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Development and Biological Analysis of a Novel Orthotopic Peritoneal Dissemination Mouse Model Generated Using a Pancreatic Ductal Adenocarcinoma Cell Line

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Objectives: Peritoneal dissemination (PD) is an important cause of morbidity and mortality among patients with pancreatic ductal adenocarcinoma (PDAC). We sought to develop and characterized a novel PD mouse model by using a previously established PDAC cell line TCC-Pan2.

Methods: TCC-Pan2 cell line was characterized for growth rate, tumor markers, histology, and somatic mutations. TCC-Pan2 cells were implanted orthotopically to produce PD. TCC-Pan2 cells from these metastatic foci were expanded *in vitro* and then implanted orthotopically in mice. This PD model was used for comparing the antitumor effect of paclitaxel and NK105.

Results: Orthotopically implanted TCC-Pan2 cells caused tumor formation and PD with high frequency in mice. A potent metastatic subline—Pan2M—was obtained. NK105 exerted a stronger antitumor effect than paclitaxel against Pan2M cells harboring a luciferase gene (Pan2MmLuc). Notably, the survival rate on day 80 in the Pan2MmLuc mouse model was 100% for the NK105 group and 0% for the paclitaxel group.

Conclusion: TCC-Pan2 cell line and Pan2MmLuc PD model can serve as useful tools for monitoring the responses to antineoplastic agents and for studying PDAC biology.

Key Words: cell line, NK105, mouse model, orthotopic implantation, pancreatic ductal adenocarcinoma, peritoneal dissemination

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Pancreatic ductal adenocarcinoma (PDAC) remains one of the deadliest malignancies worldwide, with an overall 5-year survival rate of <5%.[1–3] In Japan, mortality caused by PDAC is ranked fifth and fourth in men and women, respectively, and the PDAC prevalence rate is continuing to increase. The median survival time of patients with distant organ metastasis is extremely poor, <12 months,[4] and this high mortality rate is due to an exceptionally high aggressiveness, chemoresistance, and early occurrence of metastasis to distant organs such as the liver, lung, and peritoneum.[5] Moreover, peritoneal dissemination (PD) and liver metastasis are commonly observed at an early stage after extensive curative pancreatectomy, and the almost inevitable metastasis results in the poor prognosis of PDAC.[6] In contrast to the vast body of research that has addressed hematogenous metastasis, few studies have elucidated the biology of PD in PDAC. The development of PD involves a multistep process that includes cell shedding and transport, survival in the microenvironment of the peritoneal cavity, adhesion to the mesothelial layer, invasion of and proliferation into the submesothelial stroma, and potential access to systemic circulation.[7,8] In addition to this passive dissemination, evidence has accumulated pointing to the existence of the following 2 pathways of the PD process of gastroenteropancreatic and ovarian cancers: a lymphatic pathway via milky spots in the omentum and transport via the vascular network.[9–12] However, the mechanisms underlying this propensity for metastasis remain poorly understood.

Recent progress in chemotherapy has led to improved prognosis in the case of patients with unresectable PDAC. Patients with PD are treated with palliative systemic chemotherapy and intraperitoneal (IP) chemotherapy.[13–15] However, the impact of the treatment on primary pancreatic cancer is limited, and the prognosis of PDAC patients with PD remains extremely poor.[4,16–18] Thus, new strategies are necessary for PD management. Consequently, the development of relevant animal models of PD is crucial for the elucidation of the underlying mechanisms and the establishment of appropriate therapeutic approaches.

Orthotopic xenograft models of established PDAC cell lines, which show human-like tumor progression, facilitate the identification of the disease process and the development of novel therapeutic approaches.[19–21] Our previous report of orthotopic implantation (OI) of scirrhous-type gastric carcinoma cells in mice showed that the subsequent tumor growth results in PD and metastases to various organs, much as in human cases.[22] We recently described a PD mouse model with cachexia syndrome developed using OI of human gastric carcinoma cells; this model effectively mimicked the tumor/host interaction and pathogenesis.[23,24] Several reports have described PD from human pancreatic tumor xenografts and/or cultured cells by performing OI in immunodeficient mice.[25–29] Moreover, we previously established and characterized 10 cell lines from patient-derived PDAC xenografts.[30] However, these cell lines exhibited only weak potential for PD when implanted orthotopically into nude mice (Yanagihara et al, unpublished data). The lack of adequate experimental models for human PDAC prompted us to seek other cell lines that could be used to

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study PDAC pathogenesis and to develop an orthotopic PD model of this tumor.

