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Standardized Methods Using EUS-guided Fine-needle Biopsy and a Minimal Medium Creates Three Pancreatic Cancer Organoids

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Abstract. *Background/Aim: Recently, endoscopic ultrasound-guided fine-needle biopsy (EUS-FNB) has been conducted for diagnosing pancreatic ductal adenocarcinoma (PDAC), after which obtained samples were used in organoid cultures. However, no standardized method for PDAC organoid cultures exists. Therefore, to standardize or simplify sample collection and culture methods for PDAC organoids, we performed a floating culture using non-minced specimens obtained by EUS-FNB in a minimal medium, lacking growth factors or inhibitors for pancreatic organoids. Patients and Methods: A total of 38 patients with clinically diagnosed PDAC were enrolled in the study. First, EUS-FNB was conducted using a 22- or 25-gauge biopsy needle. Then, a surplus of samples was collected for organoid formation after rapid on-site cytological evaluations of sample adequacy. Subsequently, the established organoids were compared with clinical data and pathological diagnosis, following periodic observations and evaluations for morphology. Results: PDAC organoids were successfully created in 24 of the 38 cases (63.2%), including four cases with pathologically inconclusive EUS-FNB results. Afterward, PDAC organoid morphology was classified into ductal, dormant, and adhesive small cluster (ASC) types. Although the ductal and ASC types were seen separately, they were also seen together in other cases, which we named "mixed type". Conclusion: We propose a feasible and straightforward method for establishing organoids, especially for diagnosing PDAC, particularly when the result of EUS-FNB is pathologically inconclusive. Furthermore, PDAC organoids are morphologically classified into three types reported for the first time.*

Despite the progress in detection and treatment strategies, pancreatic ductal adenocarcinoma (PDAC) still has a poor prognosis and rising incidence (1, 2). Therefore, endoscopic ultrasound-guided fine-needle biopsy (EUS-FNB) has been adopted as a widely used diagnostic method for pancreatic diseases because of its highly positive diagnostic prediction (3, 4). Studies have observed that EUS-FNB facilitates the collection of tumor specimens before treatment. The use of EUS-FNB samples also allows not only for investigating resectable PDAC but also the study of unresectable cases for selecting the treatment methods. Besides, pancreatic cancer organoids recapitulate diseases and allow personalized drug screening (5).

Therefore, some reports on cell culture or the establishment of pancreatic organoids using materials obtained by EUS-FNB have recently been identified (6-8). However, some of these studies have revealed that PDAC has a significant heterogeneity among patients, suggesting that the treatment of PDAC requires more personalized methods (6, 9). This heterogeneity can be partly explained by the fact that various mediums for PDAC organoids are used in each study and contain several growth factors or inhibitors that allow the growth of not only cancer cells with a mutation of specific genes but also normal cells (10).

Hence, this study standardized PDAC organoid cultures using non-minced specimens obtained by EUS-FNB and created a floating culture using minimal mediums not optimized

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Key Words: Pancreatic cancer, EUS-FNB, organoid.

Table I. *Patient and procedure characteristics*.

	Organoid formation (+) n=24	Organoid formation (–) n=14	Overall n=38	*p*-Value
Median age (range), years	74.4 (49-86)	68.3 (44-81)	72.1 (44-86)	0.0378
Sex, male/female, n	15/9	8/6	23/15	0.7445
Median tumor size (range), mm	27.4 (18-60)	33.2 (14-75)	29.5 (14-75)	0.192
Location of the tumor, uncinate or head/body or tail, n	16/8	7/7	23/15	0.3106
Clinical stage, I/II/III/IV, n	4/9/0/11	3/4/3/4	7/13/3/15	0.804
Needle size, 25-gauge/22-gauge, n	8/16	2/12	10/28	0.1984
Median number of needle pass (range), n	2.2 (1-4)	1.9 (1-4)	2.1 (1-4)	0.0696

for typical pancreatic organoids. This study also revealed the feasibility and use of establishing PDAC organoid cultures using a minimal medium in diagnosis and future analyses for determining valid therapeutic targets for every PDAC type.

Patients and Methods

Study design and population. This research was a prospective observational study that the Institutional Review Board approved. It was also conducted following the Declaration of Helsinki. Written informed consent was obtained from all participants. Between December 2020 and January 2022, 38 patients with a clinical history and radiologic imaging results suggestive of PDAC were enrolled in the study. Then, to confirm that organoids cannot be established in a minimal medium from the non-neoplastic lesion samples, three patients with autoimmune pancreatitis (AIP) were assigned as a pilot cohort. They all needed EUS-FNB as a routine clinical procedure and did not receive treatment before EUS-FNB.

