

Free Fatty Acids Promote the Development of Prostate Cancer by Upregulating Peroxisome Proliferator-Activated Receptor Gamma

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Introduction: As one of the most common forms of cancer that threatens men's health, prostate cancer (PCa) is under a trend of increasing morbidity and mortality in most countries. More and more studies have pointed out that obesity is closely linked to the occurrence and development of PCa, although there are still many undiscovered molecular mechanisms between the two.

Methods: In the present study, we compare serum lipid levels in patients with PCa and normal individuals. PCa cells (PC3 and 22RV1) were cultured in vitro, the TC/TG/HDL/GLU assay kit was used to detect the glucose and lipid metabolism level of PCa cells, the flow cytometry technique was used to detect the proliferation ability of PCa cells, and the Transwell was used to detect the invasion and migration ability of PCa cells. Western blot/quantitative real-time PCR was used to detect peroxisome proliferator-activated receptor γ (PPAR γ) and vimentin/vascular endothelial growth factor-A (VEGF-A) expression levels, and immunohistochemistry was used to observe tumor-associated gene expression levels in nude mice. All data were analysed using the Independent samples *t*-test or rank sum test.

Results: We found higher levels of FFA in the serum of patients with PCa. In vitro experiments have demonstrated that high levels of FFA can promote the proliferation, migration and invasion of two PCa cells (PC3 and 22RV1) and affect the energy metabolism of PCa cells. The upregulated PPAR γ plays a key role in this process, and vimentin may be involved in this signaling pathway.

Conclusion: We infer that high levels of FFA may promote PCa development by up regulating PPAR γ expression.

Keywords: free fatty acids, peroxisome proliferator-activated receptor γ , prostate cancer, vimentin, vascular endothelial growth factor-A

Introduction

As the most commonly diagnosed form of cancer among middle-aged and elderly men, PCa ranks second in male mortality,^{1,2} and with the aging of the population, the incidence of PCa in most countries is increasing.³ Earlier studies have pointed out that there is a direct relationship between the increase of body mass index and PCa mortality.⁴ In PCa patients, tumor volume is significantly associated with body mass index, and obesity is associated with more aggressive PCa and higher post-treatment disease recurrence rates.⁵

Dyslipidemia, especially increased blood FFA levels, is an important manifestation in obese patients.⁶⁻⁸ Previous studies have shown that high levels of FFA can promote the occurrence and development of a variety of tumors.^{9,10} Existing literature has suggested

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that in the presence of palmitate, the glucose metabolism levels of PCa cells PC3 and CWR22RV1 are significantly increased.¹¹ Other literature has suggested that OA promotes an aggressive phenotype in PCa cells via FFA1/GPR40, calcium and PI3K/Akt signaling.¹² In addition, fatty acids can also act as specific receptors that affect intracellular signaling, thereby regulating castration resistance and metastasis of PCa.¹³ The above literature shows that dyslipidemia, especially the increase of FFA content, can be used as energy substance and signal molecule to promote the occurrence and development of PCa. However, its specific molecular mechanism is still not very clear.

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear receptor transcription factor superfamily that regulates the expression of target genes. According to the structure, PPAR can be divided into three types: α , β (or δ) and γ . PPAR γ is mainly expressed in the adipose tissue and the immune system.^{14,15} PPAR γ plays a major role in fat formation, glycolipid metabolism, and the immune system by regulating the transcriptional activity of target genes.¹⁶ A large amount of literature shows that PPAR γ has a comprehensive anticancer activity due to its anti-proliferative, differentiation-promoting and pro-apoptotic functions.¹⁷ The use of PPAR γ -selective ligand troglitazone can inhibit the proliferation of tumor cells such as human colon cancer cells and breast cancer cells, induce differentiation, and reduce tumor volume in nude mice.¹⁸ Recently, Orootan et al found that inhibiting the expression of PPAR γ in PCa cells can significantly reduce proliferation (53%), invasiveness (89%), and non-hormone-dependent growth rate (94%).¹⁹ These results indicate that PPAR γ has a completely opposite effect in the development of different tumors, and the role of PPAR γ in FFA-induced PCa has not been reported in the literature.

In this study, we want to evaluate the role of PPAR γ in the development of PCa caused by increased FFA due to obesity and elucidate its possible molecular mechanism. The implementation of this study will provide a new theoretical basis for explicating the possible mechanism of obesity-induced PCa. At the same time, it will provide a new experimental basis for clarifying the role of PPAR γ in the development of PCa.

Materials and Methods

Collection of General Information and Serum Samples of Clinical Individuals

From September 2017 to March 2018, in the Department of Urology, First Affiliated Hospital of Shihezi University

School of Medicine, 10 blood samples from prostate cancer patients with a clear diagnosis were collected, aged 55 to 85 years. After strict matching the age of tumor patients, 10 blood samples were collected from the physical examination in the clinic as controls that they excluded patients with prostate disease and various types of tumors. General data on height, weight, body mass index (BMI), were collected. BMI was calculated according to the formula $BMI = \text{body weight (kg)}/\text{height (m)}^2$.

To avoid circadian rhythm changes, blood samples were taken from 8:00 to 10:30 a.m. after an overnight fast of at least 8 hrs. Venous blood was immediately placed in a separate gel accelerating tube for centrifugation (4000 RPM x 10 min). Some serum was used to detect the relevant biochemical indicators. The remaining serum was immediately kept at -80°C .

Serum TC/TG/HDL/GLU Analysis

Non-esterified fatty acids Kit (A042-2-1)/Triglycerides Assay Kit (F001-1-1)/Total cholesterol Assay Kit (F002-1-1)/High density lipoprotein cholesterol Assay Kit (F003-1-1)/Low-density lipoprotein cholesterol assay kit/(A113-1-1) from Nanjing Jiancheng Bioengineering Institute, China, was used to detect the concentration of FFA/TG/TC/HDL/LDL in the serum of patients. The serum concentration of GLU in the patient's serum was measured using the Glucose Assay Kit (CAT: 361500) of Shanghai Rongsheng Biopharmaceutical Co., Ltd., China. The metabolomic platform of Tsinghua University in China used Liquid chromatography-mass spectrometry to quantitatively analyze various fatty acid components in the serum of all PCa patients and normal individuals.

Cell Lines and Cell Culture

Human PCa cells (PC3 and 22RV1) were obtained from the Shanghai Cell Bank of the Chinese Academy of Sciences. PC3 cells were cultured in F12 medium containing 10% FBS, 1% penicillin-streptomycin. 22RV1 cells were cultured in 1640 medium containing 10% FBS, 1% penicillin-streptomycin. The overexpression plasmid and siRNA interference fragment used for cell transfection were purchased from Shanghai GenePharma Company, China.

FFA Configuration

FFA in preparation for oleic acid/palmitic acid = 2/1 (OA: PA = 2:1), of which OA was dissolved in DMSO for 0.67 mol/L, and PA was dissolved in DMSO for 0.33 mol/L.

Using the culture medium containing 1% BSA to dilute FFA to 1.5mM (PC3) or 1.0mM (22RV1).

