Original Article

Coexpression of CD44-Positive/CD133-Positive Cancer Stem Cells and CD204-Positive Tumor-Associated Macrophages Is a Predictor of Survival in Pancreatic Ductal Adenocarcinoma

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BACKGROUND: The interactions between cancer stem cells (CSCs) and tumor-associated macrophages (TAMs) can promote tumor progression, maintain the CSCs population, and reduce therapeutic effects. The objective of this study was to investigate the coexpression of CSCs and TAMs and its clinical significance in pancreatic ductal adenocarcinoma (PDAC). **METHODS:** Ninety-six patients with PDAC were included in this study. Tissue microarrays were constructed for immunostaining of the CSCs markers CD44 and CD133 and the TAMs marker CD204. Correlations between the expression of CSCs and TAMs markers and clinicopathologic characteristics or disease progression were analyzed. **RESULTS:** Expression levels of CD44/CD133 and CD204 were significantly higher in tumor tissues than in normal tissues ($P < .0001$). The variables associated with survival were high coexpression of CD44/CD133 ($P = .000$), high expression of CD204 ($P = .011$), and tumor grade ($P = .014$). There was a positive correlation between CD44/CD133 and CD204 expression ($r = 0.294$; $P = .004$). Survival analysis indicated that high coexpression of CD44/CD133 and CD204 was associated significantly with shorter overall survival ($P = .000$) and disease-free survival ($P = .003$). Multivariate analysis revealed that high CD44/CD133 expression was an independent prognostic factor for disease-free survival, whereas high CD204 expression was an independent predictor for both overall and disease-free survival. **CONCLUSIONS:** Coexpression of CD44/CD133 and CD204 is a useful survival prediction marker for patients with PDAC. ***Cancer* 2014;120:2766-77.**

KEYWORDS: pancreatic ductal adenocarcinoma, cancer stem cells, tumor-associated macrophages, tissue microarrays, CD44, CD133, CD204.

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is the fourth common cause of cancer-related death in the world, with an incidence that equals mortality.[1] Less than 20% of patients have resectable PDAC, and 75% of diagnosed patients do not survive longer than 1 year.[2] The 5-year relative survival rates for the 3 periods from 1975 to 1977, from 1987 to 1989, and from 2002 to 2008 period were 2%, 4%, and 6%, respectively in the United States,[3] exhibiting the limitations of diagnosis and treatment of PDAC. The reasons for this poor prognosis include a high incidence of local invasion and distant metastasis and a largely drug-resistant phenotype.[4] Accordingly, understanding of the biologic and clinical importance of the distinct phenotypes in cancer tissues may benefit in control of PDAC.

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 antigen, CD133, aldehyde dehydrogenase 1 (ALDH1), and c-Met.[5,6] The correlations between clinical outcomes in PDAC and phenotypic CSCs remain to be investigated and are of significant interest. Increased ALDH expression in patients with resectable PDAC was associated with worse median survival (18 months for ALDH-negative tumors and 14 months for ALDH-positive tumors).[7] In another report, CD44-positive/CD133-negative expression was identified as a favorable prognostic indicator in 80 patients with PDAC.[8] However, neither CD44 nor CD133 expression played a significant role in PDAC survival.[9,10] Taken together, these findings reveal that the use of CSCs phenotypes in predicting the prognosis of patients with PDAC is still controversial.

PDAC is associated with significant intratumor and peritumor inflammation and with failure of immunosurveillance.[11] Macrophages are the major components of inflammatory cells and play an important part in tumor initiation and progression.[12]

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Macrophages can be divided into classically activated macrophages (M1 macrophages) for killing tumor cells and alternatively activated macrophages (M2 macrophages) for promoting tumor cells.[13] Circulating monocytes are recruited by tumors and differentiate into M2 tumor-associated macrophages (TAMs), acquiring protumor functions, including promotion of tumor growth, angiogenesis, metastasis, and matrix remodeling and suppression of adaptive immunity.[14,15] M2 TAMs have an IL-12^{low}/IL-23^{low}/IL-10^{high} phenotype and express high levels of class A scavenger receptor (CD204) and mannose receptor (CD163).[16] Recent studies have reported that the number of CD204-positive TAMs within a primary tumor is related to tumor progression and clinical outcome in patients with esophageal cancer, lung cancer, colorectal cancer, and PDAC.[17-20] Thus, it is anticipated that TAMs may be a useful marker for evaluating characteristics of the tumor microenvironment.

Because TAMs have been linked to CSCs maintenance,[21] we were interested in evaluating the possibility of using the expression of the pancreatic CSCs markers CD44 and CD133 and the TAMs marker CD204 as predictive markers of survival in patients with PDAC after resection. In the current study, first, we detected the expression status of these markers in PDAC tissue microarrays (TMAs); then, we clarified the correlations between these markers and clinicopathologic features and between marker status and patient survival.

MATERIALS AND METHODS

Patients and TMA Construction

Data from all patients with PDAC who underwent surgical resection between 2001 and 2011 were reviewed using electronic medical records. Patients were followed until death or up to 2012. The median follow-up was 3.7 years (range, 1.9-12.5 years). Tissue specimens were collected after acquiring approval from the Institutional Review Board of National Cheng Kung University Hospital (Tainan, Taiwan). Paraffin-embedded TMAs were mounted with 96 tumor resection specimens of pancreatic cancer selected by an experienced pathologist, including 11 normal/cancer pairs. From these representative tumor regions, 2 or 4 cores were punched out using a tissue cylinder with a diameter of 0.6 mm and were placed onto the TMA paraffin slides for immunofluorescence analysis.

