Molecular Characteristics of Pancreatic Ductal Adenocarcinomas with High-Grade Pancreatic Intraepithelial Neoplasia (PanIN) Are Different from Those without High-Grade PanIN

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Keywords

Pancreatic ductal adenocarcinoma · Pancreatic intraepithelial neoplasia · *KRAS* · p53 · SMAD4

Abstract

Aims: We reported that pancreatic ductal adenocarcinomas (PDACs) without high-grade pancreatic intraepithelial neoplasia (PanIN) in the vicinity had worse prognoses than PDACs with high-grade PanIN. However, the molecular characteristics of PDACs with and without high-grade PanIN have not been compared. The aim of this study is to clarify the molecular characteristics of PDACs with and without high-grade PanIN. **Method and Results:** We reviewed all of a consecutive series of 100 patients with PDACs and divided them into 2 groups: the PDACs with PanIN-2 or PanIN-3 in the background (the PanIN-high group, $n = 60$) and the PDACs without PanIN-2 or PanIN-3 in the background (the PanIN-low group, $n = 40$). We evaluated the p53, p16, and SMAD4 expressions in the invasive ductal carcinoma (IDC) components by immunohistochemical staining. *KRAS* mutation was also analyzed in 80 tumors. The PanIN-low group

showed significantly more frequent “high p53 expression” and “loss of SMAD4 expression” than the PanIN-high group ($p = 0.048$ and $p = 0.019$, respectively). Loss of p16 expression was not significantly different between the groups. The rate of *KRAS* wild type was significantly higher in the PanIN-low group than the PanIN-high group ($p = 0.024$). **Conclusions:** Our results demonstrated that the molecular characteristics in the PDACs with high-grade PanIN were different from those in the PDACs without high-grade PanIN. PDACs without high-grade PanIN may develop via a pathway other than the PanIN-carcinoma sequence.

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Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive cancers, with an overall survival rate of 6% in the USA [1] and 7% in Japan [2]. Risk factors for PDAC are obesity [3], tobacco smoking [4], heavy alcohol drinking (which is secondary to pancreatitis) [5], and a history of chronic pancreatitis [6]. Patients with the blood

Table 1. Primary antibodies used for immunohistochemical staining

Antibody	Clone	Source	Dilution	Antigen retrieval
p53	DO-7	Novocastra, Newcastle, UK	1:100	TRS buffer (pH 9.0), microwave 10 min
p16	E6H4	Roche MTM Laboratories, Heidelberg, Germany	1:1	PBS (pH 7.4), microwave 20 min
Smad4	B-8	Santa Cruz Biotechnology, Santa Cruz, CA, USA	1:400	TRS buffer (pH 9.0), microwave 20 min

type A or B are also at higher risk for PDAC than those with type O [7]. Diabetes mellitus is present in 70% of PDAC patients [8].

Pancreatic intraepithelial neoplasia (PanIN) is well known to be a precursor lesion of PDAC [9]. Less frequently, intraductal papillary mucinous neoplasm and mucinous cystic neoplasm can be precursor lesions of PDAC. PanIN is defined by published consensus guidelines [10]. Briefly, PanINs are microscopic papillary or flat, noninvasive epithelial neoplasms that are usually <5 mm in diameter and confined to the pancreatic ducts [9, 10]. Based on the degree of dysplasia reflected in the cytonuclear atypia and architectural changes, PanINs can be classified into 4 grades: PanIN-1A, -1B, -2, and -3 [9, 10].

Many studies have identified gene alterations of PDAC [9, 11–24]. One of the major oncogenes, *KRAS*, is mutated in >90% of PDACs [9, 11, 21–24]. In addition, 3 frequently mutated genes play critical roles in the development of pancreatic neoplasias: *CDKN2A* (*p16*), *TP53* and *SMAD4* (*DPC4*) [9, 12–27]. The *p16*, *TP53*, and *SMAD4* genes are inactivated in 95, 50–75, and 55% of PDACs, respectively [9, 12–24]. In PanIN lesions, *KRAS* activations appear to occur relatively early; *p16* inactivation occurs at an intermediate stage, and *p53* and *SMAD4* inactivations occur at a late stage [9, 13, 24–27].

We previously demonstrated that PDAC patients without PanIN-2 or PanIN-3 in the background (i.e., the PanIN-low group) had worse prognoses than those with PanIN-2 or PanIN-3 (the PanIN-high group) [28]. However, the mechanisms underlying the poor prognosis in the PanIN-low group remained unclear. In the present study we examined and compared the molecular characteristics of a PanIN-low group and a PanIN-high group.

