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ORIGINAL ARTICLE

Increased expression of CD133 is a strong predictor of poor outcome in stage I colorectal cancer patients

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Abstract

Objective. Stage I colorectal carcinomas display a highly variable behavior which is not accurately predicted by the available prognostic markers. CD133 is considered a useful marker to identify the so-called cancer stem cells in colorectal cancers (CRCs) and its expression has been shown to have prognostic significance in CRC patients. This study aimed to verify whether immunohistochemical evaluation of CD133 might correlate with the progression risk of stage I CRC patients. ***Material and methods.*** Expression levels of the CD133 molecule were analyzed and compared in two series of stage I surgically resected CRC patients showing disease progression and death for the disease and patients with no evidence of disease progression after at least 6 years after surgery. ***Results.*** A positive staining for CD133 was detected in 52% of the cases with poor prognosis and only in 9% of the group with good prognosis, and this difference was highly significant ($p < 0.001$). A significant correlation was detected between CD133 expression and histological parameters, such as tumor budding, vascular invasion, and presence of lymph node micrometastases but not tumor grading, gender, and age. Disease-free survival and cancer-specific survival of CD133 negative tumors were significantly longer compared to positive cases. In multivariate analyses, CD133 staining confirmed to be a predictor of shorter survival independent from vascular invasion but not from lymph nodes micrometastases. ***Conclusions.*** These findings demonstrate that CD133 immunostaining is a useful predictor of high risk progression in stage I CRC patients and might help to identify patients eligible for adjuvant chemotherapy.

Key Words: *adjuvant therapy, cancer stem cells, CD133, prognosis, stage I colon cancer*

Introduction

CD133, also known as prominin-1, a transmembrane pentaspan molecule, is considered a putative stem cell marker expressed in several normal and cancer tissues [1]. Numerous studies have demonstrated that surface expression of CD133 identifies a subpopulation of tumor-initiating cells, having the properties of self-renewal, proliferation, and multilineage differentiation in a variety of human cancer tissues, including colon cancer [2-4]. Although the exact role(s) of the molecule in human colorectal cancer (CRC) tumorigenesis remain unknown [5,6], CD133 is presently considered a useful marker to identify cancer stem cells (CSC) in CRCs and its expression level has been shown to have prognostic significance in colon cancer patients [7-11].

Colon cancer represents a leading cause of cancer-related deaths in Western countries and, although

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multiple therapeutic approaches are available for its treatment, its management is somehow complicated by the variable behavior of the disease. Indeed, prognostic stratification of the disease is mainly based on its anatomic extent, as assessed by the TNM staging, which strongly affects patients' survival [12]. However, TNM is not able to accurately predict clinical outcome of early lesions, such as stage I carcinomas. Stage I colorectal carcinoma indicates a disease confined within the muscular wall of the large bowel without infiltration of local structures, in the absence of lymph nodes or distant metastasis. This category can be further stratified into two subgroups: T1, tumors spreading through the muscularis mucosae into the submucosa and T2, tumors infiltrating the muscular wall. Stage I CRCs display an overall 5-year survival around 80–90% and are considered at very good prognosis. However, a small subset of stage I CRC patients display relapses and disease progression and available prognostic markers are not able to identify this subgroup of patients which might benefit from a more aggressive (i.e., adjuvant therapies) treatment [12]. Thus, the development of new prognostic markers able to identify stage I CRC patients at high risk of progression is a major topic in CRC research and is becoming increasingly needed as new treatment options become available. CD133 has been reported to be a potentially important prognostic marker in CRC patients being able to specifically mark cells responsible for tumor initiation as well as tumor relapse and metastasis. In the present study, CD133 expression levels were analyzed by immunostaining in selected specimens of stage I human primary CRCs, including patients deceased because of disease progression within 10 years after surgery and patients still alive with no evidence of disease progression after a 6 years or longer follow up. The aim of the study was to verify whether CD133 could be able to identify subgroups of stage I CRC patients with different prognosis. Moreover, the potential prognostic significance of CD133 was also evaluated on the overall population of stage I patients included in the study.