Paclitaxel exhibits strong antitumor activity in patients presenting PD in the case of several types of cancer, particularly advanced gastric cancer,[31,32] PDAC,[16] and ovarian cancer.[33] NK105 is a paclitaxel-incorporating core-shell–type micellar nanoparticle formulation comprising the novel amphiphilic block copolymer (NK105 polymer self-associated with polymeric micellar nanoparticles) in aqueous media.[34] Because of its high hydrophobicity, paclitaxel is physically entrapped within NK105 polymers and forms the inner core of the NK105 formulation. In previous preclinical studies, NK105 showed markedly stronger antitumor effects than did paclitaxel, and the associated peripheral neurotoxicity was reduced.[34,35] The increased antitumor effects of NK105 likely reflected longer retention times (relative to paclitaxel) in the plasma and enhanced permeability and retention effects in tumor tissues.[36,37] Therefore, the antitumor effects of paclitaxel and NK105 were compared in this study by using an orthotopic PD model that we established.

Here, we describe the characterization of a human PDAC cell line, TCC-Pan2, and report the development of an orthotopic PD mouse model (Pan2MmLuc). Moreover, we present the results of our investigation of the antitumor/antimetastatic effect of NK105 in this model.

MATERIALS AND METHODS

Cell Line and Culture

The cell line TCC-Pan2 was previously established from the ascitic fluid, obtained using peritoneocentesis, of a 47-year-old female Japanese patient with PDAC (moderately/poorly differentiated adenocarcinoma) in our laboratory. Cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (Gibco; Invitrogen, Carlsbad, Calif), 100 IU/mL penicillin G sodium, and 100 μg/mL streptomycin sulfate in a 5% CO_2/95% air atmosphere at 37°C.

For cell injection, the cultured cells were trypsinized for dissociation, and then the cell number was determined using a Z1 Coulter particle counter (Beckman Coulter, Fullerton, Calif) and the cells were resuspended in RPMI 1640 medium. All the cell lines used were routinely tested for *Mycoplasma* at the Central Institute for Experimental Animals (Tokyo, Japan). No contamination was detected through the entire course of the experiments.

Animal Experimentation

Four-week-old female nude mice (BALB/c nu/nu) were purchased from CLEA Japan (Tokyo, Japan) and maintained under specific pathogen-free conditions. The ambient light was controlled to maintain a 12:12-hour light/dark cycle. The mice were reared on sterile food and water in filter-protected cages, and the animals were used in experiments when they were 6 to 8 weeks old. The animal experiment protocols were approved by the Committee for Ethics in Animal Experimentation, and the experiments were conducted in accordance with the Guideline for Animal Experiments of the National Cancer Center and Yasuda Women's University.

For OI, anesthesia was induced using 5% isoflurane in room air (flow, 300 mL/min), and then the mice were maintained under 2% isoflurane anesthesia by using a face mask throughout the surgery. The abdomen was sterilized with 70% ethanol, and a small incision was made in the median abdominal wall under anesthesia. The pancreas was exposed, and 1×10^6 tumor cells in 50 μL of RPMI 1640 medium were directly injected into the pancreas by using a 30-gauge needle (Nipro Co, Tokyo, Japan). The needle was carefully withdrawn to avoid regurgitation along the needle track, and the injection orifice was pressure sealed with a dry cotton tip. The incised abdominal wall was closed using an AUTOCLIP Applier (Becton Dickinson, Sparks, Md). The mice were killed after surgery at 150 days after the tumor cell inoculation or when moribund, and the abdominal tissues were inspected macroscopically for metastasis in various organs and thereafter processed for histological examination, as described.[22]

Short Tandem Repeat Genotyping

Short tandem repeat (STR) genotyping was performed using genomic DNA extracted from all cell lines; the analysis was performed by Promega (Tokyo, Japan). This experiment was conducted using a PowerPlex 16 System (Promega, Madison, Wis) according to the manufacturer's instructions.

Next-Generation Sequencing

Genomic DNA from the patient-derived ascitic tumor and 2 cell lines was prepared using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Next-generation sequencing (NGS) analyses were performed using the NCC oncopanel (v4) for 114 cancer-related genes, which are listed in Supplementary Table 1 http://links.lww.com/MPA/A705. Targeted sequencing and data analysis have been described previously.[38]

Isolation of a Highly Peritoneally Metastatic Cell Line and In Vivo Photon Counting Analysis

A highly peritoneally metastatic cell line, Pan2M, was isolated from TCC-Pan2 by performing OI in nude mice. Briefly, we implanted TCC-Pan2 cells (1×10^6/50 μL) into the pancreas of a mouse, and then repeated 8 cycles of sequential harvesting of ascitic tumor cells and then OI of these cells into mice to establish the highly metastatic Pan2M cell line.