Immunofluorescence Staining and Measurement

Tissue sections were stained with anti-human CD44 monoclonal antibody (1:500 dilution; catalog no. M7082; DAKO, Carpinteria, Calif), anti-human CD133 monoclonal antibody (1:500 dilution; catalog no. 3663S; Cell Signaling Technology, Beverly, Mass), anti-human CD68 monoclonal antibody (1:500 dilution; catalog no. M0876; DAKO), and anti-human CD204 polyclonal antibody (1:500 dilution; catalog no. ab53566; Abcam, Cambridge, United Kingdom) according to the manufacturer's instructions, and then incubated with the appropriate fluorescently conjugated secondary antibodies. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). In this process, CD44 or CD68 is colored red, CD133 or CD204 is colored green, and the coexpression pattern of CD44 and CD133 (CD44/CD133) is colored yellow. Images were acquired from using fluorescence-activated cell sorting (FACS)-like tissue cytometry. The percentage of positive cells in each sample was further quantified using TissueQuest software (Tissue Gnostics, Vienna, Austria), as described previously.[22] Tumor heterogeneity was evaluated using Pearson correlation coefficients (r)in 2 different cores from the same tumor blocks, which revealed a significant correlation between the 2 cores for each biomarker ($r > 0.6$).

Statistical Analysis

Data were expressed as the means ± standard errors of all experiments. Clinicopathologic variables were included as adjusters in the analysis of patients who underwent resection. For intraobserver agreement, the kappa (κ) statistic[23] was used. Median disease-free survival (DFS) and median overall survival (OS) were estimated using the Kaplan-Meier method, and differences were measured using the log-rank test. The results are presented as the median survival in months with 95% confidence interval (CI), the relative risk with 95% CI, and the number of patients at risk. Cox proportional-hazards models were used for multivariate analysis, which included parameters that were identified as significant at the $P < .1$ level from the univariate analysis according to a log-rank test. The results are presented as hazard ratios (HRs) and 95% CIs. P values $< .05$ were considered significant. All statistical analyses were performed using SPSS 17.0 statistical software (IBM, Endicott, NY).

RESULTS

Clinicopathologic Characteristics and Outcomes

The clinicopathologic characteristics and outcomes of 96 patients with PDAC included in this study are summarized in Table 1. The median OS was 9.3 months, and the 1-year, 3-year, and 5-year survival rates were 39%, 10%, and 6%, respectively. Most tumors located in the head of the pancreas (62.5%), and 46.9% of tumors were larger than 3 cm. The majority of tumors were moderately differentiated (54.2%), and the remaining tumors were well differentiated (27.1%) and poorly

TABLE 1. Clinicopathologic Parameters and Clinical Outcome (n = 96)

Variable	No. of Patients (%)	OS		DFS	
		Median, mo	P[a]	Median, mo	P[a]
Sex					
Men	63 (65.6)	16.756	.921	7.622	.898
Women	33 (34.4)	13.733		7.162	
Tumor location					
Head	60 (62.5)	16.821	.336	9.035	.567
Neck	7 (7.3)	8.871		4.994	
Body/tail	16 (16.7)	14.456		6.177	
Uncinate process	13 (13.5)	8.246		7.622	
Tumor size, cm					
≤3	51 (53.1)	20.895	.151	11.039	**.011**
>3	45 (46.9)	13.634		5.979	
Lymph node status					
Negative	47 (49)	20.895	.172	11.039	**.033**
Positive	49 (51)	13.667		5.979	
Margin status					
R0	68 (70.8)	16.756	.063	9.002	.347
R1	24 (25)	13.667		5.979	
R2	4 (4.2)	9.068		6.308	
Tumor grade					
Poorly differentiated	18 (18.8)	7.294	**.014**	5.749	.180
Moderately differentiated	52 (54.2)	16.756		7.031	
Well differentiated	26 (27.1)	25.823		11.926	
Stage					
I	11 (11.5)	20.895	.066	9.002	**.000**
II	79 (82.3)	16.756		7.622	
III	4 (4.2)	7.294		4.928	
IV	2 (2.1)	2.924		0.887	
CA19-9, U/mL					
<37	20 (20.8)	32.460	.145	16.756	.333
>37	76 (79.2)	14.029		7.031	
CD44 expression					
Low	55 (57.3)	17.938	.387	9.331	.482
High	41 (42.7)	10.875		5.979	
CD133 expression					
Low	54 (56.2)	20.895	.365	10.053	.289
High	42 (43.8)	10.94		5.520	
$CD44^{Low}/CD133^{Low}$	65 (67.7)	25.593	**.000**	9.331	**.011**
$CD44^{High}/CD133^{High}$	31 (32.3)	9.068		4.928	
CD68 expression					
Low	50 (52.1)	13.405	.462	6.308	.254
High	46 (47.9)	17.938		9.035	
CD204 expression					
Low	57 (59.4)	25.593	**.011**	10.053	**.047**
High	39 (40.6)	10.94		5.979	

Abbreviations: CA19-9, carbohydrate antigen 19-9; CD133, cluster of differentiation 133 (cancer stem cells marker); CD204, cluster of differentiation 204 (tumor-associated macrophages marker); CD44, cluster of differentiation 44 (cancer stem cells marker); CD68, cluster of differentiation 68 (a glycoprotein that binds to low-density lipoprotein); OS, overall survival; DFS, disease-free survival.
[a] Values in boldface indicate $P < .05$.

differentiated (18.8%). Most patients had stage II disease (82.3%), 51% of patients had lymph node metastases, and 70.8% of patients underwent complete resection (R0). Preoperative elevated serum levels of carcinoembryonic antigen (CEA) (>5 ng/mL), carbohydrate antigen 125 (CA 125) (>35 U/mL), and CA 19-9 (>37 U/mL) were observed in 30.2%, 25%, and 79.2% of patients, respectively. Thirty-seven of 96 patients (38.5%) received chemotherapy, which included neoadjuvant therapy in 12 patients and adjuvant therapy in 25 patients; however, neither neoadjuvant therapy nor adjuvant therapy provided a significant survival benefit within this cohort of patients with PDAC. During the duration of the study, recurrent disease developed in 62 of 96 patients (64.6%; data not shown).