Intracellular TC/TG/HDL/GLU Analysis

After washing PCa cells of different groups with PBS twice, trypsinize and centrifuge at 1000 rpm for 5 mins. Wash and centrifuge for 5 mins. After resuspending the cells in 300 μ L PBS, immediately put them into liquid nitrogen for 5 s and then take out the room temperature to thaw. After repeated freezing and thawing 3 times, add the working solution according to the kit instructions. After incubation at 37°C, check the OD value and calculate the final TC/TG/HDL/GLU concentration according to the announcement. Triglycerides Assay Kit (F001-1-1)/Total cholesterol Assay Kit (F002-1-1)/High density lipoprotein cholesterol Assay Kit (F003-1-1) from Nanjing Jiancheng Bioengineering Institute, China, was used to detect the concentration of TG/TC/HDL in the PCa cells. The concentration of GLU in the PCa cells was measured using the Glucose Assay Kit (CAT: 361500) of Shanghai Rongsheng Biopharmaceutical Co., Ltd., China.

Plasmid Transfection

Transfected PCa cells with over-expressed PPAR γ plasmid constructed by GenePharma to upregulate PPAR γ in PCa cells. The Ad-PPAR γ group used pcDNA3.1 (+) PPAR γ plasmid, the mock group used pcDNA3.1 (+) NC plasmid (which does not contain the PPAR γ promoter region), and the blank group did not use pcDNA3.1 plasmid. During the transfection process, DNA-lipofectamine complex of three plasmids was formed with P3000 and Lipofectamine3000 as vectors at a concentration of 1 g/mL at room temperature, incubated in FBS-free medium for 4 h, and then cultured cells with FBS containing cell cultures for 48 h.

Flow Cytometry

PC3 and 22RV1 cells were transfected with different experimental conditions for 48 hrs, followed by trypsinization and collection. The single cell suspension was fixed with 70% ethanol (added drop-wise) and stored overnight at 4°C. The fixed cells were then washed twice with ice-cold PBS and stained with PI (33 μ g/mL with 0.1% Triton X-100) in the presence of 500 μ g/mL RNase A in the dark for 1 hr at room temperature. DNA content was analyzed on a flow cytometer. The derived data were analyzed with Software to estimate the percentages of cells in G0/G1, S, and G2/M phases.

Cell Invasion

A Transwell chamber with a pore size of 8 μ m and a diameter of 6.5 mm was used to coat the upper chamber of the Transwell chamber with Matrigel and incubated for 48hrs in a 37°C incubator. PC3 and 22RV1 cells were transfected with different experimental conditions for 48 hrs, the cells of were digested and counted, and a single cell suspension was prepared by using a serum-free medium transferred to the upper chamber of the Transwell chamber (3×10^4 cells/chamber), while a medium containing 10% FBS transferred in the lower chamber. After 48 hrs of routine culture, cells that had moved to the lower surface of the membrane were stained using Giemsa for 7 to 8 mins and counted microscopically. The mean average value was calculated based on five different observation fields.

Cell Migration

PC3 and 22RV1 cells were transfected with different experimental conditions for 48 hrs, the cells of were digested and counted, and transferred to the upper chamber of the Transwell chamber (3×10^4 cells/room) that is a pore size of 8 μ m and a diameter of 6.5 mm with a serum-free cell suspension, while the medium in the lower chamber contained 10% FBS. After 48hrs incubation, the upper surface of the Transwell membrane was gently wiped with a cotton swab to remove nonmigrating cells. Cells that had settled in the lower surface of the membrane were stained using Giemsa for 7 to 8 mins and counted microscopically. The mean average value was calculated based on five different observation fields.

Western Blot

Protein was lysed by using lysis buffer containing 1% PMSF in RIPA to extract the total cellular protein. Then, the protein concentration was tested and adjusted. The 4 \times SDS-PAGE loading buffer added to each sample was one-third of the protein volume. The protein was heated at 100°C for 10 mins. The β -actin protein expression was used as an internal control. Membranes were incubated at 4°C overnight with antibodies to β -actin (36 kDa; Zhongshan Jinqiao, China) and PPAR γ (58kDa; Abcam), at a working ratio of 1:1000. A second antibody was incubated at room temperature for 2hrs at a working ratio of 1:10,000. The chemiluminescence reagent (SuperSignal West Femto Maximum Sensitivity Substrate; Thermo Scientific) was used and detected with a ChemiScope mini-imaging system.

Quantitative Real-Time PCR

Cells were lysed with TRIzol reagent and the total RNA was extracted. RNA purity was determined by using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The amount of RNA used to reverse transcribe 20 µL of cDNA was 1 µg. The reverse transcription (Eppendorf AG, Germany) program settings were 42°C for 60 mins and 70°C for 15 mins. The mRNA expression was detected by using a qRT-PCR instrument (QIAGEN, Hilden, Germany) with the following program settings: 95°C for 3 mins, 45 cycles at 95°C for 10 s, and 60°C for 30 s. GAPDH was used as an internal control. Data were obtained as Ct values, and the $2^{-\Delta\Delta Ct}$ ($\Delta\Delta Ct$ = single sample ΔCt – average ΔCt of all samples in this group; ΔCt = single target gene Ct – single sample GAPDH Ct) method was used in the analysis. Gene expression was quantified using a relative method. Table 1 shows the primer sequences used.

Animals Feeding

Twelve 4-week old male nude mice (BALB/c-nu, Vital River, Beijing, China) were housed in SPF-level animal house laminar flow frames. After one week of adaptive feeding, 5×10^5 PC3 cells were injected into the prostate of each nude mouse.²⁰ Six days after the experiment, three were fed with normal fat containing 10% fat, and nine were fed with high-fat diet containing 60% fat (Medicine, Jiangsu, China). All food and drinking water are strictly sterilized and free to ingest. After 12 weeks, they were anesthetized, surgically harvested the intra-abdominal tumor tissue, measured tumor length and width using vernier caliper, calculated tumor volume ($V = 1/2 \text{length} \times \text{width}^2$), and weighed (g) the tumor using analytical balance.

Immunohistochemistry Staining

All specimens were set in 10% neutral formalin and embedded in paraffin, 4 µm thick sections. After dewaxing the sections, the antigen was repaired with sodium citrate buffer, washed in 1×PBS buffer, diluted 1:100 PPARγ antibody (Abcam, 41928), 1:100 VEGF-A antibody (Abcam, ab1316) and 1:100 The Vimentin antibody (Cell Signaling Technology, D21H3) was incubated overnight at 4°C, and the next day, the anti-mouse HRP secondary antibody (DAKO) was incubated at 37°C for 30 mins, and the results were observed after DAB color development. The scores were evaluated by two pathologists in the First Affiliated Hospital of Shihezi University School of Medicine. The scoring criteria were: the percentage of positive cells (0–5%, 0; 6–25%, 1; 26–50%, 2; 51–75%, 3; 76–100%, 4), positive staining intensity (negative, 0; yellow, 1; brownish yellow, 2; brown, 3), and the final total score is equal to the percentage of positive cells multiplied by the positive staining intensity.

