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Establishment of a Liver Transplant Patient-derived Tumor Xenograft (PDX) Model Using Cryopreserved Pancreatic Ductal Adenocarcinoma

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Abstract. *Background/Aim: There is rapid progression and widespread use of patient-derived tumor xenografts (PDX) in translational pancreatic cancer research. This study aimed to establish a liver transplant PDX model using cryopreserved primary pancreatic ductal adenocarcinoma (PDAC). Patients and Methods: Primary PDAC from 10 patients were cryopreserved and transplanted into immunodeficient mice using the liver pocket method. H&E staining and immunohistochemical staining, such as Ki-67, p53, Smad4, and MUC1 were used to evaluate engraftment and histological similarities. Results: Patient-derived xenograft placement was successful in six cases (60%), and 10 mice (33.3%). The Ki-67 index of primary PDAC and the cryopreservation duration were significantly related to successful engraftment (p=0.003 and p=0.007, respectively). Conclusion: In this study, we succeeded in establishing a liver transplant PDX mouse model as a preclinical platform. The successful engraftment was affected by the cryopreservation duration and could be detected by the Ki-67 index.*

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Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of death from cancer and is responsible for 45,750 deaths per year in the United States (1). The prevalence of PDAC in Japan has also increased during the last decade and has become the fifth leading cause of cancer death in men, and the third one in women (2). This malignancy is devastating, with a five-year overall survival rate of approximately 5% (3). While surgical resection is potentially curative, only a small number of patients present with a resectable tumor at the time of diagnosis (1). Chemotherapy is frequently indicated for unresectable and metastatic cases as it improves patient survival, however, it is still challenging to treat such advanced cases of the disease (4). Response to chemotherapy is highly variable, and personalized optimization of chemotherapy is critical for effective regimens.

In vitro cell killing assays using patient-derived cell lines or *in vivo* tumor growth inhibition assays using cell line-derived xenograft (CDX) models are generally available in cancer research and are used in the development of anticancer drugs. However, *in vitro* and *in vivo* preclinical study results differ from those of clinical trials (5) as CDX models lack the complex component of the human tumor microenvironment and tumor heterogeneity (6). As a representative example, although the NCI-60 cancer cell line panels, which contain 60 human cancer cell lines, are commonly used in basic and preclinical research globally, the National Cancer Institute in the United States has decided to stop screening anticancer drugs using them (7). To overcome these limitations, there has been an increasing

interest in the application of patient-derived tumor xenograft (PDX) models, which preserves tumor heterogeneity and the cancer microenvironment (8, 9).

The liver is the most frequent metastatic organ of PDAC, with half of the cases including synchronous and metachronous metastases (10). It has also been reported that the liver forms a pro-metastatic niche and creates an environment susceptible for PDAC metastasis (11). Therefore, in PDAC research, construction of a liver transplant PDX model is required as a preclinical platform, together with a pancreatic orthotopic model.

We developed a PDX model for direct transplantation into the liver using cryopreserved primary PDAC specimens. This study aimed to establish a liver transplant PDX model and determined the factors affecting its success.

Patients and Methods

An informed written consent was obtained from all patients for this research, according to an Institutional Review Board-approved protocol (approval number: 4376). The animal study was approved by the Institutional Animal Care and Use Committee of Osaka City University (approval number: 17027).

Patient-derived primary PDAC samples. Tumor samples (X0) were obtained from PDAC patients who had undergone surgery at our facility between June and December 2017. The clinical characteristics of the 10 PDAC cases are shown in Table I.

Cryopreservation and thawing procedure. The resected specimens were washed with sterile PBS twice and cut into 1mm cubes for implantation. The 1mm cube samples were transferred into sterile cryotubes containing Cellbanker® 1 plus (Zenoaq, Fukushima, Japan), and were subsequently placed in a –80˚C freezer. The median cryopreservation duration was 153 (range: 25-205) days (Table I). For thawing, cryotubes were placed in a 37˚C water bath until they were no longer frozen. Tumor samples were held on ice until implantation.

Animals. Tumors were implanted into the livers of 6–8-week-old male NSG (NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Wjl}/*SzJ*) mice (Charles River, Yokohama, Japan). Only male mice were used to prevent mating and were housed in a sterile environment.

