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ORIGINAL ARTICLE

Post-operative mortality and recurrence patterns in pancreatic cancer according to *KRAS* mutation and CDKN2A, p53, and SMAD4 expression

Yohei Masugi[1,2*†], Manabu Takamatsu[3,4†], Mariko Tanaka[5†], Kensuke Hara[1], Yosuke Inoue[6], Tsuyoshi Hamada[7,8*], Tatsunori Suzuki[7], Junichi Arita[9,10], Yuki Hirose[6], Yoshikuni Kawaguchi[9], Yousuke Nakai[7,11], Atsushi Oba[6], Naoki Sasahira[8], Gaku Shimane[12], Tsuyoshi Takeda[8], Keisuke Tateishi[7], Sho Uemura[12], Mitsuhiro Fujishiro[7], Kiyoshi Hasegawa[9], Minoru Kitago[12], Yu Takahashi[6], Tetsuo Ushiku[5‡], Kengo Takeuchi[3,4‡], and Michiie Sakamoto,[1‡] for the GTK Pancreatic Cancer Study Group in Japan

[1]*Department of Pathology, Keio University School of Medicine, Tokyo, Japan*
[2]*Division of Diagnostic Pathology, Keio University School of Medicine, Tokyo, Japan*
[3]*Division of Pathology, Cancer Institute, Japanese Foundation for Cancer Research, Tokyo, Japan*
[4]*Department of Pathology, Cancer Institute Hospital, Japanese Foundation for Cancer Research, Tokyo, Japan*
[5]*Department of Pathology, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan*
[6]*Department of Hepatobiliary and Pancreatic Surgery, Cancer Institute Hospital, Japanese Foundation for Cancer Research, Tokyo, Japan*
[7]*Department of Gastroenterology, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan*
[8]*Department of Hepato-Biliary-Pancreatic Medicine, Cancer Institute Hospital, Japanese Foundation for Cancer Research, Tokyo, Japan*
[9]*Hepato-Biliary-Pancreatic Surgery Division, Department of Surgery, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan*
[10]*Department of Gastroenterological Surgery, Akita University Graduate School of Medicine, Akita, Japan*
[11]*Department of Endoscopy and Endoscopic Surgery, The University of Tokyo Hospital, Tokyo, Japan*
[12]*Department of Surgery, Keio University School of Medicine, Tokyo, Japan*

**Correspondence to: Yohei Masugi, Division of Diagnostic Pathology, Department of Pathology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku City, Tokyo 160-8582, Japan. E-mail: masugi@z6.keio.jp; Tsuyoshi Hamada, Department of Gastroenterology, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo City, Tokyo 113-8655, Japan. E-mail: hamada-tky@umin.ac.jp*

†These authors contributed equally as co-first authors.
‡These authors contributed equally as co-last authors.

Abstract

Alterations in *KRAS*, *CDKN2A* (*p16*), *TP53*, and *SMAD4* genes have been major drivers of pancreatic carcinogenesis. The clinical course of patients with pancreatic cancer in relation to these driver alterations has not been fully characterised in large populations. We hypothesised that pancreatic carcinomas with different combinations of *KRAS* mutation and aberrant expression of CDKN2A, p53, and SMAD4 might show distinctive recurrence patterns and post-operative survival outcomes. To test this hypothesis, we utilised a multi-institutional cohort of 1,146 resected pancreatic carcinomas and assessed *KRAS* mutations by droplet digital polymerase chain reaction and CDKN2A, p53, and SMAD4 expression by immunohistochemistry. Multivariable hazard ratios (HRs) and 95% confidence intervals (CIs) for disease-free survival (DFS) and overall survival (OS) were computed according to each molecular alteration and the number of altered genes using the Cox regression models. Multivariable competing risks regression analyses were conducted to assess the associations of the number of altered genes with specific patterns of recurrence. Loss of SMAD4 expression was associated with short DFS (multivariable HR, 1.24; 95% CI, 1.09–1.43) and OS times (multivariable HR, 1.27; 95% CI, 1.10–1.46). Compared to cases with 0–2 altered genes, cases with three and four altered genes had multivariable HRs for OS of 1.28 (95% CI, 1.09–1.51) and 1.47 (95% CI, 1.22–1.78), respectively ($p_{trend} < 0.001$). Patients with an increasing number of altered genes were more likely to have short DFS time ($p_{trend} = 0.003$) and to develop liver metastasis ($p_{trend} = 0.006$) rather than recurrence at local or other distant sites. In conclusion, loss of SMAD4 expression and an increasing number of altered genes were associated with unfavourable outcomes in pancreatic cancer patients. This study suggests that the accumulation of the four major driver alterations can confer a high metastatic potential to the liver, thereby impairing post-operative survival among patients with pancreatic cancer.

Keywords: cohort studies; oncogenes; pancreatectomy; pancreatic ductal adenocarcinoma; outcome

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No conflicts of interest were declared.

Introduction

Alterations in *KRAS*, *CDKN2A* (*p16*), *TP53*, and *SMAD4* genes have been major drivers involved in pancreatic carcinogenesis [1–3]. Clinical studies suggest that each molecular alteration may be a biomarker for an unfavourable prognosis of pancreatic cancer patients with a modest increase in the mortality hazard [4–9]. Further clinical data implicate that the mortality hazard among pancreatic cancer patients is elevated according to an increasing number of the driver gene alterations, suggesting additive tumour-promoting effects of those drivers [5–10]. However, the reported associations of alterations of the four driver genes with survival outcomes have been heterogeneous largely due to a lack of data derived from large populations.

Accumulating mechanistic evidence links alterations of the four driver genes to an aggressive phenotype of pancreatic cancer, characterised by high potentials of invasion, metastasis, and immune evasion [2,3,11–17]. However, there has been a paucity of data on how combinations of the four altered genes may affect tumour behavioural patterns in pancreatic cancer populations or mouse models. For instance, it is largely unknown whether the accumulation of those molecular alterations provokes premature post-operative recurrence through a hematogenous or lymphogenic mechanism. In a pooled analysis of 20 randomised clinical trials investigating adjuvant chemotherapy, disease-free survival (DFS) was well correlated with overall survival (OS) among patients with resected pancreatic cancer [18], suggesting the importance of regulating tumour progression during the short-term post-operative period. A better understanding of tumour behaviour according to molecularly defined subtypes would help us to refine post-operative surveillance programmes and develop new treatment strategies for this highly lethal malignancy [19]. We hypothesised that pancreatic cancers with different combinations of the four major driver alterations might show distinctive recurrence patterns and post-operative survival outcomes.

To test our hypothesis, we assessed *KRAS* mutations by droplet digital polymerase chain reaction (ddPCR) and CDKN2A, p53, and SMAD4 expression by immunohistochemistry (IHC) in 1,146 patients within a large multicentre cohort of resected pancreatic tumours with comprehensive data on clinical and pathological parameters. The current study found that combinations of the four driver alterations were associated with distinct recurrence patterns and post-operative outcomes.

