



Crosstalk between Viable Targets: RUNX2 and FXR for Effective Treatment of Prostate Cancer through Regulation of the PTEN

Noor Ghalib Al-hussainy¹, InamSameh. Arif², Basma Talib Al-Sudani^{3*}

Abstract

Purpose:The aim of the project is to indicate that FXR activation can induce PTEN and BMP-2 expression through RUNX2 down expression, and FXR could be a potential therapeutic target for the treatment of prostate cancer.

Methods:Human adenocarcinoma prostate cancer cell lines (PC3) were generally cultured in RPMI1460 media, 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C and 5% CO₂ using 75 cm³ cell culture flasks and treated with Obeticholic acid INT747 (as FXR agonist). Determination of the viability of PC3 cell line after treatment with different concentrations of INT747 compound by MTT assay. The expressions level of FXR, RUNX2, PTEN, and BMP2 were measured by western blot.

Results:The results show that INT747 could inhibit PC3 cells after 72h with IC₅₀ = 7.4 μM. Western Blot analysis of FXR, PTEN, and BMP-2 expressions in PC3 cell lines revealed that INT747 significantly increases remarked of FXR, PTEN, and BMP-2 levels at concentration 7.4μM. While the expression of RUNX2 protein was decreased after being treated with INT747.

Conclusions:We expect there is a molecular relationship between FXR and RUNX2 in prostate cancer via FXR activation by increasing the expression of PTEN protein.

Key Words:PC3 cell line, INT747, FXR, RUNX2, PTEN and BMP2.

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Introduction

Prostate cancer (PCa), one of most popular male cancer, is commonly linked to bone metastases. (1). The metastatic process initiate in the primary tumor by genes activation that promote angiogenesis, tumor invasion, and migration leading to colonization of peripheral tissues involving bone(2). In prostate cancer, many genomic markers that can predict tumor behavior have been identified. One such marker is the phosphatase and tensin homolog (PTEN) gene (the most frequently deleted tumor suppressor gene in prostate cancer)(3), a tumor suppressor that down-regulates the phosphoinositide-3-kinase (PI3K)-protein kinase B (AKT) pathway(4,5). Runt-related transcription factor 2 (RUNX2) (also known as

core-binding factor subunit alpha-1 (CBF-alpha-1)) plays an important function in the regulation of cell proliferation in osteoblasts and endothelial cells. RUNX2 is also implied in human PCa(6,7) and RUNX2 overexpression has been related to elevation of matrix metalloproteinases, secreted bone-resorbing factors, and PCa cell metastasis to bone(8,9). Most studies have proven that RUNX2 was overexpression in prostate tumorigenesis, and this elevation in RUNX2 protein correlates with lowering expression of PTEN protein(10,11). Efforts to better therapy resistance against PCa are associated to elevate the expression and level of PTEN protein in PCa by elucidating the molecular mechanisms.

Corresponding author: Basma Talib Al-Sudani

Address: ¹College of Pharmacy, Department of Toxicology, Mustansiriyah University, Iraq; ²College of Pharmacy, Department of Toxicology, Mustansiriyah University, Iraq; ³College of Pharmacy, Department of Clinical Laboratory Sciences, Mustansiriyah University, Iraq.

E-mail: ³basma_alsudani@uomustansiriya.edu.iq,

ORCID:

<https://0000-0001-6253-3599>



There is a way to up-regulate the tumor suppressor gene, PTEN, by farnesoid X receptor activation (FXR, a member of the nuclear receptor superfamily). FXR is believed to play a role in the regulation of tumorigenesis development, according to previous research. and approved that the gene expression of FXR in prostate cancer was down(12). Since the activation of FXR could inhibit the proliferation of prostate cancer cell lines via the up-regulation of PTEN expression(13). The aim of the study is to indicate that FXR activation can induce PTEN and BMP-2 (bone morphogenetic protein, which play an important role in the development of bone and cartilage) expression through RUNX2 down expression, and FXR could be a potential therapeutic target for the treatment of prostate cancer. In this study, western Blot analysis of FXR, PTEN, and BMP-2 expressions in PC3 cell lines revealed that INT747 significantly increases remarked of FXR, PTEN, and BMP-2 levels at concentration 7.4 μ M. While the expression of RUNX2 protein was decreased after being treated with INT747. In conclusion, there is a molecular relationship between FXR and RUNX2 in prostate cancer by activation of the FXR.

