RESEARCH ARTICLE

Modulatory Effects of Metformin on Farnesoid X receptor and Specificity Protein 1 in Human Pancreatic Cancer BxPC-3 Cells

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ABSTRACT

Background: Pancreatic cancer is the fourth major cause of death, accounting for 7% of cancer-related deaths. Metformin (antihyperglycemic agent) users had a 62% lower risk of developing pancreatic cancer when compared to metformin non-users.

Objective: This research aims to study the role of the Farnesoid X receptor (FXR) receptor in human pancreatic cancer cell line BxPC-3 and metformin's modulatory effects on this receptor.

Material and methods: Cell viability was assessed using MTT assay for INT747 (FXR agonist) and metformin, FXR and Specificity Protein 1 (Sp1) mRNA and protein levels were measured by quantitative reverse transcription-polymerase chain reaction (RT-qPCR) and western blot, respectively.

Results: INT747 increased the growth and viability of BxPc-3 cells in addition to a significant increase in FXR and SP1 mRNA and protein levels. In contrast, BxPC-3 cells viability was significantly reduced by metformin in a dose- and time-dependent manner with downregulation of FXR and SP1 at mRNA and protein levels

Conclusion: FXR may act as an oncogenic factor in pancreatic cancer BxPC-3 cells by increasing FXR and SP1 expression, while metformin significantly reduced FXR mRNA and protein levels in BxPC-3 cells, which may be due to a reduction in SP1 expression.

Keywords: Farnesoid X receptor, Metformin, Pancreatic cancer, Specificity Protein 1.

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INTRODUCTION

Pancreatic cancer (PC) is a life-threatening condition with a poor prognosis and a rising incidence.[1] PC is the fourth major cause of death, accounting for 7% of cancer-related deaths.[2] Notably, 60 percent of PCs arise in the head of the pancreas near the bile tract, indicating that bile acids (BAs) might have a role in developing PC. BAs levels were significantly higher in PC patients' serum and pancreatic juice than in controls. Importantly, BAs serve as metabolic regulators, altering the distribution of adipose tissue, metabolism of triglyceride and insulin sensitivity. Consequently, three risk factors for PC are associated with BA: obesity, diabetes and hypertriglyceridemia.[3]

FXR is a member of the superfamily of nuclear receptors (NRs) identified as a BA receptor.[4] FXR stimulation has been shown to improve a variety of metabolic disorders, such as dyslipidemia, fatty liver disease, obesity and type 2 diabetes.[5] FXR expression is found in many gastrointestinal and extra-intestinal tissues, and its final impact on cancer initiation and growth varies from one anatomical site to another.[6] Recent research has shown that an elevated level of FXR expression in PC is associated with a poor prognosis and that Sp1 has been positively associated with FXR, giving patients the poorest prognosis with their simultaneous high expression.[7]

Sp1 is a key transcription factor for various genes involved in tumor cell survival, growth, and angiogenesis, so abnormal Sp1 expression and activation may play a role in the development and progression of human cancer including pc. High Sp1 expression is associated with aggressive biology and poor prognosis.[8]

Metformin (MET) has decreased blood glucose levels in Europe since 1957 and in the United States since 1995.[9] Diabetes has been linked to an elevation in cancer incidence by 1.2–2.0 times. MET has been shown to decrease this risk by approximately 40% compared to other antidiabetic treatments.[10] MET influences various cellular pathways in pancreatic cancer, including reduction of mammalian target of rapamycin (mTOR) signaling causing impairment in the

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protein synthesis and cell growth.[11] Sp1 transcription factor has been downregulated by using MET.[12]

This study investigates the role of the FXR receptor in human PC cell line BxPC-3 and metformin's modulatory effects on this receptor. To achieve this goal, MTT assay was used to assess cell viability of INT747 (FXR agonist) and metformin, FXR and SP1 mRNA and protein levels were measured by quantitative reverse transcription-polymerase chain reaction (RT-qPCR) and western blot, respectively.

