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CD133 expression associated with poor prognosis in ovarian cancer

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As a putative marker for cancer stem cells in human malignant tumors, including ovarian cancer, CD133 expression may define a tumor-initiating subpopulation of cells and is associated with the clinical outcome of patients. However, at this time its clinical significance in ovarian cancer remains uncertain. The aim of this study was to clarify the clinical role of CD133 expression in human ovarian cancer. Immunohistochemical staining of CD133 expression was performed in 400 ovarian carcinoma samples using tissue microarray. The associations among CD133 expression and clinical factors (diagnosis, tumor grade, cancer stage, and clinical response to chemotherapy), overall survival and disease-free survival time were analyzed. CD133 expression was found in 31% of ovarian carcinoma samples. Fisher's exact test and one-way analysis of variance suggested that CD133 expression was associated with high-grade serous carcinoma ($P=0.035$), late-stage disease ($P<0.001$), ascites level ($P=0.010$), and non-response to chemotherapy ($P=0.023$). CD133 expression was also associated with shorter overall survival time ($P=0.007$) and shorter disease-free survival time ($P<0.001$) by log-rank test. Moreover, CD133 expression was an independent predictor of shorter disease-free survival time in an unconditional logistic regression analysis with multiple covariates ($P=0.024$). Our results thus show that CD133 expression is a predictor of poor clinical outcome for patients with ovarian cancer, supporting the proposed link between CD133 and cancer stem cells.
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Cancer has long been thought of as a molecularly heterogeneous population of cells, composed of diverse cell subpopulations with different proliferation capacities and differentiation potentials. However, recent evidence suggests that only a few cells within a tumor, known as cancer stem cells, have the capacity to initiate and sustain malignancy.[1] In solid tumors, cancer stem cells have the capacity for self-renewal and multipotent differentiation and the ability to induce tumorigenesis and resistance to chemotherapy or radiotherapy.[2–4]

Ovarian cancer, the fifth leading cause of cancer-related deaths among women in the United States, was estimated to affect over 21 000 women and resulted in nearly 14 000 deaths in 2010.[5] It is the most lethal gynecologic malignancy, primarily owing to the lack of a reliable early detection method, which results in most ovarian cancers being diagnosed at an advanced stage of disease. As in other malignant tumors, cancer stem cells in ovarian cancer are responsible for tumorigenesis and tumor growth.[6]

One potential marker for cancer stem cells in ovarian and other cancers is CD133, also known as Prominin-1, a five-transmembrane glycoprotein that has a molecular weight of 97 kDa and is encoded by the *PROM1* gene on chromosome 4p15.[7,8] Although the function of CD133 is unclear, recent studies indicate that it is a marker of hematopoietic stem cells and progenitor cells[9] and possibly one of the most reliable molecular markers for cancer stem

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cells in various solid tumors, including melanoma[10] and brain,[11] lung,[12] liver,[13] prostate,[14] colon,[15] breast,[16] and ovarian cancers.[17]

As a putative marker for cancer stem cells in ovarian cancer,[17,18] CD133 expression may define a tumor-initiating subpopulation of cells. Previous studies have shown that CD133-positive cells had more tumorigenic and aggressive capacity *in vitro* and/or *in vivo* compared with their CD133-negative progeny and that they exhibited increased resistance to chemotherapeutic strategies.[17,19,20] Thus, CD133 expression may be associated with the clinical outcome of patients with ovarian cancer, but at this time its clinical significance remains uncertain. Therefore, we analyzed the association of CD133 expression in ovarian cancer with clinical factors, overall survival, and disease-free survival.

Materials and methods

Patients and Clinicopathologic Data

We analyzed ovarian carcinoma samples from 400 patients who were diagnosed with ovarian carcinoma and had undergone initial surgery at the University of Texas MD Anderson Cancer Center between 1 January 1990 and 22 February 2007, along with ovarian serous cystadenoma and normal ovarian and fallopian tube tissue samples. We obtained relevant clinical data on patients whose tissue samples we studied by retrospective review of the patients' files. These data included demographics, diagnosis, tumor grade, disease stage, ascites and serum cancer antigen 125 (CA125) level, chemotherapy regimen and response to chemotherapy. Follow-up information was updated through 31 March 2010, by reviewing medical records and the United States Social Security Index. The use of tissue blocks and the chart reviews were approved by the MD Anderson Institutional Review Board.