Materials and methods

Patient characteristics and tissue samples

The study included 95 patients, selected from 518 consecutive patients with stage I colorectal adenocarcinoma, diagnosed between January 1989 and December 2004 and collected in a specialized, population-based, Colorectal Cancer Registry of the District of Modena, Italy. Selection of patients was previously described [13]. All had undergone curative surgery for CRC at the Division of Surgery, School of Medicine, University of Modena and Reggio Emilia, Modena, Italy, and none of them had received chemotherapy. They were selected according to the following criteria: first group: all patients who died due to the progression of the disease within 10 years from the diagnosis ($n = 25$); second group: a randomized set of patients registered in the same years of cases with an excellent clinical outcome still alive after at least 6 years from surgery with no evidence of disease progression or relapses ($n = 70$). Informed consent was obtained from all subjects [13], and the study was approved by the Research Ethics Committee of the University of Modena and Reggio Emilia, Modena, Italy. Clinic-pathological data were retrieved from the archives of the Department of Pathology, University of Modena and Reggio Emilia, Modena, Italy. Histological grade was established according to the WHO classification system criteria. All cases and controls were reviewed by two expert pathologists, and in none of them metastases were detected in the lymph nodes by conventional staining with hematoxylin and eosin. Lymph nodes were analyzed for the presence of micrometastases by immunohistochemistry using a monoclonal antihuman pan-cytokeratin antibody (DAKO CK, Corpintreia, CA, USA), as previously reported [13].

Immunohistochemical analysis

Immunohistochemical analyses were performed on routinely processed, formalin-fixed, paraffin-embedded (FFPE) tissues employing an avidin–biotin complex immunoperoxidase technique, as previously described [14,15]. Briefly, successive 5 μm tissue sections were cut from blocks of the primary tumors and the lymph nodes selected for the presence of representative lesions and mounted on charged and pre-cleaned slides. Sections were de-waxed, rehydrated, and then microwave pretreated (10 mM sodium citrate, pH 6.0) for antigen retrieval. Each slide was then incubated overnight at 4°C with the primary antibody. A colon carcinoma with known positive immunostaining for CD133 served as a positive control. Positive reaction was defined by the presence of at least 50% of cancer cells displaying either membranous and/or cytoplasmic staining [14]. A specific polyclonal anti-CD133 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:100) was used for the staining. We did not notice any difference in the immunoreactivity between older and younger FFPE samples and comparable results but with a weaker staining were obtained using the monoclonal AC133 antibody (Miltenyi Biotec, Bergisch Gladbach, Germany; 1:10) (data not shown).

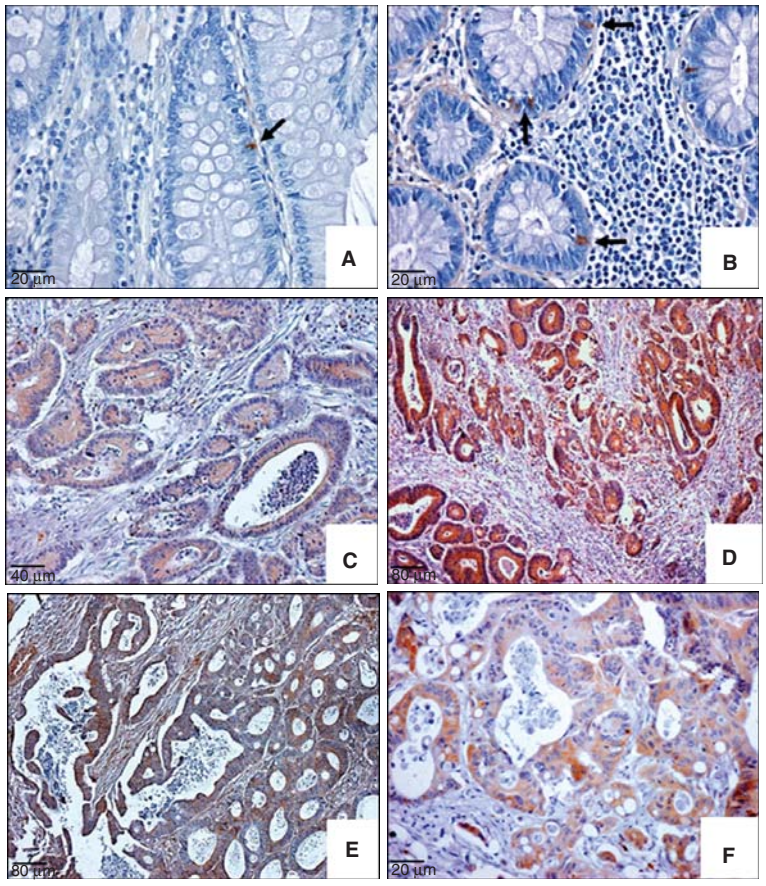

Figure 1. Examples of CD133 immunohistochemical staining in human colorectal samples. (A and B) Normal colonic mucosa. Note the rare (→) positivity for CD133 (×400). (C) A moderately differentiated adenocarcinoma displaying a diffuse staining for CD133 (×200). (D and E)

