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Xenografts Derived From Patients' Ascites Recapitulate the Gemcitabine Resistance Observed in Pancreatic Cancer Patients

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Abstract

Objectives: Most patient-derived pancreatic ductal adenocarcinoma (PDAC) xenografts have been established from surgical specimens of patients who have not received chemotherapy. However, xenografts have rarely been established from chemotherapy-resistant, advanced PDACs, because such cases are usually inoperable. The purpose of this study is to establish patient-derived xenografts using PDAC cells refractory to chemotherapy.

Methods: Clinical PDAC cells obtained from ascites of patients who had received continuous chemotherapy were implanted into the flanks of immunocompromised mice. Growth and histological features of the xenografts with and without gemcitabine treatment were then analyzed.

Results: Ascites-derived PDAC cells were successfully expanded through serial xenograft passage without changes in histological appearance. While treatment with gemcitabine substantially inhibited the growth of all PDAC xenografts tested, the tumor volume gradually increased and the tumors showed marked regrowth even under continued gemcitabine treatment. These findings are consistent with the actual clinical course of the corresponding patients for each xenograft.

Conclusions: Ascites-derived xenograft models represent a valuable experimental system for testing the efficacy of currently-available therapeutic compounds on chemotherapy-resistant PDAC cells and for elucidation of the mechanisms underlying chemotherapy resistance.

Key Words: pancreatic ductal adenocarcinoma, patient-derived xenograft, gemcitabine, chemotherapy resistance, ascites, pre-clinical model

Introduction

Pancreatic cancer is associated with a very poor prognosis, highlighted by the close parallel between disease incidence and mortality. Worldwide, pancreatic cancer accounts for more than 200,000 deaths every year. Total deaths from pancreatic cancer are currently increasing and it is predicted to be the second leading cause of cancer death in the United States by 2030.¹⁻³ Pancreatic ductal adenocarcinoma (PDAC) is the most common type of pancreatic cancer, accounting for more than 90% of cases.⁴ Surgical resection is considered to be the only potentially curative treatment for PDAC. However, most patients with PDAC are asymptomatic until the disease develops to an advanced stage and approximately 80% of cases are unresectable. Chemotherapeutic options for the treatment of patients with locally advanced unresectable or metastatic PDACs have long been limited, whereas gemcitabine has been widely used as a first-line drug.⁵ Furthermore, a high incidence of postoperative liver metastasis (57–92%), local recurrence (34–100%), and peritoneal metastasis (6–56%) have been observed in patients who received surgical resection.⁶ Despite 20 years of intensive efforts to find an effective chemotherapy,⁷ the median survival of patients with advanced PDAC (approximately 1 year from diagnosis) has not changed appreciably.¹ Current first-line regimens include gemcitabine plus nab-paclitaxel (nanoparticle albumin-bound paclitaxel) and FOLFIRINOX (fluorouracil, leucovorin, irinotecan, and oxaliplatin),⁸ and TS-1 is also employed as a first-line option in Japan.⁹ The FOLFIRINOX treatment protocol results in an improved overall survival of 11.1 months, compared with 6.8 months in controls using gemcitabine alone, but routine application of this regimen is limited due to toxic intolerance in the majority of patients.¹⁰ Therefore, gemcitabine-based therapies remain the standard first-line option for advanced-stage PDAC patients, and resistance to this drug has attracted increasing scientific interest.

Understanding how PDAC becomes resistant to gemcitabine is important to improve the efficacy of gemcitabine therapy and to develop novel therapeutic concepts. While it is conceivable

that characterization of carcinoma cells derived from gemcitabine-resistant PDAC patients is useful, it might be difficult to obtain such types of PDAC cells as surgical resection is not common for chemotherapy-resistant PDAC. On the other hand, tumor cells can be obtained via peritoneal effusion from patients receiving chemotherapy. Indeed, a few PDAC cell lines, including AsPC1¹¹ and HPAF-II,¹² were established from ascites-derived materials. However, these classical cell lines have been maintained in serum-containing medium, and thus, there is a possibility that the activity of signaling pathways important for tumor initiation and maintenance may be lost in conventional cell lines. In fact, it has been reported that cultured tumor cells in serum-containing media are very different, both genetically and biologically, from the parental tumors.^{13,14} These studies question the suitability of tumor cell lines for testing antitumor agents. Therefore, more sophisticated model systems are required to understand the biology of chemotherapy-resistant cells and to develop novel therapeutic concepts for PDAC patients suffering from recurrence caused by drug resistance.

We previously isolated PDAC cells from the peritoneal effusion of patients treated with gemcitabine and/or TS1, and succeeded in establishing series of cultured lines of chemotherapy-resistant PDAC cells by co-culturing with mouse stromal cells in serum-free conditions.¹⁵ These cells have proven to be useful for analyzing the tumor-initiating properties of chemotherapy-resistant PDAC cells and for identifying gemcitabine-induced KRAS–MEK–MMP-10 signaling pathway activation.^{15,16} In the present study, we characterize the chemotherapy-resistant PDAC cells by employing subcutaneously (s.c.)-transplanted xenografts to analyze the histopathological features and gemcitabine sensitivity of serially-passaged xenografts. Furthermore, we establish new chemotherapy-resistant PDAC lines directly from ascites-derived cells.

MATERIALS AND METHODS

Patient-derived PDAC Cells

KMC17 and KMC26 cells were established using cells obtained from peritoneal effusion of PDAC patients and maintained as described previously.¹⁵ Briefly, the KMC cells were cultured on the confluent PA6 cells at 37°C in a humidified atmosphere containing 5% CO₂. PA6 is a mouse stroma cell line¹⁷ and the cells are required for maintenance of KMC cells though the detailed mechanism has not been elucidated.¹⁵ KMC34 cells were obtained from peritoneal effusion of a 59-year-old female patient who had received conventional chemotherapy at the Kobe Medical Center (Table 1). This study was performed according to Institutional Review Board-approved guidelines at Kobe Medical Center, Kobe University School of Health Sciences, and KAN Research Institute. Written informed consent was obtained from each patient.