CD44-Positive/CD133-Positive CSCs Expression or CD204-Positive TAMs Expression in Normal and Cancer Tissues

TMAs sections were double stained with antibodies against the CSCs markers CD44 and CD133 or were

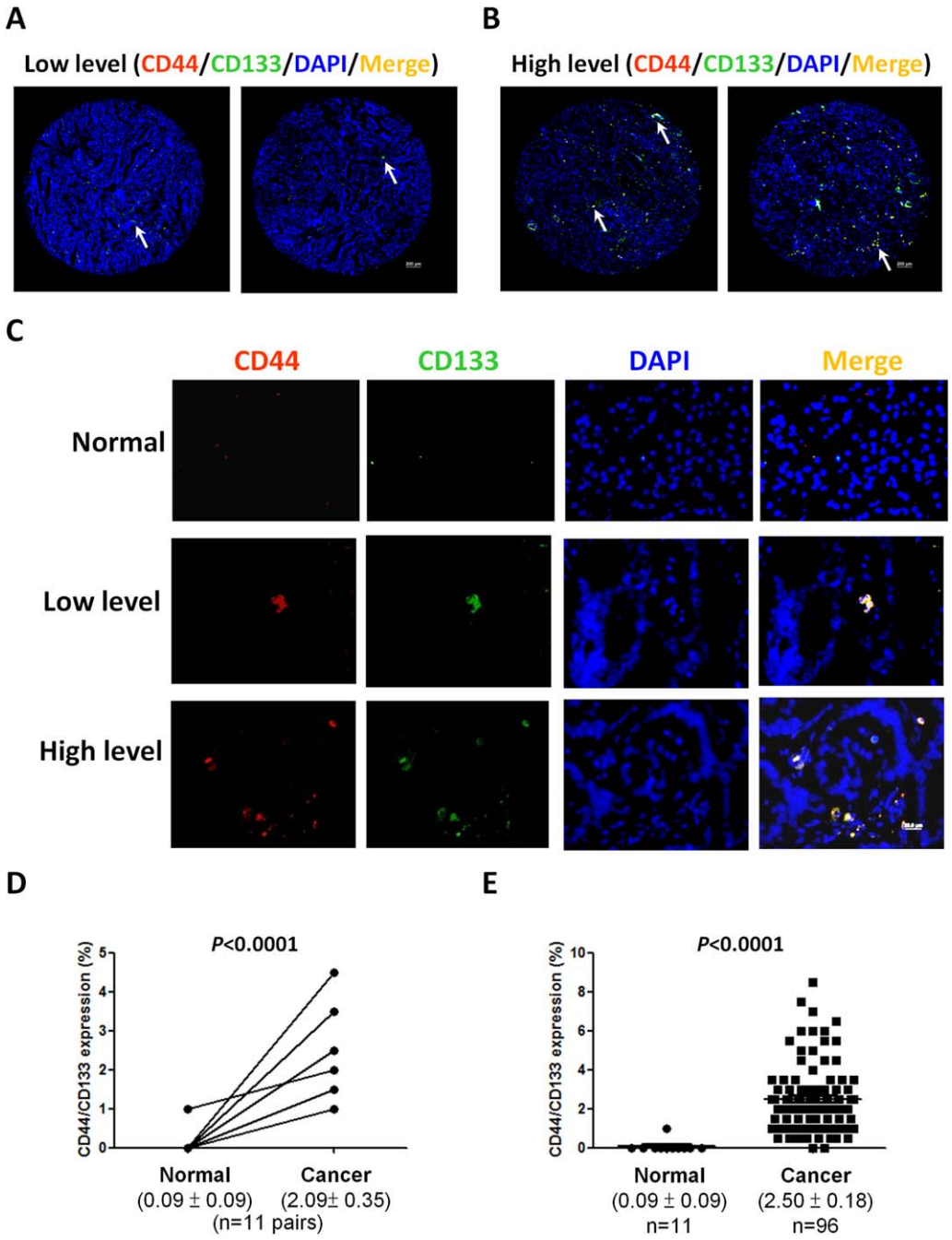

Figure 1. Expression of the cancer stem cells markers CD44 and CD133 is observed in tissue microarrays (TMAs) and in corresponding full sections of pancreatic ductal adenocarcinoma. (A) Two different TMA cores from the same tumor have a similar pattern of low CD44/CD133 expression; and (B) Two different TMA cores from the same tumor have a similar pattern of high CD44/CD133 expression. White arrows indicate CD44-positive/CD133-positive cells (original magnification ×4 in A and B; scale bars = 200 μm). (C) Part of a slide from the donor block of the same tumor is shown. Normal pancreatic tissues were obtained from the same patient as a control. Red indicates CD44 staining; green, CD133 staining. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (blue). Yellow indicates the colocalization of CD44 and CD133 (original magnification ×400; scale bar = 20 μm). (D) CD44/CD133 expression levels (mean ± standard error) were compared between pancreatic cancer specimens and their matched normal tissues (n = 11 pairs; $P < .0001$). (E) CD44/CD133 expression levels (mean ± standard error) were compared between pancreatic cancer tissues and adjacent normal tissues ($P < .0001$).

