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Establishment of organoids using residual samples from saline flushes during endoscopic ultrasound-guided fine-needle aspiration in patients with pancreatic cancer

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ABSTRACT

Background and study aims In patients with pancreatic cancer (PC), patient-derived organoid cultures can be useful tools for personalized drug selection and preclinical evaluation of novel therapies. To establish a less invasive method of creating organoids from a patient's tumor, we examined whether PC organoids can be established using residual samples from saline flushes (RSSFs) during endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA).
Methods Five patients with PC who underwent EUS-FNA were enrolled in a prospective study conducted at our institution. RSSFs obtained during EUS-FNA procedures were collected. An organoid culture was considered as established when ≥5 passages were successful. Organoid-derived xenografts were created using established organoids.
Results EUS-FNA was performed using a 22- or 25-gauge lancet needle without complications. Patient-derived organoids were successfully established in four patients (80.0 %) with the complete medium and medium for the selection of *KRAS* mutants. Organoid-derived xenografts were successfully created and histologically similar to EUS-FNA samples.
Conclusions Patient-derived PC organoids were successfully established using EUS-FNA RSSFs, which are produced as a byproduct of standard manipulations, but are usually not used for diagnosis. This method can be applied to all patients with PC, without additional invasive procedures, and can contribute to the development of personalized medicine and molecular research.

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Introduction

Pancreatic cancer (PC) is one of the most aggressive malignant diseases, with a 5-year survival rate of 9 % [1]. Because it is difficult to detect PC at an early stage [2, 3], the majority of patients with it are diagnosed when the disease is unresectable and they primarily undergo chemotherapy [4, 5]. Combination chemotherapy regimens have become the standard first-line chemotherapy in patients with unresectable PC. Fluorouracil/leucovorin plus irinotecan plus oxaliplatin (FOLFIRINOX) and gemcitabine plus nab-paclitaxel demonstrated superiority over gemcitabine monotherapy in multicenter phase III studies [4]. In patients with PC diagnosed with resectable or borderline resectable disease according to the National Comprehensive Cancer Network guidelines, neoadjuvant chemotherapy and neoadjuvant chemoradiotherapy are rapidly becoming more important. We have shown favorable treatment outcomes for neoadjuvant chemoradiotherapy followed by pancreatectomy [6–8]. Recently, neoadjuvant chemotherapy with gemcitabine plus S-1 was shown to be superior to upfront surgery in patients with resectable PC [9]. Collectively, the significance of chemotherapy is increasing regardless of resectability; however, efficient methods for drug selection in the context of personalized medicine remain to be established.

Organoids recapitulate their morphology and exhibit key features, including gene and protein expression, and cellular metabolic heterogeneity [10–12]. Recently, patient-derived organoid cultures have been demonstrated as a useful research tool for personalized drug selection and preclinical evaluation of novel therapies in patients with PC [11–14]. Tumor organoids have been generated from various sources, such as resected specimens and samples obtained from fine-needle aspiration (FNA) [12–15]. The success rate of organoid establishment ranges from 62 % to 100 % [10].

Endoscopic ultrasound (EUS) is primarily used to evaluate pancreatobiliary and mediastinal diseases [16–18]. EUS-guided FNA (EUS-FNA) has been established as an accurate method of tissue acquisition and is performed in a wide variety of patients with PC, including those who have unresectable disease [19, 20]. Tiriac et al. recently reported the successful creation of tumor organoids using aliquots of visible tissue samples obtained for pathological diagnosis by EUS-FNA in patients with pancreatic ductal adenocarcinoma [15]. Although this method is promising, serious concerns have been raised, as the amount of tissue samples obtained with EUS-FNA is limited [21, 22]. If researchers use aliquots of tissue samples for organoid creation, it may negatively affect the pathological and genetic diagnosis by significantly reducing the amount of remaining samples. An additional puncture for organoid establishment may pose an increased risk of adverse events (AEs), such as bleeding and tumor seeding [23, 24]. To establish a less invasive method of creating organoids from a patient's tumor, we examined whether PC organoids can be established using the residual samples from saline flushes (RSSFs) during EUS-FNA procedures. RSSFs are produced as a byproduct in standard manipulations to flush the inside of the FNA needle, but are usually not used for diagnosis, and have recently been used for *KRAS* mutation analysis [21]. In the present study, we examined whether PC organoids could be established using EUS-FNA RSSFs.

