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ORIGINAL ARTICLE

In vivo real-time imaging of gemcitabine-leaded growth inhibition in the orthotopic transplantation model of human pancreatic tumor

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Gemcitabine

Abstract Human xenograft mouse models, which have been used in cancer research for over a century, provided significant advances for our understanding of this multifaceted family of diseases. Orthotopic transplantation tumor models are emerging as the preference for cancer research due to the increasing clinical relevance over subcutaneous mouse models. In this study, a stable luciferase-expressed Capan-2 cell line was constructed and the expression of luciferase was tested. The results showed that the luminorescence intensity of Capan-2^{Luc} cells was associated with the number of cells and the minimal detectable cell population was 600 cells/well. We established an orthotopic transplantation model of pancreatic cancer using Capan-2^{Luc} cell line in athymic mice and investigated the inhibitory effects of gemcitabine (Gem) *in vitro* and *in vivo*. Optical imaging system was applied to evaluate the tumor growth of orthotopic transplantation model *in vivo*. The results

Abbreviations: MOH, Ministry of Health; Gem, gemcitabine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; IC_{50}, half-inhibitory concentration

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suggested that the orthotopic transplantation model of pancreatic cancer was well established and the luminorescence intensity of Gem-treated group was markedly lower than that of control group with an inhibitory rate of 56.8% ($P<0.001$). Our orthotopic transplantation model of pancreatic cancer and real-time imaging observation method established in this study could be an ideal model and a useful tool for therapeutic approaches for pancreatic cancers.

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1. Introduction

Pancreatic cancer is one of the most lethal cancers, which is incidence of occult, high grade, and has extremely poor prognosis[1]. There are great needs to develop advanced models for the preclinical investigation of pancreatic cancer. It is estimated that 43,140 people (21,370 men and 21,770 women) will be diagnosed with cancer of the pancreas in 2010, and that 36,800 men and women will die of this disease[2]. The use of orthotopic transplantation pancreatic tumor models for preclinical research reproduces its pattern of local growth and distal dissemination. It may improve the development of therapeutic strategies and diagnostic imaging modalities against this disease. Optical imaging system is a new tool which can observe tumor growth, metastasis, and other biological processes *in vivo*[3]. When tumor cells express luciferase, the optical imaging system using Living Image- software (Xenogen) can directly track the process of tumor growth *in situ*[4].

No effective early diagnosis of pancreatic cancer is available and progress in development of treatment is slow. Chemotherapy is one of the most common treatments for pancreatic cancer and Gemcitabine (Gem) is one important chemotherapy drug for this disease. Since Gemcitabine can significantly prolong survival time and improve the clinical benefit rate, US Food and Drug Administration approved Gemcitabine as the first-line drug for pancreatic cancer chemotherapy in 1998[5]. However, the benefit of Gemcitabine monotherapy in advanced and metastatic pancreatic cancer is limited[6].

In this study, an engineering modified human pancreatic cancer cell Capan-2$^{\mathrm{Luc}}$ was used to develop luciferase labeled orthotopic transplantation pancreatic cancer model. The anti-tumor effect of Gemcitabine on pancreatic cancer *in situ* was observed. We suppose that this animal model can provide an ideal preclinical tool for evaluating anti-pancreatic cancer drugs.

2. Materials and methods

2.1. Cell culture and reagents

Human pancreatic cancer Capan-2 cells were purchased from ATCC (ATCC HTB-80) and routinely grown in RPMI-1640 (Hyclone, Utah) supplemented with 10% FBS (Gibco, USA), penicillin-streptomycin (1%), and 2 mmol/L L-glutamine. Gem was from Eli Lilly (USA). D-luciferin was purchased from Xenogen (Alameda, CA).

2.2. MTT assays

Capan-2 cells were seeded in 96-well plates at a density of 3×10^3 cells/well in 200 μL medium. Then the cells were treated with various concentrations of Gem. After drug exposure for 48 h, the MTT solution (5 g/L) was added to the plates. The cells were incubated at 37 °C for another 4 h. The formazan, which is derived from MTT by living cells, was dissolved in 150 μL DMSO per well, and the absorbance was detected at 570 nm. All MTT experiments were performed in triplicate and repeated at least 3 times. The percentage of cytotoxicity was calculated as follows: cytotoxicity (%)=(1−OD_{570} of experimental well)/OD_{570} of control well. The IC_{50} (defined as the drug concentration at which 50% cell growth was inhibited) was assessed from the dose–response curves.

