Histol Histopathol (2023) 38: 1415-1427

Histology and Histopathology
From Cell Biology to Tissue Engineering

ORIGINAL ARTICLE Open Access

The combination of the low immunohistochemical expression of peroxiredoxin 4 and perilipin 2 predicts longer survival in pancreatic ductal adenocarcinoma with peroxiredoxin 4 possibly playing a main role

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Summary. Pancreatic ductal adenocarcinoma (PDAC) is a fatal disease with poor prognosis. Therefore, indicators that can be used for the early prediction of the prognosis of PDAC are needed. Peroxiredoxin (PRDX) 4 is a secretion-type antioxidant enzyme located in the cytoplasmic endoplasmic reticulum. Recent studies have reported that it is closely related to the development and prognosis of many types of cancer. Perilipin (PLIN) 2 is a lipid droplet coating protein. The high expression of PLIN2 is known to be an indicator of some types of cancer and oxidative stress management. It is highly suggestive of the interplay between PRDX4 and PLIN2 to some degree. In this study, we collected 101 patients' clinical data and paraffin-embedded specimens with PDAC and analyzed them with immunohistochemical staining of PRDX4 and PLIN2. We found that the low expression of PRDX4 predicts longer survival and a better clinical condition in PDAC patients. Moreover, when the low expression of PRDX4 is combined with the low expression of PLIN2, the 3-year survival is significantly improved. Univariate and multivariate Cox proportional hazard analyses showed that the PRDX4 expression in PDAC was an independent prognostic factor for survival. Taken together, between PRDX4 and PLIN2, PRDX4 plays a main role in prognosis and has the potential to become a clinical prognostic indicator of PDAC.

Key words: PRDX4, PLIN2, PDAC, Prognosis indicator

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a fatal disease; the 5-year survival rate is one of the lowest among all cancers (Siegel et al., 2022). Although the incidence of PDAC is low (8-12 cases per 100000 population every year, with a lifetime risk of 1.3%), it will become the second largest cause of cancer-related death in the world (Troumpoukis et al., 2022). Currently, the treatment of PDAC depends on conventional multi-

Abbreviations. PRDX, Peroxiredoxin; PLIN, Perilipin; PDAC, pancreatic ductal adenocarcinoma; H_2O_2, hydrogen peroxide; ER, endoplasmic reticulum; LD, lipid droplet; DSS, Disease-specific survival; CT, computed tomography; MRI, magnetic resonance imaging; IHC, immunohistochemistry; H&E, Hematoxylin and eosin; DAB, 3,3′-diaminobenzidine; Low-Low, low-PRDX4 with low-PLIN2; High-High, high-PRDX4 with high-PLIN2; Low-High, low-PRDX4 with high-PLIN2; High-Low, high-PRDX4 with low-PLIN2

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www.hh.um.es. DOI: 10.14670/HH-18-666

chemotherapy, but the effect is poor (Hermann and Sainz, 2018). Few patients benefit from molecular targeted treatment (Peschke et al., 2022). As a result of this, the short-term survival and prognosis of PDAC are poor (Sung et al., 2021). In view of the poor prognosis, indicators that can be used for the early postoperative prediction and identification of the severity of PDAC are needed.

The peroxiredoxin (PRDX) family is a ubiquitous and potentially medicinal antioxidant protein family composed of dimer units that metabolize hydrogen peroxide (H_2O_2) (Fujii et al., 2015). PRDXs are believed to be able to remove >90% of cell peroxides (Perkins et al., 2015). Among the PRDX family, PRDX4 is known to have an extracellular exocrine function. PRDX4 contains a hydrophobic signal sequence at the N-terminal, which is responsible for its location, whether it resides in the ER or is secreted into the extracellular space (Giguere et al., 2007). Some reports (including our own reports) suggest that PRDX4 may become a biomarker of many diseases (e.g., type 2 diabetes, sepsis, atherosclerosis and stroke) (Schulte, 2011; Yamada and Guo, 2018). However, what attracted us more is the role of PRDX4 in cancer, especially PDAC, because according to the database, the expression of PRDX4 in the pancreas is much higher than that in other tissues (Ding et al., 2010; Jia et al., 2019; Jain et al., 2021). Since the recent discovery that PRDX4 is overexpressed in a variety of human cancers, the relationship between this antioxidant protein and tumorigenesis has attracted considerable attention (Karihtala et al., 2011; Yi et al., 2014; Hwang et al., 2015; Mishra et al., 2015; Rafiei et al., 2015; Huang et al., 2017). Other reports have found that overexpression of PRDX4 was associated with poor prognosis in many kinds of cancer. Although the molecular mechanisms vary among different types of cancer, overexpression of PRDX4 may indicate the proliferation, migration, and invasiveness of cancer cells (Park et al., 2020; Gao et al., 2021). However, its role in PDAC is not fully understood. Moreover, because PRDX4 has the extracellular secretion function, we believe that it has the potential to become a prognostic indicator.

At the same time, perilipin (PLIN) 2 has also attracted our attention. PLIN2 is a lipid-coated protein (Cao et al., 2018). The PLIN family plays an important role in the formation and stability of LDs (Sztalryda and Brasaemleb., 2017). Among the PLIN family, PLIN2 is closely related to LDs and is considered to be an LD marker (Leitner et al., 2022). PLIN2 is upregulated in many types of cancer and its overexpression in cancer is considered an indicator of LD metabolism in cancer cells (Luo et al., 2022). LD metabolism disorder is an important metabolic change in cancer (Long et al., 2018) and is related to carcinogenic signal pathway activation. This activity is conducive to the survival and proliferation of tumor cells under adverse conditions (Fu et al., 2021). The PLIN2 expression in different tumor cells confers susceptibility to treatment-induced cell death (Rios Garcia et al., 2022). Therefore, overexpression of PLIN2 often indicates proliferation of cancer cells and the poor efficacy of anti-cancer drugs. However, its role in PDAC has not been clearly demonstrated. We believe that PLIN2 has the potential to become an indicator of prognosis and survival for PDAC.