Experimental

1. Materials

Chemicals were purchased from ThermoFisher Scientific and Sigma-Aldrich. Human adenocarcinoma prostate cancer cell line (PC3) was purchased from the American Type Culture Collection (ATCC). RPMI1460 medium was purchased from Capricorn, USA. BCA protein conc. Kit was purchased from the Elabscience, USA. FXR, BMP2, PTEN and RUNX2 polyclonal antibody polyclonal antibody were purchased from Elabscience, USA. Goat anti-mouse IgG secondary antibody was purchased from Invitrogen. Western blot and SDS kits were purchased from Elabscience, USA.

2. Procedures

Cell Lines and Cultures

Human adenocarcinoma prostate cancer cell line (PC3) cell lines were generally cultured in RPMI1460 media, 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C and 5% CO₂ using 75 cm³ cell culture flasks(14).

Treatment of PC3 Cell Line by FXR Agonist

Obeticholic acid INT747 was prepared at the first concentration 10Mm. This concentration was prepared by dissolved 4.5mg of INT747 in 1ml DMSO according to this equation $C1V1=C2V2$. This process had been done inside the sterile hood to avoid any contamination. The concentration of this compound which used in all experiments was depend on its IC50 in PC3 cell line.

Effect of FXR Agonist on PC3 cell Line by MTT Assay

The MTT test was used to assess the cytotoxicity of the INT747 on PC3 cells(15). A 100 μ l from PC3 cells suspensions were dispensed into 96-well flat-bottom tissue culture plates at concentrations of 5×10^3 cells per well and were incubated 24h under standard conditions; 4×10^3 cells/well for 48h incubation, and 3×10^3 cells/ well for 72h incubation(16). After 24 h, the cells was treated with a range of INT747 concentrations (1.5-100 μ M). The cell culture medium was removed after a recovery period of 24, 48, and 72h. The cultures were then incubated for 4 h at 37 °C in medium containing 30 μ l of MTT solution (3 mg/ml MTT in PBS). This medium was removed after 4h by softly inverting. 100 μ l of growth medium are just placed into control wells. All wells received 100 μ l of dimethyl sulfoxide (DMSO), which was then added, and the plates were kept at room temperature in the dark for around 15-20 minutes. Using a multiscan reader and a wavelength of 540 nm to measure the absorbance of each well and a wavelength of 650 nm to adjust for background absorbance. At 72 h, MTT test was used to determine the IC50. IC50 was evaluated by MTT assay at 72h after the cells exposure to INT747.

Western Blot Analysis

The FXR, PTEN, BMP-2 and RUNX2 proteins were separated via SDS acrylamide gel electrophoresis. By western blot technique, the specific proteins (FXR, PTEN, BMP-2 and RUNX2) were identified from a complex mixture of proteins extracted from cells (17,18). Firstly, extract the proteins from human adenocarcinoma prostate cancer cell lines (PC3) before and after treatment with INT 747 and measure the protein concentration by BCA Protein Colorimetric Assay Kit. Then, protein samples denaturated and added to the microgel (1 mm thick mini-gel). The molecular weight of FXR, PTEN, BMP-2 and RUNX2 proteins are 66, 47, 26 and



50KDa respectively. Therefore, 6% SDS gel electrophoresis was prepared. After loading the samples to the gel, the lid of the electrophoresis tank was covered and mind the positive and negative. The gel electrophoresis was run at voltage 110V around 2-3 hours until bromophenol blue reached the bottom of the gel. Secondly, wet transfer: placed the gel flat on the negative filter paper, and then placed flat the PVDF membrane (the PVDF is soaked in methyl alcohol for 5 minutes, then saturated in transferring buffer) on the gel. Loaded the transferring buffer in the transfer tank and inserted the folder. The tank was put in ice water and PVDF membrane was near the positive pole, amino acid and protein with electronegative were moved to the positive pole. The electricity of the tank was 200mA. After transferring, the transferred PVDF membrane was rinsed with a washing buffer at room temperature. After that, blocking by rinsed membrane into the blocking buffer 5% (w/v) skim milk powder (1mg skim milk powder + 9ml 1X TBST working buffer) and incubated on the shaker at room temperature for 2 hours(19). The TBST working buffer was prepared by mixing these compounds: 145.4 mM NaCl, 10 mM Tris-base, 0.1% (v/v) Tween 20, pH7.5. After blocking, the membranes were incubated overnight at 4°C with diluted primary antibody 1:100 (anti-FXR, anti-PETN, anti-BMP-2 and anti-RUNX2) to blocked membrane then washed the membrane with washing buffer for 10 minutes and repeated 3 times. After washing, incubated with diluted secondary antibody 1:5000 (anti-mouse IgG) for 2h then washed the membrane for 10 minutes and repeated 3 times. The ECL luminescence detection solution was used to detect antigen-antibody complexes. Then the

