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The combination of strong immunohistochemical mtTFA expression and a high survivin index predicts a shorter disease-specific survival in pancreatic ductal adenocarcinoma

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Summary. Mitochondrial transcription factor A (mtTFA) plays a crucial role in both the transcription and maintenance of mitochondrial DNA. A high expression of mtTFA has been demonstrated in several solid tumors, and is closely associated with cancer cell survival/apoptosis and growth. However, its expression pattern in pancreatic ductal adenocarcinoma (PAC) remains to be elucidated. Additionally, our groups have recently revealed that a subset of apoptosis-related genes is strongly regulated by mtTFA, and that two putative mtTFA binding sites are present in the promoter region of the *survivin* gene, which is a member of the inhibitor-of-apoptosis protein family. We therefore investigated the correlation of the immunohistochemical mtTFA expression and the survivin index with various clinicopathological variables and the prognosis, using 70 paraffin-embedded tumor samples from patients with surgically-resected PAC. The mtTFA expression or survivin index was considered to be strong or high when ≥30% or 10% of the PAC cells showed positive staining, respectively. Strong mtTFA expression and/or a high survivin index was revealed to have a significant relationship to a pathologically high tumor grading and advanced tumor stage. Moreover, mtTFA showed significantly high co-expression with survivin. Univariate and multivariate analyses demonstrated that both the strong mtTFA expression and high survivin index groups had significantly shorter survival rates, especially within the first two years postoperatively. The combination of strong mtTFA expression and a high survivin index may predict a poor prognosis in patients with PAC, and these new biomarkers might offer useful information for the early clinical management.

Key words: Pancreatic ductal adenocarcinoma (PDA), mtTFA, Advanced stage, Survivin, Prognosis

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Introduction

Pancreatic cancer is one of the most lethal malignancies in Japan; approximately 30,000 new cases are diagnosed each year, and more than 28,000 patients die of the disease each year in Japan alone (http://ganjoho.jp/professional/index.html, 2013). In addition, it is responsible for approximately 227,000 deaths per year worldwide. Pancreatic ductal adenocarcinoma (PAC) is the most common histopathological type of pancreatic cancer (Parkin et al., 2005). Various clinicopathological factors, such as the tumor size and vascular permeation in operable PACs, as well as the patient's initial Eastern Cooperative Oncology Group performance status (ECOG PS) and

distant metastases in cases with inoperable PACs, have been proposed as prognostic indicators, even though the results of studies evaluating these factors have been inconsistent and inconclusive to date (Li et al., 2011).

The five-year overall survival rate for patients diagnosed with pancreatic cancer is less than 4%. Additionally, long-term survival is very rare even after surgical resection, which is thought to provide the only chance of a cure, with overall five-year survival rates after surgery ranging from 10% to 25% (Clearly et al., 2004; Katz et al., 2008), since more than 80% of postoperative relapses (local or distant) occur within the first two years after surgery (Stathis and Moore, 2010). For these reasons, it is critical to predict which PAC patients are prone to develop recurrence and will have a high mortality rate after surgery, however accurate biomarkers are still lacking for this purpose.

The mitochondria are the major sites of reactive oxygen species (ROS) production in eukaryotic cells, and the accumulation of ROS causes oxidative damage, especially to the mitochondrial DNA (mtDNA) due to its lack of nucleosome structures, resulting in cell apoptosis not only due to aging, but also of cancer cells (Dröge, 2002). In addition, it is well known that mitochondrial uncoupling mediates a metabolic shift to aerobic glycolysis for energy generation in cancer cells, i.e., the Warburg effect (Samudio et al., 2009; Baffy, 2010). Thus, mitochondria are essential not only for cancer cell metabolism, but also for cancer survival/apoptosis and progression/remission (Samudio et al., 2009). Mammalian mitochondrial transcription factor A (TFAM; also known as mtTFA), which is a member of the high mobility group (HMG)-box protein family, strongly regulates the transcription of mitochondrial genes by binding to the mitochondrial D-loop region (Parisi and Clayton, 1991; Torigoe et al., 2005; Kohno et al., 2005). It has previously been reported that mtTFA plays a pivotal role in mtDNA maintenance and repair, as well as in mitochondrial gene expression (Larsson et al., 1998). Moreover, accelerated apoptotic activity has been recognized in *mtTFA*-knockout mice, which show an embryonic lethal phenotype (Wang et al., 2001; Wallace and Fan, 2009). Based on these features, mtTFA should be closely involved in the evasion of apoptosis, which is one of the hallmarks of cancer growth, as well as the aggressiveness and progression of tumors, and thus, we hypothesized that the mtTFA expression levels would be increased in cancer cells, in close association with cell proliferation. In fact, data obtained by our groups have demonstrated that mtTFA is specifically expressed in several solid tumors, and that high immunohistochemical expression of mtTFA is significantly correlated with a poor prognosis in endometrial and colorectal adenocarcinomas (Kidani et al., 2009; Toki et al., 2010; Yoshida et al., 2011; Nakayama et al., 2012).

Furthermore, our recent DNA microarray analyses have revealed that a subset of apoptosis-related genes are strongly regulated by mtTFA, and that two putative mtTFA binding sites are present in the promoter region of the survivin gene (encoded by baculoviral inhibitor of apoptosis repeat-containing 5 [BIRC5]), which is a member of the inhibitor-of-apoptosis protein family (Jiang et al., 2004; Mita et al., 2008; Han et al., 2011). However, to the best of our knowledge, there have been no previous reports of any possible associations between the mtTFA expression and the survivin index in PAC and the clinicopathological features of the disease, such as the tumor stage or patients' prognoses, even though another group has recently revealed that the immunohistochemical expression of mtTFA is an independent prognostic marker for patients with postoperative PAC (Yamauchi et al., 2014). In the present study, we show for the first time that the combination of strong mtTFA expression and a high survivin index is significantly correlated with a poor outcome, and that these findings might be promising biomarkers in patients with postoperative PAC.

Materials and methods

Patients

All the intended procedures in the present study, including the use of specimens from human subjects in UOEH in Kitakyushu, Japan, were approved especially by written consent of next of kin for research use of the materials obtained, according to the guidelines of the Japan Society of Pathology. Pathological reports were reviewed to identify patients who underwent pancreatico-duodenectomy or distal pancreatectomy for PAC between 1994 and 2010 at the hospital of the UOEH. Two patients who suffered perioperative deaths, defined as death during the patient's initial hospitalization or within 30 days of surgery, were excluded. A total of 70 patients with available follow-up data comprised the cohort of this retrospective study, after further excluding those with the following characteristics: (a) other prior or concomitant malignant tumors; (b) coexisting medical problems of sufficient severity to shorten life expectancy and (c) treatment with adjuvant chemotherapies or radiotherapies prior to the surgery.