For therapeutic studies, we introduced a luciferase gene into the Pan2M cells (Pan2MmLuc).[24] Pan2MmLuc cells stably expressing firefly luciferase were implanted subcutaneously (SC) in mice, and this was followed by measurement of the tumor volume by using the existing method and an in vivo photon counting analysis. Comparison of tumor growth patterns revealed a positive correlation between Pan2M and Pan2MmLuc tumors.[23]

The firefly luciferase-expressing cells exhibited almost the same sensitivity as the parental cells to each of the tested drugs. The OI of 1×10^6 Pan2MmLuc cells in mice (day 0) was performed as described previously.[24] To assess the tumorigenicity of Pan2MmLuc cells, bioluminescence signals from Pan2MmLuc cells implanted in mice were monitored after IP injection of D-luciferin (150 mg/kg) by using the IVIS system Lumina series (Caliper Life Sciences, Hopkinton, Mass) as described previously.[23]

Evaluation of Antitumor Activity of Paclitaxel and NK105

The antitumor activity of paclitaxel and NK105 was evaluated using nude mice. We inoculated 1×10^6 Pan2MmLuc cells into the pancreas of mice, and 15 days later, we randomly allocated the mice to paclitaxel, NK105, and (control) vehicle administration groups, each including 7 animals.

Paclitaxel was purchased from Mercian Corp (Tokyo, Japan). NK105 was supplied by Nippon Kayaku Co Ltd (Tokyo, Japan) in 20-mL glass vials containing a dose equivalent to 30 mg of paclitaxel. When reconstituted in 10 mL of 5% glucose solution and diluted with a total volume of 250 mL of 5% glucose, the reconstituted solution was stable for 24 hours at room temperature. Paclitaxel and NK105 were inoculated into tumor-bearing mice through intravenous (IV) injection on days 20, 27, and 34 after implantation. The administration doses of paclitaxel and NK105 were determined based on referring to previous reports.[34] All mice were killed at day 150. The antitumor

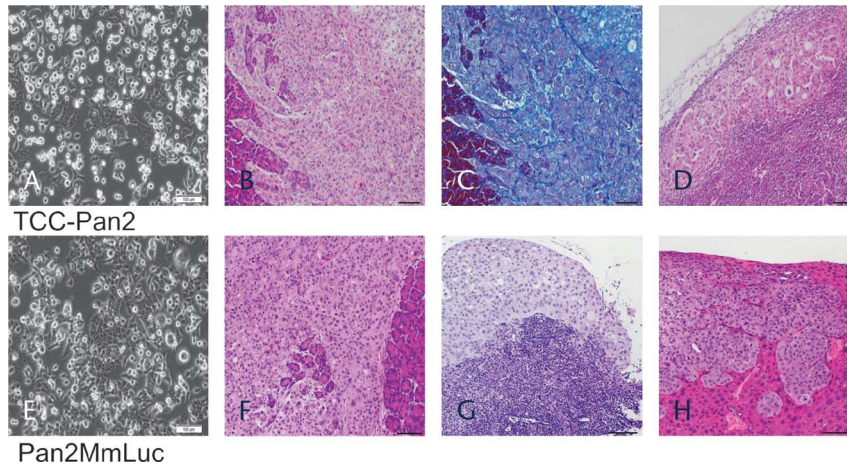

FIGURE 1. Phase-contrast micrographs of TCC-Pan2 PDAC cell line (A) and the highly metastatic subline Pan2MmLuc (E) at 20th passage. Microscopic appearance of the tumors developing in nude mice after OI of TCC-Pan2 cells (upper panels) and Pan2MmLuc cells (lower panels). B, Pancreas. C, Azan staining of the pancreas, same area as in panel B. D, lymph node metastasis. F, Pancreas. G, Lymph node metastasis. H, Liver metastasis. Hematoxylin and eosin staining, original magnification 200×. Scale bars, 100 μm.

activity of paclitaxel and NK105 was evaluated through in vivo photon counting of killed metastatic tumors at various time points after injection. We also monitored the changes in the body weight of the mice used in this study.

Statistical Analysis

All data were analyzed using unpaired *t* tests and are expressed as mean (standard deviation [SD]) $P < 0.05$ was considered statistically significant.

RESULTS

Biological Characterization of a Pancreatic Cancer Cell Line

In this study, we characterized a human PDAC cell line (TCC-Pan2) from the metastatic ascitic tumor of a Japanese patient. TCC-Pan2 cells were morphologically spherical and floated freely, and adherent cells exhibited the typical morphologic features of epithelial cells (Fig. 1A). The cell line displayed anchorage-independent growth (86% efficiency), and the doubling time was approximately 26 hours in RPMI 1640 medium supplemented with 10% fetal bovine serum (Table 1). TCC-Pan2 cells secreted duke pancreatic monoclonal antigen type 2 (DUPAN-2; mean [SD], 93.2 [41.9] U/mL) and s-pancreas-1 antigen (SPan-1; 52.0 [25.2] U/mL), but we detected no carcinoembryonic antigen and carbohydrate antigen production. When injected SC, the cell line was tumorigenic in nude mice, and the xenograft tumor was graded as "poorly differentiated adenocarcinoma" and, in histological typing, resembled the original patient tumor. Xenografts showed spontaneous metastasis to regional lymph nodes in 1 of 7 mice. These biological properties are summarized in Table 1.

