ORIGINAL ARTICLE

Prognosis of ductal adenocarcinoma of pancreatic head with overexpression of CD44

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Summary *Background:* The long-term survival rate of patients with pancreatic ductal adeno-carcinoma (PDAC) is very low. Cancer stem cells have been identified in PDAC based on the expression of the surface markers CD24, CD44, CD133, and epithelial specific antigen. The prognosis of PDAC may be related to the presence or absence of tumor cells with cancer stem cell surface markers.

Methods: Eighty-six PDAC patients (51 male and 35 female patients) who underwent surgical treatment at Chang Gung Memorial Hospital—Lin-Kou Medical Center, Lin-Kou, Taiwan between 1998 and 2007 were included in this study. The patients' ages ranged from 30 years to 84 years. All their surgical specimens showed invasive ductal cancer. Immunohistochemical staining with CD44 antibodies was performed. The differences in clinical data, cell types of tumors, tumor staging, and survival rates between patients with CD44^{-} (Group A; $n = 33$) and CD44^{+} (Group B; $n = 53$) were compared.

Results: Clinical data, cell types of tumors, and tumor staging between the two groups showed no significant differences. The 3- and 5-year survival rates were, respectively, 51.5% and 19.8% in patients with CD44^{-} tumor cells and 4.0% and 2.0% in those with CD44^{+} tumor cells. The differences were statistically significant ($p < 0.0001$). The median overall survival times of the two groups were also different (36.9 months vs. 12.2 months, $p < 0.0001$). Multivariate analysis showed that the CD44 as well as lymph node status, and differentiation of tumor cells were prognostic factors for patients with PDAC.

Conclusion: The results suggested that CD44 expression in patients with PDAC after surgery was significantly associated with decreased survival, whereas patients with CD44^{−} tumor cells survived significantly longer.

1. Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the leading causes of cancer deaths in the Western world, and is the 10th most common cancer in Taiwan. Complete surgical resection still remains the only therapeutic option for PDAC, but only 10% of patients are candidates for curative surgery.[1] The overall 5-year survival rate is 5−10%, which is much lower than that of resectable pancreatic cancer in general (25−35%).[2−4]

Malignant tumors usually comprise a heterogeneous cell population having different biologic properties, of which a small population of cancer cells, or so-called cancer stem cells (CSCs), promote tumor formation and growth.[5] The existence of CSCs was first proved in acute myelogenous leukemia[6,7] and subsequently verified in breast[8] and brain tumors.[9−11] Al-Hajj et al[8] reported a phenotypically distinct and relatively rare population having CD44^{+}, CD24^{−}, and epithelial-specific antigen^{+}, and stated that the tumor-initiating cell lineage was responsible for the propagation of human metastatic breast cancer cells. Pancreatic CSCs have not been identified until Li et al[12] described a sub-population of tumor cells from PDAC tissue with increased tumorigenic potential in mice. They also identified putative CSCs in PDAC based on the expression of the surface markers CD44, CD24, and epithelial specific antigen.

The expression of CD44 variants has been reported to correlate with a poor prognosis in colon cancer.[13] Overexpression of CD44 has also been demonstrated to be associated with poor prognoses in head and neck squamous-cell carcinoma.[14,15] In human pancreatic cancer samples, CD44 expression was reported to correlate with the histologic grade, and patients with CD44-positive tumors showed a poor prognosis.[16] The present study was designed to compare the prognosis of PDAC patients with positive or negative expression of CD44 in tumor cells after surgical resection.

2. Materials and methods

2.1. Patients and specimens

A total of 751 patients were diagnosed to have PDAC at the Department of Surgery in Chang Gung Memorial Hospital at Lin-Kou, Taiwan between 1998 and 2007, and only 370 of them (49.3%) underwent surgical resection. Tumor specimens from 86 patients with resection of the pancreatic head, who were alive after the operation during the study period, were retrieved for this study. Our pathologists have reviewed the slides to ensure that the tissues were consistent with PDAC. The study was approved by the Ethics Committee of our hospital and performed according to the Helsinki Declaration.

