



# Combination of *KRAS* and *SMAD4* mutations in formalin-fixed paraffin-embedded tissues as a biomarker for pancreatic cancer

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## Abstract

Formalin-fixed paraffin-embedded (FFPE) tissues used for routine pathological diagnosis are valuable for cancer genomic analysis; however, the association between mutation status derived from these specimens and prognosis in pancreatic ductal adenocarcinoma (PDAC) remains unclear. We analyzed 50 cancer-related gene mutations including driver genes in PDAC, using next-generation sequencing (NGS) to clarify the association between gene mutations and prognosis. DNA was extracted from FFPE tissues obtained from 74 patients with untreated resectable PDAC who underwent surgery at our institution between 2013 and 2018. Fifty of the 74 patients with DNA extracts from FFPE samples suitable for NGS were analyzed. The prevalence of driver gene mutations was as follows: 84% for *KRAS*, 62% for *TP53*, 32% for *SMAD4*, and 18% for *CDKN2A*. There were no cases of single *SMAD4* mutations; its rate of coincidence with *KRAS* or *TP53* mutations was 30% and 2%, respectively. The combination of *KRAS* and *SMAD4* mutations resulted in significantly shorter relapse-free survival (RFS; median survival time [MST], 12.3 vs. 28.9 months,  $P = .014$ ) and overall survival (OS; MST, 22.3 months vs. not reached,  $P = .048$ ). On multivariate analysis, the combination of *KRAS* and *SMAD4* mutations was an independent prognostic factor for RFS (hazard ratio [HR] 4.218; 95% confidence interval [CI], 1.77-10.08;  $P = .001$ ) and OS (HR 6.730; 95% CI, 1.93-23.43;  $P = .003$ ). The combination of *KRAS* and *SMAD4* mutations in DNA obtained from FFPE tissues is an independent poor prognostic factor in PDAC.

## KEYWORDS

*KRAS*, next-generation sequencing, pancreatic ductal adenocarcinoma, prognosis, *SMAD4*

**Abbreviations:** CA19-9, carbohydrate antigen 19-9; CHPv2, Cancer Hotspot Panel version 2; CI, confidence interval; FFPE, formalin-fixed paraffin embedded; HR, hazard ratio; IPMC, intraductal papillary mucinous carcinoma; MST, median survival time; NCCN, National Comprehensive Cancer Network; NGS, next-generation sequencing; OS, overall survival; PanIN, pancreatic intraepithelial neoplasia; PDAC, pancreatic ductal adenocarcinoma; R, residual tumor; RFS, relapse-free survival.

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## 1 | INTRODUCTION

Despite progress made in health care, PDAC retains a poor record of prognosis.<sup>1</sup> In the United States, the estimated number of newly diagnosed PDAC patients in 2019 is 56 770. Pancreatic ductal adenocarcinoma is the fourth leading cause of cancer-related death, with a mortality rate of 10% in 2019.<sup>2</sup> Due to aggressive tumor behavior and late clinical detection, the 5-year survival rate of patients with PDAC is 10%.<sup>2</sup> Although surgical resection remains the only curative treatment for patients without distant metastasis of PDAC, only 20%-30% of patients are eligible for resection at the time of diagnosis.<sup>2,3</sup> In addition, most patients who undergo curative pancreatectomy experience recurrence. Although the prognosis for patients with PDAC has improved due to multimodal treatment,<sup>4</sup> no effective molecularly targeted drug has been established. Recently, gene mutations have been reported to be effective biomarkers for the determination of therapeutic strategies in various cancers,<sup>5,6</sup> and some druggable gene mutations have been reported to improve survival rates.<sup>7,8</sup> In advanced PDAC patients with distant metastasis, germline *BRCA* mutations have been reported to be druggable genes<sup>9</sup>; however, biomarkers have not yet been established for therapeutic targets or precision medicine in resected PDAC.

