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Enriched environment housing enhances the sensitivity of mouse pancreatic cancer to chemotherapeutic agents

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ABSTRACT

Living in an enriched housing environment is an established model of eustress and has been consistently shown to reduce the growth of transplanted tumors, including pancreatic cancer. Here, we further investigate the influence of an enriched environment (EE) on the efficacy of chemotherapy in pancreatic cancer. Male C57BL/6 mice were housed in EE or standard environment (SE) conditions and transplanted with syngeneic Panc02 pancreatic cancer cells. Tumor-bearing mice were treated with 5-fluorouracil (5-FU) or gemcitabine (GEM) to examine their sensitivities to chemotherapy. The results showed that both 5-FU and GEM exerted the dose dependent inhibition of tumor growth. The tumor inhibition rates of low-dose 5-FU and GEM were improved from 17.7% and 23.6% to 46.3% and 49.9% by EE housing. Importantly, tumor cells isolated from the pancreatic cancer xenografts of EE mice had significantly enhanced sensitivities to both 5-FU and GEM (IC50 for 5-FU: 2.8 μM versus 27.3 μM; IC50 for GEM: 0.8 μM versus 5.0 μM). Furthermore, using microarray analyses, we identified the "ATP-binding cassette (ABC) transporter" that was overrepresented among EE-induced down-regulated genes in pancreatic cancer. Particularly, the tumoral expression of ABC transporter A8b (ABCA8b) was confirmed to be significantly decreased by EE. Over-expression of ABCA8b in mouse pancreatic cancer cells led to a marked decrease in the sensitivity to chemotherapeutic drugs both *in vitro* and *in vivo*. In conclusion, our data indicate that benign stressful stimulation can synergistically boost the efficiency of chemotherapeutics in pancreatic cancer, which suggests a novel strategy for adjuvant cancer therapy.

1. Introduction

Pancreatic cancer is one of the most aggressive and deadly solid malignancies [1]. Currently, gemcitabine (GEM) and 5-fluorouracil (5-FU) are the cornerstone of chemotherapy for this malignancy [2]. However, pancreatic cancer is characterized by its intrinsic chemoresistance, which contributes to the limited efficacy of chemotherapy and the profoundly poor prognosis [3]. Therefore, the development of novel strategies is warranted to overcome this resistance.

Recently, there has been emerging interest in the impact of psycho-social stress, including "distress" (negative stress) and "eustress" (positive stress), on peripheral cancer. With regard to pancreatic cancer, it has been reported that distress could promote the progression of pancreatic cancer xenografts in a mouse model [4]. The housing of laboratory animals in an enriched environment (EE) is considered to be a eustress model [5] and was able to provide a number of cerebral benefits. Cao L and her colleagues first linked EE to an anti-tumor phenotype [5]. Their study and several researches thereafter showed that EE housing significantly inhibited the growth of melanoma, colon cancer [5], breast cancer [6], and glioma [7]. Our most recent study extended the tumor−retardant activity of EE to pancreatic cancer, and revealed that EE housing could also significantly inhibit tumor growth in both subcutaneous and orthotopic models of pancreatic cancer [8]. They also encouraged us to further investigate whether such benign stress can enhance the therapeutic efficacy of common

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chemotherapeutic drugs.

Therefore, in the current study, we investigated the influence of EE housing on the chemotherapeutic efficacy of 5-FU and GEM in a syngeneic mouse model of pancreatic cancer, and demonstrated that EE housing could enhance the sensitivity of pancreatic cancer to these chemotherapeutic drugs. In addition, we identified the mouse ATP-binding cassette (ABC) transporter ABCA8b as a potential chemosensitivity regulator that might contribute to the EE-induced sensitivity enhancement of pancreatic cancer to selected anti-cancer drugs.

2. Materials and methods

2.1. Experimental animals and housing conditions

Male 3-week-old C57BL/6 mice were purchased from Sino-British SIPPR/BK Lab Animal Ltd. (Shanghai, China) and were manipulated in accordance with the ethical guidelines under the protocols approved by the Medical Experimental Animal Care Commission at Shanghai Jiaotong University. The housing conditions of EE and standard environment (SE) (Additional file 1: Fig. S1) were established strictly according to our previous report [8].

2.2. Cell culture and generation of stable Abca8b-overexpressing cells

Murine pancreatic cancer Panc02 cells (syngeneic to C57BL/6 mice) were obtained from the Division of Cancer Treatment and Diagnosis Tumor Repository, Frederick National Laboratory for Cancer Research (Frederick, MD, US), and maintained in DMEM medium (Invitrogen, Carlsbad, CA, US) with 10% fetal bovine serum (Biowest, Nuaillé, France), 100 μg/ml streptomycin and 100 U/ml penicillin in a humidified incubator at 37 °C with 5% CO_2.

