The nude mouse xenograft model has been constructed in the previous experiment. We divided the nude mice into the non-specific control group(NC) group and the IL-37 group. Nudes in the NC group were injected with untreated A549 cells subcutaneously, it in the IL-37 group were injected subcutaneously with A549 cells that was treated with IL-37. Our previous study has found that the tumor tissue in the IL-37 group was significantly reduced in size and weight compared with the NC group. The tumor tissue obtained in the later period is stored in the laboratory. The expression of IL-37 and PFKFB3 in the tissues was detected by WB technology. As shown in Figure 6, compared with the NC group, the IL-37 expression of the IL-37 group was significantly increased, and the expression of PFKFB3 was significantly decreased, which was consistent with our above cell experiment results.

Discussion

Lung adenocarcinoma is one of the most common causes of cancer death in the world[15]. With the improvement of early detection and resection treatment, the survival rate of lung adenocarcinoma increases rapidly (16). However, lung adenocarcinoma is still one of the most prevalent malignancies in the world, so it is important to continue to search for more effective targets to improve the cure rate of lung adenocarcinoma.

IL-37 plays an important role in the development of tumors (17). Tumor-associated macrophages (TAMS) play an important role in promoting tumor progression (10). Peripheral blood mononuclear cells (PBMC) obtained from patients with hepatocellular carcinoma have the characteristics of polarization to M2 type, including reduced expression of IL-37 (10). Overexpression of IL-37 can inhibit the IL-6/STAT3 signaling pathway, thereby converting M2-polarized TAMS to M1 (10). There are research findings have found that IL-37 can affect the Warburg effect (14). The Warburg effect can be observed in activated macrophages including the expression and phosphorylation of rapamycin (mTOR) was increased, and the AMP kinase (AMPK) activity was decreased; overexpression of IL-37 in macrophages can reduce the expression of mTOR and increases the activity of AMPK, thereby transforming effective aerobic glycolysis into ineffective aerobic glycolysis, thus realizing the reversal of the Warburg effect (14). At present, the effect of IL-37 on the Warburg effect of tumor cells is still unclear, so we focused on exploring the effect of IL-37 on the glucose metabolism of lung adenocarcinoma. In our research, we found that IL-37 can inhibit the glucose uptake and lactic acid production of A549 cells. We speculate that IL-37 may inhibit glycolysis through some mechanism.

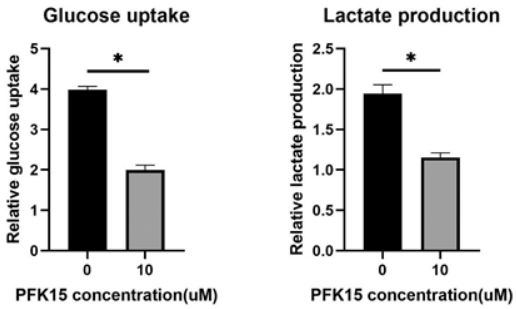

Figure 5. Glucose uptake and Lactate acid production of A549 cells with PFK15. *P < 0.05.

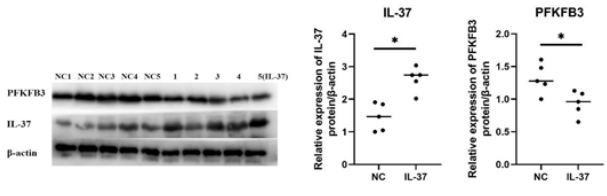

Figure 6. Expression of IL-37 and PFKFB3 in tumor tissues in vivo. *P < 0.05.

Different from normal cells, tumor cells still give priority to glycolysis to convert pyruvate into lactic acid under aerobic conditions (18). This metabolic phenotype is one of the important characteristics of malignant tumors and is known as "aerobic glycolysis", namely the "Warburg effect" (18). Previous research has found that glycolytic key protein expression in tumor cells, in order to realize the demand for high-efficiency glycolysis (19). PFKFB3 is overexpressed in tumors and is involved in tumor progression. PFKFB3 is a glycolytic enzyme that has both kinase activity and biophosphatase activity, and its kinase activity is much higher than biophosphatase activity (20). Studies have shown that PFKFB has no bisphosphatase activity in hepatocellular carcinoma, which may be the reason for its uncontrolled glycolytic metabolism (21). Lymphoxin-α (LT-α) secreted by activated lymphocytes can enhance the expression of PFKFB3 to enhance tumor endothelial cells (ECS) of glycolysis, promote the proliferation and migration of ECS, and thus promote the transfer of head and neck squamous cell carcinomas (22). Blocking PFKFB3 can inhibit the expression of VE-cadherin in ECS and tighten the vascular barrier, and due to the reduction of glycolysis, the metabolic activity of surrounding cells is reduced and the adhesion is enhanced. In addition, reducing the NF-κB signaling pathway can reduce the expression of adhesion molecules in the ECS, which can make the normalization of tumor blood vessels become (TVN), thus inhibiting tumor cell transfer (23). 1-(4-pyridinyl)-3-(2-quinolinyl)-2-propen-1-one (PFK15) is a specific PFKFB3 inhibitor that can inhibit glycolysis and proliferation of many tumors such as hepatocellular carcinoma (24), gastric cancer (25), pancreatic cancer (26), acute myeloid leukemia (27). PFK15 may become a new strategy targeting PFKFB3 to inhibit tumor growth and progression in the future (24). In our study, we found that compared with the normal lung epithelial cell line BEAS-2B, PFKFB3 mRNA is highly expressed in the lung adenocarcinoma cell line A549. IL-37 can inhibit the expression of PFKFB3 in A549 cells. Whether IL-37 inhibited the glycolysis of A549 cells by inhibiting the expression of PFKFB3 was verified by applying PFK15 to A549 cells.

In summary, our results show that rhIL-37 can inhibit the expression of PFKFB3 in A549 cells, thereby inhibiting glucose consumption and lactate production in A549 cells. This mechanism plays an important role in suppressing the malignant phenotype of lung adenocarcinoma.

Acknowledgments

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Conflict of Interests

The authors declared no conflict of interest.

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