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Utility of "liquid biopsy" using pancreatic juice for early detection of pancreatic cancer

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ABSTRACT

Background Despite advances in the diagnosis of pancreatic ductal adenocarcinoma (PDA), histological evaluation of small and poorly defined masses in the pancreas is uncomfortable and unsafe.

Methods We herein report a case of early stage PDA, in which multiple KRAS mutations were detected in the pancreatic juice preoperatively. A small hypoechoic area adjacent to the portal vein was detected through endoscopic ultrasound in the pancreatic body. KRAS mutations were evaluated using plasma, and the pancreatic juice by digital PCR.

Results Pancreatic duct biopsy and pancreatic juice cytology were performed with no evidence of malignancy; however, KRAS mutations, KRAS G12V and G12D, were detected in the pancreatic juice. Histological assessment of the resected specimen demonstrated a solid tumor with desmoplastic reaction accompanied by carcinoma in situ in the main pancreatic duct where KRAS G12V mutation was identified. More detailed analysis demonstrated KRAS G12D mutation in the cluster of low grade pancreatic intraepithelial neoplasia, implying that the lesion developed independently.

Conclusions Our study indicates the potential of "endoscopic liquid biopsy" to capture the driver gene for PDA diagnosis.

Introduction

Pancreatic ductal adenocarcinoma (PDA) is one of the most dismal types of cancer, and novel methods for early detection are highly warranted [1]. The standard diagnostics for PDA include tumor tissue collection during endoscopy and, in particular, the sensitivity and specificity values of endoscopic ultrasound-guided fine needle aspiration/biopsy (EUS-FNA/B) are over 90 % [2]. However, tissue sampling sometimes results in inadequate examination due to insufficient material. It is also challenging to fully uncover tumor heterogeneity using a small piece of specimen. Another issue of the modalities is severe complications, reported in up to 1 %–2 % and 12.0 % of EUS-

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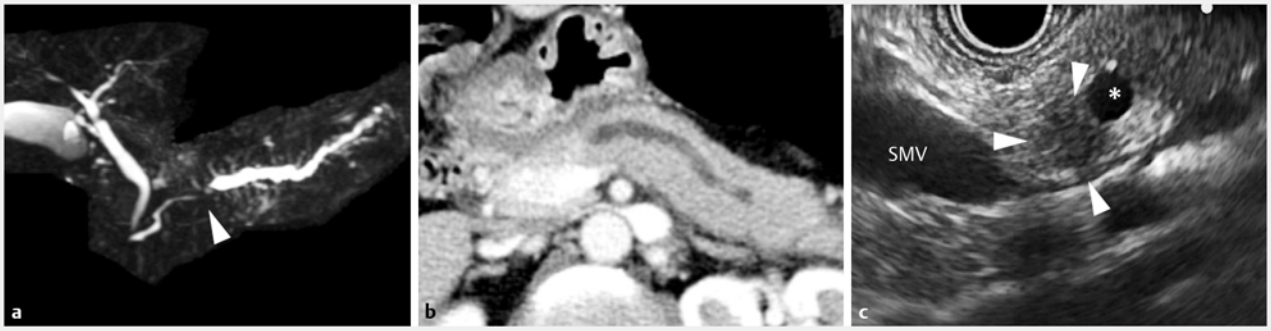


▶ **Fig. 1** Imaging diagnosis of the primary pancreatic tumor. **a** Magnetic resonance cholangiopancreatography (MRCP) demonstrates obstruction of the main pancreatic duct (MPD; arrowhead) accompanied by distal dilatation. **b** CT shows dilation of the MPD with no sign of the solid tumor. **c** Hypoechoic area is visualized by endoscopic ultrasound in the body of the pancreas (surrounded by arrowheads; 8 mm in diameter) connected to the dilated MPD (asterisk). Note that there are no other detectable tumors or cystic lesions. MPD; main pancreatic duct, SMV; superior mesenteric vein.

