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Efficacy Comparison of Traditional Chinese Medicine LQ versus Gemcitabine in a Mouse Model of Pancreatic Cancer

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ABSTRACT

Pancreatic cancer is highly treatment-resistant and has one of the highest fatality rates of all cancers and is the fourth highest cancer killer worldwide. Novel, more effective strategies are needed to treat this disease. We report here on the use of patient-like orthotopic nude-mouse models of human metastatic pancreatic cancer to compare the traditional Chinese medicine (TCM) herbal mixture LQ to gemcitabine, which is first-line therapy for this disease, for anti-metastatic and anti-tumor activity as well as safety. The human pancreatic cancer cell line, MiaPaCa-2, labeled with red fluorescent protein (RFP), was used for the orthotopic model. LQ (gavage, 600 mg/kg/day) significantly inhibited pancreatic cancer tumor growth and metastasis, as measured by imaging, with no overt toxicity. Survival of tumor-bearing mice was also prolonged by LQ. The therapeutic efficacy of LQ is comparable with gemcitabine but with less toxicity, as indicated by a lack of body-weight loss with LQ, but not gemcitabine. The results indicate that TCM can have non-toxic efficacy against metastatic pancreatic cancer comparable to gemcitabine in a clinically-relevant orthotopic mouse model. J. Cell. Biochem. 114: 2131–2137, 2013.

KEY WORDS: TRADITIONAL CHINESE MEDICINE; HERBAL MIXTURE; GEMCITABINE; ORTHOTOPIC MODEL; NUDE MICE; RED FLUORESCENT PROTEIN; IMAGING; PANCREATIC CANCER; METASTASIS

Traditional Chinese Medicine (TCM) herbal mixtures have been used to treat cancer for thousands of years. Unlike Western medicine that generally uses pure compounds and targets a single molecule or pathway, TCM compositions comprise multiple components that interact with multiple molecular targets [Wong et al., 2001; Gai et al., 2008]. There is much anecdotal evidence of the efficacy of TCM in the form of herbal mixtures in cancer patients. However, systematic preclinical evaluation of TCM is necessary.

Our laboratory has developed clinically-relevant orthotopic models of cancer [Hoffman, 1999] that can be imaged with fluorescent proteins [Yang et al., 2000; Yang et al., 2001; Hoffman, 2005; Hoffman and Yang, 2006a,b,c]. We have now begun to use these models to evaluate various formulations of TCM to further understand their mechanisms and compare them with currently-used Western cancer therapies.

Recently, we determined the efficacy of *Celastrus orbiculatus Thunb.* (COT) on tumor growth, metastasis and antiogenesis of hepatocelluar carcinoma (HCC) [Wang et al., 2012] in an orthotopic nude mouse model using fluorescent protein imaging technology. Human HCC Hep-G2 cells expressing red fluorescent protein (RFP) were orthotopically implanted onto the liver of nude mice. COT demonstrated significant efficacy in controlling tumor volume and tumor weight.

The efficacy of the TCM tubeimu, extracted from the tuber of the plant *Bolbostemma paniculatum*, on the MDA-MB-231 human breast cancer cell line was evaluated by our laboratory [Hu et al., 2012]. The

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MDA-MB-231 cell line was engineered to express RFP in the cytoplasm and green fluorescent protein (GFP) linked to histone H2B in the nucleus, which allows real-time imaging of nuclear-cytoplasmic dynamics. Apoptosis was readily visualized in these cells by nuclear shape changes and fragmentation. Cells were treated with a dichloromethane extract of fresh tubeimu. Tubeimu induced apoptosis of MDA-MB-231 cells, as observed by fluorescence microscopy, as early as 24 h of treatment in vitro.

The fluorescent RFP-expressing orthotopic MiaPaCa-2 pancreatic cancer model is highly metastatic in nude mice. Rapid tumor growth and widespread metastases developed in untreated mice within 2 weeks, leading to a median survival of 21 days. Gemcitabine, which is first-line therapy for pancreatic cancer, is active in this model [Katz et al., 2003; Tran Cao et al., 2010].

