Co-expression of CD133, CD44v6 and human tissue factor is associated with metastasis and poor prognosis in pancreatic carcinoma

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Abstract. The metastasis-related molecules CD133, CD44v6 and human tissue factor (TF) have been shown to be associated with tumor invasion and metastasis. This study aimed to determine whether co-expression of these three molecules was associated with metastasis and overall prognosis in pancreatic carcinoma. We analyzed the expression profiles of these three molecules by immunohistochemistry and evaluated the relationship of their expression profiles with metastasis and prognosis in 109 pancreatic carcinomas. The results showed that the expression levels of CD133, CD44v6 and TF were increased in pancreatic carcinoma. Co-expression of CD133, CD44v6 and TF (tri-expression) was also detected in pancreatic carcinoma. Clinical analysis showed that individual expression of CD133, CD44v6 or TF was associated with vessel invasion, lymph node metastasis and liver metastasis, while tri-expression was associated with lymph node metastasis. Survival analysis showed that patients with co-expression of CD133 and TF or tri-expression had lower and the lowest overall survival rates, respectively. Univariate analysis showed that T-factor, lymph node metastasis, TNM stage, and individual levels or tri-expression of CD133, CD44v6 and TF were survival risk factors. Multivariate analysis showed that tri-expression of CD133, CD44v6 and TF was an independent predictor of survival. These results suggest that overexpression of CD133, CD44v6 and TF is associated with pancreatic carcinoma metastasis. Tri-expression of these three molecules may be a useful predictor for pancreatic carcinoma prognosis.

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Introduction

Pancreatic carcinoma is one of the most aggressive cancers. Early metastasis to regional lymph nodes and finally hematogenous spread to distant organs are the leading causes of the low 5-year survival rate (1,2). Previous studies have shown that various distinct proteins are involved in the different steps of cancer progression. In pancreatic carcinoma, CD133, CD44 and TF are distinct proteins involved in invasion and metastasis (3-5).

CD133, as an important marker of cancer stem cells (CSCs), has been used in the identification of CSCs from several solid tumors (6-12). A $CD44^+/CD133^+$ CSC population has been identified that exhibits extensive proliferation, self-renewal, differentiation and invasion (6,13). Recent studies have shown that $CD133^+$ circulating tumor cells (CTCs) are believed to be directly involved in the metastatic process of colon cancer (14). In pancreatic carcinoma, CD133 has been shown to be associated with lymph node metastasis (3).

CD44, a transmembrane glycoprotein, has multiple variant isoforms (CD44v2-10) (15,16). CD44 standard (CD44s) can be found in most tissues in the adult organism, including the hematopoietic system, whereas the variant isoforms are expressed in specific epithelial tissues and cancers (17,18). Recent studies have indicated that CD44v6, a metastatic marker, is unregulated in aggressive pancreatic cancer CSC subpopulations, and blockage of CD44v6 suppresses the metastasis of pancreatic carcinoma cells (19,20). There is ample evidence to show that a CD44 isoform, and specifically CD44v6 as an HA binding protein, or a co-receptor for c-Met and VEGFR-2 is crucial for the establishment of primary tumors as well as for metastasis (21). Interaction of CD44 with P-selectin facilitates tumor cell survive in circulation via binding to platelets, thereby making $CD44^+$ prone to be involved in distant metastases (22-24).

Human tissue factor (TF) is the cell surface receptor of factor VII (FVII), which is responsible for triggering blood coagulation (25,26). The expression of TF in tumors can alter the tumor microenvironment, thus facilitating the survival and metastasis of CSCs (27-30). A strong correlation between TF expression and hepatic metastasis, but not lymph node

metastasis, has been recognized in colorectal carcinoma patients (31). In pancreatic carcinoma, TF expression occurs preferentially at the invasive front of the tumor and is correlated with angiogenesis, lymph node and liver metastases and a poor prognosis (5,32).

Indeed, recent studies have shown that certain types of cancer cells expressing markers of CSCs (CD133) also exhibit elevated expression of TF or CD44 (28,30,33,34). However, the role of these three molecules in tumor metastasis is unclear. To determine whether tri-expression of these three molecules is associated with metastasis and prognosis in pancreatic carcinoma, we analyzed the expression profiles of these three molecules in pancreatic carcinoma tissues by immunohistochemistry and further evaluated the relationship of their expression profiles with metastasis and prognosis of pancreatic carcinoma.