Patients and methods

Study design and patients

In a prospective study conducted at our institution, patients were enrolled from August 2020 to December 2020. Patients older than age 20 years and referred for EUS with tissue sampling of a pancreatic mass lesion suspected to be pancreatic ductal adenocarcinoma were included in the present study. Written informed consent was obtained from all patients. The study was approved by the Institutional Review Board of the Osaka International Cancer Institute and performed in accordance with the Declaration of Helsinki.

EUS-FNA was performed by or under the supervision of experienced endoscopists who were qualified by the Japan Gastroenterological Endoscopy Society. EUS-FNA was performed using a curvilinear echoendoscope (GF-UCT260; Olympus Medical Systems, Tokyo, Japan) and an ultrasound diagnostic device (ALOKA ARIETTA 850, FUJIFILM Healthcare Corporation, Tokyo, Japan). EUS-FNA was performed with a 22- and/or 25-gauge needle (EZ-Shot 3; Olympus Medical Systems; Expect Slimline, Boston Scientific Corporation, Marlborough, Massachusetts, United States). The inside of the FNA needle was flushed with 10 mL of saline onto a Petri dish. A slide for rapid on-site cytological evaluation (ROSE) and a slide for cytology were made with the smear method using a part of the visible sample. After confirming malignancy on ROSE, a visible sample was used for the histological diagnosis. Furthermore, RSSFs were used in this study.

The following patient data were collected: age, sex, location of the primary tumor, tumor size, needle gauge/type, needle manufacturer/brand, and the number of punctures. Pathological diagnoses were confirmed by an expert pathologist (S.N.), and staging was performed according to the UICC staging system (8th edition) [25].

Tumor organoid culture

The RSSF from several punctures was collected into one tube. The EUS-FNA RSSFs were allowed to settle under normal gravity on ice for 10 minutes, and the precipitate was collected in a tube. The precipitate was washed several times with cold PBS to remove blood cells. The washed precipitate was incubated with 2.5 mg/mL Liberase TH (MERCK; 5401135001) and 10 µg/mL DNase I (MERCK; DN25) for 5 to 15 minutes at 37 °C in a water bath. The digested precipitate was sheared with a 1-mL pipette tip. In cases with a visible red pellet, erythrocytes were lysed in red blood cell lysis buffer (pluriSelect; 600005110) for 5 minutes at room temperature. Isolated cells were embedded in Matrigel (growth factor reduced, phenol red-free) (Corning, 356231) on ice and seeded in 24 well plates (Corning, 3738).

Tumor organoids were cultured as described in Seino et al. and Driehuis et al., with slight modifications [13, 26]. Briefly, the culture medium comprised advanced DMEM/F12 (Thermo Fisher Scientific; 12634028) supplemented with 1× Glutamax (Thermo Fisher Scientific; 35050061), 10 mM HEPES (Thermo

Fisher Scientific; 15630080), 100 U/mL penicillin/streptomycin (Thermo Fisher Scientific; 15140122), 50 µg/mL primocin (InvivoGen; 14860–94), 1x B27 supplement (Thermo Fisher Scientific; 17504044), 50 ng/mL epidermal growth factor (EGF; Thermo Fisher Scientific; PMG8044), 50 ng/mL fibroblast growth factor-2 (FGF2; PeproTech; 100–18B), 100 ng/mL insulin-like growth factor 1 (IGF1; BioLegend; 590904), 1.25 mM N-acetylcysteine (MERCK; A9165), 10 nM gastrin (MERCK; G9145), 1 µg/mL R-spondin (PeproTech; 120–38), 5 µM A83–01 (TGF-β inhibitor) (Tocris Bioscience; 2939), 10 µM Y-27632 (FUJIFILM; 259–00613), 10 % Afamin/Wnt3a CM (MBL; J-ORMW301 R), and 100 ng/mL Noggin (BMP inhibitor) (PeproTech; 250–38). This medium was considered as the complete medium. Tumor organoids were cultured in parallel in three types of tumor organoid media that were designed to select functional mutations [13]. To select *KRAS* mutants, EGF, FGF2, and IGF1 were removed from the complete medium because *KRAS* mutants constitutively activated EGF signaling. *SMAD4* mutants were selected with the removal of Noggin, A83–01, and Wnt3a because *SMAD4* mutants suppress TGF-β/BMP or p38MAPK signaling. In cases of *TP53* mutants, 3 µM Nutlin 3 (Cayman Chemical; 10004372) was added to the complete medium because *TP53* mutation acquires resistance to MDM2 inhibitor Nutrin-3. Organoid cultures in which more than 30 % of the organoids were larger than 200 µm in diameter, or many organoids were connected to each other, were passaged. For passage, organoids were collected, washed, and disrupted by digestion with TrypLE Express (Thermo Fisher Scientific; 12604013). After passage, the organoid fragments were replated in fresh media. The organoid culture was considered as established when five or more passages were successful.

