European Review for Medical and Pharmacological Sciences 2019; 23: 4723-4729

Effects of RXRα on proliferation and apoptosis of pancreatic cancer cells through TGF-β/Smad signaling pathway

G. CHEN[1], M. HU[2], X.-C. WANG[3], S.-H. DU[4], Z.-X. CHENG[2], J. XU[2], Y.-K. QU[2]

[1]Department of Gastroenterology, First Affiliated Hospital of Jiamusi University, Jiamusi, China
[2]Department of General Surgery, First Affiliated Hospital of Jiamusi University, Jiamusi, China
[3]Department of General Medicine, Second Affiliated Hospital of Jiamusi University, Jiamusi, China
[4]Department of Emergency, Second Affiliated Hospital of Jiamusi University, Jiamusi, China

Gang Chen and Ming Hu equally contributed to this work

Abstract. – OBJECTIVE: The aim of this study was to investigate the effects of retinoid X receptor α (RXRα) on the proliferation and apoptosis of pancreatic cancer cells through the transforming growth factor-β (TGF-β)/Smad signaling pathway.

PATIENTS AND METHODS: The expression of RXRα in pancreatic cancer tissues and para-carcinoma tissues was detected via immunohistochemistry. Human pancreatic cancer PANC-1 cells were cultured and treated with RXRα *in vitro*. The apoptosis rate of cells was detected *via* flow cytometry. Furthermore, changes in the protein expression level of TGF-β/Smad signaling pathway were detected via Western blotting.

RESULTS: The protein expression level of RXRα in pancreatic cancer tissues was significantly higher than that of para-carcinoma tissues. RXRα significantly promoted the proliferation and inhibited the apoptosis of pancreatic cancer cells. Moreover, RXRα could also activate the TGF-β/Smad signaling pathway.

CONCLUSIONS: RXRα promotes the proliferation and inhibits the apoptosis of pancreatic cancer cells through the TGF-β/Smad signaling pathway.

Key Words
RXRα, TGF-β/Smad, Pancreatic cancer, Proliferation, Apoptosis.

Introduction

Pancreatic cancer is one of the most important causes of cancer-related deaths, whose 5-year survival rate is lower than 5%[1]. Some studies have demonstrated that adjuvant chemotherapy with gemcitabine or 5-fluorouracil significantly improves the overall survival and disease-free survival of patients. Recently, another study has suggested that the FOLFIRINOX chemotherapy regimen possesses a significant survival advantage. However, it also increases therapy-related toxicity in patients. At the same time, some biological agents targeting tumor-related signaling cascades have been proved to be ineffective for pancreatic ductal adenocarcinoma (PDAC) patients. This is due to the reason that the biological behaviors of pancreatic cancer are more complicated than other malignant tumors. Currently, traditional therapeutic methods, such as surgery, chemotherapy and/or radiotherapy, have little effect on pancreatic cancer[2,3]. Meanwhile, there is a lack of effective early diagnosis indexes and treatment means, leading to the poor prognosis of pancreatic cancer patients[4]. Therefore, it is necessary to search for specific diagnosis indexes for early pancreatic cancer.

Retinoic acid receptor (RAR) includes three isotypes (RARα, RARβ, and RARγ). RAR acts as a gene transcriptional regulatory factor through heterodimerization with retinoid X receptor (RXRα, β, and γ)[5,6]. Natural endogenous retinoids include all-trans retinoic acid (ATRA), which binds and activates RAR and 9-cis retinoic acid (9CRA)[7]. RXR/RAR heterodimer can bind to retinoic acid DNA response element (RARE) in the promoter region of target genes. Meanwhile, RXR also forms RXR/RXR homodimer, which binds to the target DNA sequence of retinoid X response element (RXRE). Several retinoids (such as SR11237) can induce RXR homodimer to bind to RXRE as well. It is well-known that RXR is also a heterodimeric partner for several other nuclear receptors, including peroxisome proliferator-activated receptor (PPAR), liver X

Corresponding Author: Yikun Qu, MD.; e-mail: quyikun2008@163.com

receptor (LXR), and vitamin D receptor (VDR). RXR ligands can activate the function of PPARγ/RXR with non-ligand PPARγ[8]. RXR is a nuclear receptor family involved in controlling various physiological processes, including lipid, glucose metabolism, and immune response[9]. It has been proved that some RXR subtypes can promote the induction of pluripotent stem cells. RXRα is a transcriptional regulatory molecule that regulates gene expression[10]. Furthermore, regulating RXR with natural and synthesized ligands (such as vitamin A and retinoic acid derivatives) can inhibit cell proliferation, leading to its wide application in cancer treatment. However, the exact mechanism of RXR has not been fully clarified yet. In addition, its correlation with the occurrence and development of pancreatic cancer remains unclear.