METHODS

Cell Line and Cell Culture

Human PC cell line BxPC-3 cells were provided by ATCC (American Type Culture Collection). The cells were cultured in Roswell Park Memorial Institute -1640 (RPMI-1640, Gibco, USA) supplemented by 1% penicillin, streptomycin (Gibco, USA) and 10% fetal bovine serum (FBS, Gibco, USA). The cultured cells were incubated in 5% CO_2, 95% humidified air at 37°C, and when the confluence of the cells reached 80–85%, they were ready for sub-culturing where the cells were detached using trypsin and resuspended in the complete medium.

Cytotoxicity Assay (MTT) and IC_{50} Determination

The MTT assay was used for assessing the effects of INT747 and MET on BxPC-3 viability. In 96 well plates, 5 x 10^3 cells per well, 4 x 10^3 cells, 3x10^3 cells were seeded in each well with 100 µL of complete media. 5 x 10^3 cells incubated for 24 hours, 4 x 10^3 incubated for 48 hr, and 3 x 10^3 incubated for 72 hours in 5% CO_2 at 37 °C. MTT solution prepared at 3 mg/mL in phosphate buffer saline (PBS, Gibco, USA), 30 µL of MTT solution was added for each well and incubated 4 hours at 37°C. After that, the medium containing MTT was entirely removed and 100 µL of dimethyl sulfoxide (DMSO) was added for all wells; then, plates were kept in the dark for about 1-2 hours. The optical density of the solution was read at 540 nm by the multiscan reader and corrected at 650 nm for the absorbance of the background. The (IC_{50}) was determined with cells treated for 72 hours by nonlinear regression analysis.

Quantitative Reverse Transcription-polymerase Chain Reaction (RT-qPCR)

Total RNA was extracted from BxPC-3 cells after 48 hours of treatment using the protocol that combines TRIZOL reagent and RNeasy Mini spin columns kit (Qiagen) according to the manufacturer's instructions and stored at -80°C. cDNA synthesis was performed using a reverse transcriptase kit (Bioneer). cDNA was used immediately for PCR or stored at -20°C. qPCR was performed using SYBR Green qPCR premix (intron biotechnology), an optimized ready-to-use solution incorporating SYBR Green dye, Taq DNA polymerase, ultra-pure dNTPs, MgCl2. The qPCR was carried out in a 20 µL of mixture which consisted of 3 µL of cDNA, 1-µL of forward primer, 1-µL of reverse primer, 10 µL of SYBR green qPCR premix and nuclease-free water up to 20 µL. The PCR amplification cycles were as follows: 10 minutes at 95°C for initial activation, followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 30 seconds and elongation at 72°C for 1 minute, with a final 5 minutes extension at 72°C. Normalization and quantification of the PCR signals were performed by comparing the cycle threshold value of the gene of interest, in triplicate, with the housekeeping gene GAPDH. The fold change in gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Primers were purchased from BioNeer- Korea; the following primers were used:

- FXR (forward): 5′-ACAGAACAAGTGGCAGGTC-3′
- FXR (reverse): 5′- CTGAAGAAACCTTTACACCCCTC-3′
- SP1 (forward): 5′- TTG AAA AAG GAG TTG GTG GC-3′
- SP1 (reverse): 5′- TGC TGG TTC TGT AAG TTG GG-3′
- GAPDH (forward): 5′- GAAGGTGAAGGTCGGAGT-3′
- GAPDH (reverse): 5′- CATGGGTGGAATCATATTG-GAA-3′