Histopathologic diagnoses were based on World Health Organization criteria.[21] Tumor grade for non-serous carcinomas was assigned based on the Gynecologic Oncology Group criteria;[22–24] serous carcinomas were graded on a two-tier system (low-grade and high-grade) according to the criteria proposed by Malpica *et al*[25] Disease staging was according to the International Federation of Gynecology and Obstetrics (FIGO) staging system.[26]

To analyze response to chemotherapy, we grouped patients as responders or non-responders. We defined responders as patients who entered complete clinical remission with a normal CA125 level after chemotherapy for histologically or cytologically diagnosed ovarian carcinoma and who had a treatment-free interval of $\geq$6 months.[27] Non-responder group included patients with progressive or recurrent disease. Progressive disease was defined as disease progression that occurred without any observed remission after the initiation of treatment; recurrent disease was defined as disease detected after a period of clinically documented remission of $<$6 months.[28,29]

Immunohistochemical Analysis

For this study, tissue microarray construction was performed as previously described.[30] Tissue microarray slides were subjected to immunohistochemical staining according to the manufacturer's protocol (Biocare Medical, Concord, CA, USA). After sections were deparaffinized in xylene and rehydrated in graded alcohol dilutions, antigen retrieval was performed using a universal decloaker (UD1000M; Biocare Medical) in an autoclave at 121 °C for 5 min to unmask the epitopes. Endogenous peroxidase activity was blocked using Peroxidazed 1 (PX968; Biocare Medical) at room temperature for 10 min. Non-specific binding was blocked with Background Sniper (BS966M; Biocare Medical), with slides incubated for 15 min at room temperature. Next, the slides were incubated overnight at 4 °C with primary rabbit polyclonal antibody against CD133 (ab19898, 1:300 dilution; Abcam, Cambridge, MA, USA), then for 20 min with a biotin-labeled secondary antibody (Universal Goat Link, GU600H; Biocare Medical), and finally for 20 min with 4plus HRP 1000 Universal (HP604; Biocare Medical). After being stained for 3 min with DAB Chromogen (DB801L; Biocare Medical), the tissues were counterstained with hematoxylin, dehydrated, and mounted. Negative controls were made by replacing the primary antibody with phosphate-buffered saline. One sample of human seminoma tissue was stained as a positive control. All controls yielded satisfactory results.

Two gynecologic pathologists (JL and JZ) independently analyzed the immunohistochemically stained slides for CD133 expression. Staining degree was scored, and the staining pattern—membrane and/or cytoplasmic reactivity of the cells—was noted. The degree of staining was quantified using a four-level grading system. Samples with no CD133-positive tumor cells were given a score of 0; those with $<$5% CD133-positive tumor cells, 1; those with 5–50% CD133-positive tumor cells, 2; and those with $>$50% CD133-positive tumor cells, 3. For statistical analysis, cases were divided into negative (0% CD133-positive tumor cells; Figure 1e) or positive ($>$0% CD133-positive tumor cells) CD133 expression.

Statistical Analysis

We used Fisher's exact test and logistic regression analysis to evaluate the association of CD133 expression with clinical factors. We performed one-way analysis of variance for multiple comparisons to evaluate the association of CD133 expression with response to chemotherapy. We used the Kaplan–Meier method to estimate overall survival and disease-free survival rates and the log-rank test

to compare overall survival and disease-free survival time among different groups, such as patients with negative or positive CD133 expression. To adjust for the confounding effects of age at diagnosis, tumor grade, response to clinical treatment, and FIGO stage, we performed unconditional logistic regression analysis with multiple covariates in determining the association of CD133 expression with overall survival or disease-free survival.

