

Inhibition of the breast cancer by PPAR γ agonist pioglitazone through JAK2/STAT3 pathway

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Breast cancer, especially triple-negative breast cancer, is one of the deadliest cancers in women. To date, there is a lack of a good therapeutic regimen for it. PPAR γ has been reported to be a tumor suppressor and could be activated by many agonists involved in cancer inhibition. Therefore, the expression of PPAR γ in breast cancer was analyzed by online software UALCAN whose data were from the TCGA database. The results revealed that the PPAR γ expression was reduced in breast cancer tissues. Furthermore, the methylation in the PPAR γ promoter was also assayed and the results indicated that the methylation level in the PPAR γ promoter in breast cancer tissue was higher than that in normal tissue. In order to verify the methylation in promoter involved in the regulation of gene PPAR γ expression, the 5'-Aza and fluorescence assays were performed and the results proved that methylation in promoter participated in gene PPAR γ expression regulation. Pioglitazone, a PPAR γ agonist, still was not investigated in breast cancer. Therefore, the effects of pioglitazone on breast cancer cells were tested by cell viability, scratch and transwell assays, and results indicated that the pioglitazone has the inhibition effect on the proliferation and migration of breast cancer cells by PPAR γ which was correlated with the JAK2/STAT3 pathway. In order to further confirm the inhibition effect of pioglitazone on breast cancer *in vivo*, the nude mice model was administrated by gavage with pioglitazone. And the results indicated that pioglitazone could inhibit the growth of breast cancer in the PPAR γ overexpression group *in vivo*. In summary, the expression of gene PPAR γ was decreased in breast cancer tissues, which was correlated with its methylation in the promoter region. Moreover, pioglitazone could exert its inhibition on breast cancer proliferation and migration by the JAK2/STAT3 pathway.

Key words: breast cancer, PPAR γ , pioglitazone, inhibition, JAK2/STAT3 pathway

Breast cancer, the most common cancer in women, was diagnosed in about 250 000 new cases in the United States in 2017. For women, 12% of them will face breast cancer over their lifetimes [1]. It includes several classes, with each representing unique molecular or genetic characteristics. Triple-negative breast cancer (TNBC) is a subtype of breast cancer with the heterogeneous nature that stains negatively for estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor 2 during immunohistochemistry. Anthracycline-taxane regimens remain the current standard for TNBC patients who tend to have a higher risk of relapse and worse overall survival rates with mixed outcomes [2]. On the current condition, more details on the TNBC's occurrence and development need to be understood and the innovative method for the TNBC treatment are required to be produced urgently [3].

Peroxisome proliferator-activated receptor gamma (PPAR γ), a ligand-dependent transcription factor, is a member of the nuclear receptor family that functions predominantly as a heterodimer with RXR α . Its function is involved in glucose homeostasis and adipogenesis [4]. In addition to its well-established role in adipocyte differentiation, it has also been shown to participate in differentiation in many tissues. However, its role in cancer is far less understood [5]. Historically, PPARG was considered to be a tumor suppressor. Its activation consistently induces apoptosis, inhibits cellular proliferation, and promotes cellular differentiation in both hormone-dependent and hormone-independent mammary tumors [6, 7]. PPAR γ , as a hormone receptor for estrogen and progesterone, could be modulated by many ligands including synthetic ligands such as TZDs and psammaplin A [8, 9]. Epigenetics refers

to heritable changes regulating gene expression that do not affect the DNA base pair sequence. DNA methylation is the most commonly studied epigenetic mark. PPAR γ , often found inhibited in TNBC, was suggested to be methylated in its promoter region because hypermethylation within the promoter region of a gene can reduce gene expression [10]. PPAR γ ligands, function as PPAR activators, such as pioglitazone (PGZ), still were not investigated with respect to the cell behaviors in TNBC. In addition, decitabine, as a methylation inhibitor, could reverse the inhibition of gene expression by methylation. Therefore, we think that whether we could combine pioglitazone and decitabine as a therapeutic regime to explore its effect on the TNBC cell behavior and provide a new clue for breast cancer treatment in the future [11].

Janus kinase (JAK) is a family of intracellular, non-receptor protein tyrosine kinases (PTKs), including JAK1, JAK2, JAK3, and TYK2, which selectively associate with the cytoplasmic domains of various cytokine receptors. Among them, JAK2 is a crucial intracellular mediator of cytokine and hormone signaling [12]. STAT3 is a well-characterized oncogene that affects various biological processes including to boost: (i) cell proliferation and survival by regulating cyclin D1, Bcl-2 and Bcl-xl; (ii) tumor invasion and metastasis by regulating E-cadherin, MMP-9; (iii) angiogenesis by targeting VEGF and HIF1a expression [13]. Accumulating evidence indicated that aberrant JAK2 signaling was linked to the occurrence and development of a wide variety of tumors. STAT3 is the preferred downstream targets of phosphorylated JAK2 and is constitutively activated in a variety of human tumors. The JAK2/STAT3 pathway has been intensely investigated in breast and other cancer types. When cytokines bind to a cell-surface receptor, the receptor dimerizes leading to phosphorylation and activation of JAK tyrosine kinases, which in turn phosphorylate the receptor to allow binding and phosphorylation of cytoplasmic STAT proteins, which dimerize and translocate to the nucleus to regulate transcription of various target genes [7].

