

# Combined CD133/CD44 Expression as a Prognostic Indicator of Disease-Free Survival in Patients With Colorectal Cancer

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**Hypothesis:** Because of some inconsistencies in the traditional model of human colorectal carcinogenesis, the cancer stem cell (CSC) model was recently proposed, in which tumor results from neoplastic transformation of stem cells, which become CSCs. Identification of CSCs by expression of surface antigens remains a critical issue because no biomarker has been shown to be completely reliable. CD133 and CD44 are commonly used as CSC markers, and correlation of their expression with colorectal cancer (CRC) clinicopathological features and outcomes may be useful.

**Design:** Pilot study.

**Setting:** University hospital.

**Patients:** Thirty-six consecutive patients with CRC. CD133 and CD44 expression (alone or combined) was determined in nontumor cells and in tumor cells by flow cytometry, which identified viable cells only.

**Main Outcome Measures:** Correlation of CD133 and CD44 expression with each other, with other prognostic indicators, and with disease-free survival.

**Results:** CD133 and CD44 expression was significantly higher in tumor cells than in nontumor cells, and expression of one did not necessarily correlate with expression of the other. CD133 or CD44 expression alone was variable, while combined CD133/CD44 expression identified a small subset of cells positive for CRC. CD133 or CD44 overexpression was not associated with CRC recurrence; only high frequencies of CD133<sup>+</sup>/CD44<sup>+</sup> cells were a strong indicator of worse disease-free survival and an independent risk factor for CRC recurrence.

**Conclusion:** Evaluation of combined CD133/CD44 expression could be useful to identify putative colorectal CSCs and tumors with a poor prognosis.

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POTENTIALLY CURATIVE SURGERY, with or without adjuvant chemotherapy, represents the most common treatment option for localized nonmetastatic colorectal cancer (CRC).<sup>1</sup> In metastatic CRC tumors, combinations of chemotherapeutic and biological agents have been shown to improve overall and disease-free survival

## See Invited Critique at end of article

(DFS) rates.<sup>2</sup> However, in metastatic CRC these new treatments have not translated into complete remissions, and the chance of cure is rare.<sup>3</sup> Moreover, outcomes after curative surgical resection are associated with disappointingly high rates of local recurrence or distant metastasis,<sup>4</sup> with CRC being the second most common cause of cancer-related death.<sup>5</sup>

According to the traditional model of colorectal carcinogenesis, any differentiated epithelial cell following a combination of epigenetic changes and genetic events may initiate and sustain clonal tumor growth.<sup>6,7</sup> However, this clonal model is unsatisfactory. For instance, short-lived intestinal epithelial cells may not have sufficient time to accumulate tumor-inducing genetic changes<sup>8</sup> and show poor capability of initiating tumor growth in immunodeficient mice and are resistant to treatment (chemoradiotherapy).<sup>9,10</sup> Conversely, stem cells (being long lived) may accumulate oncogenic mutations and undergo selection needed for multistep carcinogenesis, especially in such a rapidly renewing system as the gut epithelia.<sup>11</sup> In many human cancers (including CRC), it was thought that only a subset of undifferentiated cells, derived from neoplastic transformation of stem cells (so-called cancer stem cells [CSCs]), had the ability to perpetuate themselves (self-renewal), initiate tumor growth, and

cause chemoresistance, metastasis, and tumor recurrence.<sup>4,12-14</sup> However, a crucial issue with CSCs is their identification and characterization. Colorectal CSCs were initially identified by CD133 expression using an antibody specific for epitope AC133.<sup>4,15</sup> However, the use of CD133 for colorectal CSC identification is a subject of debate, and other surface markers (particularly CD44, previously identified as a stem cell marker in breast and neck cancers<sup>14,16,17</sup>) are under investigation.<sup>18-21</sup> Finally, few studies have correlated CSC surface marker expression with CRC clinicopathological features.

Using flow cytometry, we examined CD133 and CD44 expression (alone or combined) in surgical specimens of CRCs from potentially curative resections. Our study end points were (1) the frequency and pattern of CD133 and CD44 expression (alone or combined), (2) their correlation with tumor progression, and (3) their prognostic significance relative to DFS.

