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Antitumor Effect of Gemcitabine on Orthotopically Inoculated Human Gallbladder Cancer Cells
in Nude Mice

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Running head: Effect of Gemcitabine on Gallbladder Cancer

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Abstract

Background: Because the prognosis of gallbladder carcinoma is poor, investigating the efficacy of new chemotherapy agents is an important aspect of treatment for this tumor. Recently, several papers have reported clinical trials of gemcitabine treatment for advanced gallbladder cancers. However, no research has been reported studying the antitumor effect of gemcitabine on gallbladder carcinoma in an *in vivo* model system.

Methods: We examined the effect of gemcitabine against gallbladder cancers resulting from orthotopic inoculation of NOZ gallbladder tumor cells into nude mice. One week after transplantation, the mice were randomized into two groups: In Group A, mice were treated by intra-peritoneal injection of 0.9% sodium chloride for three weeks after inoculation (control). Group B mice were treated by intra-peritoneal injection of gemcitabine (125mg / kg) for three weeks. Mice were sacrificed one week after the end of treatment, and macroscopic and histological findings were evaluated. Expression levels of proliferating-cell nuclear antigen (PCNA) were examined to investigate cellular proliferation activity. Tunnel assays were performed to determine apoptotic status. Survival duration of mice after gemcitabine treatment was compared relative to untreated mice.

Results: At sacrifice, huge tumors of the gallbladder, with liver invasion and lymph node metastases, were seen in all Group A mice. However, there were no abdominal tumors in Group B mice, and microscopic gallbladder cancer could only be detected from histological findings. The mean percent of PCNA-positive tumor cells was significantly higher in tumors from mice in Group A (71.9%) compared to those of Group B (34.7%). The mean percent of Tunnel-positive tumor cells was significantly lower in mice from Group A (2.0%) than those from Group B (5.7%). Survival duration was prolonged significantly in gemcitabine-treated mice relative to untreated mice.

Conclusions: Gemcitabine treatment may inhibit tumor progression and prolong survival in gallbladder cancer by inhibiting cell proliferation and inducing apoptosis.

Synopsis

Using a newly-devised model of nude mice inoculated orthotopically with gallbladder cancer, we found that gemcitabine treatment of gallbladder cancer inhibits tumor progression and prolongs survival, perhaps by inhibiting cell proliferation and inducing apoptosis.

Gallbladder cancer is the most common malignancy in the biliary tract.¹ Because symptoms from gallbladder cancer manifest themselves late in the disease course, tumors often are detected only at an advanced stage, when patients may not qualify for surgical treatment. For this reason, successful chemotherapy is especially important for cases of advanced gallbladder cancer.

Recently, several reports have been published on gemcitabine treatment as an effective new regimen for treating gallbladder cancers.²⁻⁷ Gemcitabine is a novel nucleoside analogue that requires phosphorylation to become an active metabolite, gemcitabine triphosphate. Because gemcitabine triphosphate is a competitor of deoxycytidine triphosphate for incorporation into DNA, its presence inhibits DNA synthesis. Gemcitabine activity has been shown to function broadly in a variety of tumors, and is currently used to treat non-small-cell lung cancer and pancreatic cancer in Japan.^{8,9} Phase II studies in Western countries, as well as in Japan, of single-agent gemcitabine treatment of patients with biliary tract cancer proved efficacious to some degree, with manageable toxicity.^{2-7,10}

The murine orthotopic model is useful for determining the effectiveness and mechanism of administration of a chemotherapeutic agent.^{11,12} Previously, no animal model for examining the effects of chemotherapeutics on biliary tract cancers existed. However, our group has

recently established an orthotopic gallbladder-cancer model useful for drug tests.^{13, 14} In this paper, we examined the effect of gemcitabine *in vivo* using this gallbladder cancer model, and found that decreased cell proliferation and increased apoptosis correlated with prolonged survival.