Laparotomy and surgical orthotopic implantation using the liver pocket method. Mice were anesthetized initially using 3% isoflurane for induction and then 1-2% for maintenance. After placing mice in the supine position, the abdomen was extensively disinfected with 70 % ethyl alcohol, and a 2-cm lateral incision was made in the epigastric region. Using a cotton swab, the left lobe of the liver was maneuvered outside the body and then placed and fixed on gauze. The surface of the liver was incised horizontally using a No. 11 scalpel (AD Surgical, Sunnyvale, CA, USA) blade to form a pocket in the liver parenchyma without cutting any major vessels (12, 13). A 1 mm cube tumor sample was implanted into the liver pocket. The incision site was sealed using an absorbable hemostatic material (SURGICEL®, Johnson and Johnson, New Brunswick, USA) to stop bleeding. The left liver lobe was returned to the abdominal cavity, and the abdominal incision was closed in layers with 3-0 absorbable surgical sutures (PDS PLUS®, Johnson and Johnson).

Contrast enhanced CT imaging. Micro-CT scan was performed 16 weeks following implantation, just before sacrifice, using a LaTheta

Table I. *Clinicopathological characteristics of 10 patients with patient-derived xenograft for PDAC.*

	Number
Gender	
Men	8
Women	2
Age, median (range)	64.5 (44-85)
Tumor size, mm, median (range)	22 (18-40)
Differentiated	
Moderate	8
Poorly	2
Tumor location	
Head	6
Body/Tail	4
UICC T category	
pT1	1
pT2	1
pT3	8
pT4	0
UICC N category	
pN0	4
pN1	6
Lymphatic invasion	
Absent	6
Present	4
Vascular invasion	
Absent	5
Present	5
Neural invasion	
Absent	1
Present	9
UICC stage	
IA	1
IB	0
IIA	2
IIB	6
III	0
IV	1
Serum CEA level, ng/ml, median (range)	4.9 (1-9.9)
Serum CA19-9 level, U/ml, median (range)	250 (11-4161)
Serum SPan-1 level, U/ml, median (range)	27 (6.1-140)
Recurrence	
Yes	7
No	3
Liver recurrence	
Yes	4
No	6
Outcome	
Alive	5
Death	5
Disease free survival, days, median (range)	378.5 (71-768)
Follow up time, days, median (range)	659 (324-950)
Cryopreservation duration, days, median (range)	153 (25-205)

PDAC: Pancreatic ductal adenocarcinoma; UICC: Union for International Cancer Control; CEA: carcinoembryonic antigen; CA19-9: carbohydrate antigen 19-9; Span-1: s-pancreas-1 antigen.

Table II. *Histology and immunohistochemistry features of the primary PDAC and the xenograft tumors.*

Case	Gender	Age	Differentiated		Engraftment	Microscopic tumor size, mm×mm	Cryopreservation duration, days	Ki-67, %		p53		MUC1		Smad4	
			X0	X1				X0	X1	X0	X1	X0	X1	X0	X1
1	F	85	Mod	NA	0/3	NA	164	10	NA	–	NA	+	NA	–	NA
2	M	66	Mod	Mod	1/3	0.95×0.65	142	70	50	–	–	+	+	+	+
3	M	63	Mod	NA	0/3	NA	178	5	NA	–	NA	+	NA	+	NA
4	M	75	Mod	Mod	1/3	2.95×2.4	178	50	50	–	+	+	+	+	+
5	F	44	Mod	NA	0/3	NA	200	15	NA	–	NA	+	NA	+	NA
6	M	68	Mod	NA	0/3	NA	205	30	NA	+	NA	+	NA	+	NA
7	M	63	Por	Por	2/3	4.2×1.85	42	40	15	+	+	+	+	–	–
8	M	74	Por	Por	2/3	6.35×5.1	56	60	60	+	+	+	+	+	+
9	M	52	Mod	Mod	2/3	8.05×7.25	45	70	70	+	+	+	+	–	–
10	M	55	Mod	Mod	2/3	6.95×4.2	25	50	30	+	+	+	+	+	+

PDAC: Pancreatic ductal adenocarcinoma; X0: primary pancreatic ductal adenocarcinoma cancer; X1: xenograft tumor; F: female; M: male; mod: moderate differentiated adenocarcinoma; por: poorly differentiated adenocarcinoma; +: positive; –: negative; NA: not applicable.

