



Comprehensive Analysis of Molecular Biologic Characteristics of Pancreatic Ductal Adenocarcinoma Concomitant with Intraductal Papillary Mucinous Neoplasm

Tsujimae, Masahiro ; Masuda, Atsuhiko ; Ikegawa, Takuya ; Tanaka, Takeshi ; Inoue, Jun ; Toyama, Hirochika ; Sofue, Keitaro ; Uemura,...

(Citation)

Annals of Surgical Oncology, 29(8):4924-4934

(Issue Date)

2022-08

(Resource Type)

journal article

(Version)

Accepted Manuscript

(Rights)

This version of the article has been accepted for publication, after peer review (when applicable) and is subject to Springer Nature's AM terms of use, but is not the Version of Record and does not reflect post-acceptance improvements, or any corrections. The Version of Record is available online at:...

(URL)

<https://hdl.handle.net/20.500.14094/90009469>



Comprehensive analysis of molecular biological characteristics of pancreatic ductal adenocarcinoma concomitant with intraductal papillary mucinous neoplasm

Running title: Comprehensive analysis of PDAC with IPMN

Masahiro Tsujimae^{1*}, Atsuhiro Masuda^{1*#}, Takuya Ikegawa^{1*}, Takeshi Tanaka¹, Jun Inoue¹, Hirochika Toyama², Keitaro Sofue³, Hisahiro Uemura¹, Shinya Kohashi¹, Noriko Inomata¹, Kae Nagao¹, Shigeto Masuda¹, Shohei Abe¹, Masanori Gonda¹, Kohei Yamakawa¹, Shigeto Ashina¹, Yasutaka Yamada¹, Shunta Tanaka¹, Ryota Nakano¹, Arata Sakai¹, Takashi Kobayashi¹, Hideyuki Shiomi¹, Maki Kanzawa⁴, Tomoo Itoh⁴, Takumi Fukumoto², Yoshihide Ueda¹ and Yuzo Kodama¹

*Authors share co-first authorship.

¹Division of Gastroenterology, Department of Internal Medicine, Kobe University Graduate School of Medicine

²Division of Hepato-Biliary-Pancreatic Surgery, Department of Surgery, Kobe University Graduate School of Medicine

³Department of Radiology, Kobe University Graduate School of Medicine

⁴Division of Diagnostic Pathology, Kobe University Graduate School of Medicine

#Corresponding Author

Atsuhiko Masuda, MD, PhD

Division of Gastroenterology, Department of Internal Medicine, Kobe University Graduate

School of Medicine

7-5-1 Kusunoki-cho, Chuo-ku, Kobe, Hyogo 650-0017, Japan

Telephone: +81-78-382-6305

Fax: +81-78-382-6309

E-mail: **atmasuda@med.kobe-u.ac.jp**

Disclosure Statement: Takuya Ikegawa -JSPS KAKENHI

ABSTRACT

Background: Pancreatic ductal adenocarcinoma (PDAC) concomitant with intraductal papillary mucinous neoplasm (IPMN) is defined as PDAC occurring apart from IPMN. We comprehensively investigated the molecular biological characteristics of PDAC concomitant with IPMN in major genetic alterations, tumor microenvironment, and prognosis by contrast with conventional PDAC.

Methods: We retrospectively reviewed the data of 158 surgically resected PDAC patients. The driver gene alteration status (*KRAS*, *TP53*, *CDKN2A*, *SMAD4*, and *GNAS*), as well as the immune and fibrotic status in tumor were evaluated. The prognosis of PDAC concomitant with IPMN and conventional PDAC were also compared.

Results: No statistically significant difference was found between PDAC concomitant with IPMN and conventional PDAC in the alteration frequency analysis of the major driver genes and immune and fibrotic status in the tumor microenvironment. Overall survival and disease-free survival between patients with PDAC concomitant with IPMN and conventional PDAC did not show statistically significant differences in propensity matched subjects. Furthermore, co-existence of IPMN was not a poor prognostic factor in multivariable-adjusted Cox proportional hazards model (Hazard ratio 0.95; 95% confidence interval 0.51–1.78).

Conclusions: PDAC concomitant with IPMN had tumor characteristics similar to those of conventional PDAC in terms of the major driver gene alterations, tumor microenvironment,

and prognosis.

(202 Words)

KEY WORDS: Pancreatic ductal adenocarcinoma, Intraductal papillary mucinous neoplasm, Driver gene alteration, Tumor microenvironment, Pancreatic ductal adenocarcinoma concomitant with IPMN.

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is the most aggressive and life-threatening cancer. Intraductal papillary mucinous neoplasm (IPMN) has been recognized as a precursor lesion of PDAC [1]. According to the international consensus guidelines, IPMN-related PDAC is categorized into two groups: PDAC derived from IPMN and PDAC concomitant with IPMN. PDAC derived from IPMN is defined as IPMN transformed into PDAC. Conversely, PDAC concomitant with IPMN is defined as a carcinoma that is site-wise and pathologically distant from IPMN [2]. PDAC derived from IPMN has already been well characterized. The characteristics and prognosis of PDAC derived from IPMN correlated well with the morphological characteristics of IPMN [3]. Furthermore, the prognosis of PDAC derived from IPMN was remarkably more favorable than that of conventional PDAC (PDAC with no existing IPMN in the background pancreas) [4, 5]. *GNAS* alterations, which demonstrated the specific genetic alterations in PDAC derived from IPMN, were found in 50–70% of PDAC derived from IPMN, but not in conventional PDAC [6-8]. Based on these results, PDAC derived from IPMN was considered to have different genetic or clinical characteristics from those of conventional PDAC.

In contrast to PDAC derived from IPMN, the biological and genetic characteristics of PDAC concomitant with IPMN have not yet been fully elucidated. Previous reports have shown that PDAC concomitant with IPMN has a better prognosis than conventional PDAC. It

is pertinent to note that these studies had a discrepancy in the patient background, such as the tumor stage [4, 5]. Therefore, it is still unclear whether PDAC concomitant with IPMN is a disease entity independent of conventional PDAC. Additionally, recent studies have suggested the possibility that some PDAC concomitant with IPMN have a genetically *de novo* developmental pathway, which is independent of IPMN-related carcinogenesis [6, 9].

In the present study, we investigated the tumor characteristics of PDAC concomitant with IPMN. Initially, we investigated four major gene alterations (*KRAS*, *TP53*, *SMAD4*, and *CDKN2A*), which were reported to be associated with the postoperative prognosis of PDAC in previous reports [11]. Second, we investigated the immune and fibrotic environments of the tumor, which may influence the prognosis of PDAC [10]. An increased lymphocyte infiltration around the tumor, called tumor-infiltrating lymphocytes (TILs), is associated with a favorable prognosis in PDAC [12-14]. Furthermore, PDAC has been well characterized by an abundance of stromal cells and high degree of fibrosis in the tumor, and the stromal signature of PDAC was found to be associated with the prognosis of PDAC in some recent reports [16]. Third, we also investigated the prognosis of PDAC concomitant with IPMN using propensity score matching subjects. Prognostic factors of PDAC were also evaluated by multivariate Cox's proportional hazards model. In this comprehensive analysis, we attempt to elucidate the molecular biological characteristics of PDAC concomitant with IPMN.

