Anti proliferative and apoptotic effects on pancreatic cancer cell lines indicate new roles for ANGPTL8 (Betatrophin)

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Abstract

Despite considerable advances, the treatment of pancreatic cancer (PC) still requires much effort. Unusual regulation of the Wnt and apoptotic signaling pathways is widespread in cancer incidence. For instance, the WIF1 (Wnt inhibitory factor 1) gene is down-regulated in many cancers. The purpose of this study was to determine the effects of recombinant Betatrophin, a recently discovered hormone, on MiaPaca-II and Panc-1 pancreatic cell lines. Various concentrations of Betatrophin were added to MiaPaca-II and Panc-1 pancreatic cell lines during periods of 24, 48, and 72 h. The MTT assay was applied to investigate cell proliferation after treatment. The rate of apoptotic cells was assessed using double-staining flow cytometry, and the expression levels of the WIF1 and Bcl2 protein were observed by real-time PCR and western blotting, respectively. The findings of this study suggest that Betatrophin has an anti-proliferative effect on both MiaPaca-II and Panc-1 cell lines, in line with the up-regulation of WIF1 gene and Bcl2 protein. Thus, Betatrophin can be proposed as a potential therapeutic drug for treating pancreatic cancer.

Keywords: Betatrophin, Wnt, WIF1, Bcl2, pancreatic cancer.

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Introduction

Pancreatic cancer is one of the most fatal types of cancer. Since this cancer does not have an early prognosis, just under 20% of the patients live for more than one year after diagnosis (Bailey et al., 2016; Waddell et al., 2015). There are some factors that lead to the reduced survival rate of this disease. One is the difficulty in diagnosis during the early stages of the disease. Moreover, tumors progress rapidly while having few specific associated symptoms, and different pancreatic cancers show different responses to related drugs. Although there has been a progression towards figuring out the histological characteristics and molecular mechanisms underlying cancer development, studies showing favorable responses to available drugs continue to be rare. As a result, the survival chances of patients have not significantly improved (Siegel et al., 2013; Sahmanli et al., 2016). A major obstacle for following a better treatment plan has been the heterogeneity of these cancers. This is because of the vast amount of somatic mutations acquired during the development of a tumor, and the different consequences of these mutations on cell signaling pathways (Sousa et al., 2013; Hidalgo et al., 2015).

The Wnt signaling pathway is responsible for controlling progress such as embryonic development, cell proliferation, polarization, cell fate, and the process of renewing in stem cells (Kudo, 2010). It has been indicated that this pathway has a decisive role in numerous malignancies, including breast cancer (Geyer et al., 2011), colon cancer (Vermeulen et al., 2010), leukemia (Luis et al., 2012), gastric cancer (Yong et al., 2016), esophageal cancer (Zhang et al., 2016a), and HCC 7 (Fatima et al., 2012), for instance. A role for the Wnt signaling pathway has also been reported in pancreas development (Hebrok, 2003; Dessimoz et al., 2005; Murtaugh et al., 2005, Papadopoulou and Edlund, 2005; Heiser et al., 2006). During early pancreatic development, incorrect activation of Wnt signaling causes imperfect development of this organ (Heller et al., 2002; Heiser et al., 2006).

Expression of the Wnt inhibitory factor 1 (WIF1) gene prevents receptor interactions and induces β-catenin degradation by binding directly to Wnt ligands situating outside the cell. Down-regulation of WIF1 has been reported prostate, breast, lung, and bladder cancers. Silencing of the WIF1 gene due to promoter hyper-methylation has been revealed in gastrointestinal, lung and bladder cancers (Taniguchi et al., 2005; Urakami et al., 2006; Yoshino et al., 2009; Rahmani et al.,...
2017). It has also been observed that stimulation of WIF1 activity in cancer cells allowed to treat some malignant cancers (Ng et al., 2014). Although, activation of the Wnt pathway seems to be involved in pancreatic cancer (Wang et al., 2009), the expression and precise function of WIF1 in pancreatic cancer progression have not been determined so far.

Apoptosis is a vital biological process that controls homeostasis and the dynamic balance between cell proliferation and cell death (Tabas and Ron, 2011), and the Bcl2 gene family with about 25 members plays a key role in the regulation of the intrinsic or mitochondrial apoptotic pathway. However, for the understanding of the apoptotic effect in pancreatic cancer cell lines clarification is needed on the regulation of the pro-apoptotic gene Bax and the anti-apoptotic gene Bcl2, which play a significant role in the intrinsic pathway of apoptosis.