Statistical Analysis

SPSS (v. 17.0) computer software was used for all statistical analysis. Mean and standard deviation were determined as the main parameters, and the average of data between the experimental and control groups were compared using independent samples *t*-test or Rank sum test. Determine Sig. <0.05 as a standard of significant difference.

Results

Serum FFA Level of PCa Patients Was Higher Than Non-Cancer Individuals

Serum samples from ten PCa patients and ten non-cancer individuals were collected from the first affiliated hospital

Table 1 Primer Sequences

	Primer	Sequences (5'→3')	Fragment
Human	Human-PPARγ-F Human-PPARγ-R	AGCCTGCGAAAGCCTTTTGGTG GGCTTCACATTACAGCAAACCTGG	152 bp
	Human-VEGFA-F Human-VEGFA-R	TTGCCTTGCTGCTCTACCTCCA GATGGCAGTAGCTGCGCTGATA	126 bp
	Human-Vimentin-F Human-Vimentin-R	AGTCCACTGAGTACCGGAGAC CATTTCACGCATCTGGCGTTTC	98 bp
	Human-GAPDH-F Human-GAPDH-R	GGAGCGAGATCCCTCCAAAT GGCTGTTGTCATACTTCTCATGG	197 bp
Interference fragment	PPARγ-homo-756	GCGGAGAUCUCCAGUGAUATT UAUCACUGGAGAUCUCCGCTT	

of Shihezi University Medical College. General data of subjects were collected and blood lipid and FFA levels were measured. Our results showed that the serum TPSA and FFA level is significantly higher in PCa patients compared with non-cancer individuals ($P<0.05$), even though the body weight and BMI have been significantly over-consumed due to disease progression, as shown in Table 2.

In addition, liquid chromatography-mass spectrometry was used to detect FFA profiles in plasma of subjects, and to compare the differences between non-cancerous individuals and PCa patients. The results show that the various components of the total FFA represent a large proportion of saturated fatty acids (PA (16:0)) and unsaturated fatty acids (OA (18:1)), and in the serum of PCa patients as shown in Figure 1A.

High Concentration of FFA Promoted the Expression Level of PPAR γ and Resulted in the Change of Biological Behavior in PCa Cells

High Levels of FFA Can Promote PPAR γ Expression in PCa Cells

PC3 and 22RV1 were stimulated with different concentrations of FFA (OA: PA = 2:1) for 24hrs (Table 3). We found that FFA greater than 1.5mM inhibited both PCa cells. Next, we used oil red O staining (Figure 1B) and cell glucose and lipid metabolism kit (Table 4) to observe the effects of 0mM, 0.5mM, 1mM, and 1.5mM FFA on lipid synthesis and energy utilization capacity of two PCa cells. Finally, combined with the results of Table 3, Figure 1B and Table 4, we chose the highest concentration without inhibiting cell growth, and the strongest ability to promote cell lipid synthesis and energy utilization 1.5mM and 1mM acting on PC3 Cells and 22RV1 cells. PCa cells were stimulated with different concentrations

of FFA (OA: PA = 2:1) for 48hrs, and the expression of PPAR γ , VEGF-A, and Vimentin was detected. The results showed that the mRNA and protein expression levels of PPAR γ were significantly increased under the stimulation of high concentration FFA in PC3 and 22RV1 cells ($P<0.05$), as shown in Figure 1C–F. In addition, the mRNA expression levels of VEGF-A and Vimentin were also significantly increased under the stimulation of high concentration FFA ($P<0.01$), and the results are shown in Figure 1C and D.

High Concentration of FFA Enhanced the Ability of Proliferation, Migration and Invasion of PCa Cells

As demonstrated in Figure 1G, after high concentration FFA (OA: PA = 2:1) stimulated PC3 cells, the proportion of cells in G0/G1 phase was significantly reduced, while that in S phase was significantly increased ($P<0.05$). Same case happened after high concentration FFA stimulated 22RV1 cells, as shown in Figure 1H. The above results showed that high concentration of FFA might promote the proliferation of PCa cells by regulating the phases of the cell cycle. As shown in Figure 1I and J, after high concentration FFA stimulated PC3 and 22RV1 cells, the migration and invasion ability of cells were also significantly enhanced ($P<0.05$).

PPAR γ Can Promote the Metabolism of Glucose, Lipid, and the Biological Behavior of PCa Cells

PPAR γ Can Promote the Metabolism of Glucose and Lipid in PCa Cells

After upregulation of PPAR γ in Figure 2A and B, the levels of TC, TG and GLU in both types of cells were significantly increased ($P<0.05$), and the level of HDL in 22RV1 cells was also significantly increased ($P<0.05$), as shown in Table 5. In addition, it was found that after down-regulation of PPAR γ in Figure 3A and B, the levels of TC, TG and HDL in both types of cells were significantly decreased ($P<0.05$). In PC3 cells, the level of GLU was significantly increased ($P<0.05$), as shown in Table 6.

PPAR γ Enhanced the Ability of Proliferation, Migration and Invasion of PCa Cells

After upregulation of PPAR γ , the mRNA expression levels of VEGF-A and Vimentin of all types of cells were also significantly increased in Figure 2C. As depicted in Figure 2D and E, after upregulation of PPAR γ , the proportion of PC3 cells at G0/G1 phase was significantly decreased, and G2/M phase and

Table 2 Patient Characteristics

Characteristics	NC (n=10)	PCa (n=10)	P
Age (years)	73.80±6.94	75.10±7.96	0.702
Height (cm)	168.80±4.15	168.20±3.96	0.745
Weight (kg)	75.50±7.50	59.20±11.35	0.002
BMI (kg/m ²)	26.33±2.73	21.03±4.62	0.006
TPSA (ng/mL)	0.69±0.19	95.81±8.84	<0.001
FFA (mmol/L)	0.63±0.22	0.90±0.31	0.033
TC (mmol/L)	1.38±0.48	1.12±0.80	0.433
TG (mmol/L)	3.89±0.75	3.94±0.75	0.909
HDL (mmol/L)	1.81±0.72	1.70±0.83	0.771
LDL (mmol/L)	1.33±0.54	1.66±0.63	0.286
GLU (mmol/L)	5.06±1.04	6.43±3.18	0.216

Notes: Independent samples t- test; $P < 0.05$ was considered statistically significant.