Overall survival time was computed as the time interval from the date of first biopsy to the date of death from disease or the last follow-up date, whichever occurred first. Patients who were alive on the last follow-up date were censored from analysis. Disease-free survival time was computed as the time period from the date of first biopsy to the date of recurrence. Disease recurrence was determined clinically by the appearance of new lesions or an increase in the serum CA125 level to more than twice the upper limit of normal range (0–35 U/ml).[31,32] Patients alive without recurrence on the last follow-up date were censored from analysis. Results were considered statistically significant at the $P<0.05$ level. SPSS (version 17.0; SPSS, Chicago, IL, USA) and Stata (version 8.0; Stats Corporation, College Station, TX, USA) software were used for statistical analyses.

Results

Patient Characteristics

The mean age of the 400 patients we studied was 59 years (range, 20–89 years). At the time of this study, 67 patients were alive without clinical evidence of ovarian cancer, 53 were alive with ovarian cancer, 210 had died from ovarian cancer, 58 had died from unrelated or unknown ovarian cancer, and 12 were

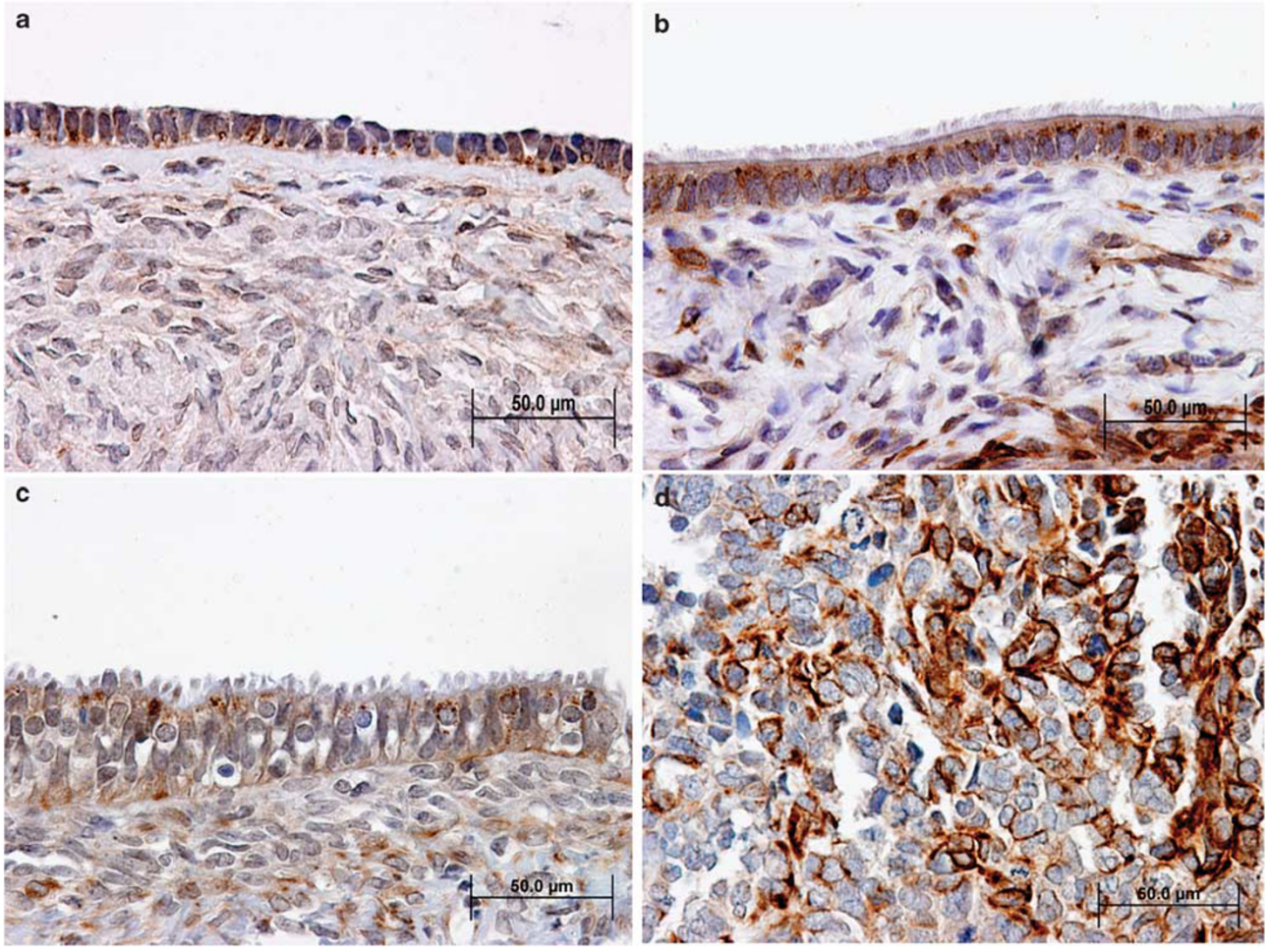

Figure 1 Pattern of CD133 immunoreactivity in normal ovarian, normal fallopian tube, ovarian serous cystadenoma, and ovarian carcinoma tissues. (**a**) CD133-positive staining in normal ovarian tissue. (**b**) Normal fallopian tube tissue showing a dot-like pattern of CD133 expression in the epithelial cells. (**c**) Serous cystadenoma tissue with a few cells showing a dot-like pattern of CD133 expression. (**d**) Cell membrane expression of CD133 in low-grade serous carcinoma. (**e**) Negative expression of CD133 in high-grade serous carcinoma. (**f**) The cytoplasmic expression of CD133 in high-grade serous carcinoma. (**g**) High-grade endometrioid adenocarcinoma cells showing CD133 expression diffusely in the cytoplasm. (**h**) Sparsely cytoplasmic staining for CD133 in malignant mixed müllerian tumor cells. (**a–h**) Original magnification ×400; see text for immunostaining procedure.