All scoring and interpretations of the results were made by two of the authors independently (MM and LRB) without knowledge of other clinicopathological variables and no cases with discrepant scoring were recorded.

Statistical analysis

The association between molecular and clinicopathological parameters were calculated using contingency table methods and tested for significance using the Pearson's chi-square test. Patients were all uniformly followed-up at our Institution and disease-free survival (DFS) was defined as the interval between surgery and the first documented evidence of recurrences in local-regional area and/or distant sites. Cancer-specific survival (CSS) was defined as the interval between surgery and death from the disease. Disease-free and cancer-specific survival curves were calculated using the Kaplan–Meier method and the log-rank test was used to compare survival curves. Univariate and multivariate relative risks were calculated using the Cox proportional hazards regression. All calculations were performed using the STATA statistical software package (Stata Corporation, College Station, Texas, TX, USA) and the results were considered statistically significant, when the p value was ≤ 0.05.

Results

The overall median age of stage I CRC patients analyzed in this study was 70 years (range, 43–90 years; mean, 69.4 ± 10.5), and they included 53 males (mean age 69.9 ± 11.2) and 42 females (mean age 68.8 ± 9.5). The first group (n = 25, *cases*) included 14 males and 11 females with a median age of 71 years (range, 55–90 years; mean, 72.04 ± 10.20) who died from the disease within 10 years after surgery (median overall survival was 50 months; range, 12–119). The second group (n = 70, *controls*) included 39 males and 31 females with a median age of 70 years (range, 43–86 years; mean, 68.51 ± 10.48) registered in the same years of cases but still alive after more

than 6 years from surgery (median follow up was 108 months; range, 72–144). The same population of patients were previously characterized [13] in detail and reported to display vascular invasion (defined as the presence of tumor cells in lymphatic or venous microvessels) in four (5.7%) controls and 13 (52%) cases ($p < 0.001$) and cytokeratin-positive micrometastasis in one (1.4%) control and in 18 of 25 (72%) stage I CRC patients who died of the disease ($p < 0.001$) [13].

Using the anti-CD133 antibody, both membrane and cytoplasmic staining was detected in positive cells (Figure 1). Scattered positive cells were detected on the bases of the crypts in normal colonic mucosa (Figure 1A–B). In cancers, a diffuse and heterogeneous staining was clearly evident in tumor cells and samples were considered positive, when at least 50% of cancer cells displayed a positive staining (Figure 1C–F). A positive staining for CD133 was detected in 13 of 25 (52%) of the cases with poor prognosis and only in 6 of 70 (9%) of the group with excellent prognosis, and this difference was highly significant ($p < 0.001$).

Overall, 76 (80%) of the 95 patients included in this study were negative for CD133, while 19 (20%) displayed a positive staining. Considering the entire subset of patients, a significant correlation was detected between CD133 expression and histological parameters such as tumor budding in the actively invasive frontal region of the tumor ($p < 0.001$), vascular invasion ($p < 0.003$), and presence of lymph node micrometastases ($p < 0.001$) but not tumor grading, gender, and age (Table I and data not shown).

Table I. CD133 expression in relation to clinical and pathological parameters in a series of 95 stage I CRCs.

	Total	Negative *n* (%)	Positive *n* (%)	*p* Value
Micrometastases				
Present	19	9 (47)	10 (53)	
Absent	76	67 (88)	9 (12)	0.001
Tumor budding				
Present	30	16 (53)	14 (47)	
Absent	65	60 (92)	5 (8)	0.001
Vascular invasion				
Present	16	8 (50)	8 (50)	
Absent	79	68 (86)	11 (14)	0.003
Tumor grading				
G1	26	22 (85)	4 (15)	
G2/G3	69	54 (78)	15 (22)	n.s.
Follow-up				
Progression	25	12 (48)	13 (52)	
Stable disease	70	64 (91)	6 (9)	0.001

Abbreviation: n.s. = not significant.