We demonstrate in this report that the TCM herbal mixture LQ has anti-cancer and anti-metastatic efficacy similar to gemcitabine without severe toxicity in the clinically-relevant orthotopic mouse model of MiaPaCa-2 human pancreatic cancer.

MATERIALS AND METHODS

CELL CULTURE

MiaPaCa-2-RFP cells were maintained in RPMI 1640 medium (HyClone, South Logan, UT) with 10% fetal bovine serum (FBS; Gemini Bio-Products, Calabasas, CA).

RFP RETROVIAL TRANSDUCTION AND SELECTION OF MiaPaCa-2-RFP

MiaPaCa-2 cells were transfected to stably express RFP, as previously described [Katz et al., 2003]. Briefly, the pDsRed-2 vector (Clontech Laboratories, Inc., Palo Alto, CA) was used to engineer MiaPaCa-2 stably expressing RFP. This vector expresses RFP and the neomycin resistance gene on the same bicistronic message. The pDsRed-2 retrovirus was produced in PT67 packaging cells. RFP transduction was initiated by incubating 20% confluent MiaPaCa-2 cells with retroviral supernatants of the packaging cells and DMEM (HyClone, South Logan, UT) for 24 h. Fresh medium was replenished at this time, and cells were allowed to grow in the absence of retrovirus for 12 h. Please refer to Katz et al. [2003] for details of transfection and efficacy.

MICE

Athymic NCR nude mice (nu/nu) (AntiCancer Inc., San Diego, CA) at 6–8 weeks of age, were used in this study. Mice were maintained in a barrier facility on high efficiency particulate air (HEPA)-filtered racks. The animals were fed with autoclaved laboratory rodent diet. Animal experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals under National Institutes of Health assurance number A3873-01.

SUBCUTANEOUS TUMOR IMPLANTATION

MiaPaCa-2-RFP cells were harvested by trypsinization and washed two times with phosphate-buffered saline (PBS; HyClone, South Logan, UT). Cells (5×10^6) were injected subcutaneously in the left flank of each study mouse in a total volume of 100 μl within 30 min of harvesting. The subcutaneous tumors were also used as the source of tissue for orthotopic implantation into the pancreas.

SURGICAL ORTHOTOPIC IMPLANTATION (SOI) OF MiaPaCa-2-RFP HUMAN PANCREATIC CANCER

MiaPaCa-2-RFP subcutaneous tumors in the exponential growth phase, in nude mice, were resected. Necrotic tissues were cut away, and the remaining healthy tumor tissues were cut with scissors and minced into 1 mm^3 pieces in RPMI 1640 medium. Mice were then anesthetized, and their abdomens were sterilized with alcohol. An incision was created through the left upper abdominal pararectal line and peritoneum. The pancreas was carefully exposed, and two tumor pieces were transplanted onto the middle of the gland using a single 8-0 surgical suture (Davis-Geck, Inc., Manati, Puerto Rico). The pancreas was then returned into the peritoneal cavity, and the abdominal wall and the skin were closed in two layers using 6-0 surgical sutures. All procedures were performed with a 7× microscope or standard surgical loupes [Fu et al., 1992; Furukawa et al., 1993; Bouvet et al., 2002; Katz et al., 2003].

PREPARATION OF CRUDE EXTRACTS OF CHINESE HERBS

LQ is a formulated TCM comprised of a mixture of Chinese medicinal herbs, including *Sinapi Alba*, *Atracty Lodes Macrocephala*, *Coix Lacryma-jobi*, and *polyporus mushrooms*. The LQ mixture was supplied by Prof. C. Wu at Nanjing University of Traditional Chinese Medicine. Herbs are derived from plants that are cultivated, harvested, selected for quality, cut, prepared, dried and stored in accordance with traditional procedures. The reference standard components (marker chemical compounds or enzyme activities) of each herb are quantitatively analyzed for quality control.