METHODS

Patients and data collection

We retrospectively analyzed 254 consecutive patients who were diagnosed with PDAC who underwent surgery between April 2008 and March 2017. A flow diagram of the study population is shown in **Figure 1**. First, patients with PDAC derived from IPMN (n = 44) were excluded from the analysis. After histologic examination, anaplastic carcinoma (n = 3), adenosquamous carcinoma (n = 6), acinar cell carcinoma (n = 1), and mucinous carcinoma (n = 1) were also excluded. Of the remaining 210 cases, those with distant metastasis at the time of surgery (n = 15) and those undergoing preoperative treatment (n = 9) were excluded, and those for whom formalin-fixed paraffin-embedded (FFPE) sections were not available were omitted. Finally, the information of 162 cases was extracted. The target amplicon sequence was performed in 162 cases, and four cases with *GNAS* alteration were excluded because the *GNAS* alteration was considered to be the characteristic of PDAC derived from IPMN. Finally, of the 158 cases analyzed, there were 21 cases of PDAC concomitant with IPMN, and 137 cases of conventional PDAC (PDAC without IPMN). We retrospectively reviewed the patients' medical records and retrieved information regarding the following factors: age, gender, body mass index, family history, alcohol consumption (regularly ≤ 50 g/day or > 50 g/day), history of smoking (presence or absence), diabetes mellitus (presence or absence), serum carcinoembryonic antigen level (> 5 ng/mL or ≤ 5 ng/mL), serum carbohydrate antigen

(CA)19-9 level (> 37 U/mL or ≤ 37 U/mL), pathological stage (Union for International Cancer Control [UICC] 8th classification), histological grade (well, moderate, or poorly differentiated), residual tumor status (R0 or R1), and prognosis. Serum absolute lymphocyte count, serum absolute neutrophil counts, and serum neutrophil-to-lymphocytes ratio (NLR) were obtained from routine laboratory data within 1 month preceding surgery in the absence of infection.

The study protocol was reviewed and approved by the ethics committee of the Kobe University School of Medicine (no.180235). The study was conducted in accordance with the principles of the Declaration of Helsinki. The requirement of informed consent was waived because of the retrospective study design. All authors had access to the study data, and reviewed and approved the final manuscript.

Definition of PDAC concomitant with IPMN and conventional PDAC (PDAC with no existing IPMN in the background pancreas)

Conventional PDAC was defined as the absence of visually co-existing IPMN on preoperative multidetector row computed tomography (MDCT), magnetic resonance cholangio pancreatography (MRCP), and endoscopic ultrasonography (EUS) and the absence of pathologically co-existing IPMN in the postoperative surgical specimen. Imaging examinations were performed at a preoperative conference with physician groups, including radiologists (K.S.), surgeons (H.T.), and endoscopists (M.T. and A.M.) to confirm the absence of co-

existing IPMNs. Postoperatively, all resected specimens, including the non-neoplastic areas, were reviewed for the presence of co-existing IPMNs. All sections were examined by three physicians, including two pathologists (M.K. and T.I.). PDAC concomitant with IPMN was defined as PDAC having a cyst apart from pancreatic cancer as assessed using a preoperative imaging method (i.e., EUS, MDCT, and MRCP) (**Figure 2**). When IPMN and PDAC were close enough to enter the surgical specimen, those with no histological transition between IPMN and PDAC were regarded as PDAC concomitant with IPMN. Furthermore, to rule out the presence of latent IPMN and the possibility of PDAC derived from IPMN, we confirmed the alterations in *GNAS*, which were reported as a specific gene alteration of IPMN and PDAC derived from IPMN [1, 6-8].

DNA extraction and quantification

DNA was extracted selectively from the pancreatic cancer portion of the FFPE section using the QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Each genomic DNA was quantified using a fluorometer (Invitrogen Qubit 2.0; Thermofisher Scientific, Massachusetts, USA) and stored at -80°C until further analysis.

Targeted amplicon next-generation sequencing

The polymerase chain reaction (PCR) amplicons were designed according to the Illumina Custom Protocol (https://www.customprotocolselector.net/cps/Out/10374_80/10374_.htm). The custom panel was designed to include KRAS, TP53, CDKN2A/ p16, SMAD4, and GNAS. The extracted DNA was subjected to PCR using a commercially available library kit (AmpliSeq for Illumina Library PLUS and Indexes, Illumina Inc., California, USA). Paired-end (2×151) sequencing was performed on a microflow cell with Reagent Kit v2 300 Cycles (Illumina Inc., California, USA) on a MiSeq instrument (Illumina Inc., California, USA). Valiant Studio software (v3.0; Illumina Inc., California, USA) was used for variant annotation and filtering. Filtering was performed as follows: a quality score of <30, variant allele frequency of <1.0%, and coverage of <500 were excluded from the analysis to correct single nucleotide polymorphisms (SNPs) and short insertions and deletions. Using public databases, such as dpSNP (<https://www.ncbi.nlm.nih.gov/snp/>), 1000 Genome Projects (<https://www.internationalgenome.org/>), and Exome Aggregation Consortium (<http://exac.broadinstitute.org/>), we extracted true alterations. In these database analyses, the frequency of population allele alterations was determined to be 1%, 0.1%, and 0.01%, respectively. All calls to the non-coding region, including 3'-UTR, 5'-UTR, downstream, and upstream, were excluded from further analysis. Alignments and putative alterations were visually verified using the Integrative Genomics Viewer (v2.3, Broad Institute). The COSMIC (<http://cancer.sanger.ac.uk/cosmic>) and ClinVar (<https://www.ncbi.nlm.nih.gov/clinvar/>)

databases were used as references to determine whether or not the detected variants were pathogenic.

Detecting copy number variations (CNVs) with droplet digital PCR (ddPCR)

CNVs were analyzed using a QX200 ddPCR system (Bio-Rad Laboratories, Munich, Germany), as described previously [17, 18]. Briefly, the purified genomic DNA was partitioned into ~20,000 droplets per sample by mixing with 70 μ L droplet generation oil in a QX200 droplet generator. Thermal cycling conditions were as follows: 95°C for 10 min, 40 cycles at 94°C for 30 s, 60°C for 60 s, 98°C for 10 min, and a 4°C hold. The absolute copy number input in the reaction and the ratio of the mutated fragments were calculated using QuantaSoft v1.7 (Bio-Rad) software based on the Poisson distribution. CNVs of <1.4 copy number values than the reference were defined as “deletion”.

Immunohistochemistry (IHC)

For immunohistochemistry, sections of 5 μ m thickness were incubated overnight at 4°C with primary antibodies. Next, these were incubated for 1 h at room temperature with ImmPRESS peroxidase-conjugated secondary antibodies (Vector Laboratories, CA, USA) and visualized with the ImmPACT DAB Substrate Kit (Vector Laboratories, CA, USA). Immunohistochemical staining was performed using the following primary antibodies: CD4

(Leica Biosystems, Wetzlar, Germany), CD8 (Roche, Ltd., Basel, Switzerland), FOXP3 (Abcam, Cambridge, UK), p53 (Dako, Santa Clara, USA), p16 (Roche, Ltd., Basel, Switzerland), and SMAD4 (Santa Cruz Biotechnology, California, USA).

Classification of the major driver gene alterations

The molecular status of each gene was obtained by integrating the targeted amplicon sequence and ddPCR analysis. The classification of driver gene alterations for *KRAS*, *TP53*, *CDKN2A/p16*, and *SMAD4* was comprehensively defined using a previously described method [11]. All cases with missense alterations in *KRAS* were identified by the sequence. *TP53*, *SMAD*, and *CDKN2A* loss pattern and *TP53* overexpression pattern in IHC was defined as “alteration” (typical IHC images are presented in **Supplemental Figure 1**). Using next-generation sequencing (NGS) or ddPCR, cases with SNV or short indels or CNV detected were defined as “molecular altered,” and cases without SNV and short indels and CNV were defined as “molecular wild”. Regarding the overexpression of TP53 protein in IHC, those without “molecular altered” were regarded as “wild type”. The reliability of IHC was confirmed by SNV, short indels, and CNVs in driver gene alteration analysis of *TP53*, *SMAD4*, and *CDKN2A*. The concordance rates of IHC and NGS/ddPCR analysis of *TP53*, *SMAD4*, and *CDKN2A* were 86%, 79%, and 86%, respectively.