The transforming growth factor-β (TGF-β)/Smad signaling pathway is involved in regulating cell differentiation. In the present study, it was found that the expression of RXRα was significantly up-regulated in pancreatic cancer tissues. Meanwhile, RXRα promoted the proliferation and apoptosis of pancreatic cancer cells *in vitro*. In addition, the results indicated that the effects of RXRα on the biological behaviors of pancreatic cancer cells were achieved through the TGF-β/Smad signaling pathway. All these findings confirmed for the first time that RXRα might be one of the key molecules for the progression of pancreatic cancer.

Patients and Methods

Pathological Tissues and Specimens

This study was approved by the Ethics Committee of First Affiliated Hospital of Jiamusi University. The signed written informed consents were obtained from all participants before the study. Pancreatic cancer patients admitted to our hospital from January 2014 to January 2017 were enrolled in this study, and tissue specimens were collected. Subsequently, collected tissue specimens were prepared into frozen sections, followed by hematoxylin and eosin (HE) staining (Boster, Wuhan, China). Immunohistochemistry (IHC) was performed to analyze the semi-quantitative expression of RXRα protein in paraffin sections.

Cell Culture

Human pancreatic cancer PANC-1 cell lines were purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA). Before the experiment, PANC-1 cells were cultured in the above medium added with 2 μM ADM in an incubator with 5% CO_2 at 37°C for at least 4 weeks. The medium was replaced once every day. Cell passage was performed when 80-90% of cell fusion.

IHC

Pancreatic cancer tissues and corresponding para-carcinoma normal tissues were fixed with formaldehyde and embedded in paraffin. Subsequently, RXRα polyclonal antibody (Abcam, 1:2000, Cambridge, MA, USA) was used for immunohistochemical SP staining according to the instructions of IHC third-generation assay kit (Invitrogen, Carlsbad, CA, USA). Positive control: the tissues containing test antigens were detected to produce strong positive expression according to the Abcam official website. Negative control: the primary antibody was replaced with PBS, and the results were all negative. Positive signals showed yellow, brown yellow or dark brown color. Five high-power fields (10×40) were randomly observed under an electron microscope. The proportion of positive cells containing yellow, brown yellow or dark brown signals and the intensity of signals were used as criteria.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA in tissues and cells was isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). Subsequently, RNA samples were purified according to the manufacturer's instructions of the RNeasy Mini Kit (Qiagen, Hilden, Germany). After that, extracted RNA was reverse transcribed into complementary deoxyribose nucleic acid (cDNA) using PrimeScript RT kit (TaKaRa, Otsu, Shiga, Japan). RT-PCR was then performed using the following specific primers (Table I). QRT-PCR reaction conditions were as follows: 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s, for a total of 40 cycles. The relative expression level of target genes was calculated by the $2^{-\Delta\Delta Ct}$ method.

Apoptosis Via Flow Cytometry

After different treatments, the culture medium was collected into a centrifuge tube. The cells were washed with phosphate-buffered saline

Table I. Primer sequences.

Gene	Forward primer sequence	Reverse primer sequence
TGF-β1	5'-CCCACAACGAAATCTATGACAAG-3'	5'-GAGGTATCGCCAGGAATTGTTG-3'
Smad2	5'-CTGGAGAATAACAGATGGGATGC-3'	5'-CCCTGGCTCCTCACTTGGC-3'
RXRα	5'-GAGTCCCTGATTGCTGTCTTC-3'	5'-AGGGTCCTTCTGGTTCTTTG-3'
β-actin	5'-AAGTACTCCGTGTGGATCGG-3'	5'-ATGCTATCACCTCCCCTGTG-3'