Materials and methods

Patients and specimens. A total of 109 patients (71 male and 38 female) with a median age 58 years (range 36-86 years) underwent surgery at the Department of Hepatobiliary Surgery Institute, Southwest Hospital, Third Military Medical University, China, for pancreatic carcinoma from January 2007 to June 2010. All the patients underwent curative resection by pancreaticoduodenectomy and pylorus-preserving pancreaticoduodenectomy with lymph node dissection. None of the patients had received neoadjuvant or adjuvant radio/chemotherapy. Formalin-fixed paraffin-embedded samples were obtained for immunohistochemical analysis. The number of patients with pT1, pT2, pT3, and pT4 tumors was 19 (17.4%), 33 (30.3%), 53 (48.6%), and 4 (3.7%), respectively. Resected primary tumors and lymph nodes were histologically examined by hematoxylin and eosin staining using the TNM (tumor-node-metastasis) classification system. Histologically, all of the tumors were invasive ductal adenocarcinomas (9 well-differentiated, 73 moderately differentiated and 27 poorly differentiated). Lymph node metastasis and vascular invasion were observed in 44 (40.4%) and 37 tumors (33.9%), respectively. All patients were assessed by radiography, ultrasonography and computed tomography every 3 months after discharge. New lesions detected by imaging were considered indicative of relapse. The median follow-up period was 13 months (range 3-46 months). During this period, 14 patients experienced recurrence of liver disease.

This study was approved by the Ethics Committee of the Southwest Hospital, and all patients provided written informed consent.

Immunohistochemistry. Specimens were fixed in formalin, embedded in paraffin and cut into 3-mm sections. Sections were deparaffinized in xylene, rehydrated in a graded series of ethanol solutions and incubated in 3.0% hydrogen peroxide in methanol for 30 min to block endogenous peroxidase action. Slides were heated at 120˚C in an autoclave in 10 mM sodium citrate (pH 6.0) for 130 sec and cooled to room temperature. After blocking with 10% goat serum for 30 min, the sections were incubated overnight at 4˚C with primary antibodies for CD133 (rabbit polyclonal; Bioss; bs-0209R, dilution 1:100), CD44v6 (mouse monoclonal; Invitrogen; 33-6700, dilution 1:50) and TF (rabbit polyclonal, Boster; BA1714, dilution 1:100). Negative controls were obtained by omitting the primary antibody. The sections were incubated with peroxidase-conjugated anti-mouse/rabbit immunoglobulins (Dako EnVision™ System; K5007) for 60 min at 37˚C. The peroxidase reaction was developed with 3,3'-diaminobenzidine as the chromogen and counterstained with hematoxylin.

Criteria for assessing immunohistochemical results. All the immunostained sections were evaluated independently by two investigators without knowledge of the clinical or pathological backgrounds of the patients. Ten random fields were selected, and expression was evaluated in 1,000 tumor cells (100 cells per field) with an image analyzer (MetaMorph Imaging System version 6.0). The immunohistochemical grade was quantified according to the proportion of stained cells. Specimens were defined as having positive expression if there were tumor cells distinctly stained by anti-CD133, CD44v6 or TF antibody. The staining intensity of CD133 was scored as 3+ (>25%), 2+ (5-25%), 1+ (<5%) or 0 (0%) respectively, according to the percentage of positively stained cells (Fig. 1A, D and G) (3,35). Similarly, the staining intensity of CD44v6 was scored as 3+ (>50%), 2+ (10–50%), 1+ (<10%) or 0 (0%), respectively (Fig. 1B, E and H) (36). The staining intensity of TF was scored as 3+ (>66%), 2+ (33-66%), 1+ (<33%) or 0 (0%), respectively (Fig. 1C, F and I) (5). For statistical analysis, as well as to reduce intraobserver variability, the immunohistochemical scores were further grouped into two categories: low (grade 0 or 1+) or high (grade 2+ or 3+).

Statistical analyses. Group differences were statistically analyzed using the χ^2 test. The Kaplan-Meier method was used to analyze survival and the log-rank test was used to estimate differences in survival. Prognostic factors were examined using univariate and multivariate analyses (Cox proportional hazards regression model). During the Cox regression, Backward LR method was applied, and values of variables not in the equation were picked from step one. P-values <0.05 were considered statistically significant. Statistical analysis was performed using IBM SPSS Statistics 19. All the statistical analyses were completed under the guidance of experienced experts in the Statistics Department.

Results

Overexpression of CD133, CD44v6 and TF in pancreatic carcinoma. It has been shown that CD133, CD44v6 and TF play important roles in the process of tumor metastasis. To analyze the expression patterns of CD133, CD44v6 and TF in pancreatic carcinoma, we applied immunohistochemistry in 109 pancreatic carcinoma and 8 normal pancreatic samples. As shown in Table I and Fig. 2, compared with the normal pancreatic tissues, the expression of CD133 in the pancreatic carcinoma was low in 44 samples and high in 65 samples. Similarly, the expression of CD44v6 was low in 59 samples and high in 50 samples. Additionally, the expression of TF was low in 42 samples and high in 67 samples. Furthermore, 46/109 pancreatic carcinoma samples showed tri-expression of CD133, CD44v6 and TF and an additional 16/109 samples showed bi-expression of CD133 and TF but were CD44v6