Evaluation of the tumor microenvironment of PDAC using TILs and IHC for alpha-smooth muscle actin (α SMA) and Elastica van Gieson stain (EVG) staining

The tumor microenvironment was evaluated in terms of the lymphocyte infiltration and fibrosis accompanying PDAC. The TILs (CD4 + T cells, CD8+ T cells, and FOXP3 positive regulatory T cells) were counted using ImageJ software (Java image processing program inspired by the National Institute of Health [NIH], USA). Lymphocytes positive for each marker were counted in three fields with maximum lymphocytic infiltration in whole slides at $\times 200$ magnification (count/mm²), which were at least 5 mm apart from each other. The mean of the three counts was calculated and categorized into high and low groups by the median split. A representative immunostaining image of TILs is attached as **Supplemental Figure 2**. EVG and α SMA immunostaining of mesenchymal cells were performed to evaluate the cancer stroma. After extracting three areas around the cancer duct where fibrosis was most strongly observed, those were quantified using Adobe Illustrator (Adobe Inc. CA, USA). The average area ratio of the three points was divided into two groups (low and high) based on the median. The typical results of EVG and α SMA staining are presented in **Supplemental Figure 3**. All slides from PDAC patients were reviewed by T.I. and were subsequently re-examined by a second pathologist (M.K.), with both pathologists blinded to the clinical features of each case.

Statistical analyses

The categorical variables were analyzed using Fisher's exact test, and the continuous variables were analyzed using the Mann-Whitney U test. Kaplan-Meier estimation was used to analyze the prognosis. The significance was estimated using a log-rank test. The prognostic factors of PDAC were analyzed using the multivariate Cox proportional hazards model. The patient characteristics were compared between the two groups—conventional PDAC and PDAC concomitant with IPMN. Propensity scores were generated using logistic regressions and given patient baselines, which were selected for backward selection. We performed a one-to-one matching analysis between conventional PDAC and PDAC concomitant with IPMN based on the estimated propensity scores of each patient using SPSS. Once the propensity score matching process was completed, the outcomes were compared to confirm the results. All statistical analyses were performed using SPSS software v27 for Windows (SPSS Inc., Chicago, IL, USA). A *P*-value of < 0.05 was considered statistically significant.

RESULTS

Comparison of the clinical characteristics between PDAC concomitant with IPMN and conventional PDAC

There were 21 [19 and 2 cases associated with branched duct (BD-) and mixed type (Mix-) IPMN, respectively] and 137 cases of PDAC concomitant with IPMN and conventional PDAC (PDAC without IPMN), respectively. The main pancreatic ductal (MD-) IPMN was not

associated with PDAC concomitant with IPMN. The PDAC concomitant with IPMN group was significantly older (72.5 ± 7.4 vs. 67.6 ± 9.2 , $P = 0.01$). The proportion of advanced cancer in patients with PDAC concomitant with IPMN was lower than in those with conventional PDAC (stage > 2b of UICC, 11/21 [52.4%] vs. 104/139 [75.9%], $P = 0.02$). There was no significant difference between the two groups in terms of gender, family history, smoking, and drinking status. In addition, there were no differences in the pathological findings and resection factors between the groups (**Table 1**).

Next, 21 patients with PDAC concomitant with IPMN and conventional PDAC with no difference in the clinical background characteristics were extracted by one-to-one propensity score matching. After matching, no significant difference was found in the background between PDAC concomitant with IPMN and conventional PDAC. Before and after propensity matching, showed no difference between the groups in terms of the serum lymphocyte count, serum neutrophil count, and serum NLR, as an indicator of the patient's systemic immune status, and carcinoembryonic antigen and CA19-9 as tumor markers (**Table 1**).

Comparison of the major driver genetic alterations between PDAC concomitant with IPMN and conventional PDAC

The four major gene alterations in PDAC concomitant with IPMN and conventional PDAC

were investigated (**Figure 3 and Table 2**). *KRAS* alteration was most commonly observed in 92.4% (146/158) of PDAC patients, 92.0% (126/137) in conventional PDAC patients, and 95.2% (20/21) in PDAC concomitant with IPMN patients. *TP53* alteration was observed in 68.4% (108/158) of patients with PDAC as the second most common genetic alteration, followed by *CDKN2A* (65.9%, 101/158) and *SMAD4* (38.0%, 60/158). *KRAS*^{G12D} was the most common amino acid substitution in PDAC, which was detected in 34.8% (55/158) of patients. When stratified by presence or absence of IPMN, *KRAS*^{G12D} was detected in 23.8% (5/21) of PDAC patients with concomitant IPMN and 36.2% (50/138) of patients with conventional PDAC. *KRAS*^{G12R} was most frequently [7/21 (33.3%)] observed in patients with PDAC concomitant with IPMN. Out of 108 cases with *TP53* alteration, 87 (80.6%) had non-silent SNVs or indels, which was the most common type of genetic alteration in *TP53*. Copy number deletion was most commonly [70/101 (69.3%)] detected in *CDKN2A* cases. Of the 60 cases with *SMAD4* alteration, SNV or indel was observed in 31 (51.7%), and deletion was observed in 20 (33.3%). Multiple driver gene alterations were observed in the majority of cases. The proportions of one, two, three, and four gene alterations were 9.5% (15/158), 32.9% (52/158), 41.8% (66/158), and 15.2% (24/158), respectively. In one case, none of the four major driver gene alterations could be detected. There was no difference between PDAC concomitant with IPMN and conventional PDAC in terms of the overview of gene alteration and the total number of gene alterations, before and after propensity matching adjusted 1:1 for age and UICC stage

(Table 2).

Comparison of the tumor microenvironment between PDAC concomitant with IPMN and conventional PDAC

Inflammation and fibrosis around PDAC were examined as the microenvironments of PDAC (Figure 3 and Table 2). Regarding local T cell infiltration, CD4 positive T cells were more frequently observed in the conventional PDAC group than in the PDAC concomitant with IPMN group [6/21 (28.6%) vs. 71/139 (51.8%), $P = 0.05$]. FOXP3 T cell infiltration and CD8 T cell infiltration around PDAC were similar in both groups. Considering the stromal reaction of PDAC, collagen fiber areas as red color in EVG staining were significantly higher in the PDAC concomitant with IPMN group than in the conventional PDAC group [16/21 (76.2%) vs. 64/137 (46.7%), $P = 0.01$].

After propensity matching was adjusted 1:1 for age and tumor stage, there was no significant difference in the inflammatory cell infiltration (Table 2). The same trend was observed for the degree of fibrosis. However, although not statistically significant, collagen fiber areas were significantly higher in the PDAC concomitant with IPMN group than in the conventional PDAC group [16/21 (76.2%) vs. 10/21 (47.6%), $P = 0.06$] (Table 2).

Prognosis of PDAC concomitant with IPMN and conventional PDAC before and after

propensity score matching

The overall survival (OS) and disease-free survival (DFS) were compared to determine the prognosis of PDAC concomitant with IPMN and conventional PDAC (**Figure4**). There was no significant difference in OS ($P=0.507$). The median OS durations of concomitant with IPMN and conventional PDAC cases were 27.5 ± 1.7 and 29.6 ± 3.7 months, respectively. Even after adjusting for the clinical background, no significant difference in OS ($P=0.959$) was observed. The median OS durations in the PDAC concomitant with IPMN and conventional PDAC groups were 27.5 ± 1.7 and 21.4 ± 7.4 months, respectively. Moreover, there was no significant difference in DFS ($P=0.749$). The median DFS durations in the PDAC concomitant with IPMN and conventional PDAC groups were 17.3 ± 2.8 and 15.5 ± 2.3 months, respectively. Furthermore, there was no significant difference in DFS between the two groups after propensity matching ($P=0.925$). The median DFS durations in the PDAC concomitant with IPMN and conventional PDAC groups were 17.3 ± 2.8 and 12.9 ± 5.0 months, respectively.