In STR genotyping, the DNA extracted from TCC-Pan2 cell line did not correspond to that of any cells in the database of the Japanese Collection of Research Bioresources (JCRB; database of 2279 cells registered in the American Type Culture Collection, the Deutsche Sammlung von Mikroorganismen und Zellkulturen, and the JCRB).

Molecular Biological Characterization of Pancreatic Cancer Cell Lines

We performed NGS analyses on the ascitic tumor of the patient and on TCC-Pan2 and Pan2MmLuc cell lines by using the

TABLE 1. Biological Characteristics of TCC-Pan2 Pancreatic Cancer Cell Line and Its Metastatic Pan2MmLuc Subline

	Origin		Growth*		Tumor Marker†				Histological Typing‡	
Cell Line	Age, y/Sex	Tumor Source	Pattern/ DT, h	In CDM/ Agar, %	CEA, ng/mL	CA 19-9, U/mL	DUPAN-2, U/mL§	SPan-1, U/mL§	Original	Xenograft, SC
TCC-Pan2	47/F	Ascites	M/26	(+)/86	<0.5	<1.0	93.2 (41.9)	52.0 (25.2)	Poor	Poor
Pan2MmLuc	47/F	Ascites	M/24	(+)/88	<0.5	<1.0	86.5 (25.1)	76.1 (33.6)	Poor	Poor

*M, mixed type (floating type and adherent type); CDM, composed of Dulbecco modified Eagle medium/Ham F-12 (1:1) medium supplemented with 0.05% bovine serum albumin; DT, doubling time; (+), positive.

†Secretion of CEA, CA 19-9, DUPAN-2, and SPan-1 was tested using radioimmunoassay and immunoradioassay at SRL Laboratories (Tokyo, Japan).

‡Tumorigenicity of the cell lines was tested through SC injection of nude mice with 1×10^6 cultured cells suspended in 0.1 mL of RPMI 1640 medium. The pancreatic cancer was histologically typed, in accordance with the "General Rules for the Study of Pancreatic Cancer (2013)," as poor (poorly differentiated adenocarcinoma).

§Data presented as mean (SD).

CA 19-9 indicates carbohydrate antigen; CEA, carcinoembryonic antigen.

TABLE 2. Genetic Characteristics of Ascitic Tumor Cells From the Patient and of TCC-Pan2 and Metastatic Pan2MmLuc Cell Lines

Tumor Cells/Cell Line	Genetic Alteration		
	Mutation	Amplification	Homozygous Deletion
Ascitic tumor	*KRAS* (G12R), *TP53* (splicing)	ND	*CDKN2A*
TCC-Pan2	*KRAS* (G12R), *TP53* (splicing)	*KRAS* (16-fold)	*CDKN2A*
Pan2MmLuc	*KRAS* (G12R), *TP53* (splicing)	*KRAS* (16-fold)	*CDKN2A*, *SMAD4*

Genomic DNA was prepared from the ascitic tumor and cell lines by using a QIAamp DNA Mini Kit according to the manufacturer's protocol. We performed NGS analyses by using the NCC oncopanel (v4) for 114 cancer-related genes (which are all listed in Supplementary Digital Content 1 http://links.lww.com/MPA/A705). Targeted sequencing and data analysis were previously described.[38]

ND indicates not detected.

NCC oncopanel (v4) for 114 cancer-related genes (Supplementary Table 1 http://links.lww.com/MPA/A705).[38] *KRAS* mutation was identified at codon 12 (Gly to Arg, G12R), and *TP53* splicing mutation was observed in both alleles. Gene amplification of *KRAS* (16-fold) was detected in the cell lines but not in the ascitic tumor. Homozygous deletion of *CDKN2A* was present in all specimens, whereas that of *SMAD4* was only detected in Pan2MmLuc cells (Table 2).