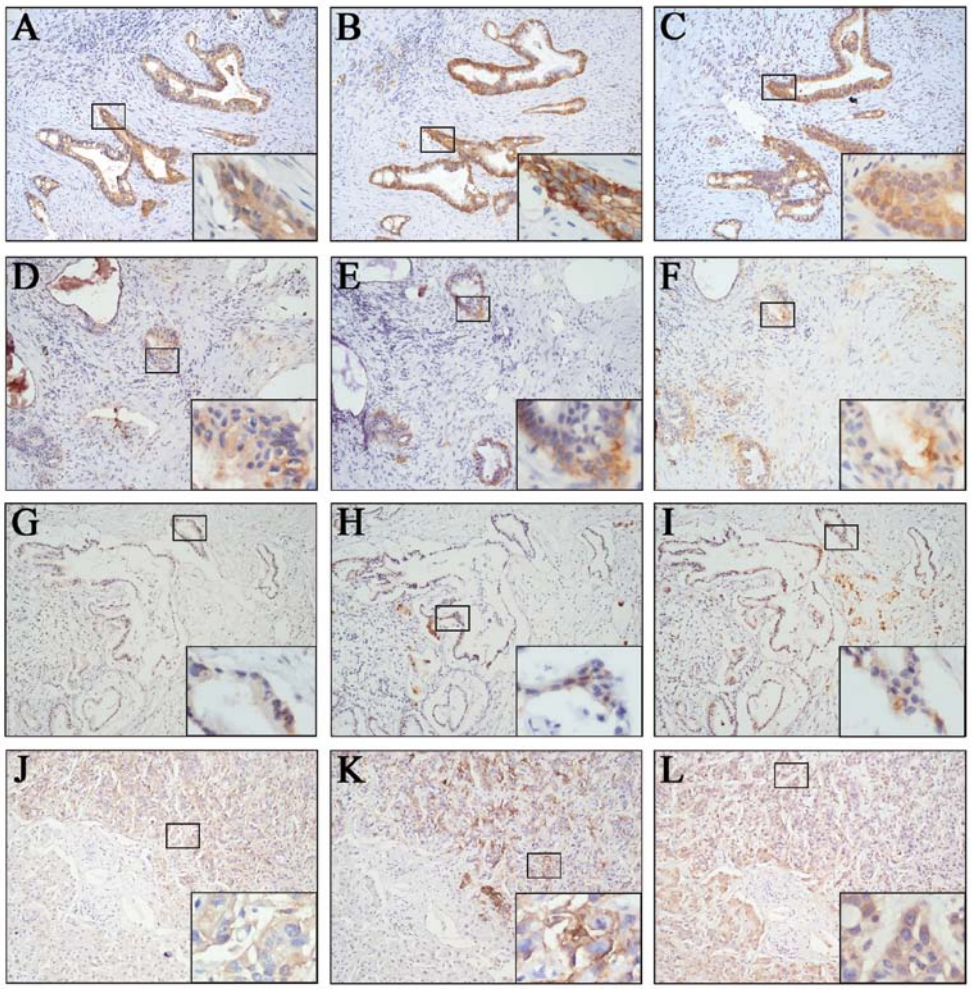

Figure 1. Immunohistochemical staining of CD133, CD44v6 and TF in pancreatic carcinoma. (A-I) Samples from primary pancreatic carcinoma. (J-L) Liver samples from metastatic pancreatic carcinoma. (A, D and G) are CD133-positive (3+), CD133-positive (2+) and CD133-positive (1+), respectively. (B, E and H) are CD44v6-positive (3+), CD44v6-positive (2+) and CD44v6-positive (1+), respectively. (C, F and I) are TF-positive (3+), TF-positive (2+) and TF-positive (1+), respectively. (J, K and L) are CD133-positive (3+), CD44v6-positive (3+) and TF-positive (3+), respectively. Original magnification, x100; x400 magnification is shown in the bottom right box.

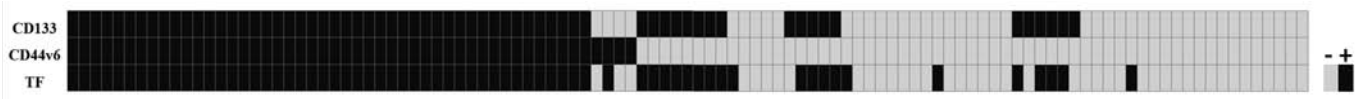

Figure 2. Schematic figure of the expression level of CD133, CD44v6 and TF. Each column represent a sample of pancreatic carcinoma (109 samples). The expression level is represented by the depth of shading: "-": low and "+": high.

low. Of the 46 tri-expression samples, 11 samples (23.9%) had hepatic metastases. However, of the 16 bi-expression samples, only 1 sample (7.1%) had hepatic metastasis (data not shown). These results suggest that the expression of CD133, CD44v6 and TF is increased in pancreatic carcinoma and tri-expression of these three molecules may be required for distant metastasis of pancreatic carcinoma.

Expression levels of CD133, CD44v6 and TF are correlated with vascular invasion and lymph node and liver metastases. To investigate whether CD133, CD44v6 and TF are involved in the metastasis of pancreatic carcinoma, we further analyzed the relationship between the clinical characteristics of the pancreatic carcinoma patients and the expression levels of CD133,

Table I. Expression of CD133, CD44v6, TF and their co-expression in the pancreatic carcinoma samples (n=109).

