CD44 Predicts Early Recurrence in Pancreatic Cancer Patients Undergoing Radical Surgery

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Abstract. *Background/Aim:* Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive types of digestive cancer. Recurrence within one year after surgery is inevitable in most PDAC patients. Recently, cluster of differentiation 44 (CD44) has been shown to be associated with tumor initiation, metastasis and prognosis. This study aimed to explore the correlation of CD44 expression with clinicopathological factors and the role of CD44 in predicting early recurrence (ER) in PDAC patients after radical surgery. *Materials and Methods:* PDAC patients who underwent radical resection between January 1999 and March 2015 were enrolled in this study. Tumor recurrence within 6 months after surgery was defined as ER. Immunohistochemical staining was performed with anti-CD44 antibodies. The association between clinicopathological parameters and CD44 expression was analyzed. Predictors for ER were also assessed with univariate and multivariate analyses. *Results:* Overall, 155 patients were included in this study. Univariate analysis revealed CA19-9 levels ($p=0.014$), CD44 histoscores (H-scores; $p=0.002$), differentiation ($p=0.010$), nodal status ($p=0.005$), stage ($p=0.003$), vascular invasion ($p=0.007$), lymphatic invasion ($p<0.001$) and perineural invasion ($p=0.042$) as risk factors for ER. In multivariate analysis, high CA19-9 levels and CD44 H-scores and poor differentiation independently

predicted ER. *Conclusion:* High CA19-9 levels, CD44 H-scores and poor differentiation are independent predictors for ER in PDAC patients undergoing radical resection. Therefore, the determination of CD44 expression might help in identifying patients at a high risk of ER for more aggressive treatment after radical surgery.

Pancreatic ductal adenocarcinoma (PDAC) is the fourth most common cause of cancer-related death worldwide, with an 8% 5-year survival rate for all stages of disease (1). Although various treatment modalities are available, only radical tumor resection can potentially provide a curative option for PDAC patients (2, 3). Nonetheless, due to the aggressive nature of the disease, recurrence within 1 year after surgery might be inevitable for many patients. Our previous study showed the 3-year and 5-year overall survival rates were 21.4% and 10.1%, respectively (4).

Cancer stem cells (CSCs), a subpopulation of tumor cells, are responsible for tumor initiation, growth, metastasis, and resistance to chemotherapy (5). Pancreatic CSCs have been identified by flow cytometry using cell markers, including cluster of differentiation 44 (CD44), CD24, epithelial-specific antigens, CD133, aldehyde dehydrogenase 1, and c-Met (5, 6). Among these markers, CD44 is a transmembrane glycoprotein receptor that binds hyaluronic acid (HA). The CD44-HA complex effectively plays an important role in cellular survival, differentiation, adhesion, and migration (7-9). Applying CD44 as a biomarker could effectively help in identifying CSCs in human cancers including breast, stomach, head and neck, pancreatic and other cancer (9, 10). Inhibition of CD44 expression can slow pancreatic cancer growth, invasion and recurrence rate (11, 12). To the best of our knowledge, no study has reported an association between CD44 expression and early recurrence (ER) in PDAC patients undergoing radical surgery. The aim of the present study was to analyze CD44 expression and clinicopathological factors for predicting ER in PDAC patients after radical resection.

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Materials and Methods

The records of PDAC patients undergoing surgical resection between January 1999 and March 2015 at the Chang Gung Memorial Hospital, Linkou, Taiwan were retrospectively reviewed. After the exclusion of 8 PDAC patients with hospital mortality, 62 patients with positive resection margins, and 11 patients with follow-up period shorter than 6 months, a total of 155 patients were recruited in this study. Tumor relapse within 6 months after surgery was defined as ER. The follow-up duration was measured from the time of surgery until death or the last follow-up time (December 2016). Clinicopathological data including sex, age, serum albumin levels, tumor markers such as carcinoembryonic antigen (CEA) and carbohydrate antigen (CA) 19-9, type of operation, tumor size, tumor location, differentiation, stage, vascular invasion, lymphatic invasion, perineural invasion, surgical complications, and the administration of chemotherapy or radiotherapy were reviewed. The tumor was staged according to the 8th edition of the American Joint Committee on Cancer (AJCC). This study was approved by the institutional review board of the Chang Gung Memorial Hospital (No. 104-2308B).