Pathological Characterization of the OI Model in Nude Mice Generated Using the Established TCC-Pan2 Cancer Cell Line

We sought to establish a physiologically relevant OI model presenting features resembling the clinical features of PDAC. When injected intrapancreatically, cultured TCC-Pan2 cells survived and showed high tumorigenicity (100%). The results are summarized in Table 3 and illustrated in Figure 1. The histology of the engrafted tumor of the TCC-Pan2 cell line revealed a poorly differentiated adenocarcinoma (Fig. 1B), resembling what was found in the original histological typing. The tumor established using TCC-Pan2 cells exhibited a rich stroma and further showed stromal hyperplasia at the OI site (Fig. 1C). We noted a high frequency of metastasis to the liver and peritoneum and to lymph nodes (Fig. 1D) in the mice (Table 3). Peritoneal dissemination of cancer cells to the peritoneum, mesenterium, diaphragm, and others, was observed in a large proportion of the mice inoculated with TCC-Pan2 cells. Ascites formation was observed frequently after OI.

Isolation of a Highly Peritoneally Metastatic Cell Line and an OI Mouse Model for Quantitative Evaluation by Using the In Vivo Photon Counting Technique

After OI of TCC-Pan2 cells, peritoneal metastasis and ascites formation occurred in 66.6% of the host mice (Table 3). Accordingly, a cell line exhibiting high PD was newly isolated from these ascitic tumors of a mouse by using the stepwise selection protocol described in Materials and Methods and was designated as Pan2M. For therapeutic studies, we introduced the firefly luciferase gene into Pan2M cells (Pan2MmLuc, Fig. 1E; see Materials and Methods). We confirmed the identity of the isolated cell line through STR analysis performed to compare specific regions of the DNA from the Pan2MmLuc subline and the TCC-Pan2 parental cell line. All the DNAs extracted from the 2 established cell lines showed identical STRs, which did not correspond to the STRs of cells in the JCRB database.

We examined the survival and tumor metastasis after OI of the tumor cells in mice (Table 3): OI of Pan2MmLuc cells resulted in the formation of bloody ascites in all the mice at 38 to 63 days after implantation, and some of the mice became moribund; a record of animal deaths revealed a median survival time after implantation of 52.9 days (Table 3 and Fig. 2A). In all animals, PD to the parietal peritoneum, mesenterium, and diaphragm was observed, and metastasis to the regional lymph nodes and liver was also frequently detected (Table 3; Figs. 1G, H, and 2A). Micrometastasis was observed in the spleen, and although only

TABLE 3. Comparison of Survival and Tumor Metastasis of Nude Mice After OI of Parental and Metastatic Cell Lines

Cell Line	Survival Days*	Tumor Formation in Pancreas	Histological Typing†	Location of Metastasis‡					Ascites Formation‖
				Peritoneum	Mesenterium	Diaphragm	Lymph Node	Liver§	
TCC-Pan2	60.4 (18.6; range, 41–103)	9/9	Poor	6/9	7/9	6/9	5/9	6/9	6/9
Pan2MmLuc	52.9 (8.9; range, 38–63)	10/10	Poor	10/10	10/10	10/10	9/10	8/10	10/10

*Data presented as mean (SD).

†Tumorigenicity of the cell lines was tested through SC injection of nude mice with 1×10^6 cultured cells suspended in 0.1 mL of RPMI 1640 medium. The pancreatic cancer was histologically typed, in accordance with the "General Rules for the Study of Pancreatic Cancer (2013)," as poor (poorly differentiated adenocarcinoma).

‡Data are shown as the number of mice with metastases at the specified site/total number of tumor-bearing mice.

§Micrometastases (including invasion through fusion with pancreatic tumor).

‖Ascite formation: >0.5 mL of ascitic fluid.

rarely, stomach and ovarian micrometastases were also noted. Lastly, hematoxylin and eosin staining of tissue sections revealed that solid tumors presented histological features consistent with poorly differentiated adenocarcinoma (Fig. 1F).

Suppression of Tumor Growth and Metastasis in Pan2MmLuc OI PD Mouse Model by NK105

We conducted an *in vivo* photon counting analysis on the Pan2MmLuc OI model to evaluate the efficacy of NK105 and paclitaxel; Figure 2B shows typical images of a mouse from each experimental group. In the control group, tumor infiltration and growth in the pancreas became marked at approximately 21 days after implantation, and the tumor gradually spread thereafter to the surrounding tissues. In the paclitaxel treatment group, a certain degree of proliferation of the surviving cells was noted, although, overall, the cell proliferative activity remained suppressed throughout the treatment period. By contrast, after the drug treatment was discontinued on day 34, the tumor began to grow rapidly. In the NK105 treatment group, Pan2MmLuc cells were detected in the implanted area, but tumor growth was found to be strongly suppressed (Figs. 2B, C). The body weights of the tumor-implanted mice in the treatment and control groups did not differ noticeably; the body weight at the end point was 21 to 22 g in each group.

