Pancreatology xxx (2018) 1–11

Contents lists available at ScienceDirect

Pancreatology

journal homepage: www.elsevier.com/locate/pan

Knockdown of AGR2 induces cell apoptosis and reduces chemotherapy resistance of pancreatic cancer cells with the involvement of ERK/AKT axis

Qing-Guo Liu [a], Yan-Ju Li [a, *], Lan Yao [b]

[a] Department of Gastroenterological Surgery, Tangshan Gongren Hospital, Tangshan, 063000, PR China
[b] Tangshan Central Blood Station, Tangshan, 063000, PR China

ARTICLE INFO

Article history:
Received 27 December 2017
Received in revised form
4 July 2018
Accepted 6 July 2018
Available online xxx

Keywords:
AGR2 gene
Pancreatic cancer
ERK/AKT axis
Chemotherapy resistance
Invasion
Migration
Proliferation
Apoptosis

ABSTRACT

Background: Pancreatic cancer (PC), an aggressive human malignancy, presents with a striking resistance to chemotherapy. Interesting, AGR2 has been found to be upregulated in various cancers and has been found to promote the dissemination of PC cells. Thereby, a series of *in-vitro* experiments were performed to investigate the relationship between AGR2 and the ERK/AKT axis, and to explore whether it affects PC cells.

Methods: Positive expression of AGR2 protein in the PC and paracancerous tissues collected from 138 patients with PC was detected using immunohistochemistry. After treatment with FGF2 (an ERK/AKT axis agonist), siRNA against AGR2 or their combination respectively, cell viability, chemotherapy resistance, radiotherapy resistance, migration, invasion and apoptosis in PC cells were detected using CCK8 assay, MTT assay, clone formation assay, wound healing assay, Transwell assay and flow cytometry, respectively. The expressions of AGR2 and ERK/AKT axis-related genes and proteins in tissues and cells were detected using reverse transcription quantitative polymerase chain reaction and Western blot assay.

Results: PC tissues exhibited highly-expressed AGR2 and abnormally activated ERK/AKT axis. FGF2 promoted the expression of AGR2, ERK/AKT axis activation, cell viability, chemotherapy resistance, migration and invasion, but decreased cell apoptosis in PC cells. However, knockdown of AGR2 resulted in inhibition of the ERK/AKT axis, reduced PC cell viability, chemotherapy resistance, migration and invasion but increased cell apoptosis in PC cells.

Conclusion: The findings reveal that AGR2 silencing could promote cell apoptosis and inhibit cell migration, invasion and chemotherapy resistance of PC cell with the involvement of the ERK/AKT axis.

1. Introduction

Pancreatic cancer (PC), the fourth most common cancerous cause of death, is one of the deadliest malignancies commonly presented with poor prognoses, and results in non-specific incipient symptoms and early metastasis [1]. Up to 2012, the incidence rate of PC was registered to be 7.28 per 100000 in registration areas in China, and the age-specific incidence rate of PC was found to be drastically increased after the age of 40-years and peaked at some the age of 80-years [2]. Common risk factors for PC include smoking, obesity, genetics, diabetes, diet, inactivity, and body mass index (BMI) in addition to pancreatitis and alcohol consumption [3,4]. The current treatments of PC include surgery, radiation therapy, chemotherapy, and palliative care, which are optioned depending on the stage of PC in a multidisciplinary approach [5]. However, the outcomes of sophisticated PC surgeries or combination therapies have improved modestly for patients, and more efficient treatments are desperately required in order to raise the quality of life of PC patients [6]. Meanwhile, PC patients eligible for surgery constitute for less than 15% of the total, making gene therapy a new widely-investigated therapeutic approach [1]. Interestingly, several genes including multidrug-resistance 1 (MDR1), murine double minute 2 (MDM2), and anterior gradient 2 (AGR2) have been found to be implicated in PC [7–9].

AGR2, a member of the endoplasmic reticulum (ER) protein

* Corresponding author. Department of Gastroenterological Surgery, Tangshan Gongren Hospital, No. 27, Cultural Road, Tangshan, 063000, Hebei Province, PR China.
E-mail address: Liyanju_YJ@163.com (Y.-J. Li).

https://doi.org/10.1016/j.pan.2018.07.003
1424-3903/

disulfide isomerase (PDI) family, works as a developmental regulator, survival factor and susceptibility gene for inflammatory bowel disease in multiple cell lineages [10]. A previous study revealed the dissemination of PC cells indicating that AGR2 plays critical roles in the pathogenesis of PC through regulation of Cathepsins B and D acting as a novel surface antigen [9]. Interestingly, AGR2 was also reported to be associated with the regulation of PC initiation [11]. Moreover, AGR2 and AKT inhibitors and ERK1/2 inhibitors have been demonstrated to work in tandem and play an important role in breast cancer cell proliferation and migration suggesting that AGR2 and AKT inhibitors might play critical roles in cancers and need to explored in PC as well [12]. Similarly, extracellular signal-regulated kinase (ERK) and protein kinase B (AKT) axis have been reported to serve to coordinate cellular responses to multiple extracellular stimuli, and are involved in cancer cell proliferation, tumorigenesis and drug resistance [13]. In addition, it has been implied that inactivation of AKT and ERK could play a vital role in regulating proliferation and apoptosis in PC cells, further warranting the exploration of the role of AKT and ERK in PC [14]. Moreover, CXC chemokine receptor 4 (CXCR4) was demonstrated to promote proliferation in PC through the AKT and ERK axis, suggesting that ERK/AKT pathway could be used as a potential treatment for PC [15].