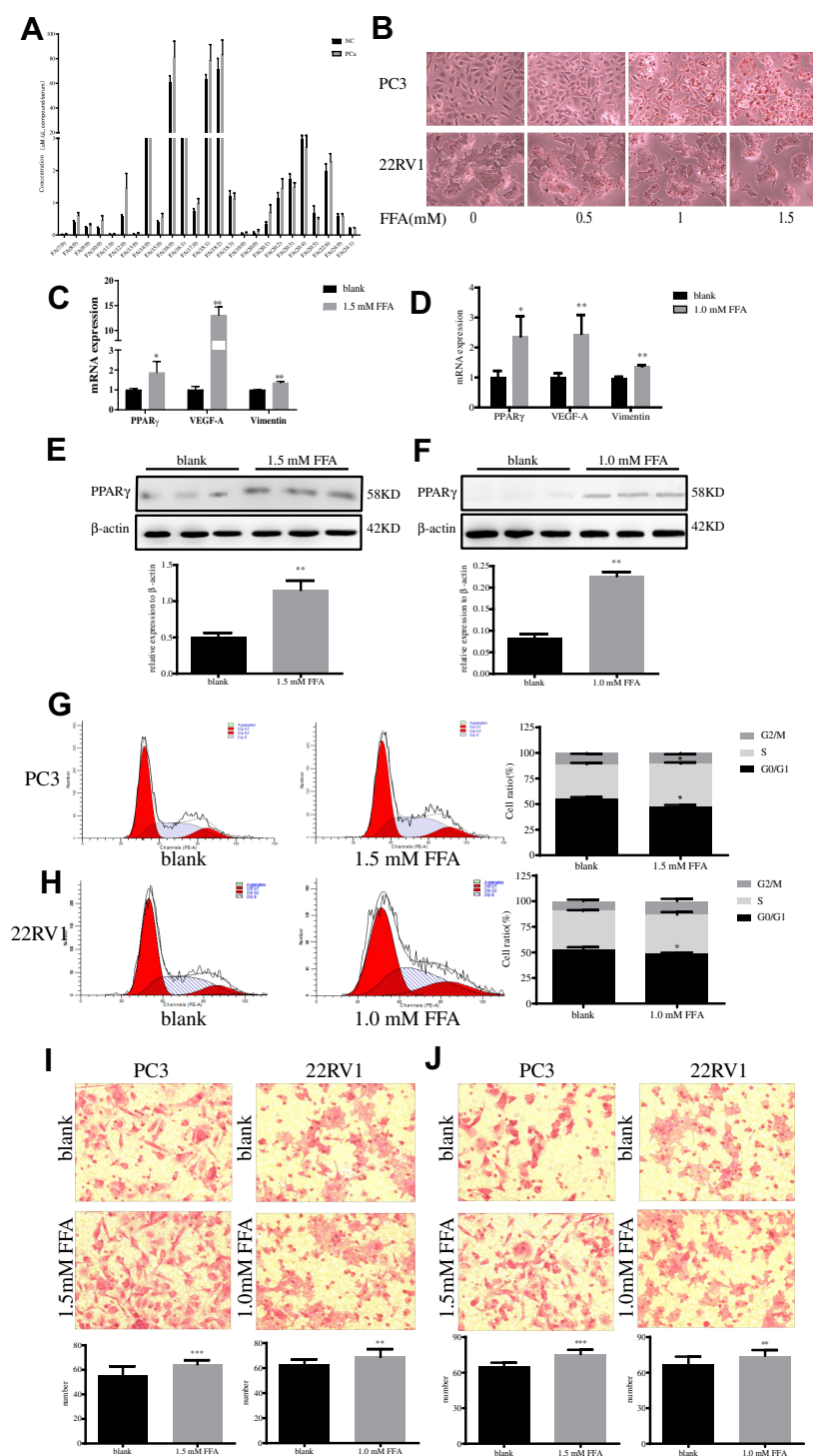


Figure 1 Elevated concentrations of free fatty acids result in elevated levels of PPAR γ and change prostate cancer cells' biological behavior: **(A)** Liquid chromatography-mass spectrometry was used to detect the level of 25 fatty acids in serum of 10 prostate cancer patients and 10 non-cancer individuals. **(B)** Oil red O staining results on PC3 and 22RV1 cells with different concentrations of FFA as 0mM, 0.5mM, 1mM and 1.5mM (200 \times). **(C)** After 48 hrs of stimulation to PC3 cells with 1.5 mM FFA, real-time quantitative PCR showed mRNA expression levels of PPAR γ , VEGF-A and Vimentin. **(D)** After 48 hrs of stimulation to 22RV1 cells with 1.0 mM FFA, real-time quantitative PCR showed mRNA expression levels of PPAR γ , VEGF-A and Vimentin. **(E)** After 48 hrs of stimulation to PC3 cells with 1.5 mM FFA, Western Bolt showed protein expression levels of PPAR γ . **(F)** After 48 hrs of stimulation to 22RV1 cells with 1.0 mM FFA, Western Bolt showed protein expression levels of PPAR γ . **(G)** Flow cytometry results of PC3 cells in blank control group without FFA stimulation, and experimental group after 1.5mM FFA stimulation. Then compare the G0/G1 phase, S phase and G2/M phase of PC3 cells between two groups. **(H)** Flow cytometry results of 22RV1 cells in blank control group without FFA stimulation, and experimental group after 1.0 mM FFA stimulation. Then, compare the G0/G1 phase, S phase and G2/M phase of PC3 cells between two groups. **(I)** PC3 and 22RV1 cells' migration results after stimulation with free fatty acids using transwell experiment (200 \times). **(J)** PC3 and 22RV1 cells' invasion results after stimulation with free fatty acids using transwell experiment (200 \times). Independent samples *t*-test. **P*<0.05, ***P*<0.01, ****P*<0.001 was considered statistically significant.

Table 3 The Inhibition Rate on PCa Cells with Different Concentrations of FFA

FFA (mM)	PC3		22RV1	
	OD (n=30)	Inhibition Rate (%)	OD (n=30)	Inhibition Rate (%)
0	1.061±0.183	–	1.202±0.025	–
0.5	1.283±0.055	–20.95	1.267±0.026	–5.49
1	1.343±0.031	–26.55	1.257±0.025	–4.73
1.5	1.431±0.049	–34.86	0.917±0.019	23.62
2	1.378±0.024	–29.84	0.514±0.020	57.20
4	1.245±0.077	–17.38	0.156±0.011	86.96

Notes: Inhibition rate (%) = ((OD value of blank group-OD value of experimental group)/OD value of blank group) × 100%.

S phase were significantly increased. In 22RV1 cells, the proportion of G0/G1 phase was significantly decreased, and the proportion of G2/M phase was significantly increased ($P<0.05$). At the same time, both the migration and invasion abilities of the two kinds of cells were significantly enhanced after upregulation of PPAR γ , as illustrated in Figure 2F and G.

After down-regulation of PPAR γ , the mRNA expression levels of VEGF-A and Vimentin of PC3 cells were significantly reduced, and the mRNA expression levels of Vimentin of 22RV1 cells were also significantly reduced in Figure 3C. The proportion of G0/G1 phase was significantly increased, and the proportion of S phase and G2/M phase was significantly reduced in PC3 cells ($P<0.05$). The proportion of G2/M phase was significantly reduced in 22RV1 cells ($P<0.05$), as shown in Figure 3D and E. At the same time, after down-regulation PPAR γ , the migration and invasion abilities of both types of cells were significantly inhibited ($P<0.01$), as demonstrated in Figure 3F and G.