Western Blot

BxPC-3 cells were seeded at a density of 6 × 10^5 cells per well in 6 well plates and treated with 10 µM INT747, 140 µM metformin, and control received medium only. Cells were lysed in RIPA (radioimmunoprecipitation assay) lysis buffer. Cell lysates were sonicated and incubated at 4°C for 30 minutes before centrifuging at 12,000 rpm for 10 minutes at 4°C. The protein concentration was determined using BCA (bicinchoninic acid) protein assay reagent (Elabscience, USA). The protein (50 µg) was electrophoresed onto 8% SDS-polyacrylamide gel, along with a 5 µL of pre-stained protein marker loaded to a reserved well to verify the target molecular weight and the extent of membrane transfer. The gel electrophoresis was started at 120V for about 2 to 3 hours till bromophenol blue reached the bottom of the gel. The separated protein bands were transferred onto a nitrocellulose membrane using a Bio-Rad Mini Trans-Blot cell. The nitrocellulose membrane was incubated in blocking buffer (5% skim milk powder in TBST buffer; tris-buffered saline + 0.1% Tween 20) on a shaker at room temperature for 2 hours, followed by overnight incubation at 4°C with the primary antibody. The antibodies against FXR, SP1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were all used at dilutions of 1:1000 (Elabscience, USA). The membrane was washed three times for 15 minutes each with TBST buffer and then incubated for 2 hours at room temperature with diluted secondary antibody 1:5000 then washed for 15 minutes, three times. The protein bands were visualized by using enhanced chemiluminescence (ECL) kit (Elabscience, USA). The band intensity of target proteins was quantified by normalization to GAPDH by using image J software.

Statistical Analysis

All statistical analysis was performed using Graph Pad Prism 8.1 software. All results are presented as the mean ± standard error of the mean. Significant differences in mean values were evaluated using one-way ANOVA with Tukey post hoc test. *$p < 0.05$ **$p < 0.01$, ***$p < 0.005$ and ****$p < 0.001$ versus corresponding control, indicated a statistically significant difference.

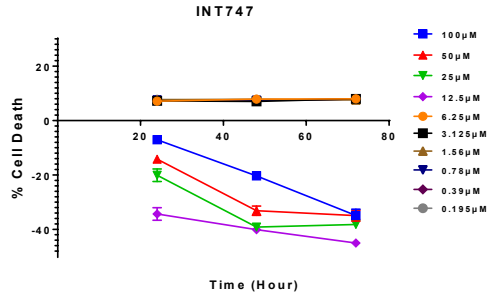

Figure 1: Percent cell death of the pancreatic cancer cell line (BxPc-3) was detected by MTT assay using a range of INT747 concentrations. The results represent the percent cell death of three independent experiments ± SEM.

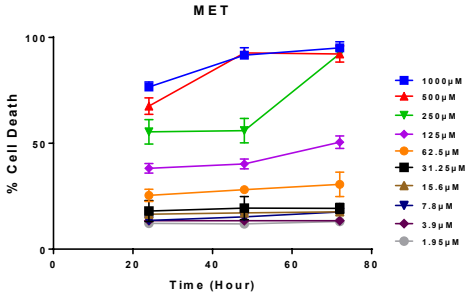

Figure 3: Percent cell death of the pancreatic cancer cell line (BxPc-3) was detected by MTT assay using a range of MET concentrations. The results represent the percent cell death of three independent experiments ± SEM.

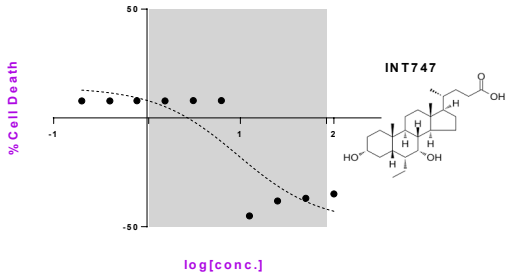

Figure 2: Dose-response curve of INT747. BxPc-3 cells were treated for 72h with 0.195, 0.39, 0.78, 1.56, 3.12, 6.25, 12.5, 25, 50 and 100 µM. The dose-response curve was plotted over log-transformed concentrations using nonlinear regression analysis.