Variables	Expression level	
	Low	High
CD133	44	65
CD44v6	59	50
TF	42	67
CD133 + CD44v6		0
CD133 + TF		16
CD44v6 + TF		1
CD133 + CD44v6 + TF		46

Table II. Clinicopathological parameters and immunohistochemical labeling of CD44v6, CD133 and TF (n=109).

	CD133			CD44v6			TF		
Variables	High n (%)	Low n (%)	P-value χ^2-value	High n (%)	Low n (%)	P-value χ^2-value	High n (%)	Low n (%)	P-value χ^2-value
Gender			0.583			0.527			0.498
Female	24 (36.9)	14 (31.8)	0.301	19 (38.0)	19 (32.2)	0.400	25 (37.3)	13 (31.0)	0.460
Male	41 (63.1)	30 (68.2)		31 (62.0)	40 (67.8)		42 (62.7)	29 (69.0)	
Age, years			0.962			0.743			0.526
<65	47 (72.3)	32 (72.7)	0.002	37 (74.0)	42 (71.2)	0.107	50 (74.6)	29 (69.0)	0.403
≥65	18 (27.7)	12 (27.3)		13 (26.0)	17 (28.8)		17 (25.4)	13 (31.0)	
Tumor location			0.083			0.237			0.112
Head	53 (81.5)	41 (93.2)	2.997	41 (82.0)	53 (89.8)	1.398	55 (82.1)	39 (92.9)	2.522
Body/Tail	12 (18.5)	3 (6.8)		9 (18.0)	6 (10.2)		12 (17.9)	3 (7.1)	
Tumor size, cm			0.908			0.805			0.702
≤2	20 (30.8)	14 (31.8)	0.013	15 (30.0)	19 (32.2)	0.061	20 (29.9)	14 (33.3)	0.146
>2	45 (69.2)	30 (68.2)		35 (70.0)	40 (67.8)		47 (70.1)	28 (66.7)	
Lymph node status			**0.002**			**0.002**			**0.001**
Negative	31 (47.7)	34 (77.3)	9.538	22 (44.0)	43 (72.9)	9.378	32 (47.8)	33 (78.6)	10.181
Positive	34 (52.3)	10 (22.7)		28 (56.0)	16 (27.1)		35 (52.2)	9 (21.4)	
Vascular invasion			**0.004**			**0.041**			**0.000**
Negative	36 (55.4)	36 (81.8)	8.177	28 (56.0)	44 (74.6)	4.165	35 (52.2)	37 (88.1)	14.803
Positive	29 (44.6)	8 (18.2)		22 (44.0)	15 (25.4)		32 (47.8)	5 (11.9)	
Neural invasion			0.261			0.302			0.792
Negative	41 (63.1)	23 (52.3)	1.264	32 (64.0)	32 (54.2)	1.064	40 (59.7)	24 (57.1)	0.070
Positive	24 (36.9)	21 (47.7)		18 (36.0)	27 (45.8)		27 (40.3)	18 (42.9)	
Duodenal invasion			0.588			0.28			0.881
Negative	52 (80.0)	37 (84.1)	0.293	43 (86.0)	46 (78.0)	1.166	55 (82.1)	34 (81.0)	0.022
Positive	13 (20.0)	7 (15.9)		7 (14.0)	13 (22.0)		12 (17.9)	8 (19.0)	
Hepatic metastases			**0.033**			**0.003**			**0.046**
Negative	53 (81.5)	42 (95.5)	4.539	39 (76.5)	56 (96.0)	8.728	55 (82.1)	40 (95.2)	3.987
Positive	12 (18.5)	2 (4.5)		12 (23.5)	2 (3.4)		12 (17.9)	2 (4.8)	
Differentiation			0.168			**0.025**			0.298
Poor	20 (30.8)	7 (15.9)	3.566	18 (36.0)	9 (15.3)	7.400	20 (29.9)	7 (16.7)	2.421
Moderate	41 (63.1)	32 (72.7)		30 (60.0)	43 (72.9)		42 (62.7)	31 (73.8)	
Well	4 (6.2)	5 (11.4)		2 (4.0)	7 (11.9)		5 (7.5)	4 (9.5)	
T-factor (UICC)			0.388			0.671			0.306
T1	11 (16.9)	8 (18.2)	3.023	9 (18.0)	10 (16.9)	1.549	12 (17.9)	7 (16.7)	3.614
T2	16 (24.6)	17 (38.6)		14 (28.0)	19 (32.2)		16 (23.9)	17 (40.5)	
T3	35 (53.8)	18 (40.9)		24 (48.0)	29 (49.2)		36 (53.7)	17 (40.5)	
T4	3 (4.6)	1 (2.3)		3 (6.0)	1 (1.7)		3 (4.5)	1 (2.4)	
Stage (UICC)			**0.002**			**0.004**			**0.001**
1A	3 (4.6)	7 (15.9)	19.132	2 (4.0)	8 (13.6)	17.479	3 (4.5)	7 (16.7)	20.563
1B	7 (10.8)	14 (31.8)		6 (12.0)	15 (25.4)		7 (10.4)	14 (33.3)	
2A	14 (21.5)	12 (27.3)		8 (16.0)	18 (30.5)		15 (22.4)	11 (26.2)	
2B	27 (41.5)	8 (18.2)		21 (42.0)	14 (23.7)		28 (41.8)	7 (16.7)	
3	1 (1.5)	1 (2.3)		1 (2.0)	1 (1.7)		1 (1.5)	1 (2.4)	
4	13 (20.0)	2 (4.5)		12 (24.0)	3 (5.1)		13 (19.4)	2 (4.8)	

Bold values indicate P-values <0.05. UICC, Union for International Cancer Control.