The product sample was ground to a powder and passed through a 40 mesh. Herbs were extracted by refluxing with boiling water for 1 h, and the solution was filtered and the filtrate collected. The residue was extracted by refluxing with boiling water once more, and filtered. The entire filtrate was concentrated and lyophilized. Herbs were also extracted with 75% ethanol (1:20 g/ml) for 4 h with a water bath maintained at 80°C. When the solution was cooled, it was filtered, and the filtrate was collected. The residue was extracted with 75% ethanol once more and then filtered while the solution was cooled. The filtrate was mixed, and the solvent was evaporated under vacuum. The dried extract was lyophilized. All extracts were combined in an appropriate proportion. The combined extract (termed LQ) appears as a fine brown powder and was stored at room temperature. Six hundred milligrams of LQ drug powder was added to 10 ml PBS. The mixture was vortexed vigorously for 1 min and then extracted at 80°C for 30 min. The sample was immediately cooled to room temperature and was then centrifuged at 1,000 rpm for 5 min. The supernatant was collected for final concentration to 60 mg/ml. This extract was then diluted for dose-ranging experiments in the mouse models or stored at −20°C up to 3 months for future use.

IN VIVO DOSING OF LQ AND GEMCITABINE

Gemcitabine (GEM) was used as a positive control, which received twice a week intraperitoneal injections of GEM (Eli Lilly, Indianapolis,

IN), at 150 mg/kg/dose, twice a week for up to four times [Braakhuis et al., 1995; Katz et al., 2003]. Mice were treated with LQ at 600 mg/kg daily gavage 5 days after orthotopic transplantation. GEM was administered 5 days after orthotopic implantation by intraperitoneal injection.

ASSESSMENT OF EFFICACY

Efficacy of treatment was determined by standard measurements of tumor volume and tumor weight in the subcutaneous models. Tumor volume was calculated using the formula (long diameter × short diameter^2)/2. In the orthotopic models, mice were sacrificed and explored after they appeared pre-morbid. After euthanasia, each mouse underwent laparotomy and median sternotomy and primary and metastatic tumors were identified by RFP fluorescence imaging.

IMAGING

The Olympus OV100 Small Animal Imaging System (Olympus Corp., Tokyo, Japan), containing an MT-20 light source (Olympus Biosystems, Planegg, Germany) and DP70 CCD camera (Olympus), was used [Yamauchi et al., 2006].

EFFECT OF LQ AND GEMCITABINE ON TUMOR WEIGHT AND BODY WEIGHT

The primary tumor was excised and its weight was measured. Body weight and general appearance of each mouse were monitored and recorded every day as evidence of systemic toxicity.

MTS CELL PROLIFERATION ASSAY

MiaPaCa-2 cells were seeded into 96-well plates at a density of 5,000 cells per well. The number of viable cells was subsequently determined using the Cell Titer 96 Aqueous One Solution Cell Proliferation assay (Promega, Madison, WI) at 24, 48, and 72 time points. Briefly, at each time point, 20 μl CellTiter 96 solution was added to each well. The plates were then incubated for 3 h, after which the absorbance of each well was read at a wavelength of 490 nm. All assays were performed in triplicate, and each assay was repeated at least twice.

STATISTICAL ANALYSIS

Differences among treatment groups were assessed using analysis of variance (ANOVA) and the Student's *t*-test using Statistic software. Kaplan–Meier analysis with a log-rank test was used to determine survival and differences between control and treatment groups. Correlating TCM treated with metastasis was carried out using the Chi-square test. A *P*-value of ≤ 0.05 was defined as statistically significant.

RESULTS AND DISCUSSION

LQ INHIBITED CANCER CELL PROLIFERATION IN VITRO

The pancreatic cancer cell line MiaPaCa-2 was sensitive to LQ. In vitro experiments showed a decrease in proliferation with increasing concentration of drug (Fig. 1). The IC_{50} (50% growth inhibitory concentration) value of LQ for MiaPaCa-2 was ~1.8 mg/ml at 24 h, ~9 mg/ml at 48 h, and ~18 mg/ml at 72 h. The results indicated that longer exposure to the drug was less efficient. The maximized efficacy was at 24 h, suggesting that LQ may have a short life-line. Therefore, in the following in vivo experiments, was administered by gavage every day.

