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ORIGINAL ARTICLE

Characterization of cancer stem cells from different grades of human colorectal cancer

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Abstract Colorectal cancer (CRC) is one of the most common solid tumors worldwide. Recent evidence suggests that a population of cancer cells, called cancer stem cells (CSCs), is responsible for tumor heterogeneity, invasion, metastasis, therapeutic resistance, and recurrence of CRC. The isolation and characterization of CSCs using cell surface markers have been reported previously with varying results. In this study, we investigated a panel of four putative CSC markers, CD44, CD24, CD166, and EpCAM, to define CRC-CSC. Paraffin embedded tissue samples from different grades of primary, untreated CRC were analyzed for the expression of four CSC markers CD44, CD326, CD24, and CD166, using immunohistochemistry. Flow cytometric analysis of CRC-CSC from HT29 (low grade) and HCT116 (high grade) human colorectal cancer cell lines was done. Marker-based isolation of CSC and non-CSC-bulk-tumor cells from HT29 was done using FACS, and tumor sphere assay was performed. There was a statistically significant difference ($p < 0.05$) in the expression of CD44, CD326, and CD166 between cases and controls. A novel cutoff distribution of CD44 and CD166 was suggested to help for better immunohistochemical analysis of CRC. Higher prevalence of CSC was seen in high-grade CRC as compared to low-grade CRC. Sorted and cultured CD44 + CD166+ cells formed tumor spheres, suggesting that these cells, having properties of self renewal and anchorage independent proliferation, were in fact CSC. Hence, CD44 and CD166 may serve as good CRC-CSC markers when used together with novel cutoff immunohistochemistry (IHC) expression levels.

Keywords Colorectal cancer · Cancer stem cells · CD44 · CD166

Introduction

Colorectal cancer (CRC) is one of the most common gastrointestinal malignancies and a major contributor to cancer-related mortality [1]. It is the third and second most common type of cancer in males and females, respectively, worldwide [2]. Changes in lifestyle, high-fat diet [3, 4], physical inactivity [5], and smoking [6] have all been implicated in CRC pathogenesis.

The primary treatment for localized CRC is surgical resection. However, approximately 25 % cases of CRC present with metastases at initial diagnosis and almost 50 % of patients with CRC will develop metastasis during their lifetime [7, 8]. The treatment outcomes for these patients are generally poor as conventional chemotherapy and radiotherapy are based on assumptions that the tumor mass is homogeneous. Emerging evidence suggests that epithelial tumors, especially solid tumors, have cellular heterogeneity within the tumor [9, 10]. This functional heterogeneity is maintained by a special subset of the tumor cells referred to as cancer stem cells (CSCs) which have an inherent capacity of 'stemness' and

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'oncogenic transformation.' CSCs self-renew and undergo asymmetric cell division to form differentiated tumor cells [11]. A direct correlation between the number of undifferentiated cells and high chances of relapse and recurrence has previously been reported in CRC. This loss of epithelial cell homeostasis contributes to tumor growth and metastasis [12, 13]. Hence, CSC may play a central role in metastasis and resistance to therapy [14–18].

There are numerous cell lines available to study human colorectal cancer. In this study, HT29 and HCT116 human CRC cell lines were used. HT29 gives rise to human well-differentiated colorectal adenocarcinoma (low grade) when xenografted into nude mice [19] and has been used to isolate CSC and study low-grade CRC previously [20]. HCT116 corresponds to poorly differentiated CRC (high grade), and numerous studies have shown its relatively undifferentiated state and association with resistance to chemotherapy [21–23].

In order to understand cancer stem cell biology in colorectal cancers, we used these cell lines as well as patient samples. However, specific markers unique for CRC-CSC are not well defined, and hence, using a panel of CSC markers rather than one seems more appropriate to study CRC-CSC. After extensive literature review, a panel of four cell surface markers including CD44, CD24, CD166, and EpCAM (CD326) were selected [24–29].

CD44 is a multifunctional transmembrane glycoprotein expressed on cancer cell surface which assists in cell adhesion, proliferation, growth, survival, migration, angiogenesis, and differentiation [30]. It has been seen to promote hematogenous spread [31] of cancer. Injection of only 100–1000 CD44+ tumor cells from colon and pancreatic cancers have been shown to promote tumorigenesis in an immunocompromised mouse model [30, 32]. CD44 is expressed on CRC-CSC and can be utilized as a marker for its isolation [25, 33].

CD24 is identified as an alternate ligand for *P*-selectin, an adhesion receptor on platelets and endothelial cells [34], through which it facilitates the passage of tumor cells in the blood stream during metastasis. It increases proliferation and adhesion of tumor cells to fibronectin, collagen, and laminin [35]. High expression of CD24 has been reported in tumor progression and metastasis [26].

CD166 is a multidomain transmembrane type 1 glycoprotein of the immunoglobulin superfamily. Its expression is pathologically correlated with aggressive diseases in a variety of cancers including melanoma, prostate, breast, ovarian, esophageal, and bladder cancers [36–41]. In human CRC, aberrant cell surface CD166 expression is strongly correlated with a shortened survival [42]. Furthermore, CD166+ cells from human CRC have been shown to induce tumorigenesis when xenografted in low numbers into immunodeficient mice [27], which is considered a hallmark of a CSC population.

EpCAM (CD326) is a type I membrane protein involved in the proliferation, differentiation, and migration of cancer cells [43, 44]. Many reports have suggested EpCAM as a CSC marker in CRC [28, 45–47].

The aim of this study was to identify valid CRC-CSC markers from a panel of four putative markers, CD44, CD24, CD166, and CD326, and to check their usefulness in distinguishing between CRC and normal tissue using immunohistochemistry and flow cytometry.

Results

Clinicopathological characteristics of patients

About 70 % of our study population ($n = 54$) was in the age group of 30–60 years, and majority of the cancers observed were located in the right colon (32 %) comprising of carcinoma of cecum, ascending colon, or hepatic flexure. The diagnosis of all patients was primary untreated colorectal adenocarcinoma. Out of the 54 patients, there were 15 (27.7 %) well-differentiated, 21 (40.7 %) moderately differentiated, and 18 (31.4 %) poorly differentiated CRC adenocarcinoma. About 60 % of the patients were tumor node metastasis (TNM) stage I and II while, 40 % were stage III and IV. Lymph node metastasis was present in 30 % of the patients and absent in 70 % of the patients (Table 1).

