Next-generation sequencing to identify genetic mutations in pancreatic cancer using intraoperative peritoneal washing fluid

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Abstract. The efficacy of next-generation sequencing (NGS) of tumor-derived DNA from intraoperative peritoneal washing fluid (IPWF) of patients with pancreatic ductal adenocarcinoma (PDAC) who intend to undergo curative resection remains unclear. The aim of the present study was to evaluate whether genomic mutations in tumor-derived DNA from IPWF samples of patients with PDAC who intend to undergo curative resection could be detected using NGS. A total of 12 such patients were included in this study. Cytology of IPWF (CY) was assessed and NGS of genomic tumor-derived DNA from the IPWF was performed to determine whether genomic mutations could be detected in these patient samples. A total of 2 patients (16.7%) had a CY(+) status and 1 patient (8.3%) showed intraoperative macro-peritoneal dissemination; 11 patients underwent radical surgery. Actionable gene alterations were detected in 8 (80.0%) out of the 10 patients with CY(-) status based on NGS of IPWF samples, and 3 (37.5%) patients among those with actionable gene mutations identified from IPWF samples underwent peritoneal dissemination after surgery within ~12 months. The most common genomic mutation was in *KRAS* (9 patients, 75.0%), followed by *TP53* (3 patients, 25.0%), *SMAD4* (1 patient, 8.3%) and *CDKN2A* (1 patient, 8.3%). These findings indicated that the genomic mutations identified in tumor-derived DNA from IPWF samples of patients with PDAC with a CY(-) status who intend to undergo curative resection are potential biomarkers for predicting the recurrence of early peritoneal dissemination.

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Abbreviations: CY, cytology of IPWF; ddPCR, droplet digital PCR; FFPE, formalin-fixed paraffin-embedded; NGS, next-generation sequencing; PDAC, pancreatic ductal adenocarcinoma; IPWF, intraoperative peritoneal washing fluid; VAF, variant allele frequency

Key words: NGS, pancreatic cancer, tumor-derived DNA, IPWF, cytology

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is among the most aggressive cancers, whose 5-year survival rate was approximately 8% in 2020; this rate was 20-30% for patients who underwent curative resection (1-3). PDAC first recurs in sites with peritoneal dissemination. Intraoperative peritoneal washing fluid (IPWF) is routinely collected, and the washing cytology of IPWF (CY) is examined. CY-positive (CY+) status for PDAC is a well-known indicator of poor prognosis and a risk factor for peritoneal dissemination (4,5), which occurs in 20% of CY-negative (CY-) patients because of the insufficient sensitivity of CY due to the presence of many noncancerous cells including mesothelial cells and leukocytes (6). Therefore, a sensitive and reliable method for predicting the risk of peritoneal dissemination is needed.

Recently, various molecular techniques for detecting genomic mutations in patients with PDAC have been reported, including PCR-based methods and next-generation sequencing (NGS) (7-9). Specifically, liquid biopsies are promising tools for quantifying minimal residual disease, monitoring treatment response, and assessing the emergence of drug resistance in patients with PDAC (10). Moreover, deep NGS can be used to sequence a genomic region multiple times, thereby enabling the detection of genomic mutations with a variant allele frequency (VAF) of <1% (11,12). This method can detect tumor-derived DNA in various sample types, such as body cavity fluids, including pleural, pericardial, and peritoneal fluid (13-17), and micrometastases, which are too small to be observed in imaging tests. Tumor-derived DNA from these samples can be evaluated before extensive metastatic spread occurs. NGS analysis of tumor-derived DNA extracted from the peritoneal fluid or IPWF of patients with PDAC is not commonly performed (13-17). Additionally, genomic mutations in tumor-derived DNA have not been evaluated using NGS of IPWF samples from patients with PDAC who intend to undergo curative pancreatectomy. Therefore, in this study,

we determined whether genomic mutations in tumor-derived DNA from IPWF samples of patients with PDAC who intend to undergo curative resection can be detected using NGS and if IPWF can be used for liquid biopsies.