Immunohistochemistry. Immunohistochemistry for CD44 was performed on formalin-fixed paraffin-embedded tissues. A single representative block from each tumor was sectioned at 3- μ m thickness and placed onto positively charged slides. The slides were then stained using a Bond-Max Autostainer (Leica Biosystems, Heidelberg, Germany). Briefly, the slides were dewaxed in Bond Dewax solution (Leica Biosystems) and hydrated in Bond Wash solution (Leica Biosystems). Antigen retrieval was performed at an alkaline pH of 9 using Epitope Retrieval 2 solution (Leica Biosystems) for 20 min at 100°C. The slides were then incubated with the primary antibody at a concentration of 1:100 for 30 min at room temperature. The Bond Polymer Refine Detection (DS9800) kit was used for detection, with incubation with post primary for 8 min, polymer for 8 min, DAB for 5 min and hematoxylin for 5 min.

Assessment of immunohistochemical staining. The staining results were independently scored by two of the authors (L.Y.L. and T.C.C.) who were blinded to all of the clinicopathological variables (Figure 1). To assess the expression of CD44 immunostaining, a semiquantitative histoscore (H-score) was used. The intensity of cytoplasmic and membranous immunostaining was scored on a scale of 0 (no staining) to 3 (strongest intensity), and the percentage of stained cells was estimated at each intensity. The percentage of cells (from 0 to 100) was multiplied by the corresponding immunostaining intensity (from 0 to 3) to obtain an H-score in the range of 0 to 300.

Treatment protocols. For tumors in the head or uncinate process of the pancreas, the standard treatment is classic or pylorus-preserving pancreaticoduodenectomy. Subtotal or distal pancreatectomy with splenectomy is performed if the tumor is located at the body or tail of the pancreas. In our study, medically fit patients usually received chemotherapy with fluoropyrimidine-based, gemcitabine or gemcitabine plus cisplatin or S-1 regimens 6-8 weeks after surgery. Selected patients underwent intraoperative radiotherapy or postoperative external beam radiation therapy coupled with gemcitabine or 5-fluorouracil.

Postoperative follow-up evaluation. The regular postoperative follow-up evaluation included physical examination, blood chemistry tests and tumor marker analysis (CEA and CA19-9). Imaging studies including abdominal ultrasonography and computerized tomography/magnetic resonance imaging were performed every 3-6 months during the first 2 years after surgery and every 6-12 months thereafter. Tumor recurrence was defined by clinical judgement based on laboratory tests, imaging findings, tissue analysis or patients' outcomes. The median follow-up time in this study was 16.1 months.

Statistical analysis. Baseline characteristics of the two outcome groups were compared using Pearson's chi-square or Fisher's exact tests. Any significant variable ($p < 0.05$) on univariate analysis was included in multivariate models for logistic regression analysis. Survival curves were depicted by the Kaplan-Meier method and compared using the log-rank test. The optimal cutoff value of CD44 H-score was defined by analyzing the receiver operating characteristic curve in terms of ER after surgery. A value of $p < 0.05$ was considered significant. All statistical analyses were performed using SPSS for Windows version 20.0 (SPSS Inc., Chicago, IL, USA).