The Expression of PPAR γ Was Significantly Higher in Nude Mice with PCa After High-Fat Diet

PC3 cells were injected in situ into the prostate of nude mice. They were fed a high-fat diet (HFD) and a normal control diet (NC) for 12 weeks, as shown in Figure 4A–C. The tumor volume and weight in HFD group ($n = 9$) are higher than the NC group ($n = 3$). As displayed in Figure 4D, the results of immunohistochemistry showed that the expression level of PPAR γ /VEGF-A/Vimentin was significantly higher than that of NC group ($P<0.01$). This further indicates that overexpression of PPAR γ in PCa tissues is associated with PCa under a high-fat diet.

Discussion

Obesity is closely related to the occurrence of tumors and has been identified as one of the causes of the occurrence and development of esophageal cancer, colon cancer, endometrial cancer, kidney cancer and postmenopausal breast cancer, as well as an important risk factor for PCa, pancreatic cancer and non-hodgkin's lymphoma.^{21,22}

Obesity promoting the development of PCa is mainly attributed to the high levels of serum FFA. Interestingly, the dominant uptake of PCa cell is fatty acid,²³ which suggests that FFA may play a significant role in the development of PCa. In our recent study, we found that although PCa patients have lower body weight and BMI than non-cancer individuals (which may be related to cachexia in the progression of tumor patients), the serum FFA levels of PCa patients were significantly higher. These results further confirm that increased FFA levels may be an important risk factor for obesity-induced PCa.

Table 4 The Lipid Content in Prostate Cancer Cells with Different Concentrations of FFA

Cell	FFA (mM)	TC (mmol/L)	TG (mmol/L)	HDL (mmol/L)	GLU (mmol/L)
PC3	0	0.104±0.022	0.544±0.021	0.025±0.008	0.023±0.000
	5	0.129±0.022	0.565±0.021	0.035±0.008	0.054±0.027
	1	0.142±0.022	0.621±0.032*	0.09±0.015*	0.108±0.027*
	1.5	0.168±0.044*	0.711±0.035*	0.104±0.025	0.279±0.046*
22RV1	0	0.177±0.000	0.720±0.020	0.057±0.020	0.029±0.012
	5	0.200±0.020	0.813±0.023*	0.064±0.011	0.059±0.013
	1	0.200±0.020	0.826±0.023*	0.071±0.011	0.088±0.022*
	1.5	0.248±0.062*	1.006±0.050*	0.090±0.011*	0.263±0.038*

Notes: Independent samples t-test. * $P < 0.05$ was considered statistically significant.