## METHODS

### PATIENTS

Between January 1, 2009, and June 31, 2009, 45 consecutive patients with CRC were observed; 9 patients who underwent a nonradical operation were excluded from this study. The study population consisted of 36 patients undergoing a potentially curative surgical resection, defined as removal of all macroscopic tumor masses, absence of microscopic residual tumor, histologically confirmed negative resection margins, and extension of lymphadenectomy beyond involved nodes.<sup>22</sup> Thirteen of our patients with metastases limited to the liver underwent simultaneous hepatic resection, including 10 segmentectomies and 3 bisegmentectomies, with negative resection margins. All the patients were discharged from the hospital and underwent adjuvant chemotherapy if appropriate (those with T4 or >N0 colon cancers and those with T3, T4, or >N0 rectal cancers).<sup>1</sup> Follow-up protocol included physical examination and carcinoembryonic antigen serum level measurement every 3 months for the first 2 years, colonoscopy at 1 year, and liver ultrasonography every 6 months for 2 years. Chest, abdominal, and pelvic computed tomography was performed annually (patients undergoing liver resection had a more strict follow-up protocol, including liver ultrasonography every 3 months and computed tomography every 6 months). If recurrence was suspected, patients underwent further diagnostic evaluation, always complemented by routine histopathological examination of biopsy specimens. No patient was lost to follow-up care, which was completed by December 31, 2010. All the patients gave their informed consent, and the study was approved by the Department of Clinical and Experimental Medicine and Surgery "F. Magrassi-A. Lanzara," Second University of Naples School of Medicine, Naples, Italy.

Several clinicopathological variables were recorded. These included patient age, sex, and performance status assessed with the Eastern Cooperative Oncology Group Performance Status Scale, as well as TNM stage,<sup>23</sup> cancer site (right vs left colon or rectum), number of resected lymph nodes (LNs), LN ratio (LNR [the ratio of metastatic LNs to resected LNs]), histologic differentiation (well, moderate, or poor), and disease recurrence rate.

### TISSUE SPECIMENS

From each patient, normal tissue and colorectal tumor samples were obtained and were immediately analyzed to minimize ex-

perimental variability and loss of cell viability, as previously detailed.<sup>5,15</sup> Briefly, samples were washed extensively in Dulbecco Modified Eagle Medium F12 (DMEM:F12) without phenol red and supplemented with 10% penicillin and 0.5% amphotericin B. Normal and tumor tissues were then minced separately in a 35-mm petri dish as much as possible using a sterile scissors. Normal and tumor solutions were then resuspended up and down for 5 minutes using a 5-mL sterile pipette and were then washed 2 times in supplemented DMEM:F12 solution. Thereafter, minced tissues were digested with collagenase type I (1.5 mg/mL; Sigma-Aldrich), hyaluronidase (20 µg/mL; Sigma-Aldrich), and deoxyribonuclease (1 mg/mL; Roche Diagnostics) using gentle agitation for 1 hour at 37°C in supplemented DMEM:F12. Cell suspensions were then washed 2 times in DMEM:F12, and red blood cells were lysed by rapid incubation for 5 minutes in ice-cold ammonium chloride. The number of cells and cell viability were determined using a Burkler chamber and trypan blue exclusion, respectively.

## FLOW CYTOMETRY ANALYSIS AND CELL SORTING

Cell suspensions were washed and reconstituted to a final concentration of  $1.0 \times 10^6$  cells/mL in 2% fetal bovine serum-phosphate-buffered saline (2% FBS-PBS). Fifty microliters of each cell suspension was pipetted into 5-mL polystyrene tubes (Falcon; Becton Dickinson) and incubated with 5 µL of each monoclonal antibody (listed herein) for 30 minutes at 4°C. Cells were then washed with 2% FBS-PBS (1 mL), resuspended in 2% FBS-PBS (500 µL), stained with sytox blue (0.5 µL; Invitrogen) for 5 minutes at room temperature, and analyzed by flow cytometry. A cell sorter (BD FACSAria; Becton Dickinson) was used for the analysis. The antibodies used in this study included CD133-APC (AC133 clone; Miltenyi Biotec), CD44-PE-Cy7 (BioLegend), and CD326-PerCP and CD45-APC-Cy7 (BD Biosciences). Sytox blue was used as viable dye. For marker expression analysis, the following gating strategy was applied. Cells in a forward scatter vs side scatter dotplot were selected, excluding debris, and then doublets were excluded from the selected cell population in a forward scatter area vs forward scatter height dotplot. Living cells were identified as sytox blue negative. Finally, among single living cells, the subset of epithelial tumor cells was selected as CD45<sup>-</sup>/CD326<sup>+</sup> cells, and expression of all other markers was evaluated in this fraction. Based on previous findings,<sup>5,15,18</sup> this allowed us to discard hematopoietic cells (CD45<sup>+</sup>) and other contaminants, such as mesenchymal or damaged autofluorescent cells, which unlike epithelial cells are CD326 negative. The background level for each fluorochrome was positioned using the "fluorescence minus 1" technique. Each surface marker expression (specifically, CD133, CD44, and CD133/CD44 positivity) was expressed as the ratio of positive cells to total sample cells.