MATERIALS AND METHODS

Cells and cell culture conditions

NOZ cells were isolated from ascites derived from a 48-year-old female patient with gallbladder cancer.¹⁵ Cells were cultured at 37°C in DMEM (Nissui, Tokyo, Japan) supplemented with 10% fetal calf serum (FCS, Sigma, St.Louis, Mo) under a humidified atmosphere containing 5% CO₂.

Animals and orthotopic implantation of tumor cells

Two-week-old athymic BALB/c male nude mice obtained from CLEA Japan Inc. (Tokyo, Japan) were used in this study. The method for inducing orthotopic gallbladder cancers in the mice was as described previously.^{13, 14} Mice were kept at the Animal Care and Use Facilities at Kobe University Graduate School of Medical Sciences under specific pathogen-free

conditions. All experiments were approved by the Animal Care and Ethics Committee of the Kobe University Graduate School of Medical Sciences.

Experimental conditions for gemcitabine therapy for established gallbladder carcinoma

Gemcitabine was supplied by Eli Lilly Japan (Tokyo, Japan). Seven days after implantation of NOZ cells into the gallbladder, five mice were killed, and the presence of cancer lesions was determined and confirmed histologically. Mice were randomized into four groups (in Groups A, B, and C, n = 10; n = 6 in Group D) as follows: (a) Group A, beginning on the seventh day after implantation of NOZ cells, twice weekly intra-peritoneal injections of 0.9% sodium chloride were continued for three weeks, and animals were then sacrificed on the 28th day after implantation, (b) Group B, beginning on the seventh day after implantation of NOZ cells, twice weekly intra-peritoneal injections of 125 mg / kg gemcitabine were continued for three weeks, and animals were then sacrificed on the 28th day after implantation, (c) Group C, beginning on the seventh day after implantation of NOZ cells, twice weekly intra-peritoneal injection of 125 mg / kg gemcitabine were continued for three weeks, and the survival duration was measured, (d) Group D, no treatment after implantation of NOZ cells and survival duration was measured.

Histological studies

When the mice were sacrificed, tumor status, the presence or absence of liver, lung and lymph node metastases, and the presence or absence of peritoneal dissemination were recorded.

Histopathology of H & E staining confirmed the identity of the disease.

Immunohistochemical determination of PCNA and TUNEL (apoptotic cells)

For immunohistochemistry procedures, the tumors were fixed in phosphate-buffered formalin, embedded in paraffin, cut in 4- μ m thickness, and stained. Immunohistochemical analysis of proliferating cell nuclear antigen (PCNA) was performed using a labeled streptavidin-biotin technique described previously.¹⁶ Anti-PCNA monoclonal antibody PC 10 (DAKO, Carpinteria, CA), which reacts exclusively with nuclei, was used at a dilution of 1:200. The number of PCNA-positive cells was counted in five high-power fields (0.135 mm² fields at X 200 magnification) selected at random, and the PCNA labeling index for each field was calculated as the percent of cells (relative to the total) positive for staining. Apoptosis in tumor cells was detected using the terminal deoxynucleotidyl-transferase-mediated d-UTP-biotin nick end-labeling (TUNEL) assay, as described previously.¹⁷ In the same manner as PCNA, five

fields (0.135 mm² fields at X 200 magnification) were selected at random, and the apoptotic index of each field was calculated as the percent of TUNEL-positive cells.

Statistical analysis

Statistical analyses were performed using StatView (Abacus Concepts Inc, Berkeley, Calif). Survival curves were computed using the Kaplan-Meier method and compared using the log-rank test. PCNA-labeling indices and apoptotic indices were assessed using unpaired Student's t-test. The incidence of metastasis was compared using Fisher's exact test. P<0.05 was considered statistically significant.

RESULTS

Effectiveness of gemcitabine in suppressing the invasion and metastasis of NOZ gallbladder cancer cells inoculated orthotopically into nude mice

Table 1 shows the incidence of gallbladder tumors and metastases with and without gemcitabine administration in mice on the 28th day after orthotopic inoculation of NOZ cells. At the time of sacrifice, macroscopically visible gallbladder tumors were present with direct liver invasion in all Group A mice. Almost all mice in Group A had metastases in multiple

organs. In contrast, no tumors were visible in Group B mice ($p<0.01$), and although a residue of cancer cells was observed histologically in 70% of Group B mice, no metastases were detected (Fig. 1).