Materials and methods

Study population

Within the GTK (Good To Know) Pancreatic Cancer consortium [20], we collected data on consecutive patients who underwent surgical resection of pancreatic carcinoma between 2005 and 2017 at Cancer Institute Hospital, Japanese Foundation for Cancer Research, The University of Tokyo Hospital, or Keio University Hospital (all in Tokyo, Japan). Among those patients, we included 1,146 cases with pancreatic cancer where surgical specimens of the tumours were available for molecular analyses. We excluded (1) patients with ductal adenocarcinoma variants, including intraductal papillary mucinous neoplasm-derived carcinoma, undifferentiated carcinoma, and colloid carcinoma, (2) patients with mixed tumours (e.g. mixed ductal-neuroendocrine carcinoma), (3) patients having advanced cancer of other origins concomitantly at the time of the index surgery, or (4) patients with 30-day or in-hospital mortality. In analyses of post-operative recurrence, we further excluded patients with a resected metastatic lesion, R2 resection margin (macroscopic residual tumour), or no available cross-sectional imaging following the index surgery. The patients were followed until death or the end of follow-up (30 April 2022), whichever came first.

The current study was designed and conducted according to the guidelines in the Helsinki Declaration. Given the retrospective study design, informed consent was obtained from all patients on an opt-out basis. The study was approved by the ethics committees at Cancer Institute Hospital, Japanese Foundation for Cancer Research, The University of Tokyo, and Keio University School of Medicine (all in Tokyo, Japan); and was registered with the UMIN registry (the University Hospital Medical Information Network registry; registration number, UMIN000044027).

Assessment of *KRAS* mutation status

As previously described [20], we conducted ddPCR for mutations in *KRAS* codons 12, 13, and 61 using the QX200 system (Bio-Rad Laboratories, Hercules, CA, USA). Variant allele frequency of *KRAS* was calculated as the ratio of the number of *KRAS*-mutant droplets to that of droplets including *KRAS*-mutant signal and/or *KRAS*-wild type signal. As previously described [20], tumours were classified as *KRAS*-mutant when the variant allele frequency was ≥1% and otherwise as *KRAS*-wild type.

Assessment of SMAD4, p53, and CDKN2A (p16) expression

Based on a standardised protocol, we constructed tissue microarrays (TMAs) from surgical specimens of the primary pancreatic carcinomas. Up to four tumour cores, approximately 2 mm in diameter, were included from each case. We conducted IHC of SMAD4 [21], p53 [21], and CDKN2A (p16) using 4-μm-thick TMA sections in an automated staining system (BOND-MAX; Leica Biosystems, Wetzlar, Germany) with the use of BOND Polymer Refine Detection Kit (Leica Biosystems). The primary monoclonal antibodies used were clone B-8 (mouse; dilution, catalogue number sc-7966, 1:100, Santa Cruz Biotechnology, Dallas, TX, USA) for SMAD4, clone DO-7 (mouse; dilution, catalogue number M700101-2, 1:2000, Agilent Technologies, Santa Clara, CA, USA) for p53, and clone EPR1473 for CDKN2A (p16) (mouse; dilution, catalogue number ab108349, 1:1000, Abcam, Cambridge, UK). A single pathologist (YM), blinded to other data, conducted all IHC assessments of the tumours based on the previously established criteria (Figure 1) [5,22,23]. For SMAD4, tumours were defined as 'intact' when there was any nuclear or cytoplasmic staining in carcinoma cells or 'lost' when there was a complete loss of staining [5,22]. Tumours showing heterogeneous nuclear p53 staining were classified as 'intact', whilst those showing diffuse nuclear staining or complete loss of nuclear staining were defined as 'aberrant' [5]. Nuclear expression levels of CDKN2A (p16) were classified as 'intact' when any ductal epithelial cells exhibited nuclear expression above the cytoplasmic background; otherwise, they were classified as 'lost' [23]. In a subset of cases ($n = 312$), expression status was independently examined by a second pathologist (KHar), and the agreements between the two pathologists were reasonable for all three markers with κ coefficients of 0.81 [95% confidence interval (CI), 0.74–0.87] for SMAD4, 0.92 (95% CI, 0.88–0.96) for p53, and 0.65 (95% CI, 0.55–0.76) for CDKN2A (p16). Based on our preliminary results suggesting no substantial intratumour heterogeneity in expression levels of SMAD4, p53, and CDKN2A in most of the invasive components of pancreatic carcinomas, we used TMA sections when available and used whole tissue sections for tumours that were not available for the TMAs or were not assessable in the TMA slides. The concordances between the scores for different cores in a single case were almost perfect with κ coefficients of 0.97 (95% CI, 0.95–0.99) for SMAD4 ($n = 837$), 0.988 (95% CI, 0.979–0.998) for p53 ($n = 854$), and 0.96 (95% CI, 0.93–0.99) for CDKN2A ($n = 857$). Regarding IHC of SMAD4, p53, and CDKN2A, we did not observe significant staining differences between surgically resected specimens and fine-needle aspiration specimens from selected cases of pancreatic cancer (supplementary material, Figure S1).

We conducted methylthioadenosine phosphorylase (MTAP) IHC in a random sample of 208 cases, as the *MTAP* gene is located at chromosome 9p21.3 close to *CDKN2A*, and thus, loss of MTAP expression has shown to be a good surrogate for homozygous deletions of *CDKN2A* [24]. The primary antibody, clone 2G4 (mouse; dilution, catalogue number H00004507-M01, 1:100, Abnova, Taipei, Taiwan), was used for MTAP immunostaining. All the tumours with complete loss of MTAP expression (65 out of 208 cases) were categorised as 'lost' expression of CDKN2A.

Targeted sequencing of genomic DNA

Previously reported sequencing data from 53 patients with pancreatic cancer were reanalysed in this study [10,25]. Detailed methods of targeted sequencing were described previously [10,25]. In brief, DNA was extracted from formalin-fixed paraffin-embedded slides of surgically resected pancreatic cancer using the QIAamp DNA FFPE Tissue Kit (Qiagen, Venlo, Netherlands) according to the manufacturer's protocol. Amplicon libraries were prepared from 10 ng DNA per sample using the Ion AmpliSeq Cancer Hotspot Panel v2 and the Ion AmpliSeq Library Kit 2.0 (Thermo Fisher Scientific, Waltham, MA, USA). Sequencing variants for point mutations, insertions, and deletions were called using the Ion Reporter Software (Thermo Fisher Scientific).

Transcriptome analysis

As previously described [21], RNA was extracted from frozen tissues of 20 patients with pancreatic cancer and was analysed using a Clariom D Pico Assay (Thermo Fisher Scientific) with a GeneChip