Results

The clinicopathological data are summarized in Table I. There were 88 (56.8%) male and 67 (43.2%) female patients. The median age of patients was 63.1 years. The median values of CEA, CA19-9 and CD44 H-score were 3.0, 158.6 and 22.0, respectively. Ninety-six (61.9%) patients underwent classic Whipple procedure, and 38 (24.5%) pylorus-preserving pancreaticoduodenectomy. The median tumor size was 3.3 cm. Stage I, II and III diseases were noted in 51, 86 and 18 patients, respectively. Sixty-six (42.6%) patients experienced surgical complications. Postoperative chemotherapy and radiotherapy were administered to 102 (65.8%) and 11 (7.1%) patients, respectively.

Table II shows the results of univariate analysis of various clinicopathological factors associated with ER in PDAC patients undergoing radical resection. CA19-9 levels, CD44 H-scores, differentiation, nodal status, stage, and presence of vascular, lymphatic, and perineural invasion significantly affected ER. No significant differences in recurrence time were observed irrespective of sex, age, albumin levels, CEA values, type of operation, tumor location, T status, surgical complications, or the administration of postoperative chemotherapy or radiation. In multivariate analysis, the independent predictors for ER were CA19-9 levels, CD44 H-scores and differentiation (Table III).

Discussion

PDAC is known for its aggressive tumor behavior such as local invasion, early metastasis, and poor prognosis. Recurrence within 1 year after surgery is inevitable and frequent for most patients. The present study is the first to

Figure 1. Representative immunohistochemistry image for CD44 staining in PDAC tissue. A-1: high power view of negative staining (H-score=0). A-2: scan view of negative staining. B-1: high power view of positive staining (H-score=270). B-2: scan view of positive staining.

Table I. Demographic characteristics of patients undergoing radical surgery.

Parameters	Patient number or median value	%
Gender		
Male	88	56.8
Female	67	43.2
Age (years), median (IQR)	62 (18)	
Albumin (g/dl), median (IQR)	3.9 (0.8)	
CEA (ng/ml), median (IQR)	3.0 (3.8)	
CA19-9 (U/ml), median (IQR)	158.6 (495.0)	
CD44 H-score, median (IQR)	20.0 (55.0)	
Type of operation		
Whipple	96	62.0
PPPD	38	24.5
Other	21	13.5
Tumor size (cm), median (IQR)	3.0 (1.5)	
Tumor location		
Head	118	76.1
Uncinate process	18	11.6
Body/tail	19	12.3
Differentiation		
No	127	81.9
Yes	28	18.1
T status		
T1	5	3.2
T2	21	13.5
T3	129	83.3
N status		
Negative	58	37.4
Positive	97	62.6
Stage		
I	51	32.9
II	86	55.5
III	18	11.6
Vascular invasion*		
No	114	75.0
Yes	38	25.0
Lymphatic invasion*		
No	80	52.6
Yes	72	47.4
Perineural invasion*		
No	36	23.7
Yes	116	76.3
Surgical complications		
No	89	57.4
Yes	66	42.6
Chemotherapy		
No	53	34.2
Yes	102	65.8
Radiotherapy		
No	144	92.9
Yes	11	7.1

IQR: Interquartile range; CA19-9: carbohydrate antigen 19-9; CEA: carcinoembryonic antigen; CD44: cluster of differentiation 44; H-score: histoscore; PPPD: pylorus-preserving pancreaticoduodenectomy. *Not all data were available.

Table II. Clinicopathological features of patients in terms of recurrence time.