Genomic biomarkers for PDAC have been identified by analyzing the association between somatic gene mutations and their clinicopathological variables.<sup>10</sup> Four driver genes, namely, *KRAS*, *TP53*, *SMAD4*, and *CDKN2A*, have been reported as representative cancer-related genes in PDAC. Among them, the *KRAS* gene mutation is most common, and is associated with poor prognosis.<sup>11,12</sup> We previously reported that *KRAS* mutations in cell-free DNA obtained from serum in the aftermath of resection are associated with poor prognosis.<sup>13</sup> However, we also previously reported that the survival rate of IPMC patients with a genetic heterogeneous primary tumor containing more than 2 types of *KRAS* mutation, was better than that of IPMC patients with genetic homogenous primary tumors with only 1 type of *KRAS* mutation.<sup>14</sup> Recently, PDAC tissues have been subjected to genome-wide sequencing, and whole-exon sequencing in particular, and approximately 40 associated gene mutations were discovered.<sup>15-18</sup> Blackford et al<sup>19</sup> utilized NGS using DNA extracts from resected cancer cells purified from cell lines or xenografts. By analyzing DNA extracts from fresh-frozen samples, Hayashi et al<sup>20</sup> revealed that the number of driver gene mutations could be a prognostic factor; however, certain gene mutations associated with prognosis were not detected. Based on NGS analysis using FFPE samples, we previously reported that multiple PDAC involves multicentric carcinogenesis and intrapancreatic metastasis, which could be distinguished by comparing driver gene mutations; the latter has a dismal prognosis.<sup>21</sup> According to the NCCN Guidelines of 2019, somatic mutational profiling of tumor tissue is recommended for PDAC.<sup>22</sup> In the future, it is expected that genetic tests from resected specimens will be routine for almost all cases of PDAC. Next-generation sequencing analysis using DNA extracted from fresh-frozen samples is well documented; however, only a few reports exist regarding DNA extracted from FFPE samples for

PDAC.<sup>23</sup> Due to formalin fixation-induced degradation, the success rate of NGS analysis using DNA extracted from FFPE tissues is lower than that obtained from fresh-frozen specimens.<sup>24</sup> However, NGS using FFPE samples is valuable, as these are used for pathological diagnosis in routine clinical practice. Therefore, we hypothesized that NGS analysis using FFPE samples could be applied to PDAC, and that this genetic analysis, including that of driver gene mutations, could identify prognostic predictive factors for PDAC and also clarify the rate of druggable gene mutations in resected PDAC. We undertook targeted deep sequencing using NGS to screen 50 cancer-related genes from surgically resected PDAC specimens at our institution. We aimed to identify the association between these mutations and clinicopathologic variables as well as the prognostic utility of these mutations.

## 2 | MATERIALS AND METHODS

### 2.1 | Patients

In total, 146 PDAC patients who underwent curative resection at our institution between March 2013 and May 2018 were retrospectively analyzed. All patients were histologically diagnosed with ductal adenocarcinoma. Among these 146 patients, 72 were excluded: for 34 of these patients, samples could not be prepared owing to a lack of consent or other reasons, 32 patients had undergone neoadjuvant treatment, 3 had received chemotherapy for remnant pancreatic cancer, and 3 had undergone R2 resection.

The pathological staging of all specimens, including residual tumors (R) was determined according to the 8th edition of the UICC TNM classification.<sup>25</sup> All participants provided written informed consent. The study was approved by the Human Experimentation Committee of Keio University Hospital (Nos. 20120443 and 20170086), and was carried out in accordance with the 1975 Declaration of Helsinki.

### 2.2 | Clinicopathological characteristics

Clinicopathological variables were extracted from medical records. The clinical variables included age, sex, presence of diabetes mellitus, amount of serum CA19-9, surgical procedure, operation time, blood loss, postoperative complications evaluated according to the Clavien-Dindo classification, surgery-related deaths, and adjuvant chemotherapy. Pathological variables included tumor size, location, and differentiation grade, lymphatic, venous, and intrapancreatic nerve infiltration, serosal, retropancreatic tissue, distal bile duct, duodenal, portal vein, arterial, and extra-pancreatic nerve plexus invasion, and invasion of other organs.<sup>26</sup>

In our institution, most patients with borderline resectable PDAC (according to NCCN resectability classification) undergo neoadjuvant treatment. In addition, some patients diagnosed with T3 or T4 disease according to the 7th edition of the UICC TNM

classification have been receiving neoadjuvant chemoradiotherapy since 2003.<sup>27,28</sup> Surgical resections included pylorus-preserving or subtotal stomach-preserving pancreatoduodenectomy, distal pancreatectomy, and total pancreatectomy. D2 lymph node dissections were undertaken in all patients.

After surgical resection of PDAC, each patient underwent standard postoperative follow-up. S-1 or gemcitabine was given as adjuvant chemotherapy at the physician's discretion. After October 2013, S-1 was primarily given based on the results of interim JASPAC01 analysis.<sup>29</sup> Successfully completed adjuvant treatment was defined as one that was given for 6 months. Recurrence was defined by definitive evidence of recurrence, which was confirmed with radiographic findings, with or without elevated serum CA19-9 levels. Physical examinations, toxicity assessments, complete blood cell counts, serum chemistry profiles, and chest-abdominal computed tomography scans were carried out approximately every 4-6 months for the first 12 months, and every 6 months thereafter.<sup>28</sup>

