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Genetic assessment of recurrent pancreatic high-risk lesions in the remnant pancreas: Metachronous multifocal lesion or local recurrence? ☆

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ABSTRACT

Background: It is difficult to determine whether a second high-risk lesion, including pancreatic ductal adenocarcinoma or high-grade pancreatic intraepithelial neoplasm, is a metachronous multifocal lesion or represents local recurrence after resection of the first high-risk lesion. This study attempts to clarify the characteristics of second high-risk lesions in the remnant pancreas using genetic analyses.

Methods: Clinicopathologic data were collected from 12 patients who underwent pancreatectomy for a second high-risk lesion in the remnant pancreas. We performed mutational and immunohistochemical analyses of 4 major genes—*KRAS, TP53, CDKN2A*, and *SMAD4*—associated with pancreatic ductal adenocarcinoma progression, as well as targeted next-generation sequencing.

Results: Mutations in the four genes in the second high-risk lesion were consistent with the first lesion in four patients but were inconsistent in the remaining eight patients, and thus we considered that the latter eight patients likely had metachronous multifocal high-risk lesions and the other four patients had local recurrence. The estimated cumulative recurrence rate after resection of the second high-risk lesion was greater in the local recurrence group compared with the metachronous multifocal group, and the estimated cumulative disease-specific survival rate was greater in the metachronous multifocal group. Targeted next-generation sequencing demonstrated that the second lesions in the metachronous multifocal high-risk lesion group showed differences in founder mutations compared with the first lesion. In the local recurrence group, the founder mutations in the second lesion were common with those in the first lesion.

Conclusion: Genetic assessment might help discriminate metachronous multifocal high-risk lesions from local recurrence.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a most lethal solid neoplasm because of its aggressive behavior and the difficulty in early detection. Despite the improvements in operative techniques and perioperative management during recent decades and the slightly increased survival time from newly developed chemotherapeutic agents, the rate of cancer-related death in patients with PDAC remains high.[1]

Genetic assessment can help evaluate the progression of PDAC and thereby provide important information for molecular targeted diagnosis and treatment. “Clonal evolution” is a theory of cancer progression in which founder mutations are passed on to any sites of the next-generation lesions, and progressor mutations vary in the primary lesion or metastatic site of the next generation. This concept of clonal evolution has been used to describe the mechanism of malignant progression of PDAC.[2,3] Mutations in four genes—*KRAS, TP53, CDKN2A*, and *SMAD4*—occur during the progression of PDAC.[4,5] Hosoda et al[6] used targeted next-generation sequencing (NGS) to show that *KRAS* mutations act as founder mutations which initiate the development of premalignant

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pancreatic intraepithelial neoplasia (PanIN), and mutations in *TP53* and *SMAD4* are later events that might accelerate the invasive behavior of PDAC.

Recent advances in diagnostics for early detection of PDAC and multidisciplinary treatment for advanced PDAC have led to a slight increase in the number of long-term survivors after resection.[1] In these patients, a second PDAC or high-grade PanIN in the remnant pancreas after partial pancreatectomy for the first lesion is often observed.[7–11] Using clinicopathologic findings alone, however, it is usually difficult to determine whether this second lesion is a new metachronous, multifocal lesion or a local recurrence of the first lesion. It may prove to be important to distinguish between these two entities, both for clinical management and to further understand the development and progression of PDAC, but most reports have combined these two entities into a single category of "recurrence."[7–9] Therefore, the aim of this study was to distinguish these two entities through molecular assessments, including NGS.

Patients and Methods

This study was approved by the Ethics Committee of Kyushu University (No. 27-126) and conducted according to Ethical Guidelines for Human Genome/Gene Research enacted by the Japanese Government and the Helsinki Declaration.

Patients and clinicopathologic data

The medical records of 411 consecutive patients who were diagnosed histologically with a high-risk lesion (HRL) involving either PDAC or a high-grade PanIN after partial pancreatectomy at the Department of Surgery and Oncology, Kyushu University Hospital, Fukuoka, Japan, between 2000 and 2014 were reviewed retrospectively. High-grade PanIN was included in this study because this lesion is considered to be equivalent to "carcinoma *in situ*" according to the recommendation from the Baltimore Consensus Meeting[12] and because many or most pancreatologists consider it as indication for resection.[6,10,11] Patients who had PDAC possibly arising from intraductal papillary mucinous neoplasm (IPMN) and those who underwent total pancreatectomy were not included in this study. We collected data on age, sex, history of smoking, history of alcohol consumption, comorbid diseases, preoperative values of serum carcinoembryonic antigen and carbohydrate antigen 19-9, tumor location, operation performed, postoperative surveillance, and pathologic findings, including tumor size, pathologic type, T factor, N factor, stage, lymphatic invasion, vascular invasion, neural invasion, and residual tumor (R). All resected specimens were rereviewed and revised, if necessary, by two pathologists (Y.O. and N.M.) experienced in the histopathology of pancreatic neoplasms. Tumor location, operation performed, and pathologic findings were determined according to the Classification of pancreatic carcinoma.[13] Chest–abdominal computed tomography (CT) and serum carcinoembryonic antigen and carbohydrate antigen 19-9 levels were examined periodically during the postoperative surveillance period according to the National Comprehensive Cancer Network guidelines for PDAC.[14]