In this study, the PPAR γ expression was analyzed based on the online database TCGA, and results showed that the PPAR γ was downregulated in breast cancer. Next, we substantiated that the gene PPAR γ was regulated by the methylation in the promoter region. In addition, the mechanism for the inhibition effect of agonist pioglitazone on the breast cancer cell line was probed and the assay revealed that the JAK2/STAT3 signal was closely connected with the inhibition effect. Our data will provide some beneficial references for a meaningful prognosis or valuable target therapy of breast carcinoma.

Materials and methods

Reagents and materials. The cell lines including MCF-7, ZR-75-1, MDA-MB-231, MDA-MB-468, and HCC-1937 were obtained from the Cell Bank of the Chinese Academy of Sciences. The rabbit polyclonal antibodies PPAR γ (#2443),

AKT (#4691), pAKT (#2965), PI3 (#4257), pPI3 (#4228), JAK2 (#3230), pJAK2 (#4406), STAT3 (#4904), and pSTAT3 (#9131) were from Cell Signaling Technology (MA, USA). The rabbit pAb GAPDH (#BA2913) was purchased from the BOSTER Biological Technology Co. Ltd (Wuhan, China). Dulbecco's modified Eagle medium: Nutrient Mixture F-12 was supplied by GIBCO (Grand Island, NY, USA). Fetal bovine serum (FBS) was from Zhejiang Tianhang Biotechnology Co. Ltd. Penicillin/streptomycin and trypsin solution were from Thermo Fisher Scientific (Shanghai, China). All other chemicals including indomethacin, pioglitazone, and ibuprofen were of the highest grade of purity commercially available.

Cell culture. The breast cell lines were all grown in DMEM/F12 basal medium containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 units/ml streptomycin. The flasks were incubated in a humidified incubator maintained at 37°C with 5% CO $_2$. The plates were observed under a phase-contrast microscope for cell adherence and morphology. In the cell viability and cell scratch assays, the concentration of the medicine on indomethacin, pioglitazone, and ibuprofen were 600 μ M, 50 μ M and 1.5 mM, respectively.

Vector construction. The gene PPAR γ has 6 transcripts and they have different transcription start sites. Taken into account the above elements together and we selected the most possible effective promoter region as the target region, which located from -1400 to 100 bp based on the transcript 3 transcription start site. Then the promoter region was synthesized and cloned into pGL3-Basic. Additionally, the gene CDS sequences were synthesized according to the canonical sequence and cloned into the vector pCDH. All the synthesized sequences were confirmed by DNA sequence analysis.

Western blot (WB) analysis. The whole protein was obtained from lysed cells using RIPA buffer with PMSF (1 mM). The extracted proteins were quantified by the BCA assay (Haimen, China) and analyzed by WB. The protein was separated by SDS polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (GE Healthcare Life Sciences, China), which was then blocked overnight with TBST containing 5% skimmed milk powder. The membranes were rinsed with PBS and treated with polyclonal primary antibody and with the HRP-conjugated secondary antibody. Lastly, protein bands were visualized by a chemiluminescent detection system (ECL, BOSTER, China). Densitometry was performed with Image Lab™ software (Bio-Rad Laboratories, CA, USA), and the band signal was normalized to the level of GAPDH protein.

Cell viability. The cell viability was measured by MTT assay. The cells were seeded into 96-well plates at the density of 3×10^5 /ml in triplicates with the volume of 100 μ l/well and allowed to adhere overnight. Forty-eight hours after transfection, 100 μ l MTT (0.5 mg/ml) was added and the cells were incubated for another 4 h. Then, the medium was removed and 100 μ l dimethylsulfoxide was added and incubated for

another 2 h. After the formazan crystal dissolved, OD values were measured at 570 nm wavelength with EnSpire-2300 multimode plate reader (Perkin Elmer, Norwalk, USA). The experiment was repeated three times.

Transwell assay. The migration ability of breast cells was determined by the number of cells passed through a polycarbonate membrane (8 μm pore size). The chamber was divided into upper and lower chambers. Breast cancer cells were seeded in the upper chamber at the density $3 \times 10^5/\text{ml}$ and incubated for 48 h. Then the cells on the upper surface of the membrane were removed with a cotton swab and cells passing through the membrane were stained with 1% crystal violet. The number of cells passing through the polycarbonate membrane was calculated under a Leica microscope in eight random fields. The experiment was repeated three times for each group.

Cell scratch. The breast cell lines were seeded on 60 mm culture dishes and incubated at 37°C with 5% CO_2 . After 24 h, the attached cells were scratched three times in parallel with a 100 μl pipette tip. The incubation continued for another 24 h and the images for wound healing were captured with a 10 \times objective lens using a Nikon camera (Nikon Corporation, Tokyo, Japan).

Luciferase assay. The plasmids were methylated by the CpG Methyltransferase (M.SssI) according to the manufacturer's manual (New England Biolabs Inc., Beijing, China). Cells (4×10^4 cells/well in 24-well plate) were transiently transfected with 0.1 μg of pRL-Tk and 1 μg of pGL3-PPAR γ luciferase construct, which has been methylated. Forty-eight h after transfection, the cells were harvested with lysis buffer and the luciferase activities of the cell extracts were measured by the dual luciferase assay system (Promega, Beijing, China). The luciferase activity was normalized for transfection efficiency with Renilla luciferase activity.