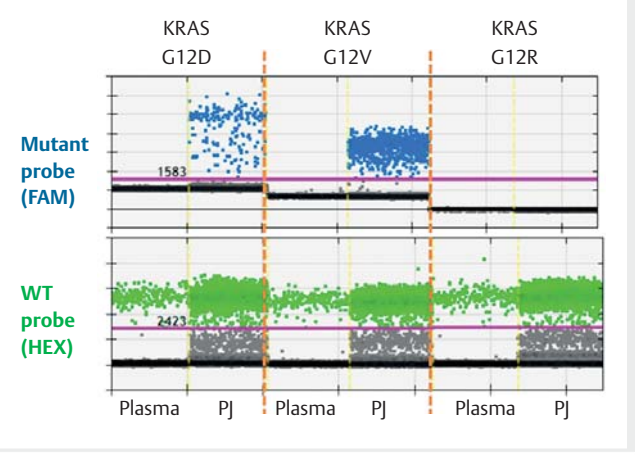
FNA/B and endoscopic retrograde cholangiopancreatography (ERCP)-related procedures, respectively [3].

In patients with cancer, the presence of cell-free DNA (cfDNA) in the blood and its potential utilities have been recognized, and their clinical relevance has attracted widespread attention [4]. The primary limitation of such "liquid biopsy" is the modest availability of circulating tumor-derived DNA. Given the extremely low tumor cellularity, detecting tumor-derived DNAs in the blood of patients is challenging [5]. Here, we describe a case of early pancreatic cancer with successful detection of mutant KRAS variants in the pancreatic juice collected through the Vater-papilla during endoscopic cytology. The latest analytical tools for tumor genotyping are powerful enough to identify the tumor-derived DNA, even from early lesions in the pancreatic juice.

Case report

A 53-year-old woman was referred to our hospital due to a complaint of epigastric and dorsal pain. Before the genetic study, written informed consent was obtained from the patient for publication of the case. The laboratory data showed a slight elevation of the pancreatic enzymes (110 U/L) and normal levels of carbohydrate antigen 19 – 9 (CA19 – 9) and IgG4. Images of the pancreas obtained using ultrasound, CT, and MRI showed dilation of the main pancreatic duct (MPD; 6 mm) with no sign of the solid tumor (▶ **Fig. 1a**). EUS revealed an 8-mm hypoechoic area in the body of the pancreas connected to the dilated MPD (▶ **Fig. 1b**).

The patient was informed about the possible malignant tumor closing the portal vein; however, she refused to undergo FNA/B testing for histological assessment. Instead, a biopsy from the stricture of the MPD and pancreatic juice cytology were performed to diagnose the tumor, resulting in no evidence of malignancy. Pancreaticoduodenectomy was selected as the treatment option for this tumor, which was highly suspected to be PDA.



▶ **Fig. 2** Droplet digital PCR confirming KRAS mutation in the pancreatic juice. KRAS (exon 2)-specific PCR fragments in cell-free DNA from the plasma and pancreatic juice are analyzed by droplet digital PCR. Mutation in KRAS is selectively detected by mutant-specific probe against mutant KRAS at codon 12/13 (upper panel). A larger p.G12V mutant frequency is demonstrated in the pancreatic juice. Additionally, the presence of a smaller proportion of p.G12D mutant alleles is also shown, whereas no signals for other KRAS variants are detected (result using p.G12R-specific probe is shown). PJ, pancreatic juice; FAM, 6-carboxyfluorescein; HEX, hexachloro-6-carboxyfluorescein.

Before the surgical intervention, we performed a genetic test in a droplet digital PCR platform to capture mutant KRAS and GNAS using the pancreatic juice (500 µL). The fresh pancreatic juice collected via the ERCP catheter was frozen immediately after the procedure (within 10 min). In the purified DNA, two major variants, KRAS G12V (13.3 %) and G12D (3.0 %), were detected (▶ **Fig. 2**), whereas mutant GNAS was not detected (see the Supplementary Method for details). We also used the pancreatic juice drained through the endoscopic nasopancreatic drainage tube, a liquid left behind out of the organ

for several hours; however, the yield of DNA was much lower than the fresh sample and too heavily fragmented for assay.

In the resected specimen, focal fibrosis was found proximal to the MPD stricture, and the size of the fibrotic area corresponded well with the hypoechoic lesion visualized by EUS (▶ **Fig. 3a**). Pathologically, well-differentiated adenocarcinoma was evident with no sign of metastasis (stage IA; UICC 8th edition).