Xenograft Propagation

All mouse studies were carried out according to protocols approved by the institutional Animal Care and Use Committee at Kobe University School of Health Sciences and KAN Research Institute, Inc. KMC17 and KMC26 cells co-cultured with PA6 cells were harvested, dissociated, and suspended in Matrigel (BD Biosciences, San Jose, Calif). The co-cultured cells (2×10^6 cells in total) were injected into the flanks of SCID mice (CB17/Icr-scid/scid Jcl; CLEA, Tokyo, Japan). The injected cells included 57% of KMC cells and 43% of PA6 cells, on average. With respect to KMC34 cells, cells collected from the peritoneal effusion were washed twice with phosphate-buffered saline, and 1×10^7 cells were resuspended in Matrigel (BD Bioscience) and injected into the flanks of athymic nude mice (BALB/cAJcl-nu/nu; CLEA).

Xenograft Passage and Gemcitabine Treatment

Portions of the KMC xenografts were fixed in 10% formalin for histological analyses, and the remainder were used to make a single cell suspension for xenograft passage. Single cell suspension was prepared as described previously.¹⁴ Briefly, KMC-xenografts were incubated with 3000 CDU/mL collagenase (SIGMA-Aldrich, St. Louis, Mo) and 530 Kunitz/mL DNase I (SIGMA-Aldrich) for 2 h, then strained using a Cell Strainer (70 µm; BD Bioscience) and resuspended in Matrigel (BD Bioscience). The suspended cells, corresponding to 0.1 g xenograft tumor, were injected into the flanks of SCID mice or aliquoted into cryotubes to be stored in liquid nitrogen. Cohorts of mice bearing passage 5 to 6 xenografts with volumes in the 140 to 350 mm³ range were consecutively treated with vehicle alone or with 100 mg/kg gemcitabine (Nihonkayaku, Tokyo, Japan) twice a week. Twenty-four hours after the last dose, mice were euthanized and xenografts were harvested. Tumor sizes were measured using calipers and tumor volumes were calculated according to the following formula: $ab^2/2$ (a, width; b, length).

Histological Analysis and Immunohistochemistry

Formalin-fixed paraffin-embedded tissues were sectioned and stained with hematoxylin and eosin (H&E) and alcian blue using standard protocols. Immunohistochemistry was carried out as described previously¹⁸ using anti-p53 monoclonal (DO7; Novocastra, Newcastle, UK) and anti-p63 monoclonal antibodies (4A4; Dako, Glostrup, Denmark).

RESULTS

Clinical Course of KMC17, KMC26, and KMC34 Patients

We previously isolated seven pancreatic tumor-initiating cell lines from seven independent PDAC patients who underwent chemotherapy, and succeeded in establishing cell lines, KMC cells, in a co-culture system using PA6 mouse stromal cells.¹⁵ While all tested KMC cells were revealed to be tumorigenic when they were transplanted into immunocompromised mice,¹⁵ the *in vivo* properties of the resulting xenografts, including sensitivity to chemotherapy, had not been addressed. To apply the established experimental models to proof-of-concept studies, it is important to confirm if the models replicate the phenotype of the original human cancers and if the models respond to therapeutic agents similarly with clinical cases.

The KMC17 patient was treated with chemotherapeutics for one year. During the first 8 months, the size of pancreatic tumor did not changed appreciably (data not shown), and carbohydrate antigen (CA) 19-9 tumor marker was maintained at a low level under gemcitabine treatment followed by chemo-radiotherapy (Fig. 1A). However, after that, the liver metastasis gradually increased, accompanied by peritoneal dissemination. At this point, CA 19-9 level dramatically increased between days 250 and 300, although gemcitabine and TS-1 were administered to this patient (Fig. 1A). The KMC26 patient received gemcitabine therapy. Tumor size in pancreatic tail was reduced by the treatment (data not shown), and the levels of CA 19-9 and another tumor marker, carcinoembryonic antigen (CEA), were almost unchanged for approximately 200 days (Fig. 1B). While tumor size in the pancreas did not increase during this period, liver and peritoneal metastasis were observed around 200 days after the first admission, and the CEA level was gradually increased by around 200 days after the first admission (Fig. 1B). The KMC34 patient was treated with gemcitabine and TS-1. Tumor size (data not shown) and CA 19-9 level (Fig. 1C) were maintained for approximately 1 year by treatment with gemcitabine followed by TS-1 (Fig. 1C). However, the levels of tumor markers increased around 400 days after

the first admission (Fig. 1C), accompanied by ascites increase, during gemcitabine and TS-1 treatment, indicating that both drugs were no longer effective to this patient. These observations indicate that gemcitabine and other agents were initially efficacious to all patients, but all patients were presumed to be resistant to the chemotherapy.

Histological Features of KMC Xenografts Through Serial Passage

KMC17 and KMC26 cells were s.c. transplanted into the flanks of SCID mice to establish patient-derived xenografts (PDXs). The resulting xenografts were then serially passaged and maintained in SCID mice, which allowed us to expand these biological resources for cohort studies to evaluate the efficacy of various chemotherapeutic agents (Fig. 2A). Histological analyses of these xenografts displayed the morphology of moderately differentiated adenocarcinomas, same as original diagnosis of patients, stage IV tubular adenocarcinoma. There was almost no difference in morphology, between early- and late-passaged xenografts, in which degree of differentiation and cellular structural atypia were maintained (Fig. 2B). Alcian blue staining indicated that mucin-producing cells were contained in xenograft tissues and that they were obviously retained after serial passages (Fig. 2B). Moderate and strong p53 staining pattern were observed in KMC17 and KMC26 xenografts, respectively, and the positivity and intensity did not change after serial passages (Fig. 2B). These data indicate that the KMC xenografts were successfully expanded without changing their histological properties through serial xenograft passage.