EUS-FNB procedure. Two experienced endosonographers (Y.I. and T.K.) conducted all EUS-FNB procedures using an oblique-viewing curvilinear echoendoscope (GF-UCT260; Olympus Medical Systems, Tokyo, Japan) under moderate sedation with intravenous midazolam or flunitrazepam and pentazocine. Then, a 22 or 25-gauge biopsy needle (Acquire; Boston Scientific, Natick, MA, USA, or SharkCore, Medtronic, Minneapolis, MN, USA) was chosen and used for the procedure at the discretion of the endosonographers. Next, a puncture was made *via* the gastric or duodenal walls after EUS evaluations, including regional vasculature with the color Doppler function. Finally, the needle was moved back and forth within the lesion 10-20 times at a 20 cc-negative pressure.

Subsequently, a cytopathologist conducted a rapid on-site cytological evaluation of sample adequacy (ROSE) for each case immediately after the aspirated material was treated, as described below. If the sample was evaluated as insufficient for diagnosis by ROSE, another sample was obtained. Considering the safety, the procedure was censored when the endosonographer judged that an additional puncture had a risk of bleeding even if ROSE could not confirm sample adequacy.

Sample preparation. The tissue sample in the FNB needle was flushed out into a Petri dish with saline after each puncture. Then, specimens were carefully examined to divide the visual tissue core from the liquid specimen. Approximately all divided visual tissue cores were fixed in formalin and processed for pathological evaluations. Afterward, while a small part of the visual tissue core was processed for ROSE, all the residual liquid specimens were processed for cytological evaluations. Finally, after the identification of epithelial cells using ROSE and the confirmation of diagnostically sufficient amount of specimens using gross examination, a small amount of the excess sample was collected for organoid formation. All procedures were conducted using sterilized tools.

Semi-solid organoid cultures. Non-minced specimens (~1 cm) obtained through EUS-FNB were rinsed in 10-ml of DMEM. Then, collected specimens were mixed with ice-cold minimal media containing DMEM/Hams F12/MCDB105 (2:1:1 ratio), 12% FBS (Gibco), B27-supplement (Gibco), and 5% Matrigel (Corning), as described in a previous report (11). Subsequently, the cells were plated immediately into two 24-well ultra-low attachment plate wells (Corning 3473). Afterward, 250 μl of 5% Matrigel in minimal media was added dropwisely to each well weekly to compensate for volume loss through evaporation, as described previously (11). Images of the organoids were finally taken using a BIOREVO BZ9000 microscope (Keyence).

Study outcome and statistical analysis. The successful establishment of organoids was defined by the recognition of the growth and morphology of organoids within one week of culture under transmitted light microscopic vision. Subsequently, established organoids were periodically observed and evaluated for morphology. Then, findings were compared with clinical data and pathological diagnosis results. Clinical stage was determined according to the 8th edition of the Union for International Cancer Control (UICC) classification (12).

Continuous variables were expressed using medians and ranges, while categorical variables were expressed as proportions. Continuous variables were analyzed using Mann Whitney *U*-test. Categorical variables were analyzed using the chi-square test. A *p*-value of <0.05 was considered statistically significant. All analyses were performed using SPSS version 25.0 (IBM Corp. SPSS inc., Armonk NY, USA).

Results

Patient and procedure characteristics. A total of 38 PDAC patients were enrolled in this study. Additionally, three patients with AIP were included as the pilot cohorts. Patients, tumor, and procedure characteristics are listed in Table I.