KRAS mutational analyses

Formalin-fixed, paraffin-embedded (FFPE) tissue samples were collected for each patient. Appropriate tissue blocks were selected, multiple serial sections (10-μm thick) were prepared, and laser microdissection was performed manually. using the Leica LMD6500 system (Leica Microsystems, Wetzlar, Germany). Genomic DNA was extracted using the QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The status of the *KRAS* mutation at codons 12 and 13 was assessed by a direct sequencing method as described elsewhere.[15–17] Briefly, the *KRAS* outer primers for genomic deoxyribonucleic acid (DNA) were 5′-AGGCCTGCTGAAAATGACT-3′ (forward) and 5′-TTGTTGGATCATATTCGTCCAC-3′ (reverse), and the sequencing primer was 5′-CCTGCTGAAAATGACTGAA-3′. After polymerase chain reaction (PCR), the amplified products were purified using the QIAquick PCR Purification Kit (Qiagen), and the sequence of *KRAS* at codons 12 and 13 (point mutation sites) was determined by the dideoxy chain-termination method, using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. The products were analyzed using a 3130*xl* Genetic Analyzer (Applied Biosystems).

In four patients, *KRAS* mutational analyses of pancreatic juice samples were also performed according to our earlier report,[16] and the results were compared with the resected specimen of the second lesions.

Immunohistochemistry for TP53, CDKN2A, and SMAD4

FFPE tissue samples from each patient were immunostained for TP53, CDKN2A, and SMAD4. A minimum of three slides from each patient were stained to evaluate heterogeneity within the first and second HRL. An antihuman, TP53, rabbit polyclonal antibody (FL-393, 1:100, Santa Cruz Biotechnology, CA, USA), an antihuman, CDKN2A, mouse monoclonal antibody (clone E6H4, 1:100, Roche MTM laboratories AG, Heidelberg, Germany), and an antihuman, SMAD4, mouse monoclonal antibody (clone B-8, 1:100, Santa Cruz Biotechnology) were used. TP53 staining was considered abnormal in lesions with diffuse (≥60% neoplastic cells) nuclear staining (overexpression) or those with no nuclear staining (lack of expression).[6] Scattered acinar and ductal cells with nuclear TP53 expression were typically present in the adjacent normal tissue and used as an internal control. Islet cells served as an internal control for positive CDKN2A expression, and CDKN2A staining in either in the cytoplasm or in the nuclei was scored as positive, indicating an intact *CDKN2A* gene, and lack of staining was scored as negative, indicating that a deletion or inactivating mutation of *CDKN2A* had occurred.[5] Normal acinar, ductal, islet, and stromal cells served as internal controls for positive SMAD4 expression. SMAD4 expression, either in the nucleus or cytoplasm, was scored as positive, indicating the presence of an intact *SMAD4* gene, and absence of staining was scored as negative, indicating that a deletion or inactivating mutation of *SMAD4* had occurred.[5,6] Samples processed with nonimmune serum instead of the primary antibodies were used as a negative control.

Estimation of progression of second lesions

To determine whether second HRLs might result from a metachronous multifocal lesion or local recurrence of the first lesion, the margin status at the time of the first operation, pathologic findings, *KRAS* mutational status, and TP53, CDKN2A, and SMAD4 immunostaining of FFPE samples from first and second lesions were compared.

Clinical characteristics, including postoperative prognosis, were also compared among patients with suspected metachronous multifocal lesion or local recurrence of the first lesion in the remnant pancreas. To assess prognosis after resection of a second HRL, the estimated disease-free survival rate for patients was compared among patients having resection of local recurrence, patients having resection of metachronous multifocal HRL, patients with diagnosis of unresectable PDAC in the remnant pancreas, including advanced local invasion and concomitant distant metastasis ($n = 10$), and patients with a diagnosis of extrapancreatic recurrence, including distant metastases and peritoneal metastases ($n = 304$, Fig. 1).

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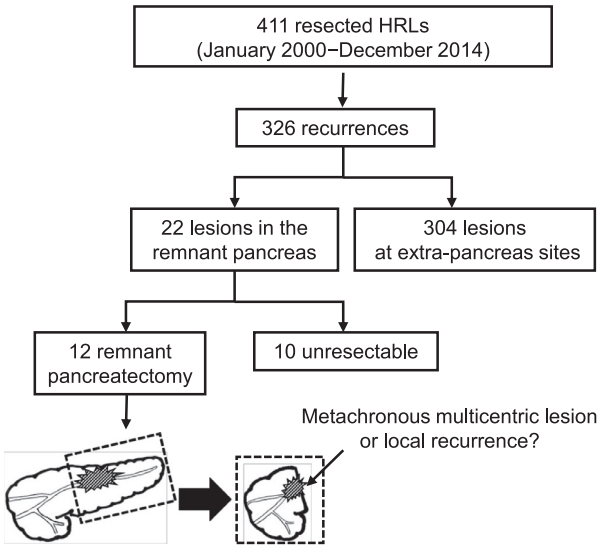