Thereby, the current study speculates the existence of a relationship between the AGR2 gene, PC and ERK/AKT; however, few researches have conducted experiments to investigate the role of AGR2 in PC via the ERK/AKT axis. Therefore, the current study aims to explore whether AGR2 silencing could influence the resistance to chemotherapy of PC through the ERK/AKT axis.

2. Material and methods

2.1. Ethical statement

The current study was approved by the Ethics Committee of Tangshan Gongren Hospital. Signed informed consents were obtained from all patients/their guardians participating in the study.

2.2. Study subjects

The current study collected PC tissues and paracancerous tissues from 138 PC male patients, aged between 20 - 62 years (mean age = 42.6 years) who were enrolled at the Tangshan Gongren Hospital from 2001 to 2013. According to the 1983 KLOPPEL classification criteria of pancreatic exocrine epithelial tumors, 40 patients were diagnosed as tubular adenocarcinoma, 36 patients were diagnosed as mucinous carcinoma, 40 patients were diagnosed as adenoid cystadenocarcinoma, and 22 patients were diagnosed as acinar cell carcinoma, and none of the study subjects underwent chemotherapy or radiotherapy previously.

2.3. Paraffin section preparation

The obtained cancer tissues were fixed with 4% polyoxymethylene, dehydrated with gradient ethanol, cleared with xylene, dipped in wax and embedded. Next, the paraffin blocks were fixed on a slicer. After rough trimming, the largest section of the blocks was exposed and sliced into thin sections with the thickness of 5 μm. The thin sections were immersed in the hot-water tank of the spreading machine in order to spread the folded sections, which were then attached to slides and baked in the drying machine at 45 °C for 5 min to dry the paraffin.

2.4. Immunohistochemistry

The streptomyces avidinperoxidase (SP) immunohistochemistry method was performed as follows: paraffin sections were rinsed with phosphate buffered saline (PBS) (3 times × 5 min), immersed in 0.01 mol/L citrate buffer (pH 6.0) for repairing under HP antigen, and then immersed in 3% hydrogen peroxide solution to block endogenous peroxidase activity. Next, each section was added with non-immune animal serum and incubated at room temperature for 20 min. Then, the samples were incubated with the rabbit anti-human AGR2 protein polyclonal antibody (dilution ratio of 1: 2000, diluted with 5% serum in PBS solution), while the negative control samples were incubated with PBS as the primary antibody at 4 °C overnight. After rinsed with PBS, the samples were added with the biotin-labeled secondary antibody and incubated at room temperature for 30 min and rinsed again with PBS (3 times × 5 min). And then, the sections were dropped with the streptavidin-peroxidase solution and incubated at 37 °C for 30 min and rinsed with PBS (3 times × 5 min). Diaminobenzidine (DAB) was used in order to develop color and the results were observed under a microscope. Next, the sections were counterstained with hematoxylin, rinsed in water, sealed with neutral gum and photographed. A total of 5 visual fields (400 ×) were randomly selected for each section, with each visual field containing more than 100 cells, and the positive rate = positive cells/total cells [16].

2.5. Cell culture

Sterile scalpels and scissors were used to slice PC tissues into 3–4 mm sections, and tissue fragments were cleared by suspension in an equilibrium salt solution without calcium and magnesium. The supernatant was removed after the tissue debris was precipitated. The tissues were washed for 2 to 3 times. The container containing tissue debris was placed on ice in order to remove the residual supernatant. The 0.25% trypsin dissolved in equilibrium salt solution without calcium and magnesium was added, with 100 mg tissues added with 1 mL trypsin. Enzymes with almost no trypsin activity were permeated as much as possible after incubation at 4 °C for 6–18 h. Subsequently, trypsin was removed from tissue fragments, and the tissue fragments containing residual trypsin were incubated at 37 °C for 20–30 min. A hot complete medium was added to tissue fragments and gently dispersed with the tissue using a pipette. If serum-free medium was used, the soybean trypsin inhibitors were added. The dispersion of all residual tissues was performed using a sterile stainless steel screen (100–200 mm). Microscopic examination revealed that most of the cells had been dispersed into a single cell, at that time, RPMI-1640 complete culture medium containing 15% calf serum was added in order to terminate digestion. A 150-mesh sterilizing screen was used to collect the single cell suspension centrifugation supernatant, and then, added with 8–10 mL RPMI-1640 complete culture medium containing 15% calf serum. Next, the cell suspension was transferred to a sterile culture dish with a sterile pipette. The cells in the logarithmic phase of growth were used for further experiments, counting and inoculating cells for culture.

2.6. Immunofluorescence assay

The cultured PC tissues were rinsed with precooled PBS (3 times × 5 min), fixed with 4% polyoxymethylene for 30 min, and then rinsed with PBS (3 times × 5 min). Next, the cells were cleared using 0.3% Triton-X for 15 min, rinsed with PBS (3 times × 5 min), and blocked with 10% goat serum for 1 h. Added with the primary antibodies of AGR2 (dilution ratio of 1: 250) and Calreticulin

Please cite this article in press as: Liu Q-G, et al., Knockdown of AGR2 induces cell apoptosis and reduces chemotherapy resistance of pancreatic cancer cells with the involvement of ERK/AKT axis, Pancreatology (2018), https://doi.org/10.1016/j.pan.2018.07.003