2.3. Transfection and selection of stably transfected Capan-2^{Luc} cells

Capan-2 cells at 70–80% confluence density were transfected with 1 μg of luciferase expression plasmid PCAGGS-NEO-luc using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. For stable expression, the cells were exposed to 500 μg/mL G418 (Gibco, USA) after 1 day transfection. After growth for 3 weeks, cells were plated at a lower density in MEM with 500 μg/mL G418 and 10% FBS in 96-wells plates until single colony was formed. Single cloned cells were isolated and expanded. To identify the positive clones, monoclonal cells were plated in 96-well plates and luciferase substrate D-luciferin was added to a final concentration of 60 mg/mL. After 30 min of exposing with the optical imaging system (IVIS200, Xenogen), fluorescence intensity of expression was tested, and the most fluorescent clone was selected and cryopreserved.

2.4. Luciferase activity test of Capan-2^{Luc} in vitro

The Capan-2$^{\mathrm{Luc}}$ cells were diluted in 96-well plates at a density of 5000, 2500, 1250, 600, 300, 150, 75 and 0 cells per well in the presence of fluorescent luciferase substrate D-luciferin. The luminous intensity was detected in the camera box.

2.5. Establishment of orthotopic transplantation human pancreatic tumor model

The 6–8 week-old female athymic nude mice (BALB/c, nu/nu) were purchased from the Institute for Experimental Animals, Chinese Academy of Medical Sciences & Peking Union Medical College (Beijing, China). The study was proceeded in accordance with the regulations of Good Laboratory Practice for non-clinical laboratory studies of drugs issued by the National Scientific and Technologic Committee of People's Republic of China. Human pancreatic cancer cells

Capan-2^{Luc} growing in log phase were diluted to 1.5×10^7 cells/mL with RPMI-1640 medium. Nude mice were anesthetized with injection of 60 mg/mL pentobarbital sodium intraperitoneally, and following by making a small incision into the abdomen along the lower left rib cage to pull out the pancreas and inject 3×10^5/0.02 mL of Capan-2^{Luc} cell suspension into it. Incision suture was operated according to conventional surgery. At 15, 30, 45, 60 days after surgery, luciferase substrate D-luciferin (150 mg/kg) was injected intraperitoneally, and the animals were placed onto the warmed stage inside the camera box (IVIS-Imaging System, Xenogen) to observe tumor growth[7]. After 60 days, all mice were weighed and sacrificed, and the tumors were excised. In addition, pancreatic tissues were taken from five nude mice on day 15 for HE staining after formalin-fixiation and paraffin-embedding.

2.6. *In vivo experiment*

Nude mice were injected with 3×10^5 Capan-2^{Luc} cells as described. On day 15 after inoculation, the animals were randomized into two groups ($n = 7$ per group): control group (saline) and Gem (80 mg/kg) treatment group. The mice were injected with either saline or Gem intravenously, once a week for two weeks. All mice were weighed and sacrificed, and the tumors were excised and weighed by the end of experiment, which was 60 days after tumor inoculation.

2.7. *Statistics*

Data were described as means ± SD of the indicated number of individual experiments. A one-way analysis of variance was carried out for multiple comparisons. If there was significant variation between treatment and control groups, the mean values were compared using Student's *t*-test. *P*-values less than 0.05 were considered statistically significant difference.

3. Results

3.1. *Inhibitory effect of Gem on human pancreatic cancer Capan-2^{Luc} cells*

To investigate the antitumor effect of Gem on Capan-2^{Luc} cells *in vitro*, cells were incubated with various concentrations of Gem for 48 h. After drug exposure, cell proliferation was determined by MTT assay. The results showed that Gem could inhibit the proliferation of human pancreatic cancer Capan-2^{Luc} cells in a dose-dependent manner (Fig. 1), and IC_{50} was 1.01×10^{-7} mol/L.

3.2. *Luciferase activity in Capan-2^{Luc} cells*

Since Capan-2^{Luc} cells can express luciferase, its activity was detected using optical imaging system in the presence of D-luciferin. When various numbers of cells were seeded in the plate, various luminescence intensities were detected by IVIS-Imaging System. The bioluminescence photon was positively correlated to the number of cells. The minimum detectable cell number was 600 cells per well according to our results (Fig. 2).

