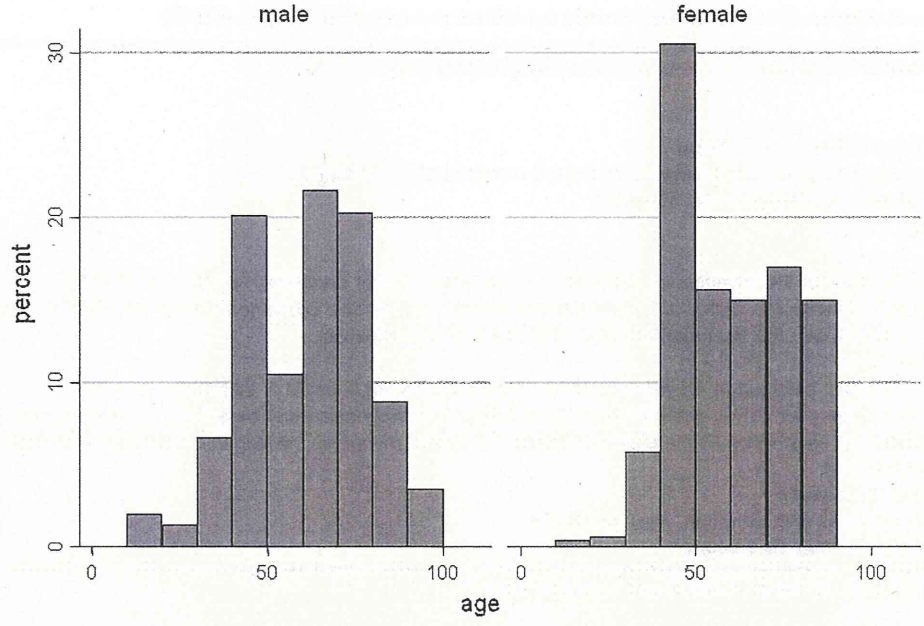

Figure. Age distribution of patients with CIPO

Table 3. Hospital visits[a] of patients with chronic intestinal pseudo-obstruction

		Male	Female	Total	*P*-value
Interval between first visit to present hospital and definitive diagnosis	Days (min/median/max)	0/28/6162	0/47/3287	0/30/6162	
	0–30 days (%)	60.7	38.1	50.5	0.005[b]
	31–90 days (%)	20.5	22.1	21.2	
	>90 days (%)	18.9	39.8	28.2	
Previous hospital(s) attended	Present (%)	12.2	29.4	20.0	0.004[c]
Present hospital attending	Same as where CIPO was diagnosed (%)	95.0	91.4	93.4	0.471[c]
Frequency of hospital visits	Visits/month (min/median/max)	0.30/1.00/6.00	0.10/1.00/6.00	0.10/1.00/6.00	

[a]Weighted mean, median, and proportions in population (n = 1148) based on answers from survey sample (n = 168; 76 males and 92 females).
[b]Difference between males and females (χ^2 test for trend).
[c]Difference between males and females (χ^2 test).

Clinical knowledge and experience of CIPO

Survey results on clinical knowledge, information source(s), and knowledge/experience of CIPO among medical facilities are shown in Table 4. Overall, 37.0% of department chiefs at surveyed medical centers responded that they had no knowledge or had never heard of CIPO, while 39.6% indicated that they knew of CIPO by name only. In more-detailed analysis, as the size of medical centers decreased, the proportion of facilities that had no knowledge of CIPO or knew it only by name significantly increased ($P = 0.001$, χ^2 test for trend). Indeed, 41.3% of hospitals with fewer than 100 beds had no knowledge of CIPO, and 90.3% were unaware of CIPO or knew it only by name. Even 27% of university hospitals/departments responded that they had no knowledge of CIPO or knew it only by name (data not shown). Concerning clinical experience of CIPO, approximately 90% of hospitals had neither diagnosed CIPO nor managed patients with the disease. Although university hospitals and special hospitals had higher rates of experience with CIPO (38.6% and 54.5% respectively), the rate was positively associated with hospital size ($P < 0.001$, χ^2 test for trend), as was the case for knowledge of CIPO. On average, less than 15% of hospitals with fewer than 300 beds had treated a patient with CIPO (data not shown).

DISCUSSION

CIPO is one of the most severe and best described gastrointestinal neuromuscular diseases. Because affected individuals are often unable to maintain normal body weight or regular oral nutrition, CIPO is an important cause of chronic intestinal failure. In this first nationwide survey of CIPO in Japan, we determined the basic epidemiologic characteristics and patterns of hospital visits of patients with

Table 4. Knowledge of and source of information on chronic intestinal pseudo-obstruction among medical professionals

	Answer	Proportion[b] (%)
Knowledge of CIPO	Do not know (DNK)	37.0
	Know only disease name	39.6
Source of information on CIPO[a]	Textbooks	15.1
	Academic periodicals	25.4
	Other periodicals	16.1
	Advising doctor	4.6
	Internet	4.1
	Others	6.7
Clinical experience of CIPO	Yes	10.7
Experience in detail	Confirmed cases	6.1
	Probable cases	2.6
	Suspected cases	2.0
Daily clinical practice	Not considering CIPO	46.3

[a]Denominator of fractions does not include those who answered DNK to the survey item on "knowledge of CIPO".
[b]Weighted proportions in population (n = 8830) based on answers from survey sample (n = 669).

CIPO. In addition, we assessed clinical knowledge of CIPO among medical professionals and the sources of their information.

Epidemiology of CIPO

We estimated the total number of patients treated for CIPO in Japan to be 1148 (95% CI, 573 to 1724) and the annual incidence of CIPO to be 0.225 per 100 000 population. The average age of patients was 63.1 years for males and 59.2 for females. In both sexes, relatively large numbers of patients were aged 40 to 49 and 70 to 79, which resulted in a bimodal age distribution. There was no statistically significant sex difference in CIPO prevalence, CIPO incidence, or mean age. Several reviews of CIPO have been published. Stanghellini et al reported that the median time between onset and recognition of CIPO was 8 years.[12] However, no study has assessed the detailed epidemiology of CIPO, and the prevalence of this intractable disease thus remains unknown.[12,24–25] Our study is the first nationwide survey of CIPO.