Expression of CD44, CD24, CD166, and EpCAM in normal tissue and CRC

Immunohistochemistry was done to check the expression, localization, and distribution of CSC markers CD44, CD24, CD166, and EpCAM in colorectal cancer and adjacent normal tissue ($n = 54$). Another group of normal colorectal samples ($n = 16$) was taken as control, from patients who were operated for non-neoplastic colorectal etiologies, to check for the presence of field cancerization.

It was observed that the distribution and localization of each CSC marker was different in different histopathological grades of CRC (Fig. 1). The CSC marker expression is majorly confined to the luminal side of crypt in well-differentiated CRC. As the loss of normal crypt architecture increases in moderately differentiated and poorly differentiated CRC, the CSC marker distribution and localization becomes more diffuse and the staining intensity also increases. Interestingly, the normal colorectal tissue obtained from patients operated for non-neoplastic conditions also showed CSC marker expression. However, in normal colorectal tissue, CD44 and CD166 were observed to be localized to the bases of intestinal crypts (Fig. 2), in contrast to their localization on the luminal side of crypt in CRC.

Statistically significant difference ($p < 0.05$) was observed in expression of CD44, CD166, and EpCAM between cases and controls (Supp Table 1a–c) in different histopathological

Table 1 Clinicopathological characteristics of patients enrolled for IHC study

Clinical data ($n = 54$)	n (%)
Gender	
Males	30 (55.55)
Females	24 (44.45)
Age (years)	
0–30	8 (14.81)
31–60	38 (70.38)
≥60	8 (14.81)
Histopathological grade	
WD	15 (27.77)
MD	22 (40.75)
PD	17 (31.48)
TNM ($n = 43$)	
I and II	26 (60.46)
III and IV	17 (39.54)
Lymph node metastasis ($n = 43$)	
Positive	13 (30.23)
Negative	30 (69.77)
Location	
Right colon	32 (59.26)
Left colon	7 (12.96)
Transverse colon	3 (5.56)
Rectum	10 (18.52)
Disseminated	2 (3.70)

WD well differentiated, *MD* moderately differentiated, *PD* poorly differentiated CRC

grades of CRC. Mean H-score in cases and controls for CD44 was found to be 9.2 ± 4.8 and 4.9 ± 2.3; for EpCAM, it was 9.2 ± 4.7 and 14.0 ± 2.3; for CD24, it was 9.0 ± 4.7 and 11.3 ± 4.5; and for CD166, it was 6.0 ± 4.8 and 2.3 ± 2.4, respectively, indicating that CD44, CD166, and EpCAM are good CSC markers for CRC (Table 2).

EpCAM expression was observed in all controls and decreased in cases suggesting that EpCAM may be a negative predictor of CSC in CRC. Statistical analysis (Table 2) as well as ROC curve analysis (Fig. 3) showed that CD24 expression did not differentiate well between cases and controls. From this study, we concluded that CD24 is not an appropriate CRC-CSC marker. We hypothesize that CD44 and CD166 are suitable CSC markers in CRC, especially when used together (Table 3) for the identification and characterization of CRC-CSC.

ROC curve analysis (Fig. 3) revealed that at a cutoff expression level of ≥25 %, distribution of CD44 had 80 % sensitivity and 82 % specificity as a CSC marker. Therefore, we considered ≥25 % distribution of CD44 as a positive expression of CD44. Likewise, distribution of CD166, at a cutoff expression level of ≥15 %, had a sensitivity of 69 % and specificity of 69 % as a CSC marker and was considered as a positive expression for CD166 for further discussion.

Correlation between CD44 and CD166 expression in human CRC

In this study, CD44 and CD166 expressions were found to be independent of the patient characteristics of age, sex, histopathological grade, TNM stage, or location of tumor (Supplementary Table 2).

Double positivity of CD44 and CD166 was identified in 33 cases, while a double-negative expression was identified in 4 cases. In addition, 12 patients were identified as CD44 positive but CD166 negative. In contrast, five patients were identified as CD44 negative and CD166 positive. Spearman's rank correlation analysis showed that CD44 expression and CD166 expression in CRC are positively correlated significantly ($r = 0.252$; $P = 0.03$; Table 4), whereas there is no correlation between the CD44 and CD166 expressions in normal colorectal tissues (Supplementary Table 3)

Characterization of HT29 and HCT116 human CRC cell lines by flow cytometry

The CSC marker-based characterization profile of HT29 (low grade) was different from HCT116 (high grade) cell line (Fig. 4a, b). Based on the IHC study, CD44 and CD166 were the most reliable CRC-CSC markers. Therefore, the CD44 + CD166+ subset most probably represented the CSC population, whereas the CD44-CD166− subset was the non-CSC-bulk-tumor cell population. The less differentiated or high-grade HCT116 cell line had a higher number of CSC (CD44 + CD166+) than the more differentiated or low-grade HT29 cell line (Table 5).

We sorted the CD44 + CD166+ and CD44-CD166− subsets from the HT29 cell line using fluorescence-activated cell sorting (FACS). Tumor sphere assay was performed on the sorted subsets to ascertain the subsets showing self-renewal and anchorage-independent proliferation, which are considered distinctive characteristics of CSC [28, 48, 49]. Both the subsets were plated in low-adherence 96-well plates with serum-free stem cell media (SCM) in dilutions from 100 cells/well to 10,000 cells/well. The wells with >5000 cells showed only clumping on the second day itself and were discarded. Optimal growth, division, and sphere formation was observed in wells plated with <500 cells. For all further assay experiments, 400 cells/well were plated on low-adherence six-well plates with serum-free SCM and microscopically observed for 3 weeks.

The CD44-CD166− subset failed to form spheres, whereas the CD44 + CD166+ subset formed spheres after 2 weeks of culture (Fig. 5).