LCT-200 scanner (Hitachi, Japan), four hours after the injection of a contrast agent. Micro-CT scan was performed at random in six cases. The contrast agent (ExiTron nano 12000®, Miltenyi Biotec, Germany) was an alkaline, earth-based, nanoparticulate contrast agent for mouse liver CT imaging (14) that accumulates in the Kupffer cells of the liver. Mice (25-30g body weight) were injected with 80 μl of the contrast agent through a lateral tail vein.

Follow-up schedule and collection of the engrafted tumor from mice. The general condition and survival of mice were observed twice a week, and weight was measured once a week. The mice were sacrificed at 16-17 weeks after implantation. Resected tumors were washed with sterile PBS, and a portion of the tumor was fixed with 4% formalin. Paraffin blocks and 4 μm slide sections were prepared for analyses of the tumor characteristics. The longest and shortest diameter of the engrafted tumors was measured by viewing the H&E-stained slide with a microscope (BZ-X710, Keyence, Osaka, Japan) (Table II). The remaining tumor was cryopreserved for analyses of tumor characteristics and for re-implantation.

Histology and immunohistochemistry. For histopathological evaluation, H&E staining of the primary PDAC and the engrafted tumor was performed on paraffin sections with a thickness of 4 μm. In the engraftment cases, immunohistochemical staining was performed using the followig antibodies: i) rabbit Ki-67 (1:400, Cell Signaling Technology, Danvers, MA, USA, 9027), ii) mouse p53 (1:100, Leica Biosystems, Buffalo Grove, IL, USA, PA0057), iii) mouse Smad4 (1:100, Santa Cruz Biotechnology, Dallas, TX, USA, sc-7966), and iv) rabbit MUC1 (1:100, Abcam, Cambridge, CB, UK, ab15481), using the Leica BOND-MAX IHC staining platform (Leica Biosystems). The Ki-67 scores were calculated using the average values of Ki-67 positive cells in three fields at 400× magnification, where 200 or more tumor cells were present. The cut-off value was set to 30%. As for the remaining antibodies, the staining intensity of tumor cells was assigned a score of 0-3 (0=no staining, 1=weak, 2=moderate, 3=strong). Expressions were considered positive when scores were 2 or 3, and negative when scores were 0 or 1.

Sequential passage. After successful engraftment of first-generation mice (X1), xenograft tumors were removed 16-17 weeks later. The resected specimens from X1 mice were washed with sterile PBS twice and cut into 1mm cubes. About 10 pieces were cryopreserved in a –80˚C freezer using Cellbanker® 1 plus. Three pieces were transplanted in the liver of second-generation mice (X2) within 2 h of obtaining the tumor, without cryopreservation. The mice were sacrificed at 16–17 weeks after implantation similar to X1 mice. Resected tumors were evaluated using immunohistochemistry.

Statistical analysis. The associations of clinicopathological characteristics with the tumor engraftment success were analyzed by Fisher's exact test. Analyses of continuous data, such as the Ki-67 index and the cryopreservation duration, were performed by the nonparametric Mann-Whitney *U*-test. Groups were considered to be significantly different at a p-Value<0.05. JMP13 (Statistical Discovery, SAS Institute, NC, USA) was used for statistical analyses.

Results

Establishment of PDX mouse model into the liver using cryopreserved PDAC. Transplantation was performed in three mice for each PDAC case, resulting in a total of 30 mice for the 10 PDAC cases. Table I shows the clinicopathological features of the 10 PDAC cases. There were no perioperative deaths of mice during this study. PDX into the liver was successful in 10 mice (33.3%) for six PDAC cases (60%) (Table II). The xenograft tumor engrafted as a solitary nodule in the liver, however, one mouse of Case 9 developed lung metastasis. Abdominal dissemination was not found in any mice. Micro-CT scan was performed at random in six cases, and it precisely detected the PDX in the liver in two cases (Figure 1). Moreover, sequential passage had a high success rate. X2 was successfully established for all six mice with an engraftment rate of 100% (6/6).