We next performed additional genetic analysis using a resected specimen; in the main tumor with dense desmoplasia, KRAS G12V mutation was demonstrated by targeted amplicon sequencing (▶ **Fig. 3b, c** and ▶ **Supplementary Table 1**; see the Supplementary Methods for details). The series of lesions continuing to the MPD possessed an identical type of KRAS mutation, suggesting a cancerization from the primary PDA (▶ **Fig. 3d, e**). Other abnormal ductal structures corresponding to acinar-to-ductal metaplasia (ADM) to low grade pancreatic intraepithelial neoplasias were noted (PanINs; ▶ **Fig. 3f, g**). These lesions were micro-dissected and sequenced. Interestingly, some lesions harbored KRAS G12D, a distinct type of variant from the main tumor, indicating an independent development of additional precursor(s). All tumors had no other mutations in the common PDA-related tumor suppressor genes, such as TP53, CDNK2A/p16, SMAD4, or RNF43. Mutations in GNAS were also not evident in the tumors including abnormal ductal structures in the normal looking pancreas.

Discussion

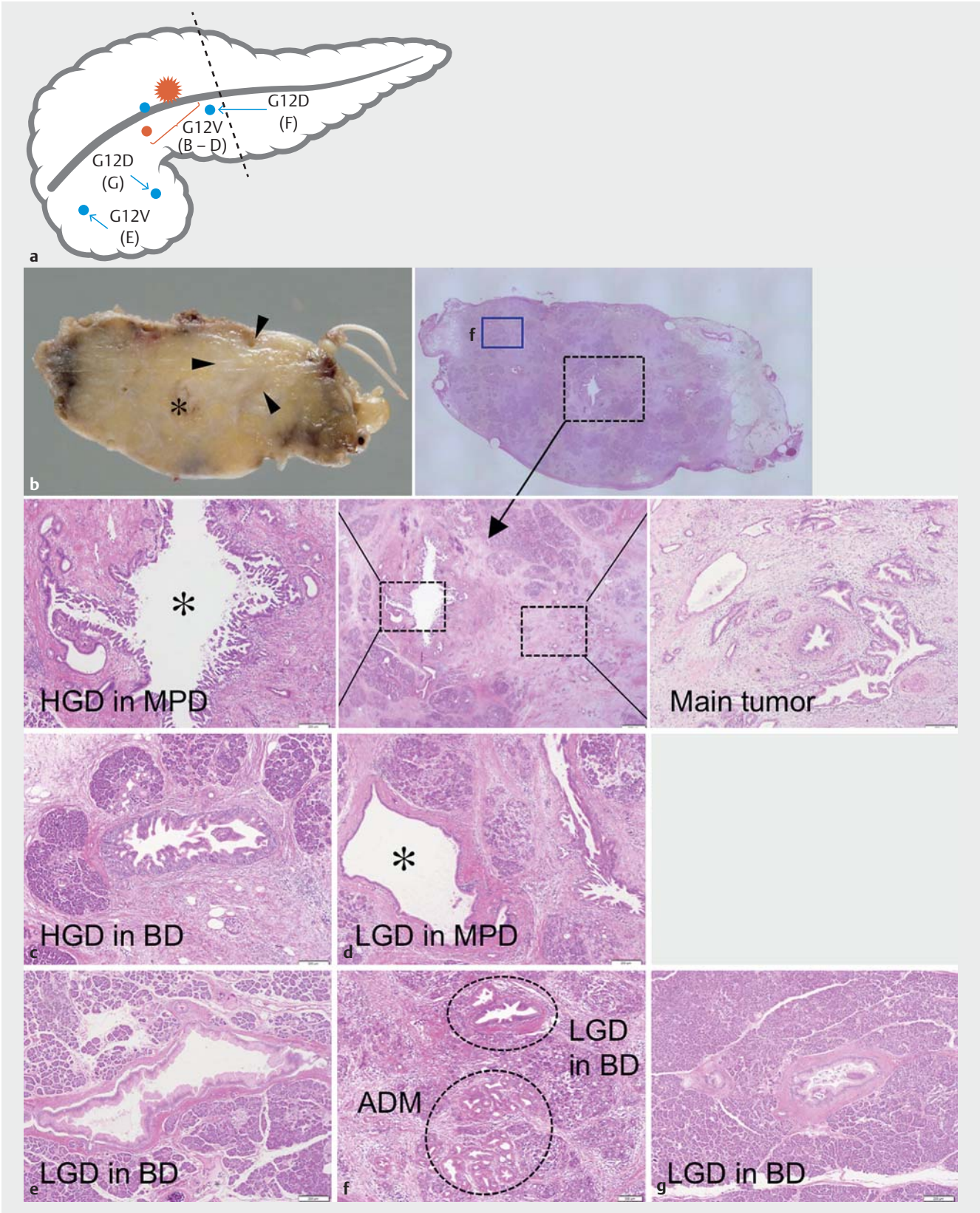
Reliable and feasible detection of early stage pancreatic cancer and premalignant lesions, such as PanIN, will greatly aid in patient survival. However, obtaining trustworthy evidence for the malignancy is not always easy, especially when a small lesion is targeted. Recently, cell-free DNA in plasma has attracted attention as a diagnostic tool for various types of cancer [4]. In human PDAs, mutations in KRAS are found in over 90 % of patients [1], and significant effort has been made to detect the driver gene in patient specimens, such as pancreatic juice or plasma [5]. In the current report, KRAS genotyping of pancreatic juice indicated the presence of "neoplasia" in the pancreas, although pancreatic duct biopsy and pancreatic juice cytology did not provide morphological evidence of malignancy. Insufficient material is a common reason for failure of cytology/biopsy, and diagnostic accuracy is hindered by the invasiveness of tissue collection. In contrast, a genetic approach can detect the tumor-associated alterations irrespective of the tumor size, providing very sensitive diagnostics relative to conventional methods.