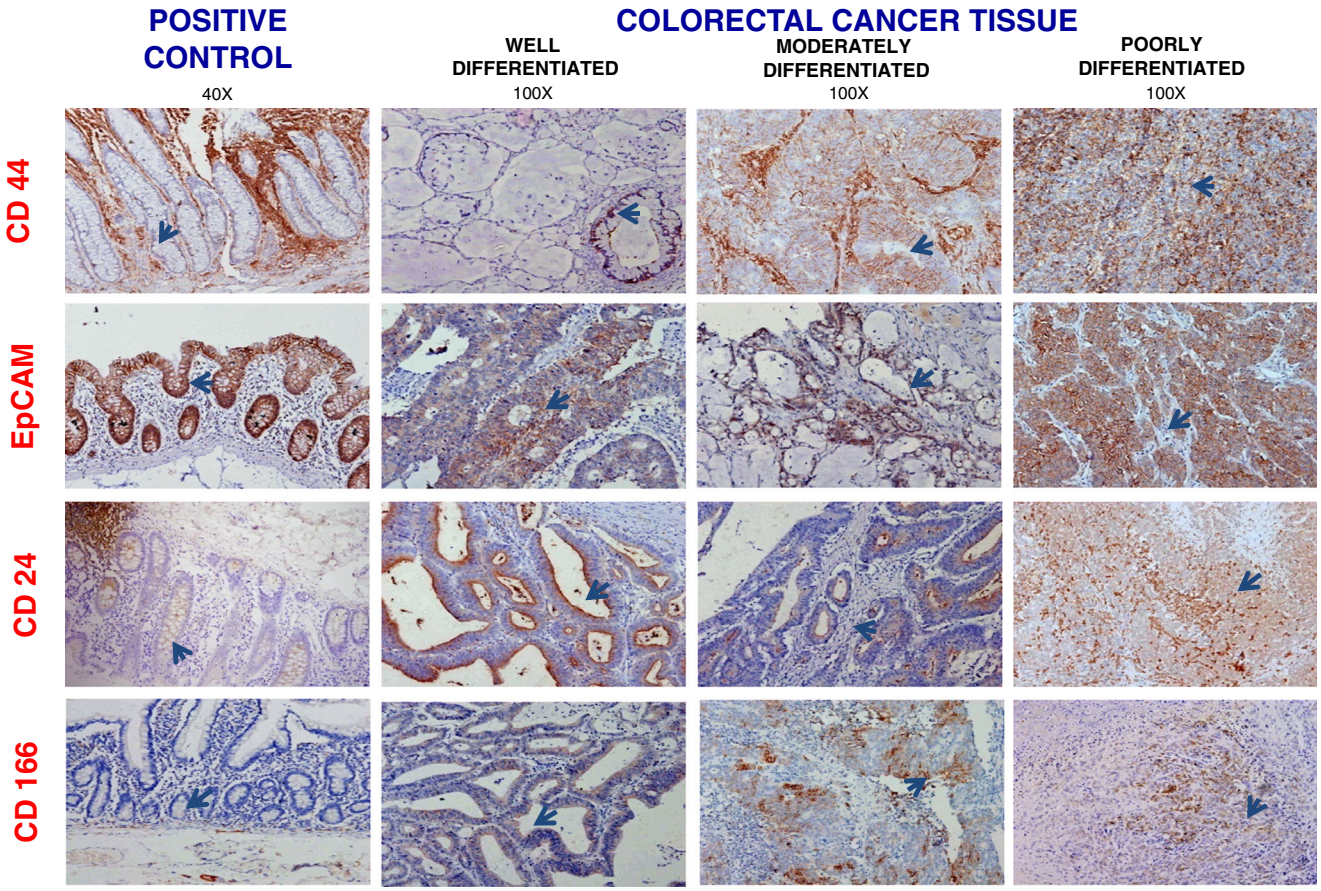

Fig. 1 IHC analysis of CSC markers shows differential distribution and localization in different histopathological grades of CRC (as indicated by *arrowheads* in the images)

Discussion

CRC is one of the most common solid organ cancers prevalent worldwide. A vast majority of research on CRC has revealed that metastasis is the major cause of mortality. The cancer stem cell (CSC) model of tumor growth assumes that a special subset of cells within the tumor, known as CSCs, have the capacity to initiate and sustain tumor growth. CSCs are also implicated in aggressiveness, metastasis, recurrence, and relapse of CRC. Thus, deciphering the biology behind CRC, especially CSCs, will give valuable insights regarding diagnosis, prognosis, and newer therapeutic targets in CRC.

Different opinions exist regarding the robustness of markers used in identifying CRC-CSC, and the functional relevance of cell surface markers used to identify CRC-CSC is important, especially when considering future therapies. A panel of putative CSC markers, CD44, CD24, CD166, and EpCAM (CD326), were analyzed by immunohistochemistry in 54 colorectal cancer cases and 70 controls (Fig. 6). Statistically significant difference was observed between cases and controls for expression of CD44, CD166, and EpCAM but not for CD24. CD44 and CD166 were able to distinguish significantly between cases and controls at a cutoff expression level of ≥25 % for CD44 and a cutoff expression level of ≥15 % for CD166 (Fig. 3). A significant correlation between the positive expressions of these markers was also observed (Table 4).

EpCAM is widely distributed on the surface of normal cells, and any decrease in surface expression is shown to be associated with cancer [44]. Our study has also shown reduced cell surface expression of EpCAM in CRC. Reduced surface expression of EpCAM has been correlated with a corresponding increase in its intracellular expression. The intracellular domain of EpCAM has been shown to regulate the activity of genes responsible for carcinogenesis [43].

Though many reports have shown convincing evidence of CD24 as a CSC marker for breast cancer, there have been conflicting reports about its usefulness as a CSC marker for CRC. CD24 in CRC did not show any differential expression, and we found no statistically significant difference between cases and controls by immunohistochemistry (IHC) analysis as suggested previously [50].