Parameters	Recurrence ≤6 months (n=61)	Recurrence >6 months (n=94)	p-Value
Gender			0.075
Male	40 (65.6)	48 (51.1)	
Female	21 (34.4)	46 (48.9)	
Age (years)			0.465
≤65	38 (62.3)	53 (56.4)	
>65	23 (37.7)	41 (43.6)	
Albumin (g/dl)			0.352
≤3.5	11 (19.6)	23 (26.4)	
>3.5	45 (80.4)	64 (73.6)	
CEA (ng/ml)			0.770
≤5	41 (74.5)	60 (72.3)	
>5	14 (25.5)	23 (27.7)	
CA19-9 (U/ml)			0.014
≤37	9 (15.0)	30 (33.0)	
>37	51 (85.0)	61 (67.0)	
CD44 H-score			0.002
≤32	27 (44.3)	65 (69.1)	
>32	34 (55.7)	29 (30.9)	
Type of operation			0.227
Whipple	36 (59.1)	60 (63.8)	
PPPD	19 (31.1)	19 (20.2)	
Others	6 (9.8)	15 (16.0)	
Tumor location			0.188
Head	46 (75.4)	72 (76.6)	
Uncinate process	10 (16.4)	8 (8.5)	
Body/tail	5 (8.2)	14 (14.9)	
Differentiation			0.010
No	56 (91.8)	71 (75.5)	
Yes	5 (8.2)	23 (24.5)	
T status			0.058
T1	8 (13.1)	18 (19.1)	
T2	37 (60.7)	65 (69.2)	
T3	16 (26.2)	11 (11.7)	
N status			0.005
N0	14 (23.0)	44 (46.9)	
N1	36 (59.0)	43 (45.7)	
N2	11 (18.0)	7 (7.4)	
Stage			0.003
I	11 (18.0)	40 (42.6)	
II	39 (64.0)	47 (50.0)	
III	11 (18.0)	7 (7.4)	
Vascular invasion*			0.007
No	38 (63.3)	76 (82.6)	
Yes	22 (36.7)	16 (17.4)	
Lymphatic invasion*			<0.001
No	21 (35.0)	59 (64.1)	
Yes	39 (65.0)	33 (35.9)	
Perineural invasion*			0.042
No	9 (15.0)	27 (29.3)	
Yes	51 (85.0)	65 (70.7)	
Surgical complications			0.095
No	30 (49.2)	59 (62.8)	
Yes	31 (50.8)	35 (37.2)	
Chemotherapy			0.322
No	18 (29.5)	35 (37.2)	
Yes	43 (70.5)	59 (62.8)	
Radiotherapy			>0.999
No	57 (93.4)	87 (92.6)	
Yes	4 (6.6)	7 (7.4)	

CA19-9: Carbohydrate antigen 19-9; CEA: carcinoembryonic antigen; CD44: cluster of differentiation 44; H-score: histoscore. Values in parentheses are percentages. *Not all data were available.

Table III. Multivariate analysis of predictive factors for early recurrence in patients undergoing radical surgery.

Parameters		Recurrence ≤6 months			<i>p</i> -Value
		Odds ratio	95%CI for odds ratio		
			Lower	Upper	
CA19-9 (U/ml)	>37/≤37	2.788	1.084	7.169	0.033
CD44 H-score	>32/≤32	2.483	1.157	5.327	0.020
Differentiation	No/yes	4.091	1.258	13.300	0.019
Stage	II/I	2.162	0.860	5.436	0.101
	III/I	3.748	0.947	14.829	0.060
Vascular invasion	Yes/no	1.610	0.656	3.953	0.299
Lymphatic invasion	Yes/no	2.072	0.873	4.914	0.098
Perineural invasion	Yes/no	0.925	0.335	2.554	0.880

CA19-9: Carbohydrate antigen 19-9; CD44: cluster of differentiation 44; H-score: histoscore; CI: confidence interval.

analyze the value of a CSC marker (CD44) and clinicopathological factors in predicting ER in PDAC patients undergoing radical surgery. Based on our results, CA19-9 levels, CD44 H-scores and differentiation were independent risk factors for ER.