## STATISTICAL ANALYSIS

Statistical analysis was performed using commercially available software (MedCalc, version 9.4.2.0; MedCalc Software), with significance set at  $P < .05$ . The equality of group means was analyzed using paired *t* test. Linear regression analysis was performed to correlate different markers with each other and with clinicopathological features. For continuous variables (such as marker expression), receiver operating characteristic (ROC) curve analysis was used to determine the value demonstrating the highest accuracy to predict outcomes. The area under the ROC curve (with its *P* value) determined the probability that the variable under study could distinguish among different outcomes. When the area under the ROC curve was significant,

the variable was grouped according to the value found by ROC curve analysis (combined CD133/CD44). Otherwise, the variable was grouped using the median value (CD133 and CD44). Univariate analysis of DFS was performed using Mantel-Cox log-rank test, and Kaplan-Meier survival curves were plotted using the product-limit method. The independent significance of prognostic variables (those with  $P < .10$  in univariate analysis) was determined in multivariate analysis using a Cox proportional hazards regression model. Finally, stepwise multivariate analysis was performed to generate a model of the best linear combination of variables that was able to predict DFS.

## RESULTS

### OVERALL FINDINGS

The clinicopathological characteristics of 36 patients are summarized in **Table 1**. Many tumors were in advanced stages, with a high probability of recurrence: 81% (29 of 36) were T3 or T4, 58% (21 of 36) were node positive, and 36% (13 of 36) had distant metastasis.

Results of flow cytometry analysis are given in **Table 2**. Significantly lower marker expression was observed in nontumor cells than in tumor cells. CD133<sup>+</sup> and CD44<sup>+</sup> cells were widely present within cancer cells, with expression ranging from 0.3% to 98.0%. In contrast, the combination of both antigens was present in much lower percentages. The mean expression of CD133<sup>+</sup>/CD44<sup>+</sup> cells was 1.5% (median, 0.6%). Linear regression analysis showed no correlation of the markers with each other: CD133<sup>+</sup> and CD44<sup>+</sup> ( $r=0.011$ ,  $P=.95$ ), CD133<sup>+</sup> and CD133<sup>+</sup>/CD44<sup>+</sup> ( $r=0.206$ ,  $P=.25$ ), and CD44<sup>+</sup> and CD133<sup>+</sup>/CD44<sup>+</sup> ( $r=0.206$ ,  $P=.25$ ). CD133 positivity has a linear relationship with node-positive tumors, distant metastasis, Dukes classification, and TNM stage. Although not significant, CD44 positivity had an inverse relationship with these factors. In contrast, CD133/CD44 positivity had no relationship with the presence of metastatic LNs, distant metastasis, or advanced tumor stages. Therefore, expression of CD133<sup>+</sup> was associated with tumor progression, whereas CD44<sup>+</sup> and CD133<sup>+</sup>/CD44<sup>+</sup> were not.

### DFS IN 36 PATIENTS WHO UNDERWENT POTENTIALLY CURATIVE SURGERY

Among 36 patients who underwent potentially curative surgery, the mean (SD) follow-up time was 20.4 (3.4) months (range, 10.5-23.9 months; median, 20.4 months). During this period, 6 patients (17%) experienced tumor recurrence. To date, 5 patients are undergoing chemotherapy, and 1 patient has died. Time to recurrence ranged from 10.5 to 18.8 months (mean [SD], 14.9 [3.2] months; median, 14.8 months). The site of tumor recurrence was peritoneal in 2 patients and liver or lung in 4 patients with previous liver resection. Two-year DFS and overall survival were 82% and 96%, respectively.

ROC curve analysis showed that the LNR and the CD133<sup>+</sup>/CD44<sup>+</sup> cell percentage were prognostic indicators of DFS. For each variable, the cutoff value with the highest accuracy was determined, which was 0.1962 for the LNR and 0.6% for the CD133<sup>+</sup>/CD44<sup>+</sup> cell percent-

age (**Figure 1**). The cutoff value for the LNR was similar to the 0.1818 that was previously reported.<sup>24</sup> In contrast, ROC curve analysis showed that CD133 and CD44 expression did not correlate with DFS; therefore, the median values were used to categorize tumors into 2 groups.