2.2. Grouping

The patients were divided into two groups. Those patients whose pancreatic CSC showed CD44^{−} ($N = 33$) were classified as Group A, and those with CD44^{+} ($N = 53$) were classified as Group B. Clinical data, and differentiation and staging of tumors using pancreatic cancer staging of AJCC (American Joint Committee on Cancer) seventh edition,[17] obtained from the pathological reports of patients, were compared between the two groups. The survival of patients between these groups was also compared.

2.3. Immunohistochemistry

Immunohistochemical staining[18] was performed on 3−4 μm sections of formalin-fixed, paraffin-embedded tissues, placed on coated glass slides and dried at 60−70°C for 30 minutes. CD44 was detected by the monoclonal body HCAM (DF1485; Santa Biotechnology Inc., Dallas, TX, USA). Using the antibody detection method, two polymer-based systems and one streptavidin−biotin system were tested. For double-labeling with cytokeratin 19 or chromogranin A, standard methods employed in routine diagnostic service were used after adjusting antibody concentrations. Antigen retrieval was performed by incubation in a pressurized heating chamber at 120°C for 1 minute in Tris-EDTA buffer (pH 9). The slides were then cooled by placing them under running tap water and incubated with primary antibody diluted 1:25 in an antibody diluent with reduced salt concentration (25 mM Tris, 75 mM NaCl, 1% BSA (bovine serum albumin), 0.01% methiolate, and 0.05% Tween 20; pH 7.4) for 60 minutes. Next, unspecific peroxidase activity was blocked by 3% H_2O_2 treatment for 5 minutes. Primary antibody detection was performed, in accordance with the manufacturer's instructions, using the MACH3 mouse probe for 20 minutes, followed by MACH3 HRP (horseradish peroxidase) polymer for 20 minutes, and the signal was developed with diaminobenzidine DAB + for 5 minutes. Between every two steps, there were two washing steps for 1 minute each on a rocking platform in washing buffer (50 mM Tris, 150 mM NaCl, and 0.05% Tween 20; pH 7.5). Finally, the slides were counterstained with hematoxylin for 1 minute, dehydrated in alcohol solutions and xylene, and mounted in Entellan.

2.4. Assessment of immunohistochemical staining

The quality of staining was judged in the control material from different organs, on the basis of the data available in

the literature regarding gene/protein expression of CD44 in various tissue types. TMA (tissue microarray) slides containing PDAC were scored independently by two of the authors (L.Y.L. and T.C.C.) as negative (0), weakly positive (1), or strongly positive (2) for CD44 expression. Cases with different scores were discussed to reach an agreement. As validation for TMA interpretation, whole sections from the border between the adenocarcinoma and nearby non-tumorous pancreatic tissue were made from 10 of the cases included in the TMA blocks, and treated and evaluated in the same way as the TMA slides. Parallels were stained with hematoxylin and eosin for controlling the tissue quality. The whole sections were also screened at $1000 \times$ magnification, looking for features such as nuclear/cytoplasmic staining, expression in vessels, etc.

2.5. Statistical analysis

Statistical analyses were performed using the SPSS (SPSS Inc., Chicago, IL, USA). The product-limit (Kaplan—Meier) analysis module was used for comparing survival rates between multiple groups. Multivariate analysis of prognostic factors was performed using the Cox proportional hazard method. Survival times versus cumulative proportion surviving, according to breakdown by the staining intensity for Group A and Group B, were plotted.

3. Results

3.1. Patients' clinical data

Patients' clinical data and tumor staging between the two groups are listed in Table 1. Differences in age, levels of albumin, GOT, ALK-P, and hemoglobin between the two groups were not significant. Tumor markers, such as CEA and CA19-9, also showed no significant differences between these groups.