The establishment for PPAR γ stable cell line. Stable cell line expressing PPAR γ protein was created during the following steps: firstly, the gene PPAR γ overexpression vector (pCDH-PPAR γ) was constructed by cloning the CDS sequence of PPAR γ gene into the plasmid pCDH; secondly, the lentivirus was produced by transient transfection of 293T cells with the vector pCDH-PPAR γ and corresponding helper plasmids; lastly, the clones were introduced into cell line MDA-MB-231 via lentiviral transfection. Cells were expanded under GFP selection and stored in liquid nitrogen.

Animal model. Four-week old female BALB/c nude mice were purchased from Shanghai SLRC Laboratory Animal Co., Ltd and maintained in the animal facilities. Six female BALB/c nude mice per group were injected subcutaneously at the armpit and hind leg area with MDA-MB-237 cells stably transfected with pCDH-PPAR γ or pCDH respectively (1×10^8 cells in 100 μl of basic medium). The mice were observed every week and after 8 weeks, sacrificed to recover the tumors. The wet weight and the volume of each tumor were determined.

Statistics. SPSS 16.0 was used for data analysis. Statistical significance was measured using Student's t-test at two treatments. If the groups were three or more, the one-way ANOVA analysis was executed for statistical analysis. A p-value <0.05 was judged to be significant.

Results

PPAR γ was frequently downregulated in triple-negative breast cancer. To determine whether the downregulation of PPAR γ was a universal phenomenon in breast cancer, the expression for PPAR γ was analyzed by using the online software UALCAN (<http://ualcan.path.uab.edu/>). The analysis was based on the TCGA database and the results indicated that compared with normal tissue, the primary tumor has a lower mRNA level of PPAR γ (Figure 1A). To further explore the expression status in subgroups, we compared the expression level of PPAR γ in normal samples and TNBC samples. The results showed that the expression of PPAR γ was markedly reduced in TNBC samples relative to that in normal samples (Figure 1B). In addition, we also measured the PPAR γ expression status between the triple-negative breast cancer cell lines (MDA-MB-231, MDA-MB-456, and HCC-1937) and non-triple negative breast cancer cell lines (MCF-7 and ZR-75-1). We found that the expression of PPAR γ in triple-negative breast cancer cells was lower than that in non-triple negative breast cancer cell lines (Figure 1C). These results indicated that the expression of PPAR γ was possibly inversely correlated with the malignant degree of the tumor. In order to verify our hypothesis, the Kaplan–Meier survival analysis was conducted based on PPAR γ expression within tumor samples, which was split by the median parameter (www.oncolnc.org). The results demonstrated that lower PPAR γ expression was significantly correlated with shorter overall survival (OS; log-rank test, p-value =0.0181, Figure 1D).

The expression of PPAR γ was inhibited by its methylation in the promoter region. We know that the methylation in the promoter region could inhibit gene expression. Based on the above results, the PPAR γ was inhibited in the tumor tissue, especially in the TNBC tissue. To probe whether the inhibition of PPAR γ expression in TNBC tissue was due to the promoter methylation, we compared the methylation status of the PPAR γ promoter region between the tumor sample and normal samples. The methylation level of gene PPAR γ in its promoter was analyzed using the online software UALCAN (<http://ualcan.path.uab.edu/>). The results indicated that the methylation level between the primary tumor and normal tissues has a significant difference (p=1.62E-12, Figure 2A). Furthermore, the methylation status was also analyzed in the samples depend on the ER, PR, and HER2 expression status by the online software MEXPRESS (<https://mexpress.be/>) and the results revealed that the methylation status only was inversely correlated with the ER status and was independent of the PR and HER2 status. These results illustrated that the