It should be noted that, in the current case, two types of KRAS variants were detected in the pancreatic juice. Among the precursor lesions of invasive pancreatic cancer, PanIN may progress to PDA sequentially from a monoclonal precursor, whereas intraductal papillary mucinous neoplasms (IPMNs) are frequently accompanied by multi-centric clones [6]. Interestingly, in our case, targeted amplicon sequencing of the resected specimen demonstrated that the primary PDA and cancerization observed in the main duct were marked by KRAS G12V, and other small areas composed of low grade PanIN (LG-PanIN) associated with ADM on the caudal side of the tumor harbored KRAS G12D. Imaging studies before the surgical intervention did not detect either IPMNs or other satellite lesions to the hypoechoic area; however, genetic tests using pancreatic juice and resected specimens provide evidence of multi-centric clones within the pancreas. Additionally, the KRAS G12V mutation was found in other LG-PanINs located in branch ducts distant from the primary PDA. Such lesions may also develop independently from the primary PDA, and the "shared" mutation in KRAS may be incidental, although the possibility of ductal spread (early dissemination via the pancreatic duct) cannot be entirely excluded [7].

Detection of a KRAS mutation alone is not sufficient for a definitive diagnosis of PDA, since it is the earliest genetic event during tumorigenesis and most precursor lesions harbor the abnormality [8]. Additional mutations and aberrant protein expression of the tumor suppressor genes, such as TP53 and SMAD4, can accumulate [1], and significant time, perhaps over a decade, may be required for progression from the emergence of the initiating mutation to the acquisition of malignant potential [9]. On the other hand, a small subset of PDAs lack the typical stepwise mutations and aberrant protein expressions [10]. Indeed, a smaller number of mutations in the major PDA-associated driver genes, KRAS, CDKN2A, TP53, and SMAD4, is an independent predictor of better overall survival [11]. Therefore, positive KRAS mutations with no other genetic abnormalities observed by liquid biopsy indicate a wide range of conditions from an occurrence of the earliest precursor to PDA with metastatic potential.

Endoscopy is an indispensable tool for the detection of small PDAs, and advanced options, such as genetic and molecular tests, may further enhance diagnostic accuracy. Although tissue collection by EUS-FNA/B is a standard procedure for providing histological evidence of the tumor, the possibility of procedure-related dissemination has to be borne in mind, specifically for candidates with a high probability of a cure [12]. A genetic test may compensate for the invasiveness with variable sensitivity and specificity depending on the source of the samples (i. e. plasma, duodenal and pancreatic juice, and other body fluids). It is necessary to integrate advanced imaging and feasible molecular profiling for precise diagnosis of the tumor.