Materials and Methods

Patients and Specimens

We retrospectively reviewed samples from 100 PDAC cases that had been diagnosed at the Department of Anatomic Pathology of Kyushu University (Fukuoka, Japan) between January 1992

and December 2009. Eighty of the 100 patients with PDAC were included in our previous study [28]. Clinical and pathological data were obtained from a retrospective database and a review of medical records. Clinical characteristics (age, sex, risk factors, diabetes mellitus, symptoms, body mass index, and tumor location) and pathological findings (lymphatic permeation, venous invasion, perineural invasion, histological grade, and UICC TNM stage) were evaluated. Alcohol intake was estimated as described [5]. Survival was measured from the time point of the surgery until death or censor. This study was approved by the Ethics Committee of Kyushu University (No. 27-242).

Pathological Examination for PanIN

The surgically resected specimens were fixed with formalin in approximately 5-mm-thick sections. All of the consecutive whole tissue sections of all resected pancreatic tissue including tumor lesions were embedded in paraffin and routinely examined microscopically with hematoxylin and eosin (HE) staining. The average number of investigated slides per case was 22.2 (range 11–44). All of the investigated slides were taken from the pancreatic tissue including the tumor lesion, and the slides taken from nonpancreatic tissue (lymph nodes, nonpancreatic margins, etc.) were not included and counted. Three pathologists (S.A., Y. Oda, and T.M.) independently evaluated PanINs that were <5 mm in size within the pancreatic duct and those that were distinctly separate from the invasive carcinoma component. We evaluated PanIN lesions accompanied by normal pancreatic tissue between PanIN lesions and the invasive ductal carcinoma (IDC) component. We histologically defined “PanIN-3” as an intraductal neoplastic lesion showing severe cellular atypia, a loss of cell polarity, and a micro-papillary structure, accompanied by the close proximity of PanIN-1A, PanIN-1B, or PanIN-2, and no involvement by an invasive carcinoma component. It was necessary for there to be at least 5 mm of normal pancreas between the PanIN-3 and the cancer. Cancerization of the duct, which is defined as carcinoma secondarily spreading into the pancreatic duct, was excluded from PanIN-3.

We counted the numbers of PanIN lesions (i.e., the numbers of PanIN-1A, PanIN-1B, PanIN-2, and PanIN-3 lesions, respectively) in each IDC. The median number of total PanIN lesions, regardless of the grade of dysplasia, was 15 (range 0–595). We classified the lesions into a <15 PanIN group ($n = 45$) and a ≥ 15 PanIN group ($n = 55$). We retrospectively investigated all slides and recorded the number of slides and the grade of all PanIN lesions in all samples.

Because PanINs can have various grades in the same samples, we classified the 100 patients with PDACs into a PanIN-low group (the PDACs with PanIN-1A, -1B or the absence of PanIN) and a PanIN-high group (the PDACs with PanIN-2 or PanIN-3) based on the highest grade of atypia. The detailed evaluation method for

Table 2. Correlations between clinicopathological characteristics and PanIN group in PDACs

	PanIN group		p value
	low (n = 40)	high (n = 60)	
Age			
<65 years	16	25	0.868
≥65 years	24	35	
Male:female	27:13	33:27	0.211
Location			
Head:body/tail	26:14	42:18	0.599
Family history			
Present/absent	35/1	53/3	1.000 ^a
Unknown	4	4	
Symptoms			
Present/absent	11/29	24/36	0.199
Smoking history			
Yes/no	23/13	36/21	0.943
Unknown	4	3	
Drinking			
Non- or occasional	22	39	0.578
Moderate	3	6	
Heavy	11	12	
Unknown	4	3	
BMI <25/≥25	31/6	53/3	0.083
Unknown	3	4	
ABO blood group			
O/others	6/30	13/43	0.449
Unknown	4	4	
Pancreatitis history			
Present/absent	4/33	3/55	0.305
Unknown	3	2	
Diabetes mellitus			
Present/absent	33/4	48/9	0.495
Unknown	3	3	
Histological grade			
G1	13	22	0.350
G2	18	31	
G3	9	7	
Vessel invasion (+)	33 (82.5%)	34 (56.7%)	0.007*
Lymphatic permeation (+)	36 (90%)	43 (71.7%)	0.027*
Perineural invasion (+)	36 (90%)	53 (88.3%)	0.794
UICC TNM category			
T1 + 2/3 + 4	2/38	5/55	0.699 ^a
N0/N1	11/29	20/40	0.537
M0/M1	38/2	59/1	0.562 ^a
UICC stage			
I + II/III + IV	37/3	58/2	0.386 ^a
Number of PanIN lesions			
≥15 PanINs/<15 PanINs	13/27	42/18	0.0002*

* Statistically significant. ^a Fisher's exact tests.

