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Co-Expression of CD133$^+$/CD44$^+$ in Human Colon Cancer and Liver Metastasis

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Although relatively good therapeutic results are achieved in non-advanced cancer, the prognosis of the advanced colon cancer still remains poor, dependent on local or distant recurrence of the disease. One of the factors responsible for recurrence is supposed to be cancer stem cells (CSCs) or tumor-initiating cells, which are a population of cancer cells with ability to perpetuate themselves through self-renewal and to generate differentiated cells, thought to be responsible for tumor recurrence. This study globally approach the possible role of tissue-derived stem cells in the initiation of colon cancer and its metastatic process in the liver. Fresh surgical specimens from colon cancer, non-tumor tissue and liver metastasis were obtained directly from the operating room, examined, and immediately processed. CSCs were selected under serum-free conditions and characterized by CD44 and CD133 expression levels. CD133$^+$/CD44$^+$ cell populations were then investigated in paraffin-embedded tissues and circulating tumor cells isolated from peripheral blood of the same group of colon cancer patients. Our data demonstrate that metastatic properties of cell populations from blood and liver metastasis, differently from primitive tumors, seem to be strictly related to the phenotype CD133 positive and CD44 positive.

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Colon cancer is the second leading cause of cancer-related death. The majority of these deaths are due to metastasis, with the liver easily the most common site of deposit (LeGolvan and Resnick, 2010).

Combining surgery and chemotherapy in the treatment of patients with colon hepatic metastases is increasingly becoming the standard of care. However, controversy remains regarding the juxtapositioning of chemotherapy and surgery, the duration of chemotherapy, and particularly, the use of preoperative chemotherapy in the treatment of patients with initially resectable metastases (Nordlinger et al., 2010).

Cancer metastasis has been explained by at least two models: a progression model and an initiating model. In the former, metastatic capacity is acquired during cancer progression in a subpopulation of cells through sequential genetic mutations or epigenetic alterations (Gray, 2010), in genes associated with proteolysis of local extracellular matrix attachments (Nagashima et al., 1997), adhesive alterations (Furger et al., 2001), angiogenesis (Weber, 2008), viable vascular dissemination, distant embolization, and survival in a new environment (Folkman, 1990; Hynes, 2003; Bird et al., 2006). However, not all of these genetic alterations occur during the process of liver metastasis (Gray, 2010). In the initiation model, cells with metastatic potential are determined by early mutational events in a progenitor cell, named cancer stem cell (CSCs) or tumor-initiating cells (TIC; Clarke and Fuller, 2006; Polyak and Hahn, 2006; Odoux et al., 2008), even if few data support the hypothesis of its role in the colon metastatic process in humans (Horst et al., 2009b; Puglisi et al., 2009; Ju et al., 2011).

CD44 and CD133 have already been validated as informative markers of stem cells in both primary tumors and xenografts (O'Brien et al., 2007; Ricci-Vitiani et al., 2007). CD133, originally known as AC133, is a glycoprotein also known in humans and rodents as Prominin 1 (PROM1; Kawamoto et al.,

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2010). It is the founding member of pentaspan transmembrane glycoproteins (5-transmembrane, 5-TM), which was specifically regarded as the colon CSC-surface markers. CD133 has been used to identify and isolate cancer initiating cells from human colon cancer and it has been demonstrated that $CD133^+$ cells are able to maintain themselves as well as differentiate and re-establish tumor heterogeneity upon serial transplantation in vivo (O'Brien et al., 2007; Ricci-Vitiani et al., 2007). CD44 is a cell surface glycoprotein involved in cell–cell interactions, cell adhesion, and migration. It was identified as the cell-surface markers of breast cancer and also recently observed to be related to the distant metastasis of colon cancer (Huang et al., 2012).