Interestingly, KMC34 cells failed to proliferate under previously reported co-culture conditions. This was unexpected as the co-culture system was used in the successful propagation of seven KMC cell lines without exception,¹⁵ suggesting that it might be difficult to encompass the full spectrum of pancreatic cancer by solely using culture-derived xenograft PDXs. Since the ascites-derived cells from the KMC34 patient could grow as xenografts and the engrafted KMC34-derived

tumor cells (KMC34 xenografts) could also be expanded via serial passage (Fig.2A), it might be possible that additional factors present in the xenograft environment and absent in the co-culture conditions contributed to the growth of the KMC34 cells. Identification of such factors missing in the co-culture microenvironment would be useful for improvement of *in vitro* propagation systems and for understanding of the mechanisms underlying pathways/molecules important for proliferation and survival of PDAC cells. KMC34 xenografts also displayed histological features of moderately differentiated adenocarcinomas containing mucin-producing cells (Fig. 2B). The intensity of the p53-staining was weaker in KMC34 xenografts compared with KMC26 xenografts, indicative of intact p53 status. Indeed, genomic analyses failed to find any hotspot mutations in *TP53* (data not shown). As neither the ratio of p53-positive cells nor intensity of p53 staining differed between early- and late-passaged xenografts (Fig.2B), it was unlikely that serial transplantation changed the *TP53* genotype. Since the staining pattern of p53 was almost indistinguishable between early- and late-passaged xenografts in all tested KMC cells, it would be considered that genetic instability can be maintained, at least to some extent, during serial passage of KMC xenografts, though minor genetic changes might be accumulated in late-passaged tumors.

While percutaneous biopsy samples were not available for all three patients, tissues from primary lesions were obtained by rapid autopsy in the case of KMC17 patient, and this patient had received chemotherapy (Fig. 1A). It would be valuable to compare the histological features between patient biopsy and xenograft tissues derived from the ascites of the matched patient. The primary lesions showed the morphology of moderately differentiated adenocarcinomas and alcian blue staining indicated mucin production (Fig. 2C), suggesting that the pathological characteristics of the KMC17 xenograft were in accordance with the original patient primary lesions of pancreatic cancer samples. However, glandular density seemed to differ between patient biopsy and xenograft, and fibrosis with concomitant pancreatitis was observed more frequently in patient biopsies (Fig. 2B and 2C). While

there are substantial discordances in the detail, histological features seem to be almost identical between patient biopsies and PDXs.

Gemcitabine Sensitivity of KMC Xenografts and Changes in Histological Appearance

Because the KMC cells used in this study have been derived from chemotherapy-resistant patients as described above, it is a reasonable assumption that the KMC xenografts have acquired, or congenitally possess, resistance to chemotherapy including gemcitabine. To explore this possibility, we examined the tumor growth rate of KMC xenografts in the presence and absence of gemcitabine. As a first step, we expanded tumor-bearing mice through serial xenograft passages (see Materials and Methods). Upon passage, we made single-cell suspension instead of the use of xenograft tumor fragments, where cell-cell interaction and tissue architecture would be retained during transplantation procedure.¹⁹ Activities of signaling pathways important for tumor growth are usually maintained in xenograft made by use of single-cell suspension,¹³ and the use of single-cell suspension would prove useful in terms of uniformity between samples and ease of storage. Using expanded tumor-bearing mice, cohort of mice were treated with 100 mg/kg gemcitabine twice weekly. Many studies used gemcitabine at the range of 25-100 mg/kg, which is considered as the clinically relevant dose, and some xenografts respond well to this concentration of gemcitabine resulting tumor shrinkage.²⁰⁻²²

The growth of KMC17 xenografts was significantly inhibited by gemcitabine treatment, indicative of the substantial efficacy of this drug in this model. However, tumor regression could not be observed: the tumor volumes of KMC17-xenografts were gradually increased, and the tumors underwent marked regrowth despite continuous gemcitabine treatment (Fig.3A). While we attempted to treat KMC17-bearing mice with higher dose of gemcitabine (200 mg/kg, twice weekly), the dose was not tolerable and severe weight loss occurred (data not shown). KMC34 xenografts also kept increasing their tumor volumes during gemcitabine treatment whereas gemcitabine was effective in this model (Fig.3C). While gemcitabine treatment failed to reduce the tumor volumes of the two KMC

xenograft models, in the case of the KMC26 xenografts, tumor regression was observed in the early phases of treatment (Fig. 3B). However, the xenograft tumors were not completely eradicated, and tumors eventually re-grew during long-term gemcitabine treatment. Tumor volume reached twice the size of their initial volume (Fig. 3B). Collectively, all KMC xenografts tested were resistant to gemcitabine in that xenograft volume increased under consecutive treatment and these findings seem to be consistent with the fact that all patients became resistant to conventional chemotherapy (Fig. 1). Since IC₅₀ values of KMC17 and KMC26 cultures were 14 nM and 7.0 nM respectively, similar to those of PDAC cell lines²³ and patients'-derived cells,²⁴ resistance to chemotherapy observed in patients and mouse xenograft models might be substantially determined by microenvironment.