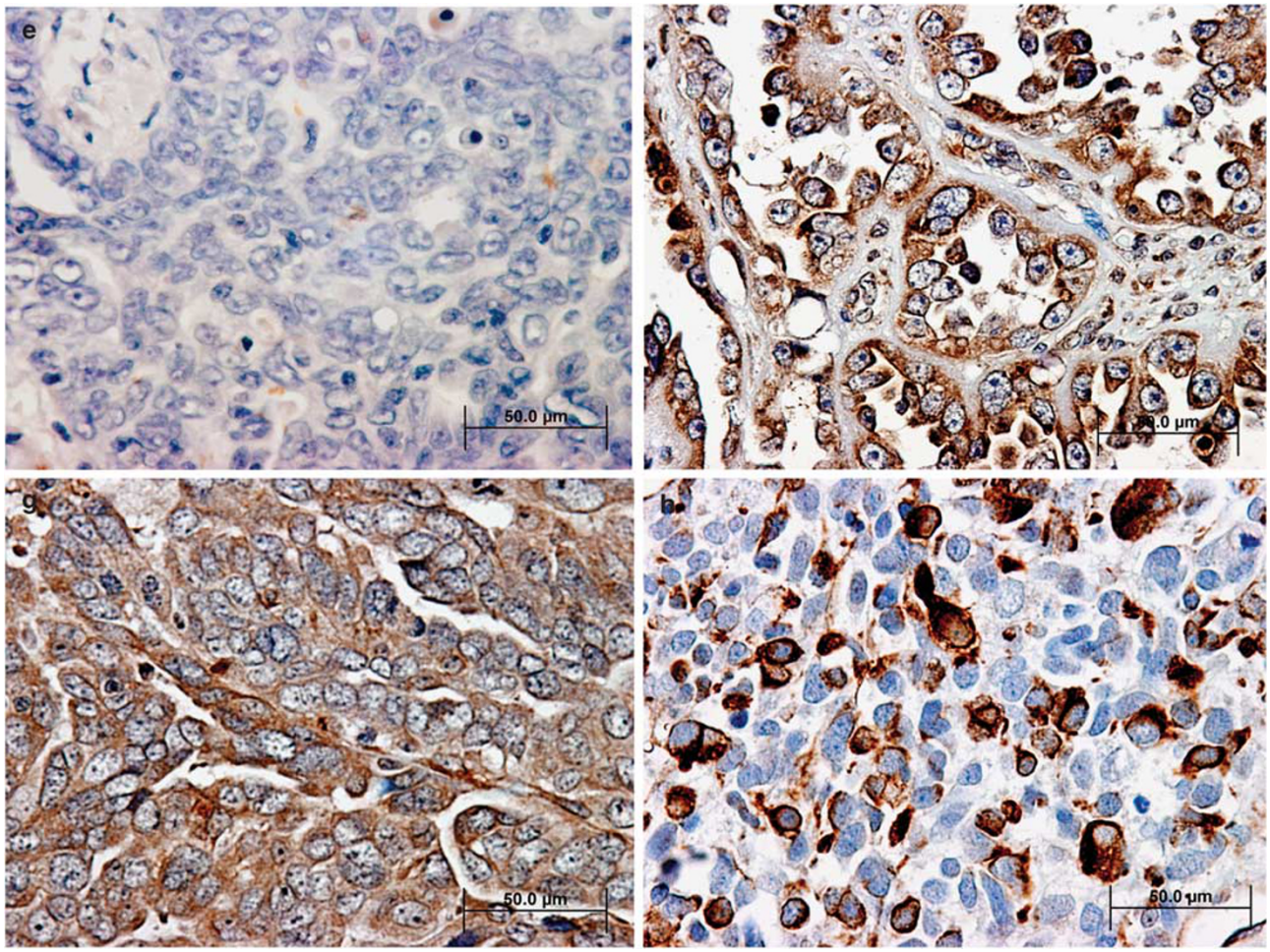

Figure 1 Continued.

lost to follow-up and were excluded from the overall survival analysis. The median overall survival time was 4.6 years (95% confidence interval (CI): 3.9–5.2 years), and the overall survival rates were 56% (95% CI: 53–59%) at 3 years, 41% (95% CI: 38–44%) at 5 years, and 33% (95% CI: 30–36%) at 10 years. In all, 51 patients had no disease relapse, 165 had disease relapse, 144 had disease progression, and 40 were lost to follow-up or unknown serum CA125 level. Only the patients with and without disease relapsed were including the disease-free survival analysis. The median disease-free survival time was 1.6 years (95% CI: 1.2–2.0 years), and the disease-free survival rates were 32% (95% CI: 29–35%) at 3 years, 25% (95% CI: 22–28%) at 5 years, and 21% (95% CI: 18–24%) at 10 years.

CD133 Expression and Localization

We analyzed CD133 expression in 400 primary ovarian carcinoma samples, 15 normal fallopian tube tissue samples, 10 normal ovarian tissue samples, and 7 ovarian cystadenoma samples. Representative samples of CD133 staining were shown in Figure 1. Staining indicated CD133-positive cells in 123 (31%) of 400 ovarian carcinoma tissues. Among these 123 cases, the CD133-positive distributions were sparse and scattered in 31%, focal in 24%, and diffuse in 45%. Although CD133 has been reported to be a cell surface marker,[7] in our samples a membrane staining pattern was evident in only 16% (20/123) of CD133-positive cases (Figure 1d). More frequently, CD133-positive cells showed moderate-to-strong, dense, brownish cytoplasmic staining (84% (103/123) of CD133-positive cases; Figure 1f–h). CD133 immunoreactivity was also found in the cytoplasm of stromal fibroblasts.