Generation of organoid-derived xenografts

Some of the established tumor organoids were suspended in a 1:1 mixture of DMEM (MERCK; D5796) and Matrigel, and 1000 organoid clusters were subcutaneously injected into the back skin of NOD.CB17-*Prkdc*^{scid}/J (NOD SCID) mice (Charles River). For pathological analysis, organoid-derived xenografts were fixed in 10 % formalin neutral buffer solution (FUJIFILM) before embedding and sectioning. Hematoxylin and eosin (H&E) staining was performed on paraffin sections of the tumors. The animal procedures were approved by the Animal Experiment Committee at Osaka International Cancer Institute.

Results

Patient characteristics

The characteristics of the five patients included in the present study are summarized in ▶**Table 1**. The median age was 68 years (range, 56 to 82 years), and three patients (60.0 %) were women. The primary tumor site was the pancreas head in three patients (60.0 %) and pancreas body/tail in two patients (40.0%). The median size of the tumors was 30 mm (range, 22 to 33 mm). The lancet needles used for EUS-FNA were 22– and 25-gauge in four cases and one case, respectively. The median number of punctures was three (range, 2 to 4). Adenocarcinoma was cytopathologically proven using EUS-FNA in all cases. No complications were observed. Four patients underwent chemotherapy, including two patients who underwent neoadjuvant chemotherapy. One patient underwent upfront surgery.

▶**Table 1** Characteristics of study patients.

Patient number	Age, years/sex	EUS-FNA			Tumor location	Tumor size, mm	Pathological diagnosis	Management	c/pTNM classification	Stage (UICC 8th)
		Needle gauge	Manufacturer/brand	No. punctures						
1	74/M	22G	Olympus/EZshot3	3	Body	22	PDAC	Chemotherapy	cT4N0M1	pStage IV
2	68/M	22G	Olympus/EZshot3	2	Head	23	PDAC	Surgery	pT3N2M0	pStage III
3	82/F	25G	Olympus/EZshot3, Boston Scientific/Expect Slimline	3	Head	33	PDAC	Chemotherapy (NAC)	ycT2N0M0 (ypT2N2M0)	ycStage IB (ypStage III)
4	56/F	22G	Olympus/EZshot3	2	Body	30	PDAC	Chemotherapy (NAC)	cT2N0M0	cStage IB
5	64/F	22G	Olympus/EZshot3	4	Head	30	PDAC	Chemotherapy	cT4N1M0	cStage III

EUS-FNA, endoscopic ultrasound-guided fine-needle aspiration; M, male; F, female; PDAC, pancreatic ductal adenocarcinoma; NAC, neoadjuvant chemotherapy.

▶ **Table 2** Tumor organoid establishment using residual samples from saline flushes during endoscopic ultrasound-guided fine-needle aspiration.

Patient number	Medium	Establishment of tumor organoid	Days to established tumor organoid
1	Complete	○	64
	KRAS selection	○	106
	P53 selection	—	—
	SMAD4 selection	—	—
2	Complete	○	54
	KRAS selection	○	54
	P53 selection	—	—
	SMAD4 selection	○	63
3	Complete	—	—
	KRAS selection	—	—
	P53 selection	—	—
	SMAD4 selection	—	—
4	Complete	○	58
	KRAS selection	○	71
	P53 selection	—	—
	SMAD4 selection	—	—
5	Complete	○	84
	KRAS selection	○	61
	P53 selection	—	—
	SMAD4 selection	—	—

○ established, — not established.

Tumor organoid establishment and xenograft creation

Tumor organoids were successfully established with the complete medium in four patients (80.0 %) (▶ **Table 2**). In these patients, organoids were also established with the *KRAS*-selection medium. In Case 2, organoids were successfully established with the *SMAD4*-selection medium, as well as the compete medium and *KRAS*-selection medium (▶ **Fig. 1**).