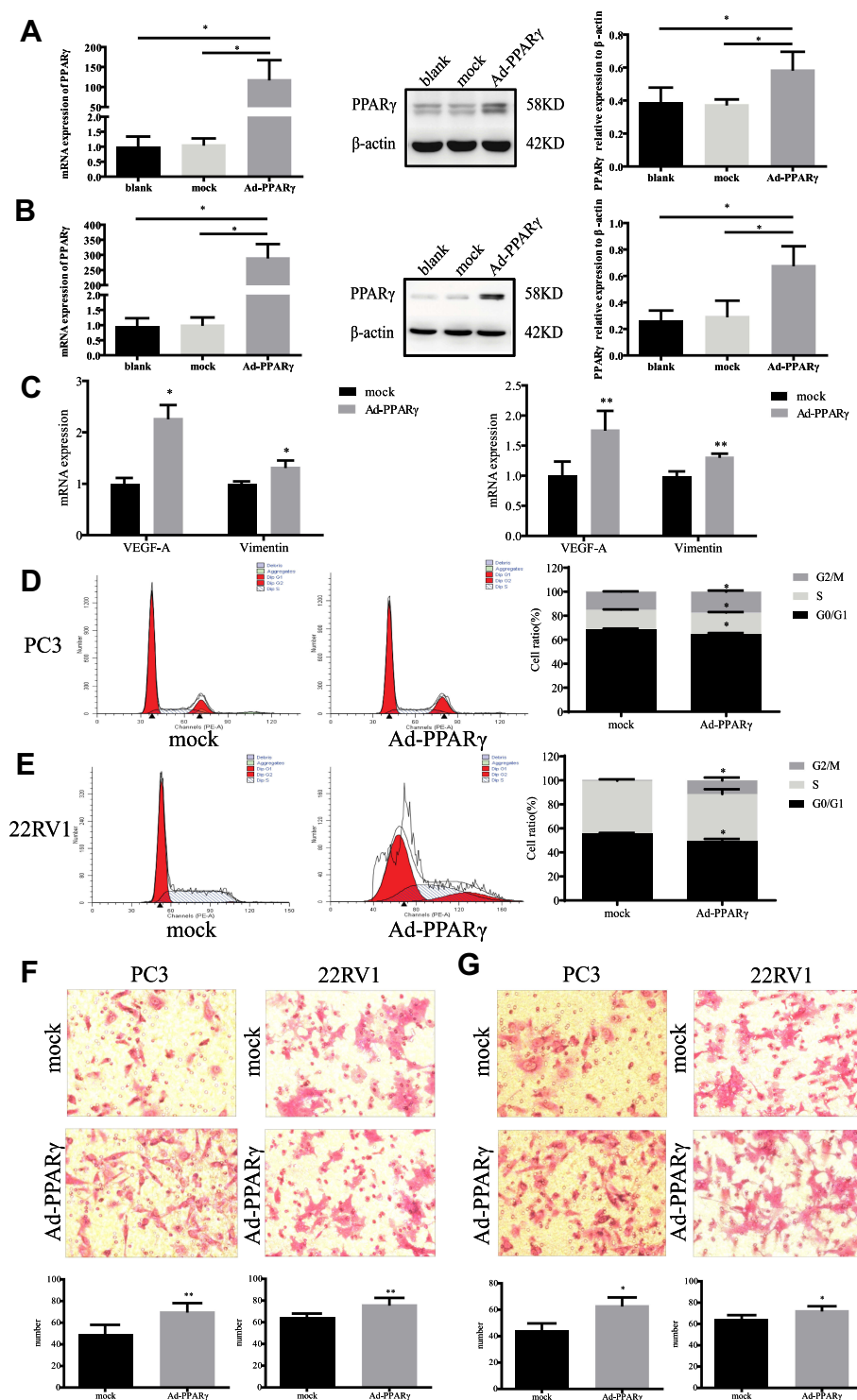


Figure 2 Changes of biological behavior in prostate cancer cells after up-regulation of PPAR γ . (A). PC3 cells were transformed with fluorescent over expression vector and an empty vector for 48 hrs, real-time quantitative PCR was used to detect the mRNA level of PPAR γ in the blank control group, the empty control group and the overexpressed PPAR γ group. Western Blot was intended to detect the protein expression level of PPAR γ among three groups. (B). 22RV1 cells were transformed with fluorescent overexpression vector and an empty vector for 48 hrs, real-time quantitative PCR was utilized to detect the mRNA level of PPAR γ in the blank control group, the empty control group and the overexpressed PPAR γ group. Western Blot was intended to detect the protein expression level of PPAR γ among three groups. (C). After overexpression PPAR γ 48 hrs, real-time quantitative PCR was utilized to detect mRNA levels of VEGF-A and Vimentin in PC3 cells (left) and 22RV1 cells (right). (D). Flow cytometry results of PC3 cells in blank control group after empty vector for 48 hrs, and experimental group after overexpression vector of PPAR γ for 48 hrs. Then compare the G0/G1 phase, S phase and G2/M phase of PC3 cells between two groups. (E). Flow cytometry results of 22RV1 cells in blank control group after empty vector for 48 hrs, and experimental group after overexpression vector of PPAR γ for 48 hrs. Then compare the G0/G1 phase, S phase and G2/M phase of 22RV1 cells between two groups. (F). PC3 and 22RV1 cells' migration results after overexpression PPAR γ 48 hrs using transwell experiment (200 \times). (G). PC3 and 22RV1 cells' invasion results after overexpression PPAR γ 48 hrs using transwell experiment (200 \times). Independent samples t-test. * $P < 0.05$, ** $P < 0.01$ was considered statistically significant.

Table 5 The Lipid Content in PCa Cells with PPAR γ Up-Expression

	PC3		22RV1	
	Mock (n=12)	Ad-PPAR γ (n=12)	Mock (n=12)	Ad-PPAR γ (n=12)
TC (mmol/L)	0.445 \pm 0.034	0.591 \pm 0.054*	1.565 \pm 0.078	1.884 \pm 0.046*
TG (mmol/L)	0.764 \pm 0.042	1.112 \pm 0.058*	0.482 \pm 0.139	0.756 \pm 0.022*
GLU (mmol/L)	0.631 \pm 0.061	0.820 \pm 0.014*	0.544 \pm 0.025	0.635 \pm 0.026*
HDL (mmol/L)	0.332 \pm 0.057	0.265 \pm 0.072	0.531 \pm 0.119	1.039 \pm 0.116*

Notes: Independent samples t-test. *P < 0.05 was considered statistically significant.

Studies have shown that fatty acids in human body can have multiple subtypes according to their carbon chain length and spatial structure, and non-similar fatty acids play different roles in tumor development and progression.^{24,25} Moreover, Omega-3 polyunsaturated fatty acids can induce apoptosis of breast cancer cells, and eicosapentaenoic acid can inhibit metastasis of colorectal cancer cells by inhibiting PGE-dependent cell movement.^{26,27} OA induces the regulatory phenotype of myeloid suppressor cells to promote immune escape of tumors.²⁸ In addition, OA and PA are both involved in the proliferation of PCa cells.^{10,29-33} The above literature suggests that OA and PA may play an important role in the development of PCa. In this study, we compared the serum fatty acid subtypes in non-cancer patients and PCa patients. The results showed that the most fatty acid subtypes in both non-cancer and tumor patients were OA and PA. And the serum levels of OA and PA in tumor patients are higher than non-cancer individuals. These results remind us that OA and PA may play an important role in the development of obesity-related PCa.

Previous literature has shown that the increased FFA level in obesity cannot only provide the energy source for tumor cells, but also act as a signal transduction molecule.¹⁰ Therefore, in our present study, we first analyzed and compared the changes in the fatty acid difference spectrum in serum of patients with PCa, combined with the existing literature, OA/PA = 2/1 was used to simulate the PC3 and 22RV1 cells.^{11,34-36} The results showed that a high concentration of FFA can significantly enhance the ability of proliferation, migration, and invasion of PCa cells. The above results suggest that the increase of serum OA and PA may be an important risk factor for the development of obesity-related PCa. However, its specific mechanism is still unclear.

Peroxisome Proliferator-activated Receptor γ (PPAR γ) plays an important role in fat formation, glycolipid metabolism and immune system.³⁷ Peroxisome proliferator-activated receptor gamma (PPAR γ) is a member of the nuclear receptor superfamily of ligand-inducible transcription factors that regulate adipogenesis, lipid metabolism, cell proliferation, inflammation and insulin sensitization.³⁸ More and more studies have also pointed out that PPAR γ plays an important role in tumors, but the role of PPAR γ in tumor development is controversial. For example: In colon cancer, cladosporol A, secondary metabolites from *cladosporium tenuissimum* characterized for their ability to control cell proliferation, inhibits proliferation of human colon cancer cells through a PPAR γ -mediated modulation of gene expression.³⁹ Pioglitazone as a PPAR γ agonist may inhibit growth and invasion of HCC cells via blockade of the RAGE signaling.⁴⁰ In esophageal cancer, rosiglitazone, an agonist of PPAR γ , can inhibit the proliferation of esophageal cancer cells and promote their apoptosis. After treatment of esophageal cancer cells with PPAR- γ antagonist GW9662, the cell proliferation ability is significantly enhanced.⁴¹ Similarly, Srivastava et al found that patients with high expression of PPAR γ in lung cancer have higher survival rates, suggesting that PPAR γ has an antitumor effect in lung cancer.⁴²

On the other hand, studies have pointed out that the PPAR γ /RXR α pathway constitutes a tumor-intrinsic mechanism underlying immune evasion in muscle-invasive bladder cancer by inhibiting CD8 + T-cell recruitment.⁴³ In addition, in a study by Chaoyang Zhu et al, it was found that PPAR γ was significantly over-expressed in tumor tissues of patients with high malignancy and lymph node metastasis, and patients with high PPAR γ expression had lower prognosis.⁴⁴ It is worth noting that although some literatures have suggested that PPAR γ has potential to modulate NF- κ B activity and oxidative stress in E2-deprived breast cancer cell lines and anti-PPAR γ therapy is a novel strategy