In our study, the prevalence of CIPO was 0.900 per 100 000 (1.004 for males and 0.801 for females). This proportion is very low as compared with other motility disorders, including gastroparesis (24.2 per 100 000; more people with gastroparesis may remain undiagnosed)[26] and chronic constipation (15 000 per 100 000).[27] Moreover, the annual incidence of CIPO (0.225 per 100 000) is much lower than that of ulcerative colitis (50–101.1 per 100 000 in 2009 in Japan), Crohn's disease (15.8–41.5 per 100 000 in 2009 in Japan),[28] and gastroparesis (6.3 per 100 000).[26] The prevalence of CIPO is nearly equal to that of primary sclerosing cholangitis (0.95 in 2007 in Japan).[29] Consequently, the estimated number of CIPO patients in Japan is much lower than that for ulcerative colitis (113 306 in 2009 in Japan) and Crohn's disease (30 891 in 2009 in Japan),[28] and nearly equal to that for primary sclerosing cholangitis (1211 in 2007 in Japan).[29]

Although the prevalence of CIPO could be considered low, the chronic and distressing nature of CIPO and its burden on individual patients and society at large are important reasons why it should not be ignored. Moreover, many patients are of working age (<65 years).

Diagnosis and clinical treatment of CIPO

Overall, approximately half of medical facilities diagnosed CIPO within 1 month after the first visit. However, more than one-third of centers required longer than 3 months to make the correct diagnosis. Diagnosis of CIPO required longer than 3 months for about 20% of males and about 40% of females, a statistically significant difference. Although more than 90% of male and female patients initially presented at the hospitals where CIPO was diagnosed, about one-third of women and one-tenth of men had initially presented at another hospital. This difference between sexes was significant.

Thus far, no studies have reported the frequencies of hospital visits and the rates of hospital change/transfer in CIPO patients. Only 1 report—a single-center study in Italy—reported the median interval between onset and diagnosis of CIPO[12]: in Italy, Stanghellini reported that the median interval between the first subocclusive episode and definitive diagnosis was 8 years (range, 0–47 years). In our study, the median interval between first visit to the present hospital and definitive diagnosis was 30 days (range, 0–6162 days), although this interval did not include the interval from onset of symptoms to first visit at the present hospital. Notably, 20% of our patients had visited other clinics or hospitals before attending their present hospital; thus, the interval from the initial symptoms may have been longer for these patients. As previously mentioned, the interval before diagnosis was longer and the rate of hospital change/transfer was higher among females than among males, possibly due to the greater difficulty of diagnosing CIPO in females. Similar motility disorders, such as chronic constipation, are more prevalent and more difficult to diagnose among females.[30]

If similar nationwide research is conducted in other countries, an international comparison of the detail epidemiology of CIPO will be possible in the future.

Knowledge and experience of CIPO

More than one-third of centers were aware of CIPO as a distinct disease entity, although more than three-quarters of centers did not know of CIPO or knew only the name of the disease, indicating that they were unaware or uncertain of the definition, characteristics, and diagnostic criteria of the disease. In particular, smaller medical facilities tended not to know about CIPO or knew it only by name. The surveyed medical facilities/departments have almost never experienced confirmed, probable, or suspected cases of CIPO.

As we reported in our 2009 pilot survey, overall recognition of CIPO among GI specialists is low in Japan.[31] Furthermore, experience with CIPO significantly correlated with knowledge of CIPO ($P < 0.001$, χ^2 test for trend). Understandably, hospitals that lacked knowledge of the disease are less likely to have clinical experience with it and vice versa. By overlooking the disease, these hospitals could have missed CIPO cases, leaving patients undiagnosed. Therefore, more than a few CIPO patients might remain undiagnosed or misdiagnosed.

Recent studies reported that CIPO patients often went undiagnosed for a long time before receiving a correct diagnosis and that they almost invariably underwent repeated, useless, and potentially dangerous surgical procedures, due to lack of specific laboratory findings and low recognition of the disease.[18,32] To overcome this tragic situation, improved recognition of CIPO is urgently required. To this end, we published a clinical guide on CIPO (written in Japanese) and sent it to all the facilities initially selected for the survey. We also presented typical CIPO cases at gastroenterology conferences in Japan, to enlighten clinicians about this intractable disease. Knowledge of CIPO is needed in order to shorten the interval from initial symptoms to correct diagnosis and minimize the rate of unnecessary surgical procedures.

Study limitations and agenda for future research

As suggested by pre-survey interviews with gastroenterologists, most patients with CIPO were being treated at hospitals. In addition, our preliminary survey found no cases of CIPO in clinics. We therefore chose not to include clinics in the main survey. The prevalence and incidence reported in this study might be underestimated if this assumption were untrue. We also assumed that the sampling weights used to estimate overall rates did not differ between survey responders and nonresponders. However, if responding centers were more likely to have CIPO patients than nonresponders, the rates reported here might be overestimates.

This nationwide questionnaire survey was conducted using stratified random sampling of Japanese hospitals and the procedure for the nationwide epidemiologic survey of intractable disease in Japan.[16] However, some of the present sampling rates were lower than those in the procedure. Thus, the precision of estimates of the total number of patients with CIPO might be slightly lower, and the results require careful interpretation.