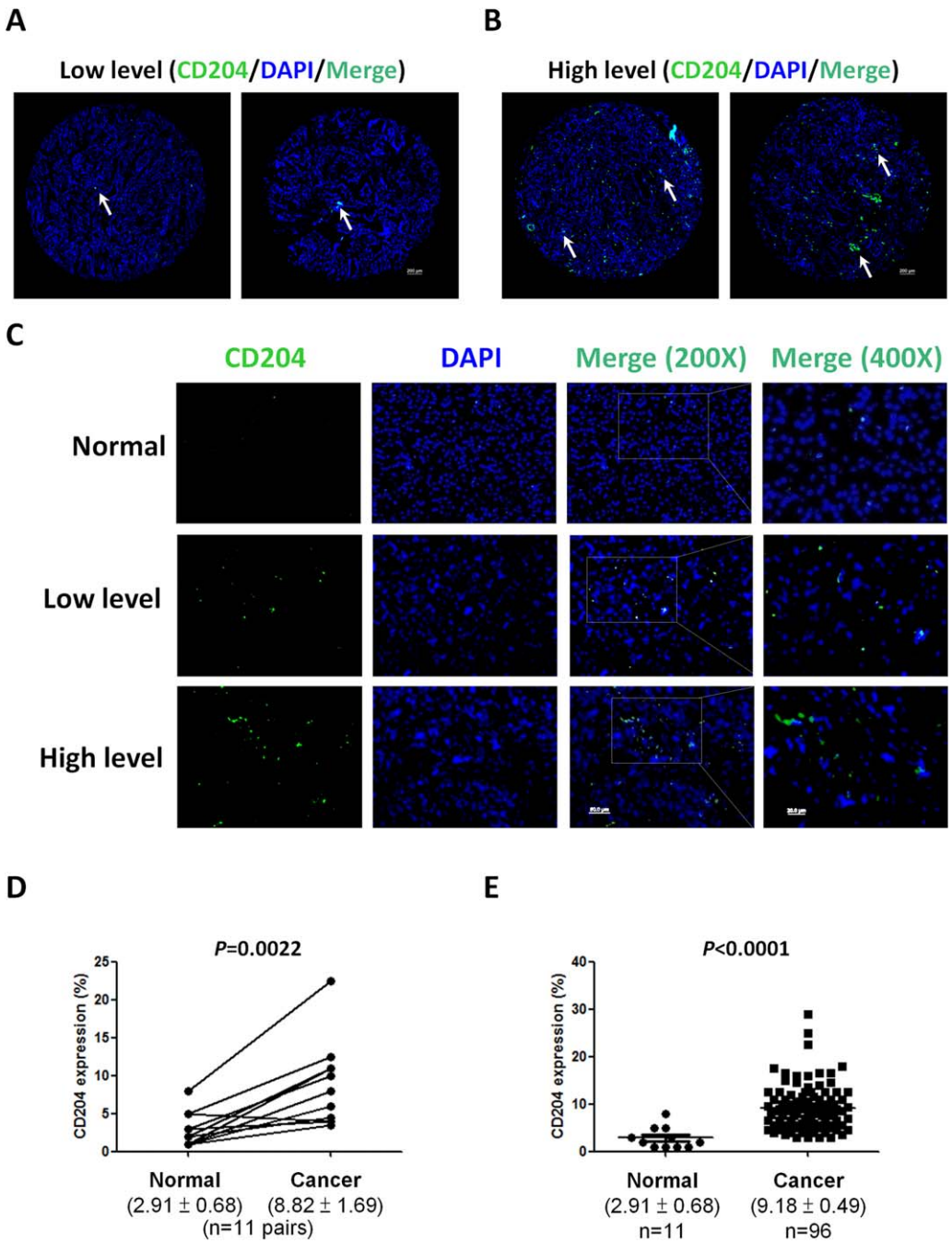

Figure 2. Expression of the tumor-associated macrophages marker CD204 is observed in tissue microarrays (TMAs) and corresponding full sections of pancreatic ductal adenocarcinoma tissue. (A) Two different TMA cores from the same tumor have a similar pattern of low CD204 expression, and (B) Two different TMA cores from the same tumor have a similar pattern of high CD204 expression. White arrows indicate CD204-positive cells (original magnification ×4 in A and B; scale bars = 200 μm). (C) Part of a slide from the donor block of the same tumor is shown at ×200 original magnification (scale bar = 50 μm), and at ×400 magnification on the right (scale bar = 20 μm). The paraffin-embedded patient tissue samples were stained with anti-CD204 antibody (green) and with 4′,6-diamidino-2-phenylindole (DAPI) for cell nuclei (blue). (D) CD204 expression was compared between pancreatic cancer specimens and their matched normal tissues (n = 11 pairs; $P < .0001$). Values indicate the mean ± standard error. (E) CD204 expression levels (mean ± standard error) were compared between pancreatic cancer tissues and adjacent normal tissues ($P < .0001$).

single stained with antibody against the TAMs marker CD204. These antibodies were also used in Western blot and immunofluorescence staining to confirm CD44, CD133, or CD204 expression in cell lines (data not shown). To verify macrophages infiltration at the tumor site, those sections were also stained with antibody against the macrophages marker CD68. Different levels of CD44 and CD133 or coexpression of CD44 and CD133 (CD44/CD133) are illustrated in Figure 1A and 1B, and different expression levels of CD204 are illustrated in Figure 2A and 2B. To validate the accuracy of our data, corresponding full sections of PDAC tumors and normal tissues were stained for these markers, as displayed in Figures 1C and 2C. CD44/CD133 expression and CD204 expression were significantly higher in tumor tissues than in their normal tissue counterparts (Figs. 1D, 1E, 2D, and 2E). The mean ± standard error expression levels of CD44 (9.71% ± 0.58%), CD133 (7.31% ± 0.44%), CD44/CD133 (2.5% ± 0.18%), CD68 (22.39% ± 0.8%), and CD204 (9.18% ± 0.49%) were used to divide patients into a high-expression group and a low-expression group. In the entire pancreatic cancer cohort, 31 of 96 patients (32.3%) had high CD44/CD133 expression, and 65 of 96 patients (67.7%) had low CD44/CD133 expression; whereas 39 of 96 patients (40.6%) had high CD204 expression, and 57 of 96 patients (59.4%) had low CD204 expression (Table 1).