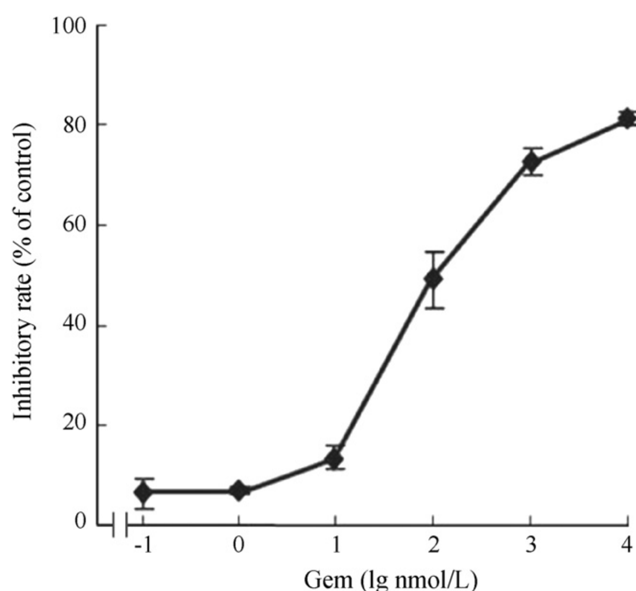

Figure 1 Analysis of Gem-leaded growth inhibition in Capan-2^{Luc} cells. Cells were treated by various concentrations of Gem for 24 h, and then were determined by MTT assay.

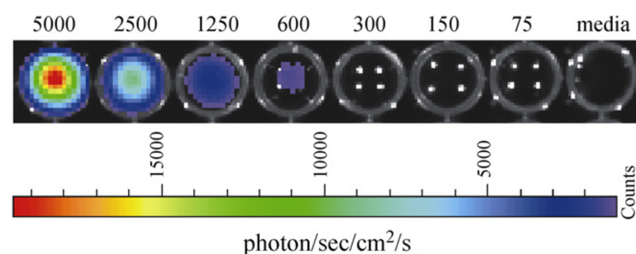

Figure 2 The positive correlation between the photons of bioluminescence and the numbers of Capan-2^{Luc} cells. Cell photons were determined by IVIS-Imaging System.

3.3. *Optical imaging in living animal*

In order to observe the real-time growth of tumors *in situ*, optical imaging system was used to track the tumor growth. On day 15 after tumor inoculation, bioluminescence in pancreas of the nude mice could be detected, indicating that Capan-2^{Luc} cells successfully grown in the pancreas (Fig. 3A). The fluorescence intensity of the pancreas *in situ* increased significantly from day 15 to day 60, suggesting that the transplanted tumor has been established and gradually grown. However, there was no convincing metastasis being observed. The amount of bioluminescence photon showed significant difference between control group and Gem treatment group from day 30 to day 60 ($P < 0.001$). The growth curve of photons emitted by Capan-2^{Luc} cells against tumor-bearing time of mice showed the proliferation of luciferase labeled Capan-2^{Luc} cells in nude mice (Fig. 3B). The results showed that Gem significantly inhibited tumor growth.

3.4. *Gem inhibited Capan-2^{Luc} cells orthotopic transplantation pancreatic tumor growth*

On day 60 after orthotopic transplantation, animals were sacrificed and the tumor were excised and weighed. The results

