Pancreatology xxx (2016) 1–10

Contents lists available at ScienceDirect

Pancreatology

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

Original Article

Nerve growth factor regulates CD133 function to promote tumor cell migration and invasion via activating ERK1/2 signaling in pancreatic cancer

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ARTICLE INFO

Article history:
Received 11 January 2016
Received in revised form
7 August 2016
Accepted 12 September 2016
Available online xxx

Keywords:
NGF
CD133
Migration
Pancreatic cancer
Perineural invasion

ABSTRACT

Background: Perineural invasion (PNI) is extremely high frequency among the various metastatic routes in pancreatic cancer. Nerve growth factor, secreted by astroglial cells, exerts effects on tumor invasion in some cancer cells, but its function on migration and invasion in pancreatic cancer is still unclear. In the present study, we determined the effects of NGF on modulating tumor cell metastatic potential and invasion activity and explored its mechanisms in pancreatic cancer.

Methods: NGF and CD133 expression were detected in tumor tissues using immunohistochemical analysis and Western blotting analysis. The effects of NGF on the regulation of CD133 expression and the promotion of cancer migration and invasion were investigated using wound healing and matrigel transwell assay. A related mechanism that NGF regulates CD133's function via activating ERK1/2 signaling also was observed.

Results: NGF/CD133 is overexpressed in human pancreatic cancer and promotes the migration and invasion of human pancreatic cancer cells through the activation of the ERK/CD133 signaling cascade. NGF/ERK signaling modulates the cancer cell EMT process, migration and invasion through the regulation of CD133 expression and its subcellular localization.

Conclusions: NGF/CD133 signaling initiated the migration and invasion of pancreatic cancer cells. NGF/CD133 might be an effective and potent therapeutic target for pancreatic cancer metastasis, particularly in PNI.

1. Introduction

Pancreatic cancer ranks as a leading cause of cancer death among solid cancers [1–4]. One of the main reasons for its high mortality rate is that pancreatic cancer cells have a high potential for metastasis [5,6]. Among the various metastatic routes, PNI has an extremely high frequency and is widely accepted to be a specific route for the spread of this disease [7]. PNI is closely related to poor prognosis and decreased survival [8]. Therefore, a better understanding of the molecular mechanisms underlying this form of metastasis and invasion is urgently needed to find effective biomarkers and therapeutic targets for improving survival rate of pancreatic cancer.

Neural invasion is characterized as invasion of intrapancreatic nerves by pancreatic cancer cells and their continuous extension into the extrapancreatic nerve plexus. Recent studies demonstrated that PNI in pancreatic cancer may involve reciprocal signaling interactions between tumor cells and neural cells, by which the tumor cells may have acquired the ability to invade or respond to proinvasive signals within the peripheral nerve [9]. NGF, as an important member of the neurotrophic factor family, and one mainly secreted by astroglial cells, is necessary to regulate the growth, development and reparation of neural cell types. Meanwhile, a number of studies have suggested that NGF exerts effects on tumor development and invasion in some tumor cells, such as lung, breast and pancreatic cancers [10–12]. However, the molecular mechanisms involved in NGF's function in tumor cell metastasis and invasion are still unclear.

Human CD133 is a transmembrane glycoprotein of 865 amino acids with a total molecular weight of 120 kDa. High expression of

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http://dx.doi.org/10.1016/j.pan.2016.09.005
1424-3903/

CD133 protein has been found to be associated with tumor development in pancreatic cancer [13]. Also CD133 has been used in the identification of putative cancer stem cells in many cancers, including pancreatic cancer [14–18]. Increasing evidence indicated that a subset of CD133+ tumor cells contribute to epithelial-mesenchymal transition (EMT) and cancer metastasis and invasion [17,19–21]. Therefore, CD133 expression might play a vital role in cancer metastasis, and additional investigations into the mechanisms underlying the expression of CD133 in pancreatic cancer are needed.

The Ras/Raf/MEK/ERK signals are major intracellular pathways and function in the propagation of many molecular signals. Transcription of CD133 was regulated by five promoters (P1, P2, P3, P4 and P5) and that among them, P5 showed the highest activity and was activated by ERK1/2 [22,23]. Moreover, studies have proved that NGF, acting through the TrkA receptor, induces a sustained activation of ERK [24]. Consequently, NGF/ERK might be involved in modulating CD133 function and expression and in promoting tumor metastasis and invasion. However, the regulatory role of NGF/ERK/CD133 signaling in pancreatic cancer progression is unknown.

In the present study, we hypothesized that NGF modulated CD133 expression, thereby affecting tumor cell invasion potential and migration activity in pancreatic cancer. We detected the overexpression of NGF/CD133 in human pancreatic cancer tissues by immunohistochemistry staining. We revealed that the mechanisms by which NGF promoted the migration and invasion of human pancreatic cancer cells are through the activation of the ERK/CD133 signaling cascade. Furthermore, we showed that NGF/ERK signaling modulated EMT processes through the regulation of CD133 expression and subcellular localization.

Our study demonstrated that NGF/CD133 signaling initiated the migration and invasion of pancreatic cancer cells, which suggested that NGF/CD133 might be an effective and potent therapeutic target for pancreatic cancer metastasis, particularly in PNI.