(PBS) twice, collected and moderately digested with trypsin. The digestion was terminated with the old culture solution previously collected. Then, the cells were blown, beaten and collected into a centrifuge tube, followed by centrifugation at 1000 rpm for 5 min. The supernatant was discarded, and the cells were re-suspended with 1 mL pre-cooled PBS and centrifuged at 1000 rpm for 5 min. After discarding the supernatant, 50 μL cells were retained and transferred into a 1.5 mL Eppendorf (EP) tube. Then the cells were washed with PBS once, and the supernatant was discarded. After treatment with rJHP0290 (100 ng/mL) and/or CPT (10 μM) (Sigma-Aldrich, St. Louis, MO, USA), the cells were stained with fluorescein isothiocyanate (FITC)-conjugated Annexin V antibody (BD Biosciences, Franklin Lakes, NJ, USA) and Propidium Iodide (PI) (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. Briefly, 1×10^5 cells in 100 μL Annexin binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl and 2.5 mM $CaCl_2$) were mixed with 1 μL FITC-conjugated Annexin V antibody. Subsequently, the mixture was incubated at room temperature for 15 min in a dark place. Before analysis, 2.5 μL PI was added into the cell suspension immediately. The relative number of Annexin V-positive and/or PI-positive cells was determined via flow cytometry. Finally, FlowJo software was used for data analysis.

Western Blotting (WB)

Total protein was extracted using radioimmunoprecipitation assay (RIPA) buffer (Beyotime, Shanghai, China). The concentration of extracted proteins was determined by the bicinchoninic acid (BCA) method. Protein samples were electrophoresed on polyacrylamide gels and transferred onto polyvinylidene difluoride (PVDF) membranes (Merck Millipore, Billerica, MA, USA). After sealing with 5% skim milk at 37°C for 2 h, the membranes were incubated with primary antibodies at 4°C overnight. On the next day, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody at 37°C for 1 h. Finally, immunoreactive bands were observed using enhanced chemiluminescence (ECL) method.

Cell Viability Assay

The cells were first inoculated into a 96-well plate at a density of 4×10^3/well. When about 70% of cell fusion, the cells were incubated in fresh medium containing ADM (0.5-8 μM) for 48 h. After that, 20 μL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (2 mg/mL) (Sigma-Aldrich, St. Louis, MO, USA) was added into each well, followed by incubation for 4 h. Meanwhile, 200 μL dimethyl sulfoxide was added to dissolve the crystals formed. Finally, optical density at 570 nm was measured using a microplate reader.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 17.0 software (SPSS Inc., Chicago, IL, USA) was used for all statistical analysis. A *t*-test was performed to compare the difference between the two groups. One-way analysis of variance was used to compare the differences among different groups, followed by a post-hoc test (Least Significant Difference). Two-sided 95% confidence interval (CI) was adopted in all tests. $p<0.05$ was considered statistically significant.

Results

Expression of RXRα in Pancreatic Cancer Tissues and Para-Carcinoma Tissues

The expression of RXRα protein in pancreatic cancer tissues and para-carcinoma tissues was detected via IHC. It was found that RXRα protein was expressed in both nucleus and cytoplasm, and positive expression displayed brown yellow or dark brown staining. Moreover, the expression of RXRα protein in pancreatic cancer tissues was significantly higher than that of para-carcinoma tissues ($p<0.05$) (Figure 1 and 2, Table II).