Table 1 Clinical data of patients in the two groups.

	Group A (n = 33) CD 44 (−)	Group B (n = 53) CD 44 (+)	p
Age (y)	61.6 ± 10.1	61.6 ± 9.4	0.988
Albumin (g/dL)	3.8 ± 0.6	3.8 ± 0.6	0.934
GOT (U/L)	156.6 ± 118.9	56.4 ± 222.9	0.997
ALK-P (U/L)	387 ± 422	356 ± 280	0.698
Hb (g/dL)	12.1 ± 1.8	12.2 ± 1.7	0.805
CEA (ng/mL)	9.1 ± 12.9	5.0 ± 5.5	0.136
CA19-9 (ng/mL)	1121 ± 2740	1021 ± 3617	0.906
Differentiation			0.352
Well	12 (46.2)	14 (53.8)	
Moderate	13 (34.7)	32 (65.3)	
Poor	4 (50.0)	4 (50.0)	
Undifferentiated	0	3 (100.0)	
Stage			0.421
I	4 (57.1)	3 (42.9)	
II	29 (36.7)	50 (63.3)	

Data are presented as n (%) or mean ± SD.
Hb = hemoglobin.

3.2. Expression in PDAC

CD44 expression was evaluated in a series blocks. Fifty-three cases (61.6%) were positive, and expression was mainly seen in the apical/endoluminal cell surface in malignant ductal structures (Fig. 1). In the positive cases, the expression area and intensity varied with the morphological heterogeneity of the tumor. Thirty-three cases (38.4%) were negative for CD44 expression.

3.3. Prognosis in the two groups

The median overall survival time of patients in the CD44$^-$ group were 36.9 months, whereas that in the CD44$^+$ group was only 12.2 months, and the difference between the two groups was significant ($p < 0.0001$). The 3- and 5-year survival rates of patients were, respectively, 51.5% and 19.8% in the CD44$^-$ group and only 4.0% and 2.0% in the CD44$^+$ group, and the differences between the two groups were also significant ($p < 0.0001$). The cumulative survival curve is shown in Fig. 2A.

For more detailed analysis, we compared between the patients with stage IIA and IIB cancers. The median overall survival time of patients with stage IIA cancer in the CD44$^-$ group ($n = 13$) was 36.9 months, whereas that ($n = 10$) in the CD44$^+$ group was only 15.7 months, and the difference between the two groups was significant ($p = 0.042$). The 3- and 5-year survival rates of patients, were, respectively, 53.8% and 34.6% in the CD44$^-$ group, whereas 10% and 0% in the CD44$^+$ group, and the differences between these groups were also significant. The cumulative survival curve is shown in Fig. 2B.

For patients with Stage IIB cancer, the median overall survival time was 17.8 months in the CD44$^-$ group ($n = 16$) and only 10.9 months in the CD44$^+$ group ($n = 40$), and the difference between these groups was significant ($p = 0.001$). The 3- and 5-year survival rates of patients were, respectively, 50.0% and 18.8% in Group A, whereas those were 0% in Group B, and the difference between the two groups was also significant. The cumulative survival curve is shown in Fig. 2C.

Results of the univariate and multivariate analyses of the relative risk factors between the two groups of patients are presented in Tables 2 and 3; only lymph node status, differentiation, and CD44 were significant.