Furthermore, because information on frequency of hospital visits was not cross-tabulated with stage of patient management (eg, diagnosis and early and late treatment), we were unable to determine the number of visits required for patients to obtain a diagnosis or any changes in hospital visits over time. After the present survey, we attempted to ascertain the details of patient clinical condition and medical treatment by sending a second survey to centers that responded to this first survey and had reported treating patients with CIPO. Analysis of the results of the second survey is ongoing and will be reported in the near future. In addition to the present results, the limitations of the present study should be carefully examined.

In summary, we conducted the first large-scale systematic survey of CIPO. We anticipate that the present results will be interpreted from an international comparative perspective when data from other countries become available.

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Original article

aPKCλ/ι is a beneficial prognostic marker for pancreatic neoplasms

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ABSTRACT

Pancreatic cancer is a lethal disease. Overall survival is typically 6 months from diagnosis. Determination of prognostic factors in pancreatic cancer that would allow identification of patients who could potentially benefit from aggressive treatment is important. However, until date, there are no established reliable prognostic factors for pancreatic cancer patients. Herein, we propose a beneficial biomarker which is significantly correlated with the prognosis in pancreatic cancer patients. Atypical protein kinase C λ/ι (aPKCλ/ι) is overexpressed and has been implicated in the progression of several cancers. We tested the expression levels of aPKCλ/ι in two types of pancreatic neoplasm, pancreatic ductal adenocarcinoma (PDAC) and intraductal papillary mucinous neoplasms (IPMNs), by immunohistochemistry. Examination of the aPKCλ/ι expression levels in surgically resected specimens of PDCA ($n = 115$) demonstrated that the expression levels of aPKCλ/ιin PDAC had prognostic implications, independent of the Tumor-Node-Metastasis classification and World Health Organization tumor grade. In the case of IPMNs ($n = 46$) also, the expression levels of aPKCλ/ιin IPMN were found to be of prognostic importance, independent of the World Health Organization histological grade or morphological type. Interestingly, high expression levels of aPKCλ/ι were significantly correlated with a worse histological grade ($p = 0.010$) and advanced stage of the tumor ($p = 0.0050$) in IPMN patients. These findings suggest that high expression levels of aPKCλ/ι could be involved in the malignant transformation of IPMNs. Based on these observations, we propose the expression level of aPKCλ/ι as a prognostic marker common to different types of pancreatic neoplasms.

1. Introduction

Pancreatic cancer is the fourth leading cause of cancer death in the United States and the-fifth in Japan, with an estimated number of annual deaths from this disease of 34,100 and 22,000 in the two countries, respectively [1,2]. Because of its aggressive growth and potential for early metastatic dissemination, the average overall 5-year survival rate of patients with pancreatic cancer is <5%, and only 20% of the patients can be treated by surgery with curative intent at the time of diagnosis [3].

To date, various treatments, including those that are highly invasive, have been tried to improve the poor prognosis of pancreatic cancer. Irradiation, chemotherapy, immunotherapy and other therapies, with or without surgery, have been applied to treat patients with pancreatic cancer [4,5]. Unfortunately, however, these modalities have failed to sufficiently control the aggressive behavior of invasive pancreatic cancer, and the overall survival of patients with pancreatic cancer remains grim, as assessed by global statistics [1].

Because pancreatic cancer is associated with a high mortality rate, aggressive treatment may be warranted, but the patients must

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be carefully chosen. Establishment of a prognostic scoring system could allow therapeutic management to be designed specifically for different sub-populations of the patients, and also identification of populations that might actually benefit from radical surgical treatment and/or chemotherapy. However, until date, there are no established reliable prognostic factors for pancreatic cancer patients whose usefulness has been proven by at least two examinations [6].

Isoforms of atypical protein kinase C, aPKCλ/ι and aPKC ζ, are PKC isozymes that are structurally and functionally distinct from other classes of this enzyme [7,8]. They play multifunctional roles in cellular maintenance and growth of epithelial cells, for example, signal transduction and cell polarity [9–15]. Studies on lung, ovary, gastric, colon, breast and prostate cancers have demonstrated a relationship between aPKCλ/ι expression and cancer progression, and have suggested that elevated aPKCλ/ι expression could be predictive of unfavorable outcomes [16–23]. Scotti ML et al. reported that high expression levels of aPKCλ/ι in pancreatic cancer were correlated with poor patient survival [24]. However, because only patients with pancreatic ductal adenocarcinoma (PDAC) were included in the aforementioned study, it remains unclear whether high aPKCλ/ι expression levels may also represent a poor prognostic factor in cases of intraductal papillary mucinous neoplasm (IPMN).

The pancreas is a complex organ comprised of three critical cell lineages; islet (endocrine), acinar, and ductal. Reflecting this variety of cell types, various types of neoplasms of the pancreas can occur in the pancreas. The most common type of pancreatic neoplasm among resected cases is PDAC, followed in frequency by IPMN, which accounts for up to 20% of all resected pancreatic neoplasms [25–27]. IPMN is characterized by the presence of dilated, frequently cystic, pancreatic ducts filled with mucus [28]. The prognosis of patients with non-invasive IPMN is excellent, and the reported 5-year survival rate is 77–100% [29–35]. Conversely, the reported 5-year survival rate of patients with IPMN-derived carcinoma ranges from 34% to 62% [25,31–33,35–40]. This survival is significantly worse than that of patients with non-invasive IPMN, but appears to be better than that of PDAC patients, which ranges from 9% to 21% [31,32,35–37,40]. It remains unclear, however, whether this difference in survival between IPMN-derived carcinoma and PDAC is related to their distinct biologies or might simply reflect the tendency of patients with IPMN-derived carcinoma to present at an earlier stage. To date, several studies have directly compared the clinicopathological features and long-term survival of patients of IPMN-derived carcinoma with those of PDAC, and conflicting results have been reported as to whether IPMN-derived carcinoma may be associated with improved survival, even in patients matched by the tumor stage [30,32,35–37,40,41].