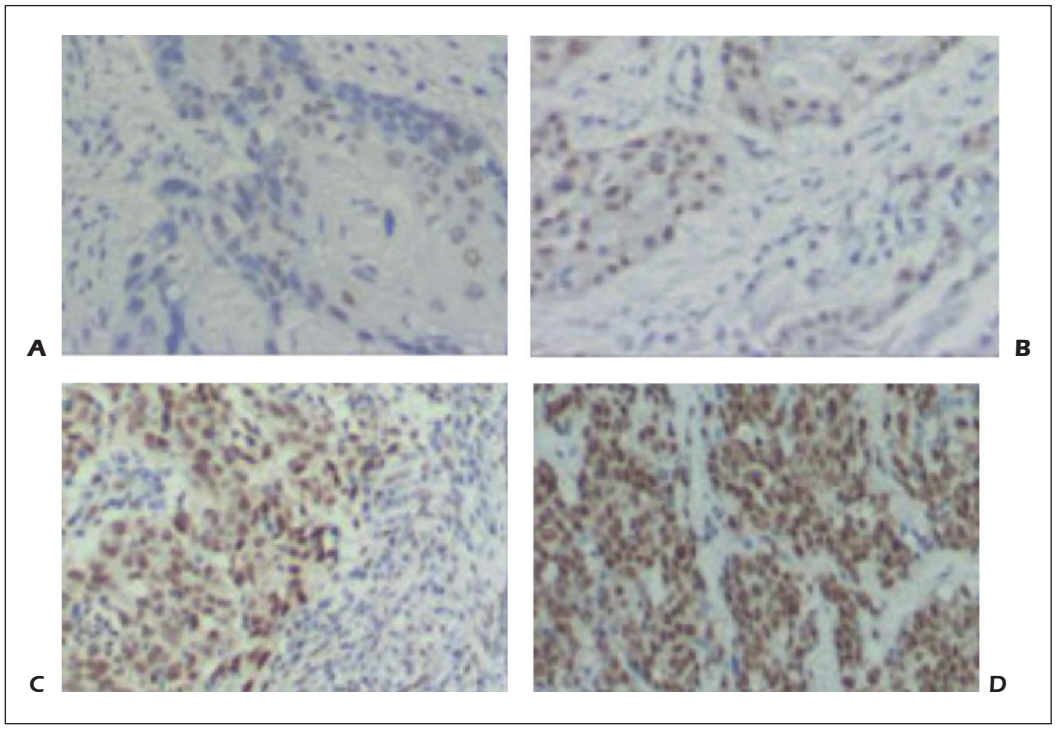

Figure 1. Expression intensity of RXRα in pancreatic cancer (Magnification × 10). **A**, IHC staining (-), **B**, IHC staining (1+), **C**, IHC staining (2+), **D**, IHC staining (3+).

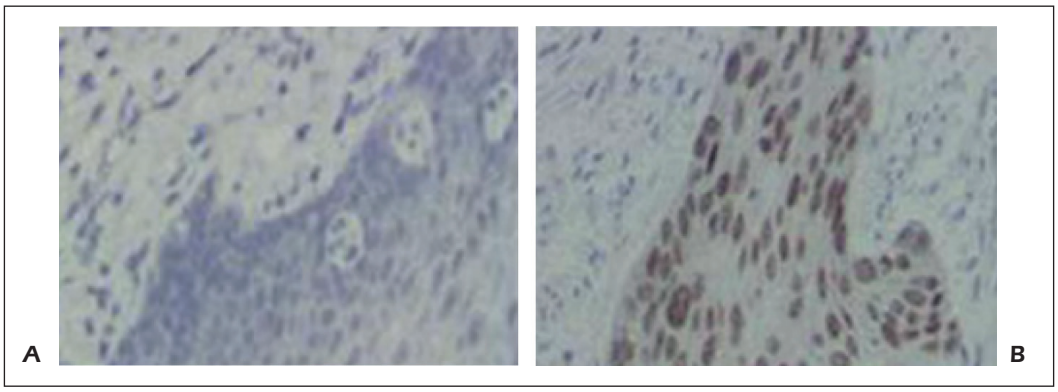

Figure 2. Expression of RXRα in pancreatic cancer tissues and para-carcinoma tissues (Magnification × 40). **A**, RXRα is negatively expressed in para-carcinoma tissues, **B**, RXRα is positively expressed in pancreatic cancer tissues.

Table II. Expression intensity of RXRα in pancreatic cancer and para-carcinoma tissues.

Tissue	Expression intensity				χ^2	*p*
	–	+	++	+++		
Cancer	10	16	18	16	15.18	0.0021
Para-carcinoma	45	11	3	1		

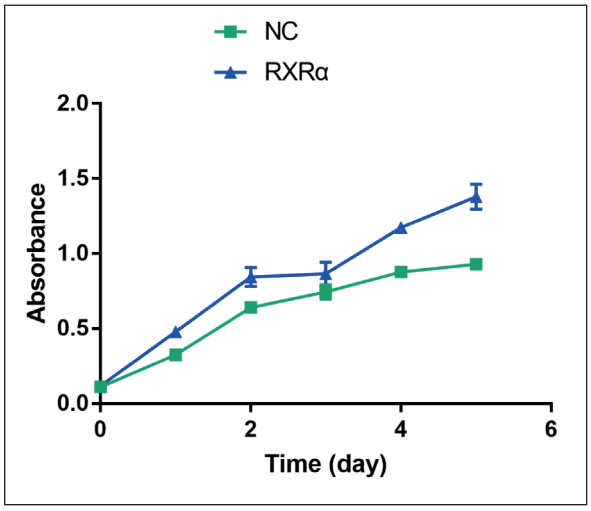

Figure 3. Cell proliferation ability. The proliferation ability of cells treated with RXRα is significantly enhanced ($p<0.01$).