Cancer cells are characterized by aggressive proliferation. This requires cells to develop strategies to obtain nutrients in the microenvironment, which lacks oxygen and a general nutrient supply (Baghban et al., 2020). Accordingly, we assume that PRDX4 with an antioxidant function may have some relationship with PLIN2, that is, the interplay between PRDX4 and PLIN2. In this way, PRDX4 and PLIN2 may both be used as indicators for the prognosis of PDAC.

We collected the data of PDAC patients from multiple centers in Japan, and statistically analyzed 101 patients' postoperative samples with immunostaining of PRDX4 and PLIN2 antibodies.

Materials and methods

Patients and specimens

Surgical specimens of PDAC patients (n=101) collected from multiple centers (Kanazawa Medical University Hospital, Independent Administrative Agency National Hospital Organization Fukuyama Medical Center and Kagoshima University Hospital) in Japan were used in the present study. The Ethics Committee of Kanazawa Medical University approved this study (NO.I233). These samples are all collected from PDAC patients who underwent surgery in the above medical institutions in 2002-2017 with pathological reports and follow-up data. The following exclusion criteria were applied: (1) perioperative death (defined as death during the initial hospitalization or within 30 days of surgery); (2) other concomitant malignant tumors; (3) coexisting medical problems of sufficient severity to shorten life expectancy; (4) adjuvant chemotherapy or radiotherapy prior to surgery.

All resected samples were formalin-fixed and paraffin-embedded and the histopathological features were examined by three pathologists (A.S., M.K. and S.Y.). For staging, the tumor node metastasis (TNM) system of the Union for International Cancer Control (UICC) 8th Edition was used. PDACs were graded based on a three-tiered histopathological scoring system from The World Health Organization (WHO), as mentioned previously (Kimura et al., 2015). No biopsy specimen was obtained from the PDAC before surgery. Disease-specific survival (DSS) was defined as the interval from the date of surgery to death (except patients who died from causes other than PDAC) or the most recent clinic visit, respectively. Patients were followed and prospectively evaluated every month within the first postoperative year, then at approximately 2-4 months intervals using chest X-ray, thoracic and

abdominal computed tomography (CT), brain magnetic resonance imaging (MRI), serum biochemistry, or measurements of tumor markers. CT, MRI, and bone scintigraphy were performed every six months for three years after surgery. Additional examinations were performed if symptoms or signs of recurrence were recognized.

Histopathology and immunohistochemistry (IHC)

Paraffin-embedded sections (thickness: 3 μm) were used for histopathology and IHC staining. Hematoxylin and eosin (H&E) staining was firstly performed to determine the pathological pattern. The representative section showing the most characteristics of PDAC components was selected for IHC staining of PRDX4 and PLIN2.

PRDX4 IHC staining was performed using a PRDX4 rabbit polyclonal antibody (Invitrogen, PA5-85252). The procedure was as follows: (1) deparaffinization and rehydration; (2) 0.5% H_2O_2 blocking for 10 minutes at room temperature; (3) 5% bovine serum albumin blocking for 30 minutes at room temperature; (4) primary antibody incubation overnight at 4°C (dilution: 1:500); (5) secondary antibody (Histofine Simple Stain MAX-PO424152) staining for 30 minutes at room temperature; (6) 3,3′-diaminobenzidine (DAB, Histofine Simple Stain SAB-PO425011) imaging and hematoxylin counterstaining. For IHC staining of PRDX4, we used pancreatic islet cells of human PRDX4 transgenic mice as a positive control and PRDX4 knock out mouse pancreatic islet cells were used as negative control (Ding et al., 2010). Also, the normal pancreatic acinar cells were used as internal quality control.

The whole PLIN2 and CD31 IHC staining process was performed using a Leica Bond-Max automatic dyeing machine (Leica, Buffalo Grove, USA) and Bond Polymer Refine Detection kit. After deparaffinization and rehydration, tissue sections went through a heat-induced (121°C) epitope retrieval process in Bond Epitope Retrieval Solution 1 (Citrate-based pH 6.0 epitope retrieval buffer) for 20 minutes. A PLIN2 IgG mouse monoclonal antibody (PROGEN, 690102; dilution, 1:100) and CD31 IgG mouse antibody (DAKO, DAKO-CD31, JC/70A; dilution, 1:200) were used as primary antibodies. Signals were visualized using a Dako REAL EnVision detection system and Peroxidase/DAB+ (Dako Cytomation), according to the manufacturer's instructions. For IHC staining of PLIN2 and CD31, we used human liver tissue as a positive control. Also, the foamy macrophages were used as internal quality control for PLIN2 and the normal vascular epithelial cells were used as internal quality control for CD31.

All H&E and IHC staining images were captured and quantitatively analyzed using NanoZoomer Digital Pathology Virtual Slide Viewer (Hamamatsu Photonics Corp., Hamamatsu, Japan). Meanwhile, preparation sections were observed under light microscopes.

Evaluation of IHC

The IHC expression was evaluated semi-quantitatively by the ratio of IHC expression-positive cells to total PDAC cancer cells. Under the assistance of a statistics expert (M.S.), we set the cut-off values based on the median IHC expression index at 12% for PRDX4 and 1% for PLIN2.

All histological and IHC slides were evaluated by two independent certified surgical pathologists in our university (A.S. and M.K.) who were blinded to the clinicopathological data. The agreement between two pathologists was very good (>85%), as measured by the interclass correlation coefficient. For cases with disagreement (<15%), an additional evaluation was performed by a third board-certified pathologist (S.Y.) in our department.

