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Journal

Annals of Surgical Oncology, 26(3)

ISSN

1068-9265

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Publication Date

2019-03-01

DOI

10.1245/s10434-018-07109-6

Ann Surg Oncol (2019) 26:807–814
https://doi.org/10.1245/s10434-018-07109-6

Annals of
SURGICAL ONCOLOGY
OFFICIAL JOURNAL OF THE SOCIETY OF SURGICAL ONCOLOGY

ORIGINAL ARTICLE – PANCREATIC TUMORS

Thrombospondin-2 is a Highly Specific Diagnostic Marker and is Associated with Prognosis in Pancreatic Cancer

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ABSTRACT

Background. Thrombospondin-2 (TSP-2) has been reported as an early diagnostic marker for pancreatic ductal adenocarcinoma (PDAC) in Caucasian populations. This study was designed to validateTSP-2 as a diagnostic marker in a large Taiwan cohort and to investigate the association of TSP-2 with the clinical outcomes of PDAC patients.

Methods. The serum TSP-2 levels in 263 PDAC patients and 230 high-risk individuals (HRIs) were measured via an enzyme-linked immunosorbent assay. The sensitivity, specificity, and accuracy of TSP-2 as a diagnostic marker to discriminating PDAC patients from HRIs and correlations between TSP-2 levels and prognosis of PDAC patients were analyzed.

Results. Serum TSP-2 levels were significantly higher in patients with PDAC (44.90 ± 40.70 ng/ml) than in the HRIs (17.52 ± 6.23 ng/ml). At a level of ≥ 29.8 ng/ml, TSP-2 exhibited 100% specificity, 55.9% sensitivity, 100% positive predictive value (PPV), and 66.5% negative predictive value (NPV) for discriminating PDAC patients from HRIs. The Cox regression analysis showed that higher serum TSP-2 levels were significantly associated with poor outcomes in PDAC patients (hazard ratio = 1.54, 95% confidence interval = 1.143–2.086, $P = 0.005$). Combining the carbohydrate antigen 19-9 (CA19-9) (cutoff value of 62.0 U/ml) and TSP-2 (cutoff value of 29.8 ng/ml) levels yielded 98.7% specificity, 90.5% sensitivity, 98.8% PPV, and 90.1% NPV for discriminating patients with PDAC from HRIs.

Conclusions. TSP-2 is a highly specific diagnostic marker and an independent prognostic marker in patients with PDAC. A combined biomarker panel, including TSP-2 and CA19-9, may facilitate future PDAC screening.

Pancreatic ductal adenocarcinoma (PDAC) remains one of the deadliest cancers, because it is notoriously difficult to diagnose early and has a poor response to treatment.[1,2] The only opportunity to improve the prognosis of patients with PDAC mainly depends on early detection to allow effective therapy. Carbohydrate antigen 19-9 (CA19-9) is the well-established blood marker for PDAC. However, CA19-9 testing is not recommended for general screening, because it might produce false-positive results in patients with cirrhosis, chronic pancreatitis, cholangitis, and other cancers and might produce false-negative results in patients with a Lewis-negative blood type.[3–5] CA19-9 has been reported to discriminate between patients with PDAC and healthy controls or patients with benign pancreatic disease with 78.2–80.3% sensitivity (SN) and 80.2–82.8% specificity (SP).[4,6] To reduce healthcare expenditures and prolong PDAC patient survival, a new diagnostic assay must be performed with an estimated minimum SN of 88% at a SP of 85%.[7,8] A search for novel PDAC diagnostic biomarkers with better performance than CA19-9 is urgently needed.

A preliminary result was presented as a poster in the AOPA&KPBA&KPSC 2018 meeting, Seoul, Korea.

Electronic supplementary material The online version of this article (https://doi.org/10.1245/s10434-018-07109-6) contains supplementary material, which is available to authorized users.