4. Discussion

The prognosis of PDAC is extremely poor, because the cancer usually invades the surrounding tissues and metastasizes to lymph nodes, liver, or peritoneum at the time of diagnosis. CD44 is an adhesion molecule and membrane receptor for hyaluronan, and is involved in cell motility and metastasis. The gene encoding CD44 generates a variety of isoforms by alternative splicing, which predominantly affects the extracellular membrane-proximal structure of CD44 proteins.[19] Prince et al[20] demonstrated that CD44 cells isolated from primary head and neck carcinoma have the ability to self-renew and differentiate in an *in vivo* mouse model, and the CD44 subpopulation in primary tumors vary from 0.1% to 42%. In other tumors, multiple cell
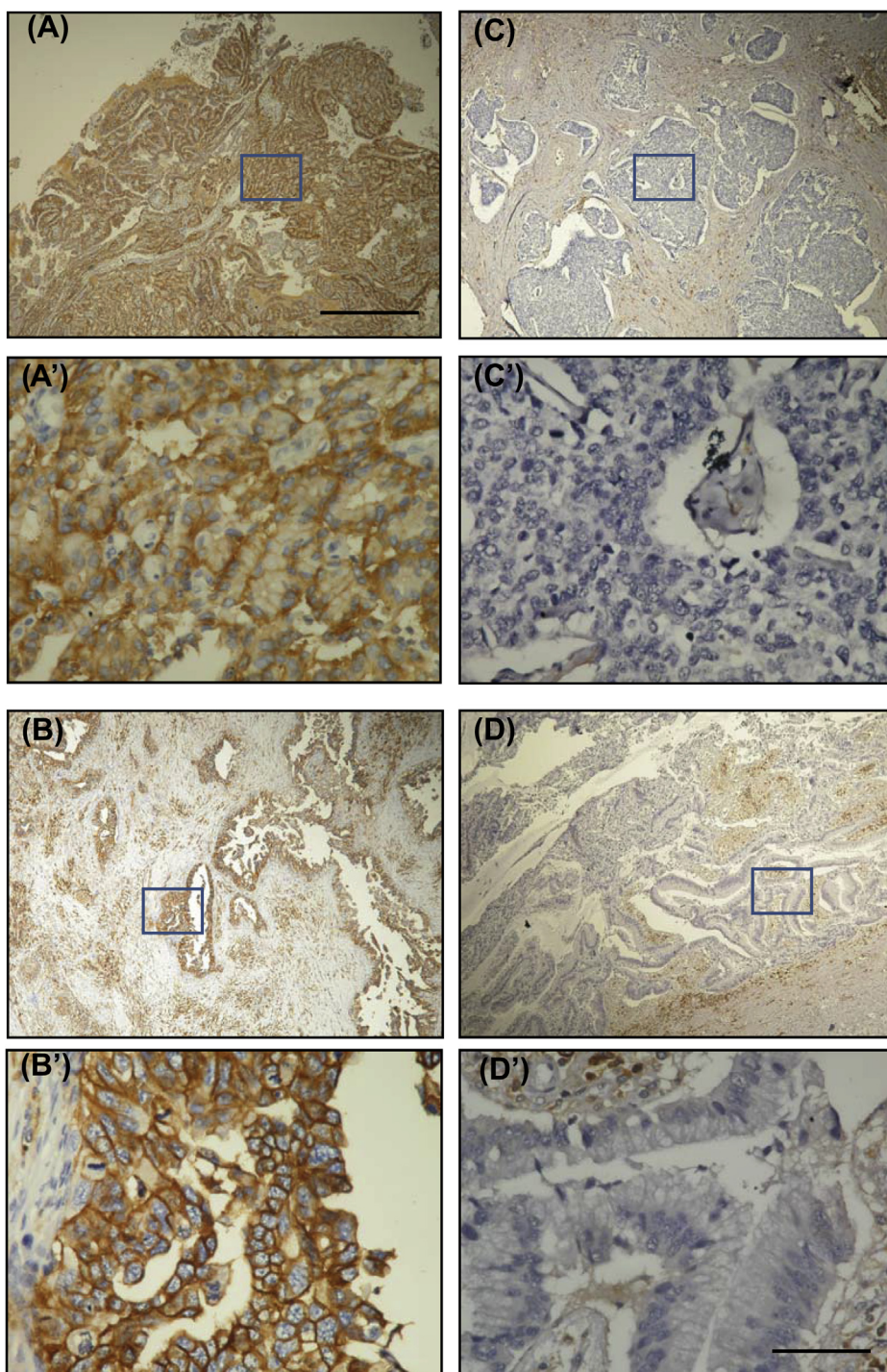
Figure 1 CD44 expressions in pancreatic ductal adenocarcinoma tumor tissues. (A and B) CD44-positive expression (brown) in exocrine tissue is most abundant in centroacinar regions and intercalated ducts in two patients. (A′ and B′) Higher magnification of the areas marked in A and B, respectively. (C and D) CD44 negative staining in two other patients. (C′ and D′) Higher magnification of the areas marked in C and D, respectively. Scale bar: (A−D) 50 μm and (A′−D′) 125 μm.