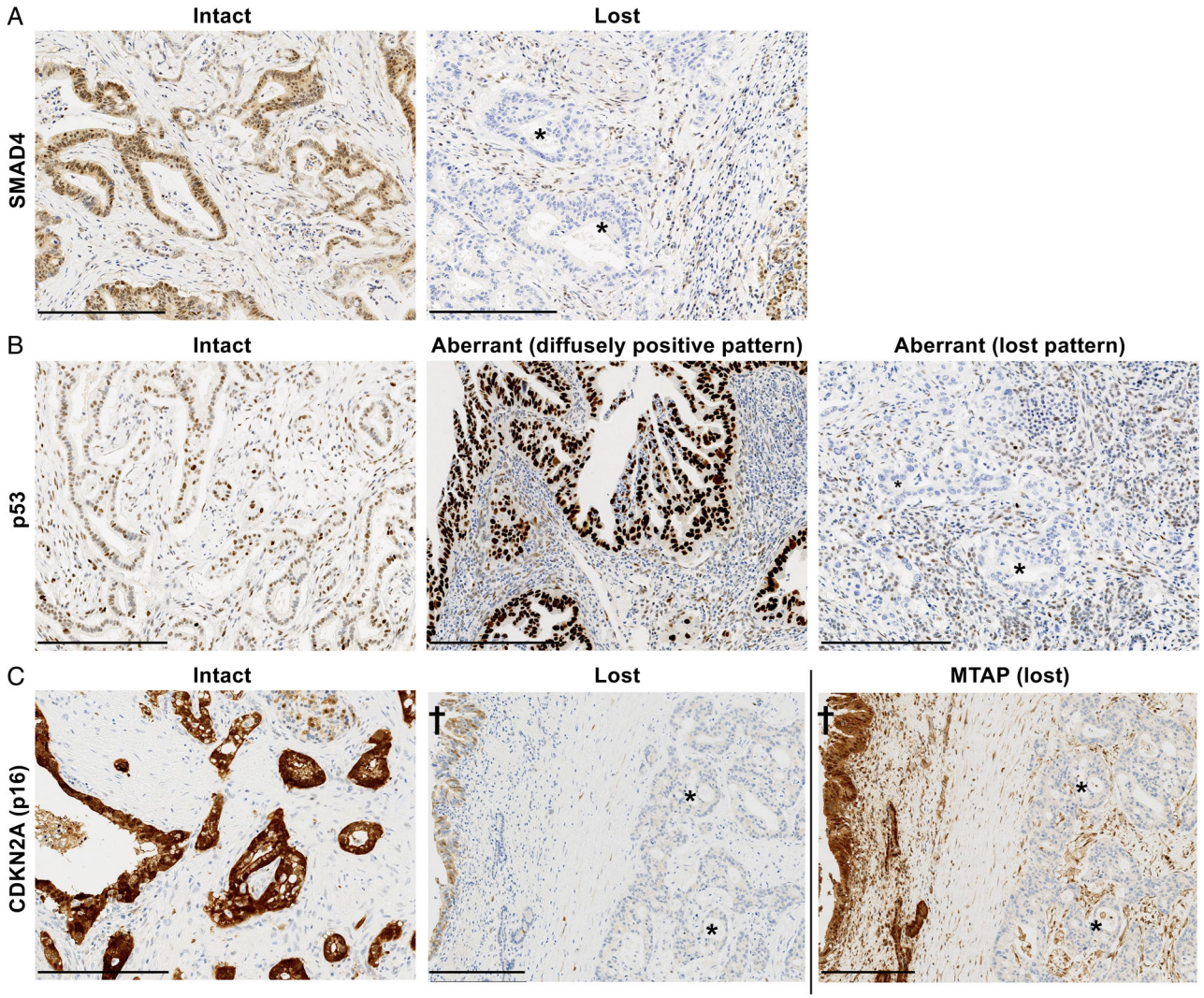


Figure 1. Representative immunohistochemistry images of (A) SMAD4, (B) p53, and (C) CDKN2A (p16) and MTAP expression. Asterisks indicate tumour cells with an aberrant 'lost' pattern of the corresponding marker. Tumours with diffuse nuclear p53 expression were classified as aberrant (B, middle). Note that cancer cells showed loss of both CDKN2A (C, middle) and MTAP (C, right) expression, whereas pancreatic intraepithelial neoplasm lesions (daggers) preserved expression for these markers. Bar = 250 μm.

Scanner 3000 7G System (Thermo Fisher Scientific). The microarray data were deposited in NCBI's Gene Expression Omnibus database (accession number, GSE157353). Tumours were categorised into classical and basal-like subtypes based on gene sets associated with Moffitt and colleagues' transcriptome subtypes [21,26].

Statistical analysis

The statistical analysis is detailed in the Supplementary methods [27–34]. All statistical analyses were performed using the SAS software (version 9.4; SAS Institute, Cary, NC, USA), and the two-sided α level of 0.005 was used for statistical significance [34].

Results

Within our multicentre cohort, we examined the tumour status of the four driver genes in 1,146 patients with resected pancreatic cancer (supplementary material, Figure S2). During the median follow-up time of

55.9 months (interquartile range, 42.8–83.7 months) for all censored cases, 812 (71%) patients were deceased. Clinical and pathological characteristics of the patients with pancreatic cancer, by alteration of each gene and the number of altered genes, are summarised in Table 1 and supplementary material, Table S1, respectively. *KRAS* was wild type and mutant in 23 (2.0%) and 1,123 (98%) cases, respectively. CDKN2A expression was intact and lost in 168 (15%) and 978 (85%) cases, respectively. p53 expression was intact and aberrant in 583 (51%) and 563 (49%) cases, respectively. SMAD4 expression was intact and lost in 512 (45%) and 634 (55%) cases, respectively. There were no statistically significant differences in clinical and pathological characteristics between the molecular subgroups at the α level of 0.005. Figure 2A demonstrates different combinations of tumour status of the four major drivers in strata of the number of altered genes. We found reasonable correlations between expression status by IHC and mutation status by targeted sequencing among 53 patients with pancreatic cancer (supplementary material, Table S2). IHC showed 95% specificity and 61% sensitivity for *TP53* mutation status. For *CDKN2A* and *SMAD4* mutations, IHC showed high sensitivities (100 and 87%, respectively). In exploratory analyses, we assessed the relationship between the Moffitt tumour subtype and the number of altered genes using transcriptome data from 20 pancreatic cancer specimens and did not observe any significant association (supplementary material, Figure S3).

In our primary analyses, we examined associations of the status of the four driver genes with survival outcomes among pancreatic cancer patients (Table 2 and supplementary material, Table S3). Loss of SMAD4 expression was associated with DFS [multivariable hazard ratio (HR), 1.24; 95% CI, 1.09–1.43] and OS (multivariable HR, 1.27; 95% CI, 1.10–1.46). The status of *KRAS* mutation, CDKN2A expression, or p53 expression was not associated with survival times in multivariable Cox regression analyses ($p > 0.06$). The number of altered genes was inversely associated with DFS and OS times: compared to cases with 0–2 altered genes, cases with three and four altered genes had multivariable HRs for DFS of 1.22 (95% CI, 1.04–1.42) and 1.32 (95% CI, 1.10–1.59), respectively ($p_{trend} = 0.003$); and multivariable HRs for OS of 1.28 (95% CI, 1.09–1.51) and 1.47 (95% CI, 1.22–1.78), respectively ($p_{trend} < 0.001$). Kaplan–Meier survival curves demonstrated similar associations of the gene alterations with DFS and OS (Figure 2B,C for the number of genes and supplementary material, Figure S4 for each gene). In meta-analyses, we observed no statistically significant heterogeneity in the survival associations of the number of altered genes between the institutional cohorts ($p_{heterogeneity} > 0.58$, Figure 2D,E) and, thus, pooled the institutional cohorts for all survival analyses. When we examined different combinations of altered genes in cases with two or three altered genes, we observed no differential associations with DFS and OS times by the combinations (supplementary material, Table S4). The limited number of *KRAS*-wild type cases precluded a robust statistical assessment of different combinations in this subgroup (data not shown). In addition, we investigated the additive effect of loss of SMAD4 expression on specific combinations of the other driver alterations (supplementary material, Table S5). Loss of SMAD4 expression was associated with shorter DFS and OS if additionally present in tumours with *KRAS* and CDKN2A alterations. Such additive effect of SMAD4 loss was not observed in tumours with other combinations of the other driver alterations. We observed no statistical interaction between the four driver alterations in relation to survival times at the α level of 0.005 ($p_{interaction} > 0.007$, supplementary material, Figure S5). Among 907 patients receiving adjuvant chemotherapy, the regimens were S-1 in 469 patients (52%), gemcitabine-based in 337 (37%), others in 93 (10%), and unknown in 8 (0.9%). In stratified analyses, similar survival associations of SMAD4 expression and the number of altered genes were observed irrespective of receipt of adjuvant chemotherapy ($p_{interaction} > 0.19$, supplementary material, Table S6). When we examined a specific regimen (S-1 or gemcitabine-based), we also observed no statistical interactions in relation to survival times ($p_{interaction} > 0.12$).