In this study, we describe, for the first time, that the aPKCλ/ι expression level in the tumor tissue may have an important prognostic impact in IPMN. In addition, we confirmed that high aPKCλ/ι expression was correlated with a poor survival in patients with PDAC. These findings suggest that the aPKCλ/ι expression level in the tumor tissue can be a good biomarker common to two or more types of pancreatic neoplasm.

2. Material and method

2.1. Patients and samples

This study was conducted with the approval of the Ethics Committee of Yokohama City University (Yokohama, Japan). Clinical and pathologic data and the specimens used for the immunohistochemical analysis were obtained through a detailed retrospective review of the medical records of all 115 patients with PDAC and 46 patients with IPMN who had undergone initial surgical resection between 1991 and 2010 at the Yokohama City University Hospital (Yokohama, Japan). In addition, 11 paired normal pancreatic tissue and pancreatic cancer tissue samples were obtained from patients who had undergone surgical resection in 2008. None of the patients had received any prior therapy. All the patients with PDAC and IPMN had received standard therapy appropriate for their clinical stages. Tumors were classified according to the WHO classification [42,43], the International Union Against Cancer Tumor-Node-Metastasis classification [44], and the classification of pancreatic carcinoma of the Japan Pancreas Society [45]. The medical records were complete for all patients, and all had been followed up by the tumor registries for survival and outcome. The latest survival data were collected on August 31, 2011. The mean follow-up was 20 months (range, 2–137 months) for patients with pancreatic ductal adenocarcinoma, and 45 months (range, 2–142 months) for patients with IPMN. The clinicopathologic features of the patients with PDAC and IPMN are summarized in Table 1 and Table 2, respectively.

2.2. Pathological examination of IPMN

Analysis of the IPMN background included the histological grade [42], type of duct involvement, and the epithelial subtype. The type of duct involvement by IPMN was determined by macroscopic and microscopic examinations, as well as by clinical imaging studies, and was classified into main-duct, branch-duct and mixed-duct type involvement [46]. Because the epidemiological and clinicopathologic characteristics of the patients with main-duct and mixed type IPMN have been reported to be similar [47], patients with the main-duct and mixed types were grouped together into the main-duct type for statistical analysis. The intraductal components were classified into four distinct epithelial subtypes (gastric, intestinal, pancreatobiliary and oncocytic) on the basis of their epithelial morphology in sections stained with hematoxylin and eosin (H&E), and where available, immunoreactivity against mucin glycoproteins, according to previously described criteria [48,49]. In cases exhibiting a heterogeneous epithelium type, the subtype was determined on the basis of the most prevalent type of epithelium of the highest malignancy grade.

Invasive carcinomas arising in the background of IPMN were classified into three histological subtypes: tubular adenocarcinoma, colloid carcinoma and oncocytic carcinoma [50,51]. In cases containing more than one histological subtype, the predominant one was considered for the purpose of this study.

2.3. RNA extraction and real-time quantitative PCR (qPCR)

Total RNA from the frozen tissues was extracted using ISOGEN (NipponGene, Tokyo, Japan), according to the manufacturer's instructions. After cDNA synthesis, qPCR was performed with an iCycler and TaqMan® Gene Expression Master Mix (Applied Biosystems, Carlsbad, CA). Primers for aPKCλ/ι were determined by the Primer-Express software (Applied Biosystems); 18S ribosomal RNA (rRNA) was used as the internal control. The primer sequences were as follows: aPKCλ/ι forward 5′- CCAATGGCCACACTTTCCA -3′, reverse 5′-CGTCCAAGTCCCCATATTCG -3′, TaqMan® probe 5′- AAGCGTTTCAA-CAGGCGT -3′. The reaction conditions consisted of preheating for 3 min at 95 °C, followed by 40 cycles of 30 s at 95 °C, 30 s at 55 °C, and 1 min at 72 °C. The quantities of the amplified products were normalized to the quantity of 18S rRNA. All PCR products were confirmed using the melt curve method for specific amplification.

2.4. Immunohistochemistry

Immunohistochemical staining for aPKCλ/ι was performed according to the method described in a previous paper [20]. Formalin-fixed, paraffin-embedded sections (5-μm thick) were

Table 1
Correlations between aPKCλ/ι expression and the clinicopathological features in PDAC.

Clinical or pathological feature	Total (N)	aPKCλ/ι				p value
		Low		High		
All cases	115	53		62		
Gender						
Male	68	30	(56.6%)	38	(61.3%)	
Female	47	23	(43.4%)	24	(38.7%)	0.61[d]
Age (years)						
<65	45	24	(45.3%)	21	(33.9%)	
≥65	70	29	(54.7%)	41	(66.1%)	0.21[d]
Pathologic tumor status[a]						
pTis	0	0	(0.0%)	0	(0.0%)	
pT1	3	1	(1.9%)	2	(3.2%)	
pT2	9	4	(7.5%)	5	(8.1%)	
pT3	103	48	(90.6%)	55	(88.7%)	0.74[e]
Pathologic node status[a]						
pN0	23	10	(18.9%)	13	(21.0%)	
pN1	92	43	(81.1%)	49	(79.0%)	0.78[d]
Pathologic metastasis status[a]						
pM0	98	45	(84.9%)	53	(85.5%)	
pM1	17	8	(15.1%)	9	(14.5%)	0.93[d]
Stage[a]						
0	0	0	(0.0%)	0	(0.0%)	
Ia	2	1	(1.9%)	1	(1.6%)	
Ib	4	2	(3.8%)	2	(3.2%)	
IIa	17	7	(13.2%)	10	(16.1%)	
IIb	75	35	(66.0%)	40	(64.5%)	
III	0	0	(0.0%)	0	(0.0%)	
IV	17	8	(15.1%)	9	(14.5%)	0.83[e]
Tumor grade[b]						
Grade 1	28	13	(24.5%)	15	(24.2%)	
Grade 2	69	33	(62.3%)	36	(58.1%)	
Grade 3	18	7	(13.2%)	11	(17.7%)	0.70[d]
Lymphatic invasion[c]						
Negative	36	21	(39.6%)	15	(24.2%)	
Positive	79	32	(60.4%)	47	(75.8%)	0.075[d]
Venous invasion[c]						
Negative	30	15	(28.3%)	15	(24.2%)	
Positive	85	38	(71.7%)	47	(75.8%)	0.62[d]
Extrapancreatic nerve plexus invasion[c]						
Negative	65	31	(58.5%)	34	(54.8%)	
Positive	50	22	(41.5%)	28	(45.2%)	0.69[d]
Tumor size						
<2.0 cm	17	5	(9.4%)	12	(19.4%)	
≥2.0 cm	98	48	(90.6%)	50	(80.6%)	0.14[d]