Tissue specimens

Three pathologists examined all resected specimens to confirm their histopathological features. The tumor node metastasis (TNM) system of the Union for International Cancer Control (UICC), 7th Edition, was used for staging (Sobin et al., 2009). All PACs were graded based on the three-tiered histological grading system from The World Health Organization (WHO) classification of tumors of the pancreas (Bosman et al., 2010); and a grade of G2 or higher was considered to indicate a high-grade tumor. Clinical information was gathered from the patients' records, and no patients had a biopsy specimen obtained from the PAC before surgery.

The duration of survival was the time from the date of surgery until the patient's death or most recent clinic visit. Patients were followed-up and evaluated every month within the first postoperative year, and at approximately two to four month intervals thereafter, using chest X-rays, thoracic and abdominal CT scans and/or measurements of tumor marker levels. CT was performed every six months for three years after surgery. Additional examinations, including brain CT, MRI and bone scintigraphy, were performed if any symptoms or signs of recurrence were recognized. The formalin-fixed, paraffin-embedded tissue blocks came from our Department of Pathology. Each patient was assigned an ECOG PS score at the time of diagnosis. Normal human tissue was taken from non-tumor portions of the surgically-resected specimens, and then stained with haematoxylin and eosin (H&E), elastica van Gieson (EVG) or were subjected to immunohistochemical analyses of sequential sections. The EVG and immunohistochemical Podoplanin (D2-40; Nichirei Bioscience Co., Tokyo, Japan; diluted 1:1) and S-100 protein (Dako, Glostrup, Denmark; diluted 1:900) staining clearly revealed whether there was vascular invasion (VI), lymphatic permeation (LI) and perineural involvement (PNI), respectively.

Preparation of an antibody against mtTFA

A polyclonal antibody was raised against human mtTFA by multiple immunizations of New Zealand white rabbits with a synthetic peptide, based on the previously published work (synthetic peptide sequence: KRTIKKQRKYGAEEC) (Yoshida et al., 2003). The specificity of our original antibody was confirmed by a Western blot analysis and immunohistochemistry with peptide competition (Yoshida et al., 2003; Toki et al., 2010). For the immunohistochemical staining of mtTFA, we used human cancer cells of well to moderately differentiated adenocarcinoma of the colon as positive controls (Nakayama et al., 2012).

Immunohistochemistry of tissue samples

Immunohistochemical staining was performed by the antibody-linked dextran polymer method for antibody-bridge labelling, with hematoxylin counterstaining (EnVision; DAKO, Glostrup, Denmark). Deparaffinized and rehydrated 4-μm sections were incubated in 10% H_2O_2 for 5 min to block the endogenous peroxidase activity. The sections were thereafter rinsed and incubated with either rabbit polyclonal anti-mtTFA (diluted 1:400) or anti-survivin (Santa Cruz Biotechnology, Santa Cruz, CA, USA; diluted 1:50) antibodies for 30 min. The secondary antibody peroxidase-linked polymers were then applied, and the sections were incubated with a solution consisting of 20 mg of 3.3-diaminobenzidine tetrahydrochloride, 65 mg of sodium azide and 20 ml of 30% H_2O_2 in 100 ml of Tris-HCL (50 mM, pH7.6). After counterstaining with Meyer's hematoxylin, the sections were observed under a light microscope. The sections were first scanned at low power for all the fields (original magnification: × 40) for tumor and non-tumor tissues, respectively, to account for the heterogeneity of distribution. The number of cells showing positive cytoplasmic (mtTFA) or nuclear (survivin) staining and the pattern of staining were recorded. Necrotic tissues, stromal cells and lymphoid cells were not included in the recording (Li et al., 2011; Wu et al., 2012; Kitada et al., 2013; Kawatsu et al., 2014).

The immunoreactivity for mtTFA in each case was assessed semi-quantitatively by evaluating the proportion of positive cells compared to the total PAC cells. Positive areas comprising less than 10% of the neoplasms were considered to be negatively stained. To assess the cytoplasmic mtTFA expression, positive areas that were equal to or more than 10% of the total were considered to be positively stained, and were graded based on four categories: weak, positive (area of 10-29%); strong (30-79%) and very strong (more than 80% positive area). We selected and validated the immunohistochemical cut-off scores for mtTFA positivity (30%), based on the performance of a receiver operating characteristic (ROC) curve analysis (Hanley, 1989). Finally, all patients were divided into two groups based on the mtTFA expression as follows: strong, when the staining was ≥30% or weak, when the staining was <30%.

The distribution of the staining for survivin in the PAC and the adjacent non-neoplastic ductal epithelium in each case was also assessed semi-quantitatively and compared. The survivin staining was also divided into categories according to the percentage of the cells stained (0-9%, 10-29%, 30-79% and 80-100%). The sections stained for survivin were then counted at high power (original: × 400) magnification. At least 1,000 nuclei were counted in each section. The survivin index was calculated as the number of positive nuclei per 1,000 nuclei counted. In addition to the survivin index, we selected and validated immunohistochemical cut-off scores for survivin positivity (10%), based on the performance of an ROC curve analysis (Hanley, 1989). All patients were divided into two groups, as follows: high when there was positive staining in 10% or more of the nuclei and low when 9% or fewer of the cell nuclei were positively stained.

All histological and immunohistochemical slides were evaluated by two independent observers (certified surgical pathologists in our department; Shohei Kitada and Sohsuke Yamada) using a blind protocol design (the observers were blinded to the clinicopathological data). The agreement between the observers was excellent (more than 90% agreement rate) for all antibodies investigated as measured by the interclass correlation coefficient. For the few (less than 1%) instances of disagreements, a consensus score was determined by a

third board-certified pathologists (Yasuyuki Sasaguri) in our department (Li et al., 2011; Wu et al., 2012; Kitada et al., 2013; Kawatsu et al., 2014).

Statistical analysis

The significance of correlations was determined by the χ^2 test to assess the relationships between the immunohistochemical expression levels and the clinicopathological variables (Li et al., 2011; Kitada et al., 2013; Kawatsu et al., 2014). Survival curves were plotted with the Kaplan-Meier method and compared with the log-rank test. Hazard ratios and 95% confidence intervals (95% CI) were estimated using univariate or multivariate Cox proportional hazard models. All statistical tests were two-tailed, with values of $P<0.05$ considered to be significant. All of the above statistical analyses were performed with the EZR (Saitama Medical Center, Jichi Medical University, Japan) graphical user interface for the R software program (The R Foundation for Statistical Computing, version 2.13.0) (Kanda, 2013; Kitada et al., 2013; Kawatsu et al., 2014). More precisely, it is a modified version of R commander (version 1.6-3) designed to add the statistical functions that are frequently used in biostatistics.