Finally, the histological changes of gemcitabine-treated xenografts were analyzed. H&E staining showed that fibrosis was increased in all three types of xenografts both with short- and long-term gemcitabine treatment (Fig. 4A–C). In xenografts with short-term gemcitabine treatment (KMC26-xenograft received four doses, in particular), a few denatured cells were observed (Fig. 4B), and these denatured cells were rarely seen in xenografts treated consecutively with gemcitabine. Instead, xenografts grown even under consecutive gemcitabine treatment often contained enlarged cancer cells (Fig. 4A–C; >10 doses) compared with cells in chemo-naïve xenografts. We also made an interesting observation that p63-positive cells increased after long-term gemcitabine treatment in KMC26 xenografts (Fig. 4B). Since p63-positive cells were diffusely detected in the untreated xenografts, it might be possible that p63-positive cells selectively survived under gemcitabine treatment. Another possibility is that the squamous transition might occur during chemotherapy. p63 is a well-known squamous marker and it has been demonstrated that the delta-N form of p63 (Δ Np63) induces squamous transdifferentiation in KRAS-driven mouse lung adenocarcinoma.²⁵ On the other hand, p63 is essential to maintain cancer cell stemness in a population of breast cancer cells²⁶ and it has been reported that squamous type of pancreatic cancers have poor prognosis.^{27,28} Therefore,

267 treatment-induced p63 expression is associated with gemcitabine resistance and poor prognosis of
268 pancreatic cancer. The KMC26 xenograft model could prove a useful means for understanding disease
269 progression under chemotherapy.
270

DISCUSSION

In genetically engineered mouse models of human cancers, tumors spontaneously arise and recapitulate the histological, spatial, and temporal context of the corresponding human disease, and these models have proven to be valuable tools not only for understanding basic cancer biology but also for proof-of-concept studies of molecular targeted therapies.²⁹⁻³² However, as genetic models recapitulate only a specific disease subset and do not encompass the full spectrum of the corresponding human cancer, it has been important to complement studies on genetic models with approaches involving human cancer cells propagated *in vitro* and *in vivo*. The majority of preclinical cancer studies have employed xenograft models established from human tumor cell lines in the flanks of immunocompromised mice.³³ Although these human tumor cell lines are often assumed to be representative of the original disease, it is unclear to what degree such experimental models replicate the phenotype of the original human cancers as the tumor cells have been removed from their native environment and selected for their ability to grow under artificial conditions. On the other hand, increasing evidence have suggested that PDXs faithfully recapitulate human tumor biology and predict patient drug response by directly comparing drug responses in patients and their corresponding xenografts.^{20,34-36}

Flank and orthotopic PDX models of PDAC have been established and have succeeded in recapitulating critical local and systemic aspects of human disease including gene expression profiles, a desmoplastic microenvironment, metastatic spread, and/or the onset of cancer cachexia.^{34,37-40} They have been proven to be useful for testing the efficacy of cytotoxic drugs⁴¹ and molecular targeted therapies,⁴² and for predicting patient prognosis⁴³ and recurrence rate.⁴⁴ Particularly, orthotopic transplantation models allows the tumor to behave more similarly to patients' tumor, and they may be strikingly different from s.c.-transplanted tumors in the aspect of tumor microenvironment.⁴⁵ Indeed, orthotopic patient-derived PDAC xenografts were reported to recapitulate the human disease including

metastasis.⁴⁰ While there is no doubt about the utility of orthotopic models, subcutaneous xenograft models, also recapitulating human disease, are particularly useful for proof-of-concept studies of molecular target therapies, because establishment of orthotopic models is usually labor-intensive. Furthermore, it has been proposed that patient-derived PDAC organoids, 3D *in vitro* culture systems, enable precise disease modeling, translational medicine, and personalized therapies.⁴⁶⁻⁵⁰ Thus, clinically relevant PDAC models have become much more sophisticated in the last decade. However, the characteristics of PDAC cells from chemotherapy-resistant patients remain unclear though there have been many reports using PDAC cells derived from patients who did not receive chemotherapy.

To deeply understand the biology of chemotherapy-resistant advanced-stage PDAC for the discovery of novel therapeutic concepts, we previously established a series of PDAC tumor-initiating cell lines, KMC cells, using PDAC cells obtained from the ascites of patients who received chemotherapy including gemcitabine.¹⁵ The KMC cells were maintained in an *in vitro* co-culture system with stroma cells. Since these cells have self-renewal capacity, differentiation ability, *in vivo* tumorigenicity, and expressed pancreatic cancer stem cell markers, they have proven to be useful for analyzing the tumor-initiating properties of chemotherapy-resistant PDAC cells. Here we extended the study to include xenografts, derived either from cultured KMC cells or directly from ascites-derived PDAC cells. As expected from the clinical courses of corresponding patients, three KMC xenografts tested in this study were resistant to gemcitabine; gemcitabine was effective in inhibiting tumor growth, but all KMC xenografts increased in size to twice that of the initial tumor volume under consecutive gemcitabine treatment (6–12 weeks). However, the time required for each KMC xenograft to doubly increase did not necessarily correlate with the clinical survival of the corresponding patient, raising concerns that flank xenografts might not completely recapitulate the microenvironment of the patient's pancreas and peritoneal cavity. It is also possible that the patient's immune status might have a critical impact on prognosis, as we used immunocompromised mice to establish KMC xenografts.

However, KMC xenografts succeeded in maintaining their PDAC features, including histological appearance, degree of mucin production, and p53 status, in the serially-passaged tumors. Furthermore, tumor cell invasion into surrounding stromal tissues, as well as increases in fibrosis, were observed in the gemcitabine-treated KMC xenografts, characteristics that are often observed in PDAC tissues of patients who have received neoadjuvant chemotherapy.^{51,52} Histological differentiation events were also found to be evident in the clinical PDAC patients by comparing biopsy samples obtained by endoscopic ultrasound fine needle aspiration and surgical specimens resected via conversion surgery.⁵³

At the early stage of drug development, many clinical trials enroll patients who do not respond to standard treatment. KMC xenografts provide great opportunities to test the candidates in the clinical relevant situation, because KMC-xenografts have been established from PDAC cells of patients treated with gemcitabine and because KMC xenografts are refractory to gemcitabine treatment, which is consistent with patients' situation. Furthermore, since endless sources for detailed analyses are available by serial *in vivo* passage, cancer cells obtained from KMC xenografts treated consecutively with gemcitabine would be useful as a powerful means to deeply understand the biology of PDAC cells growing in the presence of chemotherapy. Collectively, KMC xenografts described in this study represent a valuable experimental system for testing the efficacies of currently-available therapeutic compounds in chemotherapy-resistant PDAC cells and for elucidation of the mechanisms underlying therapy resistance. These models also should greatly contribute to the discovery of novel therapeutic approaches in this intractable cancer.