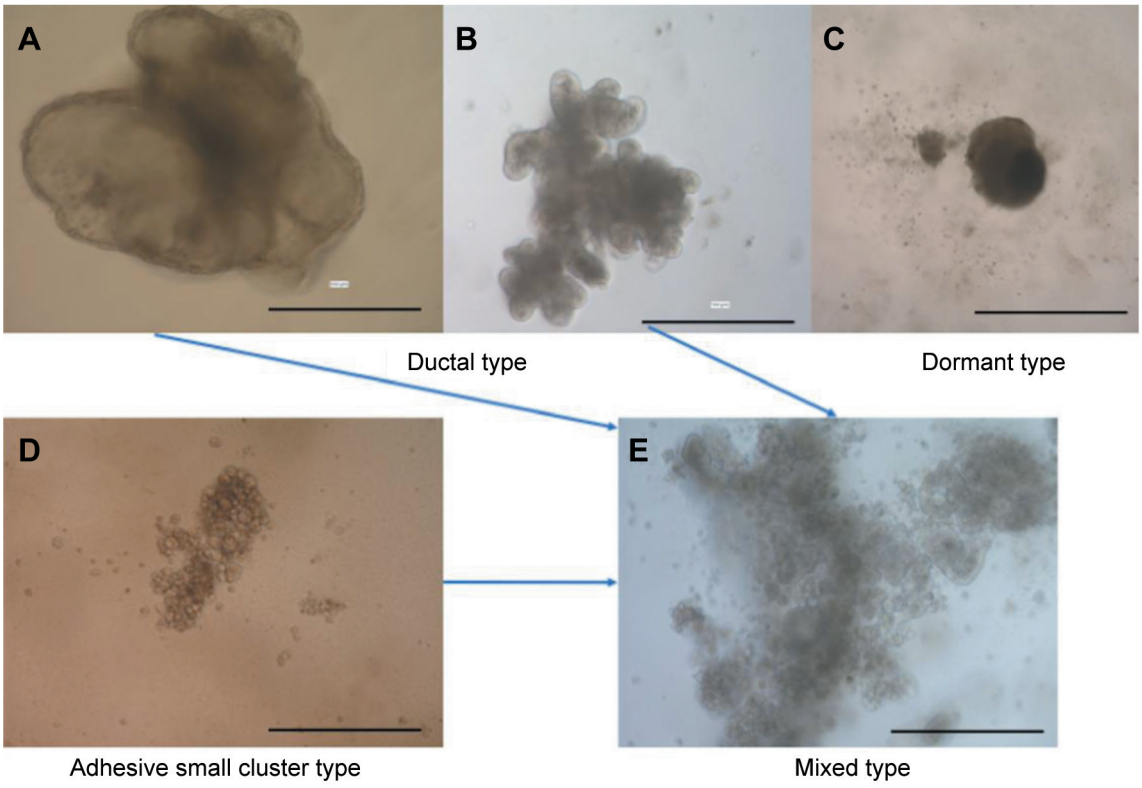

Figure 1. *Typical morphological pancreatic ductal adenocarcinoma organoid types from specimens obtained using EUS guided fine-needle biopsy. (A) Large ductal type. (B) Small ductal type. (C) Dormant type. (D) Adhesive small cluster type. (E) Mixed type. Scale bar: 500 μm.*

Table II. *Patient characteristics by morphological types of organoids.*

	Ductal type n=8	Dormant type n=5	Adhesive small cluster type n=5	Ductal + adhesive small cluster type (Mixed type) n=6
Median age (range), years	72.6 (49-86)	79.6 (79-86)	75.4 (67-82)	71.5 (66-81)
Sex, male/female, n	6/2	2/3	2/3	5/1
Median tumor size (range), mm	29.3 (18-40)	33.0 (25-48)	22.0 (18-29)	24.7 (12-60)
Location of the tumor, uncinate or head/body or tail, n	4/4	3/2	5/0	4/2
Clinical stage, I/II/III/IV, n	2/2/0/4	0/3/0/2	1/3/0/1	1/1/0/4
Rate of pathological diagnosis by EUS-FNB	100% (8/8)	100% (5/5)	60% (3/5)	66.7% (4/6)

EUS-FNB: Endoscopic ultrasound-guided fine-needle biopsy.

There was a statistically significant difference in age, suggesting that specimens of older patients may gain more mutations for the survival of the cancer cells.

Meanwhile, although it was impossible to establish normal and inflammatory organoids in the pilot cohort, PDAC organoids were successfully isolated from 24 of the 38 cases, including four patients with inconclusive results from EUS-FNB. Therefore, the success rate of the PDAC organoid culture was 63.2%.

