Prognostic significance of p16 protein in pancreatic ductal adenocarcinoma

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Abstract. The p16 gene, which is also known as *CDKN2A, INK4A, or CDK4I*, and its products that are known to be cell cycle inhibitors and tumor suppressors have been reported to be altered in various human tumor types. Altered p16 has been indicated to be correlated with negative p16 expression using immunohistochemistry (IHC). However, its association with the prognosis remains controversial because the findings of previous studies are inconsistent. The current study evaluated the relationship between the expression levels of p16 and the clinicopathological features associated with prognosis in patients with primary pancreatic ductal adenocarcinomas (PDACs). From January 2013 to December 2017, tissues of 103 PDAC patients who had undergone elective pancreatic resection were obtained and assessed for p16 expression by IHC. No correlation was observed between p16 status and clinicopathological factors (P>0.05). Notably, negative p16 expression on IHC was not significantly associated with poor prognosis using the Kaplan-Meier method.

Introduction

The pancreas consists of the pancreatic duct, acinar tissue, Langerhans islets, and mesenchymal cells. Tumors derived from the pancreatic duct epithelium account for the majority of pancreatic tumors, known as pancreatic duct adenocarcinoma (PDAC). Pancreatic cancer is extremely lethal with poor prognosis and no established survival markers. Its 5-year survival rate is only 6% and remains below 25% even after curative surgery, thereby making PDAC one of the most lethal tumors (1).

The most frequently detected gene mutation in PDAC is KRAS, which is found in more than 90% of cases. Other frequently mutated tumor suppressor genes include p16/CDKN2A, TP53/p53, and SMAD4/DPC4 (2). Inactivation of KRAS, TP53, p16, and SMAD4 is the most common genetic alteration in human PDAC (3). Next-generation sequencing has described these genetic mutations as the 'big four' genes involved in pancreatic cancer (2). p16 is an important tumor suppressor gene that has been found to affect the cell cycle (G1 to S) by inactivating cyclin-dependent kinase inhibitors (4). Inactivation of p16 is induced by mutation, homozygous deletion, and promoter methylation (1,5). Mutations in p16 are found in at least 30-50% of pancreatic cancer cases (3,6). Several reports have suggested that inactivation of p16 is significantly associated with its protein expression by immunohistochemistry (IHC) (7,8). Recent studies have also reported an association between p16 inactivation and poor prognosis (5,8). However, the correlation between p16 expression on IHC and prognosis remains controversial.

Therefore, we performed immunohistochemical staining of samples from 103 PDAC patients to assess the relationship between p16 expression and clinicopathological features, including prognosis. In some cases, we quantified p16 mRNA expression through RNA sequencing to investigate the correlation between p16 inactivation and its expression on IHC.

Materials and methods

Patients and tissue samples. From January 2013 to December 2017, 103 patients underwent elective pancreatic resection at the Division of Hepato-Biliary-Pancreatic Surgery, Chiba Cancer Center (Chiba, Japan), with a final histopathologic diagnosis of PDAC and no neoadjuvant therapy. Patients with intraductal papillary mucinous neoplasms (IPMNs) with minimal invasive component were excluded. TNM and grading were in accordance with the World Health Organization (WHO) recommendations Union Internationale Contre le Cancer (UICC) 8th edition. Freshly removed pancreatic tissue samples were

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immediately fixed in formalin for at least 12 h and embedded in paraffin. Each resected specimen was stained with hematoxylin and eosin (H&E) and subsequently, microscopically diagnosed by at least two pathologists. The present study was approved by the ethics committee of the Chiba Cancer Center, Japan. All methods were performed in accordance with the relevant guidelines and regulations.

p16 immunohistochemistry. We measured p16 levels by IHC using anti-human $p16^{INK4a}$ mouse monoclonal antibody (E6H4; Roche). Five-μm-thick sections were obtained from formalin-fixed, paraffin-embedded tissues and set aside for CINtec p16 Histology [E6H4] with a VENTANA Optiview DAB universal kit and a VENTANA BenchMark ULTRA automated slide stainer (Roche). Heat-induced antigen retrieval was carried out using Cell Conditioning 1 (CC1; Ventana Medical Systems) for 24 min at 95˚C, and the primary antibody was applied to the sample for 4 min.