Betatrophin, also known as angiopoietin-like protein (ANGPTL8), is a recently identified circulating protein that is mostly produced in the liver and adipose tissues. The human Betatrophin gene has four exons encoding a protein with 198 amino acids. In various studies, its role has been determined in glucose and lipid metabolism, metabolic diseases (Crueiras et al., 2016), polycystic ovary syndrome (PCOS) (Calan et al., 2016), adriamycin cardiomyopathy (Chen et al., 2016b), and renal dysfunction (Chen et al., 2016a).

The focus of this study was to assess its effects on cell proliferation and apoptosis in the MiaPaca-II and Panc-1 as pancreatic cancer cell lines treated with different concentrations of Betatrophin. The effects of Betatrophin on Wnt and apoptosis signaling pathways was assessed by measuring the expression level of WIF1 as a tumor suppressor gene by real-time PCR, and the expression of Bcl2 protein by western blot analysis.

Material and Methods

Cloning of Betatrophin

Betatrophin was cloned using the PET28 plasmid as vector for transformation of E. coli BL21 cells. The procedures of cloning and purification are described in our previous study (Gholami et al., 2017).

Cell culture

The human pancreatic cancer cell lines MiaPaca-II and Panc-1 were purchased from the Pasteur Institute of Iran and cultured in T-25 flasks (Jet Biofil Flask) using 4-6 mL of DMEM-high glucose medium containing 10% fetal bovine serum (Gibson,26140-079) and 1% antibiotic of penicillin–streptomycin (Gibson,15140-122). They were cultured in 5% CO₂ atmosphere. When cells reached 80% confluency in the subculture, the overlying medium was removed, and the cells were washed two times with 1 mL PBS. After adding trypsin-EDTA 0.25% (Gibson, 25200-056), the cells were incubated for 2-3 min before adding 3 mL of DMEM medium to neutralize the trypsin effect. They were transferred to 15 mL Falcon tubes and centrifuged for 7 min at 1700 rpm. The supernatant medium was removed, new medium was added to the pelleted cells, and these were then transferred to incubation flasks. For freezing, 1 mL of freezing medium (5-10% DMSO with 90-95% FBS) was used.

Cell toxicity assessment by MTT assay

Cell viability was tested using the colorimetric [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] tetrazolium reduction assay (MTT). The MiaPaca-II and Panc-1 cell lines were seeded at a concentration of 5×10⁴ cells/well and incubated for 24 h. After termination of the incubation period, the cells were exposed to 200 μL of increasing concentrations of Betatrophin (5, 10, 20, 40, 50, 75, 100, 125, 150 pg/mL) for 24, 48 and 72 h. After 24 h, the overlying medium was removed, and 180 μL of new medium was added to each well. At the end of the treatment period, the medium was removed and replaced with 200 μL of MTT (Sigma, Germany) was added to each well, followed by incubation for 4 h at 37 °C in the dark. After incubation, the MTT solution was removed and replaced with 200 μL DMSO. The cells were then incubated for 10 min in a shaking incubator. Glycine buffer was added and absorbance was evaluated at 570 nm in an ELISA plate reader. The assay was performed in triplicate.

Apoptosis assay via flow cytometry

Assessment of apoptotic cells was done using the annexin V/PI double-staining flow cytometry detection kit (Biolegend). After culturing MiaPaca-II and Panc-1 cell lines and treating them with 150M concentrations of Betatrophin for 24, 48 and 72 h, the cells were trypsinized and collected by centrifugation (350 × g, 5min). Annexin-V and PI conjugated with FITC were added to the cells, and they were incubated at room temperature for 15 min. The fluorescence distribution was recorded in a two-color dot blot analysis, and the percentage of fluorescent cells was determined.

RNA extraction and cDNA synthesis

Total RNA of treated and untreated MiaPaca-II and Panc-1 cells was extracted using the BioFACT kit (Cat.No.RP101-050/RP101-100; South Korea) according to the manufacturer’s guidelines. Quantity and purity of the extracted RNA was assessed in a Nanodrop spectrophotometer at the wavelength range of 90 – 320 nm. cDNA was synthesized using the BioFACT kit (Cat.No.BR631-096) according to the manufacturer’s instruction. The quality of the synthesized cDNA was assessed using Nanodrop spectrophotometry and gel electrophoresis.

Real-time PCR expression analysis of the WIF1 gene

The effects of Betatrophin on WIF1 gene expression were assessed by real-time PCR. Primers were obtained from Macrogen (South Korea). Their sequences were: for GAPDH 5'- CAA TGACCCTCTTACCC -3 and 5'- TGGAAGATGGTGATGGGATT-3; and for WIF-1 5'- GGAATGGAGGCTTTTGTA-3 and 5'-TGGTTGAG CAGTTTGCTTTG-3. Amplification was conducted in 20
μL of SYBR Green PCR Master Mix (qPCRBIO Syber-Green Mix Separate-Rox (NGS2X)) under the following conditions: initial denaturation at 95 °C for 15 min, 40 cycles at 95 °C for 20 s, annealing at 60 °C for the 30 s and extension at 72 °C for 30 s. Data were analyzed by the Pfaffl method, and the graphs were drawn by REST software 2009.