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FIGURE LEGENDS

FIGURE 1. Clinical course of patients. The clinical courses of (A) KMC17, (B) KMC26, and (C) KMC34 patients are shown. For each patient, time courses of CA 19-9 and/or CEA levels during treatment are demonstrated. The bars above the graph indicate the therapy received. GEM, gemcitabine; CRT, chemoradiotherapy (total 50.4 gray radiation and 7.5 g/m² of 5-fluorouracil); TS-1, combination drug Tegafur/Gimeracil/Oteracil. Standard values of CA 19-9 and CEA are < 37 U/ml and <5 ng/ml, respectively.

Figure 2. Xenograft propagation of KMC17, KMC26, and KMC34 cells. A, Schematic images of xenograft propagation are shown. KMC17 and KMC26 xenografts were initially established from co-cultured cells (culture-derived xenografts), and then serially transplanted into immunocompromised mice. KMC34 xenografts were established by direct transplantation of patient-derived ascites cells into immunocompromised mice and expanded through serial passages (direct xenografts). B, Histological features of early (passages 2–3) and late (passages 5–6) passaged KMC xenografts were analyzed. H&E (top), Alcian blue (middle), and p53 staining (bottom) are shown. Scale bar, 100 µm. C, With respect to KMC17, tissues from the primary lesion and lymph node biopsy were available from the patient who had received chemotherapy. H&E staining images of these tumor tissues are demonstrated. Alcian blue staining of primary lesion is also shown. Scale bar, 100 µm.

FIGURE 3. Response of KMC xenografts to gemcitabine. Effects of gemcitabine on (A) KMC17, (B) KMC26, and (C) KMC34 xenografts are shown. Mice bearing passages 5 to 6 xenografts with indicated tumor volumes were treated i.v. with 100 mg/kg of gemcitabine (GEM, solid lines) twice a week. Growth of vehicle-treated (Ctrl, control) xenografts is indicated by dashed lines. Tumor size

was measured using digital calipers and tumor volumes were calculated according to the following formula; $ab^2/2$ (a, width; b, length), and compared with the initial value to determine its relative volume. Results are represented by the mean (KMC17 ctrl, n = 2; KMC34 ctrl, n = 2) or mean $\pm$ SE (n = 3–6, as indicated).

FIGURE 4. Histological analysis of gemcitabine-treated KMC xenografts. The images of H&E and Alcian blue staining for (A) KMC17, (B) KMC26, and (C) KMC34 xenografts with and without gemcitabine (GEM) treatment are shown. Number of doses is also indicated ($\times 4$ –21). Arrowheads demonstrate denatured cells, indicative of gemcitabine effect. Note, p63-positive cells were increased in KMC26 xenografts that received long-term gemcitabine treatment (see the high magnification images of KMC26 xenografts, GEM $\times 16$). Ctrl, control.

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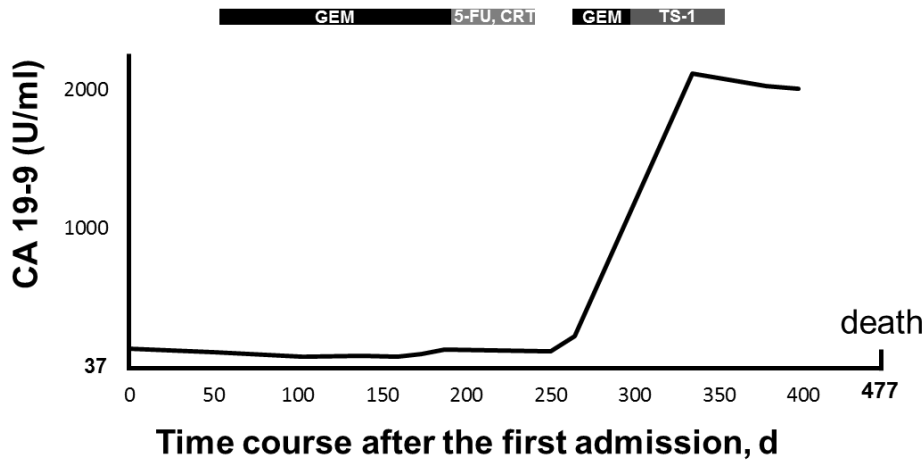
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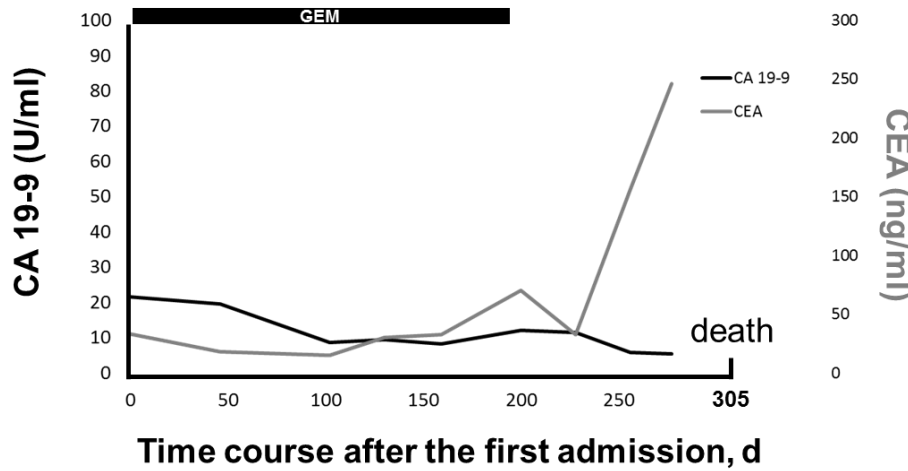
TABLE 1. Summary of Patients and Their Clinical Characteristics					
ID	Origin of KMC Cells	Sex	Age	Therapy	KRAS
KMC17	Peritoneal effusion	Male	57	Gem: 14 g/m ² , TS-1: 2.8 g/m ² , CRT	G12V
KMC26	Peritoneal effusion	Male	80	Gem: 20 g/m ²	G12V
KMC34	Peritoneal effusion	Female	59	Gem: 16 g/m ² , TS-1: 14 g/m ²	G12V
Origin of each KMC cell line and therapy received by each patient (see Figure 1 for time course) are listed with sex, age at diagnosis, and KRAS mutation status. All KMC cells used in this study were obtained from peritoneal effusion of patients who had received chemotherapy (Gem, gemcitabine; TS-1, combination drug Tegafur/Gimeracil/Oteracil). KMC17-patient received chemoradiotherapy (CRT; see Figure 1 for detail).					