Increase of Survival Time in Pan2MmLuc OI Mouse Model After Treatment With the Drug Delivery System Agent NK105

In the survival study conducted here, control mice were killed between 41 and 53 days after tumor cell OI (mean [SD], 47.6 [6.3] days; Figs. 2C, D), in accordance with the Institutional Guideline for Animal Experiments. Notably, mice in the NK105 group survived for at least 83 days after implantation (114.4 [23.6] days), and several mice exhibited normal feeding and ambulatory behavior up to 142 days after tumor cell inoculation (Figs. 2C, D). By comparison, the survival duration after OI was 73.6 (18.1) days in the paclitaxel group.

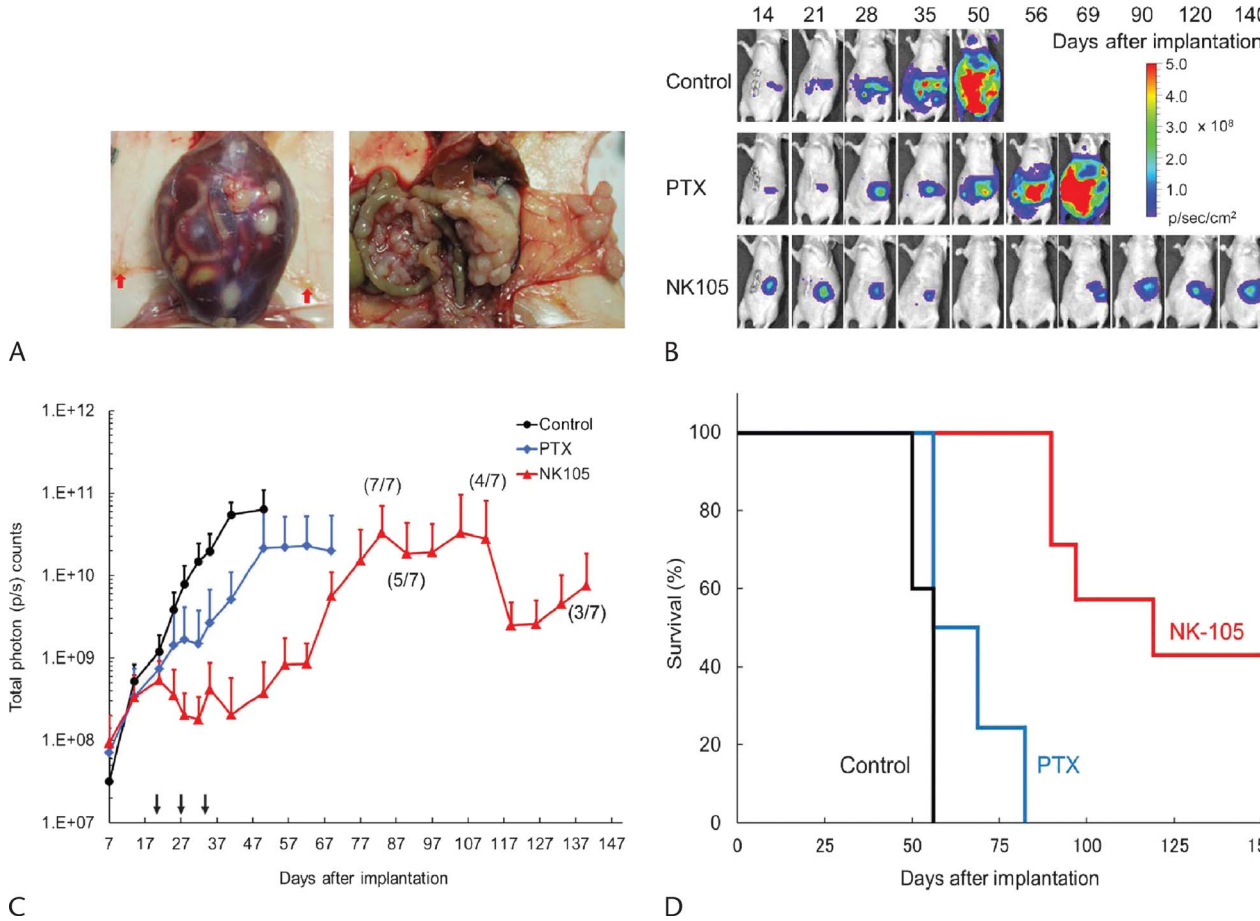

FIGURE 2. Macroscopic appearance of PD in Pan2MmLuc mouse model and in vivo photon counting analysis of the effect of NK105 and paclitaxel (PTX). A, Left, Carcinomatous peritonitis was observed at 56 days after OI of Pan2MmLuc cells. Abdominal distension because of bloody ascites was evident. Lymph node metastasis was observed in the inguinal lymph nodes (red arrows). A, Right, PD was evident from the innumerable whitish nodules visualized in the abdominal cavity, mesenterium, parietal peritoneum, diaphragm, lymph nodes, and liver. B, Effects of NK105 in the mouse model with PD established using orthotopically implanted Pan2MmLuc cells. Quantitative photon counting analysis during the process of PD progression after OI of Pan2MmLuc cells. C, Quantitative evaluation of drug-induced suppression of tumor growth. Mice treated with NK105 and PTX (black arrows) or with vehicle alone were monitored twice weekly for the development of PD. Numbers in parentheses: number of living mice/number of total mice; n = 7. D, Survival curves of the Pan2MmLuc mouse models. NK105 exhibited superior antitumor activity as compared with vehicle alone (control; $P < 0.05$); n = 7.

TABLE 4. Suppression of Peritoneal Metastasis by NK105 Treatment After OI of Metastatic Pan2MmLuc Cell Line in Nude Mice*

Drug Treatment Group	Tumor Formation in Pancreas	Tumor Volume† Photon Counts, $\times 10^8$ (%)	Location of Metastases					Ascites Formation (volume), mL§	TUNEL‖	Ki-67¶
			Peritoneum	Mesenterium	Diaphragm	Lymph Node	Liver‡			
Control	5/5	327.6 (100)	5/5	5/5	5/5	5/5	5/5	5/5 (range, >5.0)	1.0	155
NK105	5/5	3.7 (1.1)	0/5	1/5	0/5	0/5	1/5	0/5	3.7	230#
Paclitaxel	5/5	213.5 (65.2)	2/5	3/5	1/5	0/5	3/5	1/5 (range, 0.8)	3.7	147

*All mice were killed at 50 days after the OI, when the control animals became moribund. Data are shown as the number of mice with metastases at the specified site/total number of tumor-bearing mice; n = 5.

†Tumor volume was measured through in vivo photon counting analysis.

‡Micrometastases (including invasion through fusion with pancreatic tumor).