surface markers may be used to purify CSCs, and CD44 has been used as one of the CSC markers.

CD44 is a member of the transmembrane glycoprotein family, with a large number of isoforms identified in many human tissues and particularly high expression in proliferating cells and squamous cell epithelium. The CD44 variant 6 (v6) molecule has been noted as a marker for tumor metastasis and prognosis in several tumors. Although Gansauge et al[21] reported that lower serum levels of soluble CD44 v6 were significantly associated with poor prognosis in patients with PDAC, this might not be the true reflection of prognosis of CD44+ in patients with PDAC. Gotoda et al[22] first reported that CD44 v2 and CD44 v6 may be useful markers of a poor prognosis.
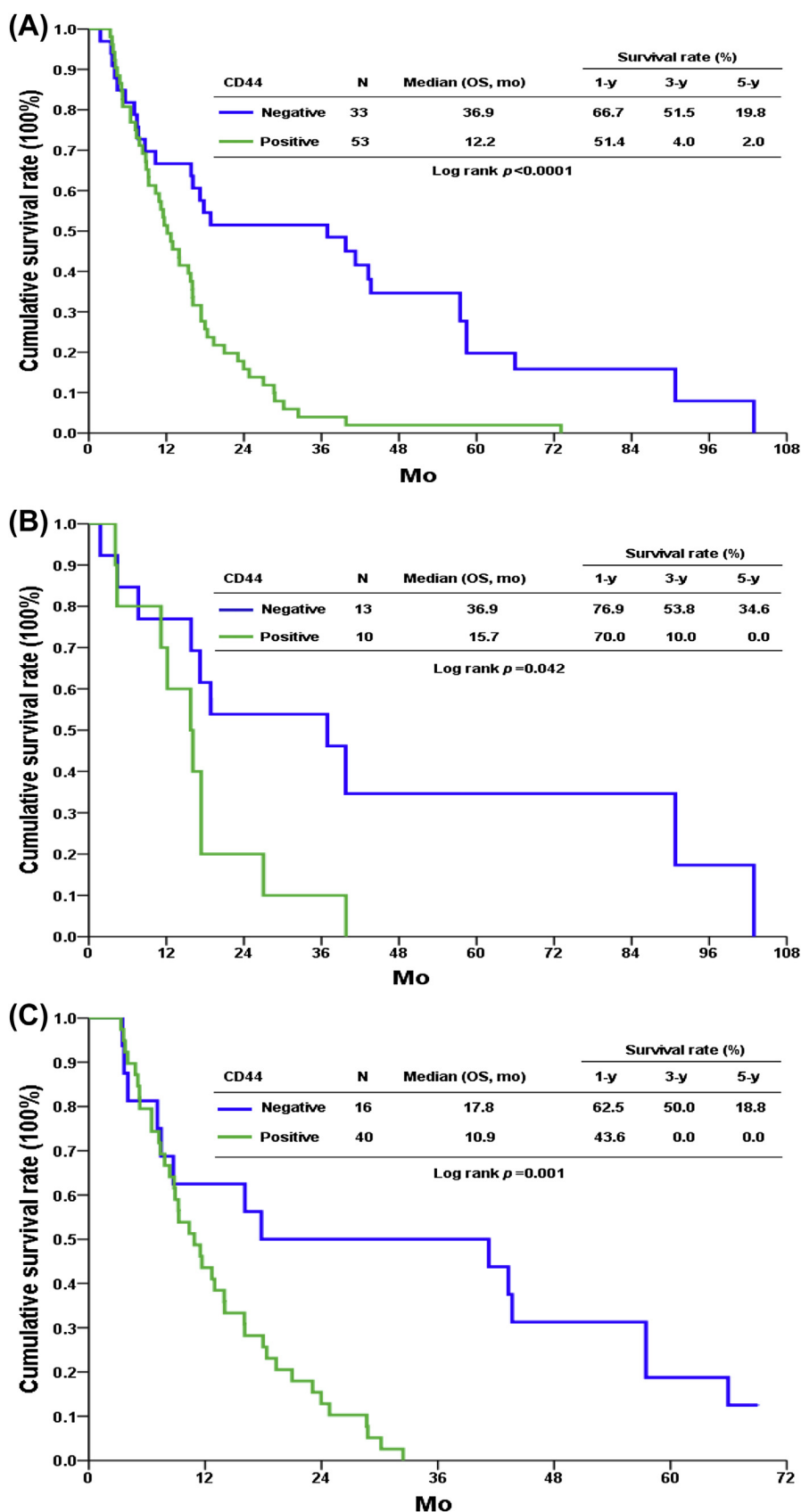
Figure 2   Kaplan–Meier 1-, 3-, and 5-year overall survival curves for (A) all pancreatic ductal adenocarcinoma patients ($n = 86$), (B) patients with stage IIA cancer, and (C) patients with stage IIB cancer. The higher curve represents the patients with negative CD44-expressing tumor, and the lower curve represents those with a strong CD44 expression. The differences in three curves between CD44$^-$ and CD44$^+$ groups were significant ($p < 0.05$). OS = overall survival.