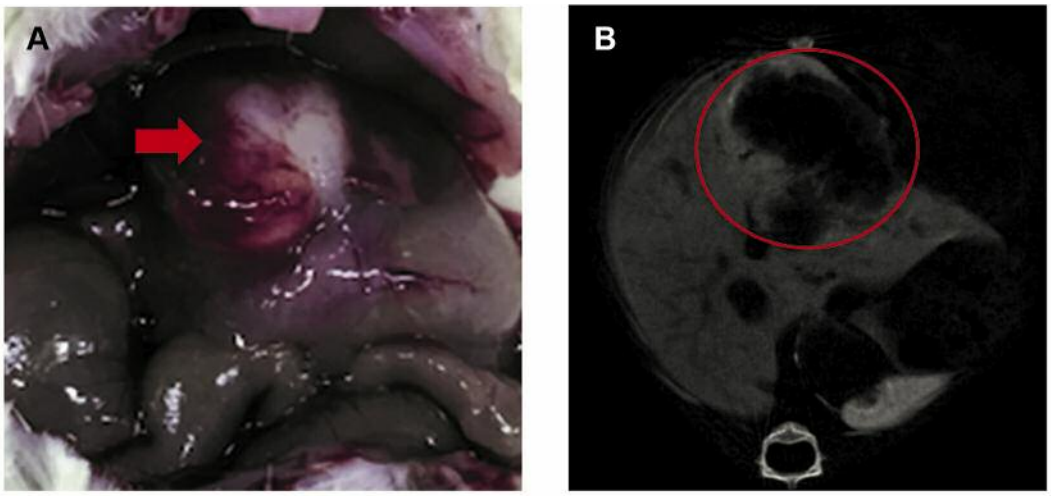

Figure 1. *A) Laparotomy image. White nodule was observed in the mouse liver (arrow). B) Contrast enhanced CT imaging was performed by ExiTron nano 12000. The contrast agent accumulates only in the normal liver not but in the liver tumor (circle).*

Clinical and pathological characteristics of the PDX model. Histological differentiation of cancer cells in X0 was maintained in X1 and X2. The histopathological features determined by H&E staining were similar among X0, X1, and X2, as well as contained stroma cells, such as fibroblasts (Figures 2 and 3). Moreover, positive staining of p53, MUC1, and Smad4, which was seen in X0, was similarly observed in X1. The ratio of Ki-67-positive cells between X0 and X1 were similar (Table II).

Factors related to successful engraftment. As shown in Table III, while clinicopathological features did not correlate to successful engraftment, the Ki-67 index and duration of cryopreservation were significantly related to successful engraftment. There was a significant difference in the Ki-67 index between the successful and unsuccessful groups, at 55% and 12.5%, respectively (p=0.003). In particular, all cases with a Ki-67 index of over 30% succeeded in engraftment. Moreover, a significant difference was seen in the median cryopreservation duration between the successful and unsuccessful groups, at 50.5 and 189 days, respectively (p=0.007).

Discussion

In this study, we succeeded in establishing a liver transplant PDX mouse model with cryopreserved primary PDAC. PDX was performed in 30 mice using the tumors from 10 PDAC cases, and the engraftment success rate was 60%. This is the first report about a liver transplant model using cryopreserved PDAC. Even though our method used cryopreserved patient-derived tumor samples and not fresh tumors, this study demonstrated a reasonable success rate of tumor engraftment under strict conditions of cryopreservation. Given our data, in the future, our mouse model may provide a standard preclinical platform in PDX research for pancreatic cancer.

In the clinical setting, liver metastases occur often in patients with PDAC and their prognosis is extremely poor (10). Creating liver transplant models using PDAC is essential to perform anticancer drug tests for patients with liver metastases. Subcutaneous PDX models are widely used as the tumors are easy to implant and it is convenient to monitor the xenograft tumors (15). According to a previous report, the tumor microenvironment plays a critical role in the responsiveness of the tumor to the anticancer drug (16). To establish treatment strategies for the recurrence of liver metastases we consider that the liver transplant model is more appropriate compared to a subcutaneous one.