### 2.3 | Preparation and extraction of DNA from FFPE specimens

Resected specimens were immediately fixed in 10% buffered formalin. The fixed specimens were embedded in paraffin within 1 week. The paraffinized sections were stained with H&E and the main tumor lesions were identified by a pathologist. Ten-micrometer sections of the primary tumor were cut from each block and placed on glass slides. One section of the main tumor was stained with H&E for orientation; this was subsequently confirmed by a faculty pathologist. Tiny fractions of the main tumor lesions were grossly dissected from the 10- $\mu$ m sections, and fractions from 3 or 4 sections were collected in sterile tubes. DNA was extracted and purified from these paraffin sections using the QIAamp DNA FFPE Tissue Kit (Qiagen) according to the manufacturer's protocol.<sup>13,14</sup> DNA concentrations were determined using the TaqMan RNase P Detection Reagents Kit (Applied Biosystems) according to the manufacturer's protocol.<sup>13</sup>

### 2.4 | Targeted sequencing of genomic DNA in FFPE

Amplicon libraries were prepared for 10 nm of individual genomic DNA using an Ion AmpliSeq CHPv2 and Ion AmpliSeq Library Kit 2.0 (Thermo Fisher Scientific). Multiplex PCR was carried out for 20 cycles according to the manufacturer's protocol. The CHPv2 targeted 2790 Catalogue of Somatic Mutations in Cancer mutational hotspot regions in the following 50 cancer-related genes: *ABL1*, *AKT1*, *ALK*, *APC*, *ATM*, *BRAF*, *CDH1*, *CDKN2A*, *CSF1R*, *CTNNB1*, *EGFR*, *ERBB2*, *ERBB4*, *EZH2*, *FBXW7*, *FGFR1*, *FGFR2*, *FGFR3*, *FLT3*, *GNA11*, *GNAS*, *GNAQ*, *GNF1A*, *HRAS*, *IDH1*, *IDH2*, *JAK2*, *JAK3*, *KDR*, *KIT*, *KRAS*, *MET*, *MLH1*, *MPL*, *NOTCH1*, *NPM1*, *NRAS*, *PDGFRA*, *PIK3CA*, *PTEN*, *PTPN11*, *RB1*, *RET*, *SMAD4*, *SMARCB1*, *SMO*, *SRC*, *STK11*, *TP53*, and *VHL*.

Sequencing adapters were joined to the amplification products using unique barcodes (Ion Xpress Barcode Adapters 1-16 Kit; Thermo Fisher Scientific) and purified using Agencourt AMPure XP Reagent (Beckman Coulter) according to the manufacturer's protocol.<sup>24</sup>

### 2.5 | Statistical analysis

Statistical analysis was undertaken using SPSS statistics version 25.0 (IBM Japan). Clinicopathological variables were compared between patients with and without driver gene mutations. Categorical variables were compared by the  $\chi^2$  test and continuous variables were compared by the Mann-Whitney *U* test. Survival duration was calculated using the Kaplan-Meier method and comparisons were made between groups using a log-rank test. A Cox proportional hazards model was used to determine independent prognostic factors among the clinicopathological and genomic variables. Variables with  $P < .10$  as determined by univariate analysis were entered into a forward, stepwise backward multivariate analysis. All statistical tests were 2-tailed, and  $P < .05$  was considered significant.