Among the non-cancerous samples, 47% (7/15) of normal fallopian tube, 50% (5/10) of normal ovarian, and 14% (1/7) of ovarian serous cystadenoma tissue samples showed CD133 expression. In each sample, only a few cells stained positively for CD133. In contrast to CD133-positive ovarian carcinoma cells, CD133-positive epithelial cells in normal fallopian tube tissue samples (Figure 1b) showed a strong punctate or dot-like pattern in the cytoplasm. The same CD133 expression pattern was

Table 1 Associations between CD133 expression and clinicopathologic factors

Characteristic	*No. of patients (%)*			P-*value*[a]
	CD133-negative expression	*CD133-positive expression*	*Total no.*	
Histologic type				0.035
High-grade serous carcinoma	203 (67)	100 (33)	303	
Low-grade serous carcinoma	11 (73)	4 (27)	15	
Endometrioid carcinoma	29 (91)	3 (9)	32	
Other[b]	34 (68)	16 (32)	50	
FIGO stage				<0.001
I	30 (100)	0 (0)	30	
II	19 (86)	3 (14)	22	
III	176 (65)	93 (35)	269	
IV	52 (66)	27 (34)	79	
Response to clinical treatment				0.386
Complete	149 (73)	54 (27)	203	
Partial	63 (69)	29 (31)	92	
No	48 (66)	25 (34)	73	
Unknown	17 (53)	15 (47)	32	
Ascites				0.010
No	52 (83)	11 (17)	63	
Yes	166 (65)	89 (35)	255	
Unknown	59 (72)	23 (28)	82	
Age at diagnosis				0.665
≤60 years	143 (70)	60 (30)	203	
>60 years	134 (68)	63 (32)	197	
CA125				0.293
<500 U/ml	117 (73)	44 (27)	161	
≥500 U/ml	125 (67)	61 (33)	186	
Unknown	35 (66)	18 (34)	53	

