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Integrin β1 promotes gemcitabine resistance in pancreatic cancer through Cdc42 activation of PI3K p110β signaling

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ABSTRACT

Pancreatic ductal adenocarcinoma (PDAC) is one of the most common malignancies with very poor prognosis due to its broad resistance to chemotherapy. Our previous study showed that integrin β1 expression is upregulated in PDAC and confers gemcitabine resistance in PDAC cells via the signaling pathway including Cdc42 and AKT activation. But the accurate signal transductions are not clear. Here, we aimed to illuminate the signal transductions of integrin β1 in the acquisition of gemcitabine resistance in PDAC. Drug-resistance (DR) cells from AsPC-1 parent cell line (PCL) were selected. Integrin β1 expression was determined using western blot assay. Changes in drug response and the activity of phosphatidylinositol 3-kinase (PI3K) signaling after knockdown of integrin β1, Cdc42 or p110β were evaluated using MTT, cleaved caspase-3 immunofluorescence and western blot assay. Western blot assays also detected the variations in Cdc42 activity and p110β expression after integrin β1 knockdown. The interaction between Cdc42 and p110β was determined by Glutathione *S*-transferase (GST) pull-down assay. The results showed that integrin β1 expression was upregulated in DR-AsPC-1 cells, and integrin β1 knockdown significantly decreased the activity of Cdc42, a target molecule of integrin β1, and p110β expression. Knockdown of anyone of integrin β1, Cdc42 and p110β inhibited the activity of PI3K signaling, and sensitized DR-AsPC-1 cells to gemcitabine. GST pull-down assay showed that GTP-Cdc42 interacted with p110β. Collectively, these data indicated that integrin β1 promoted gemcitabine resistance in PDAC through Cdc42 activation of PI3K p110β signaling. *In vivo* experiments also confirmed this conclusion. These findings contribute to a better understanding the molecular mechanism of chemoresistance and facilitate the development of more targeted and effective treatment strategy for PDAC.

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1. Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive human cancers, with an overall 5-year survival rate of <5% when all stages are combined [1]. Although the great improvements have been achieved in chemotherapy, radiotherapy, immunotherapy and molecular targeted therapy over the past few decades, surgical resection is still the most powerful therapy to cure PDAC. Unfortunately, due to lack of early signs or symptoms, most patients are diagnosed late with locally advanced stage or metastasis, and not amenable to surgery [2]. The primary treatment option for these patients includes chemotherapy, and gemcitabine, the first-line and essential drug, is the preferred therapy. However, the majority of the patients do not benefit from gemcitabine due to the rapid development of resistance to gemcitabine [3]. Thus, identification of new predictive indicators for gemcitabine resistance and elucidation of the molecular pathways involved in the acquisition of drug resistance should be high research priorities for PDAC.

Integrins are cell-adhesion molecules that lie at the interface of the cell and microenvironment and mediate the interaction between cytoskeletal elements and the extracellular matrix. Growing evidence suggests that upregulation of integrins promotes tumor progression via regulation of survival, proliferation, invasion, metastasis and angiogenesis pathways. Integrin β1, one member of integrins, is involved in much broader functional activities such as proliferation, invasion, adhesion, and inflammation. Recently, increasing evidence points that integrin β1 is linked to the resistance of some therapeutic agents in multiple solid cancer types and hematopoietic malignancies, and this integrin β1-mediated

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resistance occurs at the level of the tumor cells themselves [4,5]. Thus, integrin β1 might serve as a predictive indicator for patients with therapy resistance. For the expression and role of integrin β1 in PDAC, our laboratory has shown that integrin β1 is upregulated in PDAC and significantly associated with acquired gemcitabine resistance and poor prognosis; and downregulation of integrin β1 reduces Cdc42 and AKT activity, and relieves gemcitabine resistance, which suggests a possible drug-resistant signaling pathway of integrin β1 in PDAC [6]. Here, we aimed to illuminate the drug-resistant signaling pathway of integrin β1 in PDAC by performing *in vitro* and *in vivo* experiments.

2. Materials and methods

2.1. Drug-resistance (DR) cancer cell selection, treatments and transfections

PDAC cell line AsPC-1 was obtained from the Department of Cell Biology, Basic Research Institute, Second Military Medical University (Shanghai, China). DR-AsPC-1 cells were selected from AsPC-1 parent cell line (PCL-AsPC-1) as previously described [6]. Briefly, PCL-AsPC-1 cells were cultured in RPMI-1640 (Invitrogen Carlsbad, CA, USA) with 0.1 μM of gemcitabine (Eli Lily and Company, Indianapolis, IN, USA) for 7 days, and then cultured in normal medium for 15 days. Resistant clones were pooled, amplified and cultured by exposing them to a 24-h pulse of 1 μM of gemcitabine every other week to obtain DR-AsPC-1 cells. Integrin β1 shRNA (sh-integrin β1), sh-Cdc42, sh-p110β, and their respective controls were synthesized by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China). All transfection reactions were performed using Lipofectamine RNAi-MAX and Opti-MEM mediums (Invitrogen) in accordance with the manufacturers' instructions.