Fig. 1. Study flow of lesion identification in 411 patients who underwent pancreatectomy for high-risk lesions. A total of 411 patients with high-risk lesions (HRLs) were included in this study. The 85 patients with any sign of recurrence were excluded, and the remaining 326 patients were further analyzed. After the first partial pancreatectomy, 22 patients (5%) were diagnosed as having HRLs in the remnant pancreas, 12 of whom underwent a second pancreatectomy. Resected specimens of both initial and second HLRs were obtained to determine whether the second HLR was a metachronous multicentric lesion or local recurrence.

Targeted NGS

Frozen tissue samples were collected by tissue tablet methods according to our earlier report,[18] and 10-µm sections were cut on a cryostat and stored at −20°C until use or fixed in 5% acetic acid and stained with toluidine blue. Cancer cells were collected immediately by laser microdissection in 200-µl tubes. DNA was subsequently extracted using the DNeasy Blood & Tissue Kit (Qiagen), according to the manufacturer's instructions, and resuspended in 30 µL of nuclease-free water. DNA quantity was assessed using a Qubit photometer (Life Technologies, Carlsbad, CA, USA), and DNA quality was determined using the Qubit dsDNA High Sensitivity Assay Kit (Life Technologies) and a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions.

Libraries were prepared using the Ion AmpliSeq Library Kit 2.0-96LV (Life Technologies) and Cancer Hotspot Panel v2 (CHPv2). The minimum DNA concentration required to obtain 10 ng of total DNA input was 1.6 ng/µL. In samples containing a low amount of DNA (DNA < 1.6 ng/µL), the DNA was not diluted further, and the quantity of nuclease-free water was decreased accordingly. The number of multiplex PCRs was increased from the suggested 20 cycles to 29 cycles. The CHPv2 covers approximately 2,800 Catalogue of Somatic Mutations in Cancer mutations from 50 oncogenes and tumor suppressor genes (*ABL1, EGFR, GNAS, KRAS, PTPN11, AKT1, ERBB2, GNAQ, MET, RB1, ALK, ERBB4, HNF1A, MLH1, RET, APC, EZH2, HRAS, MPL, SMAD4, ATM, FBXW7, IDH1, NOTCH1, SMARCB1, BRAF, FGFR1, JAK2, NPM1, SMO, CDH1, FGFR2, JAK3, NRAS, SRC, CDKN2A, FGFR3, IDH2, PDGFRA, STK11, CSF1R, FLT3, KDR, PIK3CA, TP53, CTNNB1, GNA11, KIT, PTEN*, and *VHL*). The CHPv2 amplicon reference range was reported as 111–187 bp, with an average of 154 bp. Each library was barcoded with the Ion Xpress Barcode Adapters 1-16 Kit (Thermo Fisher Scientific), quantified using the Qubit dsDNA High Sensitivity Assay Kit (Life Technologies) and NanoDrop Life (Thermo Fisher Scientific), and diluted in nuclease-free water to obtain a final concentration of 100 µM.

Ion Torrent Suite Software v 5.0.4 (Thermo Fisher Scientific) was used to sequence genes involved in pancreatic cancer (*KRAS, TP53, CDKN2A*, and *SMAD4*). After alignment to the hg19 human reference genome, the Variant Caller plug-in (version 5.0.28-1) (Thermo Fisher Scientific) was applied using the Hotspot Cancer Panel file as reference. The Variant Caller plug-in reports by default a given variant when it is detected in ≥1% of the total amplicon reads with the requirement of at least the presence of one forward and one reverse read to avoid strand bias. The Ion Reporter suite (Thermo Fisher Scientific) was used to filter out known polymorphic variants. Binary Alignment Map (BAM) files were inspected visually using the Golden Helix Genome Browser (version 2.0.7; Golden Helix, Bozeman, MT, USA), and only the mutations reported in the Catalogue of Somatic Mutations in Cancer database (http://cancer.san-ger.ac.uk/) were taken into account. Postsequencing metrics (read length, the percentage of on-target reads, qPCR, and the average base depth) were recorded for each sample.

Sampling of frozen tissue and definition of local recurrence or multifocal lesion

Two categories of mutations were identified. The first category consisted of founder mutations that were in all subclones from the parental clone. The second category consisted of progressor mutations that create heterogeneity. In pancreatic cancer, the founder mutations are mutations in driver genes, shown elsewhere to drive pancreatic tumorigenesis.[2,19] In this study, DNA was isolated from frozen tissue samples taken from three regions of a primary lesion and analyzed by targeted NGS. The common mutations in these three regions were compared with mutations from one region of a second lesion that was analyzed by targeted NGS. The second lesion was likely to be a recurrence of the first lesion if there were common driver mutations in both primary and secondary lesions, and multifocal development was probable if common mutations were not observed in the second lesion.