PanIN in the background has previously been described [28]. We also determined the correlations between the PanIN groups and clinicopathological findings.

Immunohistochemical Procedures

The primary monoclonal antibodies used for the immunohistochemical analysis are listed in Table 1. In brief, 4-μm-thick tissue sections were deparaffinized in xylene and dehydrated in ethanol. Endogenous peroxidase activity was blocked by incubation in methanol containing 0.3% H₂O₂ for 30 min. Antigen retrieval was achieved as shown in Table 1.

The slides were then incubated with the primary antibodies for 90 min at room temperature (p53 and p16) or at 4°C overnight (SMAD4) and subsequently incubated with biotin-free horseradish peroxidase enzyme-labeled polymer (EnVision plus System, DAKO Cytomation, Glostrup, Denmark) for 40 min at room temperature. The labeled antigens were visualized using 3,3'-diaminobenzidine tetrahydrochloride as a chromogen. Counterstaining was performed with hematoxylin. Appropriate positive and negative controls were performed for all antibodies.

Evaluation of Immunohistochemical Staining

We analyzed both 100 IDCs and 95 concomitant PanIN components. Immunohistochemical staining of 5 of the PanIN lesions was not available because 5 PanIN lesions disappeared after the serial sectioning of the paraffin blocks. Consequently, 90 PanIN components were evaluated by immunohistochemical staining. The reactions were interpreted as positive on the basis of the presence of nuclear staining (p53) and nuclear and cytoplasmic staining (p16 and SMAD4).

For the p53 immunolabeling, focal acinar and ductal cells with nuclear p53 immunolabeling served as an internal control. The p53 immunolabeling was interpreted as "high expression" when the neoplastic cells showed robust nuclear accumulation in ≥30% of the neoplastic cells. Immunohistochemical labeling of high p53 expression corresponded to mutational inactivation [23, 29–31].

The immunohistochemical labeling of p16 and SMAD4 was defined as intact (positive) or completely lost (negative). Negative p16 and SMAD4 immunolabeling is explained by gene alterations such as homozygous deletion, promoter hypermethylation or loss of heterozygosity and intragenic mutation [16, 20, 29]. Islet cells served as an internal control for positive p16 immunolabeling. Normal acinar cells, ductal cells, islet cells, and stromal cells in each case served as internal controls for positive SMAD4 immunolabeling.

Mutational Analysis of the IDC Component

In the 100 PDACs, genomic DNA was extracted from 5 or six 7-μm sections of formalin-fixed, paraffin-embedded specimens by a macrodissection technique. We carefully macrodissected from portions containing as many carcinoma cells as possible, referring to the HE slides. The range of the percentage of carcinoma cells within the macrodissected tissue was 30–50%. Genomic DNA was subsequently extracted using the QIAamp DNA FFPE Tissue Kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). The polymerase chain reaction (PCR) and mutational analysis were performed for exon 2 of KRAS.

For 16 cases (high and low levels of p53 immunohistochemical expressions in 8 cases each), we also performed a mutational analysis of the TP53 gene (exons 5–9).

Fig. 1. Representative immunohistochemical images of p53, p16, and SMAD4 expression in the IDC component of the PanIN-low (**a–c**) and PanIN-high (**d–f**) groups. High p53 expression (**a**), p16 intact (**b**), and SMAD4 loss (**c**) were observed in the PanIN-low group, whereas low p53 expression (**d**), p16 loss (**e**), and SMAD4

intact (**f**) were observed in the PanIN-high group. p16 expression was preserved in normal islet cells (**e**, below). SMAD4 expression was preserved in normal acinar cells (**c**, below). **a–f** Original magnification, $\times 100$.

Table 3. Correlation between PanIN groups for p53, p16, and SMAD4 expression in the IDC component

	PanIN group		<i>p</i> value
	low (<i>n</i> = 40)	high (<i>n</i> = 60)	
p53			
Low expression	24	47	0.048*
High expression	16 (40%)	13 (21.7%)	
p16			
Positive (intact)	14	11	0.059
Negative (loss)	26 (65%)	49 (81.7%)	
SMAD4			
Positive (intact)	10	29	0.019*
Negative (loss)	30 (75%)	31 (51.7%)	

* Statistically significant.