Table 2 Univariate analysis of relative factors between the two groups.

Parameters		N	Median (mo)	95% CI	p
Sex	Male	50	12.16	8.60—25.73	0.272
	Female	36	17.23	14.90—19.56	
Age (y)	≤50	10	17.39	0.00—49.74	0.478
	>50	76	14.06	9.47—18.55	
Jaundice	Yes	72	16.11	12.13—20.09	0.293
	No	14	13.97	9.48—18.47	
OP type	Whipple	58	18.08	12.00—20.15	0.554
	PPPD	28	12.69	4.62—20.76	
CA19-9 (U/L)	≤37	16	32.42	3.03—61.80	0.003
	>37	70	12.99	8.74—17.23	
T status	T1	4	8.45	NA	0.731
	T2	35	12.69	8.42—16.96	
	T3	47	16.11	14.27—17.95	
Nodal status	Negative	30	17.39	13.80—20.79	0.025
	Positive	56	11.87	6.79—16.55	
Stage	I	7	58.46	29.91—87.00	0.262
	II	79	14.01	9.77—18.24	
Margin	Negative	62	16.04	13.76—18.33	0.056
	Positive	24	11.87	6.32—17.02	
Differentiation	Well	26	27.03	19.92—34.13	0.003
	Moderate	49	10.36	7.60—13.11	
	Poor	8	12.69	7.27—18.11	
	Others	3	5.06	2.96—7.17	
Vascular invasion	No	62	15.75	12.02—19.49	0.187
	Yes	24	12.99	6.09—19.88	
Lymphatic invasion	No	44	16.04	13.75—18.34	0.104
	Yes	42	11.67	9.03—14.31	
Perineural invasion	No	20	14.01	6.87—21.14	0.633
CD44	Yes	66	15.85	11.28—20.42	<0.0001
	Negative	33	36.92	11.34—62.51	
	Positive	53	12.16	8.94—15.39	
Chemotherapy	No	31	10.32	1.19—19.46	0.528
	Yes	55	16.08	10.89—21.26	

CI = confidence interval; NA = not available; PPPD = pylorus-preservation pancreaticoduodenectomy.