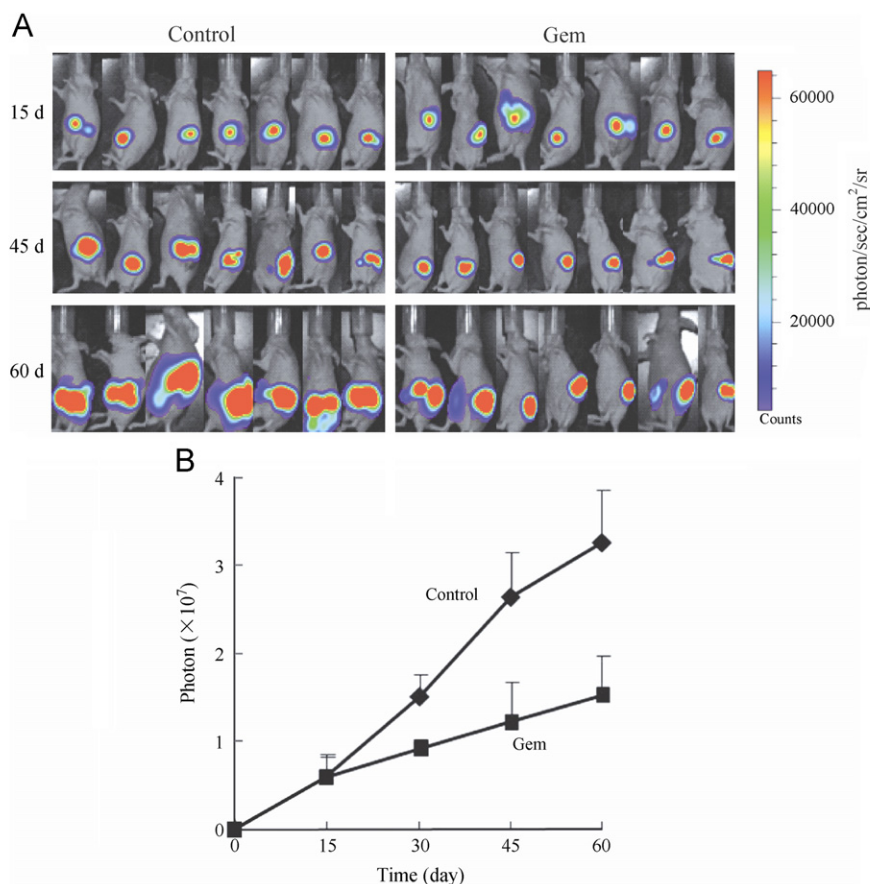

Figure 3 Gem-mediated growth inhibitions of orthotopic transplantation tumor of human pancreatic Capan-2^{Luc} cells. (A) The profile of optical imaging. (B) The growth curve of pancreas tumor.

Table 1 The inhibitory effect of Gem on orthotopic transplantation tumor of human pancreatic Capan-2^{Luc} cells.

Drug	Dose (mg/kg)	Tumor weight (g)	Inhibition (%)
Control	–	0.37±0.09	–
Gem	80	0.16±0.03*	56.8

Administration at day 7 and day 15 (iv) and detection at day 60 after tumor transplantation; $n=7$
*$P<0.001$, compared with control.

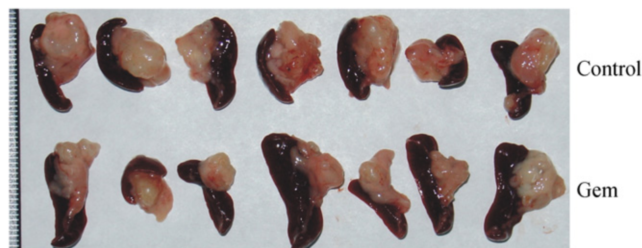

Figure 4 The profile of various treated orthotopic transplantation tumor of human pancreatic Capan-2^{Luc} cells.

Table 2 The weight changes of various treated tumor-bearing mice ($n=7$).

Group	Body weight (g)		Change of body weight (g)
	Begin	End	
Control	20.47±0.91	19.45±1.07	−1.02±0.05
80 mg/kg Gem	19.87±0.81	19.31±0.79	−0.56±0.04*

*$P<0.05$, compared with control.

showed that the orthotopic transplantation Capan-2^{Luc} tumor was significantly inhibited by Gem (80 mg/kg) with the inhibition rate of 56.8% ($P<0.001$) (Table 1). The two groups' mice body weight exhibits significant difference: Gem-treated group mildly increased while control group decreased ($P<0.05$) (Table 2). This indicated that the tumor affected mice growth by pancreatic function loss. As shown in Fig. 4, deep-colored tissue was spleen and light-colored tissue was pancreatic tumor. Compared to control group, the pancreatic tumors in Gem-treated group were smaller, which correlated with data from image system. Spleen tissues were normal, indicating Gem at the dosage used in this study was non-toxic to spleen.

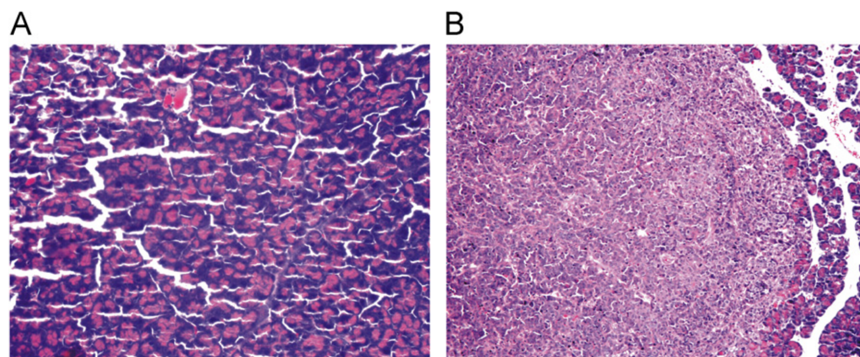

Figure 5 The pathological changes of orthotopic transplantation tumor of human pancreatic Capan-2^{Luc} cells by HE staining. Light microscope (×200). (A) The tumor cells grew nodularly, arranged in solid and nested architecture. (B) Tumor cells were polygonal conformation with clear boundary and large round nuclei, heteromorphism and mitotic phase.