The primer pairs used in the sequencing analysis are shown in the online supplementary Table 1 (for all online suppl. material, see www.karger.com/doi/10.1159/000455194).

The amplified PCR products were then purified using Montage centrifugal filters (Millipore, Bedford, MA, USA). After purifica-

tion, direct sequencing was carried out using a Perkin Elmer ABI PRISM 310 sequence analyzer (Applied Biosystems, Foster City, CA, USA). If the quality of DNA or the level of PCR amplification was insufficient for the mutation analysis, the cases were excluded from the molecular study.

Statistical Analysis

All calculations were carried out using JMP 11.0.0 software (SAS Institute, Cary, NC, USA). Statistical analyses were performed with the Fisher exact test, the χ^2 test and the Mann-Whitney U test. Survival curves were examined by the Kaplan-Meier method, and significance was examined using the log-rank test. Multivariate survival analyses were performed using the Cox proportional hazards regression model. A *p* value of <0.05 was considered significant.

Results

Frequency of PanIN Grade and the Classification of the PanIN Groups

According to the highest grade of PanIN, the 100 patients were classified as follows: no PanIN (*n* = 5), PanIN-1A (*n* = 7), PanIN-1B (*n* = 28), PanIN-2 (*n* = 38),

Table 4. Rate of p53, p16, and SMAD4 expression in PanIN and the IDC component of the PanIN group

p53	PanIN-low group			PanIN-high group		
	PanIN-1A	PanIN-1B	IDC	PanIN-2	PanIN-3	IDC
High expression	0% (0/7)	0% (0/28)*	40% (16/40)	2.9% (1/35)	15% (3/20)	21.7% (13/60)
p16 loss	28.6% (2/7)	17.9% (5/28)*	65% (26/40)	28.6% (10/35)	55% (11/20)*	81.7% (49/60)
SMAD4 loss	14.3% (1/7)	0% (0/28)*	75% (30/40)	5.7% (2/35)	25% (5/20)*	51.7% (31/60)

* $p \leq 0.05$: statistically significantly different compared to IDC.

and PanIN-3 ($n = 22$). We categorized the patients into the PanIN-low group ($n = 40$) and the PanIN-high group ($n = 60$). There was no significant difference in the number of HE-stained slides reviewed between the 2 groups.

Correlations between the 2 PanIN Groups and Clinicopathological Characteristics

We compared the correlations between the 2 PanIN groups and the patients' clinicopathological characteristics (Table 2). The PanIN-low group showed significantly more frequent vessel invasion, lymphatic permeation and a smaller number of PanIN lesions compared to the PanIN-high group ($p = 0.007$, $p = 0.027$, and $p = 0.0002$, respectively). There were no significant relationships between the PanIN group and the other clinicopathological characteristics.

The Expressions of p53, p16, and SMAD4 in the IDC Component: Comparison of the Low- and High-PanIN Groups

The IDC component immunohistochemical results are summarized in Table 3. Representative immunohistochemical images are shown in Figure 1. The frequency of high p53 expression was significantly higher in the PanIN-low group compared to the PanIN-high group (40 vs. 22%, $p = 0.048$). Loss of SMAD4 expression was observed significantly more frequently in the PanIN-low group compared to the PanIN-high group (75 vs. 52%, $p = 0.019$). The frequency of loss of p16 expression tended to be low in the PanIN-low group compared to the PanIN-high group, but there was no significant difference (65 vs. 81.7%, $p = 0.059$).

p53, p16, and SMAD4 Expression in PanIN and IDC Components

The immunohistochemical results of PanIN and the IDC components in the PanIN-low group are summa-

Table 5. Correlation between PanIN group and KRAS status in the IDC component

KRAS mutation	PanIN group		<i>p</i> value
	low ($n = 33$)	high ($n = 47$)	
–	14 (42.4%)	9 (19.2%)	0.024*
+	19 (57.6%)	38 (80.8%)	

* Statistically significant.

rized in Table 4. The rates of high p53 expression were 0, 0, and 40% in the PanIN-1A, PanIN-1B, and IDC components, respectively. The rates of loss of p16 expression were 28.6, 17.9, and 65% in the PanIN-1A, PanIN-1B, and IDC components, respectively. The rates of loss of SMAD4 expression were 14.3, 0, and 75% in the PanIN-1A, PanIN-1B, and IDC components, respectively. In the PanIN-low group, the rates of p53 high expression, loss of p16 expression and loss of SMAD4 expression were very low in the low-grade PanIN components but were notably high in the IDC component ($p = 0.0001$, $p = 0.0001$, and $p < 0.0001$, respectively).