Finally, we examined whether we could create tumor organoid-derived xenografts. Using established organoids (Cases 1 and 2), we successfully created organoid-derived xenograft tumors. Histological examination with H&E staining revealed carcinomas, featuring an irregular glandular structure with focal nuclear pleomorphism in Case 1 (▶ **Fig. 2a**, ▶ **Fig. 2b**), and well-formed papillae with abundant intracellular mucin in Case 2 (▶ **Fig. 2c**, ▶ **Fig. 2d**). The histological morphology of the xenograft tumors was highly similar to that of the tissue samples actually obtained by EUS-FNA.

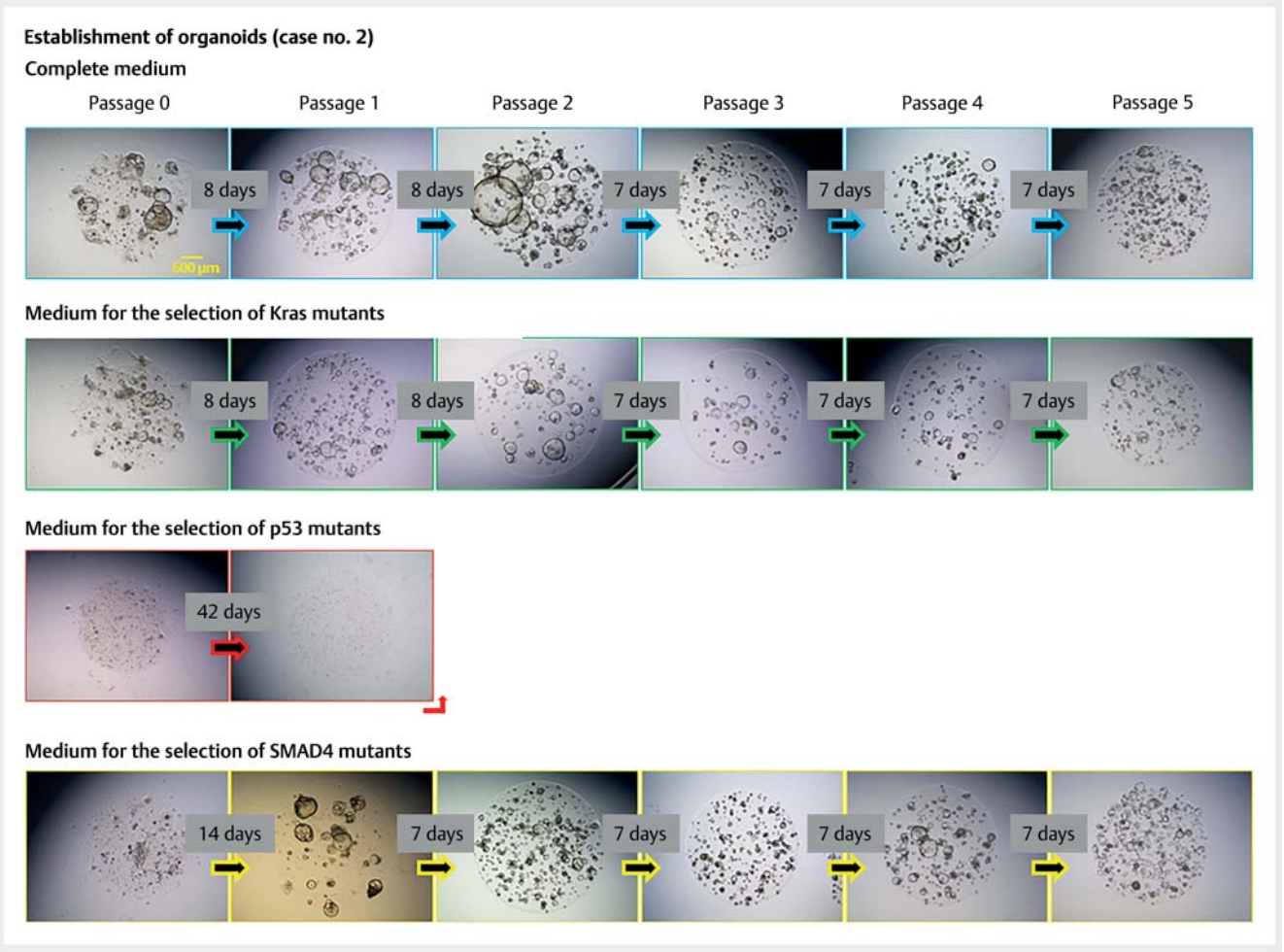
Discussion

Tumor organoid establishment is an exciting novel method for translational research and precision medicine. Tumor organoids can be used for patient-specific chemotherapeutic drug selection and basic scientific research on PC [11–14]. Tumor organoids have been primarily generated from surgically resected specimens, but this is not applicable to the majority of patients with PC who have been diagnosed with disease that is unresectable. In addition, neoadjuvant chemotherapy or chemoradiotherapy may increase the difficulty in creating tumor organoids, because neoadjuvant treatments cause shrinkage and the disappearance of PC [27–29]. Therefore, sources other than surgically resected specimens are required for the establishment of PC organoids.