Statistics

Statistical analysis was performed using JMP statistical software (version 13.0.2; SAS Institute, Cary, NC, USA). The Fisher exact probability test or the χ^2 test was used to evaluate differences in clinical factors between the two groups. The Mann-Whitney *U* test was used for continuous data, which were expressed as the median with range. The estimated disease-specific survival rate and estimated overall survival rate were analyzed using the Kaplan-Meier method with the Wilcoxon test. No formal statistics were applied to the comparison of the four patients with presumed local recurrences and the eight patients believed to have metachronous lesions because of the small *n* values in each group.

Results

Clinicopathologic characteristics of the study population

Figure 1 presents the study flow for analysis of the 411 patients in this study who underwent partial pancreatectomy for HRLs. The 85 patients with any sign of recurrence were excluded, and the remaining 326 patients were further analyzed. After the first pancreatectomy, 22 patients (5%) were diagnosed as having a second HRL in the remnant pancreas, and 12 of these patients underwent a second pancreatectomy. Resected specimens of both the first and second HRLs were obtained from these 12 patients.

Table 1 presents the clinicopathologic characteristics of the 12 patients who were diagnosed histologically with an HRL after partial pancreatectomy and underwent a second pancreatectomy.

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Table 1
Clinicopathologic and molecular characteristics of the 12 patients who underwent a second pancreatectomy of the remnant pancreas.

No	Age/Sex	Comorbidity	First lesion							Months after first operation	Second lesion						Estimated pattern of progression
			Location*	Diagnosis (T/N/P)*	Margin*	*KRAS* mut†	*TP53* IHC	*CDKN2A* IHC	*SMAD4* IHC		Distance from PCM (mm)*	Diagnosis T/N/P	*KRAS* mut	*TP53* IHC	*CDKN2A* IHC	*SMAD4* IHC	
1	63/F	DM	Ph	T3/N1/mod	R0	G12A	Abn	+	–	72	15	T3/N1/well	G12D	Abn	+	+	Multifocal
2	73/M	IPMN	Pbt	T3/N0/mod	R0	WT	N	+	+	42	20	T3/N0/mod	G12D	N	+	+	Multifocal
3	67/F	DM	Ph	T3/N1/well	R0	G12D	Abn	+	–	48	0	T3/N1/mod	G12V	Abn	+	+	Multifocal
4	65/M	IPMN, DM	Pbt	T1/N0/mod	R0	G12D	N	+	–	31	20	T3/N0/mod	G12A	Abn	+	+	Multifocal
5	71/M	Chronic pancreatitis	Pbt	Tis/N0/well	R0	WT	Abn	+	–	24	10	Tis/N0/well	G12V	N	+	+	Multifocal
6	58/F	IPMN	Pbt	Tis/N0/well	R0	G12D	N	+	+		30	Tis/N0/well	G12D	Abn	+	–	Multifocal
7	58/M	–	Pbt	T1/N0/mod	R0	G12V	Abn	+	–	53	15	Tis/N0/well	G12D	Abn	+	+	Multifocal
8	67/F	–	Ph	T3/N1/por	R0	G12V	N	–	–	37	8	T1/N0/por	G12D	Abn	+	+	Multifocal
9	66/F	IPMN	Pbt	T2/N0/por	PCM +	G12D	Abn	+	+	44	15	T3/N0/por	WT	N	+	–	Multifocal
10	79/F	–	Ph	T3/N1/mod	R0	G12D	Abn	–	+	35	0	T3/N1/por	G12D	Abn	–	+	Recurrence
11	76/M	–	Ph	T3/N0/mod	DPM+	G12A	Abn	+	+	24	5	T3/N1/mod	G12A	Abn	+	+	Recurrence
12	60/M	–	Pbt	T3/N0/mod	R0	G12D	Abn	+	+	18	0	T3/N0/mod	G12D	Abn	+	+	Recurrence
			Ph	T3/N0/mod	R0	G12V	Abn	+	+	9	0	T3/N1/mod	G12V	Abn	+	+	Recurrence

* Determined according to the Japan Pancreas Society.[13]
† *KRAS* mut: *KRAS* mutation with the highest concentration. *IHC*, immunohistochemistry; *DM*, diabetes mellitus; *Ph*, pancreatic head; *Pbt*, pancreatic body to tail; *T*, tumor factor; *N*, nodal factor; *P*, pathologic type. *Well*, *mod*, and *por* indicate well, moderately, and poorly differentiated adenocarcinoma, respectively. *R0*, negative margin; *WT*, wild-type; *PCM*, pancreatic cut end margin; *DPM*, dissected peripancreatic tissue margin; *Abn*, abnormal expression; *N*, normal expression.