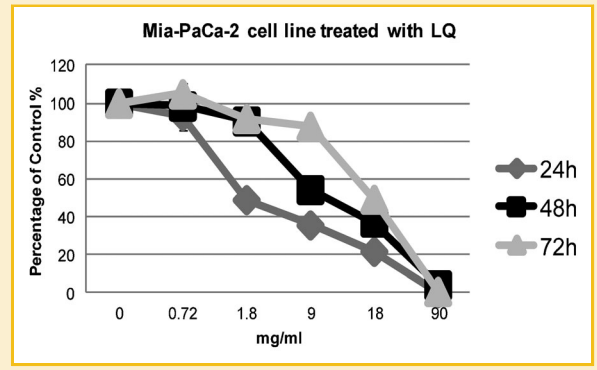


Fig. 1. Efficacy of LQ on proliferation of cancer cells in vitro. Cells were seeded in 96-well plates at a seeding density of 5,000 cells/well and left overnight. Cells were then exposed to drug for 24, 48, 72 h. The pancreatic cancer cell line MiaPaCa-2 was sensitive to LQ. The graphs show combined values from two independent experiments with each data point repeated in triplicate. All experiments showed a decrease in proliferation with increasing concentration of drug.

COMPARISON OF EFFICACY OF LQ AND GEMCITABINE ON THE SUBCUTANEOUS TUMOR MODEL

To initially determine the anti-tumor efficacy of LQ in vivo, nude mouse with subcutaneous pancreatic tumors were used. In the subcutaneous MiaPaCa-2-RFP pancreatic tumor model, LQ treatment significantly inhibited tumor growth observed by both tumor size and tumor weight compared to the PBS control group ($P = 0.029$ and $P = 0.006$, respectively; Fig. 2A,B). Gemcitabine (150 mg/kg i.p., twice a week up to 4 weeks), used as the positive control, also inhibited tumor growth and tumor weight significantly ($P < 0.001$ and $P < 0.001$, respectively; Fig. 2A,B). The body weight and general appearance of each mouse were monitored and recorded everyday as evidence of systemic toxicity. There was no significant difference in body weight and appearance between the LQ, PBS, and GEM groups (Fig. 2C).

COMPARISON OF EFFICACY OF LQ AND GEMCITABINE ON ORTHOTOPIC MODELS OF PANCREATIC CANCER

To further verify the anti-tumor and anti-metastatic activity of LQ, we tested the efficacy of LQ on patient-like orthotopic nude-mouse models. In the orthotopic model, tumor weight was also significantly inhibited by LQ and GEM compared to the PBS control group ($P = 0.036$ and $P = 0.003$, respectively; Fig. 3A). Only the GEM-treated mice had significant body weight loss compared with LQ group at the end of the experiment ($P = 0.009$; Fig. 3B).