Results

Patient characteristics

The cohort included 70 patients (40 males, 30 females) with clinicopathological variables representative of PAC (Table 1). The average age at surgery was 66 years. All patients (70/70; 100%) were ECOG 0 patients. The median tumor size was 3.5 cm (range: 1.0-7.5 cm). At diagnosis, 44 (62.9%) patients had lymph node metastases and three (4.3%) had distant metastases, the latter of which were managed palliatively. The tumor grading included 21 well differentiated (G1; 30.0%), 40 moderately differentiated (G2; 57.1%) and nine poorly differentiated adenocarcinomas (G3; 12.9%). Based on the UICC criteria, the majority of the patients (44/70; 62.9%) had stage II disease. Postoperative follow-up data was available for all 70 patients (average: 23.5 months; range: 2-200 months). The median disease-specific postoperative survival (DSS) was 14.1 months with one- and five-year survival rates of 58% and 6%, respectively. Table 2 displays each patient's information in detail.

mtTFA expression in normal pancreatic tissues and PAC specimens

The specificity of the mtTFA polyclonal antibodies was tested by immunohistochemical and Western blotting analyses (Toki et al., 2010; Yoshida et al., 2003). mtTFA showed only cytoplasmic immunohistochemical expression (Fig. 1). mtTFA expression was not detectable in the adjacent normal ductal epithelium on paraffin-embedded tissues (Fig. 1). mtTFA was weakly and strongly expressed in 43 (61.4%) and 27 (38.6%) of 70 PAC specimens, respectively (in total: 18 negative (25.7%), 25 weak (35.7%), 24 strong (34.3%) and three

Table 1. The patients' clinicopathological characteristics.

Characteristic	Patients (n = 70)
Age (years)	
Average	66
Median	67
Range	40-86
≥60 years	53
< 60 years	17
Sex	
Male	40
Female	30
ECOG PS	
0	70
≥1	0
Follow-up (months)	
Average	23.5
Median	14.1
Range	2-200
Tumor size (cm)	
Average	3.7
Median	3.5
Range	1.0-7.5
Tumor stage	
I	7
II	44
III	16
IV	3
Tumor grade	
G1	20
G2	41
G3	9
T-stage	
T1	4
T2	9
T3	41
T4	16
Regional lymph node metastasis	
N0	26
N+	44
Distant metastasis	
M0	67
M+	3
Surgical margin	
Negative	39
Positive	31
LI	
LI(-)	4
LI(+)	66
VI	
VI(-)	18
VI(+)	52
PNI	
PNI(-)	3
PNI(+)	67

very strong (4.3%)).

Association of mtTFA expression with the clinicopathological variables

To identify the association of mtTFA expression (weak vs. strong mtTFA expression) with the clinicopathological characteristics of this cohort, the variables were divided as shown in Table 3. There were no significant differences between patients with weak and strong mtTFA expressions in terms of their age, gender or the tumor location (P>0.05). However, strong mtTFA expression was closely associated with a high (G2 and G3) tumor grade, larger tumor size (>2 cm), lymph node metastases and an advanced disease stage (P=0.04, 0.04, 0.01 and 0.04, respectively) (Table 3, Fig. 1), but not with the surgical margins or presence of LI, VI or PNI (Table 3) (P>0.05) in the overall cohort. In contrast, strong mtTFA expression was evident in the LI, VI and PNI of high-grade PAC components, as shown respectively by the D2-40, EVG and S-100 protein staining (Fig. 2). The majority of the high-grade tumors (23/27) had strong mtTFA expression but only 27/43 (62.8%) of the samples with weak mtTFA expression were high-grade tumors (Table 3). In a Kaplan-Meier analysis (Fig. 3), PAC patients with strong mtTFA expression had a significantly shorter postoperative median DSS (8.3 months) compared with those who had weak mtTFA expression (18.0 months) (P=0.009, Fig. 3A).

Association of the survivin index with the clinicopathological variables

In contrast to mtTFA, survivin showed only nuclear immunohistochemical expression (Fig. 1). There was a significant relationship between the survivin index and the immunohistochemical mtTFA expression pattern (P=0.0001, r=0.46) (Fig. 1, Table 3), with strong mtTFA expression showing a significantly high rate of co-expression with survivin (a high surviving index: ≥10%) (Fig. 1, Table 3). In patients with G2 to G3 (high-grade) PACs, the survivin indices were consistently high (≥10%) (Fig. 1). This was most likely because its staining pattern was significantly more diffuse and the staining was much higher in poorly differentiated