The multivariate hazard ratios for each prognostic factor for PDAC are shown in **Figure 5**. Coexistence of IPMN was not extracted as a prognostic factor after resection of pancreatic cancer ($HR = 0.95$ 95%CI=0.51–1.78). The prognosis of PDAC was poor in patients with advanced UICC. In contrast, PDACs with high levels of CD4 or CD8 T cells infiltration and high degree of fibrosis in PDAC, which was evaluated by EVG staining, were associated with a favorable prognosis. Major gene alterations did not affect the prognosis of PDAC in this

study (**Figure 5**).

DISCUSSION

In this study, we comprehensively investigated the molecular biological characteristics of PDAC concomitant with IPMN. PDAC concomitant with IPMN had similar main driver gene alterations and immune or fibrotic microenvironments as conventional PDAC. In addition, a significant difference was not found between PDAC concomitant with IPMN and conventional PDAC in the prognosis of propensity score matched subjects. Presence of IPMN was not an independent prognostic factor for PDAC in multivariate Cox proportional hazard model. In this integrated analysis, PDAC concomitant with IPMN might exhibit molecular biological properties similar to those of conventional PDAC.

KRAS, *TP53*, *CDKN2A*, and *SMAD4* gene alterations have been reported as the four major frequent alterations in PDAC [19-21]. In our study, the frequency of *KRAS*, *SMAD4*, *CDKN2A*, and *TP53* alteration rate and its alteration status were almost the same as those reported previously [22, 23]. It is still controversial whether genetic alterations in PDAC can define the malignancy status of PDAC. It has been reported that the greater the number of alterations in the four major driver genes, the worse the prognosis, and that specific amino acid substitutions in *KRAS* are associated with worse prognosis [11, 24, 25]. In our study, specific gene alterations and their combinations were not particularly prominent in PDAC concomitant

with IPMN and conventional PDAC.

Understanding the biological properties of PDAC concomitant with IPMN could be helpful for disease monitoring after surgical resection. The tumor microenvironment of pancreatic cancer is believed to be strongly associated with the prognosis [26]. TILs and NLR were examined as indicators of the local and systemic immune status of PDAC. Previous studies have revealed that TILs and NLR may be potential predictors of prognosis in PDAC [15]. In the present study, CD4⁺ T cell infiltration was higher in patients with conventional PDAC than in those with PDAC concomitant with IPMN, but no significant difference was found after propensity score matching. PDAC is characterized by strong fibrosis around the tumor glands, which contributes to malignancy and treatment resistance. Conversely, studies using a mouse model of PDAC have revealed that the surrounding fibrosis around PDAC can suppress malignant transformation. Therefore, whether fibrosis is facilitative or inhibitory for PDAC remains controversial [27].

The present study has several limitations. First, there was a selection bias because we only examined surgical cases. Second, the total number of patients with PDAC concomitant with IPMN was limited. Therefore, examining more such cases may provide new insights. Third, the study was retrospective, and the treatment plan was decided at the discretion of the attending physicians. However, to our knowledge, none of the previous studies have investigated the biological characteristics of PDAC concomitant with IPMN and conventional

PDAC. In the present study, the prognosis of both PDAC groups was similar after adjusting for the background factors. Furthermore, the systemic immune status, local immune status, fibrotic status, and genetic alteration, which were shown to be related to the prognosis of PDAC, were not different between the groups.

In conclusion, significant biological differences were not found between PDAC concomitant with IPMN and conventional PDAC in the aspect of the major driver gene alterations, tumor microenvironment, and prognosis. PDAC concomitant with IPMN might be better treated as the same disease entity as conventional PDAC.

Acknowledgments: We would like to thank Editage (www.editage.com) for English language editing.

REFERENCES

1. Patra KC, Bardeesy N, Mizukami Y. Diversity of precursor lesions for pancreatic cancer: The genetics and biology of intraductal papillary mucinous neoplasm. *Clin Transl Gastroenterol.* 2017; 8: e86.
2. Tanaka M, Fernández-del Castillo C, Adsay V, et al. International consensus guidelines 2012 for the management of IPMN and MCN of the pancreas. *Pancreatology.* 2012; 12: 183–197.
3. Furukawa T, Hatori T, Fujita I, et al. Prognostic relevance of morphological types of intraductal papillary mucinous neoplasms of the pancreas. *Gut.* 2011; 60: 509–516.
4. Yamaguchi K, Kanemitsu S, Hatori T. Pancreatic ductal adenocarcinoma derived from IPMN and pancreatic ductal adenocarcinoma concomitant with IPMN. *Pancreas.* 2011; 40: 571–580.
5. Kang MJ, Lee KB, Jang JY, et al. Disease spectrum of intraductal papillary mucinous neoplasm with an associated invasive carcinoma invasive IPMN versus pancreatic ductal adenocarcinoma-associated IPMN. *Pancreas.* 2013; 42: 1267–1274.
6. Omori Y, Ono Y, Tanino M, et al. Pathways of progression from intraductal papillary mucinous neoplasm to pancreatic ductal adenocarcinoma based on molecular features. *Gastroenterology* 2019; 156: 647–661.
7. Tamura K, Ohtsuka T, Date K, et al. Distinction of invasive carcinoma derived from intraductal papillary mucinous neoplasms from concomitant ductal adenocarcinoma of the pancreas using molecular biomarkers. *Pancreas.* 2016; 45: 826–835.

8. Kanda M, Knight S, Topazian, et al. Mutant GNAS detected in duodenal collections of secretin-stimulated pancreatic juice indicates the presence or emergence of pancreatic cysts. *Gut*. 2013; 62: 1024–1033.
9. Felsenstein M, Noë M, Masica DL, et al. IPMNs with co-occurring invasive cancers: Neighbours but not always relatives. *Gut*. 2018; 67: 1652–1662.
10. Makohon-Moore A, Iacobuzio-Donahue CA. Pancreatic cancer biology and genetics from an evolutionary perspective. *Nat Rev Cancer*. 2016; 16: 553–565.
11. Qian ZR, Robinson DA, Nowak JA, et al. Association of alterations in main driver genes with outcomes of patients with resected pancreatic ductal adenocarcinoma. *JAMA Oncol*. 2018; 4: e173420.
12. Wang Z, Zhao J, Zhao H. Infiltrating CD4/CD8 high T cells shows good prognostic impact in pancreatic cancer. *Int J Clin Exp Pathol*. 2017; 10: 8820–8828.
13. Sideras K, Biermann K, Yap K, et al. Tumor cell expression of immune inhibitory molecules and tumor-infiltrating lymphocyte count predict cancer-specific survival in pancreatic and ampullary cancer. *Int J Cancer*. 2017; 141: 572–582.
14. Ino Y, Yamazaki-Itoh R, Shimada K, et al. Immune cell infiltration as an indicator of the immune microenvironment of pancreatic cancer. *Br J Cancer*. 2013; 108: 914–923.
15. Yang JJ, Hu ZG, Shi WX, Deng T, He SQ, Yuan SG. Prognostic significance of neutrophil to lymphocyte ratio in pancreatic cancer: A meta-analysis. *World J Gastroenterol*. 2015; 21: 2807–

2815.