Expression of of Bcl2 by western blot analysis

MiPaca-II and Panc-1 cells were seeded and incubated for 24 h and then treated with a 150 μM solution of Betatrophin. Total protein extracts of the two cell lines were produced after 24, 48, and 72 h of treatment times using cell lysis buffer. Equal quantities of protein (50 μg) were resolved by SDS-PAGE and gels were transferred to nitrocellulose membranes. Non-specific binding sites were blocked by incubation in blocking buffer (PBS containing 0.1% Tween 20 and 5% non-fat dry milk) for 24 h at 4 °C. After washing the membranes twice, they were immunoblotted using the anti-β actin, anti-Bcl-2 primary antibodies at 4 °C overnight and then incubated with the corresponding HRP conjugated secondary antibodies for 1 h at room temperature. Western blot bands were detected using an enhanced chemiluminescence (ECL) detection system (GeneGnome XRQ - Chemiluminescence imaging). Band intensities were quantified and normalized to β-actin using the NIH ImageJ software.

Results

Viability assay by MTT

MiaPaca-II and Panc-1 cell lines were treated with several concentrations of Betatrophin and their viability was assessed by MTT assay. As shown in Figure 1A-C, the cell viability of both treated cell lines was decreased at the concentrations of > 75 pg/mL at all treatment times (24, 48, and 72 h).

Apoptotic effect of Betatrophin

The apoptotic effect of Betatrophin on MiaPaca-II and Panc-1 cell lines was assessed by double-staining Annexin-V/PI flow cytometric analysis. As illustrated in Figure 2A-L the dot-plot data for 24, 48, and 72 h treatment times show the occurrence of apoptosis for both cell lines treated with 150 pg/mL of Betatrophin. The overall percentage values of Betatrophin that induced apoptosis in early and late apoptotic cell populations at the three treatment times are shown in Figure 3.

Expression changes of WIF1

Expression of the WIF1 gene was compared in both treated and untreated cells. GAPDH was considered as an internal control for normalization. As shown in Figure 4A and B, the expression of WIF1 in both cell lines increased in comparison to control in a concentration-dependent manner.

Changes Bcl-2 protein levels

For further analysis of apoptosis in Betatrophin-treated MiaPaca-II and Panc-1 cells, Bcl2 expression was assessed, as this is the main apoptosis-related gene. The protein level of Bcl2 was measured before and after treatment with 150 M Betatrophin at 24, 48, and 72 h. We found that the level of anti-apoptotic protein Bcl-2 was dramatically reduced in both pancreatic cell lines (Figure 5).
Discussion

The results of the current study showed that Beta-trophin induces anti-proliferative and apoptotic effects on the two pancreatic cancer cell lines MiaPaca-II and Panc-1. Inhibition of the Wnt signaling pathway was induced by the up-regulation of WIF1 as a tumor-suppressor gene. Betatrophin-induced apoptosis was investigated through the down-regulation of Bcl2 as an anti-apoptotic protein.

Numerous previous studies had reported that reducing the Wnt pathway targets cancer stem cells (Kim et al., 2018) and induces instant and substantial death in several cancer cell lines, including lung, breast, mesothelioma, and sarcoma, which all overexpress Wnt-1 (He et al., 2004). Inhibition of the Wnt signaling pathway resulted in suppression of cancer metastasis (Cao et al., 2017) and prevents the proliferation of cancer cells (Choi et al., 2010). Other findings suggest that this signaling pathway is crucial in pancreatic cancer and may be a target for drug therapy (Garg et al., 2017, Dehghanifard et al., 2018). Poorly regulated Wnt/β-catenin signaling has also been shown to be involved in the chemo-resistance of pancreatic cancer (Cui et al., 2012). A study done in 2017 showed that the microRNA-195 inhibits the spreading of pancreatic cancer cells by limiting the fatty acid synthase/Wnt signaling pathway. This study suggested that microRNA-195 can act as a tumor suppressor in the expansion of pancreatic cancer (Xu et al., 2017). A monoclonal antibody (OMP-18R5) that inhibits the Wnt signaling pathway in numerous tumors, including pancreatic ones, by targeting Frizzled receptors showed conspicuous synergy when combined with gemcitabine (Gurney et al., 2012). It was also demonstrated that Wnt-inhibitors, such as ethacrynic acid (EA), ciclopirox olamin (CIC), piroctone olamine (PO), and griseofulvin (GF) reduce the viability of a murine and a human pancreatic cell line (Wall and Schmidt-
Akt-GSK3 liorates the inability of insulin in increasing glucose via the et al. triglyceride lipase (Zhang/c98 ERK signal transduction pathway in hepatocytes, pancreatic al.

predictive indicators and as a new treatments for curing can-

2). and thus, the anti-proliferation effect of Betatrophin (Figure 3 and Panc-1 cell lines in a dose-dependent manner. The expression levels of WIF1 demonstrated regulatory effects of this tumor suppressor gene on the Wnt signaling pathway and thus, the anti-proliferation effect of Betatrophin (Figure 2).