The IHC results were scored based on the percentage positivity of staining. p16 protein expression was evaluated by two pathologists at a percentage of every 5% of the staining area of all tumor cells. For statistical comparisons, cases in which p16-positive cells exceeded 10% of the total tumor cells were considered positive. In normal pancreas, p16 positivity was observed in the islets of Langerhans with scattered non-specific cytoplasmic positivity in the ductal and acinar cells (Fig. 1B), and this was determined to be the positive control. The findings obtained for the normal pancreas were compared with those obtained for tumor cells. An 80% agreement between pathologists in the immunostaining evaluation was set as the criterion. When the pathologists disagreed with regard to the evaluations, a decision was reached based on consultation.

RNA sequencing (RNA-seq). Total RNA was isolated from frozen tissue blocks containing approximately 50-100 mg PDAC tissues following the manufacturer's instructions. The frozen tissues from our hospital's biobank were ground using liquid nitrogen and homogenized. RNA was extracted using the miRNeasy Mini kit (QIAGEN), and the quality, quantity, and integrity of the total RNA were evaluated using a NanoDrop One/One^c UV-Vis Spectrophotometer (Thermo Fisher Scientific) and Bioanalyzer 2100 (Agilent Technologies). Samples with an RNA quality score (RIN value) of >7.0 were used for RNA-seq. rRNA was excluded from the total RNA using RiboMinus™ Eukaryote System v2. mRNA was barcoded with Ion Xpress™ RNA-Seq Barcode 1-16 kit (Thermo Fisher Scientific), and the library was generated using Ion Total RNA-Seq kit v2 (Thermo Fisher Scientific). The libraries were constructed for next-generation sequencing (NGS) using an Ion Proton™ instrument (Thermo Fisher Scientific) with 2x75-base pair (bp) paired-end protocol. In total, 8 libraries were sequenced, generating 34-60 million pairs of reads per sample. The quantity of the sequencing data was analyzed by a bioinformatician using BAM files from NGS. The number of reads mapping to the annotated genomic features was quantified from BAM files using feature count from the Subread package (http://subread.sourceforge.net/).

Statistical variables and analyses. Age was divided into two groups with 70 as the median: ≦70 and 70<. Lymph nodes, margin status, cytology, lymphatic invasion, neural invasion, vascular invasion, differentiation, and TNM staging (UICC 8th edition) were defined based on the pathological search results. Lymph nodes were positive for lymph node metastasis or negative for lymph node metastasis. The margin status was R2, R1, and R0 for gross stump positive, histopathological stump positive, and histopathological stump negative, respectively. Cytology was defined as CY1 when cancer cells were found by peritoneal washing cytology; otherwise, it was defined as CY0. Lymphatic invasion, neural invasion, and vascular invasion were each divided into four stages: ly0, ly1, ly2, ly3; ne0, ne1, ne2, ne3; and v0, v1, v2, v3, respectively. Ly0, ne0, and v0 were defined as without lymphatic invasion, neural invasion, and vascular invasion, respectively. Vascular invasion was divided into two groups: V0, v1 and v2, v3, because there was only one patient of v0. Pancreatic cancer tissue was classified according to the degree of differentiation: Well, moderate, and poor. Here, differentiation was divided into two groups for convenience: Well/moderate and poor. Overall survival was defined as the period between the surgery and final observation (in months). For samples extracted from an infinite population, we assumed a sample ratio of 0.5 for activated p16 and 1 for inactivated p16 with 95% confidence and 5% error. The required sample size was 385, but the actual sample size might be small. For the statistical analyses, Mann-Whitney U test and chi-square test were performed. A survival curve was prepared using the Kaplan-Meier method, and log-rank test assessed significant differences. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Clinical pathological background of patients. PDAC tissues were obtained from 103 patients (59 males, 44 females), who had surgeries for pancreatic cancer, and diagnosed with PDAC by pathologists. Age ranged from 50-87 with 70 as the median. We divided PDAC patients into two groups in terms of median age. There were 50 patients older than 70 years and 53 patients younger than 70 years. There were 45 patients with well-differentiated tumors that were mainly of the tissue type, 50 with moderately differentiated tumors, and 8 with poorly differentiated tumors. Lymphatic invasion, neural invasion, and vascular invasion were each scored in 4 grades with 0 as negative. Twenty-seven PDAC patients were negative for lymphatic invasion, whereas only six patients were negative for neural invasion, and only one was negative for vascular invasion. The number of patients with weak vascular invasion (v1) was 10. There were 86 patients with negative pathological margins and 91 with negative cytology. According to the TNM classification of UICC 8th edition, there were 12 patients with stage IA disease, 14 with stage IB, 5 with stage IIA, 36 with stage IIB, and 36 with stage III (Table I).

