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Fear stress enhanced xenograft pancreatic tumor growth through activating epithelial-mesenchymal transition

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ABSTRACT

Objective: Cancer patients often experience multiple emotional distresses, particularly the fear of death. However, there are rare studies to assess the direct effect of the fear of death on disease progression.
Methods: Xenograft pancreatic cancer animal models were established in nude mice. Fear stress was induced to tumor bearing mice by closely housing with a cat and depressive behaviors were measured using open field test, forced swimming test, and sucrose consumption test. Plasma adrenaline concentration was measured using ELISA.
Results: Fear stress induced depression-like behaviors in tumor bearing mice which were accompanied with increases in tumor growth, plasma adrenaline levels as well as the protein expression of alpha 2 adrenergic receptor (α2 AR) and beta 2 adrenergic receptor (β2-AR) in tumor tissues. The β-adrenergic antagonist propranolol (Pro) treatment blocked the effect of stress on tumor growth in pancreatic cancer xenograft animal model, but had no effects on the levels of plasma adrenaline level, and α2 AR and β2-AR expression in tumor tissues. Moreover, fear stress increased Frizzled-1, Wnt1, vimentin, but decreased E-cadherin protein expression in tumor tissues, while Pro reversed the effects of fear stress on the expression of these proteins.
Conclusion: Fear of death impacted the growth of PDAC tumor though activation of epithelial-mesenchymal transition. Treating pancreatic cancer patients with β-adrenergic antagonist implicates an effective strategy to treat cancer including PDAC.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a highly lethal disease and the fourth leading cause of cancer associated death in the United States [1]. It has an estimated 5-year survival rate between 3% and 5% [2,3]. In the clinic, most PDAC patients were diagnosed at an advance stage with invasion and metastasis. PDAC is resistant to conventional chemotherapy and radiotherapy, and the current chemo- and radiotherapy for advanced PDAC is basically ineffective. Targeting therapy is still not clinically available [4]. Thus, there is an urgent need to develop an effective strategy to treat PDAC patients.

Patients suffering from cancers often face multiple stressors including fear of death. Over a quarter of cancer patients develop severe depressive symptoms, and the rate increases to about 80% in patients with advanced cancer [5]. A previous review suggests that stress is associated with higher mortality rates in cancer patients [6]. However, there are rare prospective studies that determine a clear association between fear of death and disease progression in cancer patients; while the molecular and cellular mechanisms for stress-driving tumor growth remain to be fully elucidated.

Previous studies demonstrated that chronic stress can increase the proliferation, migration, and invasion of cancer cells; enhance angiogenesis; and decrease cell death through the activation of adrenergic receptors (AR) [7,8]. For example, a recent study revealed that stress hormones activate β_2-adrenergic receptors (β_2-ARs) on the tumor cells of non-small cell lung cancer (NSCLC), which promote tumor growth and EGFR inhibitor resistance. In contrast, β-blockers can abrogate the effects of stress [9]. In pancreatic cancer animal models, chronic stress can increase tumor growth and reduce survival, which can be antagonized by β-ARs blockade [10]. A study using an orthotopic mouse model showed that both stress and pharmacological activation of β-adrenergic signaling increased primary tumor growth and induced tumor cell

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dissemination to normal adjacent pancreas. In contrast, pharmacological β-blockade reversed the effects of chronic stress on pancreatic cancer progression [11]. However, there is no clinical trial for the use of β-blockade to complement existing therapies for pancreatic cancer. Taken together, we hypothesize that as the main stress of cancer patients, fear stress may increase growth of pancreatic tumors through activation of adrenergic receptor signaling.

In this study, fear of death was induced in mice that bore xenograft pancreatic tumors by housing them closely with a cat. Behavioral changes, tumor growth, and associated molecular mechanisms were evaluated following the stress paradigm (Mice were housed close to a cat for 20 min daily for 5 days that the mice can hear, smell, and see the cat.)

Materials and methods

Cell culture

Capan-1, a pancreas adenocarcinoma obtained from a liver metastasis of a pancreas adenocarcinoma in the head of the pancreas, was purchased from ATCC (American Type Culture Collection). Capan-1 cell line was selected because Capan-1 cells possess receptors for beta-adrenergic agonists [12]. Cells were cultured in Dulbecco's Modification of Eagle's Medium (DMEM) containing 10% FBS (fetal bovine serum) at 37 °C, 5% CO_2.