First Received: 22 August 2018;
Published Online: 19 December 2018

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Thrombospondins (TSPs) comprise a family of homologous proteins that regulate the cellular phenotype and extracellular matrix structure during tissue genesis and remodeling.[9] TSPs have important functions in immune responses, inflammation, cancer, growth and development.[10–15] TSP-2 is a matricellular protein controlling the apoptosis-proliferation balance in endothelial cells[16] and associated with tumor angiogenesis.[17] TSP-2 inhibits angiogenesis through direct effects on endothelial cell migration, proliferation, survival, and apoptosis and by antagonizing VEGF activity.[18] The abnormal expression of TSP-2 has been identified in myeloma, hepatocellular carcinoma, lung cancer, and prostate cancer as a diagnostic and/or prognostic marker.[19–22] Plasma concentrations of TSP-2 at or above 42 ng/ml have been reported to discriminate PDAC patients from healthy controls with an SP of 99% (1% false-positive rate (FPR)) and an SN of 52%, and combining CA19-9 levels (> 55 U/ml) with TSP-2 levels (> 42 ng/ml) showed an SP of 98% and an SN of 87% in a large phase 2b study with 537 non-Hispanic Caucasian subjects.[23] However, the association of TSP-2 levels with the clinical outcomes of PDAC patients has not been well investigated. The purpose of this study was to validate TSP-2, in addition to or combined with CA19-9, as a diagnostic marker in a large non-Caucasian cohort and to investigate the association of TSP-2 with the clinical outcomes of patients with PDAC.

MATERIALS AND METHODS

Study Population

Between January 2005 and December 2017, peripheral blood was collected at the National Taiwan University Hospital from 263 patients with cytologically and/or pathologically confirmed adenocarcinoma of the pancreas after their written informed consent was obtained. All patients' demographic data, including age, sex, serological study results, image study results, survival data, and clinical manifestations were collected. Peripheral blood was collected from 230 individuals at high risk for PDAC (high-risk individuals [HRIs]) who had a family history of PDAC and participated in the pancreatic cancer screening program at the National Taiwan University Hospital between January 2005 and December 2015.[24] All control subjects underwent a detailed history assessment and physical examination, family history collection, magnetic resonance imaging/magnetic resonance cholangiopancreatography (MRI/MRCP) examination, and blood testing. All controls were followed up for more than 2 years and were free of pancreatic malignancy. The study was reviewed and approved by the Institutional Review Board of the National Taiwan University Hospital.

Blood Sample Collection and Measurement of TSP-2 Levels in Serum

Ten milliliters of peripheral venous blood was collected into a serum separator tube (BD Vacutainer Systems, Franklin Lakes, NJ, USA) from each patient and control under fasting conditions, and the sera were immediately separated by centrifugation at $3000 \times g$ for 10 min in a refrigerated centrifuge. The sera were stored at − 80 °C for further analysis. Serum TSP-2 levels were assayed via a quantitative sandwich enzyme-linked immunosorbent assay (ELISA) method as previously reported (DTSP20, Quantikine; R&D Systems, Inc., Minneapolis, MN, USA) in duplicate according to the manufacturer's protocol.[23] The absorbance was measured at 450 nm using a SpectraMax 250 96-well plate reader (Molecular Devices, CA, USA). The results are expressed as nanograms per milliliter. CA19-9 levels were measured in a certified clinical laboratory using a cutoff value of 37 U/ml at the time of diagnosis and before any treatment.

Statistical Analysis

The serum levels of TSP-2 and CA19-9 were summarized as the means and standard deviations and were then compared across subgroups of patients on the basis of stage using the Mann–Whitney–Wilcoxon test. Correlations were calculated with the Spearman correlation test. Clinicopathological characteristics among groups were compared by the Chi square test. Receiver operating characteristic (ROC) curves and areas under the ROC curves (AUCs) were established for discriminating PDAC patients from HRIs. The cutoff values for serum TSP-2 and CA19-9 levels were determined from the ROC curves by calculating the Youden index. The SN, SP, positive predictive value (PPV), negative predictive value (NPV), and accuracy were calculated for significant variables using the optimal cutoff as determined by the ROC analysis. A classification and regression tree (CART) analysis was conducted to test the diagnostic performance of TSP-2 levels in combination with CA19-9 levels. Survival curves were plotted using the Kaplan–Meier method and were then compared with the log-rank test. An exploratory analysis was performed using a proportional hazards model with binary indicators based on tentative cutoffs or the calculated median value. A multivariate Cox regression model was used to identify independent prognostic factors for death due to pancreatic cancer. $P < 0.05$ was considered statistically significant. Data analyses were performed using SPSS software (SPSS 15; Chicago, IL, USA),

GraphPad Prism Ver. 5.02 (San Diego, CA), and MedCalc v.5 software (Mariakerke, Belgium).