For the PanIN-high group, the immunohistochemical results of PanIN and the IDC components are summarized in Table 4. The rates of high p53 expression were 2.9, 15, and 21.7% in the PanIN-2, PanIN-3, and IDC components, respectively. The rates of loss of p16 expression were 28.6, 55, and 81.7% in the PanIN-2, PanIN-3, and IDC components, respectively. The rates of loss of SMAD4 expression were 5.7, 25, and 51.7% in the PanIN-2, PanIN-3, and IDC components, respectively. In the PanIN-high group, the rates of p53 high expression, loss of p16 expression, and loss of SMAD4 expression were gradually increased as the lesions progressed to PanIN-2, PanIN-3, and IDC (Table 4).

Fig. 2. Representative sequencing results of *KRAS* wild type in the PanIN-low group and *KRAS* mutation in the PanIN-high group. *KRAS* wild type was noted in the PanIN-low group, whereas *KRAS* mutation (G12D) was seen in the PanIN-high group.

Fig. 3. Survival analysis of patients in the PanIN-low and PanIN-high groups. Overall survival (**a**) and disease-free survival (**b**) were both significantly shorter in the PanIN-low group compared to the PanIN-high group ($p < 0.0001$, respectively).

KRAS Mutational Analysis

The results of the *KRAS* mutational analysis are summarized in Table 5 and Figure 2. Eighty of the 100 cases were successfully sequenced. The remaining 20 cases were excluded from the *KRAS* mutation analysis because of an insufficient quality of DNA or level of PCR ampli-

fication. *KRAS* wild type was noted in 14/33 (42.4%) of the PanIN-low group and in 9/47 (19.2%) of the PanIN-high group. The rate of *KRAS* wild type was significantly higher in the PanIN-low group compared to the PanIN-high group ($p = 0.024$).

TP53 Mutational Analysis

The results of the *TP53* gene mutational analysis are summarized in supplementary Table 2 in relation to the level of immunohistochemical expressions of p53 and the PanIN group. *TP53* gene mutation was observed in only 2 cases with high immunohistochemical expression of p53, and both cases were in the PanIN-low group.

Survival Analysis

Disease-free and overall survivals were significantly shorter in the PanIN-low group compared to the PanIN-high group (Fig. 3; $p < 0.0001$ and $p < 0.0001$, respectively). The median survival times for the PanIN-low group and the PanIN-high group were 16 and 42 months, respectively. Regarding the number of PanIN lesions, overall survival was significantly longer in the ≥ 15 PanIN group than in the < 15 PanIN group (online suppl. Fig. 3; $p = 0.018$). There were no significant differences in disease-free survival between the ≥ 15 PanIN group and the < 15 PanIN group (online suppl. Fig. 3; $p = 0.059$). The univariate survival analysis indicated that smoking history, heavy drinking, histological grade, lymphatic permeation, vessel invasion, lymph node metastasis, being in the PanIN-low group and the loss of SMAD4 expression were significant worse-prognosis factors (Table 6).

The multivariate survival analysis based on the Cox proportional hazards model included all parameters found to be significant in the univariate analyses and well-known prognosis factors such as UICC TNM stage and perineural invasion [9]. The multivariate survival analysis revealed that heavy drinking, lymph node metastasis and being in the PanIN-low group were independent prognostic factors (Table 6).

Discussion

There have been a few reports demonstrating the correlation between the grade of coexisting PanIN around IDC and prognosis [28, 32]. We observed previously that PDACs without high-grade PanIN led to a worse prognosis [28]. Hassid et al. reported that their patients who demonstrated an absence of PanIN in the pancreatic tissue adjacent to the resected PDAC tumor had shorter postresection survivals compared to those who demonstrated a PanIN lesion [32]. However, the molecular characteristics of PDACs based on the grade of concomitant PanIN have not been well investigated. This is the first report on differences in molecular characteristics between PDACs with and without high-grade PanIN.