Materials and methods

Patients and data collection. Twelve patients with PDAC who underwent surgical procedures between January 2020 and December 2021 were enrolled in this study. Patients who intended to undergo curative resection and with both FFPE and IPWF were included, and those with definitive distant metastasis by preoperative CT, MRI, or other imaging examination were excluded. According to the National Comprehensive Cancer Network guidelines, the patients with borderline resectable PDAC received GnP (intravenous infusion of 1,000 mg/m^2 gemcitabine and 125 mg/m^2 nab-paclitaxel, on days 1, 8, and 15 of each 28-day cycle) for 3 cycles followed by radiotherapy (45.0 Gy in 25 fractions) combined with S-1 (<1.25 m^2, 60 mg; 1.25-1.5 m^2, 80 mg; >1.5 m^2, 100 mg), or modified FOLFIRINOX (intravenous infusion of 85 mg/m^2 oxaliplatin, 150 mg/m^2 irinotecan, 2,400 mg/m^2 5-FU infusion over 46 h, no bolus 5-FU, every 2 weeks) as neoadjuvant treatment. Modified FOLFIRINOX was usually repeated for 6 to 8 cycles but the number of cycles depended on physicians' decisions or the patients' conditions. All patients were histologically diagnosed with PDAC using a standardized system consistent with WHO classification, 5th edition (18), and provided written informed consent to participate in the study. Samples were analyzed retrospectively to patient recruitment. This study was approved by the Human Experimentation Committee of Keio University Hospital (Tokyo, Japan; approval nos. 20120443 and 20170086) and performed in accordance with the tenets of the Declaration of Helsinki (2013).

IPWF. We performed routine CY immediately after laparotomy. First, 100 ml isotonic saline was introduced into the pelvic cavity. After gentle agitation, as much fluid as possible was collected using the suction tube from the pouch of Douglas. The sample was transferred to the laboratory approximately 10-20 min later and centrifuged at 1,580 x g for 2 min at 21˚C. After resuspending the pellet, the fluid was dispensed to Cyto-Tek® 2500 (Sakura Finetek, Tokyo, Japan) and centrifuged at 700 x g for 4 min at 21˚C. A pathologist examined the smears after conventional Papanicolaou and Giemsa staining. The CY results were provided to the surgeons before pancreatectomy, and normal or benign, indeterminate, and potentially malignant samples were defined as CY(-), whereas malignant samples were defined as CY(+). Another 50 ml of harvested peritoneal washing fluid was transferred to the laboratory immediately and centrifuged twice at 800 x g for 10 min at 4˚C. The pellet was stored at -80˚C until further use. The pellet was centrifuged at 10,000 x g for 5 min at 4˚C prior to DNA extraction. In this study, we collected IPWF only during the surgery.

Preparation and extraction of tumor-derived DNA from IPWF and FFPE specimens. Resected specimens from the primary tumor were immediately fixed in 10% buffered formalin at 21˚C and then embedded in paraffin within several days. Ten-micrometer-thick sections of tumor tissue were cut from each block and placed on the slide glass. The paraffinized sections were stained with hematoxylin and eosin, and an experienced pathologist identified the tumor lesions. The tumor lesions were dissected from the 10-μm sections, and fractions from five sections were collected in a sterile Eppendorf tube. Genomic DNA was extracted and purified from the stored pellet of peritoneal washing fluid and FFPE tissue samples using the QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The final elution volume was 30 μl. The concentration of the genomic DNA was measured using NanoDrop 2000c (Thermo Fisher Scientific, Waltham, MA, USA), and genomic DNA samples were stored at -80˚C (7,8).