16. Wang LM, Silva MA, D'Costa Z, et al. The prognostic role of desmoplastic stroma in pancreatic ductal adenocarcinoma. *Oncotarget*. 2016; 7: 4183–4194.
17. Lassaletta A, Zapotocky M, Mistry M, et al. Therapeutic and prognostic implications of BRAF V600E in pediatric low-grade gliomas. *J Clin Oncol*. 2017; 35: 2934–2941.
18. Mazaika E, Homsy J. Digital droplet PCR: CNV analysis and other applications. *Curr Protoc Hum Genet*. 2014; 82: 7–24.
19. Wang L, Tsutsumi S, Kawaguchi T, et al. Whole-exome sequencing of human pancreatic cancers and characterization of genomic instability caused by MLH1 haploinsufficiency and complete deficiency. *Genome Res*. 2012; 22: 208–219.
20. Biankin AV, Waddell N, Kassahn KS, et al. Pancreatic cancer genomes reveal aberrations in axon guidance pathway genes. *Nature*. 2012; 491: 399–405.
21. Jones S, Zhang X, Parsons DW, et al. Core signaling pathways in human pancreatic cancers revealed by global genomic analyses. *Science*. 2008; 321: 1801–1806.
22. Bailey P, Chang DK, Nones K, et al. Genomic analyses identify molecular subtypes of pancreatic cancer. *Nature*. 2016; 531: 47–52.
23. Witkiewicz AK, McMillan EA, Balaji U, et al. Whole-exome sequencing of pancreatic cancer defines genetic diversity and therapeutic targets. *Nat Commun*. 2015; 6: 6744.
24. Yokose T, Kitago M, Matsuda S, et al. Combination of KRAS and SMAD4 mutations in

formalin-fixed paraffin-embedded tissues as a biomarker for pancreatic cancer. *Cancer Sci.*

2020; 111: 2174–2182.

25. McIntyre CA, Lawrence SA, Richards AL, et al. Alterations in driver genes are predictive of survival in patients with resected pancreatic ductal adenocarcinoma. *Cancer*. 2020; 126: 3939–3949.
26. Murakami T, Hiroshima Y, Matsuyama R, Homma Y, Hoffman RM, Endo I. Role of the tumor microenvironment in pancreatic cancer. *Ann Gastroenterol Surg*. 2019; 3: 130–137.
27. Kobayashi H, Enomoto A, Woods SL, Burt AD, Takahashi M, Worthley DL. Cancer-associated fibroblasts in gastrointestinal cancer. *Nat Rev Gastroenterol Hepatol*. 2019; 16: 282–295

Table 1. Comparison of the clinical characteristics between PDAC concomitant with IPMN and conventional PDAC.

	Before propensity score matching				After propensity score matching (1:1 matched age and stage)			
	Total	PDAC concomitant with IPMN	Conventional PDAC	<i>p</i> -value	Total	PDAC concomitant with IPMN	Conventional PDAC	<i>p</i> -value
	(N=158)	(N=21)	(N=137)		(N=42)	(N=21)	(N=21)	
Age ± SD	68.2 ± 9.1	72.5 ± 7.4	67.6 ± 9.2	0.01	72.1 ± 7.2	72.5 ± 7.4	71.8 ± 7.1	0.74
Gender				0.89				0.36
Male	88 (56.4%)	12 (57.1%)	76 (55.5%)		21 (50.0%)	12 (57.1%)	9 (42.9%)	
Female	70 (43.6%)	9 (42.9%)	61 (44.5%)		21 (50.0%)	9 (42.9%)	12 (57.1%)	
Smoking history				0.34				0.22
Present	75 (47.5%)	12 (57.1%)	63 (46.0%)		20 (47.6%)	12 (57.1%)	8 (38.1%)	
Absent	83 (52.5%)	9 (42.9%)	74 (54.0%)		22 (52.4%)	9 (42.9%)	13 (61.9%)	
Family history				0.48				0.31
Present	14 (9.0%)	1 (4.8%)	13 (9.5%)		1 (2.4%)	1 (4.8%)	0 (0.0%)	
Absent	144 (91.0%)	20 (95.2%)	124 (90.5%)		41 (97.6%)	20 (95.2%)	21 (100.0%)	
Diabetes				0.54				0.76
Present	95 (60.1%)	12 (57.1%)	83 (60.6%)		19 (45.2%)	9 (42.9%)	10 (47.6%)	
Absent	63 (39.9%)	9 (42.9%)	54 (39.4%)		23 (54.8%)	12 (57.1%)	11 (52.4%)	
Alcohol intake				0.50				0.29
<50g/day	142 (90.0%)	18 (85.7%)	124 (90.5%)		38 (90.5%)	18 (85.7%)	20 (95.2%)	
≥50g/day	16 (10.0%)	3 (14.3%)	13 (9.5%)		4 (9.5%)	3 (14.3%)	1 (4.8%)	
Tumor size ± SD				0.60				0.77
	29.6 ± 10.3	28.0 ± 15.5	29.8 ± 9.2		28.5 ± 12.6	28.0 ± 15.5	29.1 ± 9.1	
Stage (UICC)				0.02				0.35

<IIa	43	(27.2%)	10	(47.6%)	33	(24.1%)		25	(59.5%)	11	(52.4%)	14	(66.7%)	
≥IIb	115	(72.8%)	11	(52.4%)	104	(75.9%)		17	(40.5%)	10	(47.6%)	7	(33.3%)	
Histology grade							0.71							0.15
Well/Moderate	139	(89.1%)	19	(90.5%)	120	(87.6%)		40	(95.2%)	19	(90.5%)	21	(100.0%)	
Poorly	19	(10.9%)	2	(9.5%)	17	(12.4%)		2	(4.8%)	2	(9.5%)	0	(0.0%)	
Residual tumor status							0.71							0.50
Absent	115	(72.8%)	16	(76.2%)	99	(72.3%)		30	(71.4%)	16	(76.2%)	14	(66.7%)	
Present	43	(27.2%)	5	(23.8%)	38	(27.7%)		12	(28.6%)	5	(23.8%)	7	(33.3%)	
CEA							0.55							0.74
≥5.0	44	(27.8%)	7	(33.3%)	37	(27.0%)		13	(31.0%)	7	(33.3%)	6	(28.6%)	
<5.0	114	(72.2%)	14	(66.7%)	100	(73.0%)		29	(69.0%)	14	(66.7%)	15	(71.4%)	
CA19-9							0.57							0.17
≥37.0	120	(75.9%)	17	(81.0%)	103	(75.2%)		30	(71.4%)	17	(81.0%)	13	(61.9%)	
<37.0	38	(24.1%)	4	(19.0%)	34	(24.8%)		12	(28.6%)	4	(19.0%)	8	(38.1%)	
Neutrophils count (count/mm ²)							0.48							0.75
Low (<1522)	79	(50.0%)	9	(42.9%)	70	(51.1%)		17	(40.5%)	9	(42.9%)	8	(38.1%)	
High (≥1522)	79	(50.0%)	12	(57.1%)	67	(48.9%)		25	(59.5%)	12	(57.1%)	13	(61.9%)	
Lymphocytes (count/mm ²)							0.52							0.76
Low (<3251)	78	(49.4%)	9	(42.9%)	69	(50.4%)		19	(45.2%)	9	(42.9%)	10	(47.6%)	
High (≥3251)	80	(50.6%)	12	(57.1%)	68	(49.6%)		23	(54.8%)	12	(57.1%)	11	(52.4%)	
NLR							0.48							0.75
Low (<2.1)	79	(50.0%)	9	(42.9%)	70	(51.1%)		17	(40.5%)	9	(42.9%)	8	(38.1%)	
High (≥2.1)	79	(50.0%)	12	(57.1%)	67	(48.9%)		25	(59.5%)	12	(57.1%)	13	(61.9%)	

PDAC, pancreatic ductal adenocarcinoma; IPMN, intrapapillary mucinous neoplasm

UICC, Unio Internationalis Contra Cancrum; CEA, carcinoembryonic antigen; CA19-9, carbohydrate antigen 19-9, NLR, Neutrophil-to-lymphocyte ratio

Table 2. Comparison of the major driver gene alteration between PDAC concomitant with IPMN and conventional PDAC.