In secondary analyses, we examined recurrence patterns of resected pancreatic cancer (supplementary material, Table S7) and their correlation with the number of altered genes. The number of altered genes was positively associated with the risk of distant metastasis [multivariable subdistribution HR 1.35 (95% CI, 1.11–1.64) and 1.23 (95% CI, 0.98–1.55) for three and four altered genes (versus 2), respectively; $p_{trend} = 0.096$], but not with the risk of local recurrence ($p_{trend} > 0.39$, Table 3). When we further examined specific patterns of distant metastasis, multivariable competing risks regression analyses demonstrated a stronger association of the number of altered genes for liver metastasis ($p_{trend} = 0.006$) than for non-liver metastasis ($p_{trend} > 0.35$). Cumulative incidence curves of the recurrence patterns by the number of altered genes are presented in Figure 3. To investigate whether pancreatic carcinomas harbouring only *KRAS* mutation among the four drivers showed specific recurrence patterns, we examined recurrence patterns of *KRAS*-mutated tumours with or without other

Table 1. Clinical and pathological characteristics of resected pancreatic cancer cases, overall or by the tumour status of the four drivers

Characteristic*	All cases ($n = 1,146$)	*KRAS* mutation: Wild type ($n = 23$)	*KRAS* mutation: Mutant ($n = 1,123$)	*P*	CDKN2A (p16) expression: Intact ($n = 168$)	CDKN2A (p16) expression: Lost ($n = 978$)	*P*	p53 expression: Intact ($n = 583$)	p53 expression: Aberrant ($n = 563$)	*P*	SMAD4 expression: Intact ($n = 512$)	SMAD4 expression: Lost ($n = 634$)	*P*
Mean age ± SD (years)	67.1 ± 9.7	66.0 ± 13.7	67.2 ± 9.7	0.56	66.8 ± 9.4	67.2 ± 9.8	0.57	67.2 ± 9.9	67.1 ± 9.6	0.83	67.1 ± 9.7	67.2 ± 9.8	0.87
Sex				0.50			0.13			0.011			0.16
Female	478 (42%)	8 (35%)	470 (42%)		79 (47%)	399 (41%)		222 (38%)	256 (45%)		202 (39%)	276 (44%)	
Male	668 (58%)	15 (65%)	653 (58%)		89 (53%)	579 (59%)		361 (62%)	307 (55%)		310 (61%)	358 (56%)	
Year of diagnosis				0.23			0.046			0.26			0.034
2005–2010	331 (29%)	3 (13%)	328 (29%)		57 (34%)	274 (28%)		181 (31%)	150 (27%)		135 (26%)	196 (31%)	
2011–2014	387 (34%)	9 (39%)	378 (34%)		43 (26%)	344 (35%)		192 (33%)	195 (35%)		193 (38%)	194 (31%)	
2015–2017	428 (37%)	11 (48%)	417 (37%)		68 (40%)	360 (37%)		210 (36%)	218 (39%)		184 (36%)	244 (38%)	
Tumour location				0.32			0.25			0.55			0.85
Head of the pancreas	733 (64%)	17 (74%)	716 (64%)		114 (68%)	619 (63%)		368 (63%)	365 (65%)		326 (64%)	407 (64%)	
Body to tail of the pancreas	413 (36%)	6 (26%)	407 (36%)		54 (32%)	359 (37%)		215 (37%)	198 (35%)		186 (36%)	227 (36%)	
Histological type				0.020			0.43			0.052			0.18
Adenocarcinoma	1116 (97%)	20 (87%)	1096 (98%)		162 (96%)	954 (98%)		573 (98%)	543 (96%)		495 (97%)	621 (98%)	
Adenosquamous carcinoma	30 (2.6%)	3 (13%)	27 (2.4%)		6 (3.6%)	24 (2.5%)		10 (1.7%)	20 (3.6%)		17 (3.3%)	13 (2.1%)	
Tumour differentiation†				0.23			0.045			0.26			0.31
Well to moderate	650 (58%)	9 (45%)	641 (58%)		106 (65%)	544 (57%)		343 (60%)	307 (57%)		280 (57%)	370 (60%)	
Poor	466 (42%)	11 (55%)	455 (42%)		56 (35%)	410 (43%)		230 (40%)	236 (43%)		215 (43%)	251 (40%)	
Stroma type				0.69			0.55			0.013			0.24
Non-scirrhous	753 (66%)	16 (70%)	737 (66%)		107 (64%)	646 (66%)		403 (69%)	350 (62%)		327 (64%)	426 (67%)	
Scirrhous	393 (34%)	7 (30%)	386 (34%)		61 (36%)	332 (34%)		180 (31%)	213 (38%)		185 (36%)	208 (33%)	
Lymphatic invasion				0.58			0.033			0.45			0.65
Absent/mild	735 (64%)	16 (70%)	719 (64%)		120 (71%)	615 (63%)		380 (65%)	355 (63%)		332 (65%)	403 (64%)	
Moderate/marked	411 (36%)	7 (30%)	404 (36%)		48 (29%)	363 (37%)		203 (35%)	208 (37%)		180 (35%)	231 (36%)	
Venous invasion				0.39			0.009			0.49			0.46
Absent/mild	396 (35%)	6 (26%)	390 (35%)		73 (43%)	323 (33%)		207 (36%)	189 (34%)		171 (33%)	225 (35%)	
Moderate/marked	750 (65%)	17 (74%)	733 (65%)		95 (57%)	655 (67%)		376 (64%)	374 (66%)		341 (67%)	409 (65%)	
Neural invasion				0.20			0.006			0.76			0.071
Absent/mild	404 (35%)	11 (48%)	393 (35%)		75 (45%)	329 (34%)		208 (36%)	196 (35%)		195 (38%)	209 (33%)	
Moderate/marked	742 (65%)	12 (52%)	730 (65%)		93 (55%)	649 (66%)		375 (64%)	367 (65%)		317 (62%)	425 (67%)	
Mean tumour size ± SD (cm)	3.4 ± 1.6	3.6 ± 1.5	3.4 ± 1.6	0.65	3.2 ± 1.3	3.5 ± 1.6	0.046	3.4 ± 1.6	3.4 ± 1.6	0.99	3.3 ± 1.5	3.5 ± 1.6	0.086
Positive lymph nodes				0.47			0.14			0.38			0.080
0	355 (31%)	8 (35%)	347 (31%)		63 (37%)	292 (30%)		180 (31%)	175 (31%)		176 (34%)	179 (28%)	
1–3	460 (40%)	11 (48%)	449 (40%)		62 (37%)	398 (41%)		244 (42%)	216 (38%)		197 (38%)	263 (41%)	
≥4	331 (29%)	4 (17%)	327 (29%)		43 (26%)	288 (29%)		159 (27%)	172 (31%)		139 (27%)	192 (30%)	
UICC cancer stage				0.48			0.032			0.67			0.24
I	298 (26%)	8 (35%)	290 (26%)		59 (35%)	239 (24%)		157 (27%)	141 (25%)		148 (29%)	150 (24%)	
II	499 (44%)	11 (48%)	488 (44%)		66 (39%)	433 (44%)		258 (44%)	241 (43%)		216 (42%)	283 (45%)	
III	301 (26%)	4 (17%)	297 (26%)		38 (23%)	263 (27%)		145 (25%)	156 (28%)		127 (25%)	174 (27%)	
IV	48 (4.2%)	0	48 (4.3%)		5 (3.0%)	43 (4.4%)		23 (4.0%)	25 (4.4%)		21 (4.1%)	27 (4.3%)	