We also studied the expression of these markers in two types of controls: one control group comprised of adjacent normal, healthy tissue, more than 5 cm, from the tumor margin in patients operated for CRC. The other control group was

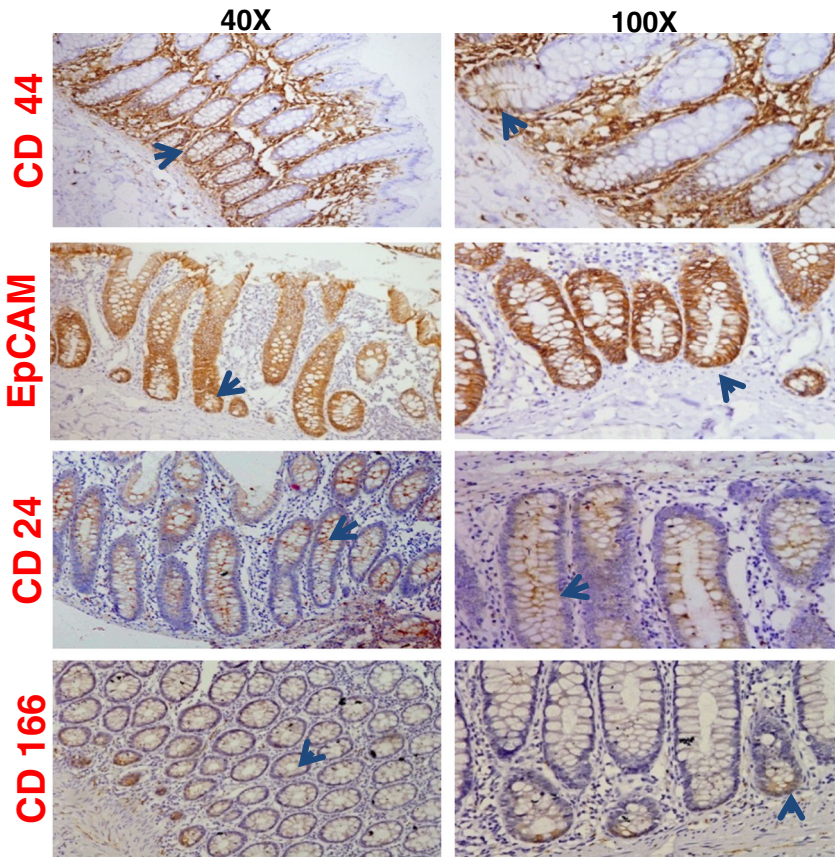

Fig. 2 Distribution and localization of CSC markers in normal colon by IHC

normal colonic tissue from patients operated for non-neoplastic colorectal conditions. EpCAM expression was detected in nearly 100 % of controls. CD24 expression was variable in all the controls.

Loss of normal crypt architecture was seen in all cases of CRC. The distribution and localization of each CSC marker varied across different histopathological grades in CRC (Fig. 1). In well-differentiated CRC, most of the CSC marker distribution was observed on the luminal side of the crypt. The luminal side of the crypt consists of differentiated cells/enterocytes [51]. However, in controls, it was interesting to note that most of the CD44 and CD166 expressions were localized to a few cells at the bases of normal colonic crypts (Fig. 2). The presence of adult stem cells residing at the bases of normal colonic crypts may be a possible explanation for the presence of CRC-CSC markers in normal colorectal tissues. The normal colonic stem cells maintain the proliferative dynamics of colonic epithelium, and this similarity in CSC marker expression suggests that normal stem cells may be a target of oncogenic transformation to give rise to CSC [29, 52, 53].

We found no significant difference in the expressions of CD44, CD24, CD166, and EpCAM between the CRC controls and the non-neoplastic controls, suggesting that there was no confounding of results attributed to the concept of field cancerization (Fig. 6). Field cancerization is a molecular concept defined as the presence of epithelial cells that have cancer-associated genetic or epigenetic alterations due to their localization in the vicinity of a tumor [54].

There was no statistically significant difference in the expression of CD44 between different histopathological grades

Table 2 Expression of CSC markers in CRC and normal tissue by IHC.

CSC markers	Colorectal cancer ($n = 54$)		Normal control ($n = 70$)		p value
	Positivity (%)	Mean H-score	Positivity (%)	Mean H-score	
CD44	45.4 ± 31.0	9.2 ± 4.8	18.1 ± 9.9	4.9 ± 2.3	<0.001
EpCAM	79.0 ± 28.0	9.2 ± 4.7	97.8 ± 9.3	14.0 ± 2.3	<0.001
CD24	70.0 ± 28.0	9.0 ± 4.7	73.1 ± 29.1	11.3 ± 4.5	>0.05
CD166	30.0 ± 29.5	6.0 ± 4.8	7.3 ± 8.5	2.3 ± 2.4	<0.001