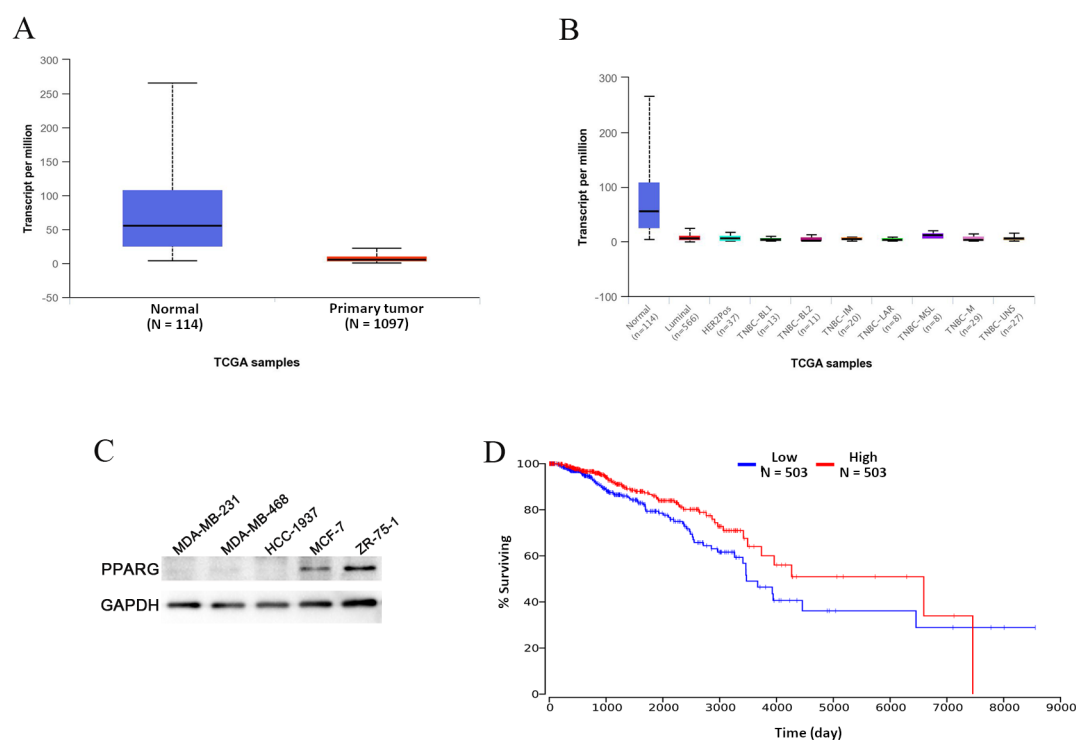


Figure 1. Downregulation of PPAR γ was associated with poor prognosis A) The expression status of PPAR γ in breast normal tissues and primary cancer tissues. B) The expression of PPAR γ in several types of TNBC and normal tissues. C) The protein level of PPAR γ in different breast cancer cell lines. D) Kaplan–Meier survival curve for breast cancer patients stratified by low and high PPAR γ expression.

inhibition of the PPAR γ expression was due to the promoter methylation and the methylation was not restricted in the TNBC tissue but in the breast invasive carcinoma tissues (Figures 2B–D). To further confirm the role of methylation in the regulation of gene PPAR γ expression, three triple-negative cell lines were selected and treated with the 5-Aza (50 μ M). The results showed that compared to the control group, the expression of gene PPAR γ in cell line MDA-MB-231 and HCC-1937 was increased while no change was observed in cell line MDA-MB-468 (Figure 2E). The results proved that the methylation participated in PPAR γ expression regulation. Whether the methylation in the promoter region plays an important role in PPAR γ expression? We synthesized the promoter region of gene PPAR γ and cloned it to the vector pGL3-basic. The recombinant plasmid was methylated by SSI methylated enzyme and transfected into the cell lines MCF-7 and MDA-MB-231. Results indicated that in cell line MCF-7, the methylation plasmid fluorescence was inhibited, but in cell line MDA-MB-231 no change was found in the fluorescence (Figure 2F). The results implied that methylation was implicated in the regulation of gene PPAR γ expression. Next, we transfected five cell lines with the promoter plasmid and in the meantime, methylation inhibitor 5-Aza was added or not to the culture medium. We found that the fluorescence was increased relative to that in the control group in

triple-negative cell lines MDA-MB-231 and MDA-MB-468 and no significant change was found in the other three cell lines (Figure 2G). Therefore, we confirmed that the methylation in PPAR γ promoter participated in the PPAR γ expression regulation and the factors beyond the methylation in PPAR γ promoter were implicated in the regulation of PPAR γ expression because the methylation plasmid only works in non-triple negative cell lines and the 5-Aza treatment only effects in part triple-negative cell lines.

The effect of ligand and methylation inhibitor 5-Aza on breast cancer cell lines. PPAR γ , as a ligand-activated intracellular transcription factor, was reported to be involved in the tumor inhibition while activated by agonists. To date, the effects of agonists such as indomethacin, pioglitazone, and ibuprofen on the breast cancer proliferation, especially combined with methylation inhibitor 5-Aza, still remain elusive. Therefore, five breast cancer cell lines were selected for analysis to confirm which agonist could exert its inhibition effect through PPAR γ . The results indicated that only pioglitazone had the inhibition effect on breast cancer cell growth through PPAR γ because in non-triple negative cell lines (MCF-7 and ZR-75-1) the inhibition effect existed while in triple-negative breast cancer cells, the inhibition effect was reduced (Figure 3A). Consequently, the pioglitazone was selected for further scratch experiments on the five cell lines