The full-length mouse *Abca8b* gene (Genechem, Shanghai, China) was subcloned into the expression lentivector pCDH-CMV-MCS (System Biosciences, Mountain View, CA, US) between the *Nhe*I and *Sal*I sites. The infectious lentiviral particles were generated and concentrated as described previously [9]. The Panc02 cells were transduced with the recombinant lentivirus carrying the mouse *Abca8b* gene or an empty virus to generate stable *Abca8b*-overexpressing cells (Panc02-ABCA8b) or control cells (Panc02-VC). The primers for cloning are listed in Additional file 2: Table S1.

2.3. Mouse pancreatic cancer models and drug treatment

Male 3-week-old C57BL/6 mice were randomized to live in EE or SE conditions for 3 weeks and then subcutaneously implanted with Panc02, Panc02-VC, or Panc02-ABCA8b cells (6×10^5 per mouse) in the right flank. The mice were then returned to their respective environments. When the tumors of EE or SE mice reached a mean volume of 20 mm^2, the mice were randomized into the anti-cancer drug treatment group (5-FU or GEM intraperitoneally for 4 consecutive days) or control group (PBS). After drug treatment, the tumor volume was measured once every 5 days according to the formula $V = \pi/6 \times \text{width}^2 \times \text{length}$. Twenty-five days after drug treatment, the tumors were harvested, weighted, and processed for the following analyses.

2.4. Ex vivo culture of xenograft tumor cells

Panc02 tumor tissues were obtained from EE- or SE-housed tumor-bearing mice. Then were minced into small pieces (~1 mm^3) and digested with collagenase, hyaluronidase, and DNase in DMEM medium (Invitrogen) with antibiotics. The following digestion was filtered through a 40-μm cell strainer (BD Biosciences, San Jose, CA, US) and centrifuged at 300 g for 10 min. The pellet was resuspended in DMEM medium (Invitrogen) containing 10% fetal bovine serum (Biowest) and streptomycin/penicillin.

2.5. Cytotoxicity assay

Cells were seeded evenly into 96-well plates (1×10^4 cells/well) by treatment medium containing the chemotherapeutic drug 5-FU or GEM in increasing concentrations ($10^{-7} - 10^{-4}$ M). Following incubation for 48 h, cytotoxicity was analyzed by the CellTiter 96 MTS Assay (Promega, Madison, WI, US). Half-maximal inhibitory concentration (IC_{50}) values were calculated by non-linear regression using GraphPad Prism v5.0 (GraphPad Software Inc., San Diego, CA, US).

2.6. Quantitative real-time PCR

Total RNA from the homogenized Panc02 tumors or tumor cells isolated from xenografts was extracted using Trizol reagent (Invitrogen), and they were subsequently reverse transcribed into cDNA using the FastQuant RT kit (with gDNase) (Tiangen Biotech, Beijing, China). A quantitative real-time PCR (qPCR) analysis of the expression of selected ABC transporters at the RNA level was performed with the FastStart Universal SYBR Green Master (Roche Diagnostics, Mannheim, Germany) on a StepOne Plus real-time PCR system (Applied Biosystems, Foster City, CA, US). The primer sequences used for quantitative PCR are also given in Additional file 2: Table S1.

2.7. Western blot

Tumor tissue lysates were prepared using RIPA buffer (Cell Signaling Technology, Beverly, MA, US). The proteins were quantified using a BCA Protein Assay kit (Thermo Scientific, Rockford, IL, US). Following blocking with 5% non-fat milk in TBS buffer with 0.1% Tween-20, the membranes were immunoblotted overnight with primary monoclonal antibodies recognizing either ABCA8b (Santa Cruz Biotech, Santa Cruz, CA, US) or β-actin (Sigma, St. Louis, MO, US) at 4 °C. The protein-antibody complex was detected using Pierce SuperSignal West Pico chemiluminescent substrate (Thermo Scientific). Protein expression was quantified using ImageJ software (available from http://rsbweb.nih.gov/ij/).

2.8. Statistical analysis

The results were presented as the mean ± SEM. The significance of difference between groups was assessed by the Student's *t* test for single comparisons or by analysis of variance (ANOVA) with Student–Newman–Keuls tests for multiple comparisons. Statistical analyses were performed using SPSS software version 19.0 (SPSS Inc., Chicago, IL, US), and the level of statistical significance was set at 0.05 (two-tailed).

3. Results

3.1. EE synergistically enhanced the anti-tumor effect of chemotherapeutic drugs on mouse pancreatic cancer in vivo

To test whether the combination of EE with chemotherapy results in a synergistic enhancement of anti-cancer activity, we treated Panc02 pancreatic tumor-bearing mice that were housed in either EE or SE conditions with 5-FU or GEM. After implantation, tumors of either EE or SE mice were allowed to reach a mean volume of 20 mm^3 prior to drug administration. Then, 5-FU and GEM

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