Table III. Clinicopathological parameters and immunohistochemical labeling of bi-expression of CD133 + TF and tri-expression of CD133, CD44v6 and TF (n=109).

	CD133 + TF			CD44v6 + CD133 + TF		
Variables	High n (%)	Low n (%)	P-value χ^2-value	High n (%)	Low n (%)	P-value χ^2-value
Gender			0.574			0.228
Female	23 (37.1)	15 (31.9)	0.316	19 (41.3)	19 (30.2)	1.454
Male	39 (62.9)	32 (68.1)		27 (58.7)	44 (69.8)	
Age, years			0.645			0.774
<65	46 (74.2)	33 (70.2)	0.212	34 (73.9)	45 (71.4)	0.082
≥65	16 (25.8)	14 (29.8)		12 (26.1)	18 (28.6)	
Tumor location			0.166			0.133
Head	51 (82.3)	43 (91.5)	1.920	37 (80.4)	57 (90.5)	2.259
Body/Tail	11 (17.7)	4 (8.5)		9 (19.6)	6 (9.5)	
Tumor size, cm			0.576			0.572
≤2	18 (29.0)	16 (34.0)	0.313	13 (28.3)	21 (33.3)	0.319
>2	44 (71.0)	31 (66.0)		33 (71.7)	42 (66.7)	
Lymphatic invasion			**0.000**			**0.000**
Negative	28 (45.2)	37 (78.7)	12.510	18 (39.1)	47 (74.6)	13.898
Positive	34 (54.8)	10 (21.3)		28 (60.9)	16 (25.4)	
Vascular invasion			**0.001**			**0.027**
Negative	33 (53.2)	39 (83.0)	10.555	25 (54.3)	47 (74.6)	4.865
Positive	29 (46.8)	8 (17.0)		21 (45.7)	16 (25.4)	
Neural invasion			0.308			0.433
Negative	39 (62.9)	25 (53.2)	1.040	29 (63.0)	35 (55.6)	0.615
Positive	23 (37.1)	22 (46.8)		17 (37.0)	28 (44.4)	
Duodenal invasion			0.755			0.470
Negative	50 (80.6)	39 (83.0)	0.097	39 (84.8)	50 (79.4)	0.521
Positive	12 (19.4)	8 (17.0)		7 (15.2)	13 (20.6)	
Hepatic metastases			**0.020**			**0.003**
Negative	50 (80.6)	45 (95.7)	5.445	35 (76.1)	60 (95.2)	8.711
Positive	12 (19.4)	2 (4.3)		11 (23.9)	3 (4.8)	
Differentiation			0.105			**0.022**
Poor	20 (32.3)	7 (14.9)	4.515	17 (37.0)	10 (15.9)	7.672
Moderate	38 (61.3)	35 (74.5)		25 (54.3)	46 (73.0)	
Well	4 (6.5)	5 (10.6)		2 (4.3)	7 (11.1)	
T-factor (UICC)			0.522			0.381
T1	10 (16.1)	9 (19.1)	2.250	9 (19.6)	10 (15.9)	3.067
T2	16 (25.8)	17 (36.2)		11 (23.9)	22 (34.9)	
T3	33 (53.2)	20 (42.6)		23 (50.0)	30 (47.6)	
T4	3 (4.8)	1 (2.1)		3 (6.5)	1 (1.6)	
Stage (UICC)			**0.000**			**0.000**
1A	2 (3.2)	8 (17.0)	22.836	2 (4.3)	8 (12.7)	28.584
1B	7 (11.3)	14 (29.8)		3 (6.5)	18 (28.6)	
2A	12 (19.4)	14 (29.8)		7 (15.2)	19 (30.2)	
2B	27 (43.5)	8 (17.0)		27 (58.7)	14 (22.2)	
3	1 (1.6)	1 (2.1)		1 (2.2)	1 (1.6)	
4	13 (21.0)	2 (4.3)		12 (26.1)	3 (4.8)	

Bold values indicate P-values <0.05. UICC, Union for International Cancer Control.

Table IV. Median survival and 1-, 2- and 3-year survival rates and CD133, CD44v6, TF and their co-expression.