[a] Classified according to the International Union against cancer tumor-node-metastasis classification.
[b] Classified according to the World Health Organization classification.
[c] Classified according to the classification of pancreatic carcinoma of the Japan Pancreas Society.
[d] χ^2 test.
[e] Mann-Whitney's *U* test.

deparaffinized and dehydrated. Then, the sections were autoclaved in 10 mM citrate buffer (pH 6.0) and incubated with normal swine serum, exposed to anti-aPKCλ/ι antibody (BD Biosciences) overnight at 4 °C, followed sequentially by incubation with biotinylated secondary antibody and avidin-biotinyl-peroxidase complex using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA). The peroxidase reaction was developed using diaminobenzidine tetrahydrochloride plus 0.02% hydrogen peroxide as the chromogen, and nuclear counterstaining was performed with hematoxylin.

2.5. Immunohistochemistry scoring

Three investigators, including a board-certified pathologist, independently assessed the stained sections. The staining intensities of aPKCλ/ι were graded as previously described, using a semiquantitative scale from 0 to 3, with 0 representing no staining, 1 representing light brown staining, 2 representing moderate brown staining, and 3 representing intense brown staining [20].

Nuclear and cytoplasmic aPKCλ/ι expression levels were independently scored on a scale of 0–3 and added to obtain a total cellular expression score of 0–6. If there were multiple staining intensities on one section, the score that defined the largest area was determined as the score for each specimen. The scores for the IPMN cases were determined in the area showing the highest degree of dysplasia. Low aPKCλ/ι expression level was defined as a total expression score of ≤3, and high aPKCλ/ι as a total expression score of >3. Sections stained with the secondary antibody alone served as the negative control.

2.6. Statistical analysis

All statistical analyses were performed using the SPSS for windows (SPSS Inc. Chicago, IL). The results on aPKCλ/ι mRNA expression were analyzed by the Wilcoxon's *t* test. The correlations between the immunohistochemistry scoring and the clinicopathologic findings were analyzed by the chi-square test; if the expected value was less than 5 in more than 20% of the cells, Fisher's exact test was used instead. For the analysis of parameters showing a significant difference, we checked the absolute value of the adjusted residuals. Because we considered that $p < 0.05$ indicated statistical significance, the cutoff value of the absolute value of the adjusted residuals was set at 1.96 for this study. The parameters including multiple ordinal scales, such as the pathologic tumor status, tumor stage, and tumor/histological grade, were analyzed by the Mann–Whitney *U* Test. We constructed Kaplan–Meier curves and used the log-rank test to determine the prognostic significances of the variables.

A Cox proportional hazards regression model was used to simultaneously examine the effects of multiple covariates on the survival. Prior to the analysis, we prepared a correlation table of the variables and confirmed that none of the variables showed any significant correlation. The effect of each variable was assessed using the likelihood test and described by the hazard ratio with a 95% confidence interval. The stepwise Cox proportional hazards models initially included age, pathologic tumor status, pathologic lymph node status, pathologic metastasis status, tumor grade, tumor size, tumor growth pattern, presence/absence of a history of chemotherapy, as well as the aPKCλ/ι expression for the PDAC patients. In the case of IPMN patients, the parameters included age, histological grade, macroscopic type, morphological types, and aPKCλ/ι expression. The final model was developed by dropping each variable in turn from the model and conducting a likelihood ratio test to compare the full and nested models. A significance level of 0.1 was used as the cutoff to exclude a variable from the model. The results of the cell invasiveness assay were analyzed by the Mann–Whitney *U* Test. All statistical analyses were two-sided; $p < 0.05$ was considered to indicate statistical significance in all the analyses.

3. Results

3.1. Overexpression of aPKCλ/ι mRNA in PDAC

To investigate the relationship between aPKCλ/ι expression and pancreatic cancer, we evaluated the expressions of aPKCλ/ι at the mRNA level in 11 pairs of surgically resected specimens of human PDAC and the surrounding non-tumor pancreatic tissue obtained from the same patients. The pathological findings in the H&E sections were confirmed by a board-certified pathologist. Quantitative real-time PCR analyses revealed that aPKCλ/ι mRNA were significantly overexpressed in the PDAC tissues as compared with those in

Table 2
Correlations between aPKCλ/ι expression and the clinicopathological features in IPMN.