Animals

Male athymic nude mice (BALB/c-nu/nu) were purchased from the Animal Facility of Central South University and housed in groups of five per cage under a 12-h light/dark cycle (07:00 to 19:00). Food and water were available *ad libitum*. Mice were acclimated for 1 week prior to experiments. All experimental procedures were approved by the Animal Care and Use Committee of Central South University; they were conducted by following the Guide for the Care and Use of Laboratory Animals of Chinese Council.

Tumor growth and stress induction

Approximately 1×10^6 Capan-1 tumor cells were subcutaneously injected into the right flank of mice. After the tumors grew to about 100 mm^3 in size, mice were randomly divided into 3 groups (10 animals per group): (1) Non-stress control (control), (2) Stress alone (stress), and (3) Stress plus β-adrenergic antagonist propranolol (Pro) treatment (Stress+Pro). On day 1–5, tumor-bearing mice in the stress alone group and Stress+Pro group were placed in cages made by metal net (five mice in one cage); that one cage was then brought to a room where a cat was housed in a similar, but bigger cage. The cat was verified to catch this kind of mice and housed in the cage for overnight with food and water available *ad libitum*. The cage with mice was placed 20 cm away from the cage with a cat for 20 min daily. The mice in the control group were placed in the same size cages and then brought to a room where there was a cage same to the cage housing the cat, but without cat. The stress and sham stress were induced at 10 a.m.–11 a.m. on day 1 to day 5. The mice in the Stress+Pro group received administration of Pro in their drinking water (4.6 mg/kg daily) from day 1–7 (Fig. 1A). Tumor size was measured twice or three times a week using a caliper. Tumor volume was calculated with the formula $(LxW^2)/2$, where L is the tumor length and W is the tumor width [13]. Same experiments were repeated with 5 animals in each group and the results were combined.

Behavioral testing in animals

The schedule for behavioral testing is presented in Fig. 1A.

Open field test

An open field test (OFT) was performed as previously described [14]. The open field arena was made from an open rectangular plastic box (100 cm × 100 cm × 30 cm) with 25 squares (20 cm × 20 cm) painted on the floor. The 25 squares included 16 peripheral squares and 9 central squares. At the time of the test, mice were coded by observers who were blind to the experimental design. Mice were then placed individually in the center of the arena and allowed to explore the area freely for 30 min. The activity of the rats was recorded by an overhanging camera that was linked to a personal computer. The total distance a mouse moved during the 30-min test was recorded. The arena was cleaned with 70% alcohol between tests to make sure the current mouse's behaviors were not affected by the imprint of previous mice.

Forced swimming test

A forced swimming test (FST) was performed 24 h after the open field test [15]. Two swimming sessions were conducted; a 15-min pretest on the first day was followed by a 5-min test the next day. At the time of the test, mice were placed individually in a Pyrex cylinder filled with 25 °C water. After swimming for 15 min on day 1, mice were dried with towels and then placed back in their home cage. The water in the cylinder was emptied and refilled between mice. Twenty-four hrs after the first trial, mice were placed in the swimming apparatus again for a 5-min test. A video camera hung above the cylinders was used to record the rats' activity. The immobility time was recorded.

Sucrose consumption test

Sucrose consumption test (SCT) was performed as described previously [16]. The whole test took 3 days. On day 1, mice were housed individually and given free access to two identical bottles of sucrose solution (1%, w/v and 100 ml). Mice were trained to adapt to sucrose solution for 24 h. On day 2, one bottle of sucrose solution was replaced with 100 ml of water. On day 3, mice were deprived of water and food for 23 h and then given free access to two pre-weighed bottles of solution: 100 ml of sucrose solution (1%, w/v) and 100 ml of water. One hour later, the consumed liquid in two bottles was measured. The sucrose preference rate was calculated using the following formula: Sucrose preference rate = sucrose consumption/(water consumption + sucrose consumption) × 100%

Enzyme-linked immunosorbent assay (ELISA)

Animals were euthanized with overdose of pentobarbital on day 31. Blood was collected into EDTA tubes via cardiac puncture. Plasma was collected by centrifugation of the blood at 1500 rpm for 5 min at 4 °C; adrenaline concentration was measured using Adrenaline Research ELISA kit (Labor Diagnostika Nord, Nordhern, Germany) by following the user's manual.