Statistical analysis

Survival was analyzed by the Kaplan-Meier method with a log-rank test using GraphPad Prism 8 (version 8.3.0). For the evaluation of clinicopathological features, categorical data were analyzed by Fisher's exact test. Hazard ratios and 95% confidence intervals (95%CIs) were determined using univariate and multivariate Cox proportional hazard models. With the exception of the survival analysis, the statistical analyses were performed with EZR (Saitama Medical Center, Jichi Medical University, Japan), a graphical user interface for R (R console version 4.0.3 [2020-10-10]). All statistical tests were two-sided. P values of <0.05 were considered statistically significant. All statistical analysis methods were carried out under the assistance of a statistics expert (M.S.).

Results

Patient characteristics

The clinicopathological characteristics of the 101 patients are shown in Table 1. The patients were 44-83 years of age (average, 69 years; median, 69 years). Most patients (n=85; 84.2%) were over 60 years of age. There was no sex difference (male, n=50; female, n=51). The postoperative survival period ranged from 2 months to 140 months (average, 40 months; median, 29 months). The pathological classifications of the 101 cases were well-differentiated (n=53; 52.5%); moderately differentiated (n=34; 33.6%) and poorly differentiated (n=14; 13.9%). 67 (66.3%) patients' tumors occurred in the head of the pancreas, while 34 (33.7%) patients occurred in body or/and tail of the pancreas. Vascular invasion and lymphatic vessel invasion occurred in most (n=91; 90.1%) patients. Meanwhile, most patients (n=92; 91.1%) had perineural invasion. More than half of the patients (n=64; 63.4%) were accompanied by

lymph node metastasis. Only 8 (7.9%) patients had distant metastasis. The clinical stages were as follows: stage 1 (n=31), stage 2 (n=37), stage 3 (n=25) and stage 4 (n=8); stage 2 was most common, accounting for 36.6% of cases.

Table 1. The clinicopathological characteristics of the patients.

Characteristic	Patients (n=101)
Age	
Average	69
Median	69
Range	44-83
>60	85
≤60	16
Gender	
Female	51
Male	50
Month after operation	
Average	40
Median	29
Range	2-140
Ductal adenocarcinoma	
Well differentiated	53
Moderately differentiated	34
Poorly differentiated	14
Location	
Head	67
Body&Tail	34
Tumor size	
Average	30
Median	28
Range	7-110
>20	69
≤20	32
Lymphatic vessel invasion	
+	91
-	10
Vascular invasion	
+	91
-	10
Perineural invasion	
+	92
-	9
pT	
T1	26
T2	46
T3	21
T4	8
pN	
N0	37
N1	39
N2	25
pM	
M0	93
M1	8
Stage	
I	31
II	37
III	25
IV	8

PRDX4 and PLIN2 expression in PDAC specimens

Low cytoplasmic expression of PRDX4 was observed in islet cells of the pre-existing pancreas. Cytoplasmic expression of PLIN2 was observed in histiocytes, including foamy macrophages (data not shown). Meanwhile, a cytoplasmic staining pattern of PRDX4 and/or PLIN2 was observed in a substantial number of PDAC cases (Fig. 1A). In this study, we used the median values as the cut-off values for the expression of PRDX4 (12%) and PLIN2 (1%). According to these cut-off values, we divided the patients into low-PRDX4 and high-PRDX4 groups as well as low-PLIN2 and high-PLIN2 groups. There was no significant correlation between the PRDX4 and PLIN2 expression levels (Table 2).

Association of PRDX4 expression with clinicopathological variables

In comparison to the high-PRDX4 group, the low-PRDX4 group had a significantly lower pN index, which showed that the low-PRDX4 group had significantly less lymph node metastasis. Although the difference was not statistically significant, the low-PRDX4 group tended to have less vascular and lymph-vessel invasion and perineural invasion (Table 3A). In the Kaplan-Meier analysis, the low-PRDX4 group obviously had significantly better three-year DSS (Fig. 2A).

Association of the PLIN2 expression with clinicopathological variables

Similar to PRDX4, in comparison to the high-PLIN2 group, the low-PLIN2 group tended to have less vascular and lymph-vessel invasion and perineural invasion, and tended to have less metastasis (Table 3B). Furthermore, the three-year DSS of the PLIN2-group tended to be better although the difference was not statistically significant (Fig. 2B).

Association of the combined PRDX4 and PLIN2 expression with clinicopathological variables

We further divided the patients into low-PRDX4 with low-PLIN2 (Low-Low) group and others, and the high-PRDX4 with high-PLIN2 (High-High) group and others according to the combined expression of PRDX4 and PLIN2. From the perspective of pathological data, vascular and lymph-vessel invasion, perineural invasion,

Table 2. The expression of PRDX4 and PLIN2.

	Low PRDX4	High PRDX4	Total	p Value
Low PLIN2	30	31	61	0.684
High PLIN2	22	18	40	
total	52	49		