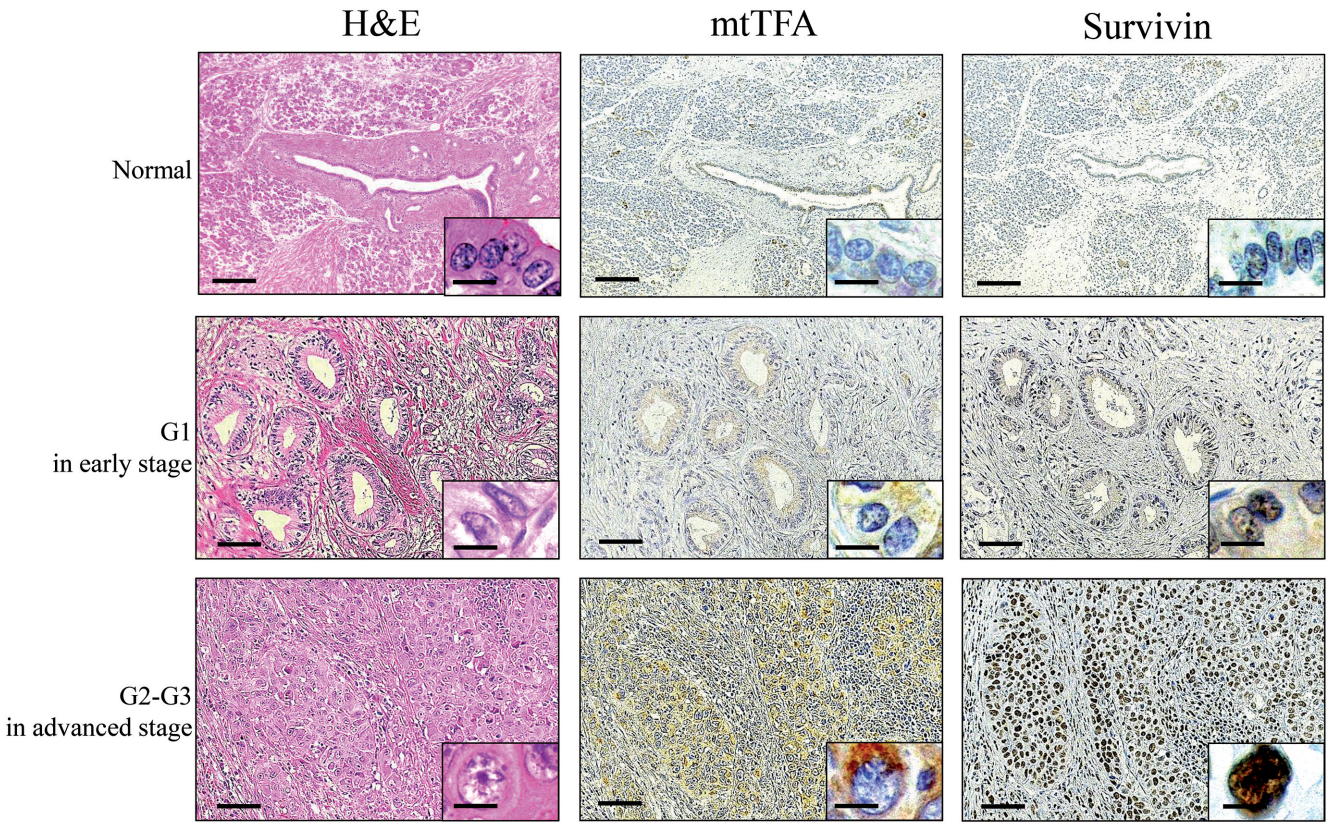

Fig. 1. Representative images of the immunohistochemical staining of both mtTFA and survivin in human PAC (G1 cancer in the early stage, weak mtTFA expression and a low survivin index; Case No. 31) (high-grade (G2 to G3) cancer in an advanced stage: strong mtTFA expression and high a survivin index; Case No. 64), displaying a cytoplasmic (mtTFA) or nuclear (survivin) staining pattern, and normal ductal specimens for comparison (negative; Case No. 31). H&E: hematoxylin and eosin. Bar: main, 100 μm; insets, 20 μm

Table 2. The detailed relationships among the mtTFA expression, the survivin index and each patient's variables

No.	Status	Months after surgery	mtTFA expression	Survivin index	Age (yrs)	Sex	Location	Grade	Size (cm)	T stage	N stage	Stage	Margin
1	dead	3.2	strong	high	80	female	head	G3	5.5	4	+	III	-
2	dead	16.9	weak	low	64	female	body/tail	G1	3.5	2	-	I	-
3	dead	28.7	strong	low	79	female	body/tail	G2	4.4	3	-	II	-
4	dead	29.3	strong	high	67	male	body/tail	G2	1.0	3	-	II	+
5	dead	32.8	weak	low	48	male	head	G1	2.0	3	+	II	+
6	dead	8.4	weak	low	71	female	head	G2	3.0	2	-	I	+
7	dead	15.4	weak	low	56	female	head	G3	5.5	4	+	IV	+
8	dead	8.7	weak	low	69	female	head	G3	2.0	3	-	II	-
9	dead	6.1	strong	high	63	female	head	G1	3.0	4	+	III	-
10	dead	11.1	weak	high	72	male	body/tail	G3	3.0	3	+	IV	+
11	dead	31.5	weak	low	62	male	body/tail	G1	3.0	3	+	II	-
12	alive	41.6	weak	low	71	male	body/tail	G1	2.5	2	-	I	-
13	dead	23.9	weak	low	67	male	head	G1	2.5	3	+	II	-
14	dead	6.6	weak	high	73	male	head	G2	3.5	4	-	III	+
15	alive	15.9	weak	low	56	male	head	G2	2.5	3	+	II	-
16	dead	6.2	strong	high	71	female	body/tail	G3	3.0	4	+	III	-
17	dead	9	weak	low	54	male	head	G1	2.5	4	-	III	-
18	alive	150.6	weak	low	75	female	head	G2	5.5	3	-	II	-
19	dead	33	strong	low	61	female	body/tail	G1	5.0	3	+	II	+
20	dead	101.6	strong	high	62	male	head	G2	2.5	3	-	II	+
21	dead	21.3	weak	low	70	male	head	G1	3.0	3	-	II	-
22	dead	9.4	strong	high	63	male	head	G2	2.5	3	+	II	-
23	dead	32.9	weak	low	68	female	head	G2	4.5	3	+	II	+
24	dead	25.1	weak	high	69	female	head	G2	2.0	1	+	II	-
25	dead	9.7	weak	low	71	male	head	G2	5.0	2	-	I	+
26	dead	18.7	weak	high	71	female	head	G2	3.0	3	-	II	+
27	dead	22.2	weak	low	69	male	head	G1	3.5	3	+	II	-
28	dead	14.5	weak	low	80	female	head	G2	4.0	1	+	II	+
29	dead	3.6	strong	high	75	male	body/tail	G2	4.4	2	+	II	-
30	dead	11.2	strong	low	59	male	head	G2	4.5	3	+	II	+
31	alive	200.5	weak	low	56	female	head	G1	1.0	1	+	II	-
32	dead	9.8	weak	low	54	male	head	G2	6.5	4	+	III	+
33	dead	3	weak	high	64	male	body/tail	G3	4.0	3	+	II	-
34	dead	5.3	strong	high	78	female	head	G2	4.0	3	+	II	-
35	dead	8.6	weak	low	60	male	body/tail	G1	4.5	3	-	II	+
36	dead	12.6	strong	low	67	female	head	G2	3.0	3	+	II	-
37	dead	36.5	weak	low	79	male	body/tail	G2	3.5	1	-	I	-
38	dead	9.4	strong	high	83	female	head	G2	4.5	4	+	III	-
39	dead	19	weak	low	60	female	body/tail	G1	5.0	3	-	II	+
40	dead	3.8	strong	high	77	female	body/tail	G2	4.0	3	+	II	+
41	dead	13.7	strong	low	49	male	head	G1	4.5	4	+	III	-
42	dead	5.1	strong	high	44	male	head	G2	7.0	4	-	III	-
43	dead	8.6	weak	low	66	male	head	G1	7.5	4	+	III	+
44	dead	3.7	strong	high	67	female	head	G3	3.5	3	+	II	+
45	dead	8.3	strong	low	40	male	head	G2	5.0	3	+	II	-
46	dead	3.1	strong	high	66	male	head	G2	5.0	3	-	IV	+
47	dead	12.4	strong	low	70	female	head	G2	4.0	4	+	III	+
48	dead	27.7	strong	high	66	male	head	G2	3.0	2	+	II	+
49	alive	94.1	weak	low	65	male	head	G2	3.0	3	+	II	-
50	dead	6	weak	low	52	female	body/tail	G2	5.0	4	+	III	+
51	dead	7.6	strong	high	71	male	body/tail	G2	6.5	4	+	III	+
52	dead	13.2	weak	low	54	male	body/tail	G1	1.0	4	+	III	+
53	dead	11.3	weak	low	53	female	head	G2	3.5	3	-	II	+
54	dead	15.8	weak	low	68	female	head	G2	3.2	3	+	II	-
55	dead	69.5	weak	low	75	female	head	G1	1.5	3	+	II	+
56	dead	23.3	weak	low	77	male	body/tail	G2	4.5	3	-	II	+
57	lost	28	weak	low	61	male	head	G2	4.0	3	-	II	-
58	alive	40.9	weak	high	67	female	body/tail	G2	2.0	2	-	I	-
59	dead	26.3	weak	high	64	male	head	G2	1.8	3	+	II	-
60	alive	32.5	weak	low	59	female	body/tail	G1	2.7	3	+	II	-
61	lost	5.5	strong	high	49	male	head	G2	3.0	3	+	II	+
62	dead	17	weak	low	70	male	head	G2	3.0	3	-	II	-
63	dead	9.7	weak	low	67	male	head	G1	2.0	3	+	II	-
64	dead	9.9	strong	high	73	female	body/tail	G2	6.2	4	+	III	-
65	alive	25	strong	high	77	female	head	G2	3.0	2	+	III	-
66	alive	22.1	weak	high	76	male	body/tail	G3	4.0	2	-	I	-
67	dead	15.7	weak	low	63	male	body/tail	G2	4.0	3	-	II	+
68	dead	4.3	strong	high	62	male	body/tail	G3	6.0	3	+	II	-
69	lost	6	strong	low	68	male	head	G1	5.0	3	+	II	-
70	alive	18	weak	low	76	male	body/tail	G1	5.0	3	-	II	+