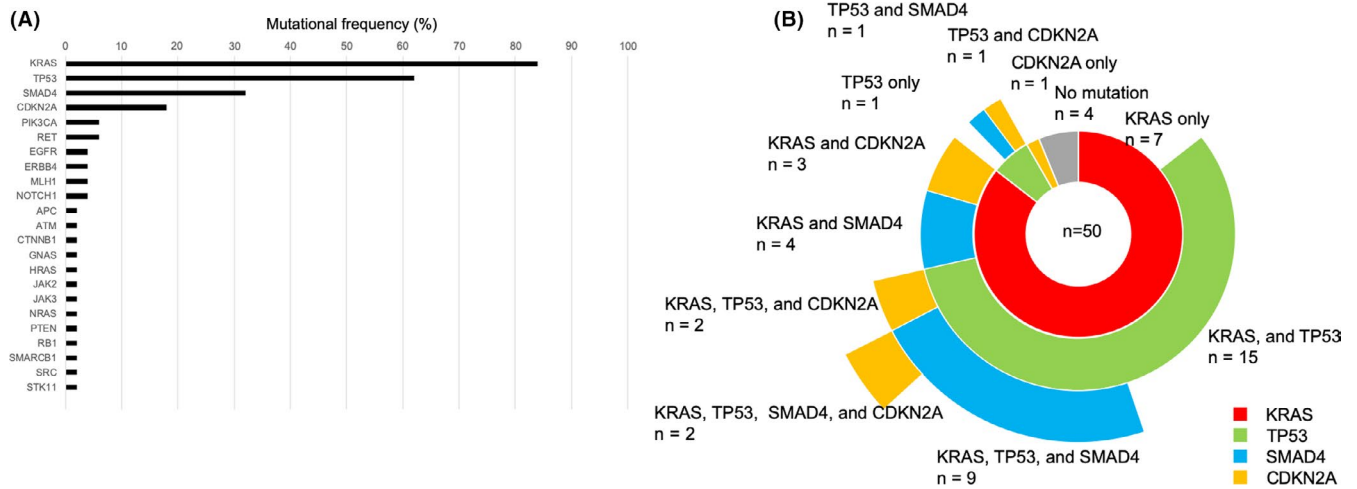
## 3 | RESULTS

### 3.1 | Mutations among 50 cancer-related genes

Seventy-four FFPE samples were used for DNA extraction, and 3 samples (4.1%) with DNA concentrations less than 0.85 ng/ $\mu$ L were excluded, as recommended by the manufacturer. Library preparation and sequencing by CHPv2 were carried out on 71 samples; however, 21 samples failed due to insufficient amplified DNA. Among the 74 samples, 50 (67.6%) were eligible for NGS analysis.

In total, 125 mutations were detected in 23 genes (Figure 1A). No mutations were detected in 3 (6.0%) cases. Detailed information on the driver gene mutations is shown in Table 1. *KRAS* was the most commonly mutated gene, with mutations observed in 42 cases (84.0%). This was followed by *TP53* in 32 (64.0%), *SMAD4* in 16 (32.0%), *CDKN2A* in 9 (18.0%), *PIK3CA* in 3 (6.0%), *RET* in 3 (6.0%), *EGFR* in 2 (4.0%), *ERBB4* in 2 (4.0%), *MLH1* in 2 (4.0%), and *NOTCH1* in 2 (4.0%) cases. Other variants detected in 1 case included hotspot mutations in *APC*, *ATM*, *GNAS*, *HRAS*, *JAK2*, *JAK3*, *NRAS*, *PTEN*, *RB1*, *SMARCB1*, *SRC*, and *STK11*. No mutations were identified within the hotspot regions of the remaining 27 genes. A single mutation was identified in 4 cases (8.0%), 2 mutations were identified in 22 cases (44.0%), 3 mutations were identified in 9 cases (18.0%), and 4 or more mutations were identified in 12 cases (24.0%).

The frequently observed mutation patterns of the driver genes included a single mutation in *KRAS*, *TP53*, and *CDKN2A* genes in 7 (14.0%), 1 (2.0%), and 1 (2.0%) cases, respectively. Mutations were observed in 2 driver genes, *KRAS/TP53* in 15 cases (30.0%) and *KRAS/SMAD4*, *KRAS/CDKN2A*, *TP53/SMAD4*, and *TP53/CDKN2A* in 4 (8.0%), 3 (6.0%), 1 (2.0%), and 1 (2.0%) cases, respectively.



**FIGURE 1** Gene mutation profile of pancreatic ductal adenocarcinoma (PDAC). A, Frequency of somatic mutations in 50 patients with PDAC. B, Combination of driver gene mutations in 50 PDAC patients

**TABLE 1** Details of driver gene mutations in 50 patients with pancreatic ductal adenocarcinoma

Parameter	KRAS (n = 42)	TP53 (n = 31)	SMAD4 (n = 16)	CDKN2A (n = 9)
Mutation, n (%)				
Missense	42 (100.0)	25 (80.6)	9 (56.3)	5 (55.6)
Nonsense	0 (0.0)	4 (12.9)	4 (25.0)	3 (33.3)
Frameshift insertion	0 (0.0)	1 (3.2)	2 (12.5)	0 (0.0)
Frameshift deletion	0 (0.0)	1 (3.2)	1 (6.3)	1 (11.1)
KRAS mutant subtype, n (%)				
G12D	20 (47.6)			
G12V	15 (35.7)			
G12R	4 (9.5)			
Q61H	3 (7.1)			

There were 9 (18.0%) and 1 (4.0%) cases with 3 mutated driver genes, namely *KRAS/TP53/SMAD4* and *KRAS/TP53/CDKN2A*, and 2 cases (4.0%) with mutations in all 4 driver genes (Figure 1B). We used CHPv2, which includes 10 druggable genes among the 50 target genes. Ten mutations were found in the druggable genes *ATM*, *NOTCH1*, *PIK3CA*, *PTEN*, and *RET*; however, these mutations were detected in only 8 cases (16.0%). Detailed information pertaining to the mutation analyses are shown in Table S1.