RESULTS

Serum TSP-2 Levels are Higher in Patients with PDAC than in High-Risk Individuals

The clinical characteristics and serum levels of TSP-2 in 263 patients with PDAC and 230 HRIs as determined by an ELISA are shown in Table 1. The mean serum concentrations of TSP-2 and CA19-9 were significantly higher in patients with PDAC than in HRIs (44.90 ± 40.70 vs. 17.52 ± 6.23 ng/ml and 4893.75 ± 75 vs. 69.02 ± 795.59; $P < 0.0001$; Fig. 1a, b; Table 1). In addition, a significant difference in serum TSP-2 levels was observed between patients with chronic calcifying pancreatitis (CCP) and patients with PDAC (Supplementary Figure 1). We next analyzed the correlation between the serum TSP-2 and CA19-9 levels and found no correlation ($r = 0.209$, $P < 0.001$; Supplementary Figure 2). This result suggests that the CA19-9 level did not affect TSP-2 expression in PDAC.

TSP-2 is a Highly Specific Diagnostic Biomarker for PDAC

The ROC curve analysis was performed to assess the SN and SP of TSP-2 as a biomarker for use in PDAC diagnosis (Fig. 2). The optimal cutoff value for the ability of TSP-2 to distinguish PDAC patients from HRIs was determined by Youden index calculation. At the cutoff value of 29.8 ng/ml, TSP-2 yielded an SN of 55.9%, an SP of 100%, a PPV of 100%, and an NPV of 66.5%, with an AUC of 0.755 (95% confidence interval (CI) = 0.715–0.793) for distinguishing PDAC patients from HRIs (Fig. 2; Table 2). Because Kim et al. reported that TSP-2 discriminated PDAC patients from healthy controls with an SP of 99% and an SN of 52% at a cutoff value of 42 ng/ml, we also analyzed the TSP-2 concentration at the cutoff value of 42 ng/ml.[23] For concentrations of TSP-2 at or above 42 ng/ml, we still observed an SP of 100% and a PPV of 100% but with a lower SN of 39.2% and a lower NPV of 66.5% for discriminating PDAC patients from HRIs (Table 2). The SN, SP, PPV, and NPV of TSP-2 and/or CA19-9 for distinguishing PDAC patients from HRIs when different cutoff values were used are summarized in Table 2. The most accurate combined biomarkers in this study were TSP-2 levels at or above 42 ng/ml with CA19-9 levels at or above 62.0 U/ml, which yielded 98.7% SP, 90.5% SN, 98.8% PPV, 90.1% NPV, and 92% accuracy for distinguishing PDAC patients from HRIs (Table 2). We performed the CART analysis using the CA19-9 level (cutoff value of 62.02 U/ml) as the first node and the TSP-2 level (cutoff value of 29 ng/ml) as the second node to distinguish PDAC patients from HRIs. The 1.4% (3/230) of the HRIs misclassified in the first node were all correctly discriminated from PDAC patients in the second node, thus ameliorating the FPR of CA19-9. Including the TSP-2 level significantly refined the diagnostic performance of CA19-9, particularly in CA19-9-positive HRIs (Supplementary Figure 3).

TSP-2 Levels were Significantly Associated with the Prognosis of PDAC Patients

We next investigated the relationship between TSP-2 levels and clinical manifestations of PDAC patients. Serum TSP-2 levels were not correlated with tumor stage ($P = 0.064$; Fig. 1c). We stratified PDAC patients into

TABLE 1 Clinical demographic data and serum levels of thrombospondin-2 and CA19-9 in pancreatic ductal adenocarcinoma (PDAC) patients and high-risk individuals

	PDAC patients ($n = 263$)	High-risk individuals ($n = 230$)
Sex (M/F)-no. (%)	123 (46.8%)/140 (53.2%)	133 (57.8%)/97 (42.2%)
Age, mean (SD), yr[a]	63.32 (13.28)	47.59 (13.63)
CA19-9, mean (SD), U/ml[a]	4893.75 (8562.26)	69.02 (795.59)
Thrombospondin-2, mean (SD), ng/ml[a]	44.90 (40.70)	17.52 (6.23)
TNM stage-no. (%)		
I–II	57 (21.7%)	
III–IV	206 (78.3%)	
Overall survival, median (SD), months	6.5(10.7)	
TNM stage I–II	13.3 (15.3)	
TNM stage III–IV[b]	5.4 (7.4)	