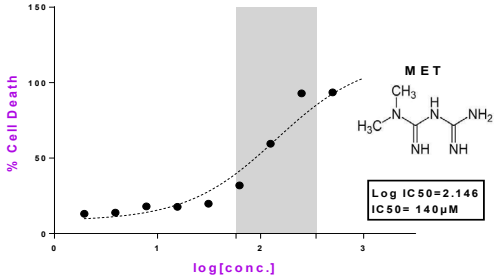

Figure 4: Dose-response curve of metformin. BxPc-3 cells were treated for 72h with 1000, 500, 250, 125, 62.5, 31.25, 15.6, 7.8, 3.9, 1.95 µM of MET. The dose-response was plotted over log-transformed concentrations. IC50 values were determined using nonlinear regression analysis.

RESULTS

Cytotoxicity of INT747 and Metformin

The MTT assay was used for assessing the cell viability of BxPC-3 cells in the presence of a range of concentrations (0.195, 0.39, 0.78, 1.56, 3.125, 6.25, 12.5, 25, 50, and 100 µM) of INT747. The results demonstrated in Figure 1 indicated that INT747 had no cytotoxic effect on the BxPc-3 cells at the low doses (0.195 to 6.25 µM), while from 12.5 to 100 µM, cell viability was enhanced and became more than 100% (cell death % value less than zero). The dose-response curve of INT747 was plotted by Graph Pad Prism 8.1 using nonlinear regression analysis, as shown in (Figure 2). The value was obtained to a range of INT747 concentrations from (0.195 – 100 µM) for 72 hr by MTT assay. The INT747 dose greater than 9.3 µM caused an increase in cell growth.

BxPC-3 cell viability was reduced by MET in a dose- and time-dependent manner, as shown in figure 3. The percentage of cell death in the lowest dose (1.95 µM) was 12.2, 12.7 and 13% at 24, 48, and 72 hours, respectively. In comparison, the highest dose (1000 µM) increased the cell death % to 77.8% at 24 hours, 92.8% at 48 hours and 95.2% at 72 hours. The IC_{50} is the concentration of drug required for 50% growth inhibition. The dose-response curve of MET was plotted by Graph Pad Prism 8.1 using nonlinear regression analysis, as shown in Figure 4. The IC_{50} value was obtained to a range of MET concentrations from (1.95–1000 µM) for 72 hours by MTT assay. The IC_{50} for MET was 140 µM.

Effects of INT747 and Metformin on the Gene Expression of FXR and SP1 in BxPC-3 cells by RT-qPCR

Figure 5 shows the RT-qPCR results of BxPC-3 cells after treatment with 120, 140 µM of MET and 5, 10 µM of INT747. MET significantly reduced FXR mRNA levels at 120 µM ($p < 0.05$) and at 140 µM ($p < 0.001$), whereas INT747 caused a significant increase of FXR expression at 10 µM ($p < 0.005$), and the effect is less pronounced at 5 µM ($p < 0.01$), compared to untreated cells.

Figure 6 shows the SP1 gene expression results of BxPC-3 cells after treatment with 120, 140 µM of METand 5, 10 µM of INT747 using RT-qPCR. MET significantly reduced SP1 mRNA level at 140 µM ($p < 0.001$) and 120 µM ($p < 0.05$), while INT747 significantly increased SP1 expression at 10 µM ($p < 0.01$), and the effect is less pronounced at 5 µM ($p < 0.05$).

Effects of Metformin and INT747 on the Protein Levels of FXR and SP1 in BxPC-3 Cell Line by Western Blot

Figure 7 shows the FXR protein bands of BxPC-3 cells after treatment with 140 µM of MET and 10 µM INT747. Western blot analysis revealed that MET significantly reduced the protein level of FXR ($p < 0.01$), whereas INT747 resulted in a significant upregulation of FXR protein levels ($p < 0.005$) compared to control.