### 3.2 | Patient characteristics

Fifty patients were successfully analyzed by NGS in this study, and their characteristics are shown in Table 2. The median age of the patients was 71.5 (38–85) years; 32 patients were male and 18 female. Pancreaticoduodenectomy, distal pancreatectomy, and total pancreatectomy were carried out in 27, 20, and 3 cases, respectively. R0 resection was undertaken in 29 cases (58%), and R1 resection in 21 cases. Adjuvant chemotherapy was given in 42 cases and successfully completed in 29 cases.

### 3.3 | Survival analysis

At the time of analysis, 26 (52.0%) patients had recurrence and 14 (28.0%) patients had died. The median time from the date of surgery to the last follow-up was 18.7 months (range, 5.1–50.0 months) for all patients. The median time of RFS was 18.2 months, but that for OS was not reached.

Patients with *SMAD4* mutations had significantly poorer RFS than those with WT *SMAD4* (MST, 12.3 vs. 23.4 months, respectively; 1- and 3-year RFS, 54.7%, 13.0% vs. 69.0%, 33.2%, respectively,  $P = .034$ ; Figure 2A). Patients with *SMAD4* mutations had poorer OS than those with WT *SMAD4* (MST, 22.3 months vs. not reached, respectively; 1- and 3-year OS, 87.1%, 30.5% vs. 93.8%, 70.9%, respectively,  $P = .063$ ; Figure 2B). The *SMAD4* WT group had a significantly higher amount of venous infiltration ( $P = .044$ ) than the *SMAD4* mutation group. There was no significant difference in the other parameters such as age, sex, diabetes mellitus, tumor location, resectability, clinical stage, tumor diameter, R0 resection, pathological TNM, and pathological stage between the 2 groups (Table S2).

**TABLE 2** Characteristics of patients with pancreatic ductal adenocarcinoma (n = 50)

Parameter	n = 50
Age, y; median (range)	71.5 (38-85)
Sex, n (%)	
Male	32 (64.0)
Female	18 (36.0)
Preoperative CA19-9, IU/mL; median (range)	100 (1-2392)
Neoadjuvant treatment, n (%)	0 (0.0)
Induction of adjuvant treatment, n (%)	42 (84.0)
Successfully completed adjuvant treatment, n (%)	29 (58.0)
Regimen of adjuvant treatment, n (%)	
S-1	26 (52.0)
Gemcitabine	1 (2.0)
Gemcitabine + S-1	2 (4.0)
Surgical procedure, n (%)	
Pancreaticoduodenectomy	27 (54.0)
Distal pancreatectomy	20 (40.0)
Total pancreatectomy	3 (6.0)
Severe complications, n (%)	7 (14.0)
Surgery-related deaths, n (%)	0 (0.0)
Tumor location, n (%)	
Head	28 (56.0)
Body or tail	22 (44.0)
Tumor size, mm; median (range)	29.5 (12-65)
Residual tumor status, n (%) <sup>a</sup>	
R0	29 (58.0)
R1	21 (42.0)
R2	0 (0.0)
Pathological T status, n (%) <sup>a</sup>	
pT1	7 (14.0)
pT2	35 (70.0)
pT3	8 (16.0)
pT4	0 (0.0)
Pathological N status, n (%) <sup>a</sup>	
pN0	10 (20.0)
pN1	21 (42.0)
pN2	19 (38.0)
Pathological stage, n (%) <sup>a</sup>	
IA	4 (8.0)
IB	6 (12.0)
IIA	0 (0.0)
IIB	20 (40.0)
III	18 (36.0)
IV	2 (4.0)

CA19-9, carbohydrate antigen 19-9.

<sup>a</sup>Based on UICC TNM classification (8th edition).