Clinical or pathological feature	Total(N)	aPKCλ/ι				p value
		Low		High		
All cases	46	35		11		
Gender						
Male	35	27	(77.1%)	8	(72.7%)	
Female	11	8	(22.9%)	3	(27.3%)	0.42[c]
Age (years)						
<65	16	13	(37.1%)	3	(27.3%)	
≥65	30	22	(62.9%)	8	(72.7%)	0.53[c]
Histological grade[a]						
IPMN with low- or intermediate-grade dysplasia	20	19	(54.3%)	1	(9.1%)	
IPMN with high grade dysplasia	9	6	(17.1%)	3	(27.3%)	
IPMN with an associated invasive carcinoma	17	10	(28.6%)	7	(63.6%)	0.010[d]
Stage[b]						
0A	20	19	(54.2%)	1	(9.1%)	
0B	9	6	(17.1%)	3	(27.3%)	
0	0	0	(0.0%)	0	(0.0%)	
Ia	1	1	(2.9%)	0	(0.0%)	
Ib	11	7	(20.0%)	4	(36.4%)	
IIa	2	1	(2.9%)	1	(9.1%)	
IIb	1	0	(0.0%)	1	(9.1%)	
III	0	0	(0.0%)	0	(0.0%)	
IV	2	1	(2.9%)	1	(9.1%)	0.0067[d]
Macroscopic type						
Branch	18	15	(42.9%)	3	(27.3%)	
Mixed	16	13	(37.1%)	3	(27.3%)	
Main	12	7	(20.0%)	5	(45.4%)	0.11[c]
Morphological types						
Gastric	19	18	(51.4%)	1	(9.1%)	
Intestinal	18	12	(34.3%)	6	(54.5%)	
Pancreatobiliary	4	2	(5.7%)	2	(18.2%)	
Oncocytic	5	3	(8.6%)	2	(18.2%)	0.025[c]
Invasion types						
None	29	25	(71.3%)	4	(36.4%)	
Tubular adenocarcinoma	10	8	(22.9%)	2	(18.3%)	
Colloid carcinoma	2	1	(2.9%)	1	(9.1%)	
Oncocytic carcinoma	5	1	(2.9%)	4	(36.4%)	0.0026[c]

[a] Classified according to the World Health Organization classification.
[b] Classified according to the International Union Against Cancer Tumor-Node-Metastasis classification. Stage 0A and Stage 0B indicate low/moderate grade dysplasia and high grade dysplasia in the primary neoplasm, respectively.
[c] Fisher's exact test.
[d] Mann-Whitney's *U* test.

the paired non-tumor pancreatic tissues obtained from the same patients ($p = 0.0044$) (Fig. 1a).

3.2. Immunohistochemical analysis of aPKCλ/ι expression

Subsequent immunohistochemical analysis of the 115 specimens of PDAC was conducted to confirm the expressions of aPKCλ/ι at the protein level. In normal pancreatic duct epithelium, expression of aPKCλ/ι protein was scarcely observed (Fig. 1b, white arrow). Although there were some specimens that showed faint aPKCλ/ι staining in the cytoplasmic region, no significant staining was observed in the membrane, junctional or nuclear regions in any of the specimens. On the other hand, in the cancer cells, aPKCλ/ι staining was present in various intensities in the cytoplasmic and nuclear regions (Fig. 1b, black arrow). Because the intensity of cytoplasmic and nuclear staining was not necessarily the same, we

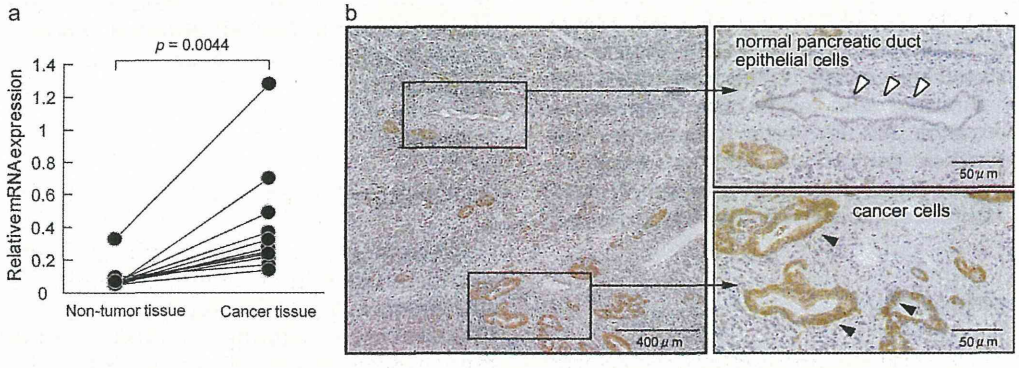

Fig. 1. **a** Quantitative real-time PCR analyses of aPKCλ/ι mRNA expression. aPKCλ/ι mRNA expression was compared between PDAC tissue and surrounding non-tumor pancreatic tissue specimens obtained from the same patients ($n = 11$ pairs). aPKCλ/ι mRNA was significantly overexpressed in the pancreatic cancer tissues ($p = 0.0044$). **b** Immunohistochemical analysis in a case of PDAC. aPKCλ/ι was overexpressed in the cancer cells (black arrow), but not in normal pancreatic duct epithelial cells (white arrow).

scored cytoplasmic and nuclear aPKCλ/ι expression levels separately on a scale of 0–3 (Fig. 2, upper row), and add the scores to obtain a total cellular expression score of 0–6. For the statistical analysis, low aPKCλ/ι expression level was defined as a total expression score of ≤3 ($n = 53$, 46.1%), and high aPKCλ/ι expression as a total expression score of >3 ($n = 62$, 53.9%).

In addition, we analyzed the protein expression of aPKCλ/ι in 46 specimens of IPMN. Consistent with the findings in the case of PDAC, protein expression of aPKCλ/ι was detected in only neoplastic lesion in the IPMN specimens (Fig. 2, lower row). The scores for the IPMN cases were determined in the areas with the highest degree of dysplasia, on the same scale as that for PDAC. In IPMN, there were 35 (76.1%) cases showing low aPKCλ/ι expression, and 11 (23.9%) showing high aPKCλ/ι expression.