Currently, the tumor-node-metastasis (TNM) staging system is not precise enough to predict patient prognosis. Recurrence time differs even in patients with the same TNM stage. Therefore, more molecular information relevant to the tumor is urgently needed to better identify patients who are at a high risk of ER, and these patients might need aggressive tailored therapies for better prognosis. CD44, a cell surface adhesion receptor encoded by a gene on the short arm of chromosome 11, is highly expressed in many cancers. Several systematic reviews and meta-analyses have reported the association between CD44 and tumor characteristics or prognosis in various cancers including head and neck, non-small cell lung, stomach, liver, kidney and ovarian cancer (13-18). For example, Chen *et al.* suggested that CD44 is related to advanced T and N status, high tumor grade and poor outcomes in pharyngeal and laryngeal cancer (13). Pre-clinical studies on the mechanism have also identified that CD44 overexpression is associated with angiogenesis, (19) which is a process of formation of new blood vessels (20). In contrast, studies have indicated that the inhibition of CD44 expression resulted in impaired endothelial function (20) and reduced the ability of endothelial cells to form vessel-like networks (21). In a clinical study, Choi *et al.* reported that the increased expression of CSC markers, especially keratin 19 (K19) and CD44, during the perioperative period can predict ER after radical surgery of hepatocellular carcinoma (22). Therefore, anti-CD44 therapy might become a new potential cancer treatment. Similar to our previous findings (23), studies

have shown that CD44 is a poor prognostic factor in pancreatic cancer (24, 25). Early in 1998, Gotoda *et al.* reported that the expression of CD44v6 and CD44v2 was a useful predictor of poor outcomes in patients with curatively resected pancreatic cancer (24). A few years ago, Li *et al.* used an antibody against CD44s (standard isoform) to inhibit pancreatic tumor initiation and post-irradiation recurrence in a mouse model (25). Although in the animal study CD44 was shown to be correlated with unfavorable prognosis in PDAC, the relationship between CD44 and ER in human PDAC had not been reported. In our present work, it was observed for the first time that increased CD44 H-scores were associated with ER in PDAC. Nonetheless, further large-scale studies are required to confirm our results, and more pre-clinical studies are needed to clarify the precise mechanism of CD44 expression in PDAC patients experiencing ER.

CA19-9 has been widely studied as a serum marker of PDAC regarding many aspects including screening, diagnosis, resectability and prognostic determination, and postoperative surveillance. Other biomarkers have also emerged from preclinical and early clinical trials with an attempt to increase the sensitivity of early detection of PDAC (26-30). However, none of these biomarkers has replaced serum CA19-9 in clinical utility to date. The serum level of CA19-9, either preoperative or postoperative, is associated with long-term survival (31-36). In addition, studies have suggested that the preoperative CA19-9 level can help in predicting occult metastasis and the likelihood of a complete (R0) resection (31, 37-41). Studies have also indicated that preoperative serum levels of CA19-9 can predict patient outcomes (41, 42). Based on this perspective, high levels of preoperative CA19-9 might be associated with ER. Recently, Shimizu T *et al.* reported that high levels of

S-pancreas-1 antigen and CA19-9 were independent risk factors for ER in PDAC patients undergoing surgical resection (43). In line with previous observations, our results showed that high preoperative CA19-9 levels were an independent predictor for ER.

Histological characteristics have also been extensively studied in relation to patient outcomes. Poor differentiation of PDAC tumors has been identified to have a negative effect on survival after resection (3, 44-48). Histological characteristics of tumors may additionally enhance the predictive value of AJCC staging in resectable PDAC (47). A post-resection nomogram including tumor differentiation has also been developed (49) and validated (50) to predict the survival of PDAC patients undergoing resection. In this study, tumor differentiation was found to significantly affect ER in PDAC patients after radical resection. Interestingly, although the TNM staging system has been well known as a reliable predictor of long-term outcomes, our results did not show a significant correlation of TNM stage with ER.

In conclusion, high CA19-9 levels, CD44 H-scores and poor differentiation were independent predictors for ER in PDAC patients undergoing radical resection. Therefore, the determination of CD44 expression may help in identifying patients at a high risk of ER for more aggressive treatment after radical surgery.

Conflicts of Interest

All Authors declare that no conflicts of interest exist regarding this study.

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