KMC17



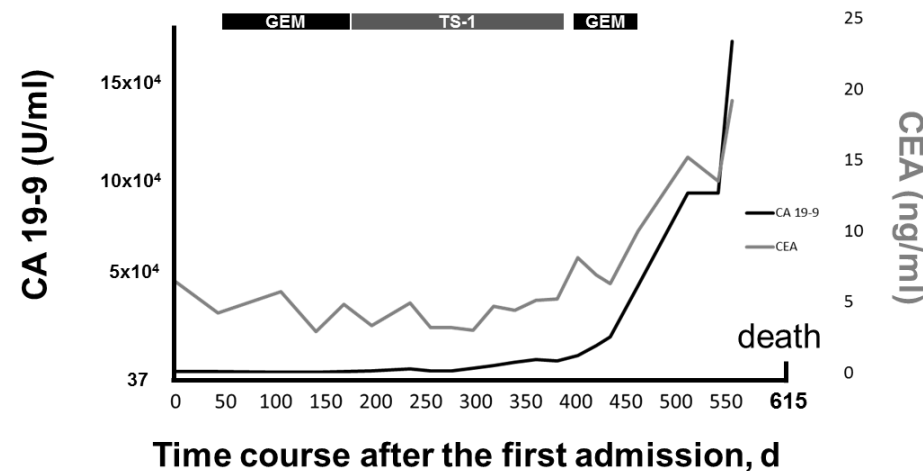
A

KMC26



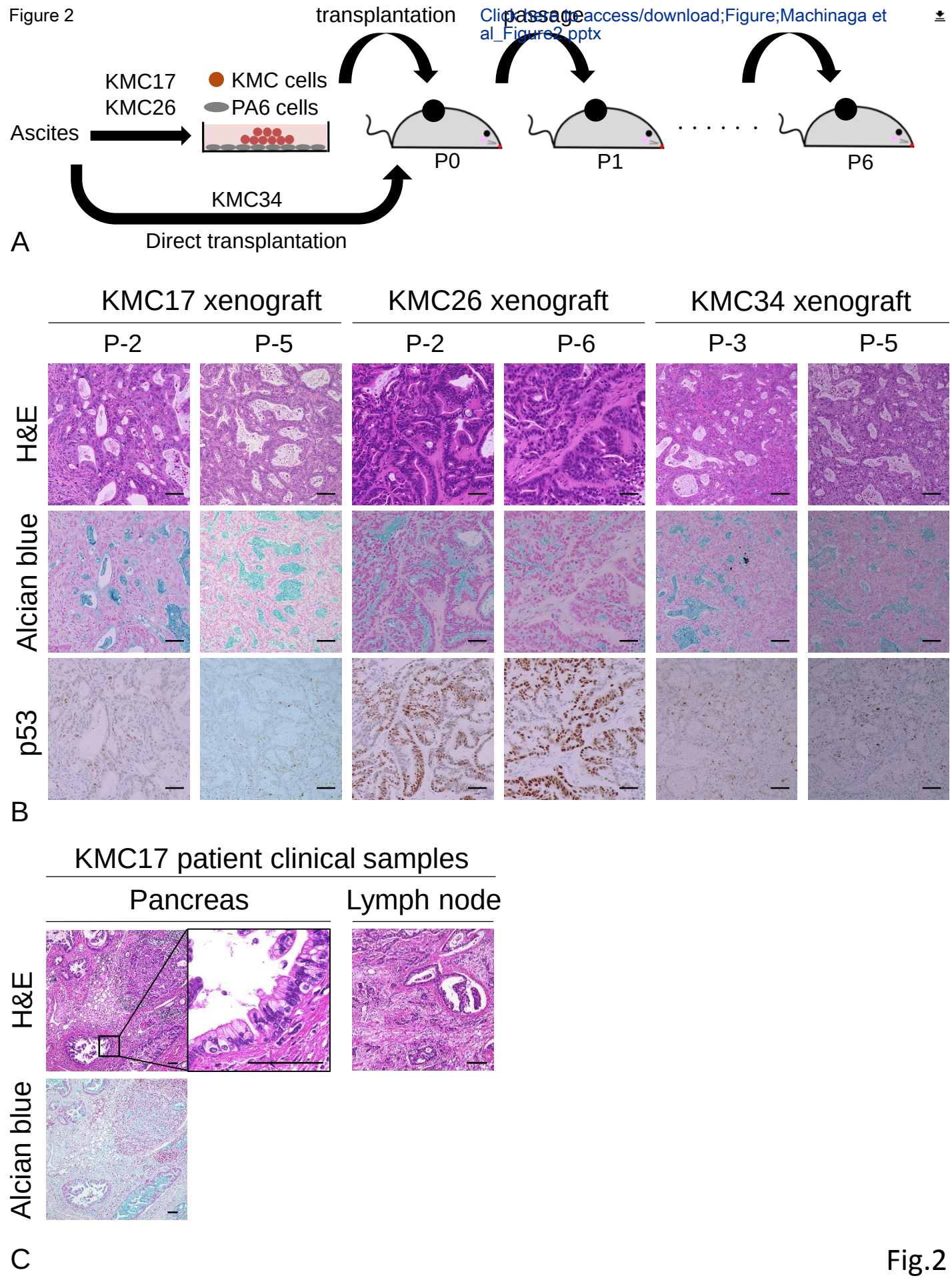
B

KMC34

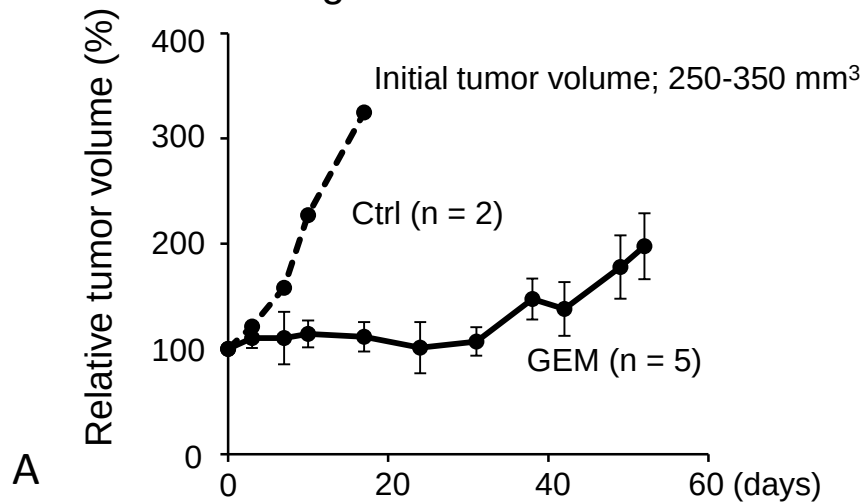