NGS. Genomic testing was performed in-house using the PleSSision testing platform (Keio University Hospital). Libraries were generated from 50 ng DNA per sample using the Human Comprehensive Cancer Panel, GeneRead DNAseq Panel PCR kit V2, GeneRead DNA Library I Core Kit, and GeneRead DNA Library I Amp Kit (Qiagen), and the library quality was assessed using Agilent High Sensitivity D1000 ScreenTape (Agilent Technologies, Santa Clara, CA, USA). Targeted amplicon exome sequencing was performed using a 160 cancer-related gene panel as described previously (19,20). The targeted regions of all 160 genes were specifically enriched using oligonucleotide probes. The enriched libraries were sequenced using a paired-end (150 bp x2) sequencing method on the Next-Seq sequencing platform (Illumina, San Diego, CA, USA), resulting in a mean depth of 500. The sequencing data were analyzed using the GenomeJack bioinformatics pipeline (Mitsubishi Space Software Co., Ltd., Tokyo, Japan) (http://genomejack.net/) as described previously (20). First, the amino acid change in FFPE samples were checked, and then the same amino acid change in IPWF as FFPE samples were checked. VAF (%)/read depth value of 0 corresponds to a lack of mutations.

Follow-up. Patients were followed up every 3-6 months post-surgery as outpatients. Clinical examinations and laboratory investigations were performed at each hospital visit, and computed tomography scans were performed every 3-6 months. When considering patterns of recurrence, only the first site was documented herein. Locoregional recurrence was defined as recurrence in the remnant pancreas or the surgical bed, such as soft tissue along the celiac or superior mesenteric artery or around the pancreatojejunostomy site. Distant recurrence was stratified into three different categories, namely liver, lung, and peritoneal recurrence. Liver, lung, and peritoneal metastases were defined as a mass detected on computed tomography during the postoperative follow-up. Recurrence was determined by a multidisciplinary team comprising radiologists, hepatobiliary-pancreatic surgeons, and physicians. If recurrence was suspected after a radiological examination, histological confirmation was deemed not mandatory. Diagnostic criteria were based on the Japan Pancreatic Society General Rules for the Study of Pancreatic Cancer, 7th edition.

Results

We performed NGS of genomic tumor-derived DNA from IPWF and formalin-fixed paraffin-embedded (FFPE) samples

of 12 patients with PDAC who underwent surgical procedures between January 2020 and December 2021. The clinical characteristics of the patients are listed in Table I. Two patients (16.7%) had CY(+) status and one patient (8.3%) exhibited intraoperative macro peritoneal dissemination. Of the 12 patients, 11 underwent radical surgery for PDAC. Except for patient No. 4, participants had genetic mutations in the primary tumor, and except for patient No. 2 and 3, the participants had genetic mutations in ascites. Among the patients with genetic mutations in ascites, except for patient No. 4, genetic mutations corresponded to those in the primary lesion.

The gene alteration profiles and clinicopathological findings of the patients are shown in Tables II and III. Actionable gene alterations were detected in two patients (100.0%) with CY(+) status and eight (80.0%) of the ten patients with CY(-) status based on NGS of IPWF samples. Three patients (37.5%) among those with actionable gene mutations in DNA extracted from IPWF samples developed peritoneal dissemination after surgery within approximately 12 months. Detailed information regarding actionable gene alterations, concentration of tumor-derived DNA, and sequence read depth of IPWF and FFPE samples is presented in Table IV. According to the NGS analysis, the most common genomic mutation was in *KRAS* (9 patients, 75.0%), which was detected in tumor-derived DNA from FFPE samples, followed by mutations in *TP53* (3 patients, 25.0%), *SMAD4* (1 patient, 8.3%), and *CDKN2A* (1 patient, 8.3%). Table V lists the driver gene mutations identified in DNA extracted from IPWF and FFPE samples.

Table I. Clinicopathological features of patients enrolled in this study (n=12).

Characteristic	Value
Sex, male/female	8/4
Median age, years (range)	68 (25-90)
Median body mass index, kg/m^2 (range)	20.5 (15.5-26.4)
Neoadjuvant therapy, n (%)	3 (25.0)
Median serum CA19-9, U/ml (range)	62 (2-1,468)
Median serum CEA, ng/ml (range)	3.3 (1.7-8.3)
Resectability[a], n (%)	
R	9 (75.0)
BR-PV	2 (16.7)
BR-A	1 (8.3)
Surgical procedure, n (%)	
Pancreaticoduodenectomy	6 (50.0)
Distal pancreatectomy	5 (41.7)
Laparotomy	1 (8.3)
Intraabdominal exploration, n (%)	
CY0P0	10 (83.3)
CY1P0	1 (8.3)
CY1P1	1 (8.3)
Pathological findings[b,c], n	
T 1/2/3/4[c]	1/0/10/0
N 0/1/2[c]	5/0/6
Stage IA/IB/IIA/IIB/III/IV	1/0/3/6/0/2