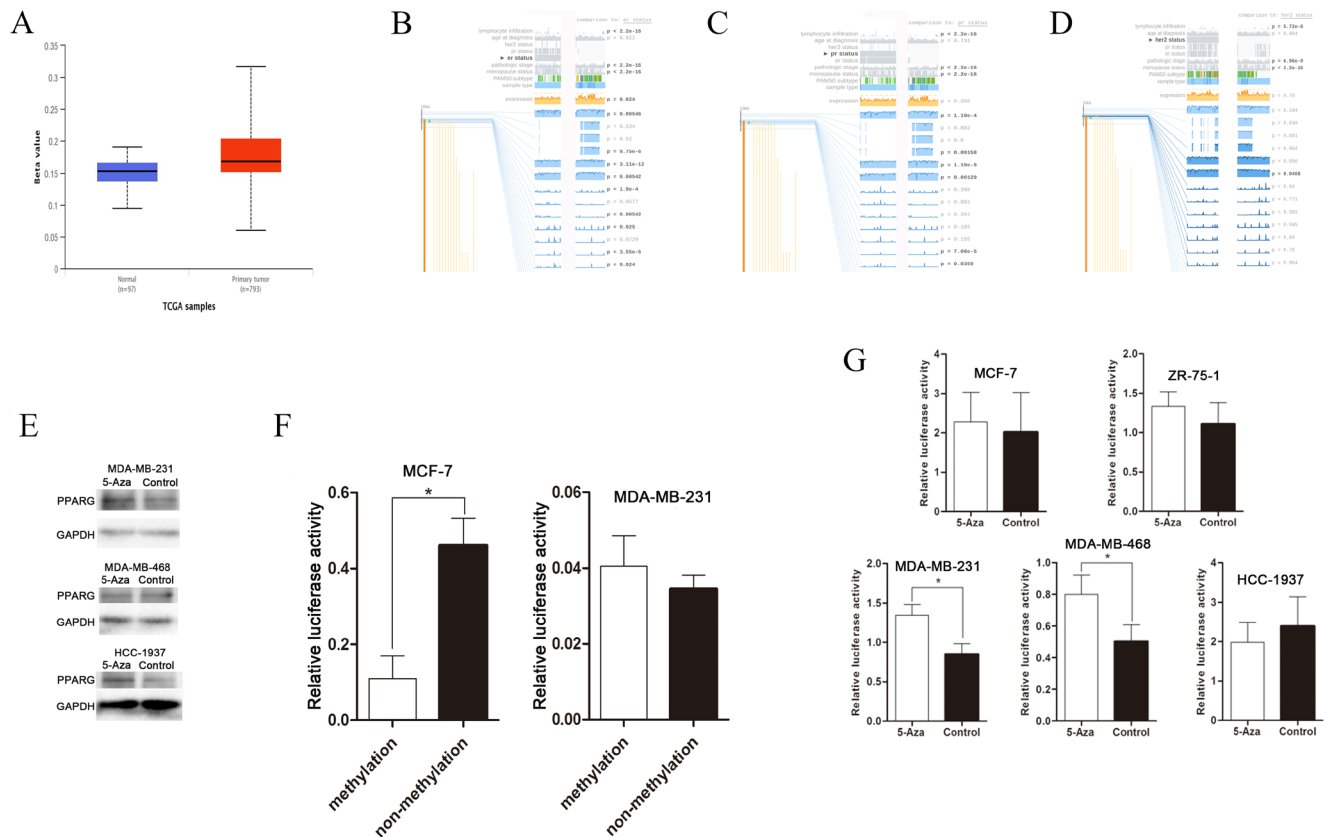


Figure 2. The expression of PPAR γ was inhibited by its methylation in the promoter region. **A)** The promoter methylation level of PPAR γ was compared between normal breast tissues and primary tumor tissues. **B)** The correlation between the ER status and the methylation status in the PPAR γ promoter. **C)** The correlation between the PR status and the methylation status in the PPAR γ promoter. **D)** The correlation between the HER2 status and the methylation status in the PPAR γ promoter. **E)** The protein level was detected by WB in cell line MDA-MB-231, MDA-MB-468, and HCC-1937 treated with 5-Aza or PBS (control). **F)** The fluorescence report assay in cell line MCF-7 and MDA-MB-231 transfected with the pGL3-PPAR γ promoter plasmids, which were methylated or not. **G)** The fluorescence report assays were performed in five different cell lines transfected with the pGL3-PPAR γ promoter plasmids and treated with 5-Aza or PBS.

and the treatments were as above. The results indicated that the agonist pioglitazone also inhibited the wound healing in the cell lines MCF-7 and ZR-75-1, and have a little inhibition effect on the migration of triple-negative cell lines (Figure 3B). The results showed that pioglitazone could influence cell migration through PPAR γ . In order to further confirm the role of PPAR γ in the inhibition of breast cancer proliferation and migration, the cell line MDA-MB-231 was selected for the following proliferation and migration assays. Cells were divided into three groups which were treated with PBS (control), 5-Aza and 5-Aza + pioglitazone, respectively, and the MTT and Transwell assays were performed. Results indicated that the cell proliferation and migration were obviously inhibited in the 5-Aza + pioglitazone group (Figure 3C). Moreover, the PPAR γ overexpression stable cell line was established by lentivirus infection and the cell viability and migration were assayed on the cell line by dividing them into three groups including the control group (normal cell line), pCDH group (transfected with blank lenti-

virus) and PPAR γ group (PPAR γ overexpressed). All three groups were treated with pioglitazone and results illustrated that the proliferation and migration were inhibited in PPAR γ group relative to that in other two groups (Figure 3D).

JAK2/STAT3 pathway is involved in the proliferation and migration promoted by gene PPAR γ . In order to confirm which pathway was possibly involved in the function of gene PPAR γ on the cell behavior, the JAK2/STAT3 and AKT/PI3 pathways were assayed in the PPAR γ and pCDH groups treated with pioglitazone and the results showed that no significant difference on the AKT/PI3 pathway existed between the two groups. However, the differences were found in the JAK2/STAT3 pathway (Figure 4A). The results indicated that in the PPAR γ -overexpression group, genes JAK2, STAT3, and their phosphorylation forms were all upregulated in contrast to that in the pCDH-control group. To further confirm the role of pathway JAK2/STAT3 in the effect of PPAR γ on the cell behavior, the PPAR γ overexpression cell line was divided into groups treated with