Currently, there are some studies addressing the CD133 and CD44 co-expression of CSC antigens in colon cancer patients (Horst et al., 2009a; Salnikov et al., 2009). CD44/CD133 co-expression was significantly higher in colon cancers with early liver metastases compared to those without early liver metastases and co-expression was also associated with poorest prognosis (Huang et al., 2012), suggesting that CD133 and CD44 proteins co-expression in colon cancer may be a potential biomarker for early liver metastases. Indeed, Chen et al. (2011) demonstrated that FACS sorted $CD133^+/CD44^+$ HCT116 cells are undifferentiated, endowed with extensive self-renewal and epithelial lineage differentiation capacity in vitro, more invasive in vitro and responsible solely for liver metastasis in vivo. However, CD133 and CD44 expression status and its relation to liver metastasis is still controversial.

A recent study (Hou et al., 2011) showed the critical role of $CD133^{+/}CD44^+$ tumor cells in hematogenous metastasis of liver cancers, underlying that CD133 is responsible for tumor growth and CD44 is important for invasion; two important factors in tumor metastasis. Their data also suggest that $CD133^+$ cells act as TIC-like populations, however were not necessarily highly invasive, and that CD44 was a marker of an invasive, but not tumorigenic, sub-population. In addition to CD133, CD44 has a critical role in tumor metastasis. Moreover, based on studies of HCC cell lines, $CD133^+$ and $CD44^+$ were proposed to be markers of TICs in liver cancers (Zhu et al., 2010). Therefore, CD133 and CD44 could be regarded as markers of metastasis-facilitating pathways through which liver tumor metastasis is jointly promoted.

Therefore, starting from these observations, in this study we have generated stem cell-enriched human colonosphere cultures from fresh samples deriving from the same group of colon cancer and liver metastasis specimens under serum-free culture conditions, comparing the sphere forming potential of the clones isolated from the primary culture coming from both compartments and the differences observed in the corresponding non-tumor counterpart, adjacent to the neoplastic lesion. We have established long-term culture spheres from the three compartments, analyzing the expression of CD44 and CD133.

Indeed, we have examined colon cancer specimens and circulating tumor cells (CTC) to investigate the CD133 and CD44 expression and localization.

Materials and Methods

Patients

Biological tissues for laboratory assays were obtained from 21 patients with a first diagnosis of primary colon cancer with (n = 11) or without (n = 10) liver metastasis. All patients were consecutively treated with primary surgery at the Gastrointestinal Surgery Unit of NCC Bari, Italy. Before undergoing routine surgery, all patients signed an informed consent authorizing the Institute to use their removed biological tissues for research purposes. Routine staging procedures were adopted for disease staging according to UICC criteria (Greene and Sobin, 2002). Just after surgical removal of the colon and, if any, of liver metastasis, tissues were macroscopically analyzed by the pathologist who performed a sampling from the primary tumor, from adjacent normal mucosa (located not less than 10 cm from the primary site), and from liver metastasis. H&E staining confirmed the diagnosis of colon cancer in all primary and metastatic sites as well the absence of cancer cells in normal mucosa. About 100 mg of tissue without fat and necrosis was placed in sterile tubes with culture medium on ice. Patients with liver metastasis were categorized as the metastatic group (M1); those without liver metastases as the non-metastatic group (M0).

Isolation and expansion of colon stem cell cultures

After sampling, biological samples were processed as described previously (Cammareri et al., 2008). The obtained tissue fragments were washed extensively with PBS and were mechanically dissociated using scalpels and vigorous trituration to yield small fragments ($<1\ mm^3$) and single cells. Enzymatic digestion was performed using collagenase/hyaluronidase (Stemcell Technologies, Vancouver, Canada) in DMEM/F12 containing 5 mM Hepes (Sigma, St. Louis, MO) for 2 h at 37°C. The suspension was then filtered through a 100-μm pore size to separate the tissue fragments from the single cells. The single cell suspension was cultured in advanced DMEM/F12 (Life Technologies, Monza, Italy) supplemented with 0.6% BSA (Sigma), 0.6% glucose (Sigma), 2 mM L-glutamine (Euroclone, SPA, Pero, Milano), 9.6 μg/ml putrescin (Sigma), 6.3 ng/ml progesterone (Sigma), 5.2 ng/ml sodium selenite (Sigma), 25 μg/ml insulin (Sigma), 100 μg/ml apotransferrin (Sigma), 5 mM Hepes (Sigma), 10 ml antibiotic–antimycotic (Life Technologies, Monza, Italy), and 4 μg/ml Heparin (Sigma). Growth factors [20 ng/ml EGF (Peprotech, London, UK) and 10 ng/ml b-FGF (Peprotech)] were added to the cell culture medium freshly each week. All cell culture was carried out in non-tissue culture treated petri at 37°C in a 5% CO_2 humidified incubator.