components, especially in the periphery of the tumor cell nests (Fig. 1), resulting in high survivin indices (≥10%). The central PAC areas and adjacent carcinoma cells were only weakly positive or negative for survivin, resulting in low survivin indices (≤9%). Furthermore, no survivin expression was detectable in the adjacent normal ductal epithelium of the paraffin-embedded tissue specimens (Fig. 1).

Corresponding to the strong mtTFA expression, high survivin indices were also evident in the LI, VI and PNI of high-grade PAC components, as shown by D2-40, EVG, and S-100 protein staining, respectively (Fig. 2). The survivin index was high or low (≥10 or ≤9%) in 27 (38.6%) and 43 (61.4%) of the 70 PAC specimens, respectively (43 (61.5%) 0-9% index; 15 (21.4%) 10-29% index; 7 (10.0%) 30-79% index and 5 (7.1%) 80-100% index). The survivin index was not related to the age of the patients, tumor location, size, margin or T stage. However, in the cases with LI, VI, PNI and/or lymph node metastases, the score was strongly correlated with the age, a high tumor grade and advanced disease stage (P=0.009, 0.0002 and 0.04, respectively) (Table 3, Fig. 1).

In a Kaplan-Meier analysis, the PAC patients with a high survivin index (≥10%) had a significantly shorter postoperative median DSS (7.6 months) compared with those who had a low survivin index (≤9%) (15.9 months) (P=0.03, Fig. 3B).

Correlations between strong mtTFA expression and a high survivin index

When the patients were divided into groups based on their mtTFA expression and survivin index, defined as

Table 3. Detailed correlations between strong mtTFA expression and/or a high survivin index and the clinicopathological variables.

Variables	Total (%)	mtTFA expression		P-value	Survivin index		P-value	Strong mtTFA+ and a high survivin index		P-value
		Weak+ (n=43)	Strong+ (n=27)		Low (n=43)	High (n=27)		Negative (n=51)	Positive (n=19)	
Age (years)				0.37			0.009			0.10
≥60	53(75.7)	31 (72.1)	22 (81.5)		28 (65.1)	25 (92.6)		36 (70.6)	17 (89.5)	
<60	17 (24.3)	12 (27.9)	5 (18.5)		15 (34.9)	2 (7.4)		15 (29.4)	2 (10.5)	
Gender				0.22			0.835			0.64
Male	40 (57.1)	27 (62.8)	13 (48.1)		25 (58.1)	15 (55.6)		30 (58.8)	10 (52.6)	
Female	30 (42.9)	16 (37.2)	14 (51.9)		18 (41.9)	12 (44.4)		21 (41.2)	9 (47.4)	
Location				0.74			0.494			0.90
Head	45 (64.3)	27 (62.8)	18 (66.7)		29 (67.4)	16 (59.3)		33 (64.7)	12 (63.2)	
Body/tail	25 (35.7)	16 (37.2)	9 (33.3)		14 (32.6)	11 (40.7)		18 (35.3)	7 (36.8)	
Pathological type				0.04			0.0002			0.008
G1	20 (28.6)	16 (37.2)	4 (14.8)		19 (44.2)	1 (4.7)		19 (37.3)	1 (5.3)	
G2 & G3	50 (71.4)	27 (62.8)	23 (85.2)		24 (55.8)	26 (96.3)		32 (62.7)	18 (94.7)	
Tumor size				0.04			0.92			0.19
≤2cm	10 (14.3)	9 (20.9)	1 (3.7)		6 (14.0)	4 (14.8)		9 (22.2)	1 (5.3)	
>2cm	60 (85.7)	34 (79.1)	26 (96.7)		37 (86.0)	23 (85.2)		42 (77.8)	18 (94.7)	
Margin				0.98			0.64			0.82
Negative	39 (55.7)	24 (55.8)	15 (55.6)		23 (53.5)	16 (59.3)		28 (54.9)	11 (57.9)	
Positive	31 (44.3)	19 (44.2)	12 (44.4)		20 (46.5)	11 (40.7)		23 (45.1)	8 (42.1)	
T stage				0.20			0.53			0.71
T1 & T2	13 (18.9)	10 (23.3)	3 (11.1)		7 (16.3)	6 (22.2)		10 (19.7)	3 (15.8)	
T3 & T4	57 (81.4)	33 (76.7)	24 (88.9)		36 (83.7)	21 (77.8)		41 (80.3)	16 (84.2)	
N stage				0.01			0.31			0.09
N0	26 (37.1)	21 (48.8)	5 (18.5)		18 (41.9)	8 (29.6)		22 (43.1)	4 (21.1)	
N1	44 (62.9)	22 (51.2)	22 (81.5)		25 (58.1)	19 (70.4)		29 (56.9)	15 (78.9)	
Stage				0.04			0.04			0.02
I-II	51 (72.9)	35 (81.4)	17 (63.0)		35 (81.4)	16 (59.3)		42 (77.8)	9 (47.4)	
III-IV	19 (27.1)	8 (18.6)	10 (37.0)		8 (18.6)	11 (40.7)		9 (22.2)	10 (52.6)	
LI			0.57			0.62			0.92	
LI(-)	4 (5.7)	3 (7.0)	1 (3.7)		2 (4.7)	2 (7.4)		3 (5.9)	1 (5.3)	
LI(+)	66 (94.3)	40 (93.0)	26 (96.7)		41 (95.3)	25 (92.6)		48 (94.1)	18 (94.7)	
V			0.25			0.60			0.59	
VI(-)	18 (25.7)	9 (20.9)	9 (33.3)		12 (27.9)	6 (22.2)		14 (27.5)	4 (21.1)	
VI(+)	52 (74.3)	34 (79.1)	18 (66.7)		31 (72.1)	21 (77.8)		37 (72.5)	15 (78.9)	
Ne			0.85			0.30			0.81	
PNI(-)	3 (4.3)	2 (4.7)	1 (3.7)		1 (2.3)	2 (7.4)		2 (4.9)	1 (5.3)	
PNI(+)	67 (95.7)	41 (95.3)	26 (96.7)		42 (97.7)	25 (92.6)		49 (96.1)	18 (94.7)	
mtTFA				----			<0.0001			----
Weak expression	43 (61.4)				35 (81.4)	8 (29.6)				
Strong expression	27 (38.6)				8 (18.6)	19 (66.7)				
Survivin				<0.0001			----			----
Low index (<10%)	43 (61.4)	35 (81.4)	8 (29.6)							
High index (≥10%)	27 (38.6)	8 (18.6)	19 (70.4)							