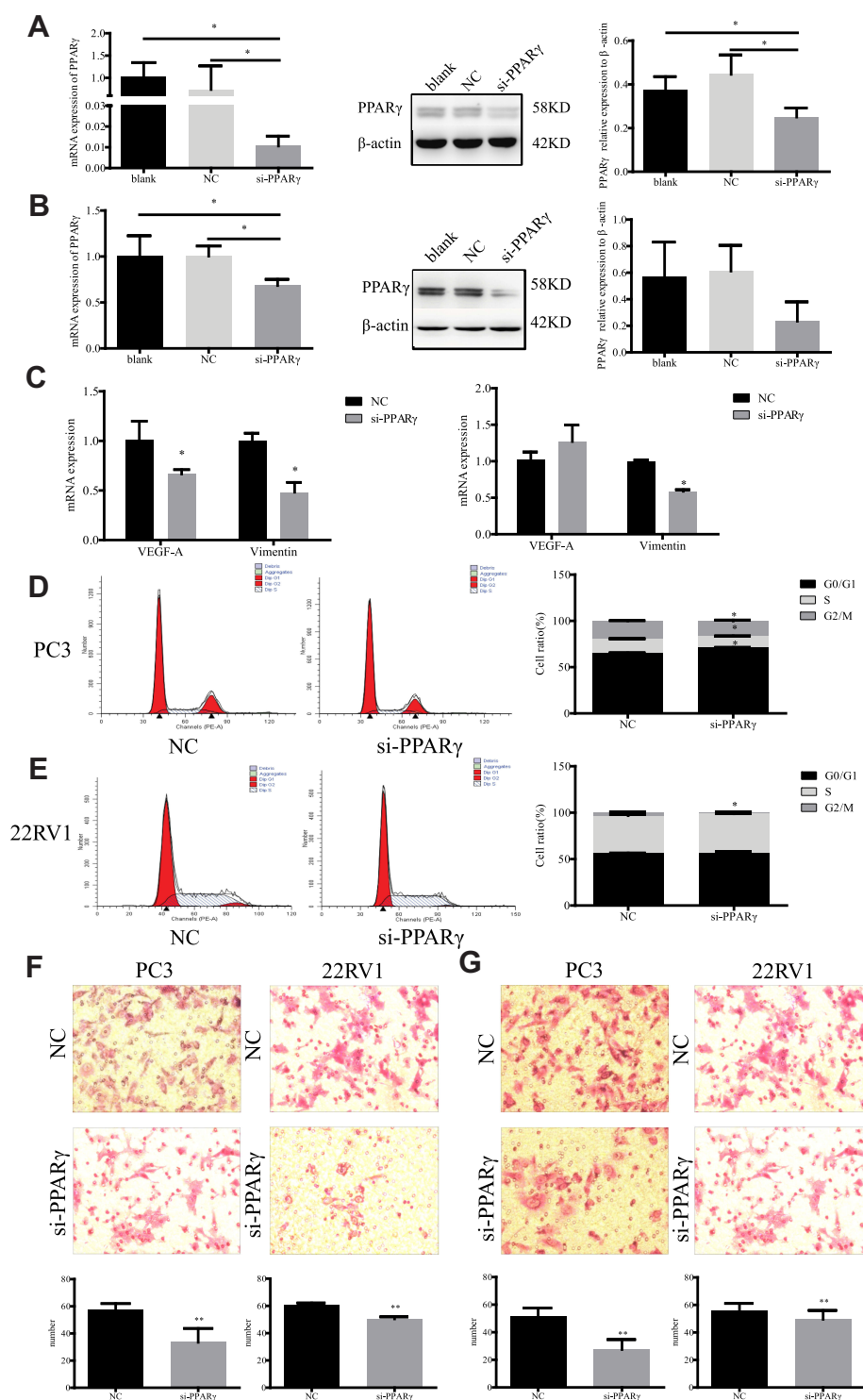


Figure 3 Changes of biological behavior in prostate cancer cells after down-regulation of PPAR γ . (A). PPAR γ was interfered in PC3 cells use si-RNA for 48 hrs, real-time quantitative PCR was used to detect the mRNA level of PPAR γ in blank group, control group and si-PPAR γ group. The Western blot was designed to detect the protein expression level of PPAR γ among three groups. (B). PPAR γ was interfered in 22RV1 cells use si-RNA for 48 hrs. Real-time quantitative PCR was used to detect the mRNA level of PPAR γ in blank group, control group and si-PPAR γ group. The Western blot was designed to detect the protein expression level of PPAR γ among three groups. (C). After si-PPAR γ 48 hrs, real-time quantitative PCR was used to detect mRNA levels of VEGF-A and Vimentin in PC3 cells (left) and 22RV1 cells (right). Then, compare the G0/G1 phase, S phase and G2/M phase of PC3 cells between two groups. (D). Flow cytometry results of PC3 cells in control group after 48 hrs, and experimental group after si-PPAR γ for 48 hrs. Then compare the G0/G1 phase, S phase and G2/M phase of PC3 cells between two groups. (E). Flow cytometry results of 22RV1 cells in control group after 48 hrs, and experimental group after si-PPAR γ for 48 hrs. Then compare the G0/G1 phase, S phase and G2/M phase of 22RV1 cells between two groups. (F). PC3 and 22RV1 cells' migration results after si-PPAR γ 48 hrs using transwell experiment (200 \times). (G). PC3 and 22RV1 cells' invasion results after si-PPAR γ 48 hrs using transwell experiment (200 \times). Independent samples t-test. * $P < 0.05$, ** $P < 0.01$ was considered statistically significant.

Table 6 The Lipid Content on PCa Cells with PPAR γ Down-Expression

	PC3		22RV1	
	NC (n=12)	si-PPAR γ (n=12)	NC (n=12)	si-PPAR γ (n=12)
TC (mmol/L)	0.721 \pm 0.017	0.569 \pm 0.065*	1.520 \pm 0.062	0.492 \pm 0.047*
TG (mmol/L)	0.298 \pm 0.023	0.162 \pm 0.025*	0.460 \pm 0.083	0.169 \pm 0.022*
GLU (mmol/L)	0.601 \pm 0.032	0.536 \pm 0.038*	0.602 \pm 0.039	0.573 \pm 0.032
HDL (mmol/L)	0.528 \pm 0.075	0.291 \pm 0.042*	0.697 \pm 0.023	0.520 \pm 0.110*

Notes: Independent samples t-test. *P< 0.05 was considered statistically significant.

to improve the therapeutic effects of E2-induced apoptosis in E2-deprived breast cancer.⁴⁵ However, most studies have pointed out that PPAR γ has an antitumor effect in breast cancer. For example, Xu et al proposed that PPAR γ can inhibit tumor cell proliferation by regulating the ptpfrf gene, and Suzuki et al proposed that PPARgamma is mainly expressed in well-differentiated and ER-positive breast cancers.^{46,47} In response to the phenomenon that PPAR γ plays different roles in tumor cells, some studies have explained that the effect of PPAR γ on tumorigenesis is determined by the cell type that activates PPAR γ . When PPAR γ is activated in epithelial cancer cells, tumor growth is inhibited, but tumor growth is enhanced when PPAR γ is activated in stromal cells.⁴⁸ Interestingly, the role of PPAR γ in the occurrence and development of PCa has also been reported in recent years. L. Qin et al found that PPAR γ agonists can induce apoptosis of PCa cells through the CXCR4/CXCL12 axis, in which its process may depend on the transduction of androgen receptors.^{49,50} Our results showed that PPAR γ could improve glucose and lipid metabolism, enhance the ability of proliferation, migration and invasion in PCa cells, which may remind that PPAR γ promoted the occurrence and development of PCa relying on the energy metabolism pathway instead of androgens. The role of PPAR γ in the development of obesity-related PCa remains unclear. Our results suggested that the increased level of FFA induced by obesity may lead to the occurrence and development of PCa by promoting the expression of PPAR γ , while the specific mechanism is not yet defined.

Vimentin, which is a major intermediate filament protein, plays an important role in cell adhesion, migration, angiogenesis and neurite extension.⁵¹ In PCa patients, the vimentin expression is positively correlated with patholo-

gical stage, poor prognosis, cell invasion and migration ability.^{52–54} Previous studies have found that vimentin interacts with PPAR γ via a proteasomal degradation process.⁵⁵ Consequently, our results showed that PPAR γ could promote the mRNA expression level of vimentin, while it could be reversed by the downregulation of PPAR γ . The above results suggest that a high level of FFA may promote the expression of vimentin by upregulating PPAR γ , which ultimately leads to the occurrence and development of PCa.