CD44-Positive/CD133-Positive CSCs or CD204-Positive TAMs Expression Versus Clinicopathologic Characteristics

Table 2 summarizes the correlations between clinicopathologic indexes and CD44, CD133, CD44/CD133, or CD204 expression. High CD44/CD133 expression was significantly related to CA 19-9 levels ($P = .017$) and had a borderline significant association with tumor size ($P = .051$). High CD204 expression was associated significantly with margin status ($P = .043$).

Clinicopathologic Features and Expression of CD44-positive/CD133-Positive CSCs or CD204-Positive TAMs Versus Survival

Clinicopathologic parameters are provided in relation to OS or DFS in Table 1. Patients with poorly differentiated carcinoma had worse OS than patients with moderately differentiated or well differentiated carcinoma (7.3 months vs 16.7 months, vs 25.8 months, respectively; $P = .014$). Larger tumor size (>3 cm), positive lymph node metastases, and advanced tumor stage also were significant risk factors for shorter DFS ($P = .011$, $P = .033$, and $P = .000$, respectively). High expression levels of CD44/CD133 and CD204 were significantly associated with shorter OS ($P = .000$ and $P = .011$, respectively). There was no difference between survival and CD44, CD133, or CD68 expression status.

Using multivariate analysis, high expression of CD204 was an independent predictor for OS or DFS (HR, 2.337 [$P = .028$] and 1.800 [$P = .048$], respectively). High CD44/CD133 expression was an independent prognostic factor for DFS (HR, 3.262; $P = .015$). Tumor size and lymph node metastases also were identified as independent factors for DFS (HR, 2.430 [$P = .015$] and 1.750 [$P = .036$], respectively). The results are summarized in Table 3.

Coexpression of CD44/CD133 and CD204 Associated With Poor Outcomes in PDAC

The close localization of CSCs and TAMs by staining with CD44/CD133 or CD204 antibodies in TMAs is illustrated in Figure 3A. Significant positive correlations were observed between expression levels of the TAMs marker CD204 ($r = 0.406$; $P = .000$), and the CSCs markers CD44, CD133 ($r = 0.344$; $P = .001$), or CD44/CD133 coexpression ($r = 0.294$; $P = .004$) (Fig. 3C), but not between expression levels of CSCs markers and the macrophages marker CD68 (Fig. 3B). On the basis of this finding, we classified the patients into 3 groups: low expression (both CD44/CD133 and CD204 expression levels were low; n = 42), intermediate expression (either CD44/CD133 or CD204 expression was high; n = 38), and high expression (both CD44/CD133 and CD204 expression levels were high; n = 16). Patients in the high-expression group had significantly worse OS ($P = .000$) and DFS ($P = .003$) compared with patients in the low-expression and intermediate-expression group in Kaplan-Meier survival analysis (Fig. 3D and 3E).

DISCUSSION

Various pathologic factors, including tumor size, resection margin status, lymph node status, and histologic grade, affect the outcomes of patients with PDAC who undergo resection.[24] In the current study, our univariate analysis indicated that tumor size, tumor differentiation, disease stage, and lymph node status could predict patient outcome, as expected. Although the number of patients enrolled in this study was not large, the 6% 5-year survival rate was consistent with a previous study in which the 5-year survival rate among 1308 patients with PDAC was 6.5% for all groups combined (resected, locally advanced, and metastatic).[25] Such low survival rates in patients with

TABLE 2. Clinicopathologic Parameters and Expression of CD44, CD133, CD44/CD133, and CD204 (n = 96)

Characteristic	CD44 Expression No. (%)			CD133 Expression No. (%)			CD44/CD133 Expression No. (%)			CD204 Expression No. (%)		
	Low	High	*P*	Low	High	*P*	Low	High	*P*[a]	Low	High	*P*[a]
Sex												
Men	37 (67.3)	26 (63.4)	.694	37 (68.5)	26 (61.9)	.499	43 (66.2)	20 (64.5)	.874	36 (63.2)	27 (69.2)	.538
Women	18 (32.7)	15 (36.6)		17 (31.5)	16 (38.1)		22 (33.8)	11 (35.5)		21 (36.8)	12 (30.8)	
Tumor location												
Head	35 (63.6)	25 (61)	.834	33 (61.1)	27 (64.3)	.955	45 (69.2)	15 (48.4)	.079	33 (57.9)	27 (69.2)	.461
Neck	4 (7.3)	3 (7.3)		4 (7.4)	3 (7.1)		5 (7.7)	2 (6.5)		6 (10.5)	1 (2.6)	
Body/tail	10 (18.2)	6 (14.6)		10 (18.5)	6 (14.3)		10 (15.4)	6 (19.4)		10 (17.5)	6 (15.4)	
Uncinate process	6 (10.9)	7 (17.1)		7 (13)	6 (14.3)		5 (7.7)	8 (25.8)		8 (14)	5 (12.8)	
Tumor size, cm												
≤3	30 (54.5)	21 (51.2)	.747	30 (55.6)	21 (50)	.588	39 (60)	12 (38.7)	**.051**	29 (50.9)	22 (56.4)	.594
>3	25 (45.5)	20 (48.8)		24 (44.4)	21 (50)		26 (40)	19 (61.3)		28 (49.1)	17 (43.6)	
Lymph node status												
Negative	28 (50.9)	19 (46.3)	.658	27 (50)	20 (47.6)	.817	33 (50.8)	14 (45.2)	.607	26 (45.6)	21 (53.8)	.428
Positive	27 (49.1)	22 (53.7)		27 (50)	22 (52.4)		32 (49.2)	17 (54.8)		31 (54.4)	18 (46.2)	
Margin status												
R0	37 (67.3)	31 (75.6)	.554	38 (70.4)	30 (71.4)	.373	45 (69.2)	23 (74.2)	.088	43 (75.4)	25 (64.1)	**.043**
R1	16 (29.1)	8 (19.5)		15 (27.8)	9 (21.4)		19 (29.2)	5 (16.1)		10 (17.5)	14 (35.9)	
R2	2 (3.6)	2 (4.9)		1 (1.9)	3 (7.1)		1 (1.5)	3 (9.7)		4 (7)	0 (0)	
Tumor grade												
Poorly differentiated	12 (21.8)	6 (14.6)	.663	12 (22.2)	6 (14.3)	.384	12 (18.5)	6 (19.4)	.938	8 (14)	10 (25.6)	.290
Moderately differentiated	29 (52.7)	23 (56.1)		30 (55.6)	22 (52.4)		36 (55.4)	16 (51.3)		34 (59.6)	18 (46.2)	
Well differentiated	14 (25.5)	12 (29.3)		12 (22.2)	14 (33.3)		17 (26.2)	9 (29)		15 (26.3)	11 (28.2)	
Stage												
I	9 (16.4)	2 (4.9)	.377	8 (14.8)	3 (7.1)	.702	8 (12.3)	3 (9.7)	.801	7 (12.3)	4 (10.3)	.960
II	43 (78.2)	36 (87.8)		43 (79.6)	36 (85.7)		54 (83.1)	25 (80.6)		47 (82.5)	32 (82.1)	
III	2 (3.6)	2 (4.9)		2 (3.7)	2 (4.8)		2 (3.1)	2 (6.5)		2 (3.5)	2 (5.1)	
IV	1 (1.8)	1 (2.4)		1 (1.9)	1 (2.4)		1 (1.5)	1 (3.2)		1 (1.8)	1 (2.6)	
CA19-9, U/mL												
<37	13 (23.6)	7 (17.1)	.433	14 (25.9)	6 (14.3)	.164	18 (27.7)	2 (6.5)	**.017**	10 (17.5)	10 (25.6)	.337
>37	42 (76.4)	34 (82.9)		40 (74.1)	36 (85.7)		47 (72.3)	29 (93.5)		47 (82.5)	29 (74.4)	