C

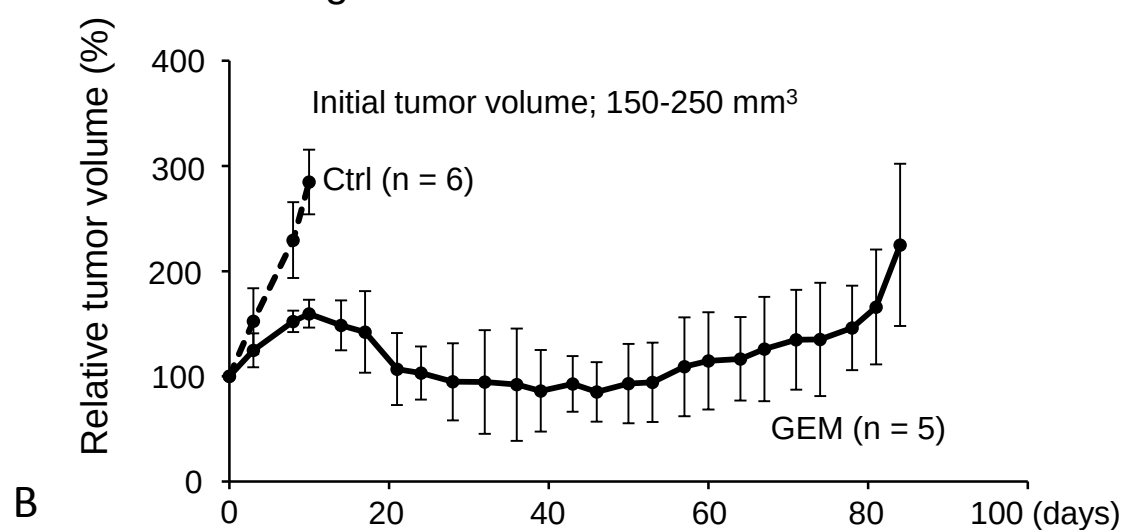
Fig.1



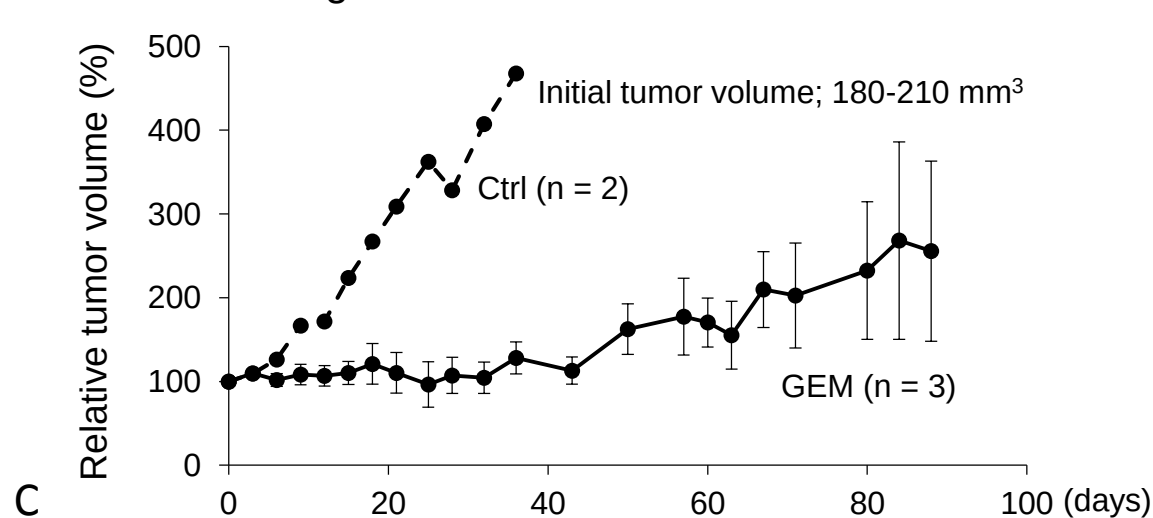
KMC17 xenograft



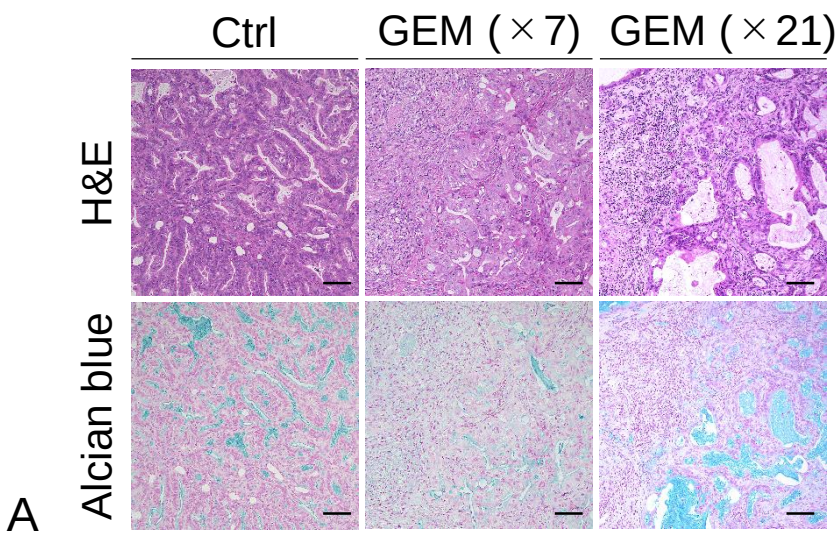