	CD133		CD44v6		TF		CD133 + TF		CD44v6 + CD133 + TF	
Variables	Low	High	Low	High	Low	High	Low	High	Low	High
Median survival time (months)	14	11	13	9	13	11	14	11	14	9
1-year survival rate (%)	67.6	41.9	55.6	31.0	56.8	35.8	59.0	33.2	58.1	25.3
2-year survival rate (%)	25.5	12.7	24.6	10.3	25.5	12.6	26.8	11.1	25.0	8.4
3-year survival rate (%)	11.3	3.2	8.9	3.9	14.2	0.0	8.0	0.0	10.4	0.0

Table V. Univariate and multivariate Cox regression of prognostic factors for overall survival in pancreatic adenocarcinoma.

	Univariate P	Multivariate P	
Independent factors	P-value	HR (95% CI)	P-value
Age (years <65/≥65)	0.281		
Gender (female/male)	0.944		
Tumor size (<2/≥2 cm)	0.860		
pN (negative/positive)	**0.000**	1.642 (1.009-2.674)	**0.046**
Pv (negative/positive)	**0.024**	1.354 (0.646-2.835)	0.422
Hepatic metastases (negative/positive)	**0.028**	1.190 (0.430-3.293)	0.737
Differentiation (poor/moderate/well)	0.672		
pT (T1,2/T3,4)	**0.017**	0.984 (0.471-2.058)	0.966
pStage (I,II/III,IV)	**0.000**	1.652 (1.081-2.524)	**0.020**
CD44v6 expression (low/high)	**0.009**	0.968 (0.278-3.372)	0.960
CD133 expression (low/high)	**0.013**	1.008 (0.227-4.486)	0.992
TF expression (low/high)	**0.018**	0.726 (0.165-3.194)	0.672
CD133 + TF expression (low/high)	**0.005**	1.305 (0.149-11.435)	0.810
CD44v6 + CD133 + TF expression (low/high)	**0.000**	1.774 (1.102-2.854)	**0.018**

Bold values indicate P-values <0.05. HR, hazard ratio; CI, confidence interval; pN, pathological node stage; pV, pathological vessel status; pT, T-factor; pStage, TNM stage (UICC).

CD44v6 and TF. As shown in Table II, overexpression of CD133 was correlated with vascular invasion (P=0.004, χ^2=8.177), lymph node metastasis (P=0.002, χ^2=9.538), hepatic metastasis (P=0.033, χ^2=4.539) and TNM stage (P=0.002, χ^2=19.132). Overexpression of CD44v6 was correlated with vascular invasion (P=0.041, χ^2=4.165), lymph node metastasis (P=0.002, χ^2=9.378), hepatic metastasis (P=0.003, χ^2=8.728) and TNM stage (P=0.004, χ^2=17.479). Overexpression of TF was correlated with vascular invasion (P=0.000, χ^2=14.803), lymph node metastasis (P=0.001, χ^2=10.181), hepatic metastasis (P=0.046, χ^2=3.987) and TNM stage (P=0.001, χ^2=20.563). As shown in Table III, co-expression of CD133 and TF was correlated with vascular invasion (P=0.001, χ^2=10.555), lymph node metastasis (P=0.000, χ^2=12.510) and hepatic metastasis (P=0.020, χ^2=5.445). The tri-expression of CD133, CD44v6 and TF was correlated with vascular invasion (P=0.027, χ^2=4.865), lymph node metastasis (P=0.000, χ^2=13.898), hepatic metastasis (P=0.003, χ^2=8.711) and TNM stage (P=0.000, χ^2=28.584), but showed greater differences in metastases to the lymph nodes and the liver. Notably, further analysis found that tri-expression and bi-expression had similar rates of lymph node metastasis and vascular invasion. Yet, in the 46 tri-expression samples, 11 samples (23.9%) had hepatic metastases. In contrast, in the 14 bi-expression samples, only 1 sample (7.1%) had hepatic metastasis. These results indicate that the expression levels of CD133, CD44v6 and TF are correlated with vascular invasion, lymph node metastasis and hepatic metastasis in pancreatic carcinoma and the co-expression of these three molecules in pancreatic carcinoma may imply a poorer prognosis.

Individual expression or tri-expression of the three molecules indicates a poor prognosis in pancreatic carcinoma patients. We further analyzed whether CD133, CD44v6 and TF expression levels affect the 1-year survival rate, median survival time and overall survival of pancreatic carcinoma patients as analyzed using Kaplan-Meier survival analyses. As shown in Table IV, patients with overexpression of CD133 had lower