Typical morphological types of the PDAC organoid from the specimens obtained using EUS-FNB. Subsequently, the PDAC organoids' morphology was classified into three types: ductal, dormant, and adhesive small cluster (ASC). Although the ductal and ASC types were seen separately, they were also seen together in other cases, which we named "mixed type" (Figure 1). Furthermore, while structures were used to define the ductal type based on the apical-basal polarity, the dormant type was defined based on

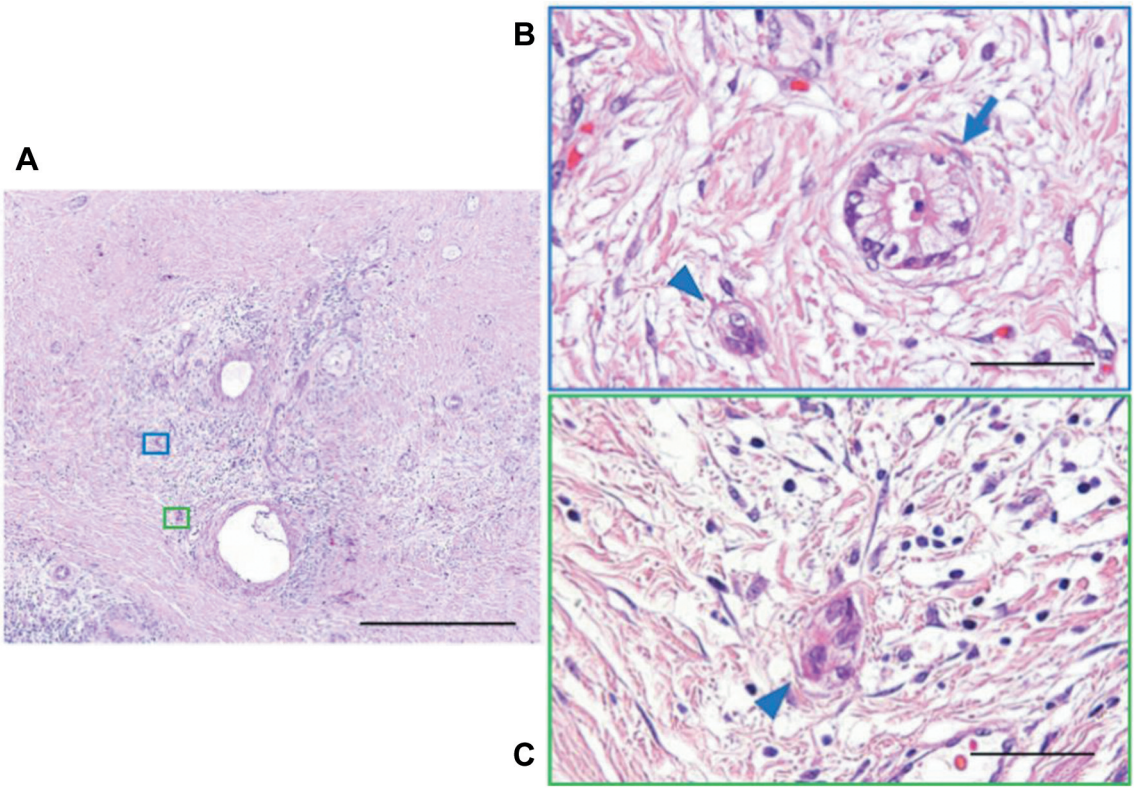

Figure 2. *Small clusters in well-differentiated adenocarcinomas of surgical specimens (Case 3 of Table III). (A) A large magnification image of hematoxylin and eosin staining, scale bar: 5 mm. (B) and (C) A small magnification of hematoxylin and eosin staining, scale bar: 500 μm. Arrowhead: Small cancer cells in the cluster, arrows: cancer cells with a ductal structure.*

Table III. *Relationship between tumor characteristics and organoid morphology.*

	Age, years	Sex	Location of the tumor	Tumor size, mm	Clinical stage	Preoperative chemotherapy	Histological diagnosis	Morphological type of PDAC organoid from EUS-FNB
1	49	Male	Pancreas head	40	Iia	GnP	Moderately differentiated adenocarcinoma	Ductal type
2	56	Male	Pancreas head	18	Ia	GS	Moderately differentiated adenocarcinoma	Ductal type
3	80	Male	Pancreas head	23	IIa	GS	Well differentiated adenocarcinoma	Adhesive small cluster
4	71	Female	Pancreas head	20	Ia	GS	Well to moderately differentiated adenocarcinoma	Adhesive small cluster
5	77	Male	Pancreas head	18	IIa	GnP	Well differentiated adenocarcinoma	Adhesive small cluster
6	82	Female	Pancreas head	20	IIa	(–)	Well differentiated adenocarcinoma	Adhesive small cluster