Effectiveness of Gemcitabine for survival duration

In Groups C and D, no apparent side effects (including loss of body weight or abnormal behavior) were observed throughout the treatment. The survival duration of mice treated with gemcitabine (Group C) ranged between 47 and 71 days (mean 59.6 days), significantly longer than the survival duration of Group D mice (range 32 – 38 days, mean 33.6 days) (Fig. 2).

PCNA expression and labeling index, and TUNEL assay and apoptotic index

Examples of staining results for PCNA immunohistochemistry and TUNEL assays in mice of Group A and B are shown in Fig. 3. The PCNA labeling indices of tumors from mice treated with gemcitabine (Group B) were significantly lower than those from mice treated with sodium chloride (Group A) ($p<0.01$) (Fig. 4A). The apoptotic indices of tumors from mice treated with gemcitabine (Group B) were significantly higher than those from mice treated with sodium chloride (Group A) ($p<0.01$) (Fig. 4B).

DISCUSSION

Our studies confirm that administration of gemcitabine inhibits the growth and metastasis of human gallbladder cancers established in the gallbladders of athymic nude mice. Using this model, we have shown that inhibiting cell proliferation and inducing apoptosis can inhibit tumor progression and prolong survival in gallbladder cancers.

Although 5-FU has been widely used in chemotherapy for gallbladder cancer, there is little evidence of its effectiveness. In previous studies, we have shown that gallbladder carcinomas acquire resistance to 5-FU *in vivo*, as well as *in vitro*, via increased expression of thymidylate synthase or dihydropyrimidine dehydrogenase.^{18, 19} Similar mechanisms are likely to contribute to the problematic nature of 5-FU-based chemotherapy for advanced gallbladder cancers.

As an alternative to 5-FU, several clinical studies which demonstrated that gemcitabine is efficacious as a single agent, or in combination with other drugs, against biliary tract cancers, including gallbladder cancer have been reported.^{2-7, 10} Despite these promising results, no experimental study of gemcitabine's efficacy for biliary tract carcinomas has been reported.

Therefore, we conducted *in vivo* gemcitabine experiments examining its effects on NOZ human gallbladder cancer cells implanted into the gallbladders of nude mice.

Several aspects of the orthotopic inoculation model make it useful for testing the effects of gemcitabine on gallbladder cancer. It has been proposed that, relative to ectopic (i.e. subcutaneous) inoculation, orthotopic inoculation is likely to provide information not only on the tumorigenicity, invasiveness and metastatic potential of tumor cells, but also on their responsiveness to drugs. Furthermore, tumors generated by orthotopic inoculation display characteristics that are similar, and unique, to human gallbladder cancers. This animal model demonstrates high incidences of lymph-node metastasis, liver invasion and peritoneal metastasis, which is similar to the natural course of human gallbladder cancers.

Gemcitabine is a nucleoside analogue that inhibits the synthesis of DNA by interfering with cytosine triphosphate production and by inhibiting the activity of ribonucleotide reductase. Gemcitabine is an effective drug approved by the FDA for the treatment of advanced pancreatic cancer.²⁰ In the present study, the therapeutic success of gemcitabine (estimated by inhibition of tumor growth and prolonged survival) correlated with decreased cell proliferation and increased apoptosis in tumor cells. These results were supported by both PCNA and TUNEL histological staining data. Our results correspond well with those of Okino *et al.*, in which pancreatic tumor

cells inoculated subcutaneously in nude mice showed decreased cell proliferation and increased apoptosis after gemcitabine therapy.²¹ Our results are compatible with the pharmacological mechanism of gemcitabine incorporating into DNA and causing a pause in DNA synthesis that subsequently induces apoptosis.²²

Studies of the benefit of gemcitabine on the survival of gallbladder cancer patients have yet to be completed. For pancreatic cancers, gemcitabine has demonstrated a survival advantage not only after resection, but also relative to treatment with 5-FU.^{20, 23, 24} The data presented here show a significant increase in survival duration resulting from gemcitabine treatment of gallbladder-cancer-model mice. Further studies involving human clinical trials will be necessary to confirm the survival benefit of gemcitabine for gallbladder cancer patients.