Expression of p16 protein in PDACs. The loss of p16 protein expression was noted in 55 out of 103 (53.4%) tumors as determined by IHC (Fig. 1). We observed 7 out of 55 ductal adenocarcinomas with weak staining (≤10%) for p16 by IHC. The 55 weakly to negatively stained tumors were grouped together in the negative p16 expression group. Meanwhile, 48 out of 103 (46.6%) tumors were stained positively with

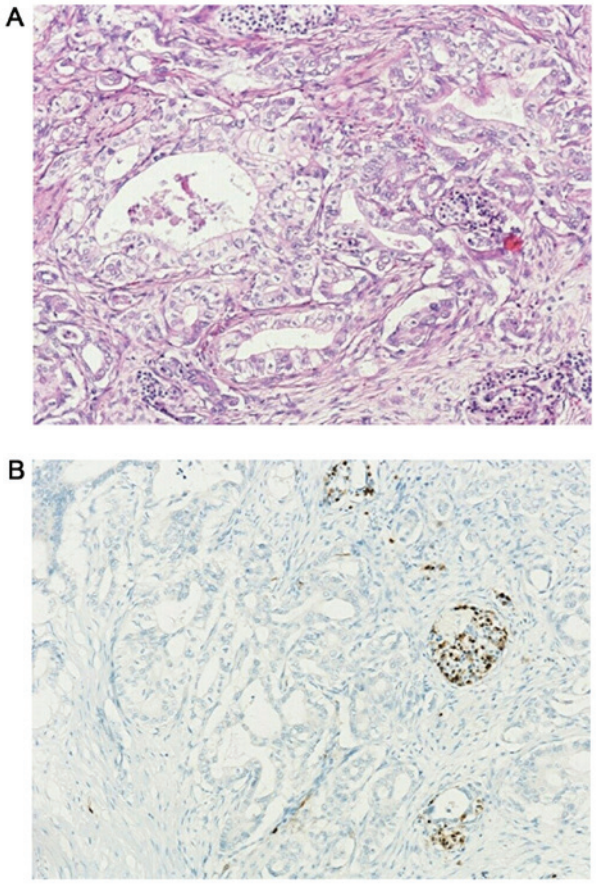

Figure 1. (A) Hematoxylin and eosin staining of PDAC with loss of p16 immunolabeling. (B) An example of PDAC with loss of p16 immunolabeling. Magnification, x400. PDAC, pancreatic ductal adenocarcinomas.