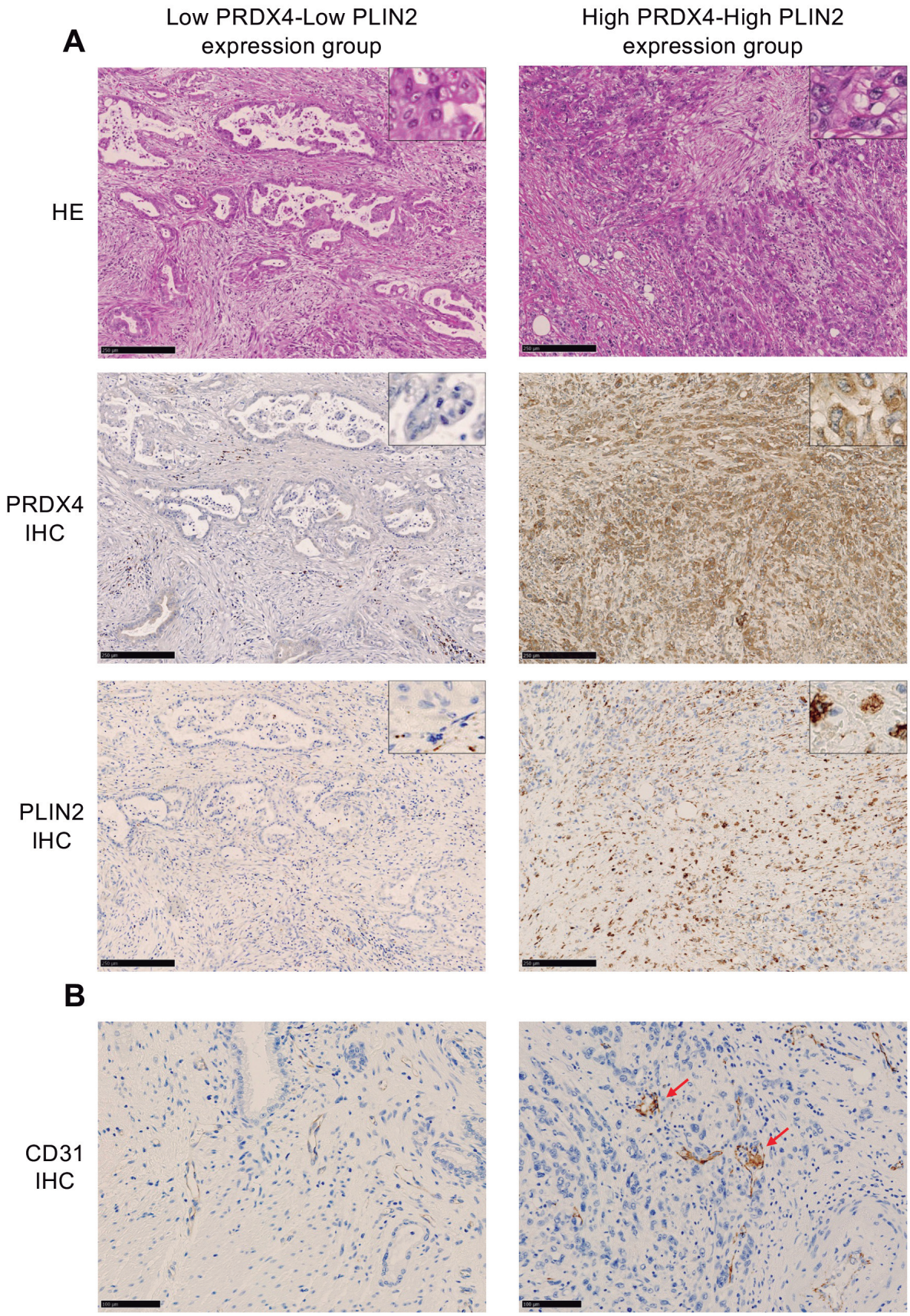

Fig. 1. The representative histological and immunostaining images of the Low-Low group and High-High group. **A.** Representative HE images (left panel) PRDX4 IHC (middle panel) and PLIN2 IHC (right panel). PRDX4, PLIN2 were detected in the cytoplasm. **B.** Representative images of CD31 IHC. Red arrow indicates vascular and lymph-vessel. CD31 was detected in the cytoplasm. Bar: A, 250 μm; B, 100 µm.

lymph node metastasis and the clinical stage of the Low-Low group were significantly lower and better than others group, but those were not significantly different between the High-High group and others group (Table 3C,D). Similarly, from the perspective of three-year DSS, the three-year DSS of the Low-Low group was obviously better than that of the others (Fig. 2C, $p<0.005$). In contrast, the three-year DSS of the High-High group was not significantly different from that of others group (Fig. 2D). Furthermore, in addition to the histological findings, the cancer invasiveness of the Low-Low group was weaker (Fig. 1A). IHC staining of CD31 clearly showed more vascular and lymph-vessel invasion in the High-High specimens (Fig. 1B). Meanwhile, the three-year DSS of the Low-Low group was obviously better than that of the High-High group (Fig. 3D).

Low expression of PRDX4 is an independent prognostic factor for PDAC and combination with the low expression of PLIN2 would further confirm the judgment

In order to further study the relationship between the expression of PRDX4 in combination with PLIN2 and the three-year DSS, we divided the patients into four groups according to the expression of PRDX4 and PLIN2: the Low-Low group, low-PRDX4 with high-PLIN2 (Low-High) group, the high-PRDX4 with low-PLIN2 (High-Low) group, and the High-High group. After comparing the Low-Low group and Low-High

Table 3. Detailed correlations between different PRDX4 and PLIN2 expression levels and clinicopathological variables.