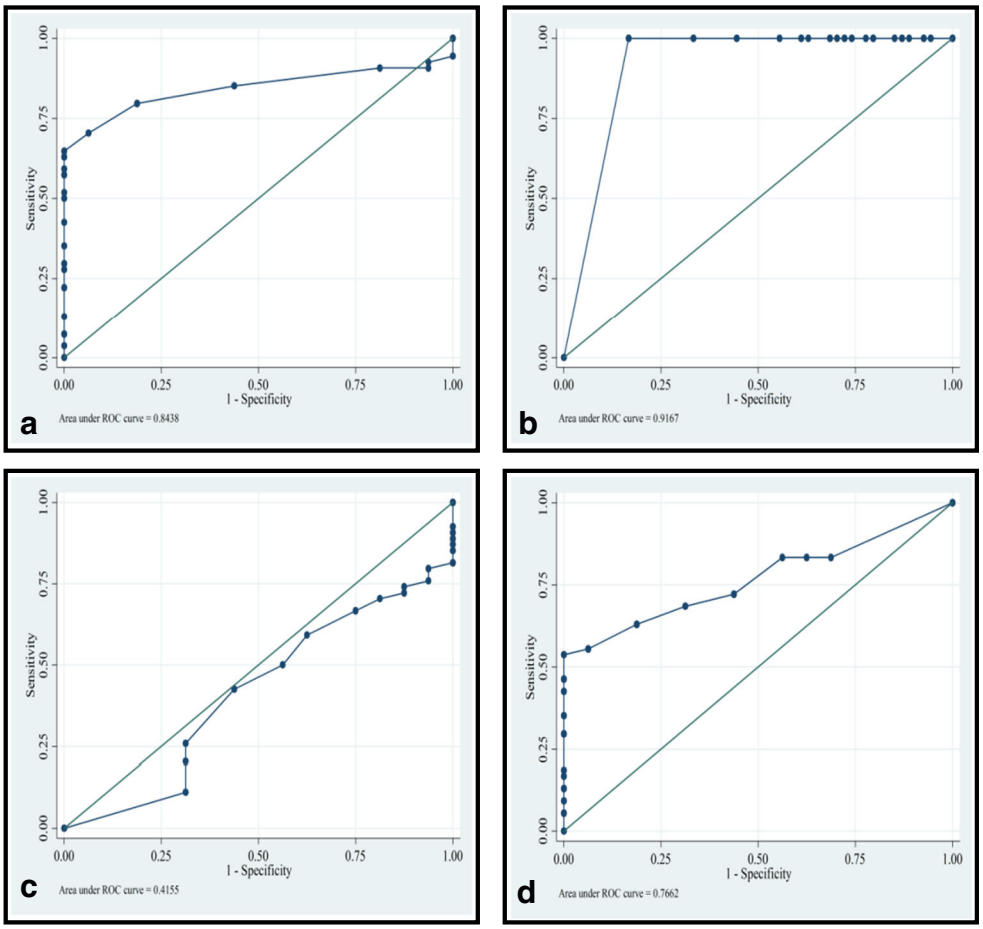

Fig. 3 ROC curve analysis for CSC markers. Area under the curve for **a** CD44 84.3 %, **b** EpCAM 91.6 %, **c** CD24 41.5 %, and **d** CD166 76.6 %

of CRC, which is in contrast to previous studies [55]. No relation between histopathological grade and CD166, CD24, or EpCAM expression was found which was in agreement with published reports [50, 56]. Further, the expression of CD44 and CD166 did not correlate with any of the studied clinicopathological parameters, eliminating any confounding effect of these on the CRC-CSC expression. Simultaneous expression of CD44 and CD166 (CD44 + CD166+) was seen in CRC but not in normal controls. This double-positive expression is a good predictor of CRC-CSC.

Based on the CD44 + CD166+ expression, we further characterized CSCs from the HCT116 human colorectal cell line, which was originally derived from a poorly differentiated or high-grade colon adenocarcinoma, and from the HT29 human colorectal cancer cell line, which was derived from a well-differentiated or low-grade colon adenocarcinoma. About 24 % of the total cancer cells were CSC (CD44 + CD166+) and only about 3 % were non-CSC-bulk-tumor cells (CD44-CD166−) in high-grade HCT116 cell line. This finding is in agreement with existing literature [22] and signifies that high-grade CRC has higher number of CSC, which further suggests a definite link between CSC and poor prognostic indicators such as differentiation grade in cancer.

Characterization of the low-grade HT29 cell line showed that about 9 % were CSCs and about 11 % were non-CSC-bulk-tumor cells out of the total cells. The increased number of CD44 + CD166+ CSC in high-grade HT116 as compared to low-grade HT29 is in agreement with previous studies [56, 57], implicating the importance of CSC in aggressive CRC. Poorly differentiated CRC with higher number of CSCs has been shown to have high metastatic potential [58, 59]. On

Table 3 Comparison of CD44 and CD166 positive expression between colorectal cancer and normal colorectal tissues

Tissue	Number	CD44+ (%)	X^2	*p* value	CD166+ (%)	X^2	*p* value
Cancer	54	43 (79.62)	45	<0.05	37 (68.51)	24	<0.05
Normal	70	13 (18.57)			17 (24.28)		

Table 4 Correlation b/w CD44 and CD166 expression in CRC ($n = 54$)

	CD166			
CD 44	Positive	Negative	*r* value	*p* value
Positive	33	12	0.252	0.03
Negative	5	4		