		Before propensity score matching							After propensity score matching (1:1 matched age and stage)						
		Total		PDAC concomitant with IPMN		Conventional PDAC		<i>p</i> -value	Total		PDAC concomitant with IPMN		Conventional PDAC		<i>p</i> -value
		(N=158)		(N=21)		(N=137)			(N=42)		(N=21)		(N=21)		
CD4 (count/mm ²)															
Low (<48)		81	(51.2%)	15	(71.4%)	66	(48.2%)	0.05	25	(59.5%)	15	(71.4%)	10	(47.6%)	0.12
High (≥48)		77	(48.8%)	6	(28.6%)	71	(51.8%)		17	(40.5%)	6	(28.6%)	11	(52.4%)	
CD8 (count/mm ²)															
Low (<140)		80	(50.6%)	12	(57.1%)	68	(49.6%)	0.52	22	(52.4%)	12	(57.1%)	10	(47.6%)	0.54
High (≥140)		78	(49.4%)	9	(42.9%)	69	(50.4%)		20	(47.6%)	9	(42.9%)	11	(52.4%)	
FOXP3 (count/mm ²)															
Low (<33)		84	(53.2%)	15	(71.4%)	69	(50.4%)	0.07	25	(59.5%)	15	(71.4%)	10	(47.6%)	0.12
High (≥33)		74	(46.8%)	6	(28.6%)	68	(49.6%)		17	(40.5%)	6	(28.6%)	11	(52.4%)	
Collagen in EVG															
Low		78	(49.4%)	5	(23.8%)	73	(53.3%)	0.01	16	(38.1%)	5	(23.8%)	11	(52.4%)	0.06
high		80	(50.6%)	16	(76.2%)	64	(46.7%)		26	(61.9%)	16	(76.2%)	10	(47.6%)	
αSMA stain															
Low		79	(50.0%)	9	(42.9%)	70	(51.1%)	0.48	19	(45.2%)	9	(42.9%)	10	(47.6%)	0.76
High		79	(50.0%)	12	(57.1%)	67	(48.9%)		23	(54.8%)	12	(57.1%)	11	(52.4%)	
<i>KRAS</i>															
Wild		12	(7.6%)	1	(4.8%)	11	(8.0%)	0.60	4	(9.5%)	1	(4.8%)	3	(14.3%)	0.29
Mutant		146	(92.4%)	20	(95.2%)	##	(92.0%)		38	(90.5%)	20	(95.2%)	18	(85.7%)	

TP53

Wild	50	(31.6%)	7	(33.3%)	43	(31.4%)	0.86	14	(33.3%)	7	(33.3%)	7	(33.3%)	1.00
Mutant	108	(68.4%)	14	(66.7%)	94	(68.6%)		28	(76.7%)	14	(66.7%)	14	(66.7%)	

SMAD4

Wild	98	(62.0%)	15	(71.4%)	83	(60.6%)	0.34	27	(64.3%)	15	(71.4%)	12	(57.1%)	0.33
Mutant	60	(38.0%)	6	(28.6%)	54	(39.4%)		15	(35.7%)	6	(28.6%)	9	(42.9%)	

CDKN2A

Wild	57	(36.1%)	9	(42.9%)	48	(35.0%)	0.49	19	(45.2%)	9	(42.9%)	9	(42.9%)	0.76
Mutant	101	(65.9%)	12	(57.1%)	89	(65.0%)		23	(54.8%)	12	(57.1%)	12	(57.1%)	

PDAC, pancreatic ductal adenocarcinoma IPMN, intrapapillary mucinous neoplasm

EVG, Elastica van Gieson; α -SMA, α -smooth muscle actin

FIGURE LEGENDS

Figure 1. Flow diagram of the study population

Of 254 PDAC patients between April 2008 and March 2017, those with PDAC derived from IPMN were excluded. Furthermore, cases with technical problems in gene analysis and those with special histological types were excluded. Finally, 158 cases were analyzed; 21 cases of PDAC concomitant with IPMN and 137 cases of conventional PDAC. Each of the 21 patients with matched age and tumor UICC stage was extracted using propensity score matching.

IPMN: intraductal papillary mucinous neoplasm, PDAC: pancreatic ductal adenocarcinoma,

UICC: Union for International Cancer Control, FFPE: formalin-fixed paraffin-embedded

Figure 2. Definitions of PDAC concomitant with IPMN and conventional PDAC

Conventional PDAC was defined as PDAC in which IPMN did not coexist in the same pancreas and did not have a *GNAS* alteration. PDAC concomitant with IPMN was defined as PDAC with IPMN in the same pancreas, but each was distant according to the radiologic images and macroscopic findings and had no *GNAS* alteration.

IPMN: intraductal papillary mucinous neoplasm, PDAC: pancreatic ductal adenocarcinoma

Figure 3. Major driver gene alterations and microenvironment of PDAC

The status of the four major driver gene alterations most commonly found in PDAC is shown.

For detailed information on alterations, amino acid substitutions were shown for *KRAS* alterations, and SNV, IHC loss, and CNV were distinguished for *TP53*, *SMAD4*, and *CDKN2A* status. Regarding the tumor microenvironment, inflammatory cell infiltration and stromal reactions are color-coded.

IPMN: intraductal papillary mucinous neoplasm, PDAC: pancreatic ductal adenocarcinoma, SNP: single nucleotide polymorphisms, IHC: immunohistochemistry, CNV: copy number variations, EVG: Elastica van Gieson, α SMA: alpha-smooth muscle actin

Figure 4. Overall survival (OS) and Disease-free survival (DFS) of PDAC concomitant with IPMN as compared to conventional PDAC

Kaplan-Meier analysis of OS and DFS stratified by the presence of IPMN (a) Overall survival of all cases, (b) Overall survival after propensity score matching, (c) Disease-free survival of all cases, (d) Disease-free survival after propensity score matching

IPMN: intraductal papillary mucinous neoplasm, PDAC: pancreatic ductal adenocarcinoma, No: number

Figure 5. Clinical prognosis and its predictors

The forest plot shows the hazard ratios of prognosis for each factor

EVG: Elastica van Gieson, α SMA: alpha-smooth muscle actin

Supplemental Figure 1. The protein expression pattern by immunohistochemistry

(a) TP53 was classified as “wild”, “lost”, and “overexpression” according to immunostaining

(b) SMAD4 and CDKN2A/p16 defined the complete loss of protein expression as “lost”

Supplemental Figure 2. Immunohistochemistry (IHC) findings of tumor-infiltrating lymphocytes