Twelve (16%) of the 76 CD133 negative and 13 (68%) of the 19 CD133 positive patients displayed a disease progression and died of disease during the period of follow-up, and this difference was significant ($p = 0.001$, see Table I). As expected, mean DFS (109.8 vs. 62.3 months) as well as CSS (113.3 vs. 80.2 months) of CD133 negative tumors were longer compared to positive cases, and the differences were significant ($p = 0.001$ for DFS and 0.002 for CSS) as confirmed by the Kaplan–Meier curves of DFS and CSS, which also displayed a significant separation between the two groups of patients ($p = 0.001$ by log-rank test) (Figure 2).

As previously mentioned, the stage I CRCs included in this study were previously characterized for several parameters (i.e., tumor dimension, pattern of growth, degree of differentiation, lymphomonocytic peritumoral infiltrate, tumor budding, vascular invasion, and the presence of cytokeratin positive micrometastasis in lymph nodes) and only the presence of micrometastasis and vascular invasion – but no other clinical or biological parameter – displayed an association with survival [13]. Indeed, 18 (95%) of the 19 patients with micrometastasis and 7 (9%) of the 76 patients in which it was not possible to detect cytokeratin positive cells in lymph nodes displayed a disease progression during the period of follow-up, and the Kaplan–Meier curves of both DFS and CSS displayed a significant separation between the two groups of patients ($p = 0.001$ by log-rank test) (data not shown). Similarly, 13 (76%) of the 17 patients with vascular invasion and 12 (15%) of the 78 patients with no vascular invasion displayed a disease progression during the period of follow-up, and the Kaplan–Meier curves of both DFS and CSS displayed a significant separation between the two groups of patients ($p = 0.001$ by log-rank test) (data not shown).

In a multivariate analysis performed by building a Cox hazards model that included all the factors (vascular invasion, micrometastase, and CD133 staining) found to be associated with disease progression in the univariate analyses, positive CD133 staining did not confirm to be an independent predictor of shorter DFS ($p = 0.102$; C.I. 0.870–4.672; RR = 2.016) nor of overall survival ($p = 0.105$; CI: 0.865–4.603; RR = 1.996) compared to vascular invasion and micrometastasis (Tables II and III). However, it is noteworthy that CD133 staining did confirm to be an independent predictor of shorter DFS ($p = 0.102$; CI: 0.870–4.672; RR = 2.016) as well as of overall survival ($p = 0.105$; CI: 0.865–4.603; RR = 1.996) compared to vascular invasion when a second Cox hazards model was built that included only these two variables but not micrometastasis (Tables II and III).