3.3. Correlations between the clinicopathological features and the aPKCλ/ι expression level

In the PDAC patients, no correlations were observed between the aPKCλ/ι expression levels and any of the clinical parameters (Table 1). By contrast, the aPKCλ/ι expression levels were correlated with some clinical features in the IPMN patients (Table 2). As shown in Table 2, the aPKCλ/ι expression level was significantly correlated with the histological grade ($p = 0.010$), advanced tumor stage ($p = 0.0050$), morphological type ($p = 0.025$), and invasion type of the tumors ($p = 0.0026$). From a comparison of the standardized residuals, a significant difference of the aPKCλ/ι expression level was observed between the gastric type and other morphological types (absolute value of the adjusted residuals = 2.5). In regard to the differences among the invasion types, significant difference of the aPKCλ/ι expression level was observed between the tumors showing no invasion and oncocytic carcinoma (absolute value of the adjusted residuals = 2.1, 3.1, respectively).

3.4. Prognostic significance of the aPKCλ/ι expression level

In the analysis of the overall survival of the PDAC patients, 8 of the 115 patients (7.0%) survived for more than 5 years after the surgical resection, and 16 patients (13.9%) survived for more than 3 years. The median survival was 17.2 months for all patients. The group with low aPKCλ/ι expression showed better survival than the group with high aPKCλ/ι expression ($p = 0.00025$, Fig. 3a). The median survival was 29.6 months in the group showing low aPKCλ/ι expression, and 14.1 months in the group showing high aPKCλ/ι expression.

In the case of IPMN also, high aPKCλ/ι expression was correlated with a worse survival ($p = 0.020$; Fig. 3b). The median survival was only 16.1 months in the group showing high aPKCλ/ι expression. Because of the good prognosis, the median survival could not be calculated in the group showing low aPKCλ/ι expression in the tumors.

Furthermore, even when only patients with invasive IPMN ($n = 17$) were extracted, aPKCλ/ι expression was found to show prognostic significance. Among the patients with invasive IPMN, 4 of the 17 patients (23.5%) survived for more than 5 years after the surgical resection, and 5 patients (29.4%) survived for more than 3 years. The median survival was 25.9 months for all patients. The group with low aPKCλ/ι expression showed better survival than the group with high aPKCλ/ι expression ($p = 0.00094$, Fig. 3c). The median survival was 99.6 months in the group showing low aPKCλ/ι expression, and 10.6 months in the group showing high aPKCλ/ι expression.

3.5. Multivariable analysis for identifying prognostic factors

We applied the Cox proportional hazards model to our dataset to compare the prognostic risk factors. Our univariate analysis revealed that in PDAC patients, high aPKCλ/ι expression had the highest prognostic significance ($p = 0.00036$). In a stepwise multivariable Cox regression model with standard parameters, high aPKCλ/ι expression ($p = 0.0000056$), presence of distant metastasis ($p = 0.00041$), high tumor grade ($p = 0.0032$), and history of chemoradiotherapy ($p = 0.043$) were identified as significant factors affecting the prognosis in all patients (Table 3). The hazard ratio for a poor prognosis was 2.71 in patients showing high expression levels of aPKCλ/ι relative to other patients.

In a univariate analysis of IPMN patients, high aPKCλ/ι expression was identified as the factor with the second highest prognostic significance ($p = 0.0053$) after the histological grade ($p = 0.0010$). In a stepwise multivariable Cox regression model with standard parameters, only high aPKCλ/ι expression ($p = 0.010$) and histological grade ($p = 0.0012$) were identified as significant factors affecting the prognosis in all patients (Table 4). The hazard ratio for a poor prognosis was 5.23 in patients showing high expression levels of aPKCλ/ι, relative to other patients.

4. Discussion

It has been reported that high aPKCλ/ι expression is correlated with a poor prognosis in many types of cancers. In relation to

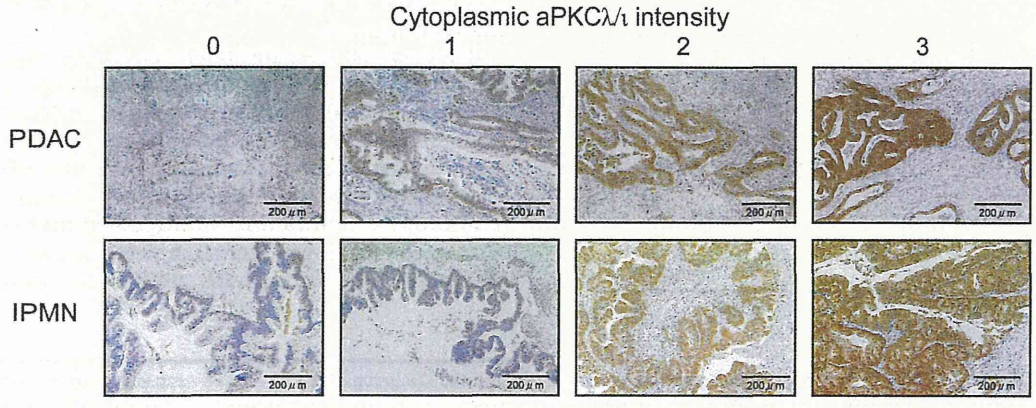

Fig. 2. Representative immunohistochemical micrographs of aPKCλ/ι expression in formalin-fixed specimens of human pancreatic ductal adenocarcinoma (PDAC; upper row) and intraductal papillary mucinous neoplasms of the pancreas (IPMN; lower row).