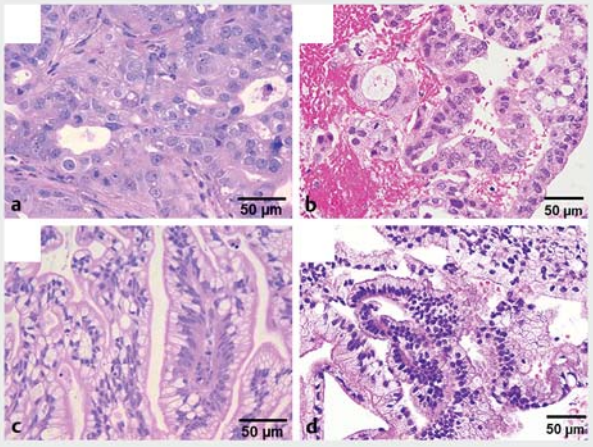
In the present study, we demonstrated that tumor organoids can be successfully established using EUS-FNA RSSFs in patients with PC. RSSFs are usually considered remnant samples because the cytopathological diagnosis can be performed with visible tissue samples. A previous study reported the successful creation of PC organoids using aliquots of visible tissue samples obtained by EUS-FNA for pathological diagnosis [15]. However, an additional puncture solely for organoid establishment is undesirable, as such additional punctures may increase the risk of AEs, including tumor seeding. Yane et al. recently reported that tumor seeding occurred in 3.4 % of cases [23]. Thus, the number of punctures should be reduced, especially in patients with resectable PC. In addition, using aliquots of visible tissue samples for organoid creation may negatively impact gene testing (microsatellite stability status and cancer multi-gene panel testing), which is of growing importance in treatment selection [30–32]. Park et al. reported that patients with PC with homologous recombination deficiency (approximately 20 % of PC) are sensitive to platinum-containing chemotherapy [33]. Although third-generation EUS-guided fine needles have recently shown a higher histologic core procurement rate and higher success rate for genetic testing, the amount of samples is still limited [34]. The application of RSSF facilitates the creation of tumor organoids in patients with PC.

To the best of our knowledge, this is the first report of tumor organoid creation using EUS-FNA RSSFs. Tumor organoids were successfully established not only in the complete medium but also in medium for the selection of mutants, including *KRAS* mutations. Furthermore, we also succeeded in creating xenografts, with histological findings similar to those of the histological images of EUS-FNA. Although only a few basic research reports exist regarding the use of EUS-FNA RSSFs [21], they could be an efficient resource for tumor organoid establishment and other molecular studies.

The present study has several limitations. The number of patients enrolled in it was small. It remains unclear whether the use of EUS-FNA RSSFs significantly decreases the number of punctures in creating PC organoids. Further studies with larger sample size are required to examine the efficacy and safety of this method. However, rate of success in establishing tumor organoids was satisfactory in comparison to previous studies of tumor organoid creation [10]. Combined with the finding that



▶ **Fig. 1** Establishment of organoids with residual samples from saline flushes (RSSFs) during endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA) (Case 2).



▶ **Fig. 2** Histological examination of tumor organoid-derived xenograft and endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA) (hematoxylin-eosin stain, original magnification × 400). **a** Case 1 tumor organoid-derived xenograft. **b** Case 1 EUS-FNA. **c** Case 2 tumor organoid-derived xenograft. **d** Case 2 EUS-FNA.

xenografts were successfully made by tumor organoids established by RSSFs, the results suggest that this method can be applied to further investigations of patients who undergo EUS-FNA for suspected malignancy.

Conclusions

In conclusion, PC tumor organoids were successfully established using EUS-FNA RSSFs, which are usually regarded as remnant samples. This method can be applied to all patients with PC without increasing AE, and has the potential to be a key method for personalized medicine in the future.

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Competing interests

The authors declare that they have no conflict of interest.

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