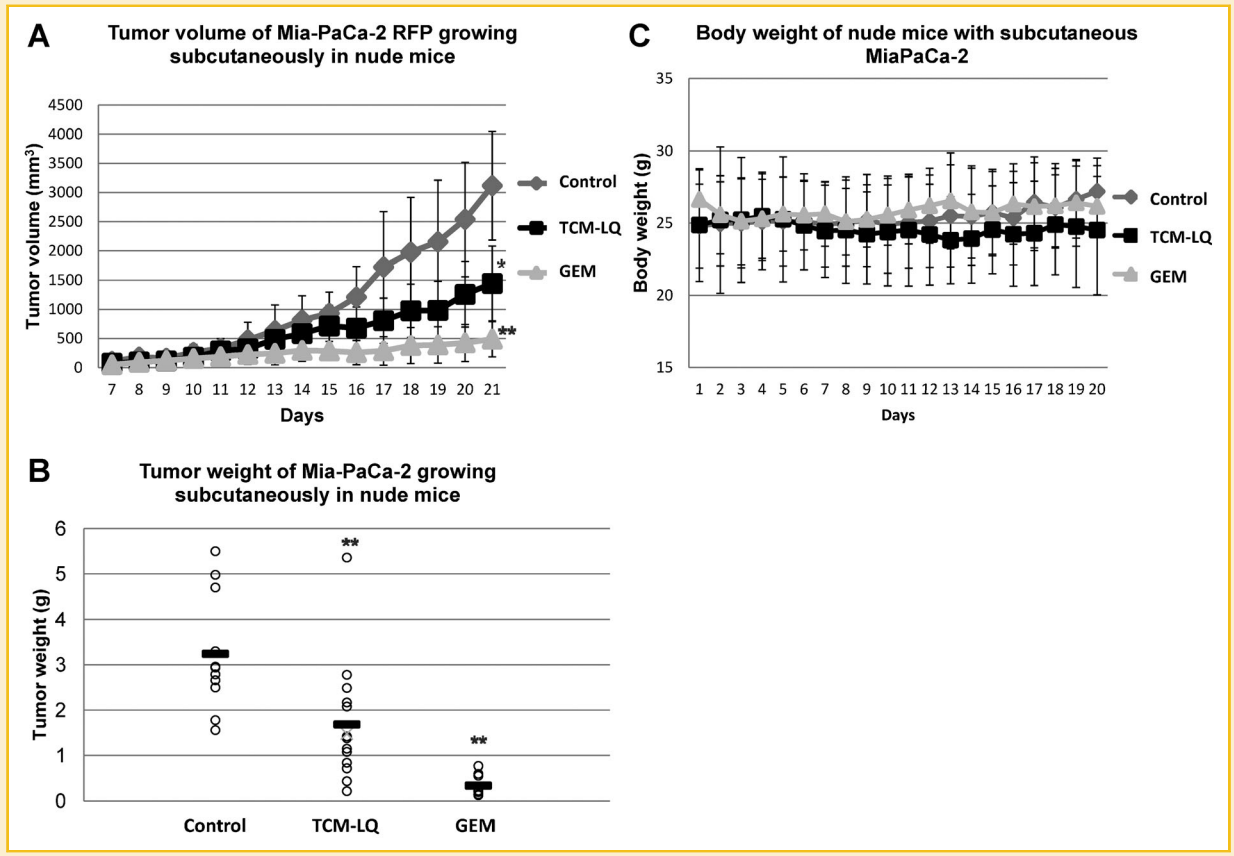


Fig. 2. Comparison of efficacy of LQ and gemcitabine on tumor volume, tumor weight and body weight in subcutaneous mouse models of MiaPaCa-2. After the subcutaneous tumors developed, the nude mice were given gemcitabine i.p injection of 150 mg/kg (twice a week) from day 5. PBS gavage was the negative control. The TCM group received LQ gavage from Day 5 at 600 mg/kg/day. Tumor volume (A), tumor weight (B), and body weight (C) were measured every day. Tumor weight was measured at the endpoint. Statistical significance between groups was determined with the Student's *t*-test. After LQ or gemcitabine treatment, pancreatic tumor volume, and tumor weight were significantly inhibited compared to the PBS control group. There was no significant difference on body weight between LQ, PBS and gemcitabine treated animals. $^{*}P < 0.05$, $^{**}P < 0.01$.