Western blot

Tumor tissues were homogenized as previously described, and the homogenate was used for Western blot analysis [16]. The protein concentration was determined by a Bradford Assay (Bio-Rad Laboratories, Hercules, CA, USA). Cell homogenate containing 20 μg protein was loaded per well. The membranes were incubated with antibodies for human alpha 2 adrenergic receptor (α2 AR), beta 2 adrenergic receptor (β2 AR), GAPDH (Santa Cruz Biotechnology, USA), E-cadherin, vimentin, Frizzled-1, Frizzled-2, and Wnt1 (Cell

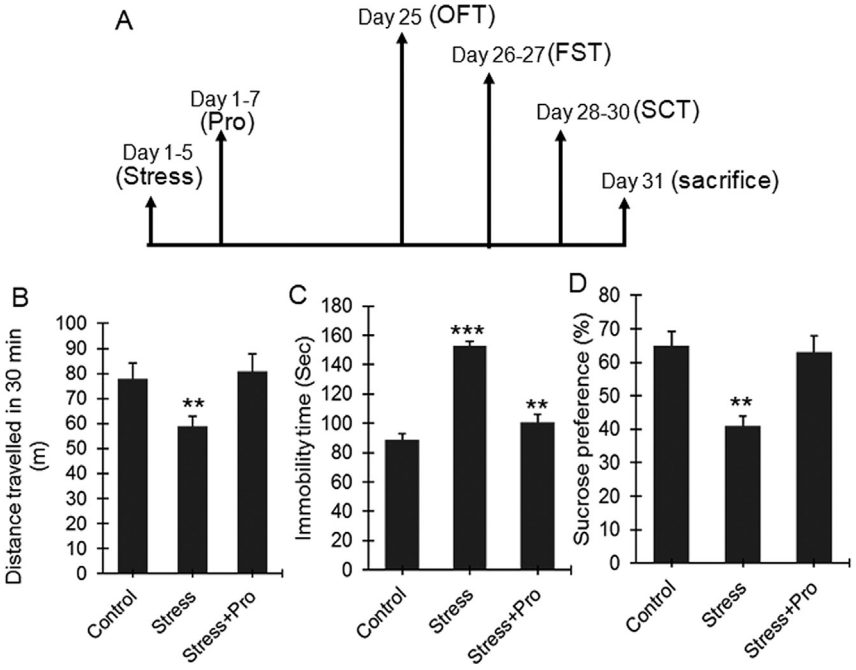

Fig. 1. Psychological stress-induced depressive behaviors in mice. Athymic nude mice bearing Capan-1 pancreatic cancer tumors were randomly divided into sham-stress (Control), stress (Stress), and stress plus propranolol (Pro) treatment groups (Stress+ Pro). **(A)** The schedule for treatment and behavioral testing. **(B)** Total distance travelled in the OFT. $^{**}p < 0.01$ *vs.* other two groups. **(C)** Floated time in FST. $^{***}p < 0.001$ *vs.* control group; $^{**}p < 0.01$ *vs.* stress group. **(D)** Percentage of sucrose preference. $^{**}p < 0.01$ *vs.* other two groups. Data are presented as mean ± SEM. N = 15.

Signaling Technology, Danvers, MA, USA) overnight at 4 °C; they were followed by incubation with horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology, USA) for 2 h at room temperature. The immunoreactive proteins were visualized using an ECL Prime kit (Amersham Sciences, USA). The images were taken using ChemiDoc XRS^{+} system and analyzed using Image Lab 4.1 (Bio-Rad Laboratories, USA).

Statistics

Data were analyzed using SPSS16.0 software (IBM, NC, USA). One-way ANOVA and *post-hoc* Newman-Keul's multiple comparison test were applied for comparison between groups in the animal study. Tumor volume and the fold of tumor growth were analyzed statistically with a repeated measure of ANOVA. A $P < 0.05$ was considered statistically significant.