[a] $P < 0.0001$ between pancreatic cancer patients and high-risk individuals

[b] $P < 0.0001$ between stage I–II pancreatic cancer patients and advanced pancreatic cancer patients

PDAC pancreatic ductal adenocarcinoma; *M* male; *F* female

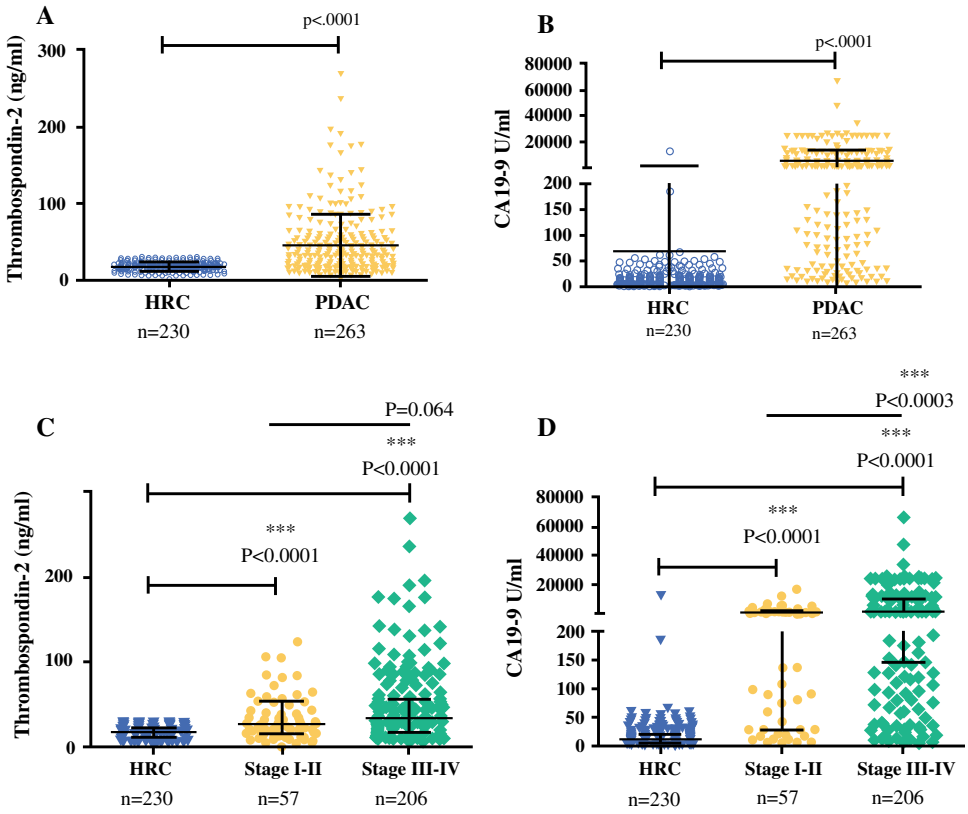

FIG. 1 Serum thrombospondin-2 (TSP-2) level in pancreatic ductal adenocarcinoma (PDAC). Serum **a** TSP-2 and **b** carbohydrate antigen 19-9 (CA19-9) levels in patients with PDAC (n = 263) and high-risk individuals (n = 230). **c** Serum TSP-2 and **d** CA19-9 levels in patients in different PDAC stages

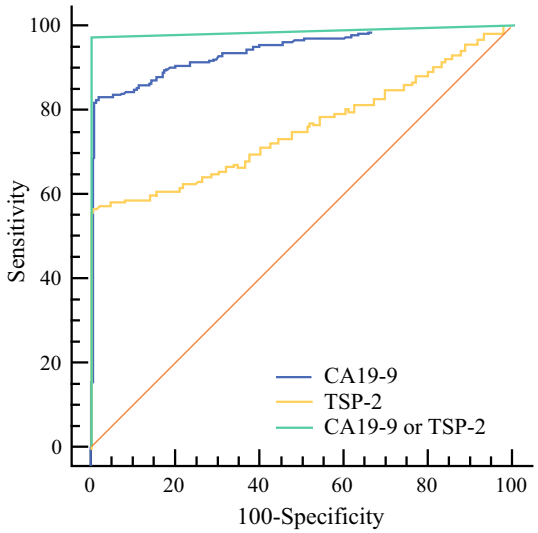

FIG. 2 Receiver operating characteristic (ROC) curves of serum thrombospondin-2 (TSP-2), CA19-9, and TSP-2 + CA19-9 concentrations in serum samples from patients with all stages of PDAC versus concentrations in control patients (PDAC, n = 263; controls, n = 230)