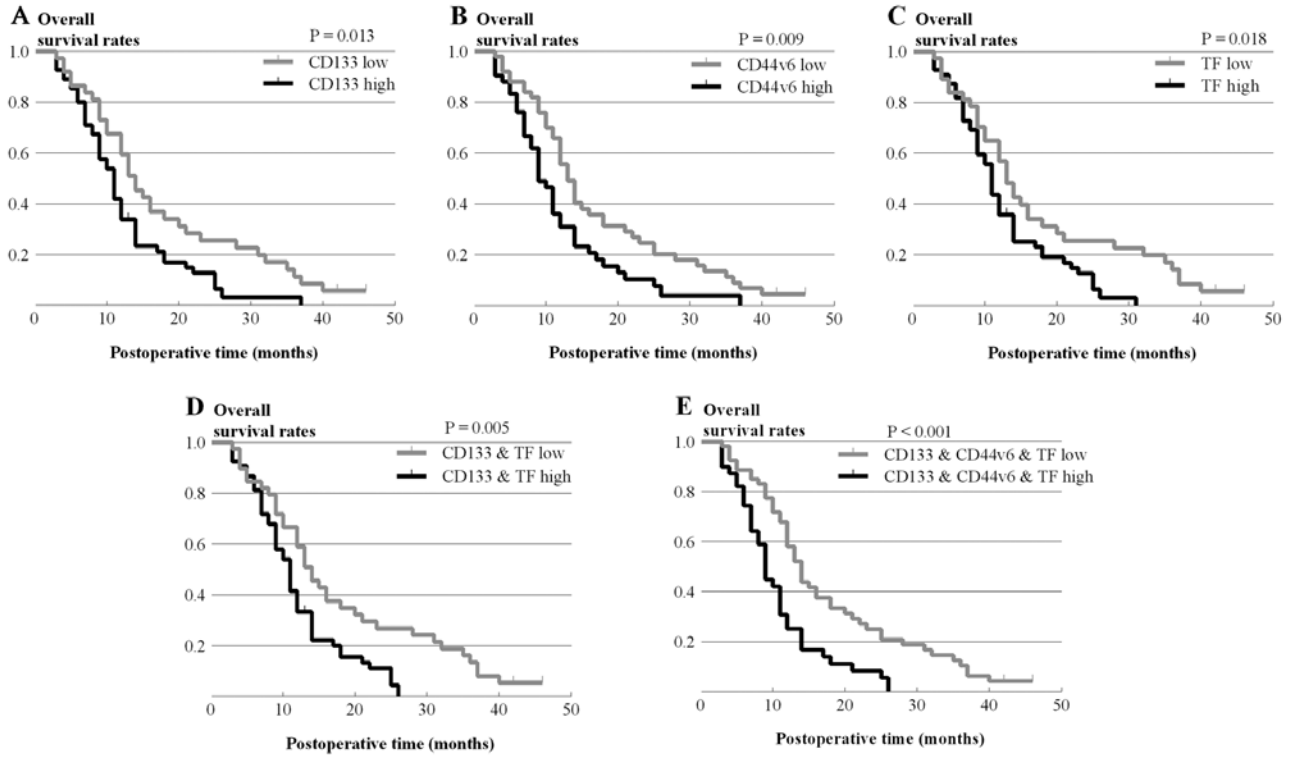

Figure 3. Kaplan-Meier survival curves for overall survival of patients with pancreatic carcinoma according to the expression status of (A) CD133, (B) CD44v6, (C) TF, (D) bi-expression of CD133 and TF, (E) tri-expression of CD133, CD44v6 and TF.

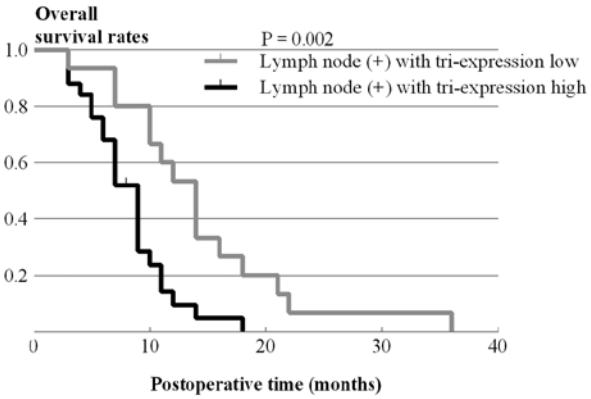

Figure 4. Kaplan-Meier survival curve of patients with lymph node metastasis stratified by tri-expression low vs. tri-expression high.

1-year, 2-year and 3-year survival rates and a shorter median survival time than patients with low expression of CD133 (67.6 vs. 41.9%, 25.5 vs. 12.7%, 11.3 vs. 3.2% and 14 vs. 11 months, respectively). Patients with overexpression of two or three molecules had even lower survival rates and shorter median survival times.

Kaplan-Meier survival curves showed that the individual overexpression of CD133, CD44v6 or TF significantly decreased overall survival (P=0.013, χ^2=6.217; P=0.009, χ^2=6.756; P=0.018, χ^2=5.622, respectively) (Fig. 3A-C). Additionally, co-expression of CD133 and TF was also associated with lower overall survival (P=0.005, χ^2=7.964) (Fig. 3D). Furthermore, patients with overexpression of all three molecules had the lowest overall survival (P=0.001, χ^2=12.021) (Fig. 3E). These data suggest that individual expression or co-expression of these three molecules indicate a poorer prognosis in pancreatic carcinoma patients.