	A. Correlations between low PRDX4 and high PRDX4 expression levels			**B.** Correlations between low PLIN2 and high PLIN2 expression levels		
	Low PRDX4 (n=52)	High PRDX4 (n=49)	p Value	Low PLIN2 (n=61)	High PLIN2 (n=40)	p Value
	Number (%)	Number (%)		Number (%)	Number (%)	
Age						
>60	41 (78.8)	44 (89.8)	0.175	52 (85.2)	33 (82.5)	0.784
≤60	11 (21.2)	5 (10.2)		9 (14.8)	7 (17.5)	
Gender						
Female	31 (59.6)	20 (40.8)	0.0741	31 (50.8)	20 (50)	1
Male	21 (40.4)	29 (59.2)		30 (49.2)	20 (50)	
Ductal adenocarcinoma						
Well differentiated	28 (53.8)	25 (51.0)	0.962	33 (54.1)	20 (50)	0.814
Moderately differentiated	17 (32.7)	17 (34.7)		19 (31.1)	15 (37.5)	
Poorly differentiated	7 (13.5)	7 (14.3)		9 (14.8)	5 (12.5)	
Location						
Head	32 (61.5)	35 (71.4)	0.4	43 (70.5)	24 (60)	0.29
Body&Tail	20 (38.5)	14 (28.6)		18 (29.5)	16 (40)	
Tumor size						
>20	37 (71.2)	32 (65.3)	0.669	41 (67.2)	28 (70)	0.829
≤20	15 (28.8)	17 (34.7)		20 (32.8)	12 (30)	
Lymphatic vessel invasion						
+	44 (84.6)	47 (95.9)	0.0934	52 (85.2)	39 (97.5)	0.084
-	8 (15.4)	2 (4.1)		9 (14.8)	1 (2.5)	
Vascular invasion						
+	44 (84.6)	47 (95.9)	0.0934	52 (85.2)	39 (97.5)	0.084
-	8 (15.4)	2 (4.1)		9 (14.8)	1 (2.5)	
Perineural invasion						
+	45 (86.5)	47 (95.9)	0.162	53 (86.9)	39 (97.5)	0.0834
-	7 (13.5)	2 (4.1)		8 (13.1)	1 (2.5)	
pT						
T1~3	47 (90.4)	46 (93.9)	0.716	58 (95.1)	35 (87.5)	0.259
T4	5 (9.6)	3 (6.1)		3 (4.9)	5 (12.5)	
pN						
N0	20 (38.5)	31 (63.3)	**0.017**	26 (42.6)	11 (27.5)	0.143
N1~2	32 (61.5)	18 (36.7)		35 (57.4)	29 (72.5)	
pM						
M0	46 (88.5)	47 (95.9)	0.271	59 (96.7)	34 (85)	0.0553
M1	6 (11.5)	2 (4.1)		2 (3.3)	6 (15)	
Stage						
0~IIA	20 (38.5)	16 (32.7)	0.678	25 (41.0)	11 (27.5)	0.205
IIB~IV	32 (61.5)	33 (67.3)		36 (59.0)	29 (72.5)	

group, as well as the Low-Low group and the High-Low group, we found that when the expression of PRDX4 was low, the expression of PLIN2 was also significantly associated with three-year DSS (Fig. 3B). Similar differences were observed with the low expression of PLIN2. When the expression of PLIN2 was low, the PRDX4 expression had a more significant association with three-year DSS (Fig .3C). However, there were no significant differences between the other groups (Fig. 4A-C), especially when PRDX4 was highly expressed, and the PLIN2 expression level had almost no effect on three-year DSS (Fig. 3F). We also performed a Cox analysis (Table 4). In the univariate analysis, tumor size, differentiation, vascular and lymph-vessel invasion, TNM index, stage and PRDX4 expression were significant prognostic factors. In the multivariate analysis, differentiation and the expression of PRDX4 were identified as independent prognostic factors.

Discussion

PDAC is difficult to treat and has a poor prognosis. However, its incidence is not high, so it is difficult to collect samples (Oberstein and Olive, 2013). In this study, we collected a large number of surgical samples from PDAC patients who were managed at multiple centers in Japan over a long time period. Our results showed that, among the overall study population, the DSS of the low-PRDX4 group in the early postoperative period (three years) was relatively good, and the incidence of related adverse events (e.g., vascular and lymph-vessel invasion) tended to be decreased. At the

(continued)

	C. Detailed correlations between Low-Low and others			**D.** Detailed correlations between High-High and others		
	Low-Low (n=30) Number (%)	Other (n=71) Number (%)	p Value	High-High (n=18) Number (%)	Other (n=83) Number (%)	p Value
Age						
>60	24 (80)	61 (85.9)	0.552	16 (88.9)	69 (83.1)	0.73
≤60	6 (20)	10 (14.1)		2 (11.1)	14 (16.9)	
Gender						
Female	18 (60)	33 (46.5)	0.277	7 (38.9)	44 (53.0)	0.309
Male	12 (40)	38 (53.5)		11 (61.1)	39 (47.0	
Ductal adenocarcinoma						
Well differentiated	17 (56.7)	36 (50.7)	0.596	9 (50)	44 (53.0)	0.936
Moderately differentiated	8 (26.7)	26 (36.6)		6 (33.3)	28 (33.7)	
Poorly differentiated	5 (16.6)	9 (12.7)		3 (16.7)	11 (13.3)	
Location						
Head	20 (66.7)	47 (66.2)	1	12 (66.7)	55 (66.3)	1
Body&Tail	10 (33.3)	24 (33.8)		6 (33.3)	28 (33.7)	
Tumor size						
>20	19 (63.3)	50 (70.4)	0.492	10 (55.6)	59 (71.1)	0.264
≤20	11 (36.7)	21 (29.6)		8 (44.4)	24 (28.9)	
Lymphatic vessel invasion						
+	22 (73.3)	69 (97.2)	**0.000811**	17 (94.4)	74 (89.2)	0.686
-	8 (26.7)	2 (2.8)		1 (5.6)	9 (10.8)	
Vascular invasion						
+	22 (73.3)	69 (97.2)	**0.000811**	17 (94.4)	74 (89.2)	0.686
-	8 (26.7)	2 (2.8)		1 (5.6)	9 (10.8)	
Perineural invasion						
+	24 (80)	68 (95.8)	**0.0179**	18 (100)	74 (89.2)	0.202
-	6 (20)	3 (4.2)		0 (0)	9 (10.8)	
pT						
T1~3	29 (96.7)	64 (90.1)	0.43	17 (94.4)	76 (91.6)	1
T4	1 (3.3)	7 (9.9)		1 (5.6)	7 (8.4)	
pN						
N0	17 (56.7)	21 (29.6)	**0.0137**	8 (44.4)	30 (36.1)	0.594
N1~2	13 (43.3)	50 (70.4)		10 (55.6)	53 (63.9)	
pM						
M0	30 (100)	63 (88.7)	0.101	18 (100)	75 (90.4)	0.344
M1	0 (0)	8 (11.3)		0 (0)	8 (9.6)	
Stage						
0~IIA	17 (56.7)	19 (26.8)	**0.00617**	8 (44.4)	28 (33.7)	0.424
IIB~IV	13 (43.3)	52 (73.2)		10 (55.6)	55 (66.3)	