In this study, we demonstrated that the overexpression of CD44 had a statistically significant association with decreased 5-year overall survival in patients with PDAC after surgery. The prognosis of patients with CD44⁻ was better with significantly improved survival, but the tumor staging between the two groups of patients showed no such difference. The main factor seems to be related to the presence or absence of tumor stem cells in their PDAC.

It has been shown that, in several cancers, only a minority of the cancer cells are able to initiate the formation of new tumors. These cancer-initiation cells are called CSCs, and in several studies the CD44 adhesion molecule has been demonstrated to be expressed specifically in these cells. It has been shown that CD44⁺ cancer cells contain cancer stem-cell like properties and can initiate *in vivo* tumor formation. Moreover, in recent studies, CSCs have

Table 3 Multivariate analysis of relative factors between the two groups (using Cox proportional hazard method).

Parameters		Hazard ratio	95% CI of HR	p
CA19-9	>37/≤37	1.649	0.871—3.124	0.125
Lymph node status	N1/N0	1.772	1.059—2.965	0.029
Margin	Positive/negative	1.289	0.771—2.153	0.332
Differentiation	Moderate/well	1.974	1.149—3.390	0.014
	Poor/well	2.954	1.250—6.981	0.014
	Undifferentiated/well	5.915	1.253—27.918	0.025
CD44	Positive/negative	2.190	1.271—3.972	0.005

CI = confidence interval; HR = hazard ratio.

also been shown to have increased resistance to drug and radiation therapies.[23,24] In the present study, we showed that CD44 overexpression is associated with poor prognosis, which may suggest that cancer cells strongly expressing CD44 have qualities linked to the CSCs. These results support the hypothesis that increased cell adhesion supports the growth of cancer cells, both in primary tumors and in metastases.

CD44^{+} cells reconstitute the resistant cell population, and CD44 could be a therapeutic target to overcome the drug resistance during gemcitabine chemotherapy for pancreatic cancer. Hence, CD44 targeted therapy is a possible option for reversing chemoresistance of pancreatic cancer cells.[16] Veapamil has been shown to be an inhibitor of ABC transporters,[25] and treatment with verapamil can resensitize resistance cells to gemcitabine therapy.

In conclusion, CD44 overexpressions in patients with PDAC after surgery are significantly associated with decreased survival, whereas patients with CD44^{−} tumor cells survive for a significantly longer time. Cancer stem-like cells play a pivotal role in acquiring multidrug resistance in pancreatic cancer, and in particular, CD44 cells, which repopulate after chemotherapy, were responsible for chemoresistance. In therapeutic application, targeted therapy against CD44 could be applied to overcome drug resistance and might be beneficial in the treatment of pancreatic cancer.