Results

Fear stress-induced depression-like behaviors in mice bearing xenograft tumors

Athymic nude mice bearing Capan-1 pancreatic cancer tumors were randomly divided into sham-stress (Control), stress (Stress), and Stress plus Pro treatment (Stress+Pro) groups. No death was observed among the three groups before euthanasia. In the OFT, mice in the stress alone group travelled a significantly shorter distance compared to the other two groups ($p < 0.01$) (Fig. 1B). In the FST, significant differences in immobility time were observed among the three groups ($p < 0.001$); mice in the stress alone group floated for a significantly longer time than control mice and Pro treated mice ($p < 0.001$ and $p < 0.01$, respectively) (Fig. 1C). In the SCT, mice in the stress alone group consumed significantly less sucrose than the control mice and Pro treated mice ($p < 0.01$) (Fig. 1D).

Psychological stress stimulated tumor growth

Tumor size was measured twice or three times per week, and tumor weights were determined at the time of sacrifice. A repeated measure of one-way ANOVA showed that stress significantly increased the tumor volume starting on day 20 compared to control mice ($p < 0.05$ for day 20 to day 24; $p < 0.01$ for day 26 to day 29; and $p < 0.001$ on day 31), and Pro treatment significantly decreased tumor volume compared to the stress group ($p < 0.01$ for day 20 to day 22; and $p < 0.001$ for day 24 to day 31) (Fig. 2A). Similarly, a repeated measure of one-way ANOVA showed significant main effect of stress and time ($p < 0.01$) on the fold of tumor growth. Specifically, Pro treatment significantly decreased the fold of tumor growth compared to the stress group ($p < 0.05$ for day 20; $p < 0.01$ for day 22; and $p < 0.001$ for day 24 to day 31) (Fig. 2B). Stress significantly increased tumor weights in mice ($p < 0.01$), but Pro treatment significantly decreased tumor weights compared to the stress alone group ($p < 0.01$) (Fig. 2C).

Fear stress elevated plasma adrenaline level and increased the expression of adrenergic receptors in pancreatic tumor bearing mice.

Plasma adrenaline (Fig. 3A) concentrations in mice were measured by ELISA, and the protein expression of alpha 2 adrenergic receptor (α2 AR) (Fig. 3B and C) and beta 2 adrenergic receptor (β2 AR) (Fig. 3B and D) in tumor tissues were measured by Western blot. A one-way ANOVA showed a significant induction effect of stress on the concentration of plasma adrenaline, α2 AR expression, and β2 AR expression in tumor tissues ($p < 0.01$, $p < 0.05$, and $p < 0.001$, respectively). In contrast, Pro treatment did not abrogate the elevated plasma adrenalin, and AR expressions by stress (Fig. 3).