[a]Radiological resectability was based on Union for International Cancer Control; [b]based on Union for International Cancer Control; [c]patients who underwent curative surgery. BR-A, borderline resectable pancreatic cancer with artery involvement; BR-PV, borderline resectable pancreatic cancer with portal vein involvement; CA19-9, carbohydrate antigen 19-9; CEA, carcinoembryonic antigen; CY, intraoperative peritoneal washing cytology; P, intraoperative peritoneal dissemination; R, resectable.

Discussion

We used NGS to investigate genomic mutations in tumor-derived DNA obtained from IPWF samples. This is the first report on actionable gene alterations, concentration of tumor-derived DNA, and sequence read depth determined using NGS of tumor-derived DNA from IPWF and FFPE samples of patients with PDAC who intended to undergo curative pancreatectomy. A comparison of the CY status revealed that some CY(-) patients had genomic mutations, particularly *KRAS* mutations, in tumor-derived DNA extracted from IPWF samples and some patients exhibited peritoneal dissemination post-surgery. Our findings suggest that the genomic mutation status of tumor-derived DNA extracted from IPWF samples is a candidate PDAC biomarker for predicting the recurrence of early peritoneal dissemination.

Several studies (13-17) have reported the effectiveness of liquid biopsy from body cavity fluids, such as pleural, pericardial, and peritoneal fluid, for performing NGS. Thus, body cavity fluids are a promising source for genomic analysis and may help expand cytomolecular practices. Here, we focused on the peritoneal fluid, as previous studies have not used peritoneal washing fluid from patients with PDAC who intended to undergo radical surgery. Chiba *et al* (14) performed NGS to analyze the ascites fluid from 13 patients. Among them, 11 patients had CY (+) status, seven showed distant metastases, and they had genomic mutations in *KRAS*, *TP53*, GNAS, SMAD4, and *CDKN2A*. Bae *et al* (16) performed NGS to analyze the ascites fluid from 13 patients whose CY was malignant or suspected of being malignant [i.e., CY(+)]; 6 patients with stage IV pancreatic cancer exhibiting distant metastases harbored genomic mutations in *KRAS*, *TP53*, and *CDH*. The concentration of tumor-derived DNA extracted from ascites fluid may be high; hence, the VAF of the genomic mutations was higher than that in CY(+) patients in our study. Our results demonstrated that genomic mutations could be detected using NGS of tumor-derived DNA extracted from IPWF samples of patients with PDAC in earlier stages of the disease.

Moreover, we used tumor-derived DNA extracted from floating cells collected from peritoneal washing fluid. Chiba *et al* used tumor-derived DNA extracted from cell-free DNA (cfDNA) and showed that the cfDNA sample obtained from IPWF samples can be used to characterize the genetic profiles of tumor cells involved in peritoneal dissemination (14). They used cfDNA from IPWF samples of patients with PDAC who underwent curative pancreatectomy and performed NGS and droplet digital PCR (ddPCR). In contrast, we focused on floating cells collected from IPWF samples owing to their high concentration and stability. The concentration of tumor-derived DNA from IPWF samples is lower

Table II. Genomic mutation profile of tumor-derived DNA from the IPWF and FFPE.