Abbreviations: CA19-9, carbohydrate antigen 19-9; CD133, cluster of differentiation 133 (cancer stem cells marker); CD204, cluster of differentiation 204 (tumor-associated macrophages marker); CD44, cluster of differentiation 44 (cancer stem cells marker); CD68, cluster of differentiation 68 (a glycoprotein that binds to low-density lipoprotein); OS, overall survival; DFS, disease-free survival.
[a] Values in boldface indicate $P < .05$.

pancreatic cancer highlighted the critical need for early diagnosis and effective treatment of PDAC. Currently, the tumor-associated antigens CEA, CA 125, and CA 19-9 are used to monitor the outcome and response to treatment of various malignancies.[26] Our results demonstrate that CA 19-9 levels were elevated in 79.2% of patients with PDAC, similar to previous reports.[27,28] However, the CA 19-9 value was not correlated with survival in patients with PDAC, indicating that CA 19-9 is not a good prognostic marker in PDAC. Therefore, the identification of accurate markers indicative of the progression of PDAC is much needed.

A potential disadvantage of TMA sections compared with full tissue sections is that donor cores may not be representative of the whole tumor. To assess these potential limitations of TMAs, different methods of validations have been used in various cancers. Camp et al suggested that 2 needle cores would adequately represent the antigen expression in a whole tissue section with accuracy >95% in breast cancer.[29] The concurrence between TMA cores and donor blocks reportedly was almost perfect, indicating that TMAs could be used to reliably appraise the expression levels of markers in colorectal cancer and esophageal squamous cell carcinoma.[30,31] Hence, we counted the mean percentages of CSCs marker-positive cells and TAMs marker-positive cells in TMAs that included duplicate or quadruplicate cores from 96 patients with PDAC, and we also validated the expression of those markers on corresponding full sections in random sampling. For the assessment of intraobserver reproducibility, we tested 48 randomly chosen patients according to each biomarker cutoff value and expression level (ie, high or low expression levels of CD44, CD133, CD44/CD133, and CD204). This procedure was repeated 10 times. Agreement between the 2 observers was in the sufficient or good range, irrespective of each biomarker cutoff value or expression level ($0.96 > \kappa > 0.6$). The results supported the applicability of this method in our study.

TABLE 3. Multivariate Analysis of Prognostic Factors for Overall and Disease-Free Survival

Variable	No. of Patients	OS			DFS		
		HR	95% CI	P^a	HR	95% CI	P^a
Tumor size, cm							
≤3	5	1.00	0.793-3.225	.221	1.00	1.191-4.957	**.015**
>3	45	1.568			2.430		
Lymph node status							
Negative	47	1.00	0.833-2.724	.175	1.00	1.039-2.948	**.036**
Positive	49	1.507			1.750		
Margin status							
R0	68	1.00	0.699-2.872	.334	1.00	0.364-1.742	.568
R1/R2	28	1.417			0.796		
CD44 expression							
Low	55	1.00	0.088-0.957	.420	1.00	0.138-2.078	.367
High	41	0.291			0.536		
CD133 expression							
Low	54	1.00	0.441-4.112	.602	1.00	0.214-3.595	.854
High	42	1.346			0.876		
CD44/CD133 expression							
Low	65	1.00	0.972-5.868	.058	1.00	1.258-8.461	**.015**
High	31	2.388			3.262		
CD204 expression							
Low	57	1.00	1.098-4.976	**.028**	1.00	1.006-3.220	**.048**
High	39	2.337			1.800		

Abbreviations: CD133, cluster of differentiation 133 (cancer stem cells marker); CD44, cluster of differentiation 44 (cancer stem cells marker); OS, overall survival; PFS, progression-free survival.
[a] Values in boldface indicate $P < .05$.