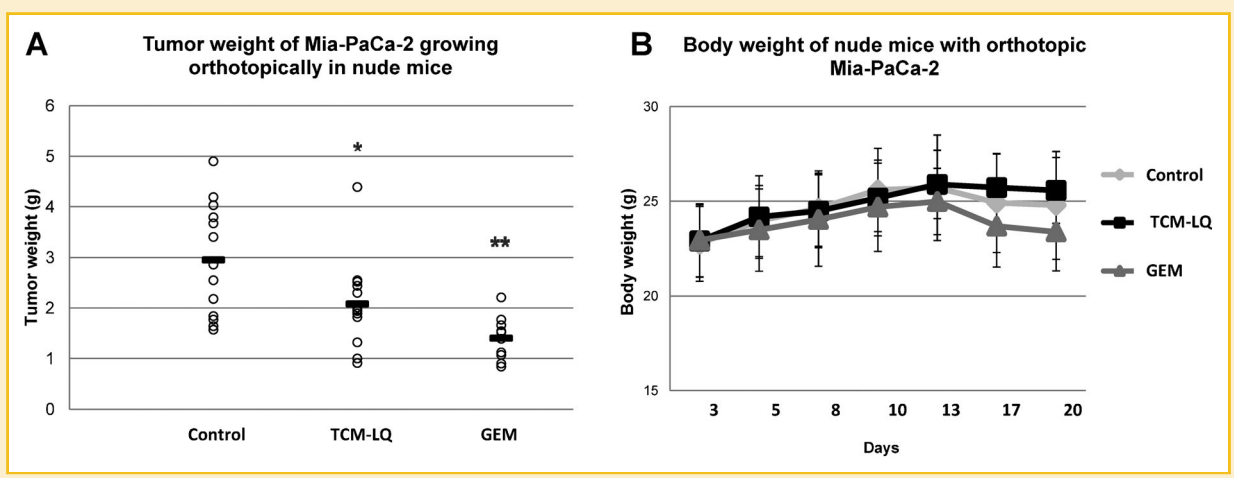


Fig. 3. Comparison of efficacy of LQ and gemcitabine on tumor volume, tumor weight and body weight in orthotopic tumor models of MiaPaCa-2. After the subcutaneously-growing MiaPaCa-2-RFP tumors grew to 1,000 mm^3 in nude mice, tumor fragments (1 mm in diameter) were harvested and implanted by surgical orthotopic implantation (SOI) into the pancreas of nude mice. The nude mice were given i.p. gemcitabine injection of 150 mg/kg (twice a week) from Day 7. PBS gavage was used as the negative control. The TCM group received LQ gavage from Day 7 at 600 mg/kg/day. A: Tumor weights were measured at the endpoint. B: Body weights were measured every day. Statistical significance between groups was determined with the Student's *t*-test. After LQ and GEM treatment, pancreatic tumor weight was significantly suppressed without body weight loss in the LQ group, but body weight loss occurred in the GEM group.

COMPARISON OF LQ AND GEMCITABINE ON SPONTANEOUS METASTASIS IN ORTHOTOPIC MODELS OF PANCREATIC CANCER

In the MiaPaCa-2-RFP orthotopic model, LQ and GEM both reduced the incidence of metastasis In the PBS control group, 12 out of 13 mice (23% incidence) had metastasis. In the GEM group, only one mouse had metastasis out of a total of 10 mice (10% incidence). In the LQ group, three out of 13 mice (23% incidence) had metastasis. Both the LQ and GEM groups showed significant reduction of metastasis incidence compared to the PBS control group (both $P < 0.001$; Fig. 4AA, Table I). In the PBS control group, the 12 mice with metastasis all had mesentery lymph node metastasis and four mice had lymph node metastasis. Four mice had metastasis in the liver and one in the spleen. In the LQ group, three mice had mesentery lymph node metastasis. In the GEM group, one mouse had mesentery lymph node metastasis (Fig. 4B).

COMPARISON OF LQ AND GEMCITABINE ON SURVIVAL OF ORTHOTOPIC MODELS OF PANCREATIC CANCER

The administration of LQ significantly increased the survival of mice with orthotopically-implanted MiaPaCa-2-RFP tumors compared to PBS control (median survival 43.2 vs. 37.5 days, respectively, $P = 0.034$). GEM also significantly increased survival of mice relative to control (median survival >52 vs. 37.5 days, respectively, $P < 0.0001$; Fig. 5A,B).