PDAC: Pancreatic ductal adenocarcinoma; EUS-FNB: endoscopic ultrasonography-guided fine needle biopsy; GnP: Gemcitabine plus nab-paclitaxel; GS: Gemcitabine plus S-1.

the presence of a polarized structure with slow growth. Also, the ASC type was defined based on the cluster of small adhesive cells without polarity. Alternatively, the ductal type was observed in eight cases (33.3%), the dormant type in five cases (20.8%), the ASC type in five cases (20.8%), and the mixed type in six cases (25.0%), respectively. A comparison between patient characteristics and morphological types in organoid cultures is shown in

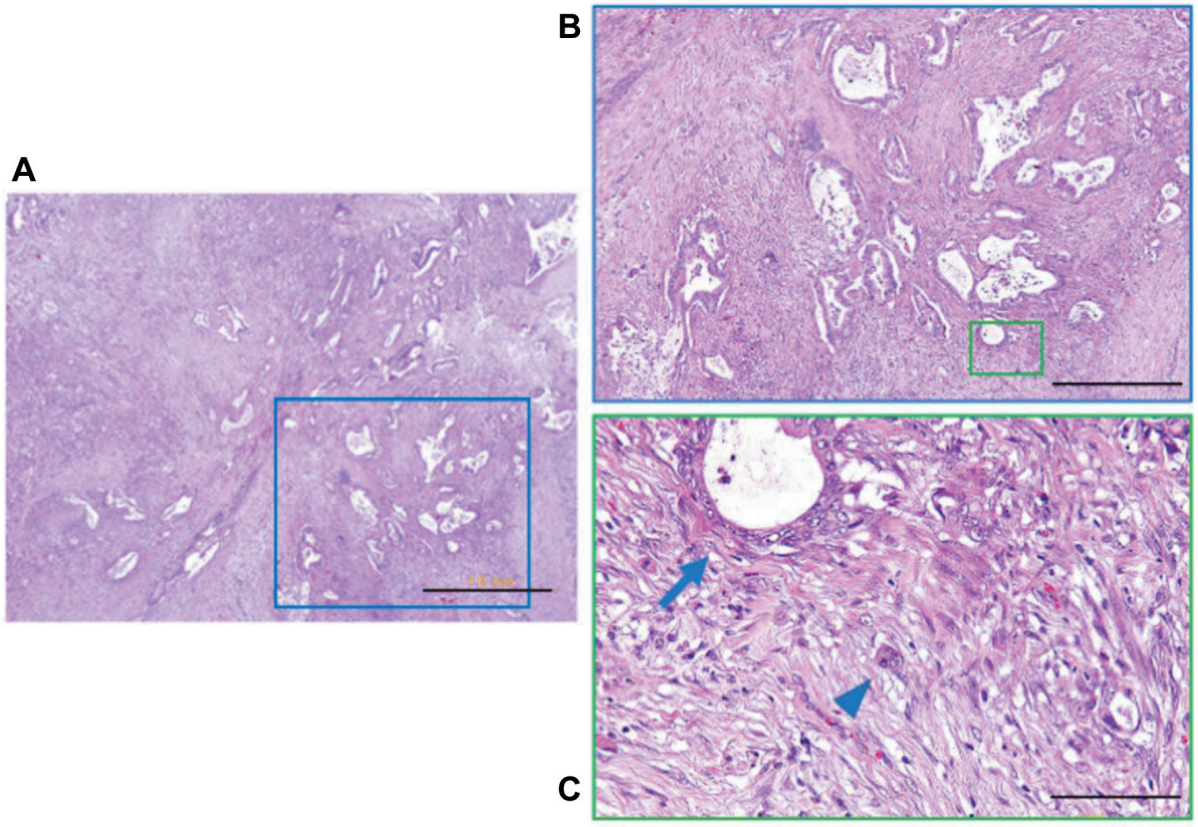

Figure 3. *Small clusters in moderately-differentiated adenocarcinomas from surgical specimens (Case 2 of Table III). (A) Hematoxylin-eosin staining ×2, scale bar: 10 mm. (B) Hematoxylin-eosin staining ×4, scale bar: 5 mm. (C) Hematoxylin-eosin staining ×20, scale bar: 1,000 μm. Arrowhead: Small cancer cells in clusters, arrows: cancer cells with a ductal structure.*

Table II. Notably, even though morphological organoids established from pathologically inconclusive cases were the ASC type or the mixed type, no ductal type in inconclusive cases was observed, indicating that ASC existed in all cases with inconclusive EUS-FNB.