We also observed that the rates of high p53 expression and loss of SMAD4 expression were significantly higher in the PanIN-low group compared to the PanIN-high group, whereas the rate of loss of p16 expression tended to be high in the PanIN-high group compared with the PanIN-low group, though not significantly (Table 3). It has been reported that the immunohistochemical labeling of p53, p16 and SMAD4 reflects the genetic status of *TP53*, *p16* and *SMAD4* [16, 20, 23, 29–31]. Thus, there may be high frequencies of *TP53* mutation and inactivation of the *SMAD4* gene in PanIN-low patients compared to the PanIN-high group. On the other hand, there may be a high frequency of inactivation of the *p16* gene in the PanIN-high group compared to the PanIN-low group.

In our PanIN-high group, the rates of high p53 expression, loss of p16 expression and loss of SMAD4 expression were increased stepwise from PanIN to IDC (Table 4). In the PanIN-low group, the rates of high p53 expression, loss of p16 expression and loss of SMAD4 expression in the PanIN component were very low, but much higher in the IDC component (Table 4). These results are consistent with previous studies [9, 12–14, 17–19, 24, 26, 27].

In this study, *TP53* gene mutation was detected in only 2 of the 16 cases examined, probably because the quality of extracted DNA was insufficient for a sequencing analysis of all of the exons of the *TP53* gene. We thus could not obtain further clarification regarding a direct relationship between *TP53* gene status and PanIN groups. However, the fact that both cases with *TP53* gene mutations showed a high immunohistochemical expression of p53 was in line with our supposition that immunohistochemical high expression corresponds to mutational inactivation [29–32].

Inactivation of *p16* in pancreatic adenocarcinomas is caused by homozygous deletion (40%), intragenic mutation in 1 allele coupled with loss of the second allele (loss of heterozygosity) (40%), and hypermethylation of a CpG island in the promoter region (15%) [14, 15]. Inactivation of *SMAD4* in pancreatic adenocarcinomas is caused by homozygous deletion (35%) and loss of heterozygosity (25%) [17–19].

Immunohistochemical labeling for the p16 and SMAD4 proteins has been shown to mirror these genes' status [16, 20]. This provides a simple method to estimate these genes' status in resected tissues. We thus chose this indirect immunohistochemical method because it was shown that immunohistochemistry for p16 and SMAD4 is a surrogate marker for these genes' status [16, 20]. However, we did not directly analyze genetic and epigen-

Table 6. Univariate and multivariate analyses of overall survival of the PDAC patients

	Univariate analysis <i>p</i> value	Multivariate analysis		
		hazard ratio	95% confidence interval	<i>p</i> value
Sex				
Male/female	0.0943			
Age				
≥65 years/<65 years	0.8801			
Location				
Head/body and tail	0.5011			
Family history				
Present	0.6732			
Symptoms				
Present	0.3707			
Smoking history				
Yes	0.0287*	1.253	0.579–2.689	0.563
Drinking				
Heavy/non- or occasional	0.0003*	3.655	1.674–7.943	0.0012*
Heavy/moderate	0.0105*	2.956	1.114–8.881	0.0287*
Moderate/non- or occasional	0.9752			
BMI				
≥25/<25	0.873			
ABO blood group				
The others/O	0.140			
Pancreatitis history				
Present	0.0568			
Diabetes mellitus				
Present	0.5466			
Histological grade				
G3/G1	0.0086*	1.201	0.468–3.083	0.700
G2/G1	0.2356			
Lymphatic permeation				
Positive	0.0005*	1.563	0.630–4.347	0.345
Vessel invasion				
Positive	<0.0001*	2.063	0.937–4.844	0.072
Perineural invasion				
Positive	0.9053	0.583	0.218–1.782	0.325
UICC pT category				
T3 + 4	0.2151	0.483	0.105–2.678	0.380
UICC pN category				
N1	0.0004*	3.261	1.451–7.873	0.003*
UICC pM category				
M1	0.0661	0.000	0–3.827	0.286
PanIN group				
Low	0.0002*	2.096	1.085–4.104	0.027*
p53				
High	0.1339			
p16				
Loss	0.845			
SMAD4				
Loss	0.0461*	1.466	0.780–2.832	0.236
* Statistically significant.				

etic alterations of *p16* and *SMAD4* genes in the present study, and we could not validate our immunohistochemical data. Further genetic and epigenetic analyses may be necessary in the future.