In univariate analysis, distant metastasis, LNR exceeding 0.1962, Dukes C or D stage, and CD133<sup>+</sup>/CD44<sup>+</sup> cell percentage exceeding 0.6% were significantly associated with worse DFS (Table 1). In addition, metastatic LNs and 13 or fewer resected LNs were associated with greater probability of tumor recurrence. The estimated relative risk of cancer recurrence associated with tumors having low CD133/CD44 expression was 13% of that in tumors having high CD133/CD44 expression (**Figure 2**). In contrast, individual CD133 or CD44 expression had no significant association with DFS.

In multivariate analysis, the number and status of resected LNs were not significant. The only independent variables associated with worse DFS were distant metastasis, LNR exceeding 0.1962, Dukes C or D stage, and CD133<sup>+</sup>/CD44<sup>+</sup> cell percentage exceeding 0.6% (**Table 3**). After backward elimination, stepwise regression analysis selected CD133/CD44 expression (hazard ratio [HR], 6.82; 95% CI, 1.64-28.30;  $P=.008$ ) and the LNR (5.48; 95% CI, 1.30-23.06;  $P=.02$ ) as the best combination of variables to predict poor long-term DFS. Indeed, the 2-year DFS was 100% among 13 patients with an LNR of 0.1962 or less and a CD133<sup>+</sup>/CD44<sup>+</sup> cell percentage of 0.6% or less but was 28% among 8 patients with an LNR exceeding 0.1962 and a CD133<sup>+</sup>/CD44<sup>+</sup> cell percentage exceeding 0.6% (HR, 0.00; 95% CI, 0.01-0.23;  $P=.001$ ) (**Figure 3**).

## COMMENT

For many years, the traditional model of human carcinogenesis assumed that stochastic genetic events and microenvironmental influences in differentiated cells could result in clonal selection promoting tumor growth.<sup>6</sup> Evident discrepancies in this model and recent progress in the field have supported the novel hypothesis called the CSC model.<sup>4,7,9-12</sup> Accordingly, human tumors would be hierarchically organized, resulting from a mutational hit involving a single stem cell. Being long-lived, stem cells may accumulate oncogenic mutations over years or decades, eventually becoming a CSC capable of promoting tumor growth.<sup>3,15</sup> Cancer stem cells, which are characterized by a slow cell cycle, have been reported to be resistant to anticancer therapies by intrinsic defense mechanisms<sup>25-27</sup> and by induction of antiapoptotic proteins, such as survivin.<sup>28-30</sup> The differences between the 2 carcinogenetic models may have great implications for therapy. If tumors arise through the clonal evolution model, all bulk cancer should be targeted therapeutically.<sup>9,10</sup> By contrast, according to the CSC model, CSCs must be eliminated to achieve definitive cure.<sup>3,31</sup> Because current chemotherapeutics interfere with the ability of rapidly growing cells to divide, CSCs might be spared, leading to tumor recurrence and metastasis.<sup>28,32</sup> Indeed, emerging data suggest that resistant CSCs are often responsible for tumor recurrence.<sup>3</sup>

**Table 1. Clinicopathological Characteristics and Results of Univariate Analysis of Disease-Free Survival in 36 Patients With Colorectal Cancer Undergoing Potentially Curative Surgery**