Table 1. Continued

Characteristic*	All cases ($n = 1,146$)	*KRAS* mutation			CDKN2A (p16) expression			p53 expression			SMAD4 expression		
		Wild type ($n = 23$)	Mutant ($n = 1,123$)	*P*	Intact ($n = 168$)	Lost ($n = 978$)	*P*	Intact ($n = 583$)	Aberrant ($n = 563$)	*P*	Intact ($n = 512$)	Lost ($n = 634$)	*P*
Resection margin status				0.82			0.11			0.47			0.89
R0	888 (77%)	19 (83%)	869 (77%)		139 (83%)	749 (77%)		448 (77%)	440 (78%)		400 (78%)	488 (77%)	
R1	253 (22%)	4 (17%)	249 (22%)		28 (17%)	225 (23%)		131 (22%)	122 (22%)		110 (21%)	143 (23%)	
R2	5 (0.4%)	0	5 (0.5%)		1 (0.6%)	4 (0.4%)		4 (0.7%)	1 (0.2%)		2 (0.4%)	3 (0.5%)	
Neoadjuvant therapy				0.52			0.27			0.17			0.19
None	999 (87%)	19 (83%)	980 (87%)		142 (85%)	857 (88%)		516 (89%)	483 (86%)		439 (86%)	560 (88%)	
Chemotherapy†	147 (13%)	4 (17%)	143 (13%)		26 (15%)	121 (12%)		67 (11%)	80 (14%)		73 (14%)	74 (12%)	
Adjuvant therapy				0.30			0.68			0.84			0.97
None	239 (21%)	7 (30%)	232 (21%)		33 (20%)	206 (21%)		123 (21%)	116 (21%)		107 (21%)	132 (21%)	
Chemotherapy†	907 (79%)	16 (70%)	891 (79%)		135 (80%)	772 (79%)		460 (79%)	447 (79%)		405 (79%)	502 (79%)	

UICC, Union for International Cancer Control.
*Percentage indicates the proportion of cases with a specific clinical or pathological characteristic in all cases or in each stratum of molecular subtypes. Total percentages may not equal 100% due to rounding.
†Tumour differentiation was assessed only for adenocarcinomas.
‡These categories include chemoradiotherapy.

driver alterations (aberrant expression of CDKN2A, p53, and/or SMAD4) and did not observe any significant difference between them (supplementary material, Table S8).

Discussion

In this large multi-institutional cohort study of patients with resected pancreatic cancer, we have shown that the number of altered genes among the four major drivers is inversely associated with post-operative DFS and OS times. These findings suggest that those driver genes play a pivotal role in pancreatic cancer progression after surgical resection as well as in pancreatic carcinogenesis. Of note, pancreatic tumours with a greater number of the altered genes were more likely to exhibit liver metastasis rather than local recurrence or recurrence at other distant sites. The survival associations of the altered genes were consistently observed in an analysis limited to patients receiving adjuvant chemotherapy. Our data underline not only the potential of those fundamental molecular alterations as biomarkers for survival among patients with pancreatic cancer but also the importance of designing specific treatment strategies for pancreatic carcinomas with the unfavourable genetic and epigenetic profile defined by the four tumour markers.

Pancreatic cancer is a collective disease entity consisting of molecularly heterogeneous neoplasms that evolve on the basis of sequential accumulation of genetic and epigenetic aberrations and represent distinctive tumour behavioural patterns according to their molecular architecture [1–3,35–37]. Experimental and clinical studies attest that alterations of the four major driver genes (*KRAS*, *CDKN2A*, *TP53*, and *SMAD4*) can individually or collectively exhibit cancer-promoting properties through accelerating cell proliferation, migration, and invasion [13–17,38–41]. For example, *SMAD4* serves as a mediator in the TGFB1 (TGF-β) signalling pathway, which has been involved in the progression of pancreatic intraepithelial neoplasia to invasive adenocarcinoma [16,42]. In addition, those molecular alterations potentially form the immunosuppressive microenvironment, as illustrated by preclinical and clinical data supporting the roles of *KRAS*- and *TP53*-related signalling pathways in immune evasion of pancreatic cancer [11–13,43]. In line with these mechanistic data, the current study points to the roles of SMAD4 expression and accumulation of the four major driver alterations in tumour progression following clinical diagnosis and surgical resection. Few large cohort