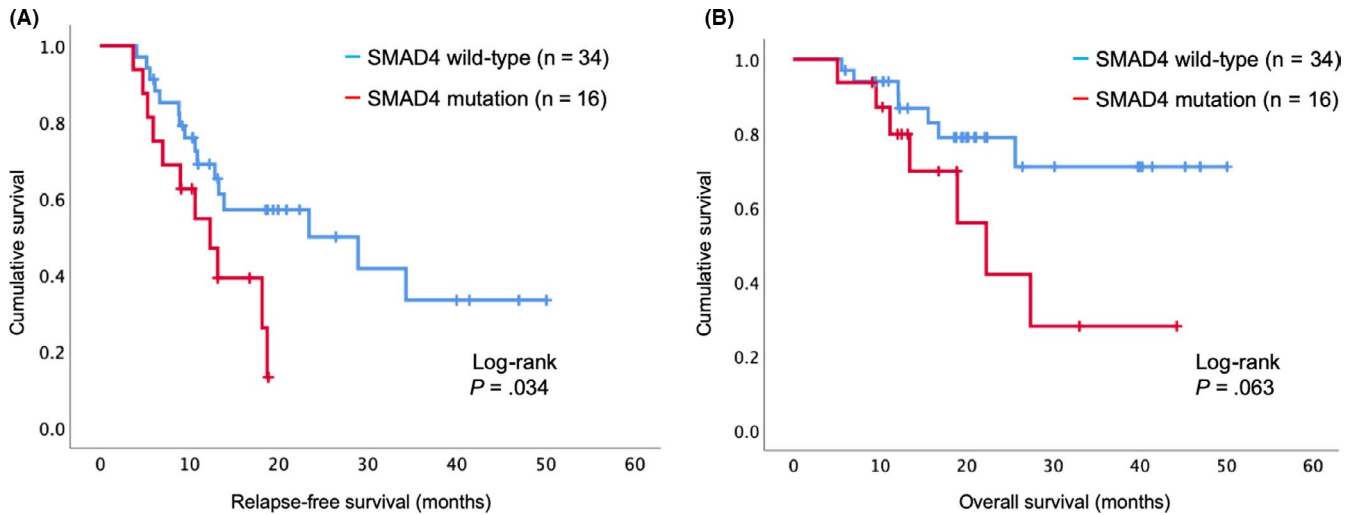
There were no single-mutation cases of *SMAD4*, and 15 (93.8%) of all cases of *SMAD4* mutations were coincident with *KRAS* mutations. Patients with a combination of *KRAS* and *SMAD4* mutations had significantly poorer RFS than those with WT *KRAS* or *SMAD4* (MST, 12.3 vs. 28.9 months; 1- and 3-year RFS, 51.4%, 11.4% vs. 69.9%, 33.8%,  $P = .014$ ; Figure 3A). Patients with a combination of *KRAS* and *SMAD4* mutations also had significantly poorer OS than those with WT *KRAS* or *SMAD4* (MST, 22.3 months vs. not reached, respectively; 1- and 3-year OS, 78.3%, 27.4% vs. 94.2%, 71.4%, respectively,  $P = .048$ ; Figure 3B).

On univariate Cox regression analysis, *SMAD4* mutation, a combination of *KRAS* and *SMAD4* mutations, lymphatic and intra-pancreatic nerve infiltration, serosal, extrapancreatic nerve plexus invasion, pathological T status, pathological N status, and successfully completed adjuvant treatment were identified as significant predictive factors for RFS (Table 3). Likewise, for OS, a combination of *KRAS* and *SMAD4* mutations, portal vein invasion, pathological T status, and successfully completed adjuvant treatment were significant predictive factors. On multivariate Cox regression analysis, a combination of *KRAS* and *SMAD4* mutations (HR = 4.218; 95% CI, 1.77-10.08;  $P = .001$ ) and successfully completed adjuvant treatment (HR = 0.226; 95% CI, 0.10-0.52;  $P < .001$ ) were independent predictive factors for RFS (Table 3), whereas a combination of *KRAS* and *SMAD4* mutations (HR = 6.730; 95% CI, 1.93-23.43;  $P = .003$ ) and successfully completed adjuvant treatment (HR = 0.068; 95% CI, 0.02-0.27;  $P < .001$ ) were identified as independent predictive factors for OS (Table 3).