strong or weak expression and a high or low index, respectively, their immunoprofiles were 50.0% weak and low (35 cases); 11.4% strong and low (8 cases); 11.4% weak and high (8 cases) and 27.2% strong and high (19 cases). The PAC patients with the strong and high profile (strong mtTFA expression and a high survivin index) had a markedly shorter postoperative median DSS (6.1 months) compared with that of the other groups (16.9 months) (P=0.006, Fig. 3C). Correspondingly, strong mtTFA expression and a high survivin index were closely associated with a high (G2 and G3) tumor grade and advanced disease stage (P=0.008 and 0.02, respectively) (Table 3, Figs. 1, 2).

Next, the DSS of the PAC patients, divided into two groups: (i) weak and low vs. strong and low (Fig. 4A) or weak and high (Fig. 4B); and (ii) strong and low vs. weak and high (Fig. 4C); then the Kaplan-Meier method was used to further examine the associations of these

Table 4. The results of the univariate and multivariate analyses of survival in 70 patients with PAC, according to the clinicopathological variables and both strong mtTFA expression and a high survivin index.

Risk factors	Univariate			Multivariate		
	Hazard ratio	95%CI	P-value	Hazard ratio	95%CI	P-value
Strong mtTFA expression and a high survivin index	2.32	1.28-4.18	0.005	3.36	1.56-7.23	0.002
Age ≥60 yrs	0.91	0.49-1.71	0.78	0.64	0.31-1.31	0.22
Sex (male)	1.17	0.68-2.01	0.56	1.08	0.60-1.92	0.80
Tumor size (>2 cm)	2.18	0.98-4.87	0.05	2.32	0.97-5.54	0.05
Surgical margin(+)	1.29	0.76-2.19	0.35	1.19	0.66-2.13	0.57
T stage pT3 & pT4	2.15	1.01-4.57	0.05	2.65	1.09-6.43	0.03
Tumor grade G2 & G3	1.61	0.88-2.95	0.12	1.42	0.67-2.99	0.36
LI(+)	1.33	0.41-4.32	0.64	0.59	0.13-2.70	0.49
VI(+)	1.19	0.65-2.19	0.57	0.88	0.41-1.86	0.73
PNI(+)	0.88	0.27-2.92	0.84	3.33	0.70-15.84	0.13
N(+)	1.32	0.77-2.28	0.31	1.30	0.67-2.53	0.44

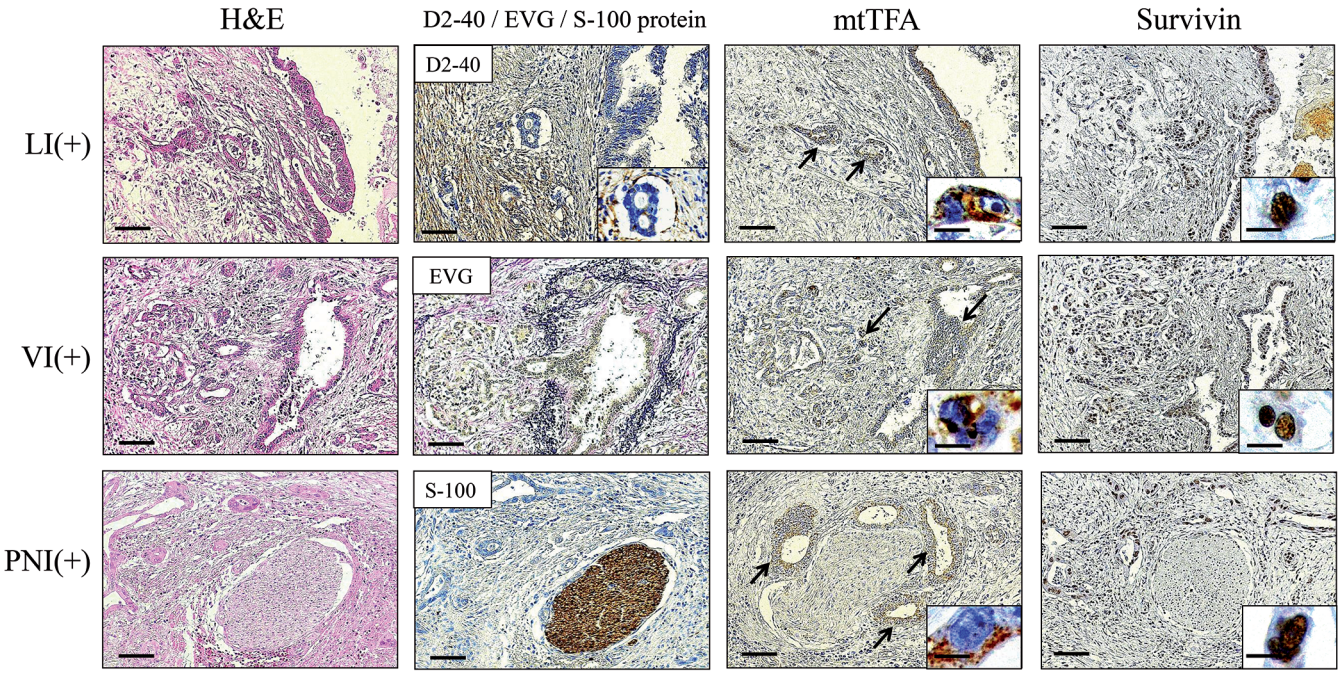

Fig. 2. Representative photograph of the H&E, elastica van Gieson (EVG) and immunohistochemical staining of mtTFA, survivin, D2-40 and S-100 protein in the vascular (VI; Case No.65), lymphatic (LI; Case No.65) or perineural (PNI; Case No.61) invasion components of advanced pancreatic adenocarcinoma (arrows; strong mtTFA expression). EVG, D2-40 and S-100 protein staining clearly revealed the elastic fibers of the vascular medial wall (VI(+)), lymphatic endothelium (LI(+)) or neuronal fibres (PNI(+)), respectively. Bars: main, 100 μm; insets, 20 μm.