In conclusion, our findings indicate that gemcitabine's mechanism for inhibition of gallbladder tumor progression and prolonging the survival of orthotopically-inoculated mice may be inhibition of cell proliferation and induction of apoptosis. The results of this study, which were derived from a new mouse model of gallbladder cancer, support further clinical testing of gemcitabine for advanced gallbladder cancer in humans.

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Figure legends

FIG 1. Macroscopic and microscopic findings from the time of sacrifice of Group A and B nude mice that were inoculated orthotopically with NOZ cells. Gallbladder tumors (black arrow with solid line) were clearly evident in Group-A mice (a), but no tumors were seen in the gallbladders (black arrow with solid line) of Group-B mice (b). Histological evidence of cancer cells (black arrow with dotted line) in the gallbladders of Group-A and -B mice.

FIG 2. Kaplan-Meier survival curves derived from Group C (thin line with Δ) and D (thick line with O). Mice treated with gemcitabine showed a significantly better long-term survival than those with no treatment.

FIG 3. Immunohistochemical staining for PCNA and Tunnel assays in the gallbladder tumors of Group-A and -B mice. Gallbladder tumors after gemcitabine treatment (Group B) show fewer PCNA-positive cells and more apoptotic cells than untreated tumors.

FIG 4. Indices of PCNA labeling (A) and apoptosis (B) in gallbladder tumor cells from mice in Group A or B. Gallbladder tumor cells after gemcitabine treatment (Group B) show a significant decrease in percent PCNA-positive cells as well as a significant increase in percent apoptotic cells. The mean index of PCNA labeling (A) for Group A (71.9 ± 3.5) and Group B (34.7 ± 10.3), and mean index of apoptosis (B) for Group A (2.0 ± 1.2) and Group B (5.7 ± 2.4) are marked by columns with $\pm$ SD error bars.

TABLE 1. Incidence of gallbladder tumor and metastases with and without administration of gemcitabine

	Group A	Group B
Macroscopic gallbladder tumor	10/10	0/10
Microscopic gallbladder tumor	10/10	7/10
Direct invasion to the liver	10/10	0/10
Metastases to the lymph nodes	10/10	0/10
Metastases to the lung	8/10	0/10
Peritoneal dissemination	10/10	0/10