same time, similarly, the low-PLIN2 group also tended to have better 3-year survival after surgery and fewer adverse events (Figs. 1, 2, Table 3). Therefore, we performed a further statistical analysis to determine the interaction between the two targets for the early prognosis of PDAC. Although in the present study there was no correlation between the expression of PRDX4 and the expression of PLIN2 (Table 2), which is to say there were not many cases of Low-Low and High-High in all, our analysis showed that the prognosis and three-year DSS of the Low-Low group was much better than that of others group (Fig. 2). Although there have been no reports indicating correlation between PRDX4 and PLIN2, there are many reports on the association between lipid droplets metabolism and oxidative stress in cancer, especially in digestive system malignancies

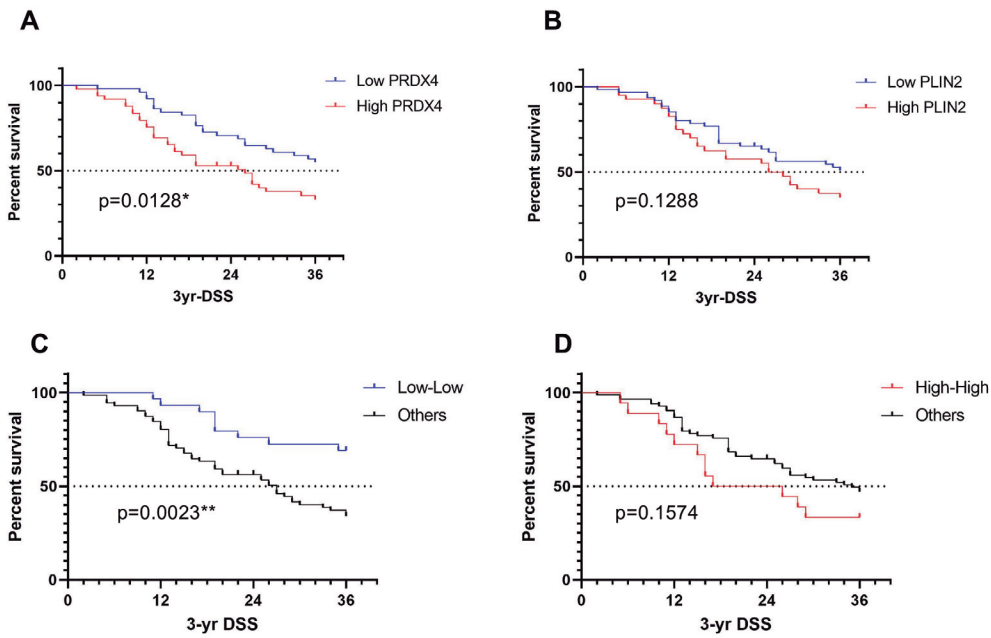

Fig. 2. Kaplan-Meier curves of disease-specific survival (DSS) in patients with PDAC at 3 years after surgery according to PRDX4 **(A)** and PLIN2 **(B)** expression. **C.** Kaplan-Meier curves of DSS at 3 years after surgery in PDAC patients with low expression of PRDX4 and low expression of PLIN2 (Low-Low) and others. **D.** Kaplan-Meier curves of DSS at 3 years after surgery in PDAC patients with high expression of PRDX4 and high expression of PLIN2 (High-High) and others. (*p<0.05. **p<0.005)