Cell Proliferation

After PANC-1 cells were treated with RXRα recombinant protein, cell proliferation ability was detected. The results showed that the proliferation ability of cells in the treatment group was significantly enhanced when compared with that of the control group. This indicated that RXRα could promote cell proliferation ability ($p<0.01$) (Figure 3).

Cell Apoptosis

Cell apoptosis rate was detected via flow cytometry. The results revealed that the apoptosis rate was (4.57±2.35)% and (10.58±2.28)% in RXRα treatment group and control group, respectively. Statistically significant differences were observed in the apoptosis rate between RXRα treatment group and control group ($p<0.05$) (Figure 4A and 4B).

The expression levels of apoptosis-related proteins in each group were detected via WB to explore the effect of RXRα on apoptosis from another aspect. The results demonstrated that RXRα treatment could significantly down-regulate the expression level of apoptotic protein Bax, and up-regulate the expression level of anti-apoptotic protein Bcl-2 (Figure 4C).

RXRÐ Regulated Proliferation and Apoptosis of Pancreatic Cancer Cells through TGF-β/Smad Signaling Pathway

To further clarify the molecular mechanism of RXRα in regulating proliferation and apoptosis of pancreatic cancer cells, related molecules of TGF-β/Smad signaling pathway were detected. The results showed that RXRα treatment could up-regulate the expression of key molecules such as TGF-β and Smad, suggesting that RXRα was able to activate the TGF-β/Smad signaling pathway (Figure 5).

Discussion

Retinoic acid derivatives have aroused great interest among researchers in cancer prevention and treatment, especially in the treatment of acute promyelocytic leukemia with ATRA. Considering the chemoprevention effect of retinoids, it is believed that retinoid therapy should also be beneficial to patients with solid tumors. Currently, patients with different types of solid tumors, including PDAC, have received retinoids. However, the effects of clinical treatment are far from satisfactory. Cancerization is an extremely complex biological process. It is a multi-factor and multi-step pathological

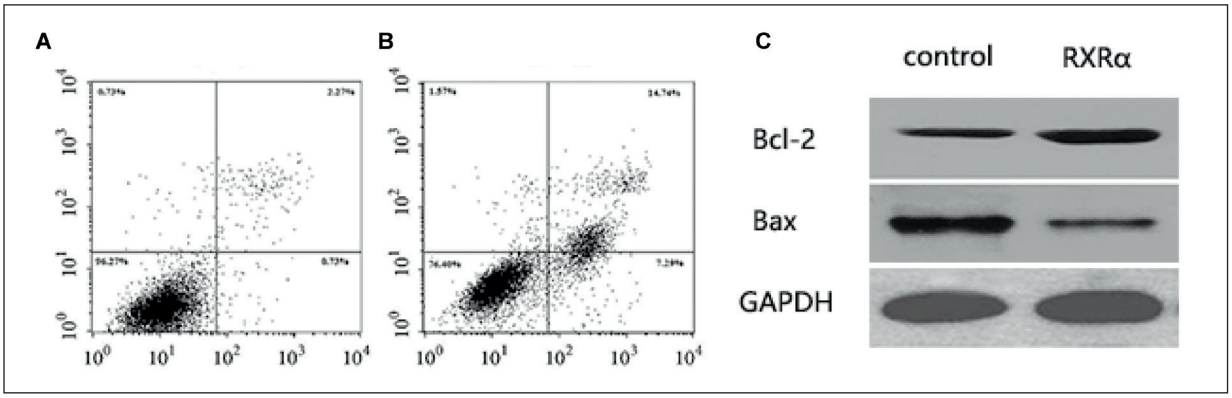

Figure 4. Cell apoptosis rate detected via flow cytometry. **A**, Apoptosis rate in RXRα group: (4.57±2.35)%, **B**, Apoptosis rate in control group: (10.58±2.28)%, **C**, Expression levels of apoptosis-related proteins detected via WB: RXRα treatment leads to decreased expression of Bax and increased expression of Bcl-2.