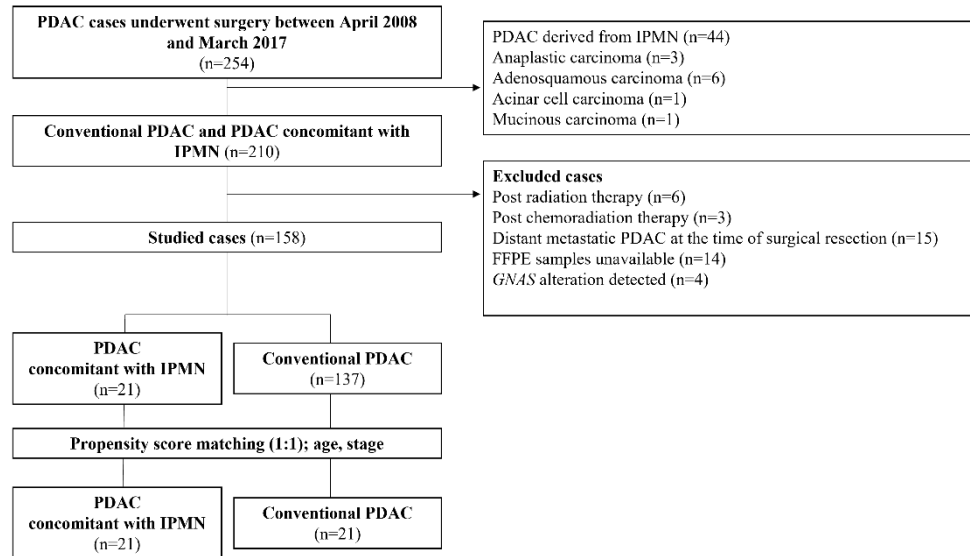
A representative IHC image of tumor-infiltrating lymphocytes of CD4, CD8, and FOXP3

Supplemental Figure 3. Evaluation of α SMA and EVG staining

A representative image of EVG staining and immunohistochemistry for α -SMA

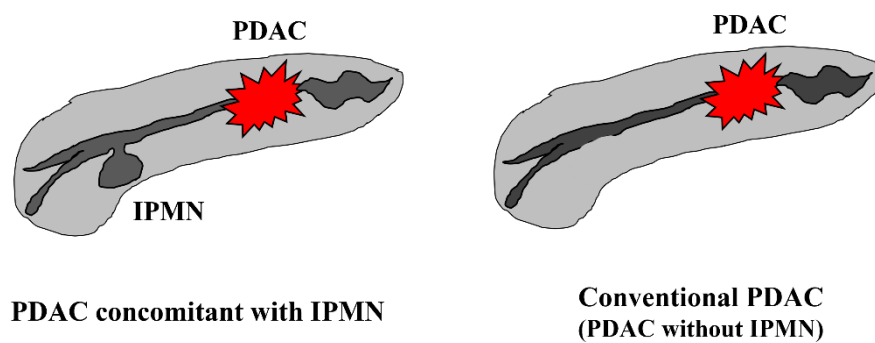
EVG: Elastica van Gieson, α SMA: alpha-smooth muscle actin

Figure 1. Flow diagram of the study population



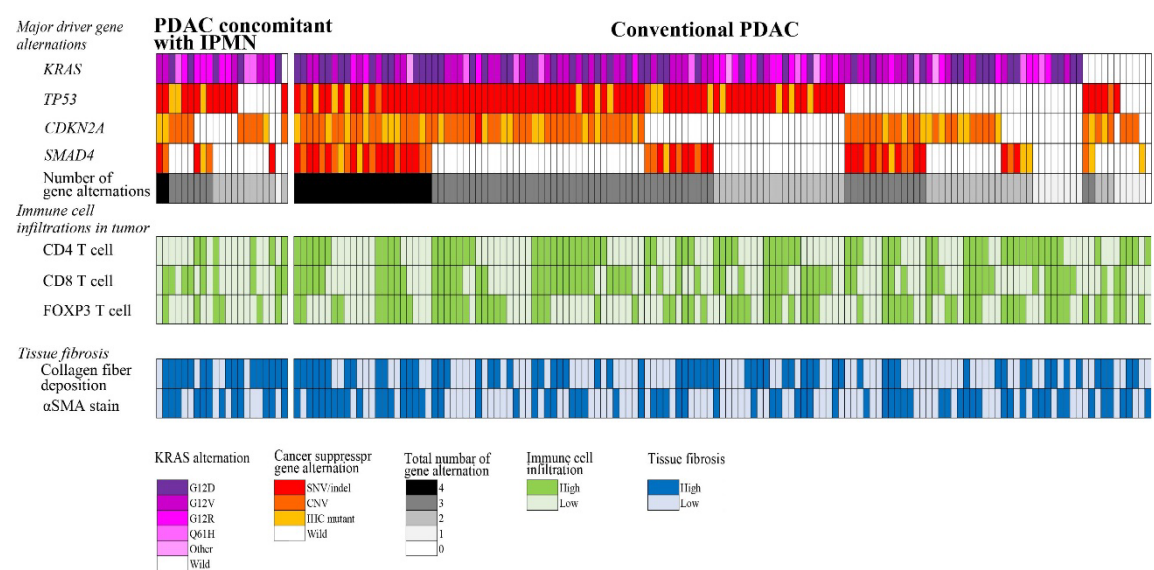
IPMN: intraductal papillary mucinous neoplasm, PDAC: pancreatic ductal adenocarcinoma, UICC: Union for International Cancer Control, FFPE: formalin-fixed paraffin-embedded

Figure 2. Definitions of PDAC concomitant with IPMN and conventional PDAC



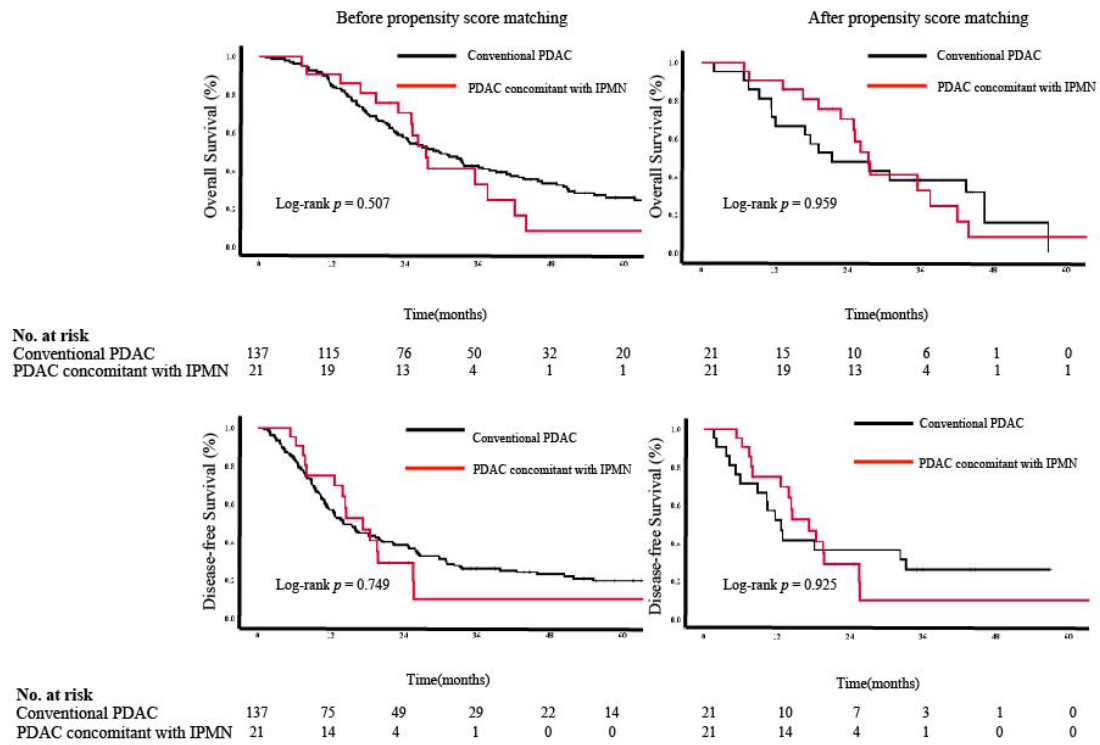
IPMN: intraductal papillary mucinous neoplasm, PDAC: pancreatic ductal adenocarcinoma