Small clusters of PDAC in surgical specimens. Additionally, we performed pathological investigations of surgical specimens to determine whether these morphological cell types existed in the obtained surgical specimens. First, surgery was conducted in six of the 24 cases where PDAC organoids were cultured. Then, results showed that while morphological PDAC organoid types were the ductal or ASC types, most of the cases were well-differentiated adenocarcinomas. Furthermore, no cases with poorly differentiated adenocarcinoma were observed (Table III). Results also showed that the resection specimens showed not only cancer cells forming ductal structures, which is considered a reflection of the ductal type PDAC organoid morphology, but also small cancer cells in clusters (SCCs in clusters) without polarity, which reflects the ASC type (Figure 2 and Figure 3). The ductal type organoids may reflect cancer cells with ductal structure; therefore, even in cases where ductal type morphology was observed in PDAC organoids obtained from EUS-FNB specimens, SCCs in a cluster were detected in the resected specimens (Figure 3), thus suggesting that SCCs in the cluster were frequently observed in several PDAC types.

Discussion

Our study shows that the rate of successful organoid cultures was 63.2%, despite the profound effect of sampling or technical errors due to using surplus samples and minimal medium use. Although the medium for PDAC organoids typically contains growth factors or inhibitors (10), a minimal medium eliminates normal and cancer-like cells (6). As a result, just cancer cells can survive even in these more severe microenvironments. Vilgeim et al. standardized organoid formation using minimal medium and FNA specimens including, melanoma, thyroid, kidney, gastrointestinal tract, and pancreas, although this study did

not show morphological properties in detail (11). Minimal medium is also beneficial in terms of cost and simplicity; therefore, their methods and the result of our study may contribute to investigation of PDAC organoids for further application. In this study, it is worth mentioning that PDAC organoids were cultured even in cases with pathologically inconclusive EUS-FNB results, contributing to the clinical diagnosis of PDAC.

Furthermore, we classified PDAC organoids into three types: ductal, dormant, and ASC type using a minimal medium. Specifically, the ductal and ASC types were occasionally seen together in the same cases, which we named the mixed type. Besides, the organoids of the cases with inconclusive EUS-FNB results were classified as the ASC type or mixed type, notably indicating that ASC was observed in all cases with inconclusive EUS-FNB results. However, although ASCs are forms in which small solitary cancer cells are found adherent to each other, similar cancer cells could be seen as SCCs in a cluster in the resection specimen (Figure 2 and Figure 3). Therefore, since these SCCs in clusters were included in the EUS-FNB specimens with inconclusive results, they are considered to have proliferated to form unpolarized organoids. Interestingly, SCCs in clusters were observed even in surgical specimens where the morphology of the PDAC organoids from EUS-FNB samples was the ductal type. In other words, while SCCs in clusters are proposed to be present in all PDACs, they may have been missed or eliminated during general pathological diagnosis. Nevertheless, it remains unclear whether SCCs in clusters exist in all PDAC, and further study is required. We are also trying to establish cell lines from separated organoids. Future studies will reveal genetic backgrounds by RNA-seq analysis.

Since our study was a pilot study, there are several limitations including no fixed criteria for specimen sampling and the small sample size. Additionally, in this study design, it is not possible to determine whether the inability to establish PDAC organoids was due to sampling error or the use of a minimal medium.

In conclusion, establishing PDAC organoids from EUS-FNB samples using a minimal medium is feasible. It can also contribute complementarily to the clinical diagnosis of PDAC, especially when the results of EUS-FNB are inconclusive. Besides, PDAC organoids can be morphologically classified into the ductal, dormant, and ASC types. However, further studies should reveal how these differences in morphology affect diagnosis, responsiveness for the treatment, and prognosis.

Conflicts of Interest

The Authors declare no conflicts of interest.

Authors' Contributions

Y.I. and T.T. designed the study and wrote the article. Y.I. and T.K. performed the EUS-FNB procedure. Y.H. and S.M. conducted the pathological and cytological diagnosis. Y.I., N.T., T.K., T.K., K.M., T.S., R.N., F.I., and M.K. collected the data. Y.I., T.T., and F.H. revised the manuscript. S.S., F.H. and S.H. critically revised the manuscript and were the supervisors.

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