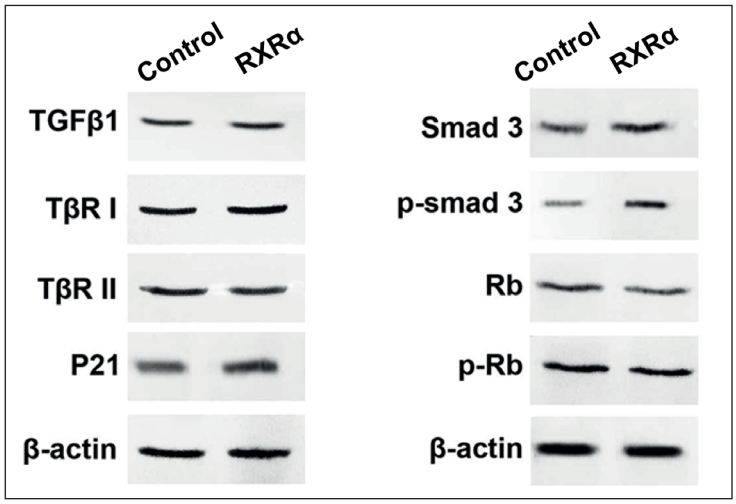

Figure 5. RXRα can up-regulate the expression of key molecules such as TGF-β and Smad.

change process that transforms normal tissue cells into cancer cells. Once successful, cancer cells will acquire the abilities of invasion and metastasis, leading to tumorigenesis in the clinic. RXR is a cofactor in nuclear extracts, whose expression has specificity in tissues, cells, and development stages. RXRγ has been proved to be associated with poor prognosis of patients with non-small cell lung cancer. In the present study, it was found that the expression level of RXRα in cancer tissues was significantly higher than that of para-carcinoma tissues. This suggested that the key signal of retinoids was dysregulated in pancreatic cancer. Therefore, the difference in the expression level of retinoids in tumor tissues may be one of the reasons for the negative results of the clinical test of retinoids.

The progression of PDAC is closely associated with repeated mutation or genetic/epigenetic changes in tumor suppressor genes (INK4/ARF, TP53)[11,12]. Knudsen et al[13] have found that TGF-β, an important core molecule of the TGF-β/Smad signaling pathway, is overexpressed in pancreatic cancer. TGF-β superfamily is involved in embryonic development, homeostasis regulation and the pathogenesis of various diseases[14,15]. TGF-β signals can bind to specific ligands through type I and type II TGF-β receptors (TβRI and TβRII), thereby activating the Smad pathway (phosphorylated Smad2 and Smad3 further accumulate in the nucleus with Smad4) and other signaling pathways (MAPK, RHOA, and PI3K/AKT)[16]. Meanwhile, TGF-β acts as a tumor suppressor or tumor promoter in malignant tumors. In fact, TGF-β is considered as a tumor suppressor in the early development of tumors. However, it displays cancer-promoting property during progression later. Furthermore, TGF-β is involved in related biological behaviors through regulating cellular plasticity, including extracellular matrix deposition, immune evasion and epithelial-mesenchymal transition (EMT) and possesses properties of tumor stem cells[17-19].

When TGF-β is activated, Smad2 is phosphorylated, and Smad3 is dimerized. After that, TGF-β is translocated in the nucleus of cancer cells, thereby promoting EMT[20]. In the present study, it was found that RXRα could activate the TGF-β/Smad pathway in pancreatic cancer cells. An overexpression of RXRα led to the up-regulation of the TGF-β/Smad signaling pathway molecules. These results indicated that RXRα could serve as an activator of the TGF-β/Smad pathway, thus regulating the proliferation and apoptosis of pancreatic cancer cells. TR3, a member of the nuclear receptor family, plays an important role in apoptosis. TR3, like RXR, is located in the nucleus under normal conditions. RXR, as a carrier, assists TR3 to enter mitochondria in the cytoplasm through nuclear pore complex in the presence of 9CRA. Regardless of the presence of 9CRA, TR3 in mitochondria can induce apoptosis. Moreover, RXR may also induce apoptosis through assisting TR3 transport.

Conclusions

We showed that RXRα can serve as an important index for clinical diagnosis and therapeutic target of pancreatic cancer. With the further deepening of research on RAR and extensive understanding of the mechanism of retinoic acid, it is necessary to search for new retinoic acid drugs with stronger selective action and less toxic side effects. In the future, RXRα-targeted drugs may play a greater role in the treatment of pancreatic cancer.

Funding Acknowledgements

The study was granted by the Project of Jiamusi University (Sz2013-006) and the Heilongjiang Natural Science Foundation of Heilongjiang Province, China (Grant No. H2017072).

Conflict of Interest

The Authors declared that they have no conflict of interests.

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