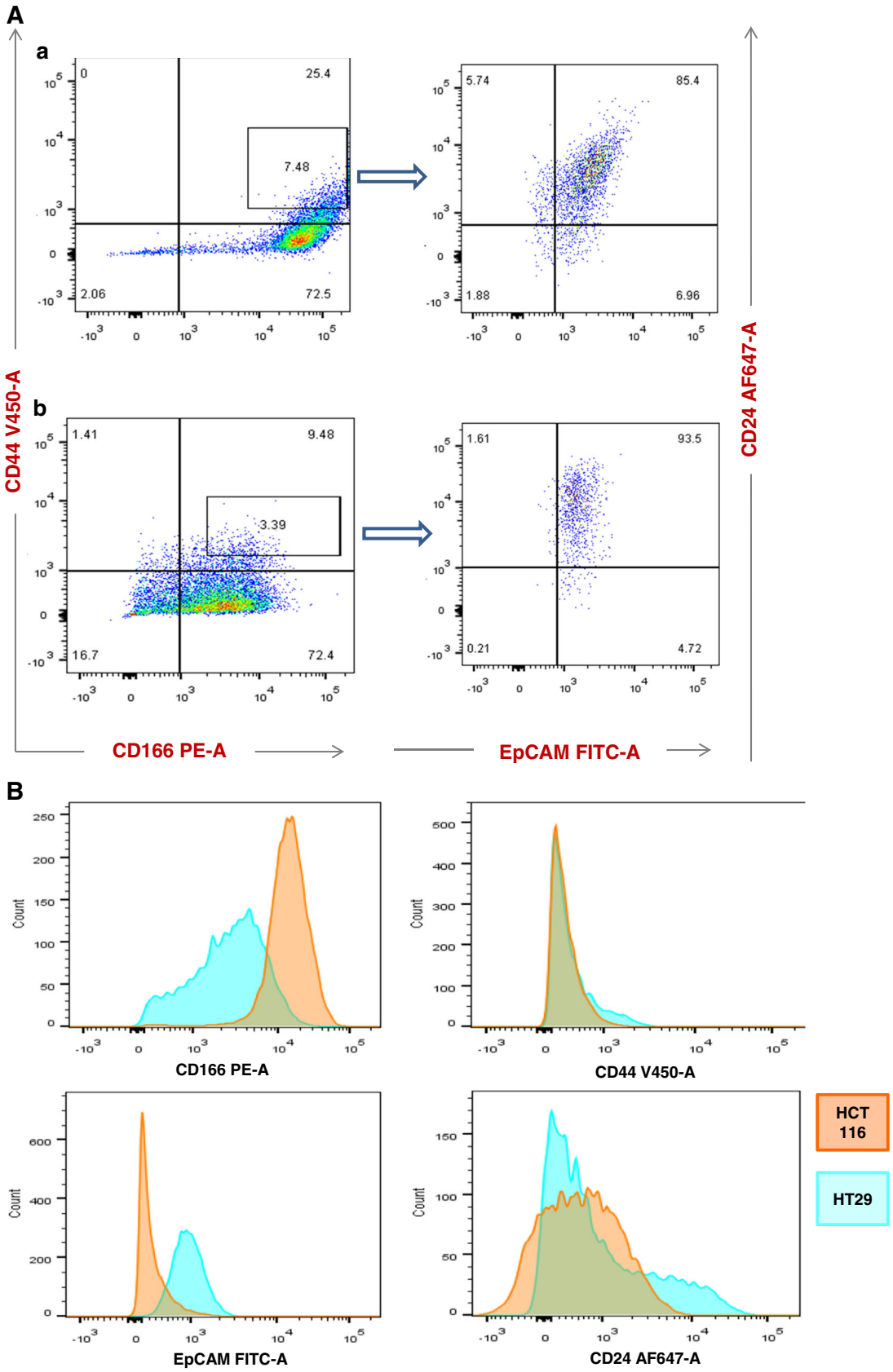

Fig. 4 **A** Characterization of CSCs from (*a*) HCT116 human CRC cell line and (*b*) HT29 human CRC cell line. **B** Expression of CSC markers in the high-grade HCT116 cell line and the low-grade HT29 cell line by flow cytometry

Table 5 Distribution of CSC (CD44 + CD166+) and non-CSC-bulk-tumor cells (CD44-CD166−) in human CRC cell lines using flow cytometry

Source		CD44+CD166+ (%)	Average (%)		CD44-CD166- (%)	Average (%)
HCT116 (Triplicates)	I.	24.3	23.2	I.	4.5	3.1
	II.	20.1		II.	2.8	
	III.	25.4		III.	2.1	
HT29 (Triplicates)	I.	8.5	9.1	I.	12.2	11.5
	II.	9.2		II.	5.5	
	III.	9.5		III.	16.7	

further analysis of these CD44 + CD166+ CSCs, the expressions of CD24 and EpCAM were found to be variable.

Tumor sphere assay performed with CSC and non-CSC-bulk-tumor cells isolated from the HT29 cell line showed sphere formation in the CSC subset as suggested in previous studies [60–63]. No sphere formation was observed with the non-CSC-bulk-tumor cells. This gives proof of principle that these CD44 + CD166+ cells within the tumor with their unique properties of self-renewal and sphere formation form the elusive CRC-CSC subset.

Conclusion

Our study suggests that CD44 and CD166 are robust CRC-CSC markers. The novel use of the cutoff expression for CD44 positivity, defined as ≥25 %, and CD166 positivity, defined as ≥15 %, on immunohistochemical analysis can significantly distinguish between cancer and normal tissue. This cutoff can be applied for better immunohistochemical analysis of CSC markers in the future.

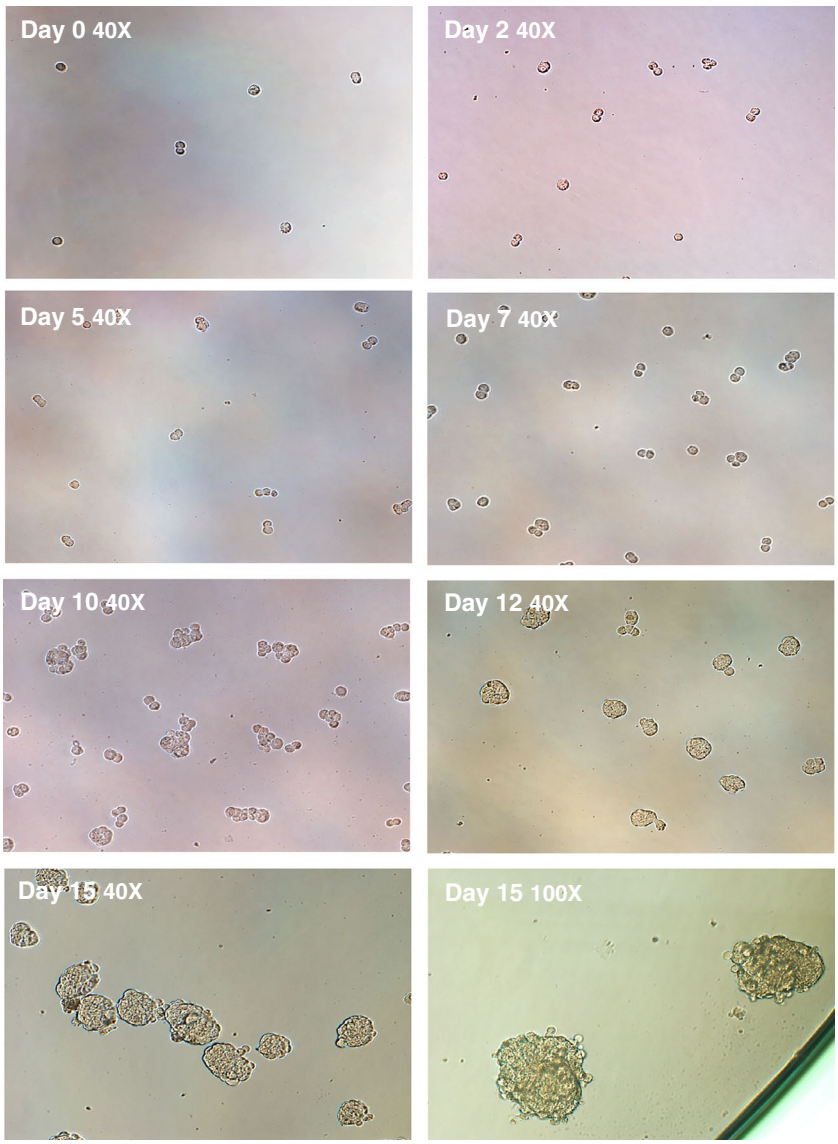

Fig. 5 Tumor sphere assay: sorted CD44 + CD166+ CSCs from HT29 cell line formed tumor spheres when grown in low-adherence Petri plates with stem cell media at the end of 2 weeks