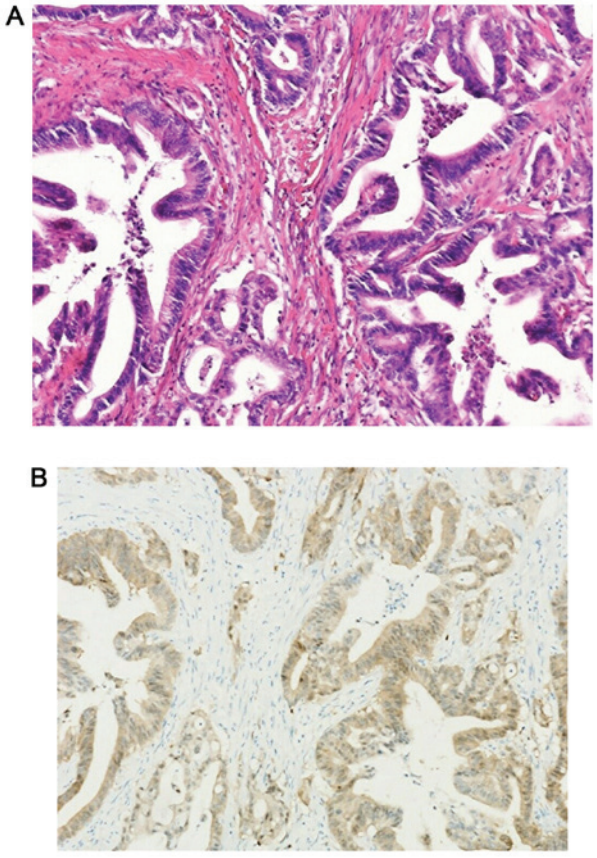

Figure 2. (A) Hematoxylin and eosin staining of PDAC with positive diffuse immunolabeling for p16. (B) An example of PDAC with positive diffuse immunolabeling for p16. Magnification, x400. PDAC, pancreatic ductal adenocarcinomas.

strong to moderate staining (>10%) for p16 by IHC (Fig. 2) and were included in the positive p16 expression group (Table I). Overall, 46.6% of the positive patients were considered positive as a result of exceeding 10% of the total tumor cells as described in Table I.

Clinicopathological outcomes. No correlation was found between p16 status and sex, age, TNM stage, or histological differentiation (Mann-Whitney U test, chi-square test, Fisher's exact test; P>0.05), as shown in Table I. The survival curves for sex, age, histological differentiation, pathological margin status, cytology, lymphatic invasion, neural invasion, vascular invasion, and TNM grade were plotted using the Kaplan-Meier method and analyzed using the log-rank test. Four factors were found to be significantly associated with prognosis (Table II): Lymph node metastasis (P<0.001), cytology (P=0.006), neural invasion (P=0.009), and T factor (UICC 8th; P=0.005). Therefore, p16-negative status on IHC was not significantly associated with poor prognosis according to the Kaplan-Meier method (P=0.181), as shown in Fig. 3. The multivariate Cox proportional regression analysis was not performed because p16 was not significantly different in the univariate analysis.

Correlation between protein expression on IHC and mRNA expression using RNA-seq of p16. Of the 103 patients, 8 were registered in our biobank, and we analyzed mRNA expression by RNA-seq of samples from these 8 patients. The relationship between protein expression level of p16 on IHC and mRNA expression level of p16 (CDKN2A) using RNA-seq was confirmed. The protein expression level of p16 on IHC was observed in percentage by every 5%. Five of the eight cases did not express the p16 protein on IHC, whereas three had mRNA expression levels below 0.5. Three of the eight cases showed p16 protein expression level over 10%, and these same cases had mRNA expression levels of over 3.5. Here, Spearman's rank correlation coefficient test showed a correlation between protein expression on IHC and mRNA expression using RNA-seq (P=0.021) as shown in Table III.

Discussion

Analyses using genetically modified mice revealed that the initiation of pancreatic tumorigenesis required KRAS gene mutation, and tumorigenesis is accelerated by the presence of p16 or TP53 mutation (9). The p16 gene product belongs to an important group of proteins that negatively regulates the G1 phase of the cell cycle. It binds to cyclin-dependent kinases, (CDK)4 and CDK6, and inhibits their interaction with cyclin D1. The inhibition of the cyclin CDK4/6 complex prevents the phosphorylation of retinoblastoma (Rb) protein and the release of E2F, subsequently leading to the inhibition of the transition from G1 to S phase in the cell cycle (4). Therefore, dysfunction in p16 induces Rb protein phosphorylation, and the cell cycle shifts from the G1 to the S phase, resulting in the synthesis of

Table I. Clinicopathological background, outcome and comparison between positive and negative p16 groups.