2.2. Colony formation assay

Colony formation assay was performed as previously described [6]. Briefly, single-cell suspensions were seeded into 6-well plates with 500 cells per plate. After 1 day, cells were treated for 24 h with different concentrations (0, 0.5 and 4.0 μM) of gemcitabine. The medium was replaced every 2 days. After 2 weeks, colonies were fixed in methanol for 10 min and stained overnight with 5% Giemsa (Sigma-Aldrich, St. Louis, MO, USA). The stained colonies were photographed, and counted.

2.3. MTT assay

Exponentially growing cells were seeded into 24-well plates at a density of 2×10^4 cells/well, and then treated with serial concentrations (0, 0.25, 0.5, 1, 2 and 4 μM) of gemcitabine for 72 h after adhesion. Then 10 μl/well of MTT (5 mg/L) was added for further incubation at 37 °C for 4 h, followed by addition of 100 μl dimethylsulfoxide (DMSO; Sigma-Aldrich) to dissolve the MTT formazan. The optical density (OD) was measured at 570 nm with a microplate reader (UVM 340, ASYS Hitech GmbH, Eugendorf, Austria).

2.4. Cell death assay

Cells were seeded into 6-well plates, and incubated until 70% confluence, followed by treatment with different concentrations (0, 0.5 and 4.0 μM) of gemcitabine for 72 h. Cells were washed in PBS and processed for caspase-3 immunofluorescence using an anti-cleaved caspase-3 antibody (Sigma-Aldrich). Positive cells were counted under a fluorescence microscope (Olympus, Tokyo, Japan). Five random fields were chosen for each gemcitabine dose, and at least 200 cells per field were counted.

2.5. Western blot assay

Cellular proteins were extracted from tissues or cells by using RIPA buffer. Protein concentration was determined by the bicinchoninic acid assay (Pierce, Rockford, IL, USA). Equal amounts of protein samples were mixed with 2× loading buffer, incubated in a boiling water bath for 5 min, separated in 12% of SDS-PAGE, and then transferred to nitrocellulose membranes. After blocking with 5% skim milk-TBS at room temperature for 1 h, the membranes were probed with the specific primary antibodies at 4 °C overnight, followed by HRP-conjugated secondary antibodies (Boster, Wuhan, China) for 2 h at room temperature. The blots were visualized using a SuperSignal West Dura kit (Pierce). The following primary antibodies were employed in this study: integrin β1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), GTP-Cdc42 (NewEast Biosciences, King of Prussia, PA, USA), AKT (Santa Cruz Biotechnology), *p*-AKT (T308, Cell Signaling, Beverly, MA, USA), Cdc42 (Cell Signaling), p110β (Santa Cruz Biotechnology) and β-actin (Santa Cruz Biotechnology).

2.6. Glutathione S-transferase (GST) pull-down assay

The recombinant GST and GST-Cdc42 fusion proteins were expressed in *Escherichia coli* strain BL21 (Tiangen Biotechnology, Beijing, China) after induction with 0.3 mM isopropyl-β-*d*-thiogalactopyranoside at 16 °C overnight. After bacteria lysis, Glutathione sepharose 4B beads were incubated with bacterial lysate for 3 h at 4 °C, washed with PBS, and then loaded with nucleotide by adding 50 μl of GTPase loading buffer containing GDP or GTPγS at 2 mM. After 20 min at 37 °C, $MgCl_2$ was added to a final concentration of 10 mM on ice. Proteins on beads were incubated with DR-AsPC-1 cell lysate expressing hemagglutinin (HA)-tagged p110β (p110β-HA) at 4 °C overnight. Then beads were washed, and proteins were incubated in a boiling water bath for 5 min, subjected to 12% of SDS-PAGE and blotted with antibodies for HA and GST.

2.7. Nude mice xenograft studies

All animal procedures were in agreement with the Guide for NIH and the institutional ethical guidelines for animal experiments. Five-week old athymic BALB/c mice were purchased from Slac Laboratory Animal Co. Ltd. (Shanghai, China), and bred in sterile laminar flow cabinets under appropriate pathogen-free conditions. DR-AsPC-1 cells (2×10^6 cells suspended in 100 μl PBS) stably transfected with sh-NC or sh-integrin β1 were subcutaneously inoculated into the dorsal scapula region of mice. The tumor size was monitored once a week with calipers, and the tumor volume was calculated using the formula: Volume (mm^3) = length × width^2 × 0.5. When the tumors were measurable, mice were injected with gemcitabine (25 mg/kg) through caudal vein every other day. Twenty-eight days after gemcitabine treatment, all mice were sacrificed and weighted. The tumor tissues were excised, weighed, imaged, and used for western blot detection.

2.8. Statistical analysis

Results were reported as mean ± standard deviation (SD) of at least three independent experiments. All statistical tests were performed on SPSS 17.0 statistical software (IBM, Chicago, IL, USA). Student's *t*-test was performed for the comparisons between groups. Statistical significance was set at $P < 0.05$.

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