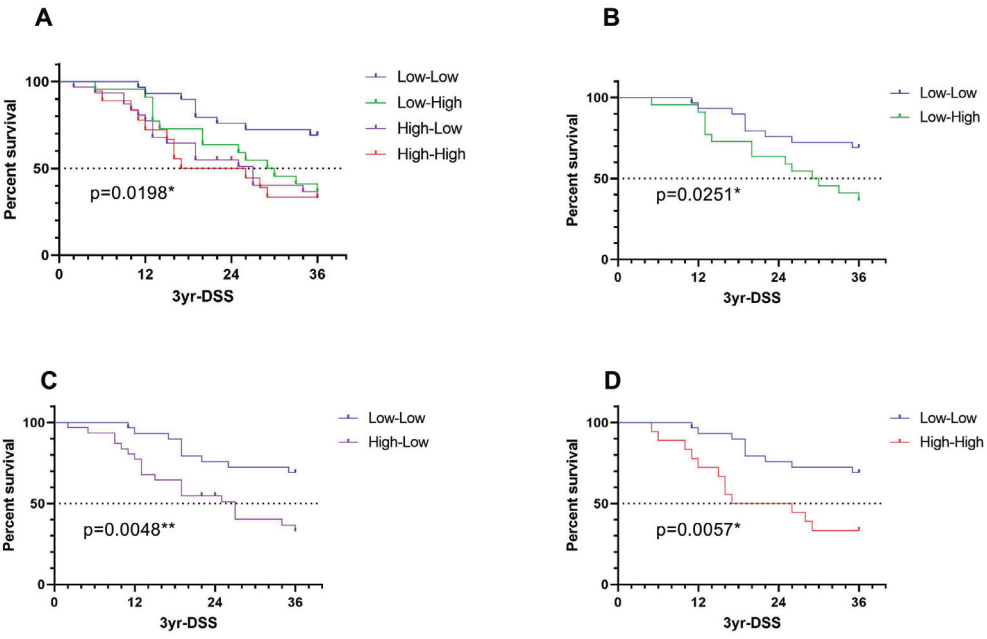

Fig. 3. Kaplan-Meier curves of DSS in patients with PDAC at 3 years after surgery according to the different expression of PRDX4 and PLIN2. **A.** Kaplan-Meier curves of the following 4 groups: Low-Lo); low PRDX4 and high PLIN2 (Low-High); high PRDX4 and low PLIN2 (High-Low); High-High. **B.** Kaplan-Meier curves of the Low-Low and Low-High groups. **C.** Kaplan-Meier curves of the Low-Low and High-Low groups. **D.** Kaplan-Meier curves of the Low-Low and High-High groups (*p<0.05. **p<0.005).

(Li et al., 2020). The low-PLIN2 group had the same tendency in terms of three-year DSS after surgery, however, in the Cox multivariate analysis, the expression of PRDX4 was one of the independent prognostic factors (Table 4).

One complex feature of PDAC is that it involves genetic changes and adapts to increase the antioxidant capacity of cells through the transcriptional upregulation of antioxidant enzymes and metabolic reprogramming, which helps it withstand the increased oxidative load (Hadi et al., 2021). Our research group recently reported that the expression of PRDX4 plays different roles in different cancers. For example, in hepatocellular carcinoma, the incidence of adverse events (e.g., portal vein and hepatic vein invasion) increased in the low-PRDX4 group (Guo et al., 2019). The same conclusion also appears in patients with early-stage of lung adenocarcinoma (Shioya et al., 2018). However, it was a little different in hepatoblastoma, which PRDX4 promoted the migration of hepatoblastoma cells as well as induced hepatoblastoma cells differentiation (Zheng et al., 2020b). In contrast, in mice with chemical-induced lung tumor, the high expression of PRDX4 may actually promote a large number of tumors probably because the extracellular exocrine PRDX4 reduces the oxidative stress in microenvironment to prove a better living environment for tumor cells (Zheng et al., 2020a). The results of this study indicate that the high expression of PRDX4 in cancer cells was related to adverse events (e.g., tumor growth, lymph node metastasis and vascular and lymph-vessel invasion) and shorter three-year DSS in PDAC patients (Table 3). Although the results of the present research are different from those previous reports

Table 4. The univariate and multivariate analyses of survival according to the clinicopathological variables and the expression of PRDX4 and PLIN2.

	Univariate		
	Hazard ratio	95%CI	p Value
Age	0.9715	0.9423-1.002	0.06315
Gender	1.701	0.995-2.909	0.05218
Tumor size	1.029	1.015-1.042	<0.0001
Location	1.592	0.879-2.882	0.1249
Differentiation	1.944	1.343-2.814	
0.0004288			
Lymphatic vessel invasion	8.719	1.205-63.09	0.03199
Vascular invasion	8.719	1.205-63.09	0.03199
Perineural invasion	3.786	0.9214-15.55	0.06483
pT	1.841	1.342-2.526	
0.0001526			
pN	2.419	1.687-3.468	<0.0001
pM	2.103	0.9505-4.655	0.06653
Stage	1.604	1.317-1.952	<0.0001
PRDX4 IHC index	1.937	1.132-3.315	0.0159
PLIN2 IHC index	1.495	0.88-2.54	0.1369
	Multivariate		
	Hazard ratio	95%CI	p Value
Tumor size	1.016	0.99450-1.039	0.1432
Differentiation	1.908	1.29100-2.818	
0.001179			
Lymphatic vessel invasion	5.292	0.67210-41.670	0.1135
Vascular invasion	5.292	0.67210-41.670	0.1135
pT	0.6355	0.20210-1.998	0.4379
pN	1.64	0.64830-4.148	0.2962
Stage	1.36	0.55330-3.345	0.5025
PRDX4 IHC index	1.907	1.06800-3.404	0.02907
PLIN2 IHC index	1.027	0.9785-1.079	0.2775