low- or high-TSP-2 groups (by TSP-2 levels of < 29.8 ng/ml or ≥ 29.8 ng/ml, respectively). The high-TSP-2 group was significantly associated with higher serum CA19-9 levels and shorter overall survival (OS) (Table 3). The median OS was 9.09 months in the low-TSP-2 group versus 5.06 months in the high-TSP-2 group (Fig. 3; Table 3). The OS was significantly shorter in patients with high serum TSP-2 levels ($P < 0.0001$, 95% CI 0.419–0.720; hazard ratio = 0.55) (Table 3). In the multivariate Cox proportional hazards model, in addition to age and tumor stage, serum TSP-2 level was a significant independent prognostic factor in PDAC patients (hazard ratio = 1.544; 95% CI 1.143–2.086, $P < 0.005$; Table 4).

DISCUSSION

PDAC remains one of the most fatal cancers due to the late stage at diagnosis of the vast majority of patients. The identification of HRIs and the ability to detect PDAC earlier are the best opportunities for improving patient outcomes. The CA 19-9 blood test is widely used for PDAC diagnosis but is not recommended for general screening because of its false-positive potential in patients with cirrhosis, chronic pancreatitis, cholangitis, etc.[3–5] In this study, we observed that serum TSP-2 levels were higher in patients with PDAC than in HRIs. In addition, we demonstrated that at the cutoff value of 29 ng/ml, the serum TSP-2 level achieves 100% SP and 100% PPV for distinguishing PDAC patients from HRIs and that the TSP-2 level combined with the CA19-9 level at the cutoff value of 62 U/ml achieves 92% accuracy for distinguishing PDAC patients from HRIs. Thus, our findings have clinical

TABLE 2 Area under the receiver operating characteristic curve (AUC) analysis of patients with pancreatic ductal adenocarcinoma (PDAC) and high-risk controls

Comparison	AUC	95% CI	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy	*P*
Thrombospondin-2								
PDAC vs. HRC (cutoff > 29.8)	0.755	0.715–0.793	55.9	100	100	66.5	76.5%	< 0.0001
PDAC vs. HRC (cutoff > 42)	0.55	0.500–0.500	39.2	100	100	59.0	67.6%	< 0.0001
Carbohydrate antigen 19-9 (CA19-9)								
PDAC vs. HRC (cutoff > 37 U/ml)	0.86	0.849–0.871	85.6	88.7	89.6	84.3	87.0%	< 0.0001
PDAC vs. HRC (cutoff > 62 U/ml)	0.942	0.918–0.961	82.5	98.7	98.6	83.2	90.1%	< 0.0001
CA19-9 ≥ 62.0 or TSP-2 ≥ 29.8								
PDAC vs. HRC	0.948	0.9.35–0.955	90.5	98.7	98.8	90.1	92.0%	< 0.0001
CA19-9 ≥ 62.0 and TSP-2 ≥ 29.8								
PDAC vs. HRC	0.778	0.747–0.808	55.5	100	100	66.3	76.3%	< 0.0001

Cutoff point determined by the Youden index. Thrombospondin-2 levels, ng/ml, CA19-9 levels, U/ml

PPV positive predictive value; *NPV* negative predictive value; *CI* confidence interval; *PDAC* pancreatic ductal adenocarcinoma patients, *HRC* high-risk controls; *SN* sensitivity; *SP* specificity

TABLE 3 Clinical demographic data of PDAC patients with low and high TSP-2 levels

	TSP-2 low *n* = 116	TSP-2 high *n* = 147
Age, mean (SD), yr	62.95 (13.40)	63.62 (13.22)
Sex (M/F) -no.	50/66	73/74
Stage-no.		
I–II	31	26
III–IV	85	121
CA19-9, mean (SD), U/ml[a]	2729.96 (5546.05)	6601.24 (10,033.81)
TSP-2, mean (SD), ng/ml[a]	16.36 (5.91)	67.42 (42.28)
OS, median (SD), months[a]	9.1 (11.98)	5.06 (8.99)