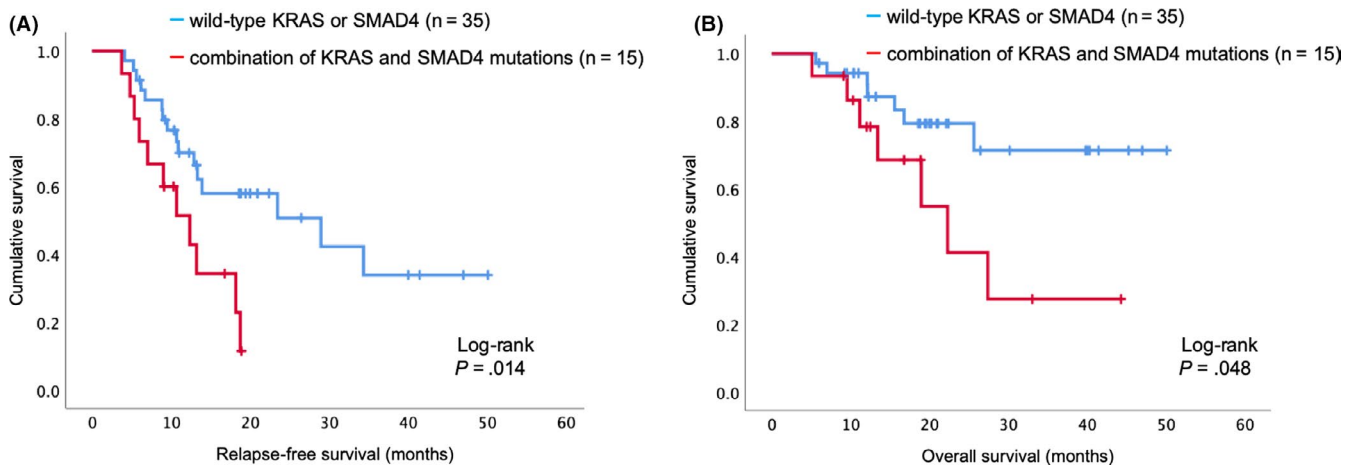
## 4 | DISCUSSION

Among the 4 driver genes, *SMAD4* mutations were recognized as a prognostic factor for RFS. Furthermore, the combination of *KRAS* and *SMAD4* mutations was found to be an independent prognostic factor for RFS and OS. DNA extracted from FFPE tissues was suitable for analysis by NGS. Druggable gene mutations were detected in 16% of resected FFPE samples of PDAC.

In the current study, 68% of FFPE samples were successfully sequenced. Nakagaki et al<sup>24</sup> reported that all fresh-frozen specimens were successfully sequenced, but 40% of FFPE specimens were eligible for NGS analyses. The report by Nakagaki et al used samples that were up to 10 years old, although details on the proportions of sample age are unknown; the quality of those samples might have been poorer than those used in the current study, which only included samples up to 5 years of age. However, in the current study, the success rate of analysis decreased when the time to analysis exceeded 3 years (data not shown). The Guidelines for Validation of Next-Generation Sequencing-Based Oncology Panels recommend that NGS analysis using DNA extracted from older FFPE blocks (eg older than 3 years) could increase background noise due to deamination.<sup>30</sup> Hence, DNA was extracted from newer samples as per the



**FIGURE 2** Survival curve using the Kaplan-Meier method for all patients with pancreatic ductal adenocarcinoma (PDAC) ( $n = 50$ ). A, Relapse-free survival curve according to *SMAD4* gene mutation status. B, Overall survival curve according to *SMAD4* gene mutation status



**FIGURE 3** Survival curve generated by the Kaplan-Meier method for patients with pancreatic ductal adenocarcinoma. A, Relapse-free survival curve based on the combined *KRAS* and *SMAD4* gene mutation status. B, Overall survival curve based on the combined *KRAS* and *SMAD4* gene mutation status

recommendation. However, this introduces the potential limitation of a shortened follow-up period. Establishing a biobank for FFPE specimens might not be necessary, as for fresh-frozen specimens, because FFPE is routinely used for pathological diagnosis and thus, genomic testing can be carried out retrospectively using resected specimens. Using NGS with FFPE should therefore aid in the introduction of genomic testing in daily clinical practice. The current study indicated that DNA extracts from FFPE tissues are eligible for NGS analysis and elicit an accurate prognosis.

In the current study, the mutation rates in the four driver genes were similar to those described in previous reports.<sup>17,18,20</sup> Among these genes, *SMAD4* mutations were found to be an independent prognostic factor for RFS. Furthermore, the combination of *KRAS* and *SMAD4* mutations was an independent prognostic factor for both RFS and OS. However, other mutations, including those in

*KRAS* alone, as well as those in *TP53* and *CDKN2A*, did not stratify cases by RFS or OS. Although *KRAS* mutations in PDAC occur too frequently to be considered appropriate prognostic factors, previous reports mention that the *KRAS* mutant subtype G12V or G12D is associated with poor prognosis<sup>31,32</sup>; however, which exact subtype is associated with a worse prognosis is controversial. In the current study, differences in the *KRAS* mutation subtype had no impact on prognosis.

The transforming growth factor- $\beta$ /*SMAD4* signaling pathway suppresses tumors that induce cell cycle arrest and apoptosis.<sup>33,34</sup> Loss of heterozygosity or the homozygous deletion of *SMAD4* was first reported in PDAC,<sup>35</sup> but has since been identified in various types of cancer.<sup>36,37</sup> In PDAC, the decreased expression of *SMAD4* identified by the immunolabeling of resected specimens has been reported to be associated with poor prognosis.<sup>14,38-40</sup> In NGS,

**TABLE 3** Univariate and multivariate Cox regression analysis of prognostic factors for relapse-free survival and overall survival in patients with pancreatic ductal adenocarcinoma