result recorded with chemiluminescence imaging system. Analyzed the bands with BandsCan software. Relative levels of proteins expression were obtained by comparing ratios of intensities of a reference band (glyceraldehyde-3-phosphate dehydrogenase [GAPDH]).

3. Statistical Analysis

GraphPad Prism 8 software was used for all statistical analysis of the data. IC50 was calculated using software Origin 9.1. A comparison between all groups within the same plate of MTT was evaluated by one-way ANOVA with Tukey (Prism 8 software). Statistically significant values considered $p < 0.05$.

Results

1. Effect of FXR Agonist in PC3 Cell line by MTT Assay

PC3 cells were pre-treated with arrange concentrations (1.5, 3.12, 6.25, 12.5, 25, 50 and 100 μM) of INT747 for 24, 48 and 72h to determine the effect of INT747 on PC3 cell viability. INT747 was decreased significantly the cell viability at 25-100 μM , $p < 0.005$, at 24, 48 and 72h as shown in **figure 1**. To compare between INT747 groups (at different time), after 24h of cell incubation with 50 and 100 μM the percentage of cell death reach to 62.03% and 82.9% respectively. At 48h incubation treatment of PC3 with 12.5, 25, 50 and 100 μM of INT747 increased cell death % to 61.15%, 61.15%, 83.36% and 90.55% respectively. The cell death of PC3 at 72h incubation after treated 12.5, 25, 50 and 100 μM of INT747 was 67.41%, 77.55%, 88.08%, 91.35% respectively.

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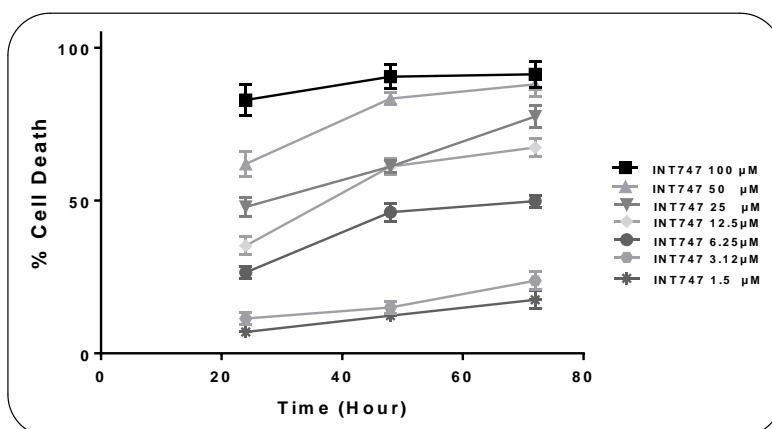


Figure 1. Percentage of cell death induced by different concentrations of INT747. The results of PC3 cells after 24, 48 and 72h treatment of 1.5, 3.12, 6.25, 12.5, 25, 50 and 100 μM INT747. The results represent the mean absorbance \pm SEM of 3 independent experiments using Prism Pad 8.1 software to draw bars

The dose-response curve generated by Origin 9.1 using nonlinear regression analysis for INT747 in PC3 cells is shown in **figure 2**. The IC50 value was obtained to a range of INT747 concentrations (1.5-100 μM) by MTT assay for 72 h. IC50 of INT747 in PC3 cells = 7.4 μM .

in Prostate Cancer (PC3) Cell Lines After Treated with INT747 and Guggulsterone by Western Blot

Western Blot analysis of FXR expression in PC3 cell lines (**Figure 3**) revealed that INT747 caused increase in FXR expression at concentration 7.4 μM . After treatment with INT747, the amount of expressions relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) significantly enhanced (**Figure 3A and B**) as compared with control (FXR level in PC3 cell lines without INT747 treated), ($p < 0.05$).