Variable	Number (%)	Positive p16 expression group	Negative p16 expression group	P-value
Number (%)	103	48 (46.6)	55 (53.4)	
Sex				0.11[a]
Male	59 (57.3)	23 (23.3)	36 (35.0)	
Female	44 (42.7)	25 (24.3)	19 (18.4)	
Median age	70	71.5	69	0.33[b]
Age range	50-87	50-83	51-87	
Lymph nodes				1.00[a]
Negative	31 (30.1)	14 (13.6)	17 (16.5)	
Positive	72 (69.9)	34 (33.0)	38 (36.9)	
Margin status				0.06[a]
R0	86 (83.5)	44 (42.7)	42 (40.8)	
R1/R2	17 (16.5)	4 (3.9)	13 (12.6)	
Cytology				0.18[a]
CY0	91 (88.3)	40 (38.8)	51 (49.5)	
CY1	12 (11.7)	8 (7.8)	4 (3.9)	
Lymphatic invasion				0.37[a]
Negative	17 (16.5)	15 (14.6)	12 (11.7)	
Positive	76 (73.8)	33 (32.0)	43 (41.7)	
Neural invasion				0.41[a]
Negative	6 (5.8)	4 (3.9)	2 (1.9)	
Positive	97 (94.2)	44 (42.7)	53 (51.5)	
Vascular invasion				1.00[a]
Negative (v 0/1)	21 (20.4)	10 (9.7)	11 (10.7)	
Positive (v 2/3)	82 (79.6)	38 (36.9)	44 (42.7)	
Differentiation				0.72[a]
Well/Moderate	95 (92.2)	45 (43.7)	50 (48.5)	
Poor	8 (7.8)	3 (2.9)	5 (4.9)	
T factor (UICC 8th)				1.00[a]
T1/2	79 (76.7)	37 (35.9)	42 (40.8)	
T3	24 (21.4)	11 (10.7)	13 (12.6)	
Stage (UICC 8th)				0.83[c]
IA	12 (11.7)	4 (3.9)	8 (7.8)	
IB	14 (13.6)	7 (6.8)	7 (6.8)	
IIA	5 (4.9)	3 (2.9)	2 (1.9)	
IIB	36 (35.0)	18 (17.5)	18 (17.5)	
III	36 (35.0)	16 (15.5)	20 (19.4)	

[a]Chi-square test, [b]Mann-Whitney U test, [c]Fisher's exact test. UICC, Union Internationale Contre le Cancer; T1, tumor maximum diameter ≦2 cm; T2, tumor maximum diameter ≦24 cm; T3, 4 cm <tumor maximum diameter; R0, microscopic surgical margin negative; R1, gross surgical margin negative and microscopic surgical margin positive; R2, gross and microscopic surgical margin; CY0, Peritoneal washing cytology negative; CY1, Peritoneal washing cytology positive.

deoxyribonucleic acid (DNA). Consequently, genetic abnormalities induce the inactivation of the p16 gene and provide a growth advantage to cells involved in tumorigenesis (10).

The inactivation of the p16 gene occurs through intragenic mutations with loss of heterozygosity (40%), homozygous deletion (40%), and methylation-associated transcriptional silencing (15%) (5) and has been reported in approximately 95% of PDAC cases (11-13). An examination of 25 PDAC cases showed that p16 was inactivated or mutated in 80% of tumors (6). Another examination with the same sample size showed that the inactivation of p16 was significantly associated with a negative p16 expression on IHC (7). Ohtsubo *et al* found that p16 inactivation tended to be more detected in patients with immunohistochemically negative p16 expression than in those with positive expression, after the examination of 60 pancreatic carcinoma cases (8).

Table II. Univariate analysis of prognostic factors for overall survival.