Co-expression of CD133, CD44v6 and TF is an independent predictor of survival in pancreatic carcinoma patients. To further identify the independent risk factors of survival, we applied univariate and multivariate analyses to investigate the survival rate for pancreatic adenocarcinoma. In the univariate analysis, we found that age, gender and differentiation were not risk factors of survival. However, T-factor (pT), lymph node metastasis (pN), vascular invasion (pV), TNM stage and individual expression or co-expression of CD133, CD44v6 and TF were all risk factors for survival (Table V). Multivariate analysis showed that lymph node metastasis (pN) and TNM stage were independent predictors of survival rate (P=0.046 and P=0.020, respectively). Yet, the individual expression levels of CD133, CD44v6 and TF were not independent predictors. Importantly, the co-expression of CD133, CD44v6 and TF was also found to be an independent predictor of survival (P=0.018) (Table V). Therefore, although CD133, CD44v6 and TF all had an effect on the survival rate, only co-expression of CD133, CD44v6 and TF was found to be an independent predictor of survival in pancreatic carcinoma patients.

Discussion

We first characterized the expression patterns of three tumor metastasis-related molecules, CD44v6, CD133 and TF, in 109 pancreatic carcinoma samples using immunohistochemistry. In this study, we applied antibodies that specifically reacted with CD44-v5, v6 and v7/v8, respectively. We found that,

except for CD44v6, the other CD44 variants were all negative in the pancreatic carcinoma samples (data not shown). Therefore, among the different splice variants of CD44, CD44v6 is most likely a reliable marker in pancreatic carcinoma (20). Our study showed that the individual expression levels of CD44v6, CD133 and TF were increased in pancreatic carcinoma compared with normal pancreas. These results are consistent with previous studies that have shown that CD44v6, CD133 and TF are increased in pancreatic carcinoma and are associated with metastasis (3,32,37).

CD133, CD44v6 and TF have been shown to play a very important role in the process of metastasis (3,37,38). CD133 and CD44v6 are markers of CSCs (11,39,40). Previous studies have shoen that a subset of CSCs with $CD133^+/CD44^+$ has stronger invasive and metastatic capabilities (33,41). TF is unregulated in many cancer cells and impacts tumor progression by altering the tumor microenvironment (27,28,42). Various recent reports have shown that either cancer cells with increased expression of TF or cancer cells with the CSC marker CD133 are important in cancer progression (43,44). However, the relationship between tri-expression of $CD133^+/CD44^+/TF^+$ and tumor progression are still unknown. In the present study, we found that tri-expression of CD133, CD44v6 and TF may be involved in the metastasis of pancreatic carcinoma. First, 46/109 pancreatic carcinoma samples showed tri-expression of CD133, CD44v6 and TF and an additional 16/109 samples showed bi-expression of CD133 and TF but were CD44v6 low. Of the 46 tri-expression samples, 11 (23.9%) presented with hepatic metastasis. However, of the 14 bi-expression samples, only 1 (7.1%) presented with hepatic metastasis, suggesting that tri-expression of these three molecules may be associated with distant metastasis of pancreatic carcinoma. Indeed, we found strong tri-expression of these three molecules in liver samples from metastasis of pancreatic carcinoma (Fig. 1J-L). Furthermore, the tri-expression of these three molecules showed stronger association with tumor metastasis compared with each molecule individually. These findings may suggest that TF probably alters the tumor microenvironment and facilitates survival and metastasis of pancreatic carcinoma $CD133^+/CD44v6^+$ CSCs. Therefore, tri-expression of these three molecules may imply a poor prognosis for pancreatic carcinoma patients.

It has been shown that many invasion-related molecules are implicated in the prognosis of pancreatic carcinoma. In our study, univariate analysis showed that T-factor (pT), lymph node metastasis (pN), vascular invasion (pV), TNM stage, individual CD133, CD44v6 and TF expression levels, bi-expression and tri-expression were all significantly correlated with patient survival. In this study, although bi-expression, tri-expression and individual CD133, CD44v6 and TF expression showed similar patient survival curves, bi-expression and tri-expression were associated with reduced survival times in comparison with individual CD133, CD44v6 and TF expression. The 'heat map' of expression indicated that these three markers were frequently co-expressed and that bi-expression or even uni-expression were uncommon, suggesting that tri-expression of these three markers may be more important in the prognosis of pancreatic carcinoma. Consistent with the above results, multivariate analysis also showed that tri-expression was an independent prognostic factor. Therefore, tri-expression of CD133, CD44v6 and TF has significant predictive value for overall survival and a poor prognosis. Therefore, we believe that tri-expression is important in predicting prognosis in pancreatic carcinoma patients. In fact, among patients with lymph node metastasis, patients with tri-expression tumors had a markedly poorer prognosis (P=0.002) (Fig. 4). Thus, our findings suggest that tri-expression of these three molecules correlates with the aggressiveness of the pancreatic carcinoma. Taken together, we demonstrated that three tumor metastasis-related molecules, CD133, CD44v6 and TF were overexpressed in pancreatic carcinoma and were associated with tumor metastasis and we initially detected the internal relationships among these three molecules in pancreatic carcinoma tissues. Tri-expression of these three molecules may be a useful predictor for pancreatic carcinoma prognosis. This novel finding may provide insight into a novel metastatic mechanism for pancreatic carcinoma.

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