The results presented here, including from, orthotopic mouse models of pancreatic cancer, demonstrate that LQ can have significant anti-tumor and anti-metastatic efficacy. Not only is the TCM herbal mixture tested in this study efficacious, but it is non-toxic. The results indicate that TCM has important potential as an effective paradigm to treat metastatic pancreatic cancer, a highly treatment-resistant disease. Gemcitabine has only a 10~15% response rate in the clinic. Future

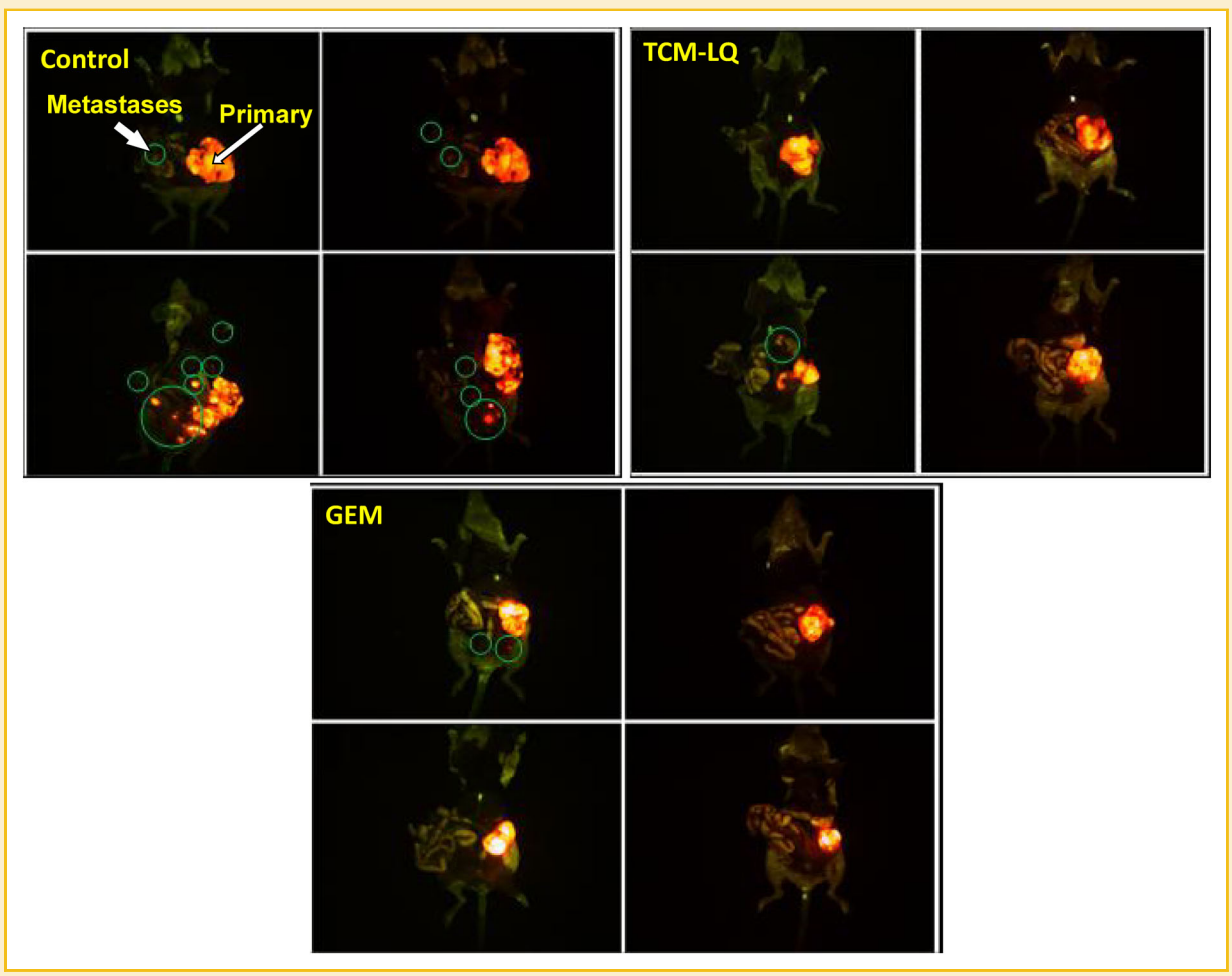
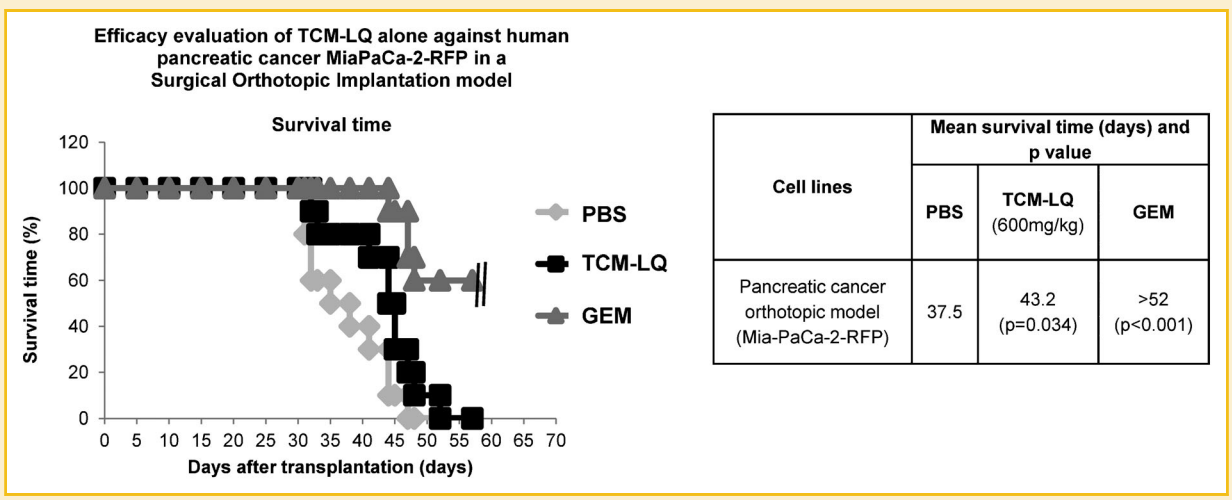