Pancreatic CSCs can be distinguished from bulk tumor cells by unique markers. CD44, CD133, and epithelial-specific antigen are frequently used to isolate CSCs in different types of tumors.[32] Therefore, we used CD44 and CD133 to isolate CSCs within PDAC tissues. Correlations of the CSCs markers CD44 and CD133 with clinical outcomes have been examined in different gastrointestinal tumors. In 80 patients with PDAC, there was no significant difference in 5-year survival rate based on CD44 expression, but the 5-year survival rate for CD133-positive patients was lower than that for CD133-negative patients.[8] CD44 and CD133 expression levels were significantly higher in tumor cells than in nontumor cells, but individual CD44 or CD133 expression did not correlate with DFS in patients with colorectal cancer.[33] It is noteworthy that another group reported on patients who had CD44-negative but high CD133-expressing tumors that were associated with a significantly worse survival rate than those with CD133-low tumors.[34] In our study, neither CD44 expression nor CD133 expression was correlated significantly with survival, similar to the results reported by Immervoll et al.[9,10] The use of a single CSCs marker (CD44 or CD133) as a predictor of prognosis remains controversial, suggesting that the combination of CD44 and CD133 may be a more specific CSCs marker.

We explored the correlations between CD44, CD133, or CD44/CD133 expression levels and clinicopathological features or survival in patients with PDAC and observed that high CD44/CD133 expression was an independent predictor for shorter DFS, consistent with previous results, which indicated that the combination of CD44 and CD133 expression was a useful marker for predicting survival in patients with pancreatic or colorectal cancer.[8,33] We note that Immervoll et al[10] reported the observation of CD44 at the apical cell membrane adjacent to, but never overlapping with, CD133 expression in some malignant pancreatic ducts. To avoid the immunohistochemical dislocalization of CD44 and CD133 expression, double immunofluorescence staining was performed to assess the CD44-positive/CD133-positive CSCs. We clarified that the clinical significance of CD44-positive/CD133-positive CSCs was associated with survival and malignant tumor behavior in patients with PDAC.

The association between CD204 and worse survival has been observed in patients with esophageal, lung, and colorectal cancers, indicating an important role of CD204-positive TAMs in disease progression.[17-19] In PDAC, patients with high CD163 or expression had a significantly worse prognosis than those with low CD163-positive or CD204-positive expression, but there was no significant difference between the survival rate and the number of CD68-positive macrophages.[20] Furthermore, M2 macrophages were the predominant tumor-