Fig. 1

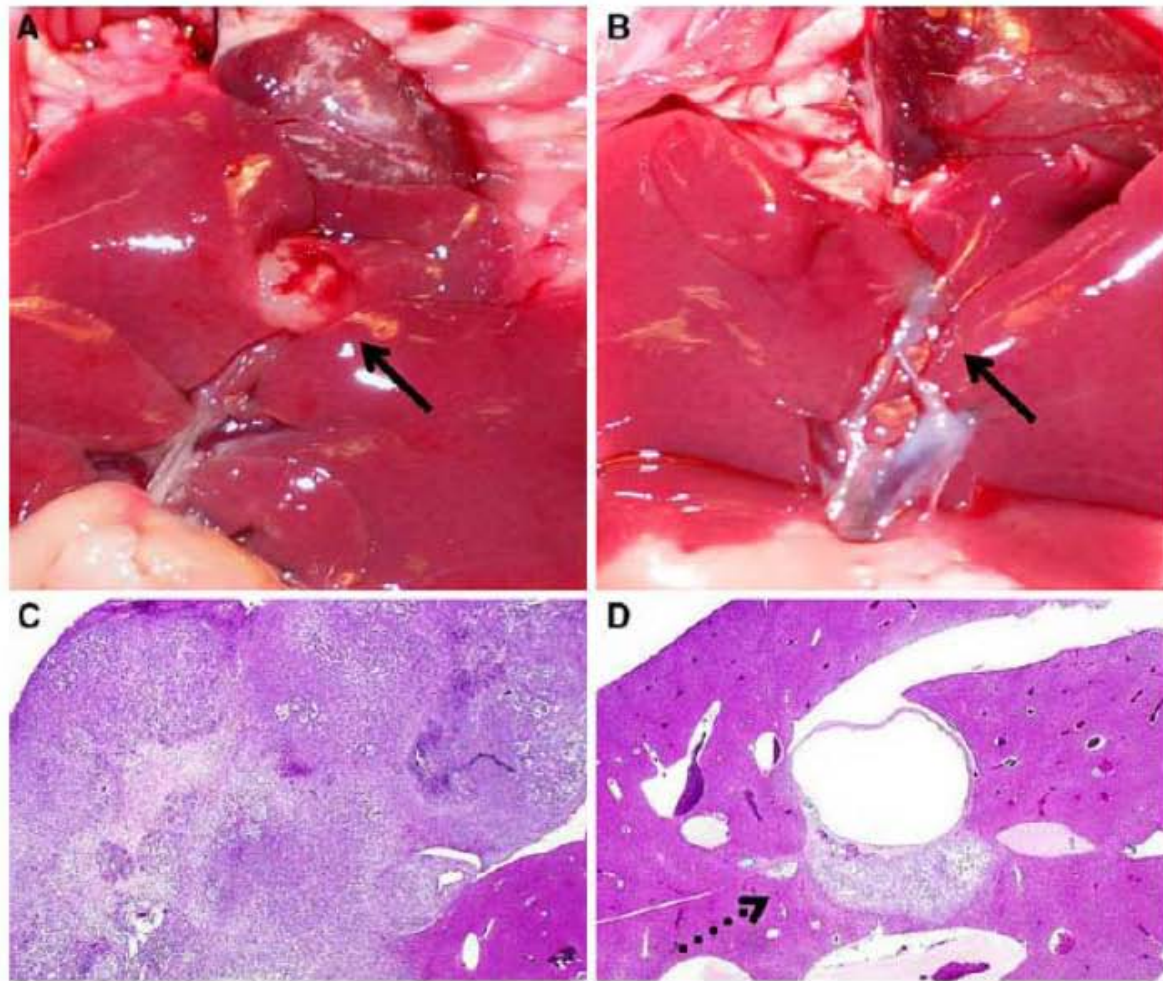


Fig. 2

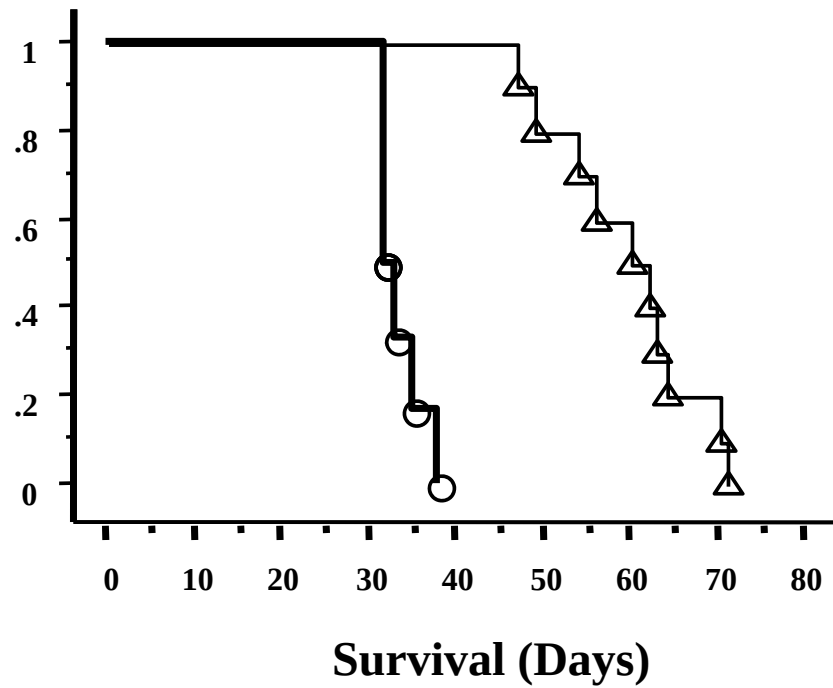