Abbreviations: FIGO, International Federation of Gynecology and Obstetrics; CA125, cancer antigen 125 (normal range, 0–35 U/ml).
[a]*P*-values were calculated using Fisher's exact test.
[b]Other includes mucinous carcinoma (8 cases), clear cell carcinoma (12 cases), transitional cell carcinoma (10 cases), malignant mixed müllerian tumor (9 cases), undifferentiated carcinoma (8 cases), and mixed type carcinoma (3 cases).

observed in ovarian cystadenoma samples (Figure 1c) and in three samples of CD133-positive normal ovarian epithelial cells (Figure 1a).

Association of CD133 Expression with Clinicopathologic Variables

The results of CD133 immunohistochemical staining in the ovarian carcinoma tissue microarrays, organized according to the clinicopathologic characteristics of the patients, were summarized in Table 1. Expression of CD133 was associated with high-grade serous carcinoma ($P=0.035$), late-stage disease ($P<0.001$), and ascites level ($P=0.010$). We analyzed the association of CD133 expression with the grade of serous or endometrioid carcinoma separately because of their different grading systems and found no association between CD133 expression and tumor grade in either serous or endometrioid carcinoma (data not shown).

Table 2 Associations between CD133 expression and response to chemotherapy

Response to chemotherapy	*No. of patients (%)*		*Total no.*
	CD133-negative expression	*CD133-positive expression*	
Unknown response	12 (57)	9 (43)	21
Responders[a]			
Cisplatin-based regimen			
Postsurgery[b]	119 (74)	41 (26)	160
Presurgery	9 (90)	1 (10)	10
Other regimen	1 (100)	0 (0)	1
Non-responders[a]			
Cisplatin-based regimen			
Postsurgery[b]	83 (61)	53 (39)	136
Presurgery	28 (76)	9 (24)	37
Other regimen	2 (67)	1 (33)	3
No chemotherapy	16 (73)	6 (27)	22
Unknown chemotherapy regimen	7 (70)	3 (30)	10
Total	277 (69)	123 (31)	400

[a]Responders *vs* non-responders: $P=0.023$.
[b]Responders *vs* non-responders in cisplatin-based postsurgery subgroup: $P=0.014$.
P-values were calculated by one-way analysis of variance.

The association of CD133 expression with response to chemotherapy was shown in Table 2. A total of 378 patients (95%) were known to have received chemotherapy. Two hundred ninety-six patients (74%) received postsurgical cisplatin-based treatment, either alone or in combination with other drugs. In 47 patients (12%), cisplatin-based treatment was administered before debulking surgery. Four patients (1%) received other forms of treatment (GOG182 or 5-fluorouracil plus folinic acid). Compared with the responders group and its postsurgical cisplatin-based treatment subgroup, CD133 expression was observed more often in the non-responder group than in the responder group ($P=0.023$) and in the postsurgical cisplatin-based treatment non-responders than in the postsurgical cisplatin-based responders ($P=0.014$).