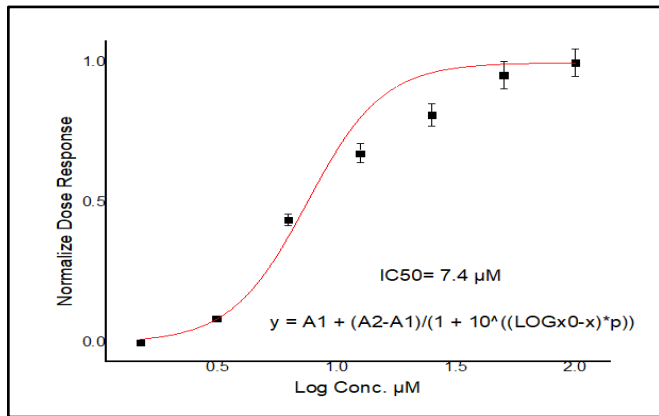


Figure 2. Dose-response curves of INT747 for PC3 cells. PC3 cells were treated for 72h with 1.5, 3.12, 6.25, 12.5, 25, 50 and 100 μM dose ranges of INT747. IC₅₀ values were determined using nonlinear regression analysis (Origin 9.1). Error bars represent the standard error of the mean (SEM) for triplicate data

2. Protein Expression of FXR, RUNX2, PTEN and BMP2

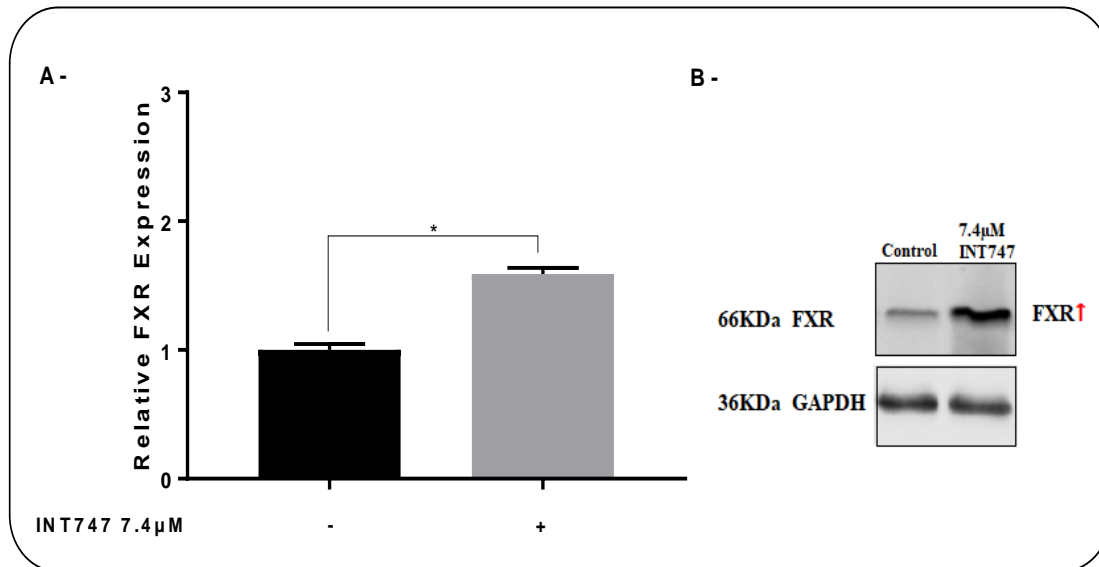


Figure 3. Effect of INT747 on FXR by Western blot. A-INT747 induced the increasing of FXR expression. INT747 concentration is labeled at the bottom (μM), $p < 0.05$. B-Western blot analysis result of FXR expression. Significant difference compared with the control group. Y-axis: FXR expression versus GAPDH

As shown in (**Figure 4**), PTEN expression of PC3 cells increased significantly after treated with INT747 ($p < 0.005$), suggesting that PTEN levels

responded actively to the up regulation of the expression and function of FXR.