ANGPTL proteins became attractive as prognostic or predictive indicators and as new treatments for curing cancers (Carbone et al., 2018). ANGPTL8 (Betatrophin) ameliorates the inability of insulin in increasing glucose via the Akt-GSK3β or Akt-FoxO1 pathway in HepG2 cells (Guo et al., 2016). It has been reported that this protein activates the ERK signal transduction pathway in hepatocytes, pancreatic β-cells, and adipocytes, causing down-regulating adipose triglyceride lipase (Zhang et al., 2016b). Betatrophin probably makes use of the macrophage receptor for regulating lipid/triglyceride metabolism, and the neuronal receptor mediating the signaling to pancreatic beta cells via nerves(Yi et al., 2014). It seems that increased levels of Betatrophin in serum in pancreatic cancer-associated diabetes (Susanto et al., 2016) may be a protective mechanism limiting the proliferation of cancer cells.

Applying flow cytometry to evaluate the apoptotic role of Betatrophin on MiaPaca-II and Panc-1 cell lines (Figures 3 and 4) showed that it increased the overall percentage of early and late apoptosis compared to control untreated cells, especially after 72 hours treatment time. Apoptosis related genes, including Bcl2, Bcl-xl, and caspase-3 can be regulated by NF-κB, thus inhibiting the apoptosis of pancreatic cells (Banerjee et al., 2005; Kumnumakkara et al., 2007). We found a significant decrease in the level of Bcl2 protein (Figure 5) indicating that induction of apoptosis by ANGPTL8 (Betatrophin) was achieved by down-regulation this protein. Higher expression of Bcl2 in cancer cells is known to lead to tumor progression by preventing apoptosis (Florou et al., 2013). Since the mitochondrial membrane potential is preserved by Bcl2, and its overexpressing causes a less pronounced decrease of mitochondrial depolarization, it is reasonable to assume that mitochondrial fission and fusion occur by reducing the level of Bcl2 in ANGPTL8-treated cells.

In the current study, higher expression of WIF1 was observed after treatment with Betatrophin in the MiaPaca-II and Panc-1 pancreatic cancer cell lines. Decreased expression of WIF1 was reported in many cancers, such as gastrointestinal tract, kidney, glioblastoma, osteosarcoma, lung, pituitary, bladder, and oral cavity (Mazieres et al., 2004; Taniguchi et al., 2005; Urakami et al., 2006; Elston et al., 2007; Kawakami et al., 2009; Rubin et al., 2010; Lambiv et al., 2011; Paluszczak et al., 2015). When re-expressed, WIF1 can down-regulate the Wnt pathway and prevent cancer cell growth (Gao et al., 2009; Kawakami et al., 2009; Yee et al., 2010; Hirata et al., 2011; Ramachandran et al., 2012; Jiang et al., 2016). In line with our study, down-regulation of the WIF1 gene was observed in pancreatic cancer tissues, and this was attributed to hypermethylation of the WIF1 promoter region. Treatment with the demethylating agent 5-aza-20-deoxycytidine (5-aza-dC) re-established WIF1 expression in cancer cell lines. It was suggested that managing the Wnt pathway would be a probable target for treatment and/or prevention of gastrointestinal cancers like pancreatic cancer (Taniguchi et al., 2005; Azad et al., 2013). Furthermore, in pancreatic ductal adenocarcinoma (PDA), HOX transcript antisense intergenic RNA (HO-TAIR) regulates theexpressionof WIF1, affecting the radiosensitivity of pancreatic ductal adenocarcinoma (Jiang et al., 2016).

Taken together, it is plausible to say that Betatrophin has an anticancer effect on the pancreatic cancer cell lines used in this study, MiaPaca-II and Panc-1, by inhibiting cell growth and increasing WIF1 gene expression, which subsequently reduces Wnt signaling as a decisive pathway in proliferation. Also, its anti-pancreatic cancer effect was shown by its induction of apoptosis and down-regulation of Bcl-2 as an anti-apoptotic protein.

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Conflict of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicial to the impartiality of the reported research.

Author Contributions

All authors contributed in all the steps of this study as followed: NG and KGC conceived the study and wrote the manuscript; FT and SZ conducted the experiments; KMH...
analyzed the data, and all authors read and approved the final manuscript version.

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