| Variable   | No. of Patients |                 | 2-y Disease-Free Survival, % | Hazard Ratio (95% CI) <sup>a</sup> | P Value |
|--|-----------------|-----------------|------------------------------|------------------------------------|---------|
|  | Total           | With Recurrence |                              |                                    |         |
| Age, y <sup>b</sup>  |                 |                 |                              |                                    |         |
| ≤66  | 19              | 3               | 82                           | 0.84 (0.16-4.21)                   | .83     |
| >66  | 17              | 3               | 82                           |                                    |         |
| Sex  |                 |                 |                              |                                    |         |
| Male   | 21              | 3               | 84                           | 0.67 (0.12-3.45)                   | .63     |
| Female   | 15              | 3               | 80                           |                                    |         |
| Cancer site <sup>c</sup>   |                 |                 |                              |                                    |         |
| Right colon  | 13              | 2               | 84                           | ...                                | .25     |
| Left colon   | 14              | 4               | 70                           |                                    |         |
| Rectum   | 9               | 0               | 100                          |                                    |         |
| ECOG Performance Status Scale                                      |                 |                 |                              |                                    |         |
| 0  | 15              | 4               | 70                           | ...                                | .23     |
| 1  | 17              | 1               | 94                           |                                    |         |
| 2  | 4               | 1               | 75                           |                                    |         |
| TNM stage  |                 |                 |                              |                                    |         |
| T  |                 |                 |                              |                                    |         |
| 1  | 2               | 0               | 100                          | ...                                | .67     |
| 2  | 5               | 0               | 100                          |                                    |         |
| 3  | 14              | 2               | 84                           |                                    |         |
| 4  | 15              | 4               | 73                           |                                    |         |
| 1-2 vs 3-4   | 7 vs 29         | 0 vs 6          | 100 vs 78                    |                                    |         |
| N <sup>d</sup>   |                 |                 |                              |                                    |         |
| 0  | 15              | 1               | 93                           | ...                                | .08     |
| 1  | 6               | 0               | 100                          |                                    |         |
| 2  | 15              | 5               | 63                           |                                    |         |
| M  |                 |                 |                              |                                    |         |
| 0  | 23              | 1               | 96                           | 0.09 (0.01-0.54)                   | .008    |
| 1a, Liver only   | 13              | 5               | 58                           |                                    |         |
| Histologic differentiation   |                 |                 |                              |                                    |         |
| Well   | 2               | 0               | 100                          | ...                                | .28     |
| Moderate   | 33              | 5               | 84                           |                                    |         |
| Poor   | 1               | 1               | 0                            |                                    |         |
| No. of resected lymph nodes <sup>b</sup>                           |                 |                 |                              |                                    |         |
| ≤13  | 17              | 5               | 69                           | 6.19 (0.96-24.52)                  | .06     |
| >13  | 19              | 1               | 94                           |                                    |         |
| Lymph node ratio <sup>e</sup>                                      |                 |                 |                              |                                    |         |
| ≤0.1962  | 21              | 1               | 95                           | 0.12 (0.02-0.77)                   | .02     |
| >0.1962  | 15              | 5               | 63                           |                                    |         |
| Dukes stage  |                 |                 |                              |                                    |         |
| A or B   | 14              | 0               | 100                          | 0.00 (0.03-0.87)                   | .03     |
| C or D   | 22              | 6               | 70                           |                                    |         |
| CD133 <sup>+</sup> cell percentage <sup>b</sup>                    |                 |                 |                              |                                    |         |
| ≤5.0%  | 17              | 2               | 85                           | 0.52 (0.10-2.65)                   | .44     |
| >5.0%  | 19              | 4               | 79                           |                                    |         |
| CD44 <sup>+</sup> cell percentage <sup>b</sup>                     |                 |                 |                              |                                    |         |
| ≤8.0%  | 17              | 1               | 94                           | 0.24 (0.06-1.58)                   | .16     |
| >8.0%  | 19              | 5               | 74                           |                                    |         |
| CD133 <sup>+</sup> /CD44 <sup>+</sup> cell percentage <sup>f</sup> |                 |                 |                              |                                    |         |
| ≤0.6%  | 20              | 1               | 95                           | 0.13 (0.02-0.80)                   | .03     |
| >0.6%  | 16              | 5               | 64                           |                                    |         |

Abbreviation: ECOG, Eastern Cooperative Oncology Group.

<sup>a</sup>For cancer recurrence.

<sup>b</sup>The median value was used to categorize tumors into 2 groups.

<sup>c</sup>Right colon indicates cecum and ascending and proximal transverse colon; left colon indicates distal transverse colon and descending and sigmoid colon.

<sup>d</sup>N1 includes N1a (metastasis in 1 node) and N1b (metastasis in 2-3 nodes); N2 includes N2a (metastasis in 4-6 nodes) and N2b (metastasis in ≥7 nodes).

<sup>e</sup>The lymph node ratio is the ratio of metastatic lymph nodes to resected lymph nodes, as calculated by receiver operating characteristic curve analysis.

<sup>f</sup>The median value and cutoff value as calculated by receiver operating characteristic curve analysis were equal.

The first evidence for CSCs was by Park et al<sup>33</sup> in mouse multiple myeloma. In 1997, CSC existence was confirmed in human acute myeloid leukemia<sup>34</sup> and thereafter in many human tumors, including CRC.<sup>5,14,15</sup> Large se-