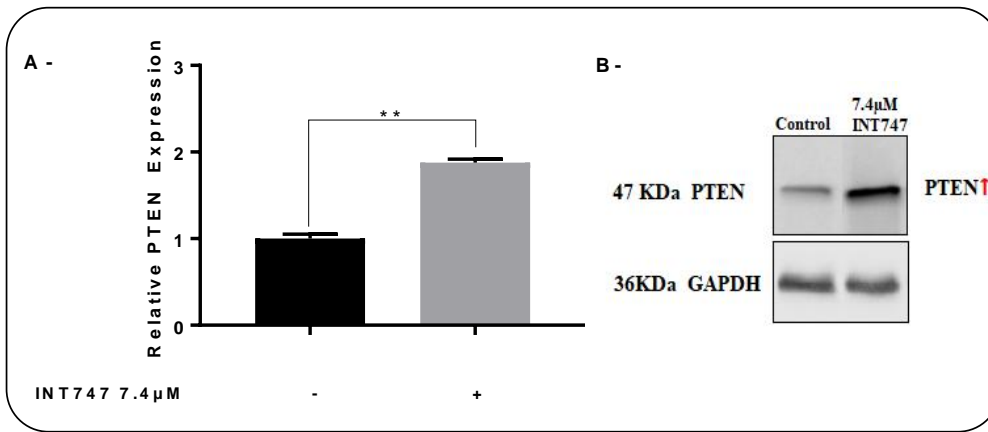


Figure 4. Effect of INT747 on PTEN by Western blot. A-INT747 induced the increase in PTEN expression. INT747 concentration is labeled at the bottom (μM), $p < 0.005$. B-Western blot analysis result of PTEN expression. Significant difference compared with the control group. Y-axis: PTEN expression versus GAPDH

(Figure 5) shows the BMP-2 protein expression of PC3 cells after treatment with 7.4 μM INT747. Western blot analysis revealed that INT747 significantly increases remarked of BMP-2 protein level ($p < 0.05$) as compared with its low level in the

PC3 cell line before treatment. While the expression of RUNX2 protein was decreased after treated with INT747 as shown in **(Figure 6)** ($p < 0.005$), after the marked increase in expression before treatment.

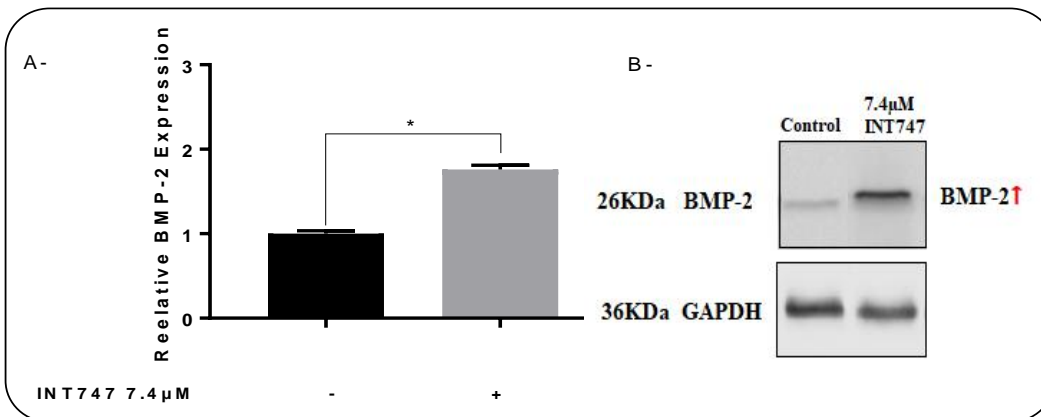


Figure 5. Effect of INT747 on BMP-2 by Western blot. A-INT747 induced the increasing in BMP-2 expression. INT747 concentration is labeled at the bottom (μM), $p < 0.05$. B-Western blot analysis result of BMP-2 expression. Significant difference compared with the control group. Y-axis: BMP-2 expression versus GAPDH