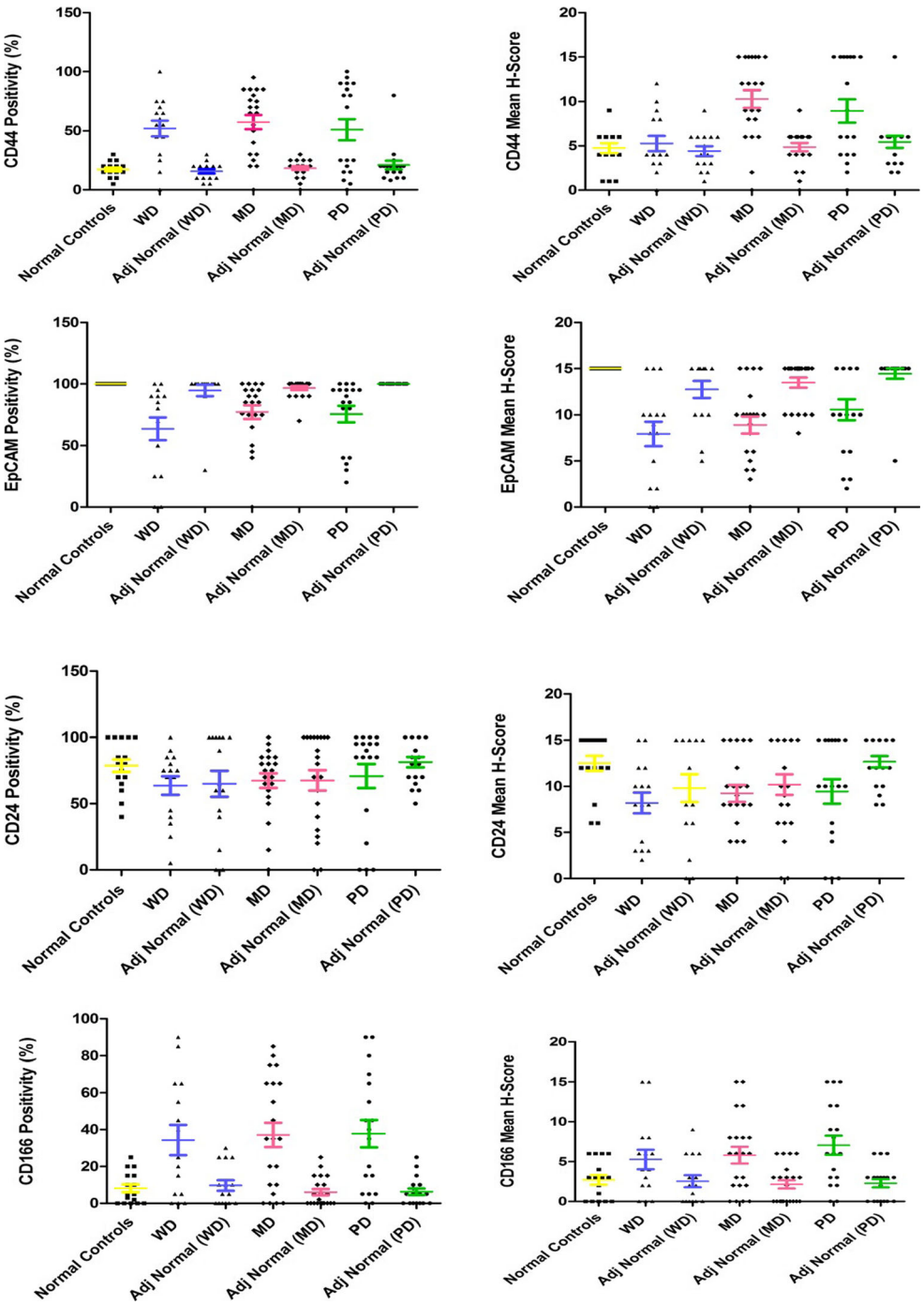

Fig. 6 Expression of CSC markers in different histopathological grades of CRC and normal controls by immunohistochemistry

We observed CSC marker expression in normal colonic crypts which is the site of normal intestinal stem cells. Normal stem cells may be a possible target for accumulating mutations that are necessary for stepwise malignant transformation in CRC. The number of CSC (CD44 + CD166+) is higher in HCT116 as compared to HT29 which further validates that high-grade tumors have increased number of CSC responsible for metastasis, relapse, and recurrence.

Our study has highlighted the advantage of using CD44 and CD166 together to isolate, characterize, and study CRC-CSC instead of using a single CSC marker. However, a larger study is required to further establish the relationship of tumor grade with CD44 + CD166+ CSC.

Materials and methods

All protocols were reviewed and approved by the Ethics Committee, All India Institute of Medical Sciences, New Delhi, India. All tissue samples were collected after obtaining

written, informed consent from the patients. Differential expressions of CD44, CD24, CD166, and CD326 were studied in 70 patients by immunohistochemistry. Characterization of CSC from two different human colorectal cancer cell lines was done using flow cytometry and tumor sphere assay.

Immunohistochemistry

Patients and tissue samples

Paraffin-embedded colorectal cancer tissues and controls from primary, untreated, sporadic colorectal cancer patients were obtained from the Department of Pathology, All India Institute of Medical Sciences. All patients receiving preoperative radiotherapy or chemotherapy, with secondary colorectal cancer, with family history of colorectal cancer or hereditary and genetic associations were excluded from the study. A total of 54 colorectal tumors were studied out of which 15 were well differentiated, 21 were moderately differentiated, and 18 were poorly differentiated. Normal mucosal specimen, incised more than 5 cm away from the primary tumour margin, was taken as adjoining control for each patient. Another set of 16 healthy colorectal tissue specimens were obtained as controls from patients whose colon was removed for non-neoplastic indications.