When using our liver transplant model, it is important to conduct the anticancer drug screening test on tumors from patients with liver metastases. Go *et al*. have reported that orthotopic pancreatic PDX models generate spontaneous metastases in the liver by hematogenous metastasis, which involves many processes such as invasion, adhesion, and settlement (17). However, the rate of liver metastasis was very low, only 8%, and there were very small and multiple metastatic nodules in the liver (17). Although the spontaneous liver metastatic model is a theoretical mimic model that reflects real patients, it is labor-intensive and cost-intensive to produce with an engraftment rate less than 10%. In this study, we established the liver transplant PDX model of PDAC. Although our model skipped the actual metastasis process, our method achieved a high rate of

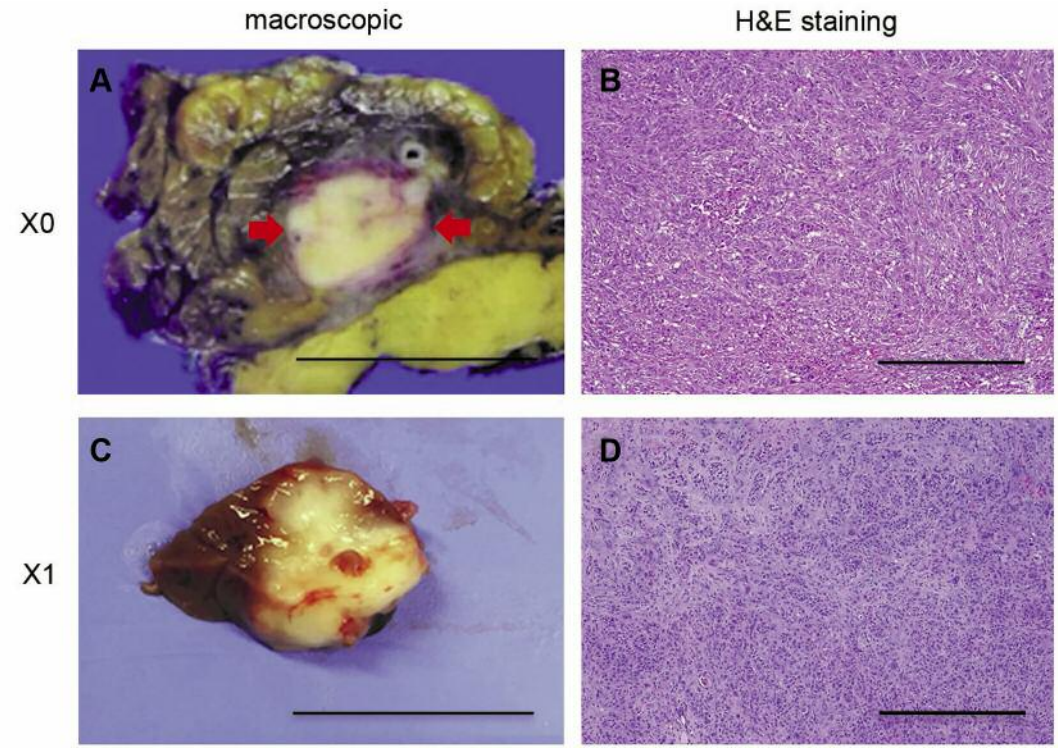

Figure 2. *A) Macroscopic finding of primary tumor (arrow). B) Histological finding of xenograft tumor. C) Macroscopic finding of xenograft tumor. D) Histological finding of xenograft tumor. Scale bar=40 mm (A), 200 μm (B and D), and 10mm (C). PDAC: Pancreatic ductal adenocarcinoma; X0: primary PDAC; X1: xenograft tumors of first generation.*