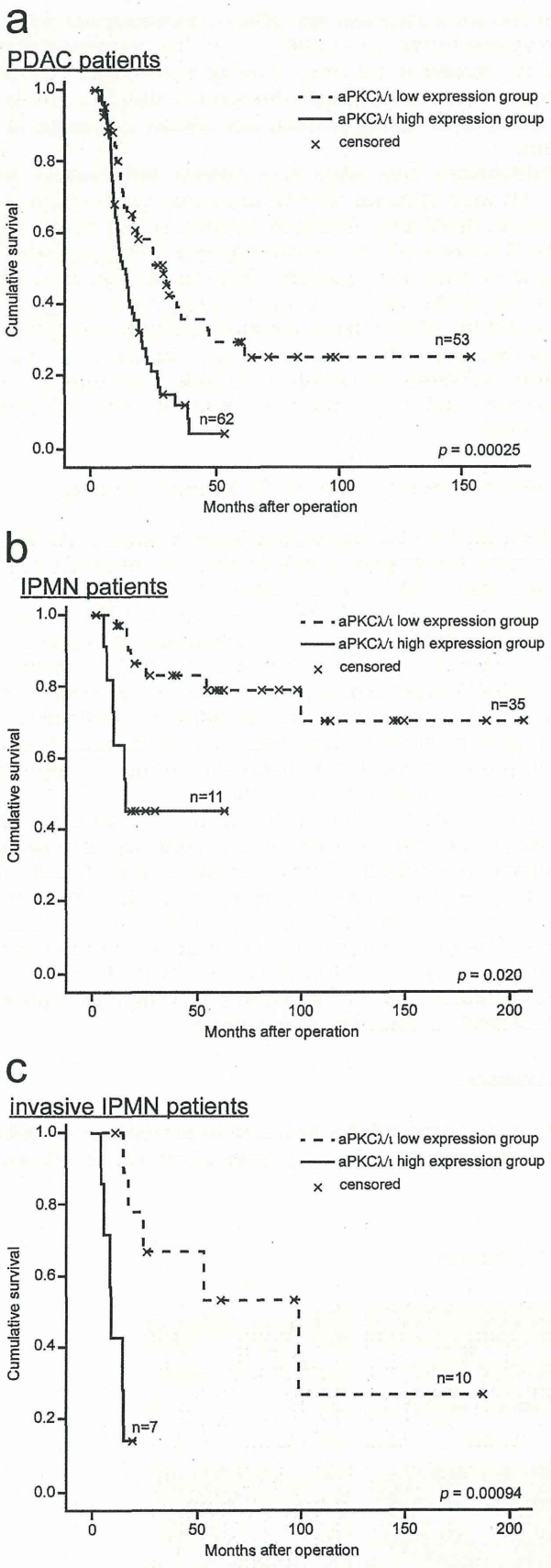

Fig. 3. **a** Prognostic significance of aPKCλ/ι expression in PDAC patients. The group with low aPKCλ/ι expression showed better survival than the group with high aPKCλ/ι expression ($p = 0.00025$). **b** Prognostic significance of aPKCλ/ι expression in IPMN patients. The group with low aPKCλ/ι expression showed better survival than the group with high aPKCλ/ι expression ($p = 0.0020$). **c** Prognostic significance of aPKCλ/ι expression in patients with invasive IPMN. The group with low aPKCλ/ι expression showed better survival than the group with high aPKCλ/ι expression ($p = 0.00094$).

cancers of the digestive system, such an association has been reported for gastric cancer, colon cancer, cholangiocarcinoma, and PDAC [21,23,24,52]. In this study, we obtained evidence of the existence of a statistically significant correlation between high aPKCλ/ι expression and the prognosis in patients with two types of pancreatic neoplasms.

IPMN has been reported as the second common type of pancreatic neoplasm among resected pancreatic tumor specimens, after PDAC [25–27]. When the prognosis of patients with pancreatic neoplasms is analyzed, IPMN and PDAC can hardly be analyzed under comparable conditions. One reason is that the prognosis of patients with non-invasive IPMN is excellent. Even if patients with invasive IPMN are extracted, the prognosis is different between invasive IPMN and PDAC [32,35–37,40]. Thus, in this study, we analyzed the prognostic significance of the aPKCλ/ι expression level separately in PDAC and IPMN, and our results revealed the prognostic significance of aPKCλ/ι expression in PDAC, IPMN, as well as invasive IPMN. Moreover, multivariable analysis to identify significant prognostic factors revealed that the aPKCλ/ι expression level was a prognostic factor independent of standard clinical parameters. These findings suggest that the aPKCλ/ι expression level can be used as a common prognostic marker for different pancreatic neoplasms.

Although there were no correlations between high aPKCλ/ι expression and any clinical features in PDAC patients, high aPKCλ/ι expression was associated with some clinical features in IPMN patients. In IPMN patients, high aPKCλ/ι expression was significantly correlated with the histological grade ($p = 0.010$) and advanced stage of the tumor ($p = 0.0050$). IPMN is a rare neoplasm of the pancreas in which the course can be followed up from adenoma to carcinoma [42]. These findings suggest that aPKCλ/ι overexpression could be involved in the malignant transformation of IPMN.

In human PDAC, it is difficult to analyze the process of carcinogenesis in detail. One possible way is to analyze pancreatic intraepithelial neoplasia (PanIN), one of the distinct precursors to invasive pancreatic cancer that can be detected by pathological studies [53]. However, surgical specimens containing only PanIN are very rare. Scotti ML et al. reported that overexpression of aPKCλ/ι in human PDAC was correlated with poor patient survival [24]. They also demonstrated that aPKCλ/ι is required for the transformed growth of pancreatic cancer cells *in vitro* and their tumorigenesis *in vivo* [24].

In contrast to the case for PDAC, the course of malignant transformation of human IPMN can be easily analyzed in pathological specimens. In this study, we demonstrated the possible role of aPKCλ/ι in malignant transformation of human IPMN. Moreover, taken together with the results for PDAC, it is speculated that aPKCλ/ι may be involved in the carcinogenesis of several types of pancreatic cancers.

Although the mechanism remains unclear, two possibilities are speculated. One is K-ras mediated signaling, and the other is interlukin-6 (IL-6) mediated signaling. Oncogenic KRAS mutations are found in >90% of all advanced pancreatic cancers [54]. It has been demonstrated that antisense inhibition of oncogenic K-ras expression in pancreatic ductal adenocarcinoma (PDAC) cell lines blocks cellular transformation, suggesting continued requirement of oncogenic K-ras mediated signaling to maintain the transformed phenotype [55]. Furthermore, aPKCλ/ι has been reported as an