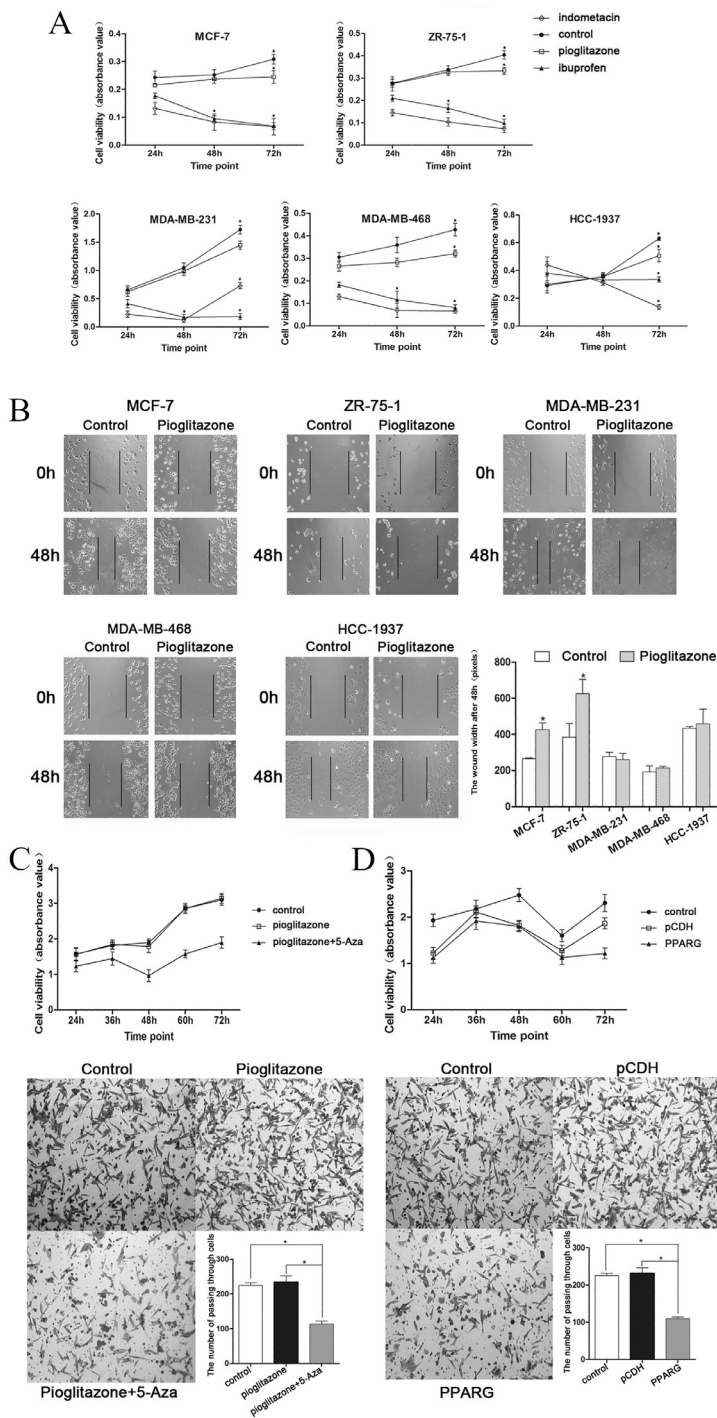


Figure 3. The effect of agonists and methylation inhibitor 5-Aza on breast cancer cell lines A) The cell viability assays in different cell lines treated with PPAR γ agonist indomethacin, pioglitazone, ibuprofen, or PBS (control), respectively. B) The scratch assays were performed in five different cell lines treated with PBS (control) or pioglitazone. C) The cell viability and transwell assays were performed in the MDA-MB-231 cell line treated with PBS, pioglitazone, or pioglitazone+5-Aza, respectively. D) The cell viability and transwell assays were performed in stable cell line MDA-MB-231 established by PPAR γ overexpression lentivirus, control lentivirus, or PBS, respectively, which were treated with pioglitazone.

pioglitazone+fedoratinib (JAK2 inhibitor, 3 nM, treatment group) or pioglitazone (control group). The results revealed that the cell proliferation (proliferation index, PI) was enhanced and the ratio of apoptosis cells was reduced relative to that in the control group (Figures 4B–D). In addition, the transwell assay was also performed and the results showed that the fedratinib treatment could augment the cell migration relative to that in the control group (Figure 4E).

PPAR γ activation inhibits breast cancer growth *in vivo*. In order to test whether the gene PPAR γ could exert its effect on the cell proliferation *in vivo*, the nude mice model was established and the pioglitazone was infused to stomach every day. The results indicated that the tumor size in the overexpression group was significantly smaller than that in the control group ($p < 0.05$) (Figure 5A, B). The average tumor weight in the overexpression group was also significantly lower than that in the control group (Figure 5C). The volume also differs in the two groups (Figure 5D). These findings further demonstrate that PPAR γ was involved in the inhibition of proliferation for breast cancer by agonist activation.