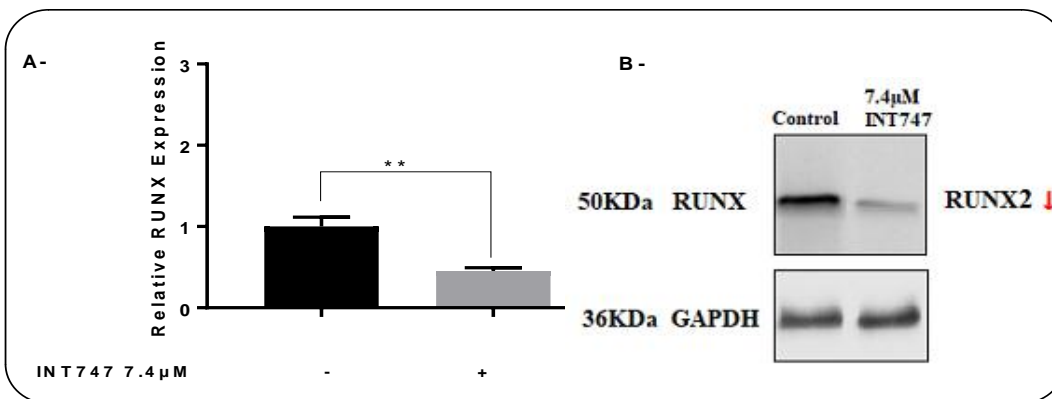


Figure 6. Effect of INT747 on RUNX2 by Western blot. A- INT747 was decreased the RUNX expression. INT747 concentration is labeled at the bottom (μM), $p < 0.005$. B-Western blot analysis result of RUNX2 expression. Significant difference compared with the control group. Y-axis: RUNX2 expression versus GAPDH



Discussion

Understanding the specific role performed by FXR is expected to increase our understanding of the biology of prostate cancer, which could be essential for treatment in the future. In the current study, the results show that FXR expression was downregulated in PC3 cell lines. While, after treating the PC3 cells with an FXR agonist (INT747), the FXR expression was elevated (Figure 3) and this overexpression induced inhibition of PC3 cell proliferation (Figure 1). Therefore, this study 7.4M INT747 showed that FXR activation may function as a tumor suppressor in the development of prostate cancer (Figure 2). However, the mechanisms of FXR down-regulation in prostate cancer remain unknown. Some researchers have demonstrated that insulin and proinflammatory cytokines as well as microRNAs are able to regulate FXR in cells(20,21). Therefore more studies about the down-regulation of FXR expression in prostate cancer should be performed. Modica et al, claimed that FXR can protect against intestinal tumor genesis and other studies showed that FXR can inhibit cell proliferation in hepatocellular carcinoma and colon cancer(22,23). The findings of this study showed that activation of the FXR increased the expression of the tumor suppressor gene PTEN (Figure 4). In this work, BMP-2 levels were elevated in PC3 cells with INT747 treatment (figure 5), and these results agree with previous studies which described the loss of BMP-2 expression is linked with prostate cancer progression (24). More recently studies demonstrated that decreased BMP-2 level in PCa tissue is linked to progression to a more aggressive phenotype and worsened Gleason grade which is similar to the result of the western blot, but other researchers suggested inhibition of BMP-2 level is related to control of cell proliferation so, "the mechanism of BMP-2 on prostate cancer is not completely understood"(25). In prostate tumorigenesis, most studies have proven that RUNX2 is overexpressed, and this elevation in RUNX2 protein links with decreased expression of PTEN protein(11). So, our results confirmed that down-regulation of PTEN by FXR activation could inhibit the RUNX2 level as shown in (figure 6). Based on the experimental results, the proposed therapy showed high level of resistance against PCa, which is has been achieved by increasing the expression and the level of PTEN protein in PCa

associated with elucidating the molecular mechanisms.

Therefore, the FXR/PTEN/RUNX2 signaling pathway may be a novel pharmaceutical target for the treatment of prostate cancer.

Conclusions

This study revealed that PTEN regulates Human adenocarcinoma prostate cancer cell line (PC3) cell lines resistance to cancer via FXR protein activation and down regulation the RUNX2 protein. FXR activator could potentially be used in the future to prevent the proliferation of prostate cancer.

Conflicts of Interest

There are no conflicts to declare.

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