Fig. 3

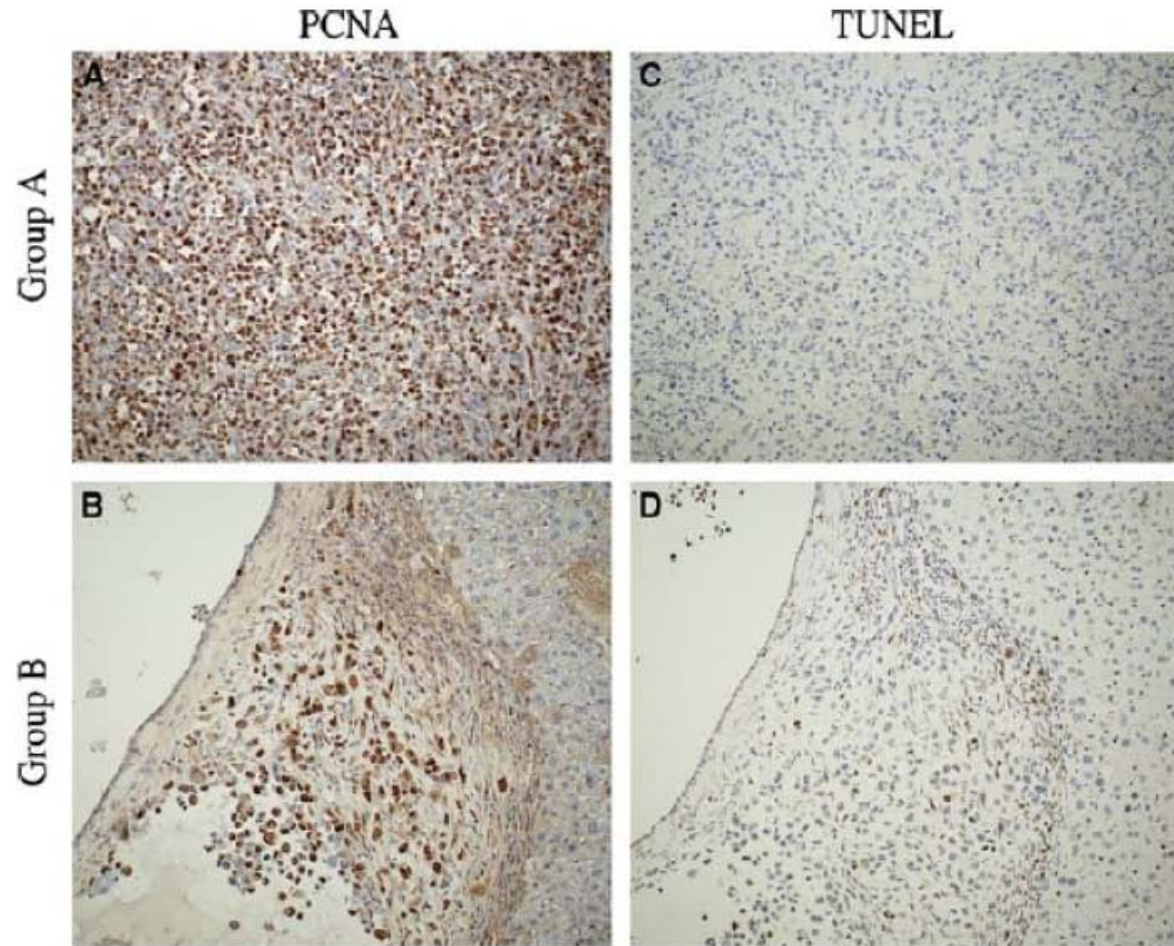


Fig. 4