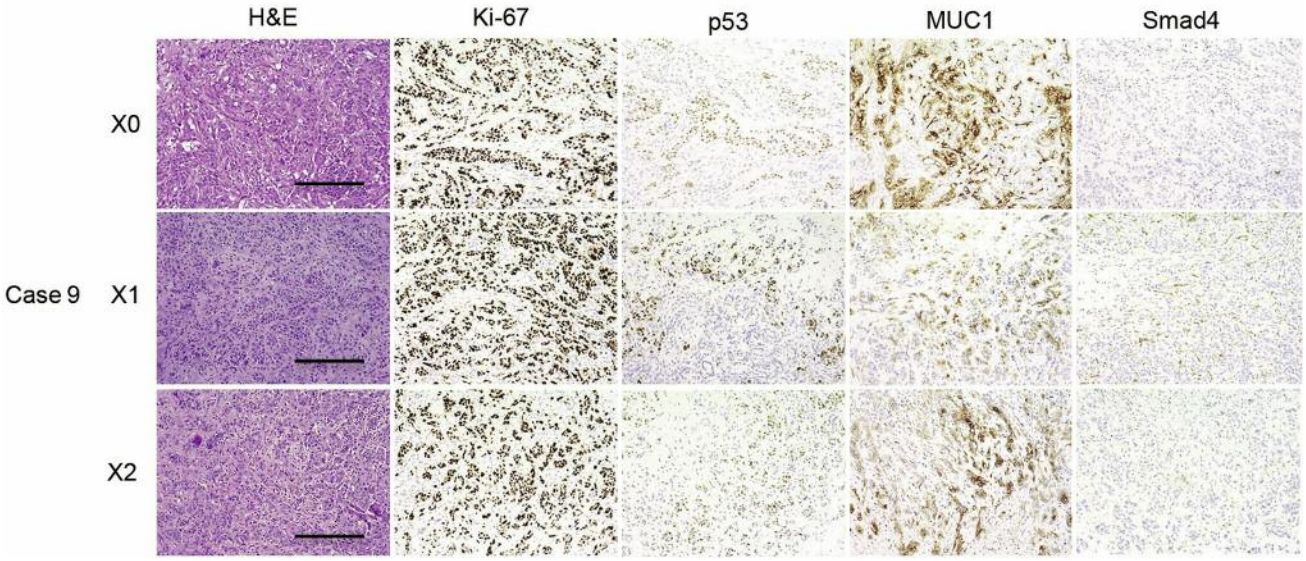

Figure 3. *H&E and immunohistochemical staining of primary (X0) and xenograft tumors (X1, X2). The histological features of the primary tumor correlated to those of the xenograft tumors. In Ki-67 and p53, the nucleus of cancer cells was stained brown. In MUC1, the cytoplasm of the cancer cells was stained brown. These were positive in Case 9. In Smad4, stromal fibroblasts around cancer cells were stained brown, and cancer cells were not stained. In Case 9, Smad4 was negative. Scale bar=100 μm. PDAC: Pancreatic ductal adenocarcinoma; X0: primary PDAC; X1: xenograft tumors of first generation; X2: xenograft tumors of second generation.*

Table III. *Factors of successful engraftment of PDX.*

	Successful engraftment n=6	Unsuccessful n=4	*p*-Value
Gender			
Men	6	2	0.13
Women	0	2	
Age, median (range)	64.5 (52-75)	65.5 (44-85)	0.93
Tumor size, mm, median (range)	22 (18-40)	23.5 (22-26)	0.54
Differentiated			
Moderate	4	4	0.33
Poorly	2	0	
Tumor location			
Head	4	2	0.55
Body/tail	2	2	
UICC T category			
pT1	1	0	0.60
pT2	1	0	
pT3	4	4	
pT4	0	0	
UICC N category			
pN0	3	1	0.45
pN1	3	3	
Lymphatic invasion			
Absent	4	2	0.55
Present	2	2	
Vascular invasion			
Absent	2	3	0.26
Present	4	1	
Neural invasion			
Absent	1	0	0.60
Present	5	4	
UICC stage			
IA	1	0	0.67
IB	0	0	
IIA	1	1	
IIB	3	3	
III	0	0	
IV	1	0	
Serum CEA level, ng/ml, median (range)	5.5 (1-9.9)	4.75 (1.6-7.9)	0.99
Serum CA19-9 level, U/ml, median (range)	181 (11-4161)	250 (46-1811)	0.59
Serum SPan-1 level, U/ml, median (range)	24.1 (6.1-140)	55 (24-120)	0.71
Recurrence			
Yes	5	3	0.67
No	1	1	
Liver recurrence			
Yes	3	1	0.45
No	3	3	
Outcome			
Alive	3	2	0.73
Deceased	3	2	
Disease free survival, days, median (range)	340.5 (117-768)	378.5 (71-950)	0.75
Follow up time, days, median (range)	659 (224-838)	673 (324-950)	0.73
Cryopreservation duration, days, median (range)	50.5 (25-178)	189 (164-205)	0.007*
Ki-67, %, median (range)	55 (30-70)	12.5 (5-30)	0.003*
p53			
Positive	4	1	0.52
Negative	2	3	
MUC1			
Positive	6	4	1
Negative	0	0	
Smad4			
Positive	4	1	0.52
Negative	2	3	

UICC: Union for International Cancer Control.