Association of CD133 Expression with Overall Survival and Disease-Free Survival

Overall survival and disease-free survival rates at 3, 5, and 10 years were shown in relation to CD133 expression in Table 3. Overall, CD133 expression was associated with both overall survival and disease-free survival. Patients who had ovarian

Table 3 Association of CD133 expression with overall and disease-free survival

CD133 status	*Overall survival* (n = *388*)		*Disease-free survival* (n = *216*)	
	Negative	*Positive*	*Negative*	*Positive*
No. of patients	268	120	161	55
Median survival (95% CI), years	5.4 (4.0–6.7)	4.0 (3.0–4.9)	1.9 (1.1–2.6)	1.1 (0.8–1.3)
Survival rate (95% CI)				
3 years	0.67 (0.61–0.73)	0.59 (0.50–0.68)	0.43 (0.35–0.51)	0.19 (0.09–0.30)
5 years	0.50 (0.44–0.57)	0.37 (0.28–0.47)	0.33 (0.25–0.40)	0.12 (0.03–0.21)
10 years	0.38 (0.30–0.45)	N/A (<<0.23)	0.27 (0.20–0.35)	N/A (<<0.07)
P-value[a]	0.007		<0.001	

Abbreviation: CI, confidence interval.
[a]*P*-values were derived from the log-rank test.

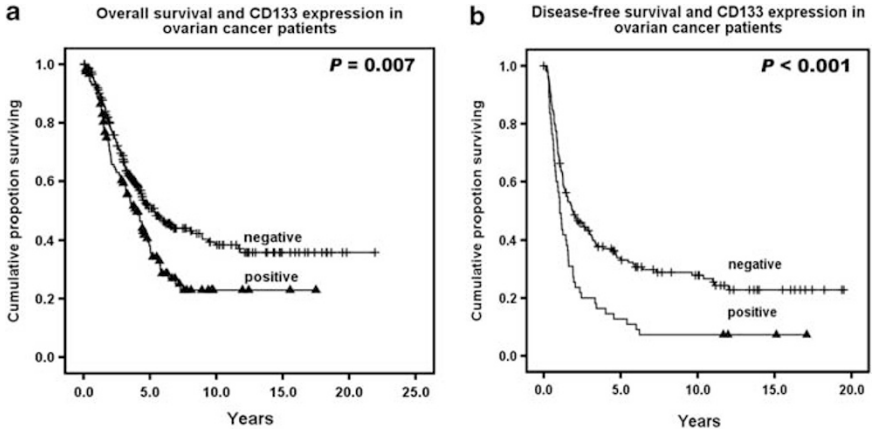

Figure 2 Kaplan-Meier survival curves for patients with ovarian carcinoma with (positive) or without (negative) CD133 expression in tumor cells: (**a**) overall survival ($n = 388$) and (**b**) disease-free survival ($n = 216$).

carcinoma with CD133 expression had shorter median overall survival ($P = 0.007$) and disease-free survival times ($P < 0.001$) than patients who had ovarian carcinoma without CD133 expression (Figure 2). There were no significant associations among the different patterns of CD133 expression (cytoplasm or membrane) and overall survival ($P = 0.132$) or disease-free survival ($P = 0.880$) or among the different degrees of CD133 expression (<5, 5–50, or >50%) and overall survival ($P = 0.891$) or disease-free survival ($P = 0.774$).

Unconditional logistic regression analysis with multiple covariates revealed that CD133 status was an independent predictor of disease-free survival in patients with ovarian carcinoma (Table 4). CD133 expression was associated with shorter median disease-free survival time and higher risk increased for disease recurrence (adjusted odds ratio (OR): 1.50; 95% CI: 1.06–2.13; $P = 0.024$), especially in the stratified analysis according to age at diagnosis ≤60 years old (adjusted OR: 2.07; 95% CI: 1.27–3.37; $P = 0.004$), high-grade tumor (adjusted OR: 1.51; 95% CI: 1.06–2.16; $P = 0.022$), complete and partial response to clinical treatment (adjusted OR: 1.53; 95% CI: 1.08–2.18; $P = 0.017$), and late stage (adjusted OR: 1.43; 95% CI: 1.00–2.05; $P = 0.048$). However, CD133 expression was not an independent predictor of overall survival (data not shown).

Discussion

Our results demonstrated that the CD133 expression was a predictor of poor clinical outcome in patients with ovarian cancer. CD133 expression was associated with high-grade serous carcinoma, late-stage disease, ascites level, and shorter overall survival and disease-free survival time. Moreover, multivariate logistic regression analysis showed that CD133 status was an independent predictor of disease-free survival.