Gene mutations	Patient No.																							
	1		2		3		4		5		6		7		8		9		10		11		12	
VAF %	IPWF	FFPE	IPWF	FFPE	IPWF	FFPE	IPWF	FFPE	IPWF	FFPE	IPWF	FFPE	IPWF	FFPE	IPWF	FFPE	IPWF	FFPE	IPWF	FFPE	IPWF	FFPE	IPWF	FFPE
KRAS	0.21	12.0		5.4		16.4	0.14		0.43	27.7		14.6	0.07	17.5	0.03	23.5	0.12	12.6	1.39	19.7	1.65	31.4	1.47	17.6
TP53		10.1		5.1						28.4	0.07	15.4		35.9			0.09	13.5		22.1		25.8	1.27	18.6
SMAD4				6.5								16.4											0.90	18.8
CDKN2A																							N/A	10.9
NF1										21.2							0.32	33.3						
KMT2D																			1.49	16.3				
KDM6A											N/A	41.0												
SETD2												13.1												
SRC																16.2								

DNA, deoxyribonucleic acid; FFPE, formalin-fixed and paraffin-embedded; IPWF, intraoperative peritoneal washing fluid; VAF, variant allele frequency; N/A, not applicable.

than that from peritoneal washing fluid samples, and cfDNA concentration from IPWF samples is also low. The concentration of tumor-derived DNA in floating cells from IPWF samples is typically higher than that of cfDNA (14), enabling the evaluation of the former in IPWF samples and obtaining a sufficient concentration of tumor-derived DNA with high quality and purity suitable for NGS. Hence, tumor-derived DNA from floating cells in IPWF may be clinically useful.

In the present study, the most frequently mutated gene was *KRAS*, which encodes a member of the RAS family of GTPases. Four driver genes, *KRAS*, *TP53*, *SMAD4*, and *CDKN2A*, were identified as representative cancer-related genes in PDAC, with *KRAS* being the most commonly mutated gene and associated with a poor prognosis (21,22). Although the VAF of these genes varied among patients, *KRAS* was the easiest gene to detect using inexpensive equipment (23). For detecting *KRAS* mutations, ddPCR is highly sensitive, cost-effective, and rapid, and requires only a small amount of DNA. Liquid biopsy using ddPCR for detecting *KRAS* mutations in patients with PDAC using FFPE or blood samples has been reported (24). Our NGS results revealed *KRAS* mutations in tumor-derived DNA from floating cells obtained from IPWF samples of patients with PDAC who intended to undergo curative pancreatectomy; however, it is an expensive analysis. Therefore, ddPCR analysis of *KRAS* mutations using tumor-derived DNA extracted from floating cells in IPWF samples is an innovative and direct strategy that is highly sensitive and cost-effective in clinical situations.

The present study was a case series and could not reveal whether genomic mutations, particularly *KRAS* mutations, identified in tumor-derived DNA from IPWF samples could predict survival rate, early recurrence, or treatment response. However, three (37.5%) of the eight patients who were CY(-) and harbored actionable gene mutations in their IPWF sample DNA developed peritoneal dissemination within approximately 1-year post-surgery. Thus, genomic mutations in tumor-derived DNA from IPWF samples may be useful biomarkers for predicting early recurrence of peritoneal dissemination and determining whether chemotherapy should be performed or continued. Recently, a phase I/II study in Japan evaluated the efficacy of adding intraperitoneal paclitaxel to the new treatment strategy for patients with PDAC and peritoneal dissemination (25); this treatment showed clinical efficacy with acceptable tolerability. Moreover, in this phase I/II study, a peritoneal access port was implanted in the lower abdomen of participants and multiple tests were made possible by the implantation of this port. Using this type of port, the status of ascites can be evaluated many times throughout the treatment course for PDAC. Further studies are needed to identify the efficacy of NGS for ascites multiple times.

In the present study, peritoneal washing fluid was evaluated via cytology to determine the treatment response and inclusion criteria for conversion surgery. Liquid biopsy is used to monitor minimal residual disease in various malignant diseases (26); therefore, if the genomic status is used to determine the treatment response or inclusion criteria for conversion surgery, it may be possible to prevent or inhibit the progression of peritoneal dissemination and thus, improve the survival rate of patients with PDAC. Moreover, for patients at high risk for early recurrence, adjuvant chemotherapy should be improved

Table III. Clinicopathological findings of each patient.