In studies of ovarian serous and colorectal carcinomas, the carcinomas without a precursor lesion had worse prognoses and usually the *KRAS* wild type [33, 34]. It has been proposed that these carcinomas may arise via a *de novo* pathway rather than an adenoma-carcinoma sequence [33, 34]. In the present study, there was a high rate of *KRAS* wild type in the PanIN-low group (Table 5), and thus the PanIN-low group was more likely to include tumors that develop via a distinct pathway other than the PanIN-carcinoma sequence. Hassid et al. [32] noted that the subset of tumors lacking PanIN may represent a biologically distinct group arising independently from the usual PanIN route in an as yet undefined pathway. Some cases of the PanIN-low group in our study may be included in such a biologically distinct group.

Vascular and lymphatic invasions, which are well known as poor prognostic factors for pancreatic cancer [9, 29], were more frequent in the PanIN-low group compared to the PanIN-high group (Table 2). This may be 1 reason for the poor prognosis of the PanIN-low group.

Our multivariate survival analysis showed that being in the PanIN-low group was an independent prognostic factor (Table 6). The carcinogenic pathway of the PanIN-low and PanIN-high groups may correspond to “*de novo*” and “adenoma-carcinoma” sequences, respectively.

In conclusion, the molecular characteristics in some PDACs without high-grade PanIN may be different from those of the PDACs with high-grade PanIN. There is a possibility that some PDACs without high-grade PanIN may develop via a pathway other than the PanIN-carcinoma sequence.

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Disclosure Statement

The authors declare that there are no conflicts of interest to disclose.

References

- 1 Siegel R, Naishadham D, Jemal A: Cancer statistics, 2013. *CA Cancer J Clin* 2013;63:11–30.
- 2 Wakao F, Nishimoto H, Katanoda K, et al (eds): 5-Year Relative Rate (7 Registries), Both Sexes. Cancer Statistics in Japan 2014. Tokyo, Foundation for Promotion of Cancer Research, 2015, p 80.
- 3 Marmot M, Atinmo T, Byers T, et al (eds): Cancers. World Cancer Research Fund/American Institute for Cancer Research. Food, Nutrition, Physical Activity, and the Prevention of Cancer: A Global Perspective. Washington, American Institute for Cancer Research, 2007, pp 271–274.
- 4 Iodice S, Gandini S, Maisonneuve P, et al: Tobacco and the risk of pancreatic cancer: a review and meta-analysis. *Langenbecks Arch Surg* 2008;393:535–545.
- 5 Tramacere I, Scotti L, Jenab M, et al: Alcohol drinking and pancreatic cancer risk: a meta-analysis of the dose-risk relation. *Int J Cancer* 2010;126:1474–1486.
- 6 Lowenfels AB, Maisonneuve P, DiMaggio EP, et al: Hereditary pancreatitis and the risk of pancreatic cancer. International Hereditary Pancreatitis Study Group. *J Natl Cancer Inst* 1997;89:442–446.
- 7 Amundadottir L, Kraft P, Stolzenberg-Solomon RZ, et al: Genome-wide association study identifies variants in the ABO locus associated with susceptibility to pancreatic cancer. *Nat Genet* 2009;41:986–990.
- 8 Chari ST, Leibson CL, Rabe KG, et al: Probability of pancreatic cancer following diabetes: a population-based study. *Gastroenterology* 2005;129:504–511.
- 9 Hruban RH, Boffetta P, Hiraoka N, et al: Ductal adenocarcinoma of the pancreas; in Bosman FT, Carneiro F, Hruban RH, Theise ND, et al (eds): WHO Classification of Tumours of the Digestive System, ed 4. Lyon, IARC, 2010, pp 281–291.
- 10 Hruban RH, Takaori K, Klimstra DS, et al: An illustrated consensus on the classification of pancreatic intraepithelial neoplasia and intraductal papillary mucinous neoplasms. *Am J Surg Pathol* 2004;28:977–987.
- 11 Hruban RH, van Mansfeld AD, Offerhaus GJ, et al: K-ras oncogene activation in adenocarcinoma of the human pancreas. A study of 82 carcinomas using a combination of mutant-enriched polymerase chain reaction analysis and allele-specific oligonucleotide hybridization. *Am J Pathol* 1993;143:545–554.
- 12 DiGiuseppe JA, Hruban RH, Goodman SN, et al: Overexpression of p53 protein in adenocarcinoma of the pancreas. *Am J Clin Pathol* 1994;101:684–688.
- 13 Boschman CR, Stryker S, Reddy JK, et al: Expression of p53 protein in precursor lesions and adenocarcinoma of human pancreas. *Am J Pathol* 1994;145:1291–1295.
- 14 Caldas C, Hahn SA, da Costa LT, et al: Frequent somatic mutations and homozygous deletions of the p16 (MTS1) gene in pancreatic adenocarcinoma. *Nat Genet* 1994;8:27–32.
- 15 Schutte M, Hruban RH, Geradts J, et al: Abrogation of the Rb/p16 tumor-suppressive pathway in virtually all pancreatic carcinomas. *Cancer Res* 1997;57:3126–3130.
- 16 Geradts J, Hruban RH, Schutte M, et al: Immunohistochemical p16INK4a analysis of archival tumors with deletion, hypermethylation, or mutation of the CDKN2/MTS1 gene. A comparison of four commercial antibodies. *Appl Immunohistochem Mol Morphol* 2000;8:71–79.
- 17 Hahn SA, Schutte M, Hoque ATMS, Moskaluk CA, et al: DPC4, a candidate tumor suppressor gene at human chromosome18q21.1. *Science* 1996;271:350–353.