Ovarian cancer has a high risk of recurrence and metastasis; tumor cells remaining after surgery often escape chemotherapy- and/or radiotherapy-induced cell death. Cancer stem cells are relatively resistant to these therapeutic methods because of abnormalities in the cell death pathway, such as inhibition of NF-κB activation,[33] or overexpression of the inhibitor of apoptosis family proteins,[34] Bcl-2 proteins,[35] or TRAIL-receptor proteins.[36] In our study, CD133 expression was more often observed in patients who did not respond to chemotherapy in general and

Table 4 Multivariate analysis of the association between CD133 expression and disease-free survival ($n = 214$)[a]

CD133 status	*Relapse, no. of patients (%)*	*No relapse, no. of patients (%)*	*Odds ratio (95% CI)*[b]	*Median survival, years*	P-*value*[c]
Overall					0.024
Negative	47 (30)	112 (70)	1 (Reference)	1.9	
Positive	4 (7)	51 (93)	1.50 (1.06–2.13)	1.1	
Stratified by age at diagnosis (≤60 years)					0.004
Negative	30 (34)	58 (66)	1 (Reference)	2.9	
Positive	1 (3)	28 (97)	2.07 (1.27–3.37)	1.1	
Stratified by tumor grade (high-grade)					0.022
Negative	43 (29)	104 (71)	1 (Reference)	1.8	
Positive	4 (7)	50 (93)	1.51 (1.06–2.16)	1.1	
Stratified by clinical response (complete and partial)					0.017
Negative	41 (28)	107 (72)	1 (Reference)	1.8	
Positive	3 (6)	48 (94)	1.53 (1.08–2.18)	1.1	
Stratified by FIGO stage (stage III and IV)					0.048
Negative	23 (20)	92 (80)	1 (Reference)	1.2	
Positive	3 (6)	51 (94)	1.43 (1.00–2.05)	1.1	

Abbreviations: CI, confidence interval; FIGO, International Federation of Gynecology and Obstetrics.

[a]Excluding two patients unknown clinical response.

[b]Odds ratios are adjusted for age, tumor grade, chemotherapy response, and FIGO stage.

[c]*P*-values were derived from unconditional logistic regression analysis with multiple covariates.

postsurgical cisplatin-based treatment in particular than in responders. These results are consistent with the hypothesis that the cells expressing CD133 are cancer stem cells and, thus, are resistant to cisplatin-based chemotherapy.

Ferrandina *et al*[37,38] were the first to identify CD133 expression in human ovarian cancer tissue. In a study of 160 cases, they found that CD133 expression seemed not to provide additional prognostic information for ovarian cancer patients.[37,38] However, our study, which found an association between CD133 status and prognosis, was much larger, with 400 well-characterized patients, and included long-term patient follow-up data. Our results showing that CD133 expression is associated with various clinicopathologic characteristics of primary ovarian cancer patients, with shorter disease-free survival time, and with lack of response to chemotherapy are in agreement with findings that CD133 expression has prognostic value in hepatocellular carcinoma,[39,40] colon and rectal adenocarcinoma,[41,42] invasive ductal breast carcinoma,[43] glioma,[44] and non-small cell lung carcinoma.[45] Our study suggests the need to establish a novel strategy to kill CD133 + cancer cells in the future, especially in the treatment of chemoresistant or radioresistant in order to decrease disease recurrence.

There is much histologic heterogeneity in ovarian cancer. Based on clinicopathologic and genetic molecular analyses, at least a subset of ovarian high-grade serous carcinoma,[46,47] may originate in the epithelium of the distal fallopian tube, although most ovarian cancers arise from various tissues of the ovaries. Therefore, we detected CD133 expression in normal ovarian and fallopian tube tissues as well as in the cancerous tumors. Only a few cells in these non-cancerous samples showed CD133 expression, and they had a strong punctate or dot-like staining pattern in the cytoplasm, which contrasted with the more diffuse staining pattern in the membrane and/or cytoplasm of the cancerous cells. This may indicate that CD133 has different locations or functions during ovarian tumorigenesis and progression, but its specific biologic mechanisms need further study.

In conclusion, CD133 expression is a predictor of poor response to chemotherapy and of reduced disease-free survival time for patients with ovarian cancer. These findings lend support to the proposed link between CD133 and cancer stem cells.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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