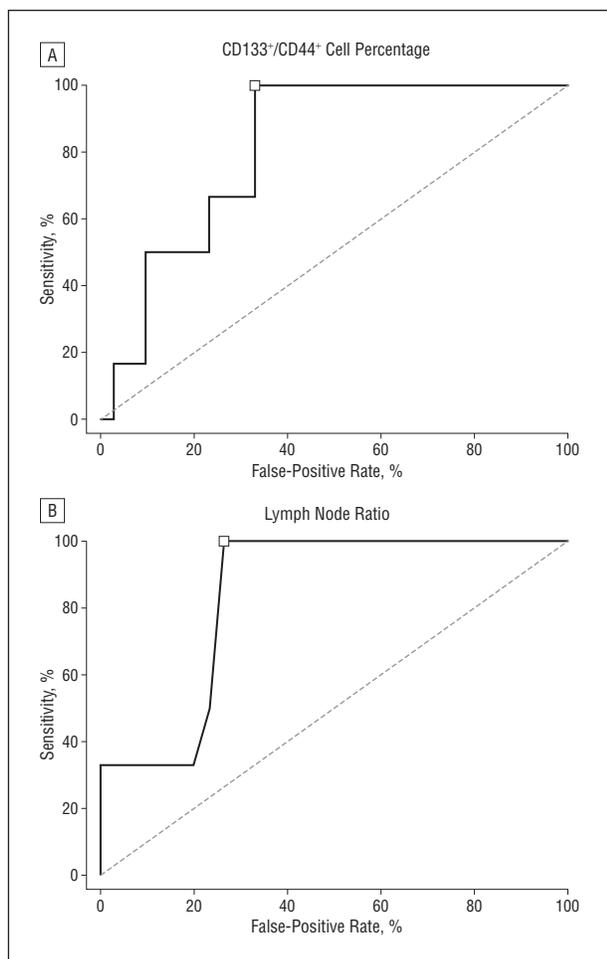
ries have documented the existence of colorectal CSCs and their specific cell surface biomarkers. Using an antibody directed against the CD133 glycoprotein, 2 studies<sup>5,15</sup> identified a small subset of CD133<sup>+</sup> cells (mean [SD], 2.5%

**Table 2. Results of Flow Cytometry Analysis<sup>a</sup>**

| Variable                              | Mean (SD)   | Median (Range) | P Value <sup>b</sup> |
|---------------------------------------|-------------|----------------|----------------------|
| CD133 <sup>+</sup>                    |             |                |                      |
| Nontumor cells                        | 0.8 (1.7)   | 0.1 (0.0-9.0)  | <.001                |
| Tumor cells                           | 9.9 (12.5)  | 5.0 (0.3-60.0) |                      |
| CD44 <sup>+</sup>                     |             |                |                      |
| Nontumor cells                        | 20.2 (28.0) | 7.0 (0.3-99.0) | .002                 |
| Tumor cells                           | 26.8 (33.0) | 8.0 (0.4-98.0) |                      |
| CD133 <sup>+</sup> /CD44 <sup>+</sup> |             |                |                      |
| Nontumor cells                        | 1.13 (2.1)  | 0.4 (0.0-11.0) | .004                 |
| Tumor cells                           | 1.54 (2.8)  | 0.6 (0.1-15.0) |                      |

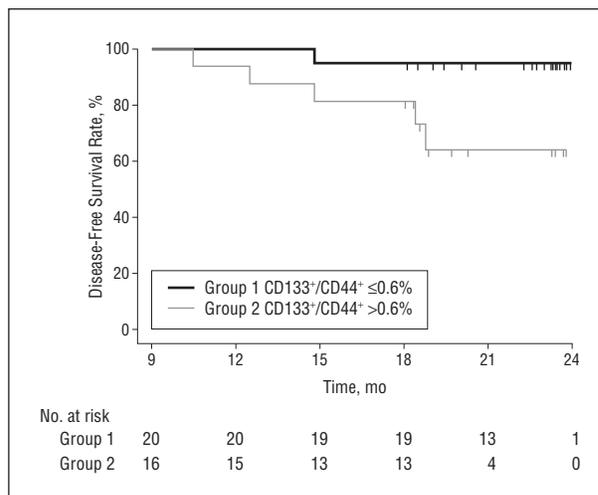
<sup>a</sup>Values are expressed as the ratio of positive cells to total cancer cells.

<sup>b</sup>Paired *t* test.



**Figure 1.** Receiver operating characteristic (ROC) curves for the CD133<sup>+</sup>/CD44<sup>+</sup> cell percentage (A) and the lymph node ratio (B) to predict colorectal cancer disease-free survival. The points with the highest accuracy are marked. Diagonal lines indicate equivalence of the distributions. A, The cutoff with the highest accuracy is 0.6% (100% sensitivity and 67% specificity). Global predictive accuracy (area under the ROC curve) is 81% (95% CI, 64%-92%; *P* = .006). B, The cutoff with the highest accuracy is 0.1962 (100% sensitivity and 73% specificity). Global predictive accuracy is 84% (95% CI, 67%-93%; *P* = .001).