Method

Formalin-fixed, paraffin-embedded human colorectal tissue blocks were sectioned at 5-μm thickness on aminopropyltriethoxysilane (APES, Sigma, St. Louis, Missouri, USA) coated slides. Tissue sections were deparaffinized with xylene and rehydrated with alcohol. Antigen retrieval was achieved by microwaving in citrate buffer for 20 min. All sections were rinsed with Tris-buffered saline and blocked with peroxide in methanol for 30 min at room temperature. Sections were then blocked using Super block (CRF™ Scy Tek Laboratories, Utah, USA) for 5 min at room temperature. Primary antibodies anti-CD24 (1:200 dilution, cat. no. 31622 Abcam, Cambridge, UK), anti-CD44 (1:1000 dilution, cat. no. MS668P Thermo Scientific, CA, USA), anti-CD166 (1:1000 dilution, cat. no. 109215 Abcam, Cambridge, UK), and anti-EpCAM (1:800 dilution, cat. no. 32392 Abcam, Cambridge, UK) were added to the individual sections and incubated at 4 °C in a humidity chamber overnight. Primary antibody was detected using an anti-polyvalent HRP polymer kit (CRF^{TM} Scy Tek laboratories, Utah, USA) and diaminobenzidine as the chromogen. Counterstaining was done by Mayer's hematoxylin followed by dehydration, permanent mounting using DPX Mountant (Sigma, St. Louis, Missouri, USA), and the slides were allowed to dry overnight. Finally, the slides were examined by light microscopy.

A section from a reactive lymph node, esophageal carcinoma, prostate cancer, and stomach cancer was used as a positive control for CD24, CD44, CD166, and EpCAM, respectively, in each batch of IHC.

Evaluation of IHC

For each of the four CSC markers, i.e., CD24, CD44, CD166, and EpCAM, slides were evaluated using a scoring system for both staining intensity and percentage positivity. Immunopositive staining was evaluated in randomly selected five areas of the tissue section. Percentage positivity grading was done as follows: 0–10 % = 1, 11–25 % = 2, 26–50 % = 3, 51–75 % = 4, and >76 % = 5. Semiquantitative grading for staining intensity was done as follows: negative = 0, mild = 1, moderate = 2, and intense = 3. Finally, an H-score was calculated by multiplying the intensity grade and percentage positivity grade for each slide. In cases where both surface and cytoplasmic immunostaining was observed, both were scored independently and a combined score assigned. All slides were scored by three independent observers/pathologists who were blinded to the diagnostic or prognostic history and clinical details of the patient (slides were coded). The scoring was discrepant in about 5 % cases where consensus was reached by reevaluation and discussion.

Statistical analysis

All experimental data was analyzed using SPSS software version 20.0 (SPSS Inc., Chicago, IL, USA). One-way ANOVA test with Bonferroni correction and Wilcoxon signed-rank test was used for multiple comparisons. Paired *t* test was used for comparisons between cases and controls. ROC curve analysis was done to evaluate the sensitivity and specificity of the CRC-CSC markers. p value of <0.05 was taken as statistically significant.

The correlation between the immunohistochemical staining of CD44 and CD166, and the clinicopathological parameters was evaluated by the Chi-square test. The correlation between the expression of CD44 and CD166 was assessed by Spearman's rank test.

Cell culture and flow cytometry

The HT29 (low grade) and HCT116 (high grade) human colorectal cancer cell lines used in this study were obtained from NCCS, Pune, India. The cell lines were grown in DMEM with 10 % fetal calf serum (FCS) at 37 °C in 5 % CO_2. All experiments were repeated at least three times.

Immunophenotyping

Log-phase cells from HCT116 and HT29 cell lines were utilized for all experiments. Antibodies were procured from BD Biosciences, San Jose, CA, USA: Alexa Fluor 647 mouse anti-human CD24 (cat. no. 561644) with isotype control (cat. no.

557715), V450 mouse anti-human CD44 (cat. no. 561292) with isotype control (cat. no. 560374), PE mouse anti-human CD166 (cat. no. 559263) with isotype control (cat. no. 555749), and monoclonal anti-EpCAM FITC (cat. no. 347197) with isotype control (cat. no. 349041). Flow cytometry was performed using BD FACS ARIA III using appropriate compensation and fluorescence minus one (FMO) controls.

FACS and tumor sphere assay

The differential distribution of the CSC markers, CD44, CD24, CD166, and EpCAM, in HT29 and HCT116 was analyzed using FlowJo V10 software.

Two subsets of tumor population, CD44 + CD166+ (CSCs) and CD44 − CD166− (non-CSC-bulk-tumor cells), from the HT29 cell line were sorted into tubes containing receiving media (high-glucose DMEM with 50 % FCS). For FACS experiments, compensation controls were used and appropriate gating was done using unstained, single stained, and FMO controls. Post-acquisition analysis of the fluorescence-activated cell sorting data was accomplished using the third-party flow cytometry software, FlowJo V10.

Tumor sphere assay was performed for the sorted population of CSCs from the HT29 cell line. The CD44 + CD166+ cells (CSCs) and CD44 − CD166− cells (non-CSC-bulk-tumor cells) from HT29 were centrifuged and washed with PBS after sorting. Cell viability and cell count was checked using Trypan blue dye exclusion and hemocytometer. Triplicates of different cell numbers, 100 cells, 200 cells, 400 cells, 600 cells up to 10,000 cells were plated on low-adherence 96-well plate using serum-free, stem cell media (SCM). All the wells were inspected under a microscope daily for clumping and cell growth. The wells showing evidence of clumping during the first few days of observation were excluded from further observation. About 100 μl of SCM was added every alternate day, after day 3, in the wells being observed for tumor sphere formation. SCM consists of high-glucose DMEM (Thermo-Fisher Scientific, MA, USA) and Ham's F12 (Thermo-Fisher Scientific, MA, USA) 1:1, Glutamax (Thermo-Fisher Scientific, MA, USA), non-essential amino acids (NEAA) (Gibco, New York, USA), B27 supplement (Gibco, New York, USA), growth factors (Thermo-Fisher Scientific, MA, USA), and penicillin-streptomycin (Sigma, St. Louis, Missouri, USA). The tumor sphere formation and propagation was observed for 3 weeks.

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Compliance with ethical standards

Conflicts of interest None

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