One patient (number 5) had 2 synchronous and metachronous multifocal HRLs. One of the high-grade PanIN lesions was diagnosed at the time of first operation by brush cytology (class V) for a stricture in the main pancreatic duct, and the other high-grade PanIN was found by chance during pathologic assessment of the resected specimen of the first pancreatectomy. We often perform follow-up endoscopic retrograde pancreatography (ERP)/pancreatic juice cytology in patients with high risk for the development of a second HRL in the remnant pancreas, such as patients with multiple, high-grade PanIN lesions or with HRL concomitant with IPMN, because ERP/pancreatic juice cytology is the only way to diagnose early stage HRL that cannot be detected by other imaging modalities.[10,20] Thus, a second pancreatectomy was performed in 2 patients (numbers 5 and 6) because follow-up ERP/pancreatic juice cytology showed class V even without any sign of the HRL by three-dimensional radiologic findings.

KRAS mutation status assessed in FFPE samples was different between the first and second lesions in 8 patients (numbers 1–8) but was the same in the remaining 4 patients (numbers 9–12). Notably, all *KRAS* mutations detected in this study were at codon 12 and not at codon 13. In addition, patterns of immunohistochemical staining for TP53, CDKN2A, and SMAD4 were not always in complete accord between the first and second HRLs in patient numbers 1–8 but were consistent in patient numbers 9–12. Together these results show that the mutation status or expression of these 4 major genes differed between the first and second lesions in patient numbers 1–8, and there were no such differences in patient numbers 9–12.

Distinction of second HRLs and comparison of their clinicopathologic characteristics

Based on the mutational and immunohistochemical analyses and clinicopathologic findings discussed earlier in this report, we considered the progression pattern of the second HRLs in patient numbers 1–8 to be metachronous multifocal lesions, and the HRLs in patient numbers 9–12 were determined as local recurrence (Table 1). The time to diagnosis of the second HRL after resection of first lesion tended to be less in the local recurrence group (median 21 months, range 9–35 months) compared with the metachronous multifocal group (median 43 months, range 24–72 months) The distance from the pancreatic cut margin to the second HRL in the remnant pancreas tended to be greater in the metachronous multifocal group (median 15 mm, range 0–30 mm) compared with the local recurrence group (median 0 mm, range 0–5 mm).

No differences in clinicopathologic characteristics of the first HRLs were found between the metachronous multifocal and local recurrence groups (Table 2). In contrast, after resection of the second HRL, the estimated cumulative recurrence rate tended to be greater in the local recurrence group compared with the metachronous multifocal group (Fig. 2, *A*). In addition, the estimated cumulative disease-specific survival rate after resection of the second HRL tended to be greater in the metachronous multifocal group compared with the local recurrence group (Fig. 2, *B*). The disease-specific survival rate of the patients with local recurrence after second pancreatectomy was not different from the 10 patients with unresectable second PDAC in the remnant pancreas ($P = 0.71$) or the 304 patients with extrapancreatic recurrence ($P = 0.15$).

Assessment of KRAS mutations in pancreatic juice

Some patients underwent surveillance ERP and subsequent collection of pancreatic juice before the second pancreatectomy. To clarify whether the status of the *KRAS* mutation in the second lesions could be predicted and to determine whether the second le-

Table 2
Characteristics of patients in the possible metachronous multifocal and local recurrence groups.

	Metachronous multifocal ($n=8$)	Local recurrence ($n=4$)
Sex (M / F)	4 / 4	2 / 2
Age in years (range)	65 (58–73)	70 (68–79)
History of smoking, positive	4 (50%)	1 (25%)
History of alcohol consumption, positive	2 (25%)	3 (75%)
Comorbidity		
IPMN	3 (37.5%)	1 (25%)
Chronic pancreatitis	1 (12.5%)	0 (0%)
Diabetes mellitus	3 (37.5%)	0 (0%)
Tumor marker		
CEA, ng/mL (range)	3.0 (0.5–19.1)	7.4 (2.8–15.3)
CA19-9, U/L (range)	30 (0.6–62)	855 (0.9–3352)
Operation		
PD / DP	2 / 6	2 / 2
Pathologic findings*		
Tumor size (mm)	17 (13–35)	25 (13–45)
T factor, ≥ T3	4 (50%)	4 (100%)
N factor, ≥ N1	3 (38%)	1 (25%)
Stage, ≥ IIB	4 (50%)	1 (25%)
Lymphatic invasion +	4 (50%)	2 (50%)
Vascular invasion +	2 (25%)	2 (50%)
Neural invasion +	4 (50%)	4 (100%)
Residual tumor, R1	1 (13%)	1 (25%)

* Pathologic findings were determined according to the Japan Pancreas Society.[13] *IPMN*, intraductal papillary mucinous neoplasm; *CEA*, carcinoembryonic antigen; *CA19-9*, carbohydrate antigen 19-9; *PD*, pancreatoduodenectomy; *DP*, distal pancreatectomy.