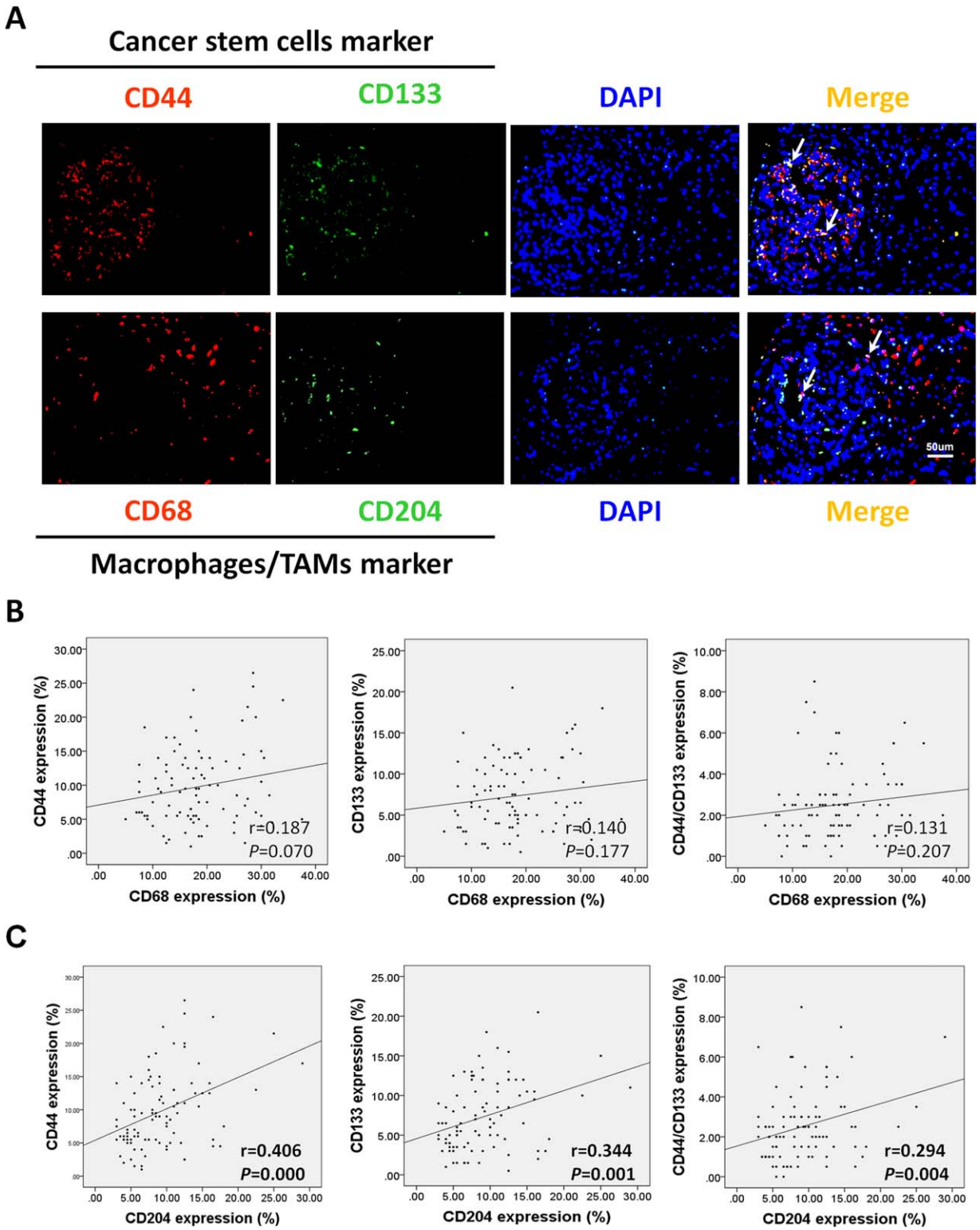

Figure 3. The correlation between CD44-positive/CD133-positive cancer stem cells (CSCs) and CD204-positive tumor-associated macrophages (TAMs) in pancreatic ductal adenocarcinoma is illustrated. (A) CD44/CD133 colocalized with CD68/CD204 in pancreatic cancer specimens. White arrows indicate CD44-positive/CD133-positive or CD68-positive/CD204-positive cells. Red indicates CD44 or CD68 staining; green, CD133 or CD204 staining. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (blue) (original magnification ×400; scale bar = 50 μm). Charts illustrate correlation analyses of CSCs marker expression and macrophages marker (B) or TAMs marker (C) expression level (Pearson test). (D,E) The presence of CD44-positive/CD133-positive CSCs and CD204-positive TAMs had a positive correlation with overall survival and disease-free survival in patients with pancreatic ductal adenocarcinoma.

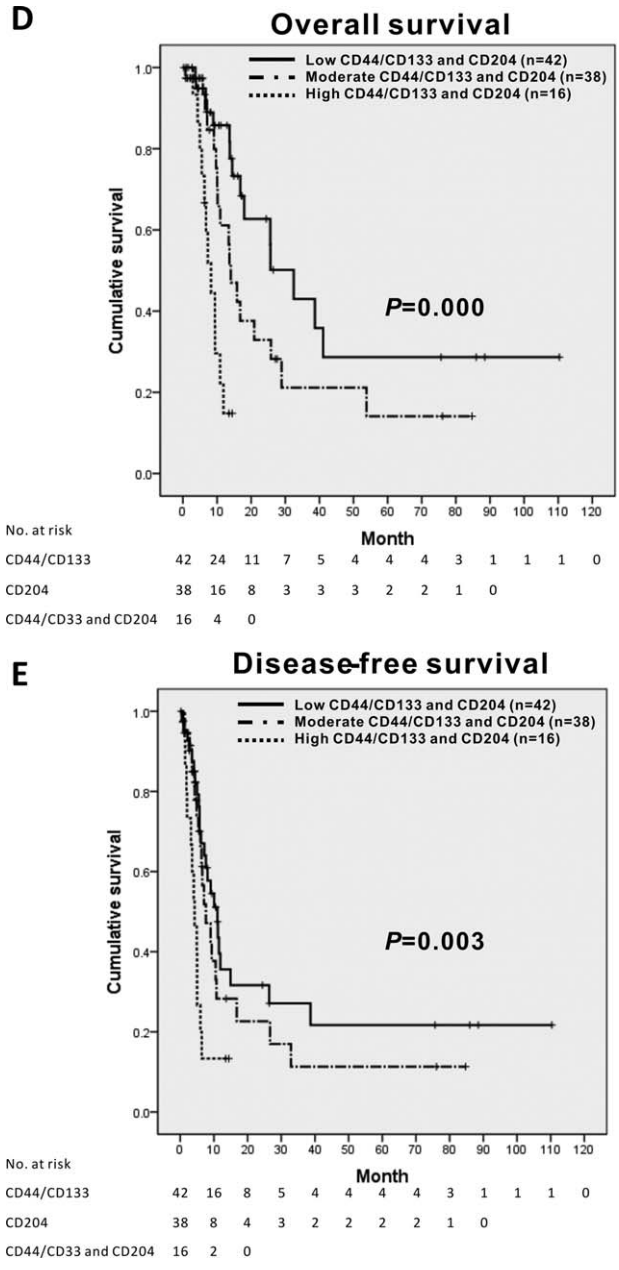

Figure 3. Continued.

infiltrating macrophages in PDAC, whereas M1 macrophages infiltrated predominantly in chronic pancreatitis.[35] In the current study, we observed that CD204 expression was significantly linked to margin status and was associated with poor patient outcomes, consistent with previous studies.

The tumor microenvironment comprises not only cancer cells, which interact with other cells, but also stroma cells, such as stellate cells, panendothelial cells, and infiltrating immune cells.[5] Macrophages were the chief component of the cellular-infiltrating immune cells during pancreatic oncogenesis in genetically engineered mouse models.[36,37] Glioma CSCs recruit circulating monocytes through colony-stimulating factor-1 and mediate their differentiation and polarization into M2 TAMs.[38] Macrophage infiltration in gastric tumor tissues is also an important component of CSCs in promoting Wnt/β-catenin signaling through the TNF-α pathway.[39] TAMs reportedly increase the CSC-like properties of colon and lung cancer cells to promote tumorigenicity and resistance to chemotherapy.[40]

In PDAC, the inhibition of macrophages recruitment into the tumor resulted in a reduction of pancreatic cancer cells that expressed high levels of the CSCs marker ALDH.[21] Taken together, these studies revealed that the interactions between pancreatic CSCs and TAMs may maintain the population of CSCs, promote tumor growth and progression, and alter the stromal compartment to reduce the efficacy of chemotherapy. Therefore, the conjunction of CD44/CD133 and CD204 expression may be a better predictor of survival in PDAC than CD44/CD133 or CD204 expression alone.

In conclusion, not only expression of the CSCs markers CD44 and CD133 but also expression of the TAMs marker CD204 is associated with malignant behavior of PDAC. The clinicopathologic significance of CD44/CD133 and CD204 expression is interrelated. Therefore, the combination of CD44/CD133 expression and CD204 expression is an ideal prognostic marker for PDAC treatment.

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CONFLICT OF INTEREST DISCLOSURES

The authors made no disclosures.

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