Vascular endothelial growth factor-A (VEGF-A) can promote the new blood vessel formation and increase vascular permeability resulting in the growth and metastasis of tumor cells. As one of the most effective tumor angiogenesis factors, VEGF-A plays a key role in the process of the formation and metastasis of the malignant tumor.⁵⁶ Previous literature has shown that PPAR γ can promote the ability of proliferation and invasion by upregulating VEGF-A expression in PCa cells.¹³ Our study suggested that high-level FFA may promote the expression of VEGF-A by upregulating PPAR γ in PC3 and 22RV1 cells, which also ultimately leads to the occurrence and development of PCa.

In addition, in our present study, we built a BALB/c-nu mice model with PCa in situ, feeding on a high-fat diet (HFD) and normal control diet (NC). Then, we compared the protein expression of PPAR γ /Vimentin/VEGF-A in PCa tissues of the two groups of mice. The results showed that the expression level of PPAR γ /Vimentin/VEGF-A was all significantly higher in the HFD group than the NC group. These results further support that high levels of PPAR γ , Vimentin, and VEGF-A may be involved in the development and progression of PCa in obese conditions.

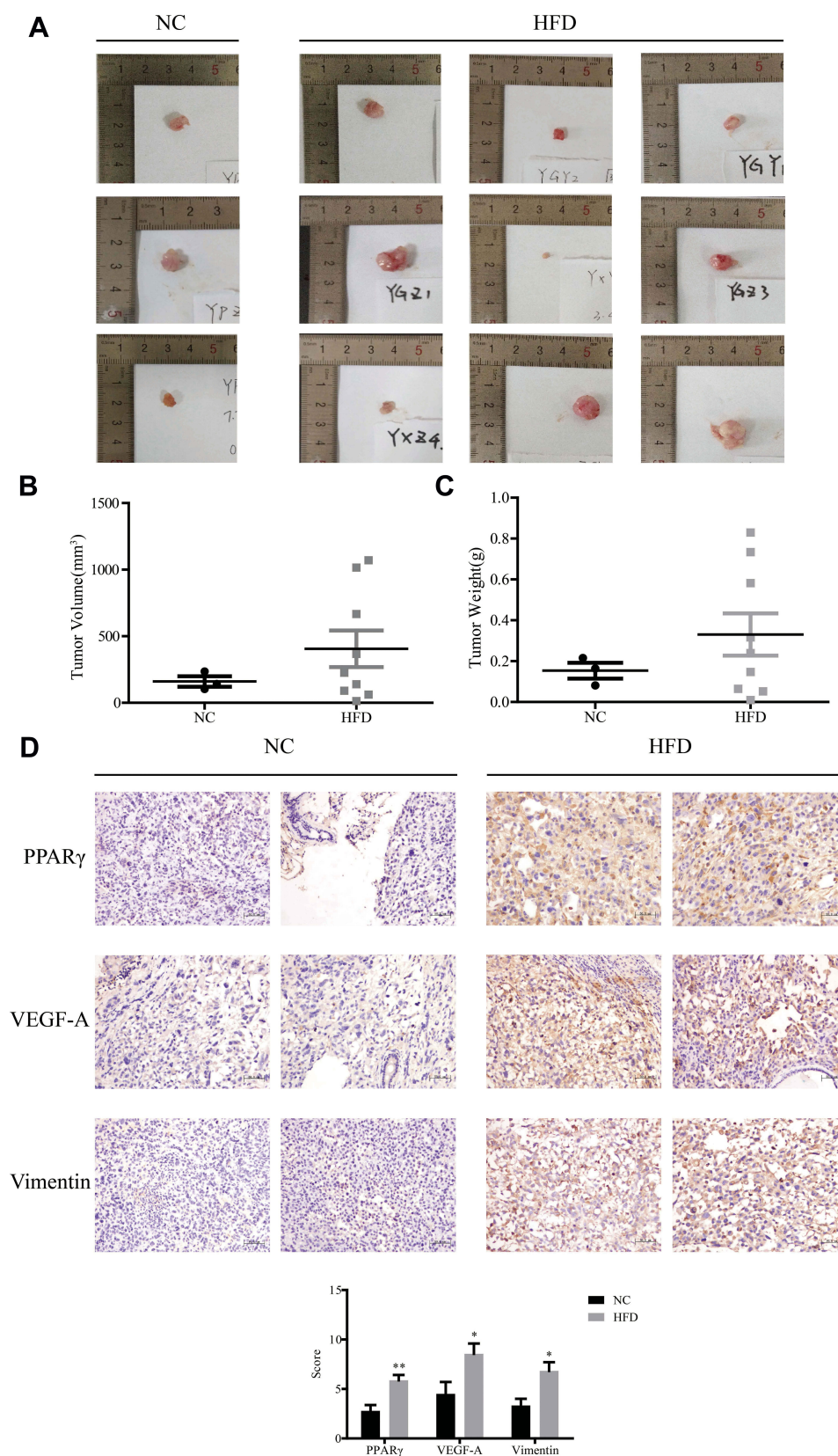


Figure 4 PPAR γ may promote prostate cancer in vivo after a high-fat diet. Prostate cancer was induced by injected PC3 cells into the prostate of nude mice. **(A)**. After 12 weeks of feeding, the tumor took out. Compared two groups' tumor volume **(B)** and weight **(C)**. **(D)**. The expression of PPAR γ /VEGF-A/Vimentin in the tissues was observed by immunohistochemistry (200 \times). Finally, the results of immunohistochemistry were scored, and the difference of PPAR γ , VEGF-A, vimentin expression between the high-fat diet group and the normal diet group was compared. Independent samples *t*-test. **P*<0.05, ***P*<0.01 the difference was statistically significant.

Conclusion

Our study found that under the condition of obesity, high level of FFA can promote the expression of VEGF-A and Vimentin by upregulating PPAR γ , which ultimately leads to the occurrence and development of PCa. PPAR γ may become a new target for the development and progression of obesity-related PCa.

Abbreviations

PCa, prostate cancer; FFA, free fatty acid; OA, oleic acid; PA, palmitic acid; PPAR γ , peroxisome proliferator-activated receptor γ ; VEGF-A, vascular endothelial growth factor-A.

Ethical Approval and Consent to Participate

Ethical approval was given by the medical ethics committee of First Affiliated Hospital, Shihezi University School of Medicine with the following reference number: 2017-049-01, A2017-115-01. In addition, animal models are based on the guidance given by the Department of Urology, University of Michigan, Ann Arbor, MI, USA (Dai, J., 2016).

Data Sharing Statement

The datasets generated and/or analysed during the current study are not publicly available (the data belongs to our group) but are available from the corresponding author on reasonable request.

Author Contributions

All authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that they have no competing interests.

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