§Ascite formation: >0.5 mL of ascitic fluid.

‖Apoptosis was detected by TUNEL assay, with an *in situ* cell death detection Kit (Roche Diagnostics, Risch-Rotkreuz, Switzerland). Data are shown as percent of positive cells.

¶Ki-67 (mean number of positive cells/high-power field).

#$P < 0.03$, compared with values for control.

TUNEL indicates terminal transferase-mediated deoxyuridine triphosphate nick end-labeling.

Inhibition of Primary Tumor Growth and PD in Pan2MmLuc OI Mouse Model by NK105

In view of the findings presented earlier, we also compared the effects of different drug treatments on tumor growth and metastasis in the Pan2MmLuc OI mouse model. All mice were killed at 50 days after the OI (the end point), when the control animals became moribund. Detailed necropsy analysis revealed that all the mice harbored tumors in the pancreas. Table 4 lists the quantified photon counts indicating the degree of tumor growth suppression by each tested drug. According to the photon counts, the spread of tumor (tumor mass) in the NK105 treatment group was strongly suppressed, to 1.1% of that in the control group, and macroscopic examination revealed no metastasis to the peritoneum, diaphragm, lymph node, or liver in the NK105 treatment group; moreover, no mice developed ascites in their peritoneal cavity (Table 4). In the paclitaxel treatment group, the frequency of metastasis was significantly lower than that in the control group ($P < 0.05$), and 1 (20%) of the 5 mice developed ascites (Table 4).

Lastly, to investigate the growth kinetics of the primary tumor (intrapancreatic tumor) in mice necropsied on day 50 after Pan2MmLuc OI, immunohistochemical analysis was performed for cell proliferation and apoptosis markers. Although terminal transferase-mediated deoxyuridine triphosphate nick end-labeling staining revealed no statistically significant difference in the number of apoptotic cells between each drug treatment group and control (vehicle) group, numerous Ki-67–positive cells were scored in the NK105 group, and the cell number was significantly different from that in the control group ($P < 0.03$; Table 4). Based on these findings, we conclude that NK105 exposure suppressed the cell proliferation occurring during the process of tumor regrowth.

DISCUSSION

Our study makes 3 major contributions to the PDAC research field. First, we characterized a previously established patient-derived cell line from a PDAC. Second, we isolated a highly metastatic subline and developed an orthotopic PD mouse model. Third, we demonstrated the potent antitumor efficacy of NK105, which could eradicate metastasis to the peritoneal cavity in this orthotopic PD model.