	Relapse-free survival					Overall survival				
	Univariate		Multivariate			Univariate		Multivariate		
	HR	P value	HR	95% CI	P value	HR	P value	HR	95% CI	P value
KRAS mutation	0.847	.763				0.525	.336			
KRAS mutant subtype	0.861	.464				1.016	.958			
TP53 mutation	1.154	.720				1.245	.716			
SMAD4 mutation	2.337	.040				2.611	.074			
CDKN2A mutation	1.157	.770				2.496	.131			
Combination of KRAS and SMAD4 mutations	2.639	.019	4.218	1.765-10.079	.001	2.767	.057	6.730	1.934-23.426	.003
Preoperative serum CA19-9	1.000	.214				1.001	.146			
Tumor size ≥20 mm	1.703	.387				2.176	.454			
Tumor differentiation grade	0.670	.313				1.182	.785			
Lymphatic infiltration ≥2	2.786	.061				2.020	.359			
Venous infiltration ≥2	1.388	.548				2.375	.405			
Intrapancreatic nerve infiltration ≥2	2.928	.080				29.19	.230			
Serosal invasion	2.116	.059				1.146	.814			
Retro pancreatic tissue invasion	1.332	.601				26.98	.349			
Portal vein invasion	1.895	.100				3.213	.052			
Arterial invasion	0.572	.449				0.653	.684			
Extrapancreatic nerve plexus invasion	2.428	.061				1.547	.577			
Pathological T status	2.107	.041			.092	2.339	.078			.332
Pathological N status	1.713	.061			.561	1.275	.501			.863
Pathological stage	1.189	.259				1.163	.474			
Residual tumor status	1.640	.208				1.137	.824			
Severe complications	0.433	.560				0.592	.614			
Successfully completed adjuvant treatment	0.295	.002	0.226	0.098-0.521	<.001	0.228	.013	0.068	0.017-0.274	<.001

Abbreviations: CA19-9, carbohydrate antigen 19-9; CI, confidence interval; HR, hazard ratio.

SMAD4 mutations are considered a poor prognostic factor in the analysis of DNA extracts from resected PDAC cells purified from cell lines or xenografts,<sup>21</sup> whereas they were not a prognostic factor in the analysis of DNA extracts from FFPE or fresh-frozen specimens. To our knowledge, the current study is the first to report SMAD4 mutations as a prognostic factor for PDAC in NGS analysis using DNA extracts from FFPE. Wilentz et al reported that SMAD4 mutations could be detected at a rate of 30% in high-grade PanIN, a precancerous lesion of pancreatic cancer. However, there were no SMAD4 mutations in low-grade PanIN (non-PanIN and PanIN 1).<sup>41</sup> In a mouse model, KRAS mutations alone could slow the progression of PanIN to cancer; however, combining KRAS mutations

and SMAD4 deletions was found to cause the rapid progression of tumors.<sup>42</sup> In the examination of autopsy cases, SMAD4 gene mutations were found to be a predictor of multiple distant metastases.<sup>43</sup> These previous reports suggest that SMAD4 mutations could lead to cancer progression and, therefore, poor prognosis; this supports the results of the current study. The combination of KRAS and SMAD4 could potentially be used as a prognostic indicator for the selection of appropriate adjuvant treatment regimens, such as multidrug combination therapy or extension of the treatment period.

Similar to previous reports, druggable gene mutations were detected in only 16% of cases in the current study.<sup>18,20</sup> Cancer Hotspot

Panel version 2 does not contain *BRCA* mutations, which have been reported to be effective targets for treatment with a poly (adenosine diphosphate-ribose) polymerase inhibitor.<sup>9</sup> In addition, no mutations were detected in 27 genes of CHPv2 in the current study. Therefore, the development of a specific multigene panel might be needed for PDAC.

The current study had some limitations. First, CHPv2 could only detect 50 cancer-related genes, and could not identify the presence or absence of other related and targetable mutations. Second, the current study had a retrospective design; thus, the results should be verified in more cases, and in a prospective study. Finally, approximately 30% of NGS analyses were excluded due to unsuccessful sequencing.

In conclusion, based on NGS analysis using the FFPE tissue of resected PDAC, the current study revealed that the combination of *KRAS* and *SMAD4* mutations is an independent poor prognostic factor for recurrence and survival. Further prospective investigations based on this analysis are needed in the future.

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## CONFLICT OF INTEREST

MS has received research funds from Taiho Pharmaceutical, Shionogi, and Eisai. YK has received scholarship endowments from Taiho Pharmaceutical, Chugai Pharmaceutical, Tsumura & Co., Otsuka Pharmaceutical, AsahiKASEI, Eisai, Otsuka Pharmaceutical Factory, Takeda Pharmaceutical, Medicon, Pfizer Japan, Ono Pharmaceutical, Shionogi & Co., and Daiichi Sankyo, and received lecture fees/honoraria from AsahiKASEI, Ono Pharmaceutical, Ethicon, Taiho Pharmaceutical, and Chugai Pharmaceutical, and is an officer/advisor for medical corporations Keiyoukai Keiai Clinic, Hirata Clinic, and Ageo Central General Hospital. The other authors declare that they have no conflicts of interest.

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#### SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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