PDX: Patient-derived xenograft; UICC: Union for International Cancer Control; CEA: carcinoembryonic antigen; CA19-9: carbohydrate antigen 19-9; Span-1: s-pancreas-1 antigen. *p-Value<0.05.

engraftment of tumors and the solitary formation of tumors in the liver. Our model provides a better environment for testing anticancer drugs. This study can contribute to real patient care using a liver transplant PDX model of PDAC. For example, our mouse model may be shown that miR-related therapeutic agents eradicate metastasis *in vivo* (18).

Generally, fresh tumor specimens from patients are used to generate PDX mouse models as soon as possible following tumor removal at surgery (9). The sequential passage should also be performed using fresh tumors, because the characteristics between the patient's original tumor and the passaged xenograft tumor differ (19). Moreover, the characteristics of xenograft tumors after several passages differ from those of primary tumors, as the mouse microenvironment modifies the clonal composition of the PDX and causes it to diverge from the clonal composition of the parental human tumors (19). Therefore, PDX models are required to reduce passages to help the xenograft tumors maintain characteristics similar to those of the original tumors. Cryopreservation is a useful method for reducing unnecessary sequential passages. However, this study revealed that the cryopreservation duration was a factor important for the success of the engraftment. A shorter cryopreservation duration led to a higher PDX engraftment rate. Vaeteewoottacharn *et al.* have reported that the longest cryopreservation duration of successful engraftment was 134 days but did not mention differences in duration (20). Linnebacher *et al.* have reported that colorectal carcinoma

specimens can be successfully xenografted following an up to 643 days cryopreservation (21), suggesting that the PDX engraftment could succeed regardless of the cryopreservation duration (22). Ivanics *et al*. have reported an association between the cryopreservation duration and successful tumor engraftment. In particular, over 52 weeks of cryopreservation with standard cryoprotectant has been reported to have a significantly lower engraftment rate compared to one containing specialized cryoprotectant (23). Cryopreservation causes cell apoptosis and decreases cell viability (24). To improve the advantages of cryopreservation, we consider that further investigations are required to test a shortened storage period, modify the storage solution, and adjust the freezing and thawing methods (23, 24).

The Ki-67 index was closely associated with successful engraftment in this study. Generally, the Ki-67 index is associated with cell proliferation (25), and it has been reported as an independent prognostic factor in pancreatic cancer (26). Kageyama *et al*. have reported that the Ki-67 index was significantly correlated with serial passaging and successful engraftment (12), while Pergolini *et al*. have reported successful engraftment of PDAC associated with adverse clinicopathological features and poor survival (27).

In PDAC, there have been no reports on the relationship between the Ki-67 index and a successful engraftment in PDX. Generally, in the liver transplant model it is difficult to monitor the engraftment tumor. In this preliminary study we performed CT scanning and successfully detected tumors in the liver. We did not perform CT scanning in all of the cases, because we solely focused on establishing the methods of CT scanning. This contrast enhanced CT technique can detect tumor at least 300 μm in size (28). If we performed anticancer drug tests in this PDX model using CT we should be able to detect shrunk tumors following drug therapy.

As a first limitation of this study, the number of PDAC series was small. Here, we set a protocol of creating PDX only for 10 PDAC cases. In the future it will be necessary to perform the same procedure for a large number of PDAC cases to achieve a more accurate success engraftment rate. Second, the timing of the sacrifices might have been inappropriate. In previous reports of subcutaneous PDX models, the period of tumor engraftment was about 17 weeks (9), so we decided to sacrifice at 16 weeks, however, the engraftment tumor had not yet increased sufficiently. For this reason, we consider that prolonged observation in our PDX models might have enhanced the success of the engraftment rates.

For future perspective, we will conduct anticancer drug screening tests using our liver transplant PDX model to determine whether it resembles that of patients with liver metastases.

In conclusion, we succeeded in establishing a liver transplant PDX model using cryopreserved PDAC. The duration of cryopreservation affected the success of the engraftment, which could be observed using the Ki-67 index. This model might be useful for preclinical trials using PDX for PDAC.

Conflicts of Interest

The Authors declare no conflicts of interest for this article. It is the responsibility of the corresponding author to review this policy with all authors.

Authors' Contributions

KK, KK and AY conceived this study. KK, GO, SY and RA performed the surgeries. RT did the animal experiments and analyzed the data. RT, KK, KK and MO were involved in the drafting of the manuscript. KK, KK and MO supervised this study.SE, JT, HS, GO, SY, AY, ST, RA, HT, MY and SK were involved in the critical revision of the manuscript.

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