[a]$P < 0.0001$ between TSP-2 Low pancreatic cancer patients and TSP-2 High pancreatic cancer patients

relevance. First, the combination of serum TSP-2 and serum CA19-9 levels is a more accurate diagnostic marker, with high SP (nearly 100%), than is CA19-9 alone for PDAC diagnosis. Our results are similar to those in Kim et al.'s report stating that plasma TSP-2 concentrations at or above 42 ng/ml could discriminate PDAC patients from healthy controls with a high SP of 99% (1% FPR) and that combining CA19-9 levels (> 55 U/ml) with TSP-2 levels (> 42 ng/ml) yielded an SP of 98% and an SN of 87% in a large phase 2b study with 537 non-Hispanic Caucasian subjects.[23] Both studies, one in the West and the other in Asia, showed that TSP-2 is a highly specific (nearly 100% SP) diagnostic marker for PDAC in two different large cohorts (approximately 500 cases in both studies). In our study, the CART analysis further showed that TSP-2 levels correctly discriminated patients with PDAC from HRIs with elevated serum CA19-9 levels, thus ameliorating the FPR of CA19-9. The prevalence of PDAC in the general population is low, so a test with high SP is important to ensure low numbers of false positives. Second, in the report by Kim et al., the mean serum levels of TSP-2 in the patients with PDAC and in the controls are similar to those in the PDAC patients and HRIs in our study using the same ELISA. Our findings suggest that the difference in serum TSP-2 levels between Caucasian and Taiwanese populations is not significant. The consistency in the findings from different laboratories in different study populations suggests that TSP-2 is a convincing diagnostic marker for PDAC and is not influenced by ethnic factors. Compared with that in Kim et al.'s study, the optimal serum TSP-2 cutoff value for differentiating PDAC patients from controls is lower in our study (29 ng/ml vs. 42 ng/ml). This

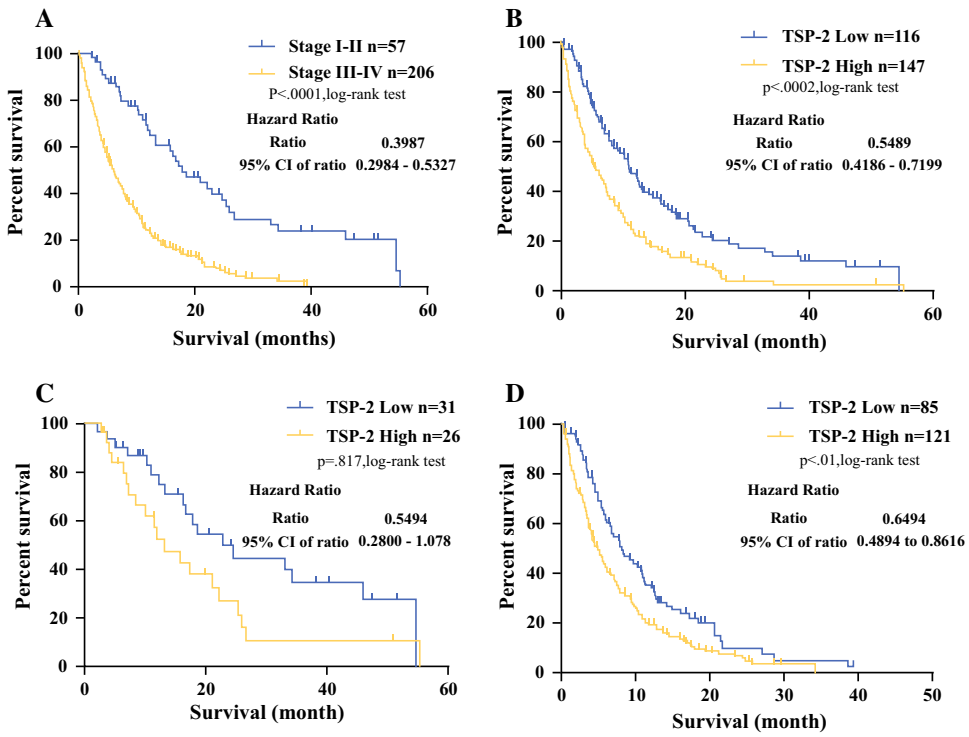

FIG. 3 **a** Kaplan–Meier analysis of overall survival in patients with early-stage and advanced pancreatic ductal adenocarcinoma ($P < 0.0001$, log-rank test). **b** Kaplan–Meier analysis of overall survival in all patients with low or high serum thrombospondin-2 (TSP-2) levels ($P < 0.0002$, log-rank test). **c** Kaplan–Meier analysis of overall survival in stage I/II patients with low or high serum TSP-2 levels. **d** Kaplan–Meier analysis of overall survival in stage III/IV patients with low or high TSP-2 levels