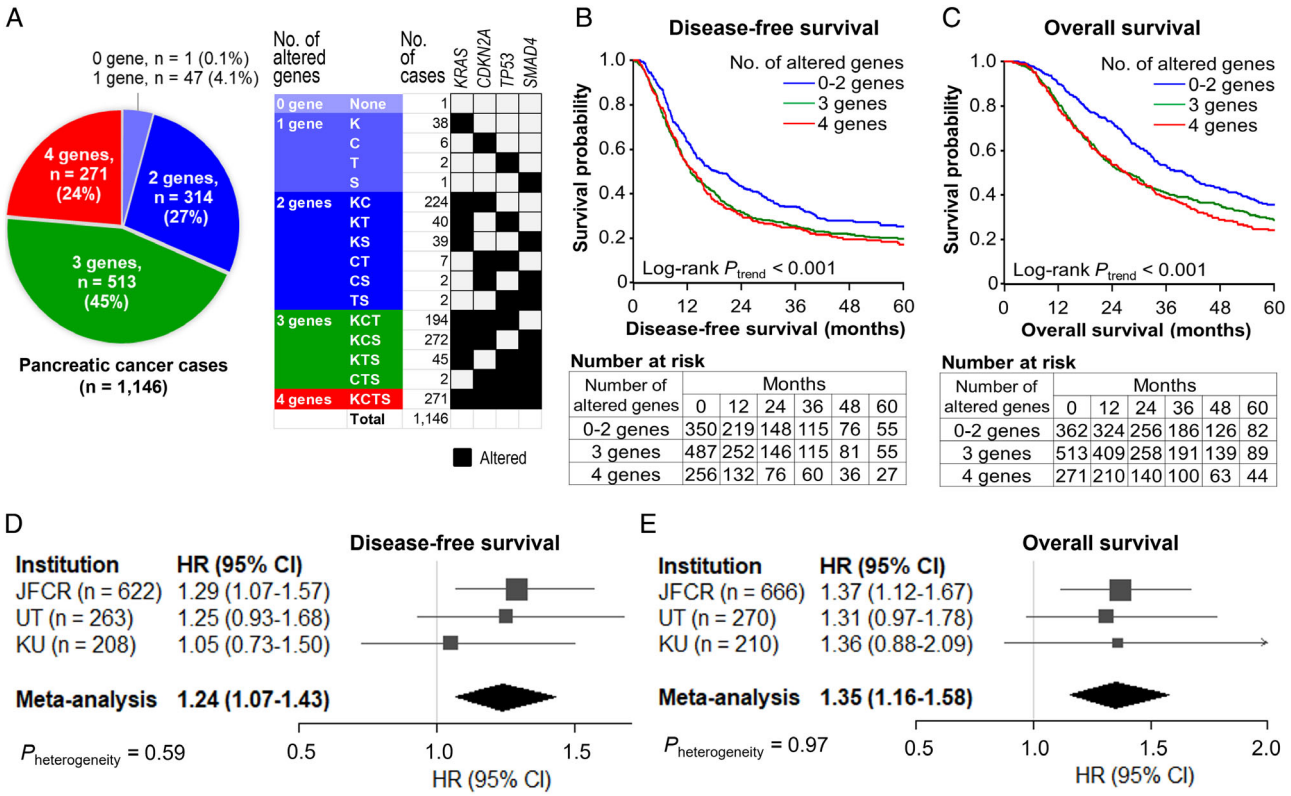


Figure 2. Associations of the number of altered genes with survival times among patients with resected pancreatic cancer. (A) Different combinations of tumour status of the four major drivers in strata of the number of altered genes. (B and C) Kaplan–Meier survival curves of DFS and OS times, respectively, according to the number of altered genes (0–2 and 3 versus 4 genes). (D and E) Meta-analyses of associations of the number of altered genes (3–4 genes compared to 0–2 genes) with DFS and OS times, respectively. Squares and horizontal lines indicate multivariable-adjusted HRs and 95% CIs, respectively. The area of the square reflects cohort-specific weight (inverse of the variance). Diamonds indicate pooled multivariable-adjusted HRs (centre) and 95% CIs (width). HRs were adjusted for the same set of covariates as Table 2. JFCR, Japanese Foundation for Cancer Research; KU, Keio University; UT, The University of Tokyo.

studies have investigated the responsiveness of pancreatic cancer to conventional chemotherapy regimens (i.e. 5-fluorouracil- or gemcitabine-based regimens) by the status of the four major driver alterations [44–46]. In the current study, the high mortality hazard associated with those driver alterations appeared to be unamenable to the administration of standard adjuvant chemotherapy. The four major driver alterations have long been untargettable, and no molecular-targeted agents have been approved, resulting in poor prognosis of pancreatic cancer patients. However, inhibitors selectively targeting *KRAS* G12C mutation (e.g. sotorasib) have emerged as promising therapeutics [47,48] and have opened opportunities for precision oncology for pancreatic cancer. In addition, investigations are underway to examine the effectiveness of agents on tumours harbouring other driver mutations, such as *TP53* mutations [49,50]. Our findings implicate the importance of developing agents targeting the signalling pathways altered by the four primary drivers.

In our study, pancreatic cancer with an increasing number of driver alterations was more likely to represent hepatic metastasis at an earlier stage of the post-operative course. Our large multicentre study likely provides robust evidence supporting the roles of the four major driver genes in determining post-operative tumour progression patterns as well as overall mortality hazard. Interestingly, the number of altered driver genes was not associated with any histopathological features of pancreatic cancer, including the number of positive lymph nodes and histopathological lymphatic invasion. Therefore, it is considered that pancreatic tumours with accumulated molecular drivers may metastasise through the hematogenous mechanism rather than the lymphogenic mechanism. Accumulating biallelic loss of cell-cycle regulation genes, including *CDKN2A*, *TP53*, and *SMAD4*, has contributed to a stepwise acceleration in cell cycle progression in pancreatic cancer [51]. A previous study has suggested

Table 2. The tumour status of the four major drivers and survival among patients with resected pancreatic cancer

	DFS				OS			
	No. of cases	No. of events	Univariable HR (95% CI)	Multivariable HR* (95% CI)	No. of cases	No. of events	Univariable HR (95% CI)	Multivariable HR* (95% CI)
KRAS mutation								
Wild type	23	15	1 (reference)	1 (reference)	23	13	1 (reference)	1 (reference)
Mutant	1,070	850	1.23 (0.74–2.05)	1.06 (0.63–1.78)	1,123	799	1.27 (0.73–2.20)	1.07 (0.62–1.86)
P			0.43	0.83			0.40	0.81
CDKN2A (p16) expression								
Intact	163	125	1 (reference)	1 (reference)	168	113	1 (reference)	1 (reference)
Lost	930	740	1.22 (1.00–1.47)	1.10 (0.91–1.33)	978	699	1.32 (1.08–1.61)	1.21 (0.99–1.48)
P			0.046	0.35			0.006	0.065
p53 expression								
Intact	557	438	1 (reference)	1 (reference)	583	404	1 (reference)	1 (reference)
Aberrant	536	427	1.10 (0.96–1.26)	1.07 (0.94–1.23)	563	408	1.13 (0.98–1.30)	1.12 (0.98–1.29)
P			0.17	0.32			0.082	0.10
SMAD4 expression								
Intact	490	373	1 (reference)	1 (reference)	512	346	1 (reference)	1 (reference)
Lost	603	492	1.20 (1.05–1.38)	1.24 (1.09–1.43)	634	466	1.25 (1.08–1.43)	1.27 (1.10–1.46)
P			0.008	0.002			0.002	0.001
No. of altered genes								
0–2	350	263	1 (reference)	1 (reference)	362	238	1 (reference)	1 (reference)
3	487	391	1.29 (1.10–1.51)	1.22 (1.04–1.42)	513	367	1.36 (1.15–1.60)	1.28 (1.09–1.51)
4	256	211	1.36 (1.13–1.63)	1.32 (1.10–1.59)	271	207	1.52 (1.26–1.83)	1.47 (1.22–1.78)
P_{trend} †			<0.001	0.003			<0.001	<0.001

*The multivariable Cox regression model initially included age, sex, year of diagnosis, tumour location, histological type, cancer stage, resection margin status, receipt of neoadjuvant chemotherapy, and receipt of adjuvant chemotherapy. Backward elimination with a threshold *p* of 0.05 was conducted to select variables for the final models. The variables that remained in the final models are described in supplementary material, Table S2.
†P_{trend} was calculated by entering the number of altered genes (continuous, 0–4) in the Cox regression model.

that an increasing number of genetic alterations of *KRAS*, *CDKN2A*, *TP53*, and *SMAD4* confer niche independency in engineered organoid models of pancreatic cancer [52]. Another mechanistic study using pancreatic cancer organoids has shown that *SMAD4* inactivation is required for the collective invasion of tumour cells [53]. These lines of evidence suggest that the four driver alterations in pancreatic carcinoma cells may co-ordinately drive highly metastatic phenotype. Future basic research is warranted to better understand the mechanisms underlying the development of liver-specific metastasis of pancreatic cancer.