Figure 3. Major driver gene alterations and microenvironment of PDAC



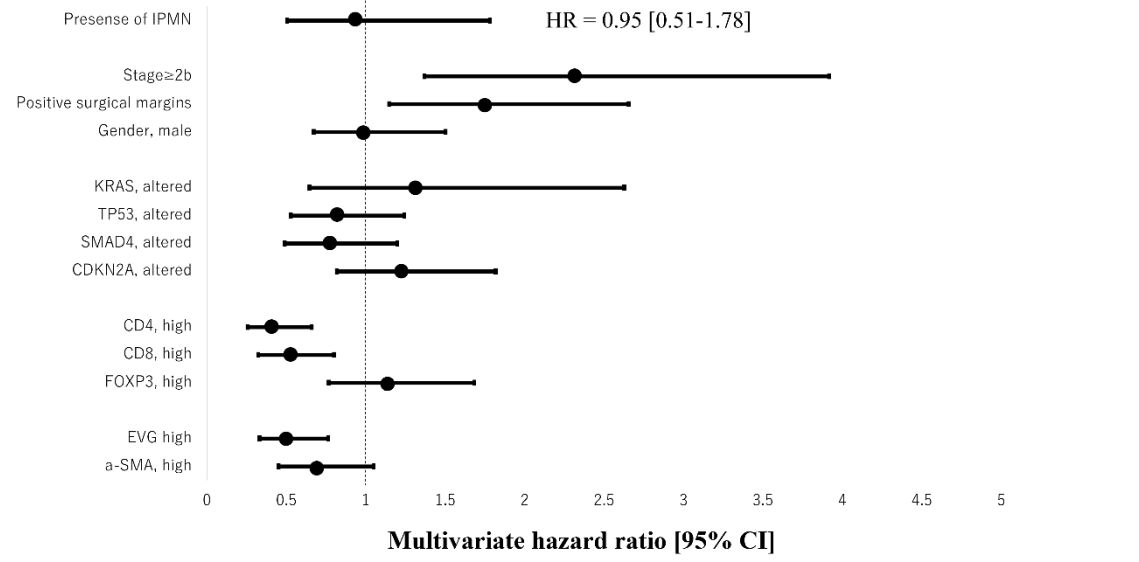
IPMN: intraductal papillary mucinous neoplasm, PDAC: pancreatic ductal adenocarcinoma, SNP: single nucleotide polymorphisms, IHC: immunohistochemistry, CNV: copy number variations, EVG: Elastica van Gieson, α SMA: alpha-smooth muscle actin

Figure 4. Overall survival (OS) and Disease-free survival (DFS) of PDAC concomitant with IPMN as compared to conventional PDAC



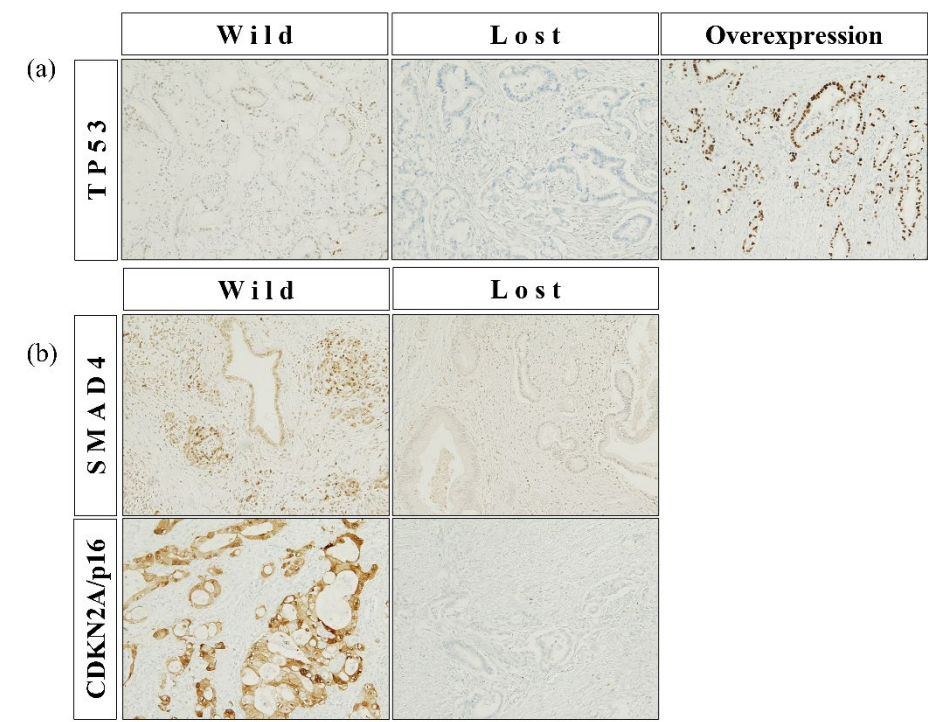
IPMN: intraductal papillary mucinous neoplasm, PDAC: pancreatic ductal adenocarcinoma, No: number

Figure 5. Clinical prognosis and its predictors

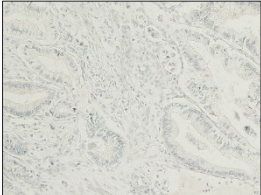
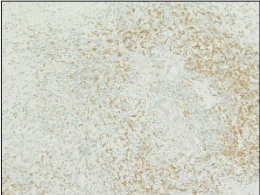
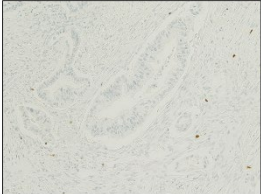
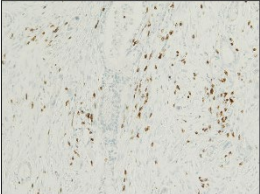
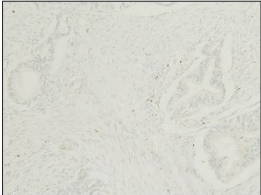


EVG: Elastica van Gieson, α SMA: alpha-smooth muscle actin

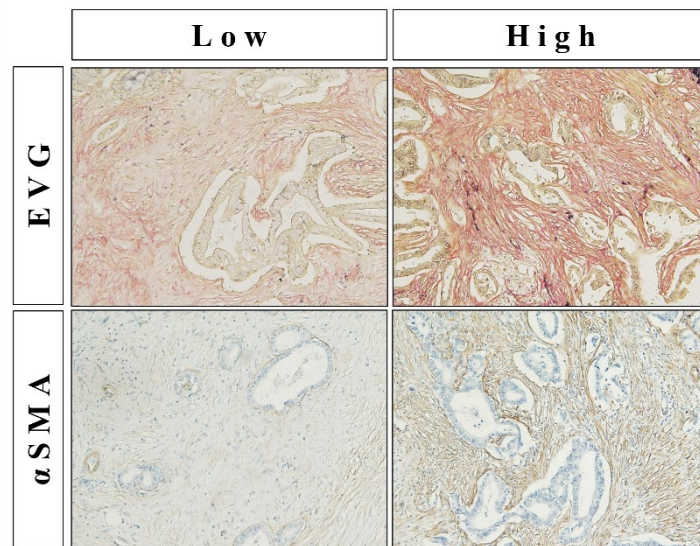
Supplemental Figure 1. The protein expression pattern by immunohistochemistry



Supplemental Figure 2. Immunohistochemistry (IHC) findings of tumor-infiltrating lymphocytes

	Low	High
CD4		
CD8		
FOXP3		

Supplemental Figure 3. Evaluation of α SMA and EVG staining



EVG: Elastica van Gieson, α SMA: alpha-smooth muscle actin