TABLE 4 Cox regression analysis of predictors of overall survival

	HR	95% CI	Multivariate *P* value
Age	1.466	1.113–1.931	0.006*
Sex	1.303	0.985–1.725	0.064
PDAC stage (I–II vs. III–IV)	3.055	2.093–4.459	0.000*
TSP-2, ng/ml (cutoff ≥ 29.8)	1.544	1.143–2.086	0.005*
CA19-9, U/ml (cutoff ≥ 62.0)	1.324	0.887–1.975	0.170

*$P < 0.05$

TSP-2 thrombospondin-2; *PDAC* pancreatic ductal adenocarcinoma; *HR* hazard ratio; *CI* confidence interval; *CA19-9* carbohydrate antigen 19-9

discrepancy probably arose, because we selected HRIs with a family history of PDAC instead of heathy individuals from the general population as our control group. The different TSP-2 cutoff value influences only the SN and does not change the SP (Table 2) of TSP-2 as a diagnostic marker for PDAC. Thus, the optimal cutoff value of TSP-2 for screening different high-risk populations might vary.

In addition to identifying TSP-2 as a diagnostic marker for PDAC, we found that a high serum level of TSP-2 was an independent, poor prognostic factor in PDAC patients. TSP-2 is an extracellular matrix glycoprotein involved in tumor progression and angiogenesis regulation.[17,25,26] TSP-2 is upregulated and is correlated with cell proliferation, migration, invasion, and bone metastasis in prostate cancer.[22] In addition, TSP-2 overexpression is an independent predictor of adverse outcomes in urothelial carcinomas.[27] These findings demonstrate that TSP-2 is associated with the malignant progression of cancer. Nevertheless, the role of TSP-2 in PDAC pathogenesis remains to be investigated.

This study has some limitations. First, the SN of TSP-2 alone as a diagnostic marker for PDAC is not acceptable, so TSP-2 needs to be combined with CA19-9 or another new biomarker to increase its SN for PDAC screening. Second, blood-based screening tests are most useful in high-risk populations where disease prevalence is higher. The targeted screening of individuals at risk for PDAC would reduce the screening burden and capacity demand. The first step in PDAC screening is to define the risk cohorts, including individuals with new-onset diabetes, chronic pancreatitis, and familial pancreatic cancer. In this study, only HRIs with a family history of PDAC were analyzed. However, the preliminary data show that serum TSP-2 levels in individuals with late-stage chronic

pancreatitis are similar to those in the HRIs and lower than those in the PDAC patients (*Supplementary data*). The diagnostic power of TSP-2 in other risk populations including patients with new-onset diabetes, chronic pancreatitis, and pancreatic introductory papillary mucinous neoplasm (IPMN) needs further study in a large cohort. Third, combining biomarkers is a potential strategy for the early detection of cancer; however, not all PDAC patients in this study were in the early stage. Last, this study is not a prospective study. There is much work to be done until the clinical value of TSP-2 as a diagnostic marker for PDAC can be validated in a prospective study of a high-risk population.

In summary, the serum TSP-2 level is an independent prognostic factor in PDAC. The combination of TSP-2 with CA19-9 is a blood-based biomarker for PDAC diagnosis with very high SP in both Western and Asian populations. The inclusion of TSP-2 also decreases the false positivity associated with the use of CA19-9 alone to screen for PDAC in HRIs with abnormal CA19-9 levels. The development of a combined biomarker panel that includes TSP-2, CA19-9, and/or another highly sensitive marker may facilitate future PDAC screening.

ACKNOWLEDGMENT Taiwan Pancreas Foundation; MOST106-2321-B-002-033 and 107-2321-B-002-013.

AUTHOR CONTRIBUTIONS Y-TC had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. *Study concept and design:* H-YP, M-CC, C-MH, W-HL, and Y-TC. *Acquisition, analysis, or interpretation of data:* H-YP, M-CC, C-MH, H-IY, and Y-TC. *Drafting of the manuscript:* H-YP and Y-TC. *Critical revision of the manuscript for important intellectual content:* W-HL and Y-TC. *Statistical analysis:* H-YP, M-CC, and H-IY. *Study supervision:* W-HL and Y-TC.

DISCLOSURE All authors indicate no potential conflicts of interest.

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