Clinicopathological findings	Patient No.											
	1	2	3	4	5	6	7	8	9	10	11	12
Neoadjuvant treatment	-	+	-	-	-	-	+	-	-	-	-	+
Preoperative CA19-9, U/ml	38	<2	646	86	887	16	14	6	219	141	1468	7
Preoperative CEA, ng/ml	5.3	3.1	8.3	2.8	4.3	3.5	2.2	7	2.7	3.9	6.8	2
Genomic mutations in tumor-derived DNA from FFPE	+	+	+	-	+	+	+	+	+	+	+	+
Genomic mutations in tumor-derived DNA from IPWF	+	-	-	+	+	+	+	+	+	+	+	+
Conventional cytology status	-	-	-	-	-	-	-	-	-	-	+	+
Intraoperative macro peritoneal dissemination	-	-	-	-	-	-	-	-	-	-	-	+
Pathological stage	IA	IIA	IIA	IIA	IIA	IIB	IIB	IIB	IIB	IIB	IIB	IV
Recurrence			+		+		+	+	+	+	+	
Peritoneal dissemination			+		+			+		+	+	
Liver					+			+	+			
Lung								+			+	
Remnant pancreas			+									
Extraregional lymph node metastasis									+			
Locoregional							+					
Postoperative observation period, months	4.2	2.5	9.7	15.4	3.9	4.5	8.9	12.2	14.7	6.5	10.7	

CA19-9, carbohydrate antigen 19-9; CEA, carcinoembryonic antigen; DNA, deoxyribonucleic acid; FFPE, formalin-fixed and paraffin-embedded; IPWF, intraoperative peritoneal washing fluid; VAF, variant allele frequency.

by administering modified-FOLFIRINOX or gemcitabine plus nab-paclitaxel (27,28). In the era of precision medicine, genomic mutations identified in tumor-derived DNA from IPWF samples can be used to identify patients at a high risk of early recurrence of peritoneal dissemination. Genomic mutations identified in tumor-derived DNA from blood samples or body cavity fluids may offer various treatment options based on the risk factor for the recurrence type.

In the present study, we used an amplicon-based NGS method, which has numerous advantages compared to hybridization capture methods (29). Amplicon-based approaches offer a simpler, faster workflow with high PCR specificity, allowing the enrichment of target gene regions using a low sample input. Genetic material from limited sample sources, such as IPWF, can be sequenced for biomarker discovery. Furthermore, amplicon-based approaches are useful for sequencing specific regions of interest, such as hotspots in *KRAS*, at high coverage depths. Here, the read depth of the *KRAS* mutation hotspot was approximately 6,400 although the mean depth was lower than 500.

Among patients with genetic mutations in ascites, except for patient No. 4, genetic mutations corresponded to the mutations in the primary lesion, that is, we did not observe heterogeneity between FFPE and IPWF samples. Hashimoto *et al* (30) also revealed no heterogeneity between primary tumors and lymph node metastases in patients with PDAC. Baldus *et al* (31) also reported heterogeneity in KRAS between primary tumors and lymph node metastases in 31% of cases of colorectal adenocarcinoma. PDAC might be homogenous in terms of KRAS mutation and oncogenic activation of KRAS can be the first driver mutation in PDAC (30,32).

This study has some limitations. First, we conducted a retrospective study of a case series at a single institution and no statistically significant analysis was found in this study due to the small sample size because the cost of NGS of IPWF or some liquid samples is relatively high. This examination is not covered by the national health insurance program. A multi-center validation and prospective study is needed to investigate whether these genomic mutations identified in tumor-derived DNA from IPWF samples are candidate PDAC biomarkers for predicting survival rate, early recurrence, or treatment response and to obtain statistically significant data from the prospective study about these biomarkers in clinical situation. Our findings should help us take the first step to ensure that NGS for IPWF from the patients who intend to undergo curative resection becomes a common procedure if the cost issue is overcome. Second, in amplicon-based sequencing, the read depth of variants, except for hotspots in *KRAS*, may be low, leading to false-negative results. Thus, *KRAS* should be targeted because it is the most commonly mutated gene and may be useful as a pancreatic cancer biomarker (7,8). However, other genomic mutations in tumor-derived DNA from IPWF samples may also be suitable biomarkers for PDAC. For instance, mutations in *TP53*, *SMAD4*, and *CDKN2A* were detected at higher read coverages of hotspots in these genes. According to the restriction of the amplicon-based NGS, the detection rate of these genomic mutations differs. Considering the read coverage distribution, *TP53*, *SMAD4*, and *CDKN2A* are driver genes that are potential PDAC biomarkers. However, focusing only on *KRAS* may be cost-effective in clinical situations. Driver genes for PDAC can be evaluated using amplicon-based enrichment techniques if the read coverage

Table IV. Details of actionable gene alterations, concentration of tumor-derived DNA, and sequence read depth of IPWF and FFPE samples (n=12).