We report a case of an early stage PDA with genetic evidence of neoplasia (i. e. KRAS mutation) in pancreatic juice before surgical intervention. Sequencing study of the resected specimens indicated the utility of "liquid biopsy" for diagnosis when the findings from pathological examination are not informative. Early detection of PDA is still challenging, and more accurate and less invasive options that can uncover both tumor grade and heterogeneity may bring out the full potential of endoscopic diagnostics without hampering the possibility of a cure.



▶ **Fig. 3** Gross appearance of the resected pancreas and pathological mapping of the main tumor and microscopic lesions. **a** The whole resected specimen is used to define the distribution of the lesions. The primary ductal adenocarcinoma (PDA; red star) is surrounded by multiple low grade (LGD; blue circles) and high grade dysplasias (HGD; red circles) as illustrated. Dashed line indicates resection margin. **b** Macroscopic findings for the main tumor. **c** – **g** Microscopic lesions associated with the main tumor. The main PDA and closely located HGD in the MPD harbor KRAS G12V (**b**). In the surrounding normal-looking pancreas, LGD and HGDs with KRAS G12V are identified (**c** – **e**), whereas LGDs, one of which is accompanied by acinar-ductal metaplasia, are marked by KRAS G12D (**f**, **g**). MPD; main pancreatic duct, BD; branch duct. Asterisk indicates MPD.

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Competing interests

None

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▶ **Supplementary Table 1** Result of multiregion sequencing (targeted amplicon sequencing).

	Main tumor (B)	HGD in MPD (B)	HGD (C)	LGD in MPD (D)	LGD (E)	LGD (F)	ADM (F)	LGD (G)
KRAS	G12V (10.3 %)[1]	G12V (11.5 %)[1]	G12V (19.2 %)[1]	G12V (1 %)[1]	G12V (2 %)[1]	G12D (5.2 %)[1]	G12D (2 %)[1]	G12D (1 %)[1]
TP53	WT	WT	WT	WT	WT	WT	WT	WT
CDKN2A	WT	WT	WT	WT	WT	WT	WT	WT
SMAD4	WT	WT	WT	WT	WT	WT	WT	WT
GNAS	WT	WT	WT	WT	WT	WT	WT	WT
RNF43	WT	WT	WT	WT	WT	WT	WT	WT
BRAF	WT	WT	WT	WT	WT	WT	WT	WT
PIK3CA	WT	WT	WT	WT	WT	WT	WT	WT
STK11	WT	WT	WT	WT	WT	WT	WT	WT
IDH1	WT	WT	WT	WT	WT	WT	WT	WT
CTNNB1	WT	WT	WT	WT	WT	WT	WT	WT
MAP2K4	WT	WT	WT	WT	WT	WT	WT	WT
TGFBR1	WT	WT	WT	WT	WT	WT	WT	WT
TGFBR2	WT	WT	WT	WT	WT	WT	WT	WT
ARID1A	WT	WT	WT	WT	WT	WT	WT	WT
SF3B1	WT	WT	WT	WT	WT	WT	WT	WT
RBM10	WT	WT	WT	WT	WT	WT	WT	WT
KDM6A	WT	WT	WT	WT	WT	WT	WT	WT

LGD, low grade dysplasia; HGD, high grade dysplasia; ADM, acinar-to-ductal metaplasia; WT, wild-type.
[1] Values in parentheses indicate the multiregions shown in ▶ **Fig. 3**.

Supplementary methods

Pancreatic juice collection and cfDNA extraction

Pancreatic juice (< 1 mL) was used, immediately frozen (– 80 °C) after collection via the transpapillary route using an ERCP catheter and drained through an endoscopic nasopancreatic drainage tube. DNA was isolated using the QIAamp Circulating Nucleic Acids Kit (Qiagen; Hilden, Germany) according to the manufacturer's instructions. The sample was quantified using the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific; Waltham, Massachusetts, United States) and a Qubit2.0 fluorometer (Thermo Fisher Scientific).