Fear stress induced epithelial-mesenchymal transition in pancreatic tumors

Western blots showed that fear stress significantly increased

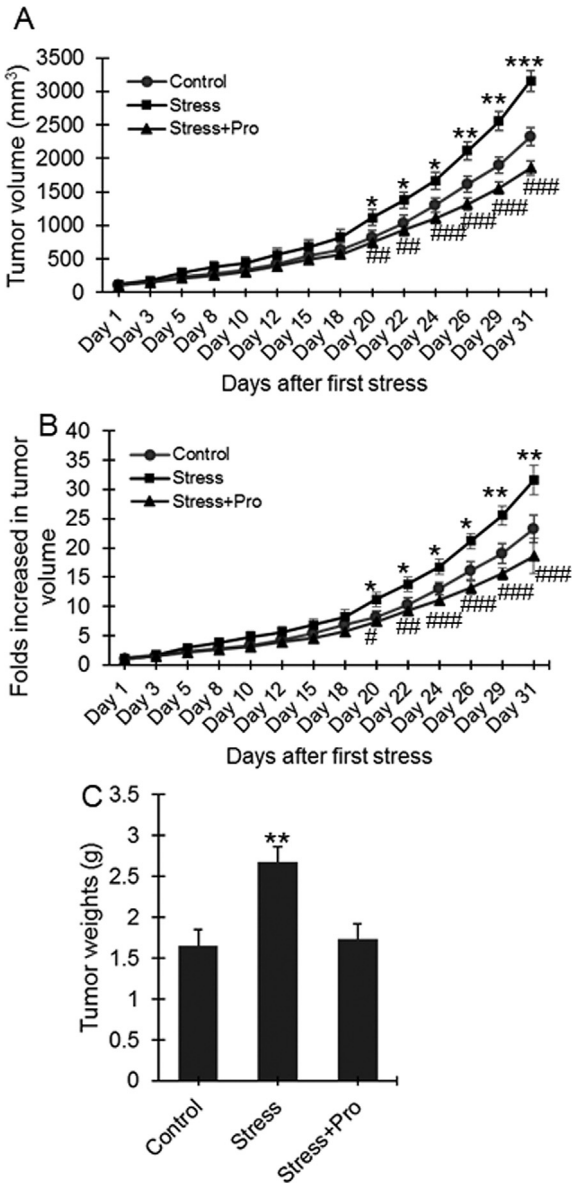

Fig. 2. Psychological stress-stimulated tumor growth in mice. **(A)** Tumor volumes between 3 groups. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ *vs.* control; $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ *vs.* stress group. **(B)** Fold increase in tumor volume. $^{*}p < 0.05$, and $^{**}p < 0.01$ *vs.* control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ *vs.* stress group. **(C)** Tumor weights between groups.$^{**}p < 0.01$ *vs.* other two groups. Data are presented as mean ± SEM. N = 15.

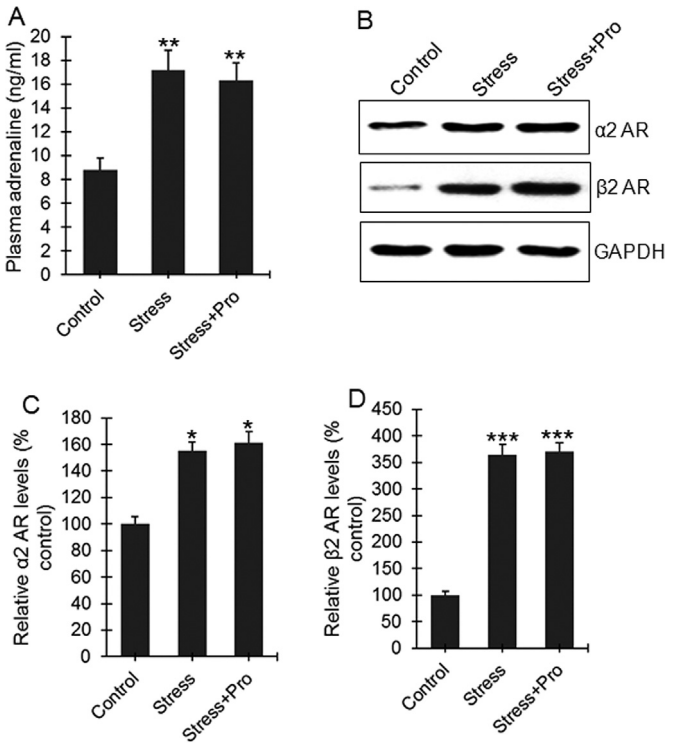

Fig. 3. Plasma adrenaline levels in mice and adrenergic receptor expression in xenograft tumor tissues. **(A)** Plasma adrenaline concentration. **(B)** Representative Western blots of alpha 2 adrenergic receptor (α2 AR) and beta 2 adrenergic receptor (β2 AR) expression in tumor tissues. **(C)** Quantitative analysis of α2 AR expression in (B). (D) Quantitative analysis of β2 AR expression in (B). $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ *vs.* control. Data are presented as mean ± SEM. N = 15.

Frizzled-1 (Fz1), Wnt-1 and vimentin protein expression, but decreased E-cadherin protein expression in xenograft PDAC tumor tissues. In contrast, Pro normalized the expression of these proteins in tumor tissues. Both stress and Pro had no effects on Frizzled-2 (Fz2) protein expression (Fig. 4).

Discussion

Pancreatic ductal adenocarcinoma (PDAC) is a highly aggressive tumor with poor prognosis, and long-term survival of patients is rather low. This study investigated the effect of fear stress on behavioral changes, tumor growth, plasma adrenaline level, and the expression of adrenergic receptors, and associated molecular mechanisms in xenograft tumor tissues in mice. Our study validated the role of fear of death in the progression of xenograft PDAC and proposed the strategy to treat cancer patients with β-adrenergic antagonist plus psychological intervention.