- 18 Hahn SA, Hoque ATMS, Moskaluk CA, et al: Homozygous deletion map at 18q21.1 in pancreatic cancer. *Cancer Res* 1996;56:490–494.
- 19 Schutte M, Hruban RH, Hedrick L, et al: DPC4 gene in various tumor types. *Cancer Res* 1996;56:2527–2530.
- 20 Wilentz RE, Su GH, Dai JL, et al: Immunohistochemical labeling for dpc4 mirrors genetic status in pancreatic adenocarcinomas: a new marker of DPC4 inactivation. *Am J Pathol* 2000;156:37–43.
- 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 Biankin AV, Waddell N, Kassahn KS, et al: Pancreatic cancer genomes reveal aberrations in axon guidance pathway genes. *Nature* 2012;491:399–405.
- 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 Hruban RH, Wilentz RE, Kern SE: Genetic progression in the pancreatic ducts. *Am J Pathol* 2000;156:1821–1825.
- 25 Yanagisawa A, Ohtake K, Ohashi K, et al: Frequent c-Ki-ras oncogene activation in mucous cell hyperplasias of pancreas suffering from chronic inflammation. *Cancer Res* 1993;53:953–956.
- 26 Wilentz RE, Geradts J, Maynard R, et al: Inactivation of the p16 (INK4A) tumor-suppressor gene in pancreatic duct lesions: loss of intranuclear expression. *Cancer Res* 1998;58:4740–4744.
- 27 Wilentz RE, Iacobuzio-Donahue CA, Argani P, et al: Loss of expression of Dpc4 in pancreatic intraepithelial neoplasia: evidence that DPC4 inactivation occurs late in neoplastic progression. *Cancer Res* 2000;60:2002–2006.
- 28 Oda Y, Aishima S, Morimatsu K, et al: Pancreatic intraepithelial neoplasia in the background of invasive ductal carcinoma of the pancreas as a prognostic factor. *Histopathology* 2014;65:389–397.
- 29 Oshima M, Okano K, Muraki S, et al: Immunohistochemically detected expression of 3 major genes (CDKN2A/p16, TP53, and SMAD4/DPC4) strongly predicts survival in patients with resectable pancreatic cancer. *Ann Surg* 2013;258:336–346.
- 30 Baas IO, Mulder JW, Offerhaus GJ, et al: An evaluation of six antibodies for immunohistochemistry of mutant p53 gene product in archival colorectal neoplasms. *J Pathol* 1994;172:5–12.
- 31 Melhem MF, Law JC, el-Ashmawy L, et al: Assessment of sensitivity and specificity of immunohistochemical staining of p53 in lung and head and neck cancers. *Am J Pathol* 1995;146:1170–1177.
- 32 Hassid BG, Lucas AL, Salomao M, et al: Absence of pancreatic intraepithelial neoplasia predicts poor survival after resection of pancreatic cancer. *Pancreas* 2014;43:1073–1077.
- 33 Vang R, Ie Shih M, Kurman RJ: Ovarian low-grade and high-grade serous carcinoma: pathogenesis, clinicopathologic and molecular biologic features, and diagnostic problems. *Adv Anat Pathol* 2009;16:267–282.
- 34 George SM, Mäkinen MJ, Jernvall P, et al: Classification of advanced colorectal carcinomas by tumor edge morphology: evidence for different pathogenesis and significance of polypoid and nonpolypoid tumors. *Cancer* 2000;89:1901–1909.