	IPWF				FFPE			
No.	DNA concentration, ng/μl	Gene	Amino acid change	VAF, %[a]	DNA concentration, ng/μl	Gene	Amino acid change	VAF, %[a]
1	139.9	KRAS	G12D	0.21 (8/3,900)	53.0	KRAS	G12D	12.0 (45/376)
		TP53	R158H	0 (0/479)		TP53	R158H	10.1 (37/368)
2	262.3	KRAS	G12R	0 (0/6,422)	167.8	KRAS	G12R	5.4 (32/590)
		TP53	R175H	0 (0/160)		TP53	R175H	5.1 (22/428)
		SMAD4	P318Afs*4	0 (0/1,594)		SMAD4	P318Afs*4	6.5 (36/556)
3	52.8	KRAS	G12V	0 (0/7,214)	403.8	KRAS	G12V	16.4 (98/596)
4	529.6	KRAS	G12D	0.14 (9/6,512)	96.0	KRAS	wt	0 (0/590)
5	43.1	KRAS	G12V	0.43 (3/691)	223.7	KRAS	G12V	27.7 (423/1,527)
		TP53	R196*	0 (0/596)		TP53	R196*	28.4 (201/707)
		NF1	R1325K	0 (0/341)		NF1	R1325K	21.2 (11/52)
6	176.2	KRAS	G12V	0 (0/5,600)	469.1	KRAS	G12V	14.6 (62/425)
		TP53	Y220C	0.07 (2/3,000)		TP53	Y220C	15.4 (21/136)
		SMAD4	R361Afs*23	0 (0/2,253)		SMAD4	R361Afs*23	16.4 (80/489)
		KDM6A	S121Lfs*6	N/A		KDM6A	S121Lfs*6	41.0 (59/144)
		SETD2	R1407fs*8	0 (0/2,100)		SETD2	R1407fs*8	13.1 (41/313)
7	78.5	KRAS	G12V	0.07 (4/6,062)	379.2	KRAS	G12V	17.5 (255/1,453)
		TP53	R175H	0 (0/133)		TP53	R175H	35.9 (14/39)
8	1,185.6	KRAS	G12C	0.03 (2/6,948)	167.1	KRAS	G12C	23.5 (338/1,436)
		SRC	T542H	0 (0/702)		SRC	T542H	16.2 (24/148)
9	207.8	KRAS	G12D	0.12 (8/6,463)	113.7	KRAS	G12D	12.6 (185/1,467)
		TP53	R273C	0.09 (4/4,265)		TP53	R273C	13.5 (138/1,026)
		NF1	R1325K	0.32 (2/628)		NF1	R1325K	33.3 (16/48)
10	137.1	KRAS	G12R	1.39 (76/5,471)	322.7	KRAS	G12R	19.7 (222/1,127)
		TP53	V173M	0 (0/57)		TP53	V173M	22.1 (102/461)
		KMT2D	R2734*	1.49 (6/401)		KMT2D	R2734*	16.3 (15/92)
11	29.8	KRAS	G12D	1.65 (121/7,344)	153.7	KRAS	G12D	31.4 (796/2,535)
		TP53	R175H	0 (0/73)		TP53	R175H	25.8 (17/66)
12	303.3	KRAS	G12D	1.47 (96/6,523)	90.9	KRAS	G12D	17.6 (96/545)
		TP53	E271K	1.27 (54/4,255)		TP53	E271K	18.6 (16/86)
		SMAD4	E538*	0.90 (26/2,880)		SMAD4	E538*	18.8 (83/441)
		CDKN2A	P72Rfs*100	N/A		CDKN2A	P72Rfs*100	10.9 (13/119)

[a]Data in brackets show sequence reads observed matching a specific DNA variant/the overall coverage at that locus. FFPE, formalin-fixed and paraffin-embedded; IPWF: intraoperative peritoneal washing fluid; VAF, variant allele frequency; N/A, not applicable; Wt, wild type.