The psychological state of cancer patients may significantly affect disease prognosis and therapeutic effect. The accumulated evidence suggests the adverse effects of psychological stress on the progress of cancer patients [17,18]. In the present study, fear stress was induced in tumor-bearing mice *via* close housing with a cat. We showed that this stressor significantly shortened the travel distance in mice in the open field test, increased the immobility time in the forced swimming test, and decreased the sucrose consumption in the sucrose consumption test. These behavioral changes suggest that this stressor induced a depressive phenotype in mice bearing pancreatic tumor, which was associated with enhanced tumor growth. This is the first study to induce depression in pancreatic tumor bearing mice by a cat. These behavioral alterations were accompanied by a persistent dysregulation of the adrenergic signaling, such as the increased plasma adrenaline levels and increased beta 2 and alpha 2 adrenergic receptor levels in tumor tissues of stressed mice. Adrenaline is normally produced by both the adrenal glands and the sympathetic neurons of the autonomic nervous system and plays an important role in stress response through activating the adrenergic receptors [19]. Adrenergic receptors have gained a lot of interest as potential targets for the treatment of several types of cancer [20]. Our mouse model with fear stress may support a more crucial role of β-AR in fear stress-induced tumor growth than α-AR (There was a 3.6-fold increase in β2-AR expression vs. 1.5-fold increase in α2-AR expression after stress and Pro can abrogate stress-induced EMT and tumor growth). This model therefore has several advantages: 1) fear stress is established by housing with a cat that the mice can hear, smell,

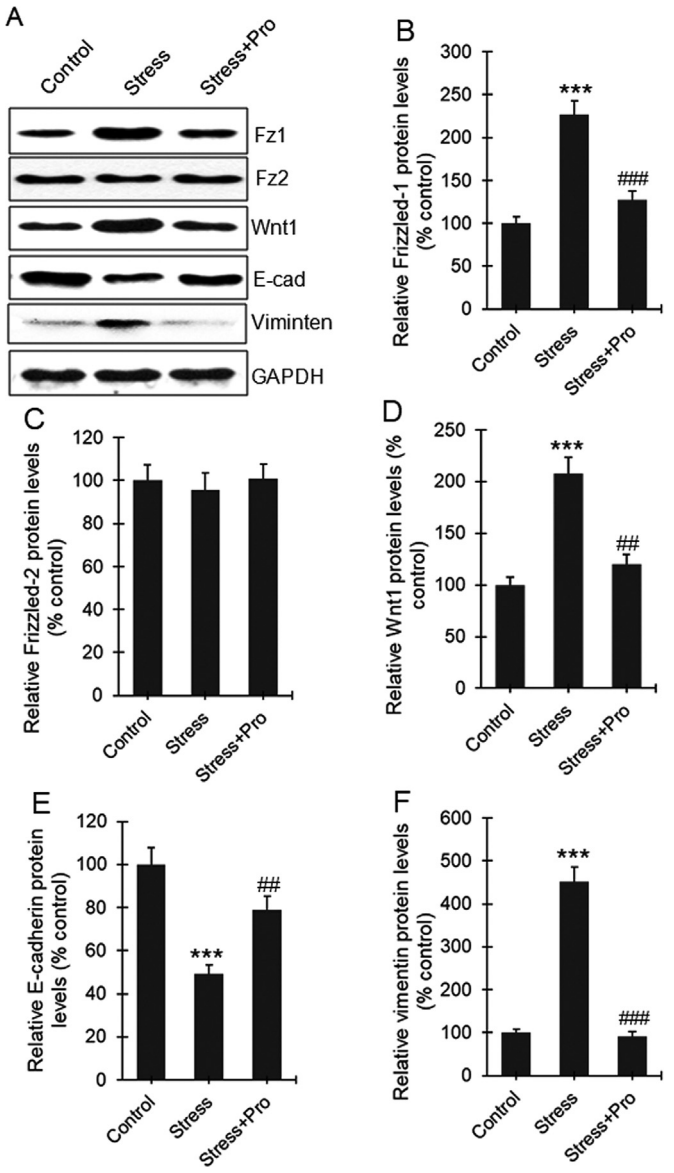

Fig. 4. Western blots of protein expression in xenograft tumor tissues. (A) Representative Western blots of Frizzled-1 (Fz1), Frizzled-2 (Fz2), Wnt1, E-cadherin (E-cad), vimentin, and GAPDH protein expression. **(B, C, D, E, F)** Quantitative analysis of Frizzled-1, Frizzled-2, Wnt1, E-cadherin, and vimentin expression in (A), respectively. $^{**}p < 0.01$, and $^{***}p < 0.001$ *vs.* control. $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ *vs.* Stress group. Data are presented as mean ± SEM. N = 15.

and see the cat; 2) fear stress did not cause physical injury or pain in mice; and 3) this stressor induced a depressive phenotype in tumor-bearing mice, which well mimics the emotional disturbance in cancer patients.