Discussion

Breast cancer is one of the deadliest cancers, especially triple-negative breast cancer (TNBC), and to date, we still do not have a good therapeutic regimen for TNBC [14]. Therefore, an evolving understanding of the genetic and molecular alterations in TNBC is urgently needed. PPAR γ is highly expressed in fatty tissues and many human cancers, including breast cancer [15]. However, the TCGA database analysis indicated that the PPAR γ expression in human breast cancer or TNBC was reduced compared to that in normal tissues. To further confirm the online analysis, the five breast cancer cell lines were tested including triple-negative breast cancer cell line (MDA-MB-231, MDA-MB-468, and HCC-1937) and non-triple-negative breast cancer cell lines (MCF-7 and ZR-75-1). Our results demonstrated the expression of PPAR γ in triple-negative breast cancer cell lines was significantly lower than that in non-triple-negative breast cancer cell lines. These results documented that with the malignancy increased, the expression of PPAR γ is decreased in breast cancer and substantiated the online analysis.

In addition, we have known that hypermethylation in the promoter region could inhibit the gene expression [16, 17]. In order to clarify whether the methylation was involved in the gene PPAR γ expression regulation, the methylation inhibitor 5-Aza was added in triple-negative breast cancer cell lines and the results indicated that the expression of PPAR γ could

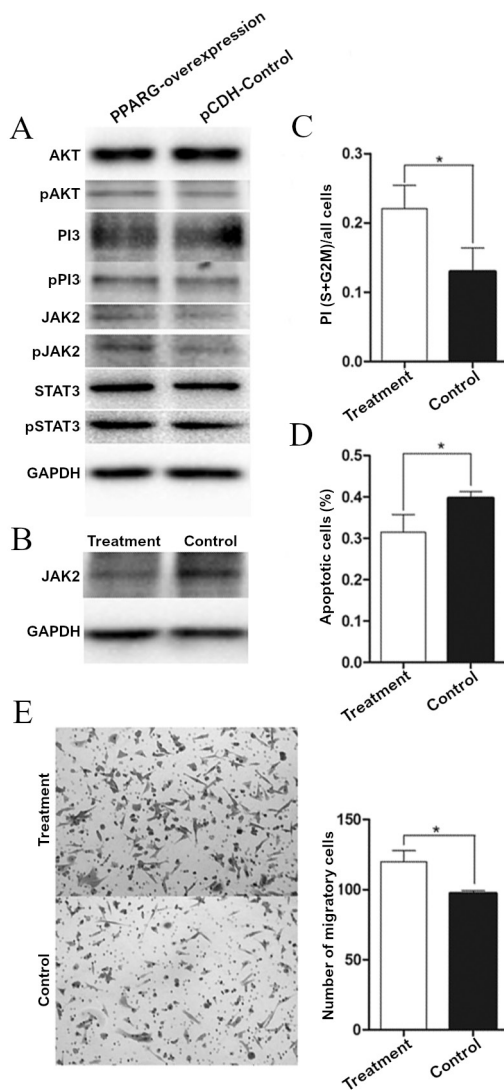


Figure 4. JAK2/STAT3 pathway was involved in the proliferation and migration inhibited by gene PPAR γ agonist pioglitazone. A) The protein levels of AKT, PI3, JAK2, and STAT3 and their phosphate forms were measured by western blot in the stable cell line whose gene PPAR γ was overexpressed and treated with pioglitazone. B) JAK2 protein was measured in PPAR γ overexpression stable cell line treated with JAK2 inhibitor fedratinib or PBS. C) The proliferation analysis by PI in the stable cell line treated with JAK2 inhibitor fedratinib or PBS. D) Apoptosis analysis in the stable cell line by the percentage of apoptosis cells treated with JAK2 inhibitor fedratinib or PBS. E) Transwell assays in the stable cell line by the number of passing through cells treated with JAK2 inhibitor fedratinib or PBS.

be upregulated in the treatment group relative to that in the control group. In order to further verify the methylation in the PPAR γ promoter region involved in the gene PPAR γ expression regulation, the fluorescence reporter system assays were conducted in the cell lines including MCF-7 and MDA-MB-231. There was an obvious difference in the fluorescence signals between the treatment group and the control group in the MCF-7 cell line. However, as for the triple-negative cell