KMC26 xenograft



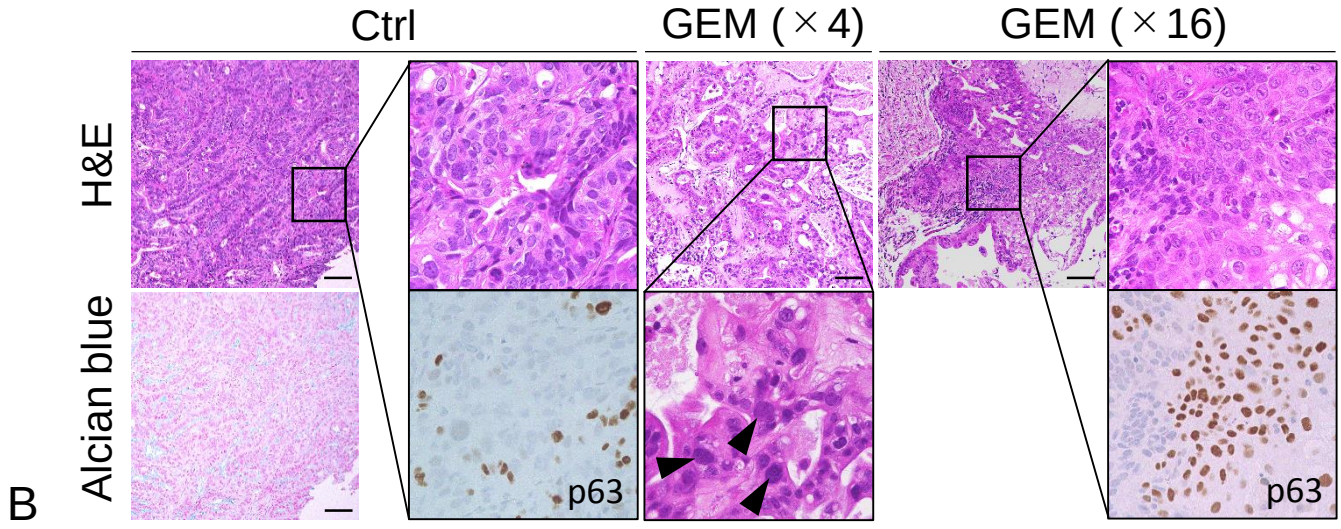
KMC34 xenograft



KMC17



KMC26



KMC34

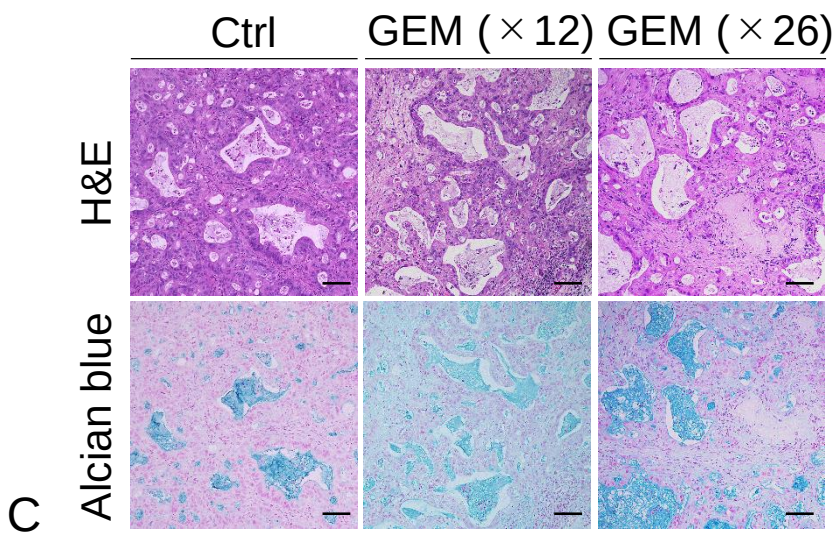


Fig.4