2. Materials and methods

2.1. Patient and sample preparation

Samples of fresh pancreatic adenocarcinoma and paracarcinoma tissues were obtained from patients with pancreatic cancer and who received radical pancreatectomy without chemotherapy or radiation. The pancreatic cancer surgical specimens were cut into 1 cm^3 pieces, quickly fixed in 100 g/L of formaldehyde solution, embedded in paraffin and then stored at 4 °C for further study. Histological slides were reviewed by two experienced pathologists without consideration for the clinical data. And the study involved human samples was approved by the Human Research Committee of Nankai University and China Anti-Cancer Association (CACA) and had been performed in accordance with the Helsinki Declaration.

2.2. Immunohistochemistry

Paraffin sections of tumor and paracarcinoma tissues were sliced into sections of 4 μm thickness by a slicing machine, and then a routine 3-step immunohistochemical stain was used. This process consisted of: sectioning, dewaxing and hydrating the tissues; incubation with 3% H_2O_2 at room temperature for 10 min; antigen repairing with Tris-EDTA; sealing with 5% goat serum (diluted in PBS); incubating overnight at 4 °C with primary antibody against CD133 at a 1:150 dilution (rabbit polyclonal antibody, Proteintech, USA) or anti-NGF at a 1:150 dilution (rabbit polyclonal antibody, Cell Signaling Technology, USA); and then incubating with secondary antibody and rinsing with PBS. Chromogen 3, 3'-diaminobenzidine tetrachloride (DAB) (Serva, Heidelberg, Germany) was used as a substrate. The cell nucleus was dyed with Harri's hematoxylin solution.

The expression levels of NGF and CD133 were scored by the extent and intensity of the staining. The extent of staining was scored by the percentage of the positively stained area. Stained area in each region of interest was scored using the following scale: 0 for a percentage <5%, 1 for 5–25%, 2 for 25–50%, 3 for 50–75%, and 4 for≥75%. The staining intensity was scored as 0, 1, 2 and 3 for the representation of negative (no staining), mild (weak), intermediate (distinct) and intense (strong) staining, respectively. The staining intensity and stained area percentage were multiplied to make a weighted score. The scoring was determined by three independent evaluators without any knowledge of the pathological and clinical characteristics of the patients [25].

2.3. Cells culture and reagents

Human pancreatic cancer cell lines MIA PaCa-2, Capan-2, PANC-1 and SW1990 were purchased from the Cancer Institute & Hospital of the Chinese Academy of Medical Sciences (Shanghai, China). MIA PaCa-2 and PANC-1 cell lines were grown in RPMI-DMEM medium (Biological Industries, Kibbutz BeitHaemek, Israel) containing 10% fetal bovine serum (BI, Australia), 100 μg/ml of penicillin and 100 μg/ml of streptomycin. The Capan-2 and SW1990 cell lines were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum and 100 μg/ml penicillin/streptomycin. All cells were cultured at a constant temperature of 37 °C in a humidified incubator with 5% CO_2. For CD133 expression reactivation, all of the cell lines were treated with 100 ng/ml of Rh-β-NGF (No. GF307, Millipore, USA).

2.4. RNA interference and cell transfection

The small interfering RNAs (siRNAs) targeting the human NGF gene were purchased from Santa Cruz Biotechnology, Inc. (No.43970, USA), siRNAs targeting human CD133 gene were purchased from OriGene Technologies, Inc. (No.SR305838), and siRNAs targeting ERK1/2 gene were synthesized by Cell Signaling Technology (No.6560). MIA PaCa-2, Capan-2, PANC-1, SW1990 were transfected with siNGF and their controls using INTERFERin® transfection reagent (Polyplus, France) according to the manufacturer's instructions. A total of 2×10^5 cells were transfected with 110 pmoles of siRNA. The transfection effects of siNGF and siERK1/2 were detected by Western blotting 48 h after transfection. The silencer negative control siRNA was used as a control.

The NGF plasmid (No.RC221463) and an empty vector plasmid (purchased from OriGene) were transformed into Trans 10 cells (TranGen Biotech, Beijing, China) and plated on Kanamycin-LB agar plates overnight at 37 °C. Then, the two plasmids were transfected into cells using INTERFERin® transfection reagent (Polyplus, France).

2.5. Plasmid construction and luciferase activity assay

The CD133/P5 promoter-Luc plasmid was synthetized by the Public Protein/Plasmid Library (China) by inserting the human CD133/P5 promoter region into the pGL4.10 reporter vector. As previously reported, the region between −98 and −25 contains the minimal elements required for CD133 transcription through the P5 promoter [23]; thus, only the active region (from −98 to −25) of CD133/P5 promoter-Luc was included.

For the luciferase assay, SW1990 cells were transiently transfected using INTERFERin® transfection reagent with CD133/P5 promoter-Luc plasmid and Psv-β-galactosidase (Promega, WI, USA) as control. After 48 h, the luciferase and β-galactosidase activities

Please cite this article in press as: Xin B, et al., Nerve growth factor regulates CD133 function to promote tumor cell migration and invasion via activating ERK1/2 signaling in pancreatic cancer, Pancreatology (2016), http://dx.doi.org/10.1016/j.pan.2016.09.005