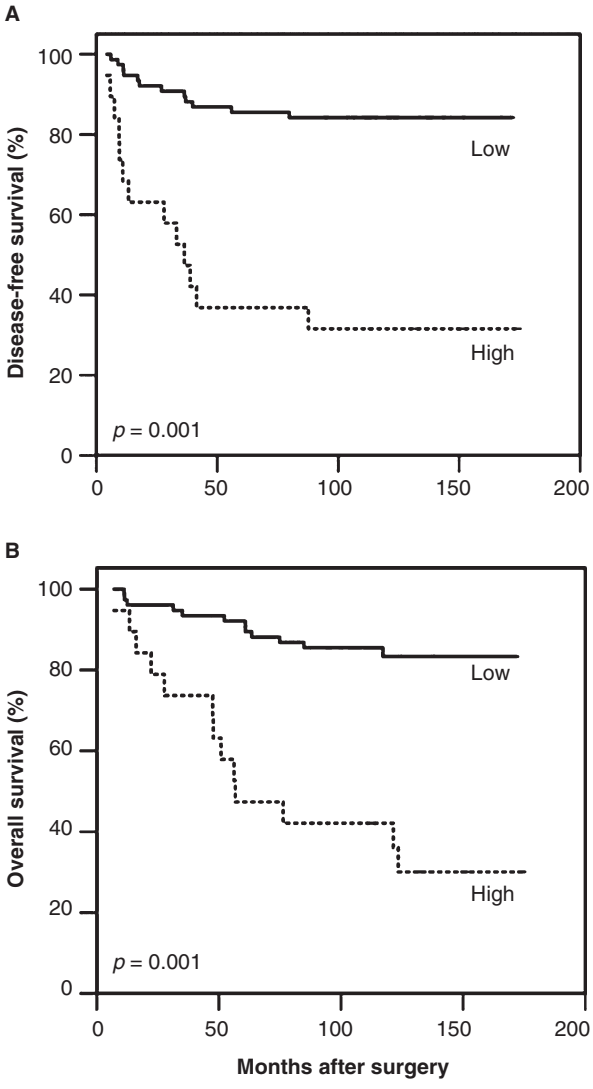

Figure 2. Kaplan–Meier curves for disease-free (*upper panel*) and overall (*lower panel*) survival in a series of 95 stage I CRC patients. Patients were stratified by CD133 expression (see text for details).

Table II. Contribution of various potential prognostic factors to DFS by Cox regression analysis in stage I CRC patients.

Variable	Risk ratio	95% Confidence interval	*p* Value
Model 1			
Micrometastasis*	12.157	4.465–33.105	0.001
Vascular invasion#	3.316	1.413–7.784	0.006
CD133**	2.016	0.870–4.672	0.102
Model 2			
Vascular invasion#	5.691	2.347–13.797	0.001
CD133**	3.534	1.471–8.489	0.005

*The risk ratio is given as presence versus absence of micrometastases.
#The risk ratio is given as presence versus absence of vascular invasion.
**The risk ratio is given as positive versus negative tumors.

Discussion

The present study represents an extension of a previous report focused on the identification of prognostic factors useful for a better stratification of stage I CRC patients [13]. Indeed, stage I (Dukes' A) carcinomas usually show an excellent clinical outcome and are solely treated by surgical resection since, at present, the accepted guidelines recommend adjuvant chemotherapy only for treatment of stage III and selected cases of stage II patients with CRC. However, a small fraction of stage I patients die of recurrence or metastases and available prognostic factors do not allow the identification of high risk patients [12]. Thus, new markers are needed for a better prognostic stratification of stage I patients. To this aim, we took advantage of a specialized CRC registry to identify all patients with stage I CRC and, among them, those who died of the disease, which undoubtedly represent a small fraction of all incident cases. We believe that the patients ($n = 25$) with a worse prognosis included in this study are fully representative of the subset of stage I patients at high risk of disease progression and they were matched to patients registered in the same years of cases but still alive after more than 6 years from surgery.

To our knowledge, this is the first report analyzing CD133 expression in a subset of stage I CRC patients selected on the basis of clinical outcome. We found that 52% of the cases with poor prognosis and 9% of the group with good prognosis displayed a positive staining for CD133, and this difference was highly significant ($p < 0.001$). Moreover, considering the overall population of patients included in this study, positive staining for CD133 correlated strongly with the occurrence of lymph node micrometastases and unfavorable histological features, including tumor budding and vascular invasion (Table I), and displayed a significant relationship with high risk of disease progression in univariate analyses (Figure 2). These observations are consistent with the CSC model of tumorigenesis suggesting that tumors are hierarchically organized like normal tissues and that only a rare subpopulation of undifferentiated cells has the unique biological properties necessary for tumor initiation, maintenance, and spreading [16]. As previously mentioned, CD133 is presently considered a useful marker to identify CSC in CRCs, and it is expected to identify cells which have the ability to initiate tumor growth and would represent the majority responsible of tumor progression, including colonization of distant organs and metastasis [1]. Moreover, the results of the present study are in

agreement with previous reports suggesting a prognostic significance of CD133 immunohistochemical evaluation in CRC patients [7-11] and the observed relationship between CD133 expression and histological parameters, such as vascular invasion and the presence of lymph node micrometastases, warrants further studies to analyze whether cancer cells in vascular invasion and micrometastases actually express CD133.