Animal models resembling clinical cases are now widely accepted to be indispensable for investigations into the mechanisms underlying metastasis. To date, numerous cancer cell lines have been established, but these differ substantially from tumors under clinical conditions, and the cells are frequently nonmetastatic in xenotransplantation. Although PDAC frequently metastasizes to the regional lymph nodes, liver, and peritoneum in the early stages of the cancer, only a few cell lines have been reported to metastasize spontaneously in vivo into regional lymph nodes or distant organs such as the liver, lung, and peritoneum in immunodeficient mouse SC implantation models.[39,40] In this study, we characterized a previously established cell line, TCC-Pan2, derived from a PDAC (moderately/poorly differentiated adenocarcinoma), and it was found to exhibit the potential for spontaneous metastasis and PD after SC implantation or OI in nude mice. TCC-Pan2 cells were positive for pancreatic tumor markers (DUPAN-2 and SPan-1) and were identified as being derived from PDAC, and the histology of the transplanted tumors resembled that of poorly differentiated adenocarcinoma.

Pancreatic ductal adenocarcinomas exhibit gene derangements that are defined by point mutations, amplifications, deletions, translocations, and inversions.[41,42] Multiple genetic alteration steps have been widely demonstrated to be associated with malignant progression in PDAC. Activation of the oncogene *KRAS* and inactivation of the tumor suppressor gene *TP53* have been proposed to play a pivotal role in PDAC progression.[42,43] Pancreatic ductal adenocarcinoma cell lines have been considered to poorly represent the tumor of origin.[44] However, mutation of *KRAS* and *TP53*, gene amplification of *KRAS*, and homozygous deletion of *CDKN2A* were present in the original ascitic tumor and in TCC-Pan2 and metastatic Pan2MmLuc cell lines (Table 2). These data indicate that the established cancer cell lines present strong genetic conservation with the pancreatic tumor of origin.

Establishment of suitable xenograft models of human PDACs that show human-like tumor progression is essential for understanding the disease and developing novel approaches for treating the disease. As noted previously, the OI model is well established to be more clinically relevant than heterotopic inoculation models (such as SC, IP, and IV models) because of the interaction between tumor cells and the specific organ microenvironment and the eventual extensive local tumor growth and/or high incidence of metastasis.[23–25] However, differences also exist in tumor biology and morphology in the xenograft site.[20,21,26,27] To provide useful models for preclinical evaluation of new

experimental therapeutics, OI mouse models of PDAC have been developed.[19–21] Although OI models are preferred over SC models, few reports have been published concerning PD after OI of cultured cells derived from human PDACs.[26–29] Therefore, in this study, orthotopic PD models in mice were developed using the previously established cell lines, and their molecular pathological characteristics were analyzed. For the isolation of a PD cell line, TCC-Pan2 was selected because of its aggressiveness and because of metastasis development in the liver and lymph nodes and PD in the OI model. After 8 cycles of stepwise selection, a highly metastatic subline was obtained, Pan2MmLuc, which was successfully used in developing our PD model mice. This mouse model could be useful for designing new therapeutic modalities.

A drug delivery system (DDS) designed for delivering only the required quantity of a drug can act when and where necessary, and the use of such a DDS is expected to provide an optimal drug dosage.[35,45,46] For examining the efficacy and pharmacological effects of a DDS preparation, NK105 was used in this study; preclinical trials for various carcinomas have already shown that NK105 exerts strong antitumor effects at low toxicity doses, as compared with paclitaxel, because of enhanced accumulation, distribution, and retention within tumor tissues and because of the sustained release of paclitaxel from NK105.[34] The safety and pharmacokinetic advantages of NK105 use in humans were demonstrated in a phase I trial,[47] whereas a phase II trial of NK105 for advanced gastric cancer with failure of first-line chemotherapy reported an overall response rate of 25% and a median overall survival of 14.4 months.[48] Emoto et al[49] showed that IP administration of NK105 was more effective than paclitaxel administration in an IP inoculation mouse model against gastric cancer with PD because of its enhanced penetration into peritoneal nodules and higher and longer retention in systemic circulation. In this study, we evaluated the effects of IV administration of NK105 by using the newly developed Pan2MmLuc orthotopic PD mouse model of PDAC, and our results demonstrated the potent antitumor efficacy of NK105, which eradicated metastatic foci in the peritoneal cavity. The strong suppression of local tumor growth/invasion (at the OI site) by NK105 led to a delay of the PD process, and no PD or ascites formation was detected. Consequently, survival was substantially increased in the NK105 treatment group relative to that in the control group.

In conclusion, we developed an animal model by using the OI of patient-derived PDAC cell lines into the pancreas of immunodeficient mice and showed that this model was useful as a system for quantitative evaluation of anticancer drugs and for studying the biopathology of PDAC. The findings we obtained using this system suggest that NK105 treatment might represent a promising therapy for patients with PDAC.

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