Mutation detection by digital PCR

Mutant KRAS variants (codons 12 and 13) or mutant GNAS variants (codon 201) were analyzed using a QX200 Droplet Digital PCR System (Bio-Rad; Hercules, California, United States) as described previously [1]. Custom probes and primers were designed for eight major mutations in KRAS codons 12 and 13 or GNAS codon 201 [1]. The reaction mixture was prepared as described in ▶ **Supplementary Table 2**. Purified DNA was partitioned into ~22 000 droplets per sample by mixing with 70 µL of Droplet Generation Oil in a QX200 droplet generator (Bio-Rad). Droplets were then subjected to thermal cycling, as detailed in ▶ **Supplementary Table 3**. Samples were transferred to a QX200 droplet reader (Bio-Rad) for fluorescence measurement of 6-fluorescein amidite and hexachlorofluorescein probes. Droplets were scored as positive or negative based on their fluorescence intensity, which was determined by the gating threshold defined using positive and negative controls. Finally, absolute copy number input in the reaction and the ratio of mutated fragments were calculated by QuantaSoft (ver 1.7; Bio-Rad) software based on the Poisson distribution.

Mutation profiling of tumors and abnormal lesions

Each specimen was prepared as formalin-fixed paraffin embedded (FFPE) blocks and slides. Genomic (g) DNA was then purified and isolated using the GeneRead DNA FFPE Kit (Qiagen; Hilden, Germany) according to the manufacturer's instructions, and finally eluted with 30 µL of elution buffer.

Mutation profiles were determined by target amplicon sequencing using a next generation sequencer as described previously [2]. A 20- to 60-ng portion of gDNA was amplified by PCR using an IPMN/PDA-related gene panel which we designed, which consisted of 18 genes, and 220 amplicons (Ion AmpliSeq Custom DNA Panel) (▶ **Supplementary Table 4**). Sequencing was performed using an Ion Personal Genome Machine System and the Ion PGM 200 Sequencing Kit (both from Thermo Fisher Scientific) according to the manufacturer's instructions. Sequence reads were demultiplexed, quality-filtered, and aligned to the human reference genome (GRCh37) using Torrent Suite software (ver. 5.0.4; Thermo Fisher Scientific). Variants were identified with the Variant Caller software (ver. 5.0.4.0; Thermo Fisher Scientific). To identify somatic mutations, independent genotyping of each lesion and normal sample (duodenum) was subtracted; variants found in the normal sample were excluded from the molecular profiling. The variant calling analysis was operated using the somatic variant calling mode optimized to detect low-frequency variants, which was set with the following parameters: minimum allele frequency of 0.02 and minimum coverage of 100. We also excluded putative false-negative variants by evaluating the Phred-scaled variation call quality calculated by this plugin, and by manually confirming the alignment with IGV software (version 2.3.59).

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▶ **Supplementary Table 2** Preparation of ddPCR reaction mixture.

Component	Final concentration
ddPCR Supermix for Probes (no dUTP)	1×
Template DNA[1]	–
Additional dNTP mixture	0.91 mM
Primers (forward and reverse primer)	0.45 µM each
Probes (input as a pair of WT and each mutant probe)	
▪ KRAS WT	0.45 µM
▪ KRAS G12C	0.77 µM
▪ KRAS G12D	0.45 µM
▪ KRAS G12V	0.45 µM
▪ KRAS G13 D	0.05 µM
▪ KRAS G12A	0.68 µM
▪ KRAS G12 R	0.07 µM
▪ KRAS G12S	0.68 µM
▪ KRAS G13C	0.68 µM
▪ GNAS WT	0.45 µM
▪ GNAS R201C	0.34 µM
▪ GNAS R201H	0.45 µM

Total, 22 µL reaction volume.
[1] 1 – 4 µL of purified DNA were utilized.

▶ **Supplementary Table 3** ddPCR thermal cycling conditions.

Step	No. of cycles	Temperature, °C	Time, min
1	1	95	10
2	40	94	0.5
		58 (KRAS)/60 (GNAS)	1
3	1	98	10

▶ **Supplementary Table 4** Targeted regions of the 18 genes explored by the AmpliSeq custom panel.

Gene	No. of amplicons	Total amplicon length, bp
KRAS	4	309
TP53	14	1317
CDKN2A	3	307
SMAD4	11	912
GNAS	2	170
RNF43	36	3349
BRAF	4	342
PIK3CA	4	311
STK11	6	553
IDH1	2	153
CTNNB1	2	152
MAP2K4	12	978
TGFBR1	21	1712
TGFBR2	12	1071
ARID1A	47	2934
SF3B1	8	628
RBM10	15	1371
KDM6A	17	1394