Acknowledgments

References

1. Ouaissi M, Giger U, Louis G, Sielezneff I, Farges O, Sastre B. Ductal adenocarcinoma of the pancreatic head: a focus on current diagnostic and surgical concepts. *World J Gastroenterol*. 2012;18:3058–3069.
2. Gudjonsson B. Cancer of the pancreas: 50 years of surgery. *Cancer*. 1987;60:2284–2303.
3. Nitecki SS, Sarr MG, Colby TV, van Heerden JA. Long-term survival after resection for ductal adenocarcinoma of the pancreas is it really improving. *Ann Surg*. 1995;221:59–66.
4. Allema JH, Reinders ME, van Gulik TM, et al. Prognostic factors for survival after pancreaticoduodenectomy for patients with carcinoma of the pancreatic head region. *Cancer*. 1995;75:2069–2076.
5. Jemal A, Murray T, Ward E, et al. Cancer statistics, 2005. *CA Cancer J Clin*. 2005;55:10–30.
6. Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat Med*. 1997;3:730–737.
7. Lapidot T, Sirard C, Vormoor J, et al. A cell initiating human acute myeloid leukemia after transplantation into SCID mice. *Nature*. 1994;17:645–648.
8. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. *Proc Natl Acad Sci U S A*. 2003;100:3983–3988.
9. Singh SK, Hawkins C, Clarke ID, et al. Identification of human brain tumour initiating cells. *Nature*. 2004;432:396–401.
10. Galli R, Binda E, Orfanelli U, et al. Isolation and characterization of tumorigenic, stem-like neural precursors from human glioblastoma. *Cancer Res*. 2004;64:7011–7021.
11. Hemmati HD, Nakano I, Lazareff JA, et al. Cancerous stem cells arise from pediatric brain tumors. *Proc Natl Acad Sci U S A*. 2003;100:15178–15183.
12. Li C, Heidt DG, Dalerba P, et al. Identification of pancreatic cancer stem cells. *Cancer Res*. 2007;67:1030–1037.
13. Ponta H, Sherman L, Herrlich PA. CD44: from adhesion molecules to signalling regulators. *Nat Rev Mol Cell Biol*. 2003;4:33–45.
14. Lin JT, Chang TH, Chang CS, et al. Prognostic value of pretreatment CD44 mRNA in peripheral blood of patients with locally advanced head and neck cancer. *Oral Oncol*. 2010;46:e29–e33.
15. Han J, Kioi M, Chu WS, Kasperbauer JL, Strome SE, Puri RK. Identification of potential therapeutic targets in human head & neck squamous cell carcinoma. *Head Neck Oncol*. 2009;14:1–27.
16. Hong SP, Wen J, Bang S, Park S, Song SY. CD44-positive cells are responsible for gemcitabine resistance in pancreatic cancer cells. *Int J Cancer*. 2009;125:2323–2331.
17. Tempero MA, Arnoletti JP, Behrman SW, et al. Pancreatic adenocarcinoma, version 2.2012: featured updates to the NCCN guidelines. *J Natl Compr Canc Netw*. 2012;10:703–713.
18. Immervoll H, Hoem D, Steffensen OJ, Miletic H, Molven A. Visualization of CD44 and CD133 in normal pancreas and pancreatic ductal adenocarcinomas: non-overlapping membrane expression in cell populations positive for both markers. *J Histochem Cytochem*. 2011;59:441–455.
19. Wielenga VJ, Heider KH, Offerhaus GJ, et al. Expression of CD44 variant proteins in human colorectal cancer is related to tumor progression. *Cancer Res*. 1993;53:4754–4756.
20. Prince ME, Sivanandan R, Kaczorowski A, et al. Identification of a subpopulation of cells with cancer stem cell properties in head and neck squamous cell carcinoma. *Proc Natl Acad Sci U S A*. 2007;104:973–978.
21. Gansauge F, Gasnuage S, Rau B, et al. Low serum levels of soluble CD44 variant 6 are significantly associated with poor prognosis in patients with pancreatic carcinoma. *Cancer*. 1997;80:1733–1739.
22. Gotoda T, Matsumura Y, Kokawa A, Kondo H. The prognostic factors of pancreatic cancer—adhesion molecule CD44. *Gon To Kagaku Ryohu*. 1998;25:2036–2042.
23. Bourguignon LY, Peyrollier K, Xia W, Gilad E. Hyaluronan—CD44 interaction activates stem cell marker Nanog, stat-3-mediated MDR1 gene expression, and ankyrin-regulated multidrug efflux in breast and ovarian tumor cells. *J Biol Chem*. 2008;283:17635–17651.
24. Ischenko I, Seeliger H, Schaffer M, Jauch KW, Bruns CJ. Cancer stem cells: how can we target them? *Curr Med Chem*. 2008;15:3171–3184.
25. Dean M, Fojo T, Bates S. Tumor stem cells and drug resistance. *Nat Rev Cancer*. 2005;5:275–284.