Variable	No. of patients (%)	Median (95% confidence interval)	P-value
Sex			0.270
Male	59 (57.3)	27.1 (20.2-32-2)	
Female	44 (42.7)	42.5 (23.4-N/A)	
Age			0.518
≤70	53 (51.5)	30.2 (21.6-40.8)	
>70	50 (48.5)	24.9 (19.7-43.5)	
Range	53-87		
Follow-up (M)			
Median	19.1		
Range	2-61.2		
Lymph nodes			<0.001
Negative	31 (30.0)	47.5 (29.9-N/A)	
Positive	72 (70.0)	24.5 (18.4-29.7)	
Margin status			0.812
R0	86 (83.5)	28.8 (24.0-40.8)	
R1-R2	17 (16.5)	24.5 (14.9-N/A)	
Cytology			0.006
CY0	91 (88.3)	38.5 (24.1-40.8)	
CY1	12 (11.7)	15.1 (8.15-N/A)	
Lymphatic invasion			0.151
Negative	27 (26.2)	41.4 (24.5-N/A)	
Positive	76 (73.8)	27.1 (20.2-32.2)	
Neural invasion			0.009
Negative	6 (5.8)	N/A	
Positive	97 (94.2)	26.7 (21.6-32.2)	
Vascular invasion			0.081
Negative (v0/1)	21 (20.4)	N/A	
Positive (v2/3)	82 (79.6)	27.1 (23.4-32.2)	
Differentiation			0.975
Well/Moderate	95 (92.2)	28.8 (24.0-37.0)	
Poor	8 (7.8)	11.6 (1.91-N/A)	
T factor (UICC 8th)			0.005
T1/2	79 (76.7)	31.4 (24.5-43.5)	
T3	24 (23.3)	19.1 (11.9-28.4)	
Stage (UICC 8th)			0.13
IA	12 (11.7)	39.3	
IB	14 (13.6)	22.1	
IIA	5 (4.9)	22.3	
IIB	36 (35.0)	18.2 (20.2-39.0 (I,II))	
III	36 (35.0)	17.5 (13.2-28.4 (III))	
p16 IHC (Median: 10%)			0.18
Positive (>10%)	48 (46.6)	26.7 (23.7-47.5)	
Negative (≦10%)	55 (53.4)	32.6 (20.0-38.5)	
p16 IHC (Average: 32%)			0.08
Positive (>32%)	37 (35.9)	24.5 (18.4-29.9)	
Negative (≦32%)	66 (64.1)	32.2 (24.1-46.8)	

M, month; IHC, immunohistochemistry; UICC, Union Internationale Contre le Cancer; T1, tumor maximum diameter ≦2 cm; T2, tumor maximum diameter ≦4 cm; T3, 4 cm <tumor maximum diameter; R0, microscopic surgical margin negative; R1, gross surgical margin negative and microscopic surgical margin positive; R2, gross and microscopic surgical margin; CY0, Peritoneal washing cytology negative; CY1, Peritoneal washing cytology positive.

Table III. Comparison of p16 expression by IHC and RNA-seq.

Case no.	P16 expression by IHC[a]	P16 expression by RNA-seq	P-value	r[b]
			0.021	0.784
1	0	2.728		
2	0	0.269		
3	0	3.349		
4	90	3.787		
5	0	0.265		
6	0	0.233		
7	40	4.399		
8	40	4.521		

[a]Stain range of p16 protein expression by IHC; [b]Spearman's rank correlation coefficient value; IHC, immunohistochemistry; UICC, Union Internationale Contre le Cancer; T1, tumor maximum diameter ≦2 cm; T2, tumor maximum diamete ≦4 cm; T3, 4 cm <tumor maximum diameter; R0, microscopic surgical margin negative; R1, gross surgical margin negative and microscopic surgical margin positive; R2, gross and microscopic surgical margin; CY0, Peritoneal washing cytology negative; CY1, Peritoneal washing cytology positive.

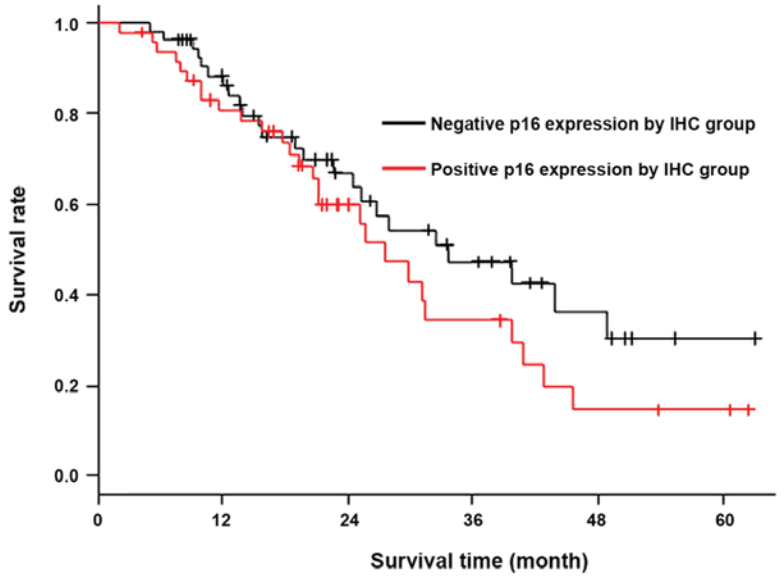

Figure 3. Kaplan-Meier survival analysis of the overall survival in the negative p16 expression groups. IHC, immunohistochemistry.