Figure 8 shows the SP1 protein bands of BxPC-3 cell lines after treatment with 140 µM of MET and 10 µM of INT747 using western blot analysis. MET significantly reduced the protein levels of SP1 ($p < 0.01$), whereas INT747 significantly

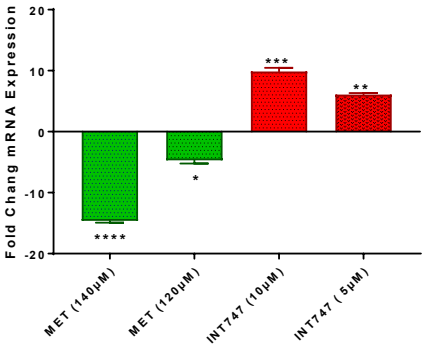

Figure 5: Fold change of FXR gene expression in BxPc3 cells after treatment with MET, INT747 relative to untreated cells by RT-qPCR. Data were normalized to reference gene GAPDH. The baseline shows expression in control samples. Values above zero indicate an upregulation of the gene compared to untreated cells, while values below zero indicate the downregulation of the gene. *p < 0.05 **p < 0.01, ***p < 0.005 and ****p < 0.001 versus corresponding control.

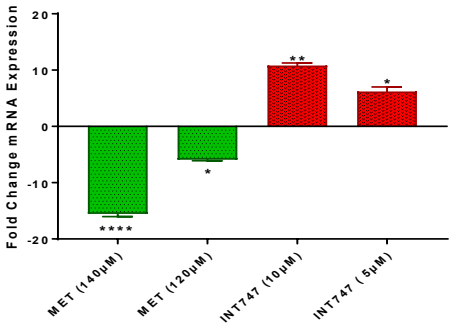

Figure 6: Fold change of SP1 gene expression in BxPc3 cells after treatment with MET, INT747 relative to untreated cells by RT-qPCR. Data were normalized to reference gene GAPDH. The baseline shows expression in control samples. Values above zero indicate an upregulation of the gene compared to untreated cells, while values below zero indicate the downregulation of the gene. *p < 0.05 **p < 0.01, ***p < 0.005 and ****p < 0.001 versus corresponding control.

increased the SP1 band density ($p < 0.005$) compared to control.

DISCUSSION

Recent studies showed paradox hypotheses for the role of FXR overexpression in PC, some suggesting an antitumor effect and others an oncogenic effect.[13] This controversial role led us to search for the FXR overexpression role in PC using a selective FXR agonist. In the present study, the role of FXR in PC BxPc-3 cells has been investigated. Our results showed that INT747 increased the BxPc-3 cell growth and viability, as shown in (Figure 1), which indicates that FXR could act as an oncogene in PC cells. Lee *et al.* showed that FXR functions as an oncogene because it is associated positively with metastasis of the lymph node, migration, and PC invasion that causes poor patient survival and outcome.[14]

In the current study, to evaluate the ability of INT747 for FXR activation in BxPC-3 cells and determine whether this activation is attributed to FXR oncogenic effects, mRNA and

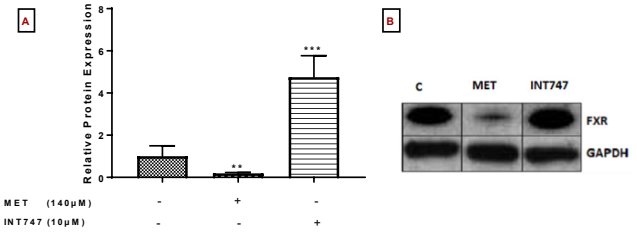

Figure 7: FXR protein levels were assessed by western blot in BxPc3 cells after treatment with MET and INT747. **p < 0.01 and ***p < 0.005 versus corresponding control.