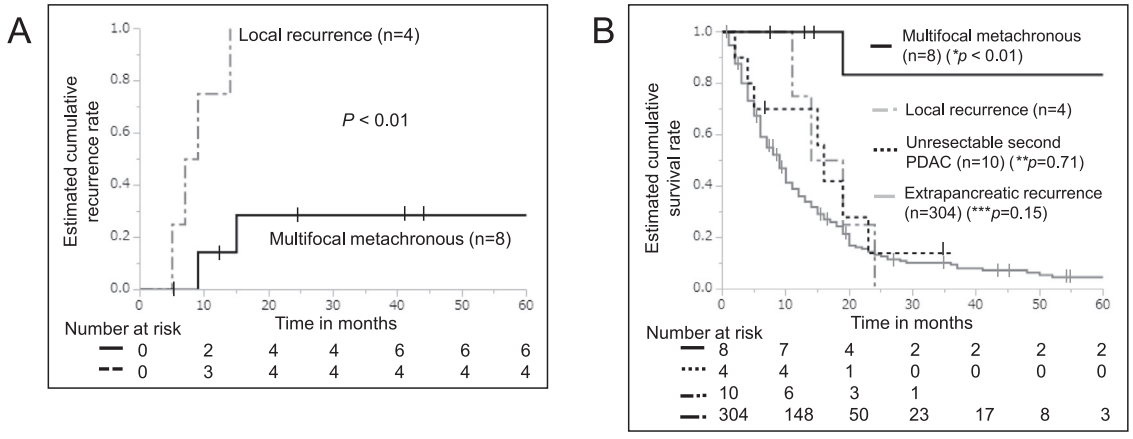

Fig. 2. Clinical course after resection of first or second lesion. (*A*) Estimated cumulative recurrence rate after resection of the second high-risk lesion (HRL) tended to be greater in the local recurrence group compared with the metachronous multifocal group. (*B*) Estimated cumulative disease-specific survival rate after resection of the second HLR tended to be greater in the metachronous multifocal group compared with the local recurrence group (*$P < .01$). Disease-specific survival rate of the patients with local recurrence after second pancreatectomy was not different from patients with unresectable second PDAC in the remnant pancreas ($n=10$; **$P=.71$) or patients with extrapancreatic recurrence ($n=304$; ***$P=.15$).

Table 3
KRAS mutation status in the resected second high-risk lesion and pancreatic juice before the second operation.

Patient number	Second lesion			Pancreatic juice		
	G12A	G12D	G12V	G12A	G12D	G12V
4 (possible multifocal)	42%	4%	0%	46%	8%	0%
5 (possible multifocal)	0%	40%	6%	0%	54%	10%
11 (possible recurrence)	10%	18%	0%	14%	22%	0%
12 (possible recurrence)	6%	0%	26%	8%	0%	66%
Patient with chronic pancreatitis	0%	0%	0%	0%	0%	0%

sion might be recurrence or a metachronous multicenteric lesion before the second pancreatectomy, we compared *KRAS* mutational analyses of pancreatic juice samples with the resected specimen of the second lesions. Mutations in *KRAS* at codon 12 and 13 were determined in pancreatic juice obtained from 4 patients (numbers 4, 5, 11, and 12) before their second operation. A similar mutational status at codon 12 was observed in the second HRLs and pancreatic juice samples for all 4 patients (Table 3). Patients who underwent pancreatectomy for mass-forming pancreatitis served as a control and had wild-type *KRAS* in both resected specimens and pancreatic juice.

Targeted NGS

Targeted NGS was performed in frozen samples from three patients (numbers 8, 9, and 12). In patient number 8, the status