In pancreatic carcinomas, *SMAD4* has been inactivated through homozygous deletion in 35% and a monoallelic mutation with loss of the other allele in 20% [54]. *CDKN2A* alterations in pancreatic carcinomas occur based on homozygous deletion in 40%, a monoallelic mutation with loss of the other allele in 40%, and hypermethylation in the gene promoter in 15% [54,55]. In our NGS (next-generation sequencing)-based analysis of 53 pancreatic cancer specimens, the prevalence of mutations in *SMAD4* and *CDKN2A* genes was 28 and 18%, respectively. This result is likely reasonable because the mutation calling by targeted sequencing approach underestimates the alterations in *SMAD4* and *CDKN2A* as this assay cannot detect uncommon mutations, large genomic deletion, or hypermethylation in promoter regions. In our IHC-based assessment, loss of expressions of SMAD4 and CDKN2A was found in 55 and 85%, respectively. Of note, IHC of SMAD4 and CDKN2A could detect genetic mutations in corresponding genes with high sensitivity (87 and 100%, respectively). SMAD4 IHC has been a powerful tool for detecting the genetic status of *SMAD4* [5,22]. Previous studies have also suggested that IHC may be a surrogate method to detect *CDKN2A* promoter methylation and subsequent gene silencing in various cancer types. For example, a previous study of 766 colorectal carcinomas has shown that loss of immunohistochemical expression of CDKN2A is more common in *CDKN2A*-methylated carcinomas than in *CDKN2A*-unmethylated carcinomas (61% versus 8.7%, respectively) [56]. Collectively, IHC may be a simple and useful method in assessing *SMAD4* and *CDKN2A* alterations in pancreatic cancer.

On the other hand, we should acknowledge a limitation of the IHC-based approach for assessing *TP53* status. The *TP53* gene is inactivated in 75% of pancreatic adenocarcinoma cases by loss of one allele coupled with an intragenic mutation in the second allele [54]. In the current study, the prevalence of aberrant p53 status

Table 3. The number of altered genes in relation to incidences of specific recurrence patterns among patients with resected pancreatic cancer

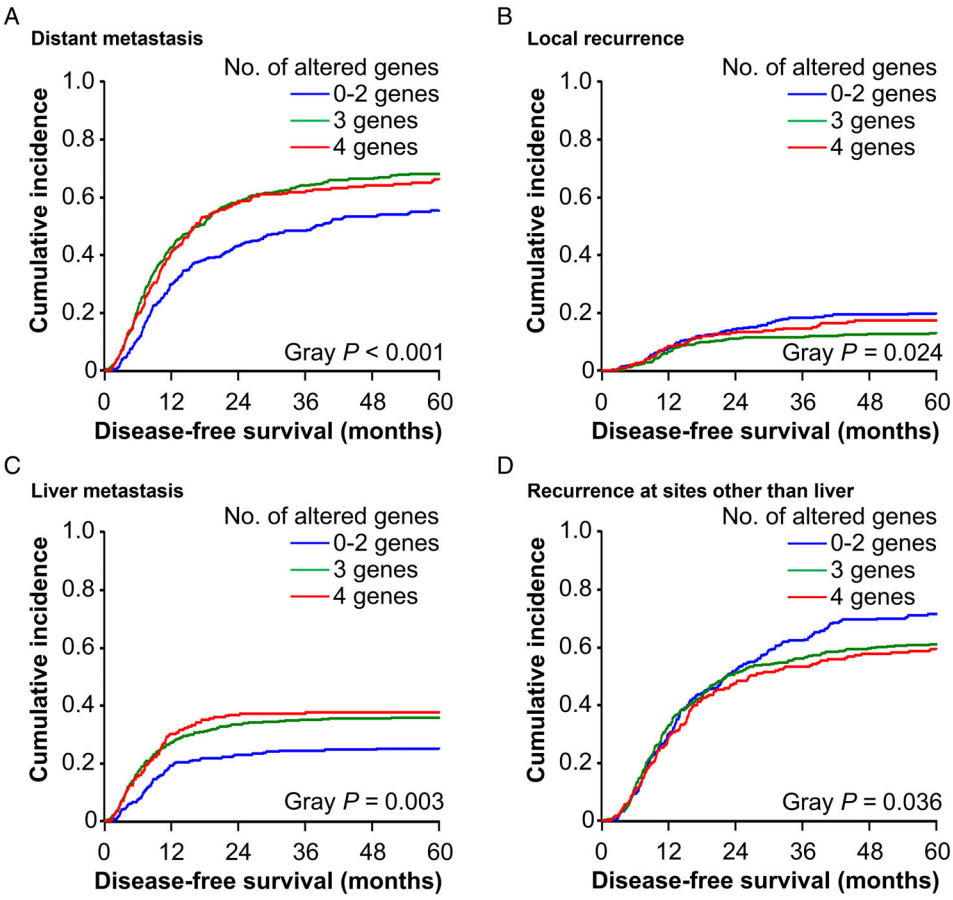
	No. of altered genes			
	0–2	3	4	P_{trend}*
Distant versus local†				
Distant only ($n = 563$)				
n	159	271	133	
Univariable SHR (95% CI)	1 (reference)	1.40 (1.16–1.68)	1.27 (1.02–1.59)	0.037
Multivariable SHR (95% CI)‡	1 (reference)	1.35 (1.11–1.64)	1.23 (0.98–1.55)	0.096
Distant + local ($n = 128$)				
n	36	57	35	
Univariable SHR (95% CI)	1 (reference)	1.30 (0.86–1.95)	1.41 (0.90–2.23)	0.11
Multivariable SHR (95% CI)‡	1 (reference)	1.28 (0.86–1.93)	1.39 (0.88–2.19)	0.12
Local only ($n = 174$)				
n	68	63	43	
Univariable SHR (95% CI)	1 (reference)	0.63 (0.45–0.88)	0.87 (0.60–1.25)	0.47
Multivariable SHR (95% CI)‡	1 (reference)	0.61 (0.43–0.85)	0.84 (0.58–1.22)	0.40
Specific site of distant metastasis				
Liver ($n = 283$)§				
n	65	137	81	
Univariable SHR (95% CI)	1 (reference)	1.55 (1.17–2.05)	1.63 (1.19–2.23)	0.003
Multivariable SHR (95% CI)‡	1 (reference)	1.44 (1.08–1.92)	1.58 (1.15–2.16)	0.006
Peritoneum ($n = 146$)§				
n	39	76	31	
Univariable SHR (95% CI)	1 (reference)	1.37 (0.95–1.98)	1.01 (0.64–1.58)	0.72
Multivariable SHR (95% CI)‡	1 (reference)	1.37 (0.95–1.98)	1.01 (0.64–1.58)	0.72
Lung ($n = 150$)§				
n	49	70	31	
Univariable SHR (95% CI)	1 (reference)	0.93 (0.65–1.33)	0.76 (0.49–1.18)	0.36
Multivariable SHR (95% CI)‡	1 (reference)	0.94 (0.65–1.34)	0.76 (0.49–1.19)	0.36
Lymph node ($n = 131$)§				
n	37	63	31	
Univariable SHR (95% CI)	1 (reference)	1.22 (0.83–1.79)	1.10 (0.69–1.73)	0.52
Multivariable SHR (95% CI)‡	1 (reference)	1.22 (0.83–1.79)	1.10 (0.69–1.73)	0.52

SHR, subdistribution hazard ratio.
*P_{trend} was calculated by entering the number of altered genes (continuous, 0–4) in the competing risks regression model.
†Three recurrence patterns (distant only versus distant and local versus local only) were treated as competing risk events.
‡The multivariable competing risks regression model initially included age, sex, year of diagnosis, tumour location, histological type, cancer stage, resection margin status, receipt of neoadjuvant chemotherapy, and receipt of adjuvant chemotherapy. Backward elimination with a threshold *p* of 0.05 was conducted to select variables for the final models.
§In an analysis of a specific site of distant metastasis, recurrence at other sites only was treated as a competing risk event.

examined by IHC was lower (51%) than expected. Indeed, our IHC-based assessment could predict *TP53* mutation status with high specificity (95%) but with moderate sensitivity (61%), suggesting that some *TP53* mutated tumours might be misclassified as intact by IHC. This result is in line with previous studies reporting the correlations between IHC-based findings and *TP53* mutation status in several types of malignancies [57,58]. Taken together, IHC is not a perfect method but a surrogate for estimating intragenic mutations of the *TP53* gene.