3.5. Pathological analysis of Capan-2^{Luc} orthotopic transplantation pancreatic cancer

HE staining results showed the tumor cells in pancreatic tissue were polygonal conformation with clear boundary and large round nuclei, heteromorphism and mitotic phase (Fig. 5B). In addition, the tumor cells grew nodularly, arranged in solid and nested architecture. Meanwhile, small blood sinus and rarely gland-like structure were in line with poorly differentiated pancreatic carcinoma comparing to normal tissue (Fig. 5).

4. Discussion

In recent years, the incidence of pancreatic cancer was rising and was still an important unsolved health problem in the world. It was estimated that pancreatic cancer acted as the seventh or eighth cause of cancer death in China, and fourth lethal human cancer in the United States. As the growth of pancreatic cancer, it was easy to infiltrate into pancreas surrounding tissues and blood vessels, and developed lymph node metastasis. Almost 100% of patients with pancreatic cancer develop metastases and die of the debilitating metabolic effects of their unrestrained growth[8]. In addition, pancreatic cancer could directly invade perineurium space and spread along the nerve bundle.

Gem is a deoxycytidine analog with structural and metabolic similarities to cytarabine. Gem must be phosphorylated to its active metabolites: Gem diphosphate and Gem triphosphate. Gem diphosphate inhibits ribonucleotide reductase and depletes intracellular pools of all the deoxynucleotide triphosphates necessary for DNA synthesis. Gem triphosphate may be incorporated into an elongating DNA chain and leads to premature chain termination which may lead to apoptosis[9]. Although Gem is currently the first choice chemotherapy drug for pancreatic cancer, its efficacy is not inspiring, only about 20%.

Despite many efforts made in the past decades, pancreatic cancer is resistant to currently available conventional treatment approaches, such as surgery, radiation, chemotherapy, or combination therapies. Therefore, new drugs discovery to multiple and comprehensive treatments are highly necessary[10,11]. The establishment of appropriate animal models of pancreatic cancer could improve the development of therapies and drugs discovery against this disease. Heterotopic subcutaneous models are implanted between the dermis and underlying muscle, and are typically located on the flank, on the back or the footpad of the mouse. For over 30 years, the subcutaneous xenograft model has been the most widely used preclinical mouse model for cancer research. It is rapid, inexpensive, easily reproducible. Subcutaneous model could also provide visual confirmation that mice used in an experiment have tumors prior to therapy, and provide a means of assessing tumor response or growth over time[12].

However, a major disadvantage of subcutaneous xenograft models is that are curative in mouse subcutaneous xenograft models often do not have a significant effect on human disease. The primary cause of this failure may be due to the observation that the subcutaneous microenvironment is not relevant to that of the organ site of primary or metastatic disease. These observations suggest that heterotopic tumor models do not represent appropriate sites for human tumors[13,14]. Advantages of orthotopic tumor models include use of the relevant site for tumor–host interactions, the emergence of disease-relevant metastases, the ability to study site-specific dependence of therapy, organ-specific expression of genes and that clinical scenarios can be replicated, e.g. surgical removal of primary tumor, or adjuvant therapy of occult metastasis[13]. Therefore, orthotopic human pancreatic cancer xenografts models are used as the preference for cancer research due to the increased clinical relevance and similar to the ideal of the "anthropomorphic" (patient-like) pancreatic cancer model[15].

We have established a luciferase labeled orthotopic transplantation human pancreatic cancer model in athymic nude mice. Furthermore, we used the optical imaging system (IVIS200, Xenogen) to observe the growth of Capan-2^{Luc} cells in live animals and track the process of the animal construction. The current model overcomes the deficiencies of the traditional animal model, in which was difficult to observe *in vivo* dynamic process of tumor growth, invasion and metastasis.

5. Conclusions

For the first time, a human pancreatic cancer Capan-2^{Luc} cells orthotopic transplantation xenograft model was successfully established in nude mice in this study. This orthotopic xenograft pancreatic tumor model has several advantages,

such as high rate of tumorigenesis, stable tumor histopathology, etc. In this human pancreatic cancer orthotopic transplantation xenograft model, the efficacy of Gem was evaluated using optical imaging system to real-time observe the inhibitory effect of the drug, and the results were consistent with clinical treatment, which indicated that this *in vivo* model is a convincing evaluation model, which could accelerate preclinical investigation of new therapies and drugs against pancreatic cancer.

Acknowledgments

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