[1.4%] of total cells) showing CSC properties. However, not every CD133<sup>+</sup> cell seemed to have stem cell properties; limiting dilution assays revealed that only 1 of 262 CD133<sup>+</sup> CRC cells could promote tumor growth.<sup>9</sup> There-



**Figure 2.** Colorectal cancer 2-year disease-free survival among 20 patients with a CD133<sup>+</sup>/CD44<sup>+</sup> cell percentage of 0.6% or less (group 1) and among 16 patients with a CD133<sup>+</sup>/CD44<sup>+</sup> cell percentage exceeding 0.6% (group 2) (hazard ratio, 0.13; 95% CI, 0.02-0.80; *P* = .03).

**Table 3. Results of Multivariate Analysis of Disease-Free Survival in 36 Patients With Colorectal Cancer Undergoing Potentially Curative Surgery**

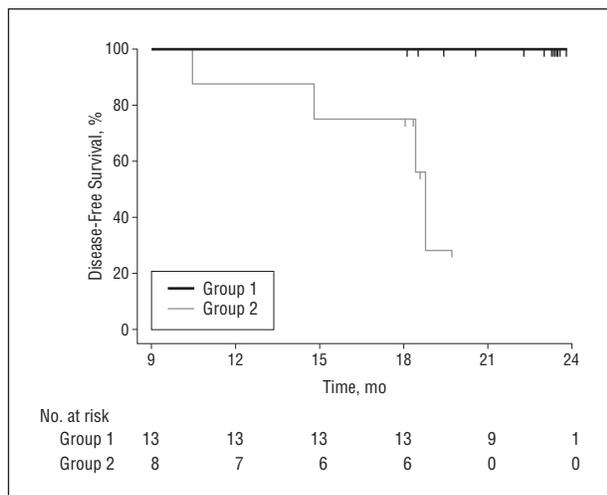
| Variable  | Coefficient (SE) | P Value <sup>a</sup> | Hazard Ratio (95% CI) <sup>b</sup> |
|---|------------------|----------------------|------------------------------------|
| TNM stage >N0   | 1.0658 (1.3100)  | .42                  | 2.90 (0.22-37.84)                  |
| No. of resected lymph nodes ≤13                             | 1.5840 (1.4422)  | .27                  | 4.87 (0.28-82.32)                  |
| Distant metastasis  | 0.1466 (0.0674)  | .03                  | 1.15 (1.01-1.32)                   |
| Lymph node ratio >0.1962                                    | 2.2270 (0.8933)  | .01                  | 9.27 (1.60-53.40)                  |
| Dukes stage C or D  | 1.0814 (0.4985)  | .03                  | 2.94 (1.11-7.83)                   |
| CD133 <sup>+</sup> /CD44 <sup>+</sup> cell percentage >0.6% | 2.2889 (0.8327)  | .006                 | 9.86 (1.92-50.45)                  |

<sup>a</sup>Cox proportional hazards model.

<sup>b</sup>For cancer recurrence.

fore, CSC identification remained to be clarified. In 2008, Shmelkov et al<sup>35</sup> demonstrated that CD133 expression was not restricted to stem cells and that CD133<sup>+</sup> metastatic CRC cells could initiate tumors. Simultaneously, Du et al,<sup>19</sup> confirming previous findings by Dalerba et al,<sup>18</sup> showed that CD44<sup>+</sup> cells but not CD133<sup>+</sup> cells had CSC properties. However, Haraguchi et al<sup>20</sup> and Chu et al<sup>21</sup> observed that only CD133<sup>+</sup>/CD44<sup>+</sup> CRC cells displayed tumorigenic potential, suggesting that the use of both markers could identify colorectal CSCs much more accurately.

In this study, we evaluated CD133 and CD44 expression (alone or combined) in 36 CRCs using flow cytometry. Unlike the use of immunohistochemistry,<sup>4,5,18,35,36</sup> this technique allowed us to isolate only viable colorectal cells, representing 20% of the sample composition, with 80% comprising other cells (30% hematopoietic, 20% nonepithelial, and 50% damaged cells). Marker expression was significantly higher in tumor than in nontumor colorectal cells, supporting the CSC model of colorectal carcinogenesis.<sup>5,15,19,35</sup> However, CD133 and CD44 positivity varied widely, suggesting that each marker taken separately may not accurately identify CSCs. In con-



**Figure 3.** Colorectal cancer 2-year disease-free survival among 13 patients with a lymph node ratio of 0.1962 or less and a CD133<sup>+</sup>/CD44<sup>+</sup> cell percentage of 0.6% or less (group 1) and among 8 patients with a lymph node ratio exceeding 0.1962 and a CD133<sup>+</sup>/CD44<sup>+</sup> cell percentage exceeding 0.6% (group 2).

trast, CD133/CD44 positivity ranged from 0.1% to 15.0% (mean, 1.5%), allowing us to identify a much more defined subset of tumor cells that may correspond to CSCs. Moreover, the absence of correlation between markers indicated that CD133<sup>+</sup>/CD44<sup>+</sup> cells differed from single CD133<sup>+</sup> and CD44<sup>+</sup> cells, as demonstrated in part by Du et al,<sup>19</sup> who observed that CD44<sup>+</sup> cells did not colocalize with CD133<sup>+</sup> cells.