Fig. 4. Comparison of efficacy of LQ and gemcitabine on site-specific metastasis. Metastases in the MiaPaCa-2 pancreatic cancer orthotopic nude mouse models were determined at endpoint. The nude mice were given gemcitabine (150 mg/kg, twice a week i.p.) as the positive control or PBS gavage as the negative control. The TCM group received LQ gavage from Day 1 at 600 mg/kg/day. The incidence of metastasis in relevant organs was evaluated under fluorescence microscopy of fresh tissue. Statistical significance for anti-metastatic efficacy was determined with the Chi-square test. In the PBS control group, 12 out of 13 mice had metastasis. The gemcitabine (1/10) and LQ (3/13) group showed significant reduction of metastasis incidence compared to the PBS control group (both *P*-value <0.001). Green circles are metastasis.

TABLE I. Metastasis incidence

Group	Animal ID	Metastasis (+/−)	L.N.	Liver	Spleen	Group	Animal ID	Metastasis (+/−)	L.N.	Liver	Spleen	Group	Animal ID	Metastasis (+/−)	L.N.	Liver	Spleen
A Control	1	+	+			B TCM-LQ	1	+	+			C GEM	1	−			
	2	+	+				2	−					2	−			
	3	+	+				3	−					3	−			
	4	+	+				4	−					4	−			
	5	+	+				5	−					5	+	+		
	6	+	+	+			6	−					6	−			
	7	−					7	−					7	−			
	8	+	+				8	−					8	−			
	9	+	+				9	−					9	−			
	10	+	+				10	−					10	−			
	11	+	+	+			11	+	+								
	12	+	+	+			12	−									
	13	+	+	+	+		13	+	+								



Cell lines	Mean survival time (days) and p value		
	PBS	TCM-LQ (600mg/kg)	GEM
Pancreatic cancer orthotopic model (Mia-PaCa-2-RFP)	37.5	43.2 (p=0.034)	>52 (p<0.001)

Fig. 5. Survival curve of LQ alone or GEM alone against human pancreatic cancer Mia-PaCa-2 in a Surgical Orthotopic Implantation model.

experiments will evaluate combinations of LQ with gemcitabine and other drugs on pancreatic cancer mouse models.

ACKNOWLEDGMENT

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