Previous studies demonstrated that psychological stresses affect cancer cell survival, tumor progression, and therapeutic response [20]. Su et al. study in severe combined immunodeficient mice bearing breast tumors demonstrated that restraint stress induced chemoresistance by activating the α_2-adrenerdric receptor through upregulation of the expression of the multi-drug resistance gene MDR1 [21]. Braadland et al. study showed that the use of β-blockers reduced prostate cancer-specific mortality through inhibiting β-adrenergic receptor signaling, subsequently increasing apoptosis, as well as decreasing epithelial-mesenchymal transition and metastasis of prostate cancer cells [22]. Powe et al. study showed that beta-blocker treatment significantly reduced the risk of metastasis, tumor recurrence, and mortality [23]. In this study, fear stress stimulated xenograft pancreatic tumor growth in athymic nude mice through the activation of adrenergic signaling; β-blockers can block the adverse effect of fear stress.

The β2-AR forms a chimeric receptor with Frizzled-1 (Fz1) or Frizzled-2 (Fz2). A previous study demonstrated that beta-adrenergic agonists can activate the Wnt/beta-catenin and Wnt/Ca^{2+} or cyclic GMP pathways through the β2-AR/Fz1 and β2-AR/Fz2 chimeric receptor, respectively [24]. The activation of the Wnt/beta-catenin signaling has been revealed to induce EMT and subsequent invasiveness and proliferation of prostate cancer cells [25]. Wnt/β-catenin signaling can enhance the transcription of EMT-promoting genes [26]. Loss of E-cadherin and increase in vimentin expression are major markers for the activation of EMT. The present study showed that fear stress increased Frizzled-1, Wnt-1, and vimentin protein expression, but decreased E-cadherin protein expression in xenograft PDAC tumor tissues. Also, the elevated Wnt1 levels further supported the changes in blood adrenaline level and the expression of α_2 and β_2-AR in tumor tissues. In addition, a non-specific beta-AR decreased Frizzled-1, Wnt-1 and vimentin protein expression and increased E-cadherin protein expression. In this study, the fear-stress for death up-regulated the plasma adrenaline level and cancer AR expressions, while Pro did not alter these up-regulation, but exerted the suppressive effect on EMT and consequently suppressed the tumor growth. Thus, the fear-stress is one of the factors to up-regulate adrenaline signaling that activates the β_2-AR/Fz1 chimeric receptor, and in turn stimulates tumor growth through EMT and associated genes. However, this study did not test whether administration of adrenaline can stimulate xenograft CAPAN-1 tumor growth and whether Pro can abrogate it. This is a limitation of the current study. Besides the possible effects of Pro on EMT, a recent study demonstrated that low and high doses of Pro produced vasoconstriction and vasodilation of tumor respectively in melanoma-bearing mice [27]. In addition, previous studies also demonstrated that Pro can markedly reduce glycogenolytic action of norepinephrine in cultured Ewing's sarcoma cells [28], and block the suppressive effect of beta-adrenergic agonist on NK activity in an NK-sensitive tumor model [29]. Thus, multople mechanisms may be involved in the fear-stress induced tumor growth.

In conclusion, this study introduced a fear stress that induced depressive-like behaviors in an animal model of pancreatic cancer and then re-evaluated the efficacy of fear stress on tumor growth. The present study found that fear-stress induced emotional impairment increases tumor growth via an EMT. This study also suggests that the applications of beta-blockers and psychological interventions may benefit patients' progression when patients do not aware the existence of tumor. Also, the application of beta-blocker might be more effective in pancreatic cancer patients at an early stage of cancer progression.

Acknowledgement

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pan.2019.01.002.

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