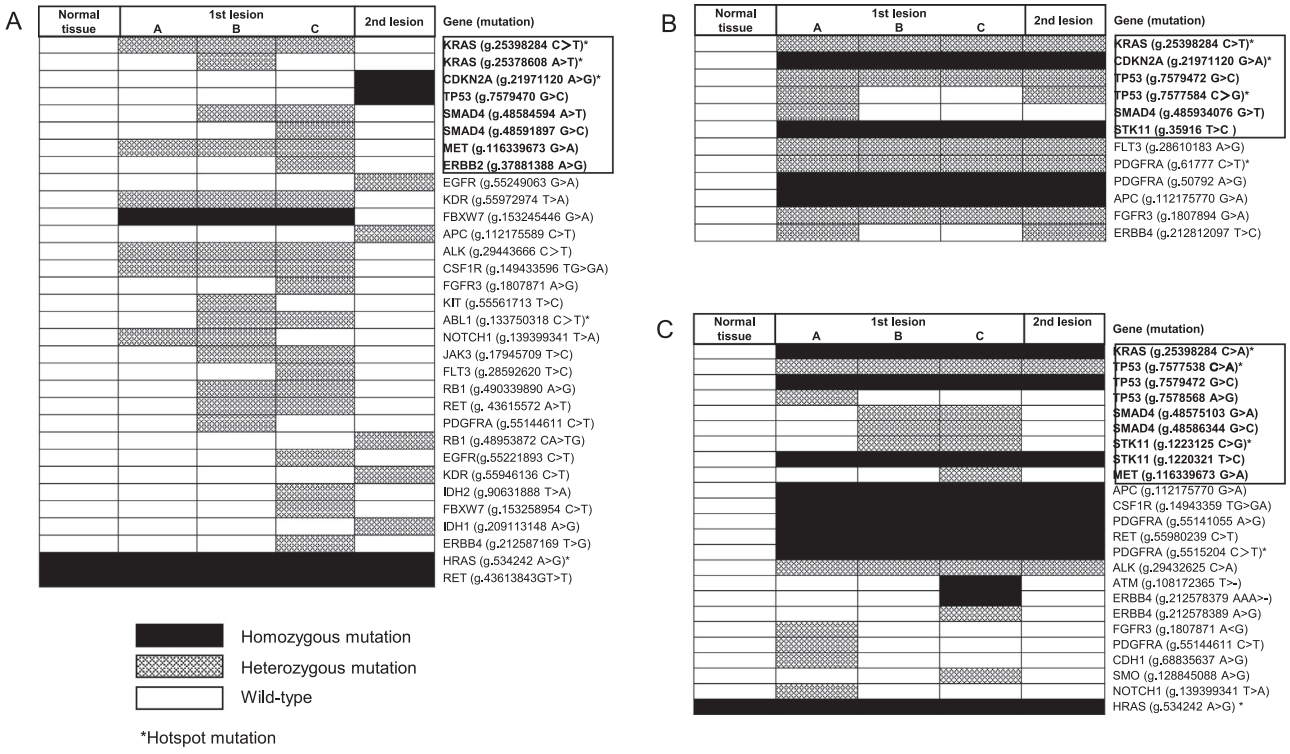

Fig. 3. Next-generation sequencing results. Next-generation sequencing was performed in three patients in frozen samples from three sampling regions (columns A, B, and C) from the first lesion, the second lesion, and normal tissue. (*A*) In patient number 8, the main founder mutations in *KRAS, CDKN2A, TP53*, and *SMAD4* were different between the first and second lesions, indicating that these two lesions were metachronous independent lesions. Mutations in ***bold*** and ***within the box*** indicate founder mutations of pancreatic ductal adenocarcinoma. *Hotspot mutations include *KRAS* at codon 12 and 61, *CDKN2A* at codon 80, *TP53* at codon 233 and 248, and *STK11* at codon 354. (*B*) In patient number 9, the *KRAS, CDKN2A, TP53*, and *STK11* founder mutations were detected in the primary and second lesion, indicating that the second lesion was likely to be a recurrence of the first lesion. (*C*) In patient number 12, the founder mutations in *KRAS, TP53*, and *STK11* were detected in both primary and secondary lesions, indicating that the second lesion was likely to be a recurrence of the first lesion.

of the four main founder mutations in *KRAS, CDKN2A, TP53*, and *SMAD4* was different in the first and second lesions, indicating that the two lesions were likely to be independent metachronous lesions. In patient number 8, two *KRAS* mutations were observed in the first HRL, with a mutation at codon 12 (g.25398284 C > T) detected in all three sampling regions and a mutation at codon 61 (g.25378608 A > T) detected in only one sampling region (Fig. 3, *A*). In patient number 9, founder mutations in *KRAS* at codon 12, *CDKN2A, TP53*, and *STK11* were the same in both the first and second lesion, indicating that the second lesion was likely to be a recurrence of the first lesion (Fig. 3, *B*). In patient number 12, the founder mutations in *KRAS* at codon 12, *TP53*, and *STK11* in the first lesion were the same as those in the second lesion, indicating that the second lesion was also likely to be a recurrence of the first lesion (Fig. 3, *C*). Other mutation patterns in patient numbers 9 and 12 were also similar between the first and second HRLs.

Discussion

This study demonstrates that the clinical course of metachronous multifocal HRLs appears to be different from that of local recurrence in the remnant pancreas after resection of a first HRL and that genetic assessment using NGS may possibly discriminate between these two entities. Assessment of *KRAS* mutation status in pancreatic juice may be helpful for preoperative differentiation between metachronous multifocal HRLs and local recurrence in the remnant pancreas, which would inform appropriate management of the second HRL in the remnant pancreas.

Genomic assessment using NGS provides a detailed process of progression of PanIN to PDAC, indicating the process of multistage carcinogenesis via *KRAS→CDKN2A→TP53→SMAD4* mutations in a stepwise manner.[2–6,21,22] Mutations in these four genes are termed "founder mutations" for PDAC and are consistent during the progression of PanIN to PDAC. These mutations can lead to malignant characteristics of PanIN that drive progression to PDAC. Multiple IPMNs have been reported to show two potential progression patterns, namely monoclonal skip progression via intraductal dissemination[23] and multicentric occurrence via field defect theory.[24] This study is the first molecular assessment of two possible progression patterns in second HRLs in the remnant pancreas.

Despite the limited size of this study population, our results suggest that mutational and immunohistochemical assessments of the four major genes may possibly discriminate synchronous multifocal HRL from local recurrence in the remnant pancreas. Among the PDAC mutation hotspots in *KRAS*, only mutations at codon 12 were detected in the FFPE samples of the examined patients, and these results that were confirmed by NGS. In patient number 8, *KRAS* mutation at codon 61 was also detected in one of the three sampling regions. This discovery was in addition to mutations at codon 12 in all three sampling regions of the first HRL, indicating the possibility that, with the exception of codon 12 mutation, *KRAS* point mutations might occur as late events during the progression of PDAC.