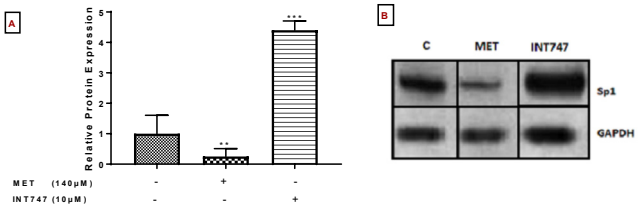

Figure 8: SP1 protein levels were assessed by western blot in BxPc3 cells after treatment with MET and INT747. **p < 0.01 and ***p < 0.005 versus corresponding control.

protein levels of FXR were measured after treatment of cells with INT747 using RT-qPCR and western blot, respectively. As shown in Figures 5 and 7, INT747 caused a significant upregulation in the FXR mRNA and protein levels in the BxPC-3 cells. These results were compatible with Lew *et al.,* who found that all bile acids and the synthetic FXR agonist GW4064 increased FXR protein levels in human hepatocellular carcinoma cells (HepG2).[15] Xu *et al.* also revealed that deoxycholic acid (DCA) and cholic acid (CA) enhanced FXR mRNA expression in rabbits. The ability of endogenous and synthetic FXR agonists to effectively induce FXR indicates that this auto-regulatory loop is essential in FXR-mediated gene regulation.[16]

To reveal the cascade of FXR overexpression in the BxPc-3 cell line, Sp1, a key transcription factor involved in tumor cell survival, growth, and angiogenesis, was assessed. Sp1 is upregulated and associated with a poor prognosis in breast, gastric, pancreatic, brain, and lung cancers. Several studies found that Sp1 can be used as a cancer treatment target.[17] In the present study, activation of the FXR receptor by INT747 caused a significant increase in the SP1 protein and mRNA expression, indicating a positive correlation between FXR and SP1 in BxPC-3 cells partially attributed to the increase in cell viability of BxPC-3 cells by INT747. These results are consistent with Hu *et al*., who discovered that FXR and Sp1 had been positively correlated in mRNA levels, with a high expression indicating a poor prognosis for PC patients, also predicted that Sp1 expression would be downregulated when FXR was knockdown, implying that the positive correlation also found in PC cells at the protein level.[7]

MET (antihyperglycemic medication) displayed a significant inhibitory effect on the growth of BxPc-3 cells in a dose and time-dependent manner, as shown in Figure 3. These results indicate that MET may have antitumor activity in

PC cells. In the present study, to investigate the potential effect of MET on FXR and SP1 in BxPC-3 cells and determine whether this modulatory effect is contributed to the growth inhibitory effect of MET, mRNA and protein levels of FXR and SP1 were measured after treatment of cells with MET using RT-qPCR and western blot, respectively. Our results showed that MET caused a significant downregulation of FXR mRNA and protein levels, and the mechanism for this reduction may be due to a downregulation in Sp1 expression in BxPC-3 cells by MET. These results were consistent with Sun *et al.,* who found that FXR mRNA expression was significantly reduced in the ileum of mice treated with MET through gut microbiota modulation.[18] Tauroursodeoxycholic acid (TUDCA) levels in the ileum were significantly higher in MET-treated mice compared to vehicle-treated mice.[19] MET enhanced glycoursodeoxycholic acid (GUDCA) levels in the intestines of T2DM patients. *In vitro* and *in vivo*, GUDCA and TUDCA were potent FXR antagonists.[20] As a result, MET inhibits the growth and viability of BxPC-3 cells due, in part, to the downregulation of FXR and Sp1 expression.

CONCLUSIONS

From the data above, we can conclude that FXR may act as an oncogenic factor in pancreatic cancer BxPC-3 cells by increasing FXR and Sp1 expression, while metformin significantly reduced FXR mRNA and protein levels in BxPC-3 cells, which may be due to a reduction in Sp1 expression.

CONFLICTS OF INTEREST

There are no conflicts to declare.

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