In multivariate analyses performed by building a Cox hazards model including all the factors (vascular invasion, micrometastase, and CD133 staining) found to be associated with disease progression in the univariate analyses, positive CD133 staining did not confirm to be an independent predictor of worse prognosis compared to vascular invasion and micrometastasis but did show an independent association with survival when micrometastasis was not included in the hazards model (Tables II and III). We previously reported that lymph nodal micrometastases detected by immunostaining using an anti-cytokeratin monoclonal antibody could be detected in the majority of the resected specimens of patients with stage I tumors who died of the disease within 10 years from surgery and that by Cox regression analysis, micrometastasis, and vascular invasion, but no other clinical or biological parameter, showed an independent association with survival when both are included in the model, with the presence of micrometastasis displaying the highest RR (see Tables II and III) [13]. Thus, micrometastasis can be considered a useful parameter for the identification of high risk stage I CRC patients for which adjuvant chemotherapy could be a valuable option. However, conflicting results have been reported on the prognostic significance of micrometastases for stage I patients [17-19,13,20]. Several factors can explain such discrepancies, including different techniques used for their identification (IHC or DNA-based techniques), different antibodies (i.e., different anticytokeratin antibodies) as well as differences in the number and methods (i.e., number of sections for node) used to search for occult metastases. In our study, pathologists performed a complete analysis of all lymph nodes which could be detected and the isolated nodes were cut entirely at intervals of 200 μm until no more lymph node was available [13]. This meticulous approach, however, is extremely tedious and time-consuming and likely not easy to be performed on a routine basis. Thus, the availability of a prognostic marker which could be performed directly on the surgical tumor specimen using a simple, immunohistochemical technique appears as an extremely useful and promising factor for the identification of stage I CRC patients at high risk of disease progression who might benefit of an adjuvant therapy following surgical resection. The introduction of CD133 immunostaining on a routine basis, of course, is not expected to be easily achieved. Several problems remain to be resolved, and first of all it will be important to reach a consensus agreement on the antibody to be used for the analysis as well as the criteria for the evaluation of the staining, since different approaches have been reported in the literature [7-11]. Once these problems will be solved, CD133 has the potentiality to become an important prognostic factors in stage I (and maybe stage II which also represent a challenging group) CRC patients useful for a better management of such patients.

Table III. Contribution of various potential prognostic factors to overall survival by Cox regression analysis in stage I CRC patients.

Variable	Risk ratio	95% Confidence interval	*p* Value
Model 1			
Micrometastasis*	11.006	4.125–29.362	0.001
Vascular invasion#	3.751	1.597–8.812	0.002
CD133**	1.996	0.865–4.603	0.105
Model 2			
Vascular invasion#	6.260	2.620–14.959	0.001
CD133**	3.331	1.407–7.888	0.006

*The risk ratio is given as presence versus absence of micrometastases.
#The risk ratio is given as presence versus absence of vascular invasion.
**The risk ratio is given as positive versus negative tumors.

In conclusion, we investigated CD133 expression in a subset of stage I CRC patients and demonstrated a clear correlation of its expression with other prognostic indicators as well as with the clinical outcome of patients. Stage I CRC patients are considered a good prognosis at diagnosis and undergo potentially curative surgery. However, a subset of them (up to 10%) [12] experience cancer progression with the development of recurrences/distant metastases which are associated with a significantly worse prognosis and shorter survival. The results of the present study suggest that increased CD133 expression is a useful prognostic marker for stage I CRC patients. These findings will certainly be of interest, considering the need of useful prognostic indicators that can accurately predict the clinical outcome of patients with localized CRC. However, it has to be underlined that the population studied was too small to allow a conclusive and definitive evaluation of the prognostic significance of CD133 expression level in these patients. Thus, additional studies on a larger series of cases are warranted to confirm these results and to further elucidate the role(s) of CD133 in the development and progression of CRC and its suitability as a prognostic biomarker predictive of cancer

aggressiveness and patient outcome. If confirmed on larger cohorts of patients, these findings could have therapeutic implications by helping to identify patients at higher risk of progression and death from the disease who would benefit most from a systemic adjuvant treatment after surgical resection. Moreover, CD133 might well be a candidate molecular target for the development of new therapeutic interventions if a direct role of the molecule in the process of tumor development and progression will be demonstrated in colon tumorigenesis.

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References

[1] Mizrak D, Brittan M, Alison M. CD133: molecule of the moment. J Pathol 2008;214:3–9.

[2] Corbeil D, Roper K, Hellwig A, Tavian M, Miraglia S, Watt SM, et al. The human AC133 hematopoietic stem cell antigen is also expressed in epithelial cells and targeted to plasma membrane protrusions. J Biol Chem 2000;275: 5512–20.

[3] O'Brien C, Pollett A, Gallinger S, Dick, JE. A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. Nature 2007;445:106–10.