Although the details remain unclear, p16 inactivation may not be necessary to achieve p16 negative expression on IHC. In this study, we divided the cases into two groups with the median value (10%) of p16 expression range on IHC and evaluated the relationship between the two groups. There were no significant differences in the clinicopathological factors between the groups (Tables II and IV). The results did not change when average values were used. p16 inactivation has been significantly associated with poor prognosis, lymphatic metastasis, and lymphatic invasion (5,8). However, the correlation between p16 expression on IHC and prognosis remains controversial because of inconsistent results. Some studies have reported an association between negative p16 expression on IHC and poor prognosis (14), whereas others have found no significant relationship (15,16). In the present study, a negative p16 expression was not significantly associated with a poor prognosis; in fact, rather than a poor prognosis, negative p16 expression was associated with better prognosis. In other words, positive p16 expression tended to be associated with poor prognosis (Fig. 3).

In other tumors, such as laryngeal squamous carcinoma, positive p16 expression has been significantly associated with poor prognosis (17). Zhao *et al* (18) suggested that positive p16 expression in non-small-cell lung carcinoma was associated with poor outcome. In colon adenocarcinomas, p16 overexpression has been shown to correlate with the clinical features of poorer prognosis, such as sex, distal location, tumor grade, and stage (19). Meanwhile, in breast cancer, p16 overexpression was detected in approximately 20% of tumors and was significantly associated with unfavorable prognostic factors (20).

Among the four gene mutations frequently found in pancreatic cancer, KRAS, TP 53, and SMAD 4 have also been related to prognosis (5,8,14,15). Positive lymph node metastasis and the presence of KRAS mutation have been identified as independent

Table IV. Clinicopathological background, outcome and comparison between positive and negative p16 groups (average).

Variable	Number (%)	Positive p16 expression group	Negative p16 expression group	P-value
Number (%)	103	37 (35.9)	66 (64.1)	
Sex				0.41[a]
Male	59 (57.3)	19 (18.4)	40 (38.8)	
Female	44 (42.7)	18 (17.5)	26 (25.2)	
Median age	70	69	71	0.46[b]
Age range	50-87	50-80	51-87	
Lymph nodes				0.38[a]
Negative	31 (30.1)	9 (8.7)	22 (21.4)	
Positive	72 (69.9)	28 (27.2)	44 (42.7)	
Margin status				0.28[a]
R0	86 (83.5)	33 (32.0)	53 (51.5)	
R1/R2	17 (16.5)	4 (3.9)	13 (12.6)	
Cytology				0.11[a]
CY0	91 (88.3)	30 (29.1)	61 (59.2)	
CY1	12 (11.7)	7 (6.8)	5 (4.9)	
Lymphatic invasion				0.11[a]
Negative	17 (16.5)	11 (10.7)	6 (5.8)	
Positive	76 (73.8)	26 (25.2)	60 (58.2)	
Neural invasion				0.24[a]
Negative	6 (5.8)	4 (3.9)	2 (1.9)	
Positive	97 (94.2)	33 (32.0)	64 (62.1)	
Vascular invasion				0.80[a]
Negative (v 0/1)	21 (20.4)	8 (7.8)	13 (12.6)	
Positive (v 2/3)	82 (79.6)	29 (28.1)	53 (51.5)	
Differentiation				0.71[a]
Well/moderate	95 (92.2)	35 (44.0)	60 (58.2)	
Poor	8 (7.8)	2 (1.9)	6 (5.8)	
T factor (UICC 8th)				0.63[a]
T1/2	79 (76.7)	27 (26.2)	52 (50.5)	
T3	24 (21.4)	10 (9.7)	14 (13.6)	
Stage (UICC 8th)				0.48[c]
IA	12 (11.7)	3 (2.9)	9 (8.7)	
IB	14 (13.6)	3 (2.9)	11 (10.7)	
IIA	5 (4.9)	3 (2.9)	2 (1.9)	
IIB	36 (35.0)	15 (14.6)	21 (20.4)	
III	36 (35.0)	13 (12.6)	23 (22.3)	