Transcriptome-based subtypes of pancreatic cancer (i.e. basal-like subtype, squamous subtype, and quasi-mesenchymal subtype) have been associated with poor prognosis and liver metastasis [26,51,59–66]. In our exploratory analysis of 20 pancreatic carcinomas, we did not observe any significant association between the number of altered genes and basal-like tumour subtypes. However, Bailey *et al* have attested to the importance of *TP53* mutation in increasing squamous subtype-related gene expressions in pancreatic cancer [66], although *TP53* mutation is not necessarily accumulated in pancreatic cancer with squamous or basal-like features [67,68]. Integrated whole-genome analysis has demonstrated that *TP53* mutation, *CDKN2A* loss, and mutated-*KRAS* amplification are associated with basal-like pancreatic cancer subtype, whereas complete *SMAD4* loss is correlated with classical subtype [62]. Future comprehensive analyses of genetic and epigenetic alterations of *KRAS*, *CDKN2A*, *TP53*, and *SMAD4* genes may unveil a potential link between transcriptome-based subtypes and those driver alterations in pancreatic cancer.

The use of reliable assays for *KRAS* mutation was a strength of our study. Low tumour cellularity due to



Number at risk

Number of altered genes	Months					
	0	12	24	36	48	60
0-2 genes	350	219	148	115	76	55
3 genes	487	252	146	115	81	55
4 genes	256	132	76	60	36	27

Figure 3. Cumulative incidence curves of recurrence at specific sites among patients with resected pancreatic cancer according to the number of altered genes. (A) Distant metastasis (with or without local recurrence), (B) local recurrence only, (C) liver metastasis, and (D) recurrence at sites other than the liver.

abundant desmoplastic stroma characteristic of pancreatic cancer [69,70] has hampered DNA-based molecular annotation in clinical tumour samples [14]. As previously described [20], the use of ddPCR allowed for the sensitive detection of *KRAS* mutations in decades-old FFPE (formalin-fixed paraffin-embedded) tissue samples of pancreatic cancer. The current study has other notable strengths, including the large sample size based on three independent patient populations. The large sample size allowed us to conduct various subgroup analyses with sufficient statistical power. In this multicentre study, we observed no statistically significant heterogeneity between the institutional cohorts in our main findings, supporting the generalisability of our data. The availability of comprehensive clinical data on pre- and post-operative chemotherapy regimens and various survival outcomes (e.g. various recurrence types and all-cause death) was another strength.

The current study has several limitations. First, there might be unmeasured confounding factors in our survival analyses. Nonetheless, our multivariable models included a variety of clinical and pathological characteristics, including established prognostic factors in patients with pancreatic cancer, and the adjustment did not alter the results materially. Second, a lack of gold-standard criteria for IHC assessments of CDKN2A, p53, and SMAD4 expression is another limitation; nonetheless, it is more likely that misclassifications of the expression

status would have driven the survival associations of the altered expression levels toward the null hypothesis. Third, our study population consisted of patients with resected pancreatic ductal adenocarcinoma. Therefore, the usefulness of the driver alterations as prognostic biomarkers should be validated in patients with locally advanced or distant adenocarcinomas and other adenocarcinoma variants (e.g. intraductal papillary mucinous neoplasm-derived carcinoma and colloid carcinoma). Finally, a vast majority of the study population was Japanese. Therefore, our findings should be validated in independent populations with racial diversity.

We have demonstrated the feasibility of IHC-based assessments of these driver alterations in small tissue samples obtained via fine-needle aspiration. As described, ddPCR has been shown to provide a reliable mutation calling in specimens with low DNA amounts. In the current practice, an increasing number of patients diagnosed with pancreatic cancer are referred to neoadjuvant chemotherapy. A further investigation is warranted to examine the potential of those simple and cost-effective analyses in identifying patients with highly aggressive tumours who are less likely to benefit from neoadjuvant chemotherapy and thus are candidates for upfront surgery.

In summary, the current study suggests that the accumulation of the four major driver alterations involved in pancreatic carcinogenesis can confer a high metastatic potential to tumours, thereby impairing post-operative survival among patients with pancreatic cancer. The elevated mortality hazard associated with an increasing number of the altered genes appeared not to be offset by the standard chemotherapy regimens used in the current clinical practice. Therefore, further research is warranted to identify targetable molecular and immune parameters in resected pancreatic cancer with the accumulated molecular alterations and, thereby, improve clinical outcomes of patients with this highly lethal tumour subgroup.

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Author contributions statement

YM, MTak, MTan and TH planned the study. All authors collected, analysed and interpreted the data. YM and TH drafted the manuscript, and all authors participated in revising the manuscript. YM, MTak, TH, TS, YN and TT obtained funding. YM, TH, TU, KTak and MS supervised the study. All authors approved the final submitted manuscript.

Ethics approval and consent to participate

This study was designed and conducted according to the guidelines in the Helsinki Declaration and was approved by the ethics committees at Cancer Institute Hospital, Japanese Foundation for Cancer Research, The University of Tokyo, and Keio University School of Medicine (all in Tokyo, Japan). Informed consent was obtained from all patients on an opt-out basis.

Data availability statement

The data sets generated and/or analysed during the current study are available from the corresponding authors upon reasonable request. The microarray data were deposited in NCBI's Gene Expression Omnibus database (accession number, GSE157353).

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SUPPLEMENTARY MATERIAL ONLINE

Supplementary methods

Figure S1. Microscopic images of H&E and immunohistochemistry staining in a fine-needle aspiration specimen from a patient with pancreatic cancer

Figure S2. Flow diagram of the selection of patients with resected pancreatic cancer in a multi-institutional cohort

Figure S3. Association between the Moffitt tumour subtype and the number of altered genes

Figure S4. Kaplan–Meier survival curves of patients with pancreatic cancer according to altered genes/proteins

Figure S5. Interactions of altered genes in relation to survival patients with pancreatic cancer

Table S1. Clinical and pathological characteristics of pancreatic cancer cases by the number of altered genes

Table S2. Correlations of tumour status based on immunohistochemical expression of CDKN2A (p16), p53, and SMAD4 with mutation calls by targeted sequencing

Table S3. The tumour status of the four drivers and survival among patients with pancreatic cancer (the final multivariable models)

Table S4. Specific combinations of the four major driver alterations and survival among patients with resected pancreatic cancer

Table S5. SMAD4 expression status in addition to specific combinations of the other three major driver alterations and survival among patients with resected pancreatic cancer

Table S6. The tumour status of the four drivers and survival among patients with pancreatic cancer, stratified by administration of adjuvant chemotherapy

Table S7. Recurrence patterns of resected pancreatic cancer by the number of altered genes

Table S8. Recurrence patterns of resected pancreatic cancer harbouring *KRAS* mutation with or without other driver alterations