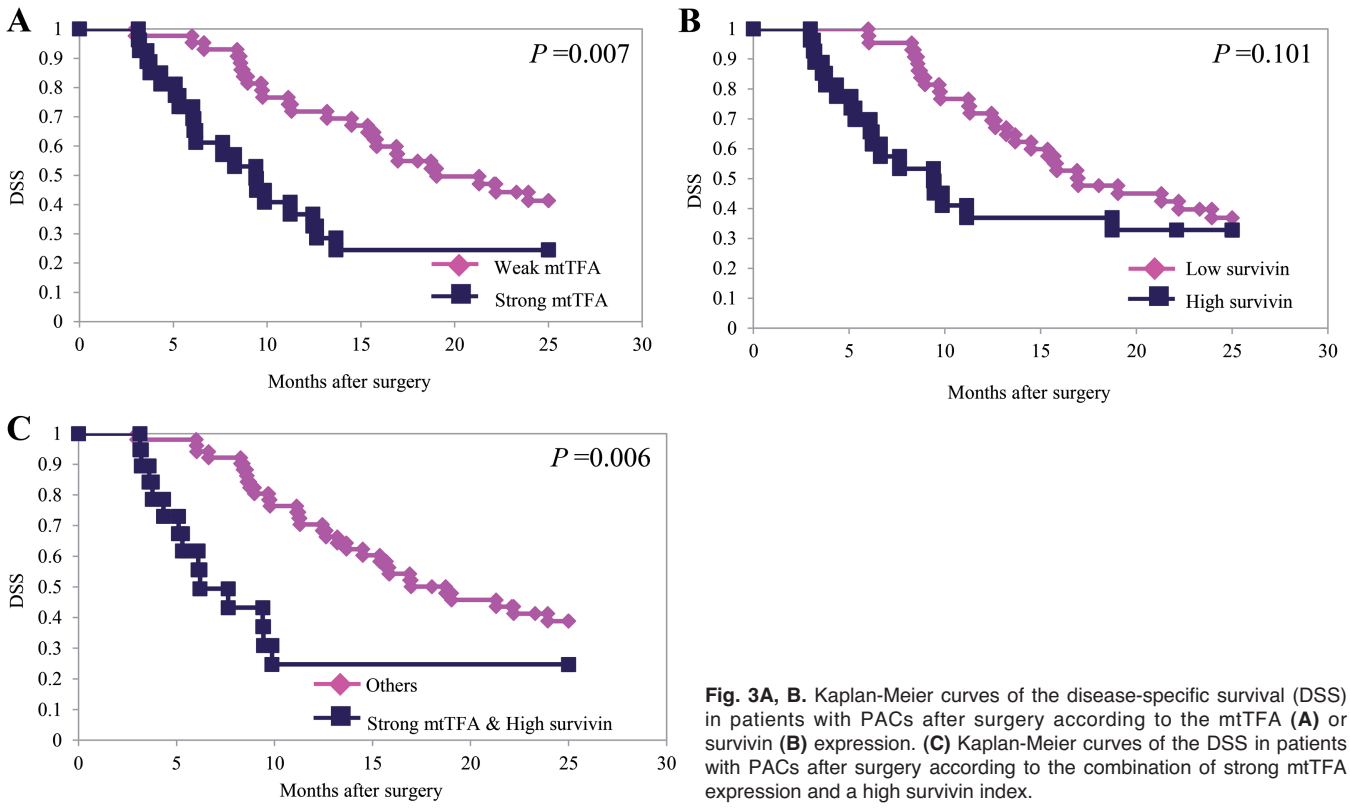

Fig. 3A, B. Kaplan-Meier curves of the disease-specific survival (DSS) in patients with PACs after surgery according to the mtTFA **(A)** or survivin **(B)** expression. **(C)** Kaplan-Meier curves of the DSS in patients with PACs after surgery according to the combination of strong mtTFA expression and a high survivin index.

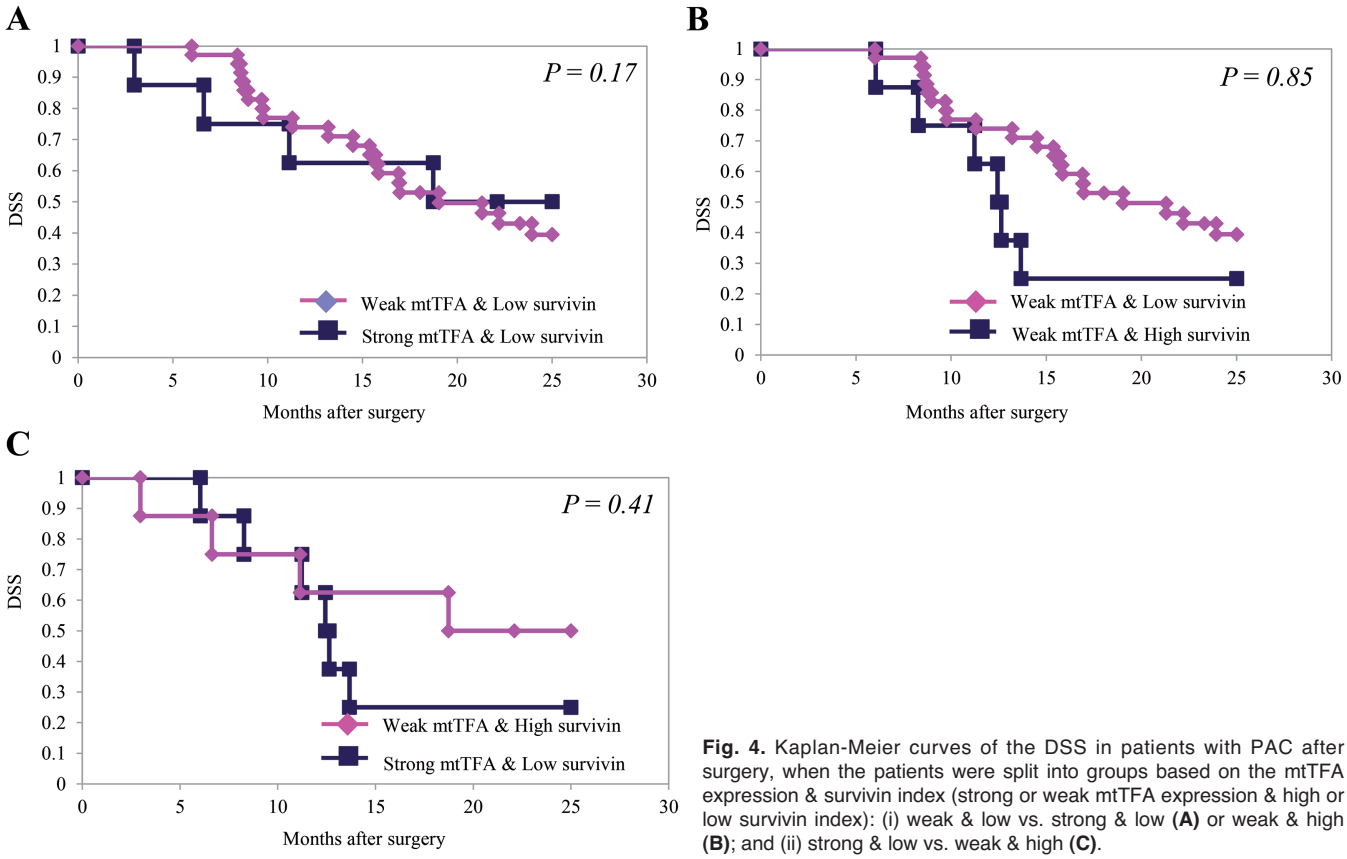

Fig. 4. Kaplan-Meier curves of the DSS in patients with PAC after surgery, when the patients were split into groups based on the mtTFA expression & survivin index (strong or weak mtTFA expression & high or low survivin index): (i) weak & low vs. strong & low **(A)** or weak & high **(B)**; and (ii) strong & low vs. weak & high **(C)**.