A Japanese, multicenter study of stage 0/I HRL[11] showed that, although the estimated overall survival rates after resection of stage 0 high-grade PanIN, stage I (TS1a) and stage I (TS1b) PDAC were 94.7%, 93.8%, and 78.9%, respectively, 31 of 200 patients (15.5%) with stage 0/I PDAC experienced a second HRL in the remnant pancreas during the postoperative surveillance period. There are many types of PanIN other than HRL, and therefore it is likely that HRL may potentially develop multifocally in the same pan-

creas. We have not been able to confirm this phenomenon to date because most PDAC patients die before the development of a second HRL. Further examination is necessary to clarify whether such lesions are really multicentric independent, and careful attention should be paid to possible occurrence of a distinct second HRL in the remnant pancreas in long-term survivors after resection of HRL, even if there is no finding of extrapancreatic recurrence.

A total of 7 of the 12 patients (58%) in our study had one or more pancreas-related comorbid diseases, such as IPMN, chronic pancreatitis, or diabetes mellitus. These diseases are predictive factors for the early detection of HLRs,[10,11] and many early stage HLRs described in the literature were found during the assessment or surveillance of these comorbid diseases.[10,11] In addition, Date et al[25] demonstrated recently that patients who underwent partial pancreatectomy for HRL concomitant with IPMN had a high risk of developing a second concomitant HLR in the remnant pancreas. Detailed molecular and genetic assessments of HLR might lead to further understanding of the mechanisms of development of PanIN and PDAC in patients with such comorbidities and to the selection of patients for whom preventive total pancreatectomy would be beneficial.

The present study has shown that assessment of *KRAS* mutations in pancreatic juice of patients with second HRLs and subsequent comparison with the *KRAS* mutations in the first HLR may contribute to the distinction of first and second HLRs preoperatively. We prefer to use pancreatic juice rather than endoscopic ultrasonographic-guided fine-needle aspiration samples for potentially resectable HLR[26] because the latter has a risk for needle-tract implantation or dissemination. In contrast, endoscopic ultrasonographic-guided fine-needle aspiration can also be used for *KRAS* mutational assessment. Despite the small study population, our current study demonstrates that the prognosis of recurrent HLR in the remnant pancreas is poor, and therefore, the management strategy for recurrent HLR in the remnant pancreas might be different from that of metachronous multifocal HLR (ie, upfront resection would be chosen for metachronous multifocal HLR, and neoadjuvant chemoradiotherapy and subsequent resection would be chosen for recurrent lesions).

Although it remains unclear whether PanIN lesions will progress to invasive PDAC, we have taken the position that high-grade PanIN is compatible for carcinoma in situ, as stated in the recommendation by the Baltimore Consensus Meeting.[12] In addition, we often cannot determine whether the small lesion of interest is high-grade PanIN or invasive PDAC when only ERP/pancreatic juice cytology provides evidence for the presence of carcinoma cells (class V), and the final diagnosis of high-grade PanIN (carcinoma in situ) can only be obtained after resection, as presented in our current study. Prognosis after resection of high-grade PanIN is favorable, and therefore we consider that resection at the time of high-grade PanIN would be ideal. Further investigation is necessary to clarify whether a stepwise progression process from PanIN to invasive PDAC exists, as in IPMN.

This study has several limitations. First, as described earlier, the study population was small because patients undergoing a second pancreatectomy for second HLR in the remnant pancreas represent a small population. Therefore, the present study does not provide valid statistical evidence to help determine the management of this rare condition. Second, this is a retrospective study, and frozen sections from first and second HLRs for NGS were only available for three patients, and there were no stage 0 or I HLR samples. In addition, multiple samplings could not be performed for any of the second HLRs. It is difficult to obtain large samples from small HLRs, especially from high-grade PanIN (carcinomas in situ), because noninvasive lesions can only be identified microscopically. We have also been attempting to purify DNA suitable for NGS from FFPE or pancreatic juice samples; however, we have not yet been able to obtain stable, high-quality DNA samples. Third, although immunohistochemistry for TP53, CDNK2A, and SMAD4 was performed to evaluate the mutation status for the clinical differentiation of the second HRL from the first lesion, the protein expression level does not always reflect the precise mutation status. In addition, assessment of *KRAS* status in only pancreatic juice may also be insufficient for differentiation because *KRAS* is a mutational hotspot, and there is a possibility that the same mutation may occur by chance in the second lesion; however, comparison of the combination of the mutational and immunohistochemical assessments between first and second lesions might give some information to help discriminate the second lesion from the first lesion. A prospective study design, including an adequate sampling method and further efforts to improve the quality of DNA samples from FFPE or pancreatic juice for NGS, is needed to overcome these limitations.

In conclusion, careful attention should be paid to the possible development of metachronous multifocal second HRLs in the remnant pancreas in addition to the recurrence of the first PDAC after partial pancreatectomy. Genetic assessment may possibly discriminate multifocal HLR from local recurrence.

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