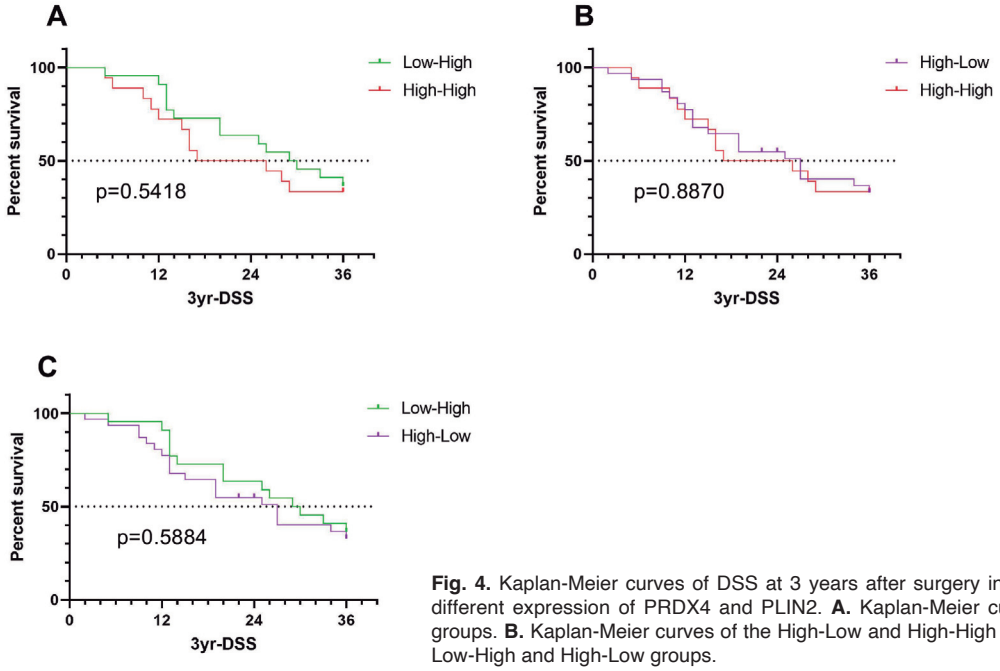

Fig. 4. Kaplan-Meier curves of DSS at 3 years after surgery in patients with PDAC according to the different expression of PRDX4 and PLIN2. **A.** Kaplan-Meier curves of the Low-High and High-High groups. **B.** Kaplan-Meier curves of the High-Low and High-High groups. **C.** Kaplan-Meier curves of the Low-High and High-Low groups.

of our group in other malignant tumors, we still found reports that could support the present results (Jain et al., 2021). Although the report did not study the prognosis of PDAC, it indicated that the growth and survival of pancreatic cancer cells depended on the antioxidant effect of PRDX4. This conclusion also supported the poor prognosis of the high-PRDX4 group in the present research. We hypothesize that the high expression of PRDX4 can upregulate the extracellular exocrine PRDX4 level and reduce the oxidative stress of the microenvironment around cancer cells to make it within a suitable range for proliferation. Meanwhile, the intracellular PRDX4 created an antioxidative cellular environment to ensure the cancer progression. However, we noticed that although there was a significant difference in lymph node metastasis (pN) between the low-PRDX4 group and the high-PRDX4 group, there was no significant difference in lymphatic vessel invasion which is often considered as the previous process of lymph node metastasis (Table 3). We inferred that the reason for this phenomenon was that the symptoms of PDAC were non-specific (Perelló-Reus et al., 2022), so only very few patients undergoing surgery did not have lymphatic vessel invasion (about 10% in the present report) which made it difficult to evaluate the statistical differences.

At the same time, PLIN2 is reported to be related to the development of type 2 diabetes, which can also indicate that it is related to pancreatic diseases (Conte et al., 2016). In this study, although the expression of PLIN2 is not an independent determinant, it can be seen that the overexpression of PLIN2 is positively related to a poor prognosis in PDAC (Fig. 2, Table 4). As a reliable protein marker of LDs, the PLIN2 level can reflect lipid accumulation. Increased intracellular LDs can be observed in many kinds of cancers to maintain better cancer cell survival (Liu et al., 2022). Although, PLIN2 played complex roles in hepatoblastoma according to the studies of our group (Azukisawa et al., 2021), its overexpression still indicated the proliferation of malignant tumor cells. In other types of cancer, such as breast cancer and lung adenocarcinoma, the accumulation of LDs which could be reflected by the expression of PLIN2, may support tumor progression by supplementing unsaturated fatty acids rather than potentially toxic saturated lipids (Vidavsky et al., 2019). Also, in colorectal cancer, the high expression of PLIN2 helps to obtain the lipid storage required for ER homeostasis to make a better environment for cancer cells (Tirinato et al., 2015). In fact, there was one report revealing that the high IHC expression of PLIN2 predicts poor prognosis in PDAC (Hashimoto et al., 2019). Although it was no further molecular mechanism research in this report, its results showed that PLIN2 can be as a decisive prognostic indicator for PDAC, which was different from our results. Definitely, the results of this report also supported our results. According to these reports, we could infer that PLIN2, as a reliable marker of LDs, was related to adequate lipid supplement for cancer cell proliferation, which was related to PDAC prognosis. However, in the present study the expression of PLIN2 had only positive trends for early postoperative survival and it was not an independent factor for survival.

The present study was associated with some limitations, including its retrospective design and use of clinical data. In particular, some samples were obtained long before the time of the study. Although we strictly formulated the exclusion principle, the retrospective data analysis still had time limitations. Secondly, we did not perform double IHC staining for PRDX4 and PLIN2, so we could not compare their expression patterns within the cancer cells. Furthermore, we only performed IHC staining and the statistical analysis of clinical data, without analyzing the underlying molecular mechanisms. This will be investigated in future research.

Despite these limitations, the results of our analysis supported that PRDX4 and PLIN2 were both related with the prognosis of PDAC patients. In addition, because of the extracellular exocrine characteristics of PRDX4, it is more conducive to quantitative measurement, has the potential to be used for targeting, and is a specific prognosis indicator. In conclusion, PRDX4 is an independent potential target that is associated with the postoperative prognosis of PDAC patients. Moreover, the low expression of PRDX4 in combination with the low expression of low PLIN2 was more closely related to a good postoperative prognosis and 3-year DSS in PDAC patients while PRDX4 played the main role in PDAC.

Acknowledgements. We would like to thank Shinichi Kinami from Kanazawa Medical University for the helpful clinical and surgical assistance. We would also like to thank Yoshimi Nakajima and Asari Tanaka for their expert technical assistance.

Informed Consent. Written informed consent was obtained from the patients for the publication of their anonymized information in this article.

Conflict of interest. The authors declare no conflicts of interest in association with the present study.

Funding. This work was supported in part by Grants-in-Aid for Scientific Research 20K07454 to S.Y. and 19K16783 to X.G., Shibuya Science Culture and Sports Foundation in 2021 to T.I.

Author Contributions. J.H., T.I. and S.Y. conceptualized and designed the experiments; J.H., A.S., M.K., H.T., M.O., T.M. M.I., M.H. and X.G. carried out the experiments; J.H., M.S., H.K., X.G. and S.Y. analyzed the data; J.H. and S.Y. wrote the manuscript; X.G., S.Y. and T.I. edited the manuscript.

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Accepted September 27, 2023