groupings with the survival. The DSS of the PAC patients showed no significant differences (Fig. 4A: P=0.15; Fig. 4B: P=0.74, respectively) in either the former (i) classification approach, or the latter approach (ii) (P=0.69; Fig. 4C), thus indicating that there were neither any complementary nor competitive correlations between mtTFA and survivin.

The combination of strong mtTFA expression and a high survivin index represents a significant independent prognostic indicator for PAC

To assess whether the combination of the mtTFA expression and survivin index was an independent predictor of the postoperative DSS, a Cox proportional hazards model was created in a forward fashion including only covariates that had statistically significant correlations with the DSS, using an inclusion threshold of P<0.05 (Table 4). A univariate analysis showed that the tumor size (>2 cm), advanced T stage (T3 to 4), and both strong mtTFA expression and a high survivin index were significant predictors of a poorer survival (P=0.05, 0.05, and =0.005, respectively). Furthermore, a multivariate analysis demonstrated that, after correction for confounding variables, the combination of strong mtTFA expression and a high survivin index remained an independent prognostic indicator for the DSS (P=0.002), in addition to the tumor size and advanced cancer stage (P=0.05 and 0.03, respectively) (Table 4), but neither strong mtTFA expression nor a high survivin index alone was a significant factor (data not shown).

Discussion

The present study revealed, for the first time, that the combination of the mtTFA expression with the survivin index represents a powerful and potentially independent negative indicator of the DSS in patients with postoperative PAC, and by extension, as a novel prognostic marker for the disease, especially within the first two years after surgery. Further supporting our findings, another group recently reported that the expression of mtTFA played a pivotal role in worsening the clinical course of postoperative PAC patients through the induction of anti-apoptotic effects in PAC cells (Yamauchi et al., 2014). The authors of that study used a commercially available anti-mtTFA mouse polyclonal antibody for their immunohistochemical studies, unlike our original rabbit polyclonal antibody, but they did not identify any specific apoptosis-related molecules associated with mtTFA.

The present study supports the previous studies showing that approximately 80% of the patients experience postoperative recurrence (local or distant) and die within the first two years after surgery (50 or 70 (71.4%) of the patients in our study) (Clearly et al., 2004; Katz et al., 2008; Stathis and Moore, 2010). There have been no reliable predictors of the progressive potential of PAC to date. In that sense, the mtTFA expression and survivin index patterns in surgical specimens of primary PAC might allow for improved patient selection with regard to postoperative adjuvant therapies and the prediction of appropriate clinical postoperative courses, especially in the early phase. Collectively, our data are in agreement with in vitro studies of epithelial cancers. For example, the overexpression of mtTFA in carcinoma cells results in significantly more rapid growth than in control cells, whereas mtTFA-knockdown significantly suppresses the cell growth (Han et al., 2011). In addition the mtTFA protein expression levels were shown to be increased after treatment with cisplatin, one of the major anticancer drugs, and upregulation of mtTFA expression could contribute to the ability of cells to avoid cisplatin-induced apoptosis, i.e., cisplatin resistance (Yoshida et al., 2003). Taken together, these data indicate that it is conceivable that mtTFA plays a critical role in cancer cell survival/anti-apoptosis and subsequent growth, via the orchestrated regulation of many transcription factors, including the mtTFA/survivin pathway. In fact, co-localization of strong mtTFA expression and a higher survivin index were found especially at the invasive fronts of PAC, potentially being related to poor differentiation.

Furthermore, we herein demonstrated for the first time that the survivin index (a high index is considered to be present when ≥10% of the nuclei are stained) could be a useful adjunctive aid for identifying worse clinicopathological features, such as a higher tumor grade or shorter DSS, in patients with PACs, in addition to the strong mtTFA expression. It has been proposed that there are at least two kinds of interactions possible among the two representative proteins: competition or complementation, similar to the GalNAc-T family members (Bennett et al., 1998; Li et al., 2011; Kitada et al., 2013). We hypothesized that, if the relationship between strong mtTFA expression and a high survivin index was complementary, the DSS would be different between the weak and low and strong and low or weak and high groups. On the other hand, if strong mtTFA expression and a high survivin index competed with each other, there would be differences in the DSS between the strong and low group and the weak and high group. The patient cohort was divided in two groups based on the strong/weak and high/low patterns, and the Kaplan-Meier methods were applied to verify the interactions between the proteins. Unexpectedly, the DSS of the patients with PAC demonstrated no significant differences for either potential classification of the interaction, indicating that there were neither apparent competitive nor complementary relationships between the mtTFA expression and survivin index. Despite the fact that our analyses were in a relatively smaller cohort from a single institution, these two cancer cell growth-related proteins were clearly demonstrated to be co-expressed, but appear to function separately. In this context, it is noteworthy that the combination of the two biomarkers significantly predicted higher

clinicopathological aggressiveness of the tumor or a poor outcome in patients with postoperative PAC, but neither mtTFA nor survivin alone was significantly predictive in the present study. Further follow-up in larger cohorts of patients will be necessary to confirm the relationships between the mtTFA and survivin pathways. Of note, we have recently reported that there are two putative mtTFA binding sites present in the promoter region of the survivin gene, indicating that mtTFA plays a key role in regulating survivin, further supporting the importance of the expression of these molecules (Han et al., 2011).

In conclusion, the present cohort study indicates, for the first time, that the combination of mtTFA and survivin expression is an independent, novel and powerful marker for a poor prognosis in PAC patients after surgery. Collectively, our data demonstrate that strong mtTFA expression and/or a high survivin index in PAC (1) has a significant relationship with a high tumor grade or advanced tumor stage, manifesting as tumors with poor differentiation, together with more invasive/aggressive behaviors; and (2) show significantly high co-expression, and potentially regulate the cell survival and/or growth of PAC. Finally, evaluating the expression of both mtTFA and survivin might be useful for guiding the clinical management of PAC patients, especially in the early postoperative phase.

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