To date, few studies have correlated marker expression with both clinicopathological features and survival rates. Inconsistencies and contrasting results have been reported. A high CD133<sup>+</sup> cell percentage was significantly associated with tumor progression,<sup>4,17,36-39</sup> poor DFS,<sup>4,36-39</sup> and a low overall survival rate.<sup>4,36-38,40</sup> In multivariate analysis, a high frequency of CD133<sup>+</sup> cells was an independent prognostic factor of worse CRC outcomes.<sup>4,37,38,40</sup> In contrast, according to other investigators, CD133 overexpression was unassociated with tumor progression<sup>40,41</sup> or survival rate<sup>17</sup> and was not an independent risk factor for recurrence.<sup>17,36,39</sup> In 2 patient series, CD44 expression correlated with neither clinicopathological features nor long-term outcomes.<sup>17,41</sup> To date, no previous data were available on the combined analysis of these 2 markers.

These discordant results may be explained in part by differences in cutoff values, primary end points (overall survival vs DFS), and study methods (immunohistochemistry vs flow cytometry analysis).<sup>39</sup> In the present study, we aimed to overcome these discrepancies by using flow cytometry, which allowed more accurate identification of tumor cells and avoidance of arbitrary cutoff values. When necessary, ROC curve analysis was applied to identify the best cutoff value. Moreover, because CSCs are thought to promote tumor recurrence, the prognostic significance of marker expression was correlated with DFS rather than with overall survival. Furthermore, the many advanced tumor stages (with potential recurrence) in the present series allowed confidence in the statistical analyses. The results showed that CD133

and CD44 expression did not correlate with CRC recurrence, indicating that the use of these markers individually failed to identify colorectal CSCs. In contrast, combined CD133/CD44 expression significantly correlated with DFS and was an independent risk factor for cancer recurrence. In addition, CD133/CD44 expression and the LNR were the best linear combination of variables predicting DFS. Therefore, evaluation of combined CD133/CD44 expression could be useful to identify putative colorectal CSCs because of segregation of the combined markers to a small subset of tumor cells and because of its prognostic significance related to DFS. Further studies are warranted to elucidate the CSC model of colorectal carcinogenesis, to enhance isolation of purified CSCs, and to assess the potential of CD133 and CD44 antigens as therapeutic targets for CRC.

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**INVITED CRITIQUE**

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## Colorectal Cancer Stem Cells—Hype or Real?

The article by Galizia et al<sup>1</sup> is an intriguing, well-designed, and timely study that lends credence to the controversial concept of colorectal cancer stem cells (CSCs). In recent years, the traditional model of colorectal carcinogenesis is being challenged by the CSC model. In the traditional clonal model of carcinogenesis, each differentiated cell in a cancer has the potential to form a cancer, whereas the CSC model holds that only the long-lived stem cells have the potential to accumulate all the needed mutations. Cancer stem cells are characterized by self-renewal and pluripotency whereby each CSC can differentiate into mature and diverse cancer cells, which are then capable of tumor initiation, growth, invasion, and metastasis.<sup>2-4</sup> This paradigm shift has obvious clinical connotations because CSC may also account for the failure of current chemotherapeutic regimens to cure metastatic colorectal cancers. Conventional cytotoxic chemotherapy targets only rapidly di-

viding cells, while the slowly proliferating CSCs may escape cell death, resulting in eventual cancer recurrence and metastasis. These CSCs are also enriched with multidrug-resistant proteins that may allow them to survive. On the bright side, a better understanding of CSCs will allow us to target these subpopulations and potentially eradicate tumors. The evidence for the CSC model is strongest in acute myeloid leukemias,<sup>3,4</sup> but results of recent studies<sup>3</sup> in brain, breast, and colorectal cancers have been promising. However, there is widespread controversy within the cancer field because the marks that identify these CSCs keep evolving and because most investigators have used mouse xenograft models rather than primary human models.

The study by Galizia et al<sup>1</sup> is one of the first studies in colorectal cancer that not only seems to identify the fraction of colorectal CSCs in human samples but also correlates it with clinical outcomes. In this study, the au-