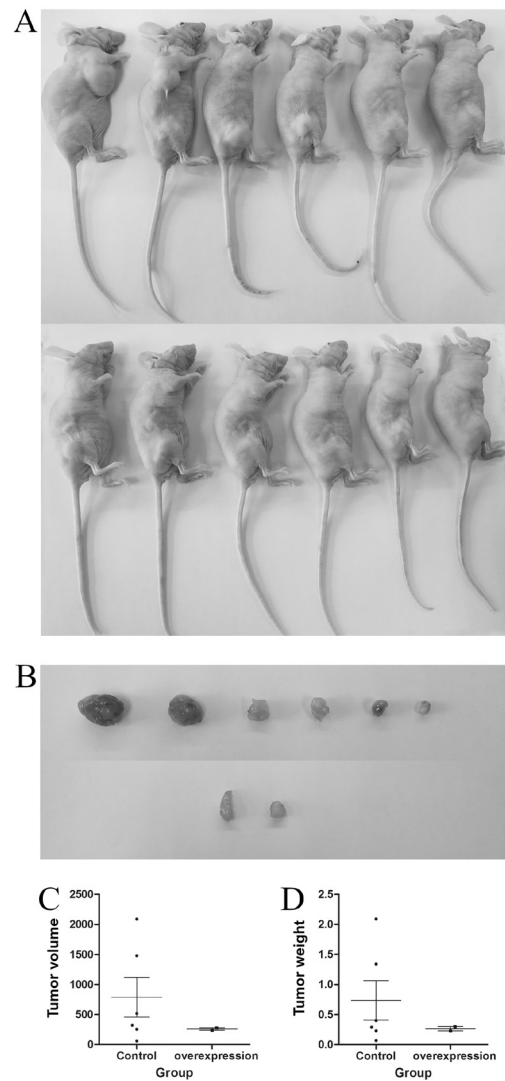


Figure 5. PPAR γ activation inhibits breast cancer growth *in vivo*. A) The subcutaneous xenografts model on nude mice showed that the inhibition of the proliferation of stable cell lines by pioglitazone whose gene PPAR γ was overexpressed. B) The tumor tissues from nude mice indicated that the inhibition effect on the proliferation of breast cancer by pioglitazone. C) The column graph for tumor size from nude mice. D) The column graph for tumor mass from nude mice.

line MDA-MB-231, there is no difference between the treatment group and the control group. With the above results in mind, we could speculate that methylation was implicated in the regulation of the gene PPAR γ expression. Given the different results between the MCF-7 and MDA-MB-231, we thought that many other factors such as some transcription factors besides the methylation in the promoter region were involved in the gene PPAR γ expression regulation. The detailed mechanism needs to be further explored.

Many biological functions of PPAR γ have been reported such as promoting terminal differentiation of adipocytes,

inducing differentiation and apoptosis of tumor cells, and inhibiting tumor angiogenesis [18]. Several reports said that PPAR γ acted as a tumor suppressor in many cancers and could be a target in an adjuvant therapeutic plan by combining with some agonists [19, 20]. However, whether these agonists such as ibuprofen, pioglitazone, and indomethacin have the potential in the treatment for breast cancer still remain unknown. Therefore, the three agonists were selected and used for the assay on the effect of breast cancer treatment. The results showed that only pioglitazone has the inhibition effect through PPAR γ based on the cell viability difference between the triple-negative cells and non-triple-negative cells and the variation trend from 24 h to 72 h. Then, we performed scratch experiments with the agonist pioglitazone. Results revealed that in the non-triple negative cell lines the wound healing was inhibited relative to that in triple-negative cell lines. These results further proved that pioglitazone could exert its inhibition effect through PPAR γ . However, whether the PPAR γ expression could be reversed in triple-negative breast cancer and become a target for pioglitazone in the treatment plan. So the cell line MDA-MB-231 was selected for analysis of the combination of pioglitazone and 5-Aza. The results indicated that the combination has more inhibition on proliferation and migration than that in the other two groups. In order to further ascertain the effect of PPAR γ on cell proliferation and migration by pioglitazone, the PPAR γ overexpressed stable cell line was established and was assayed by treating with pioglitazone. The results were the same as the above. All these results documented that pioglitazone could be used as an adjuvant therapy choice by gene PPAR γ .

The PI3K/AKT signal pathway is often involved in the regulation of cellular metabolism, tumor development, growth, proliferation, and metastases. As a part of a complex intracellular cell signal cascade, it was often selected as a target in cancer therapeutics [21, 22]. To probe the mechanism of pioglitazone by PPAR γ , the signal pathway was investigated and the results indicated that there was no obvious difference between the treatment group and the control group. Then, another JAK2/STAT3 pathway was probed in our study. We know that the JAK2/STAT3 pathway widely participates in tumor metastasis and survival in various cancers, including colorectal cancer, breast cancer, and skin cancer [23]. Our results indicated that between the treatment group and the control group, a marked difference was found not only in protein level but also in their phosphorylation form. In order to further clarify the role of signal pathway JAK2/STAT3, the inhibitor fedratinib for JAK2 was used in the cell cycle, cell apoptosis, and cell migration assays. The inhibitor was proved to reverse the effect exerted by the pioglitazone in the PPAR γ overexpressing stable cell line. Taken the above results together, we concluded that the signal pathway JAK2/STAT3 participated in the pioglitazone effect by PPAR γ on the reduction of cell proliferation and migration in breast cancer.

In order to further probe the inhibition effect of pioglitazone on the breast cancer cells *in vivo*, the xenograft

tumor model in nude mice was established by inoculating the breast cancer cells subcutaneously or by tail vein injection. However, the rate for tumor formation by subcutaneous injection was relatively lower than expected, and the tail vein injection model almost did not succeed. Therefore, in the formal experiment, the xenograft model was developed at the armpit and hind leg area, and the metastasis model was abandoned. Our results indicated that the group treated with pioglitazone had the inhibition effect on tumor proliferation. The nude mice assay meant that the ligand pioglitazone could be an alternative choice in breast cancer adjuvant therapy. The results also provide a clue for clinical practice in breast cancer therapy by a combination of methylation inhibitor 5-Aza and pioglitazone.

In summary, the PPAR γ expression both on mRNA level or protein level was reduced in breast cancer tissues compared to that in normal breast tissues. Its expression regulation was dependent on methylation level in the PPAR γ promoter region in a non-independent way. And the inhibition of pioglitazone on the proliferation and migration in breast carcinoma by PPAR γ was closely related to the JAK2/STAT3 pathway. The combination of 5-Aza and pioglitazone could be an alternative choice for the treatment of breast cancer. Of course, many detailed mechanisms are not fully understood yet and further research is needed to make a good understanding of its function and mechanism.

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