Table V. Detection, VAF and read depth of KRAS, TP53, SMAD4, and CDKN2A among tumor-derived DNA from IPWF and FFPE samples (n=12).

Category	Sample	KRAS	TP53	SMAD4	CDKN2A
Detection, n (%)	IPWF	9 (75.0)	3 (25.0)	1 (8.3)	N/A
	FFPE	11 (91.7)	9 (75.0)	3 (25.0)	1 (8.3)
Median VAF, % (range)	IPWF	0.21 (0.03-1.65)	0.09 (0.07-1.27)	0.90	N/A
	FFPE	17.0 (0-31.4)	18.6 (5.1-35.9)	16.4 (6.5-18.8)	10.9
Median read depth (range)	IPWF	6,443 (691-7,344)	479 (57-4,265)	2,253 (1,594-2,880)	N/A
	FFPE	862 (376-2,535)	368 (39-1,026)	489 (441-556)	119

FFPE, formalin-fixed and paraffin-embedded; IPWF, intraoperative peritoneal washing fluid; VAF, variant allele frequency; N/A, not applicable.

of these genes is improved. Third, a few cases in which the primary tumor is negative for KRAS mutation but positive for cytology have been documented as NGS can identify genetic mutations other than those in KRAS and cytology can identify morphological changes in tumor cells by microscopic examination. This morphological change is due to nuclear and structural atypia, so the patient may have CY(+) even if the primary tumor is negative for KRAS mutation because there is a possibility that the primary tumor is positive for other genomic mutations, such as in *TP53*, *SMAD4*, or *CDKN2A* (8). Fourth, no specific measures were taken to address bias during this study. This study included consecutive patients who were required to meet inclusion criteria which demanded the availability of DNA extracted from both FFPE specimens and IPWF. Finally, we acknowledge that validation using an independent method can provide additional confidence in the results. However, given the high accuracy of modern NGS technologies and the additional cost and time associated with performing additional validation experiments, we opted to not perform independent validation in this study. We believe that the quality control measures and bioinformatics analyses employed provide sufficient certainty for the accuracy of our results.

In conclusion, we identified genomic mutations in tumor-derived DNA from IPWF samples of patients with PDAC who were CY(-) and intended to undergo curative resection. These may be candidate PDAC biomarkers for predicting the recurrence of early peritoneal dissemination. Further studies are warranted to determine the clinical relevance of these findings and investigate whether these genomic mutations are predictive clinical biomarkers of PDAC.

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Availability of data and materials

The raw sequence data generated in the present study may be found in the Genome Sequence Archive (Genomics, Proteomics & Bioinformatics 2021) in National Genomics Data Center (Nucleic Acids Res 2022), China National Center for Bioinformation/Beijing Institute of Genomics, Chinese Academy of Sciences under accession number GSA-Human: HRA006272) or at the following URL: https://bigd.big.ac.cn/gsa-human/browse/HRA006272. The other data generated in the present study may be requested from the corresponding author.

Authors' contributions

YN, GS, KN, YM, MK, HN and YK conceived and designed the study. YN and GS drafted the manuscript. RT, EA, HY, YA, YH, SH and MT analyzed the data and critically revised the manuscript. KN and HN confirm the authenticity of all the raw data. All authors were involved in the data interpretation and drafting of the manuscript and have read and approved the final version of the manuscript.

Ethics approval and consent to participate

This study was approved by the Human Experimentation Committee of Keio University Hospital (Tokyo, Japan; approval nos. 20120443 and 20170086) and performed in accordance with the tenets of the Declaration of Helsinki (2013). All participants provided written informed consent to participate in the study. Samples were analyzed retrospectively to patient recruitment.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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