[a]Chi-square test, [b]Mann-Whitney U test, [c]Fisher's exact test.

prognostic markers according to a multivariate analysis (5,14). Another multivariate analysis found that the number of driver gene alterations among these four genes remained independently associated with overall survival (21). Consistent with other reports, our findings revealed the significant association between lymph node metastasis and poor prognosis. However, negative p16 expression was not necessarily associated with poor prognosis; instead, it was associated with better prognosis. The examination of p16 in combination with other genes such as KRAS, p53, and SMAD4 may find correlations with prognosis and other clinicopathological factors (14). In addition, we evaluated the inactivation state of p16 through the expression level of p16 mRNA using RNA-seq and found a correlation with protein expression level on IHC in a small number of cases (Table III). However, we could not evaluate the inactivation state of p16 using other factors. If the relationship among the factors causing inactivation of p16, i.e., mutation, homozygous deletion, and promoter methylation, expression level using RNA-seq, and protein expression on IHC can be examined in more samples, more insights into the inactivation of p16 and expression on IHC can be obtained.

Here are some limitations of our methods. We found no significant difference in p16 expression status, and it was not

associated with poor prognosis. This can be due to our small sample size, i.e., 103, as the statistically required size was 385. Moreover, confounding factors, such as mutated KRAS, might worsen the prognosis, but we have not investigated the relationship between KRAS and prognosis. Unfortunately, we have not evaluated the KRAS protein by immunostaining, which is one of the limitations of our study.

Therefore, to confirm the relationship between the inactivation of p16 and p16 expression on IHC, we evaluated p16 expression using RNA-seq. From a small sample of 8 cases, we presented a correlation between p16 expression on IHC and mRNA expression using RNA-seq (Table III). In pancreatic cancer, inactivation of p16 has been assessed by exon sequencing and has been reported to occur by mutation, homozygous deletion, and promoter methylation of the p16 gene (1,5,22). In this study, the number of samples might again be too small, and therefore, the correlation between the factors causing inactivation of p16 gene, namely mutation, homozygous deletion, and promoter methylation, and mRNA expression could not be confirmed. In this study, we considered the inactivation of p16 as a decrease in mRNA expression level, extracted it from the information of RNA-seq, and obtained a correlation with the range of staining on IHC. However, p16 inactivation did not correlate with clinicopathological data. As a limitation of RNA-seq, we do not use controls because we did not analyze expression fluctuations and did not detect differentially expressed genes, only expression level analysis and normalization of p16 (CDKN2A).

As far as we searched, there were no reports examining p16 inactivation and p16 immunostaining at the same time in pancreatic cancer, and it was considered a novel report.

The inactivation status of p16 was evaluated using mRNA expression level, and it was related to protein expression level on IHC. If the p16 protein expression on IHC was low, p16 would have been inactivated. We defined low p16 protein expression level on IHC (<10%) as negative p16 expression (Table III). The p16 expression status, i.e., positive or negative, on IHC was not significantly associated with clinicopathological factors including Overall survival (Tables I and II).

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

YI and IH analyzed and interpreted the patient data regarding the pancreatic cancer was a major contributor in writing the manuscript. FI, SC, HA, HYa, HN, HYo, WT collected tissue samples and extracted mRNA from pancreatic tissue. MI performed the pathological examination of the pancreatic cancer and interpreted the tissue on IHC. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Chiba Cancer Center Review Board (H29-006). All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation and with the Helsinki Declaration of 1964 and its later amendments. Informed consent was obtained from all patients in this study.

Patient consent for publication

Written informed consent was obtained from the patients for publication of this study and accompanying clinicopathological data.

Competing interests

The authors declare that they have no competing interests.

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