[4] Ricci-Vitiani L, Lombardi D, Signore M, Biffoni M, Pallini R, Parati E, et al. Identification and expansion of human colon--cancer-initiating cells. Nature 2007;445:111–15.

[5] Shmelkov S, Butler J, Hooper A, Hormigo A, Kushner J, Milde T, et al. CD133 expression is not restricted to stem cells, and both CD133+ and CD133- metastatic colon cancer cells initiate tumors. J Clin Invest 2008;118:2111–20.

[6] Yang K, Chen X, Zhang B, Yang C, Chen HN, Chen ZX, et al. Is CD133 a biomarker for cancer stem cells of colorectal cancer and brain tumors? A meta-analysis. Int J Biol Markers 2011;26:173–80.

[7] Horst D, Kriegl L, Engel J, Kirchner T, Jung A. Prognostic significance of the cancer stem cell markers CD133, CD44, and CD166 in colorectal cancer. Cancer Invest 2009;27: 844–50.

[8] Horst D, Kriegl L, Engel J, Kirchner T, Jung A. CD133 expression is an independent prognostic marker for low survival in colorectal cancer. Br J Cancer 2008;99: 1285–9.

[9] Horst D, Scheel S, Liebmann S, Neumann J, Maatz S, Kirchner T, Jung A. The cancer stem cell marker CD133 has high prognostic impact but unknown functional relevance for the metastasis of human colon cancer. J Pathol 2009;219:427–34.

[10] Kojima M, Ishii G, Atsumi N, Fujii S, Saito N, Ochiai A. Immunohistochemical detection of CD133 expression in colorectal cancer: a clinicopathological study. Cancer Sci 2008;99:1578–83.

[11] Li C, Li B, Liang Y, Peng RQ, Ding Y, Xu DZ, et al. Higher percentage of CD133+ cells is associated with poor prognosis in colon carcinoma patients with stage IIIB. J Transl Med 2009;7:56.

[12] O'Connell J, Maggard M, Ko C. Colon cancer survival rates with the new American Joint Committee on Cancer sixth edition staging. J Natl Cancer Inst 2004;96:1420–5.

[13] Reggiani Bonetti L, Di Gregorio C, De Gaetani C, Pezzi A, Barresi G, Barresi V, Roncucci L, Ponz de Leon M. Lymph node micrometastasis and survival of patients with Stage I (Dukes' A) colorectal carcinoma. Scand J Gastroenterol 2011;46:881–6.

[14] Arena V, Caredda V, Cufino V, Stigliano E, Scaldaferri F, Gasbarrini A, Cittadini A, Sgambato A. Differential CD133 expression pattern during mouse colon tumorigenesis. Anticancer Res 2011;31:4273–5.

[15] Sgambato A, De Paola B, Migaldi M, Di Salvatore M, Rettino A, Rossi G, et al. Dystroglycan Expression is Reduced During Prostate Tumorigenesis and is Regulated by Androgens in Prostate Cancer Cells. J Cell Physiol 2007; 213:528–39.

[16] Vermeulen L, Sprick M, Kemper K, Medema JP. Cancer stem cells – old concepts, new insights. Cell Death Differ 2008;15:947–58.

[17] Cutait R, Alves V, Lopes LC, Cutait DE, Borges JL, Singer J, da Silva JH, Goffi FS. Restaging of colorectal cancer based on the identification of lymph node micrometastases through immunoperoxidase staining of CEA and cytokeratin. Dis Colon Rectum 1999;34:917–20.

[18] Lee M, Hong C, Yoon S, Lim SB, Park KJ, Lee MJ, Kim WH, Park JG. Isolated tumor cells in lymph nodes are not a prognostic marker for patients with stage I and stage II colorectal cancer. J Surg Oncol 2006;93:13–18.

[19] Oberg A, Stenling R, Tavelin B, Lindmark G. Are lymph node micrometastases of any clinical significance in Dukes' Stages A and B colorectal cancer? Dis Colon Rectum 1998; 41:1244–9.

[20] van Schaik P, Hermans E, van der Linden J, Pruijt JR, Ernst MF, Bosscha K. Micro-metastases in stages I and II colon cancer are a predictor of the development of distant metastases and worse disease-free survival. Eur J Surg Oncol 2009;35:492–6.