The viable cells were counted using a light microscope by trypan blue dye exclusion test at day 7. Surviving immature tumor cells slowly proliferate, and grow as non-adherent spheres. The formation of such spheres, containing about 50 cells, takes 2 months. Sphere formation was scored as follows: (−) no sphere observed; (+) two spheres per field.

Cytospin cell preparation and immunocytochemical staining of CD44 and CD133

Cells were centrifuged at 800 rpm for 5 min, washed with PBS, and re-suspended in PBS. The cells were attached to coated microscope slides (Bio Optica, Milano, Italy) in a Cyto-Tek centrifuge (Sakura Finetek, Alphen Aan Den Rijn, Netherlands) at 2,000 rpm for 10 min and dried overnight on a slide warmer at 37°C. They were then fixed with acetone and stained. Primary antibodies specific for CD133 (Abcam, Cambridge, UK), CD44 (DakoCytomation, Glostrup, Denmark) were used, with primary antibody binding detected using corresponding Dako EnVision System-HRP Labelled Polymer secondary antibodies (DakoCytomation) and AEC Substrate Chromogen (DakoCytomation). The cells were counterstained with Mayer's hematoxylin (DakoCytomation). After incubation, cells were washed with PBS, and incubated with biotinylated link for 30 min, peroxidase-labeled streptavidin for 30 min and 3-amino-9-ethylcarbazole substrate-chromogen (Labelled Streptavidin-Biotin2 System-Horseradish Peroxidase; DakoCytomation) for 15 min in the dark. After PBS washing, the slides were counterstained with hematoxylin and mounted with aqueous mounting medium (DakoCytomation). For negative controls, the primary antibody was omitted and replaced by PBS.

Photomicrographs of cytospin preparations were acquired under bright field illumination with a Leica DMLB optical microscope (Leica, Cambridge, UK) and analyzed with Leica IM1000 software.

Immunohistochemical staining of CD44 and CD133

Double immunostaining with antibodies for detection of CD44 and CD133 was performed on paraffin-embedded tissue sections and cytospins, using primary antibodies specific for CD133 (Abcam), CD44 (DakoCytomation), and Alexa 488 goat anti-rabbit and the Alexa 568 goat anti-mouse immunoglobulin G1 secondary antibodies conjugate (Invitrogen). Tissue sections were then mounted with aqueous mounting medium (DakoCytomation). For negative controls, the primary antibody was omitted and replaced by PBS. Images were obtained on a BX40 microscope (Olympus, Tokyo, Japan) with a SenSys 1401E Photometrics CCD camera.

Immunohistochemical evaluation

The scoring was performed twice by two persons in a blinded fashion. CD133 and CD44 staining was detected mainly in the membrane and was evaluated as the percentage of immunoreactive cells with respect to the total analyzed. The result was the mean of 15–20 random field at 40× magnification.

Collection of blood samples

Fifteen milliliters of peripheral blood were collected from each participant in a vacutainer system with lithium-heparin before surgery. A written consent should be obtained from all patients prior to enrolment in the study, and the Ethical Committee of the NCI approved the protocol which was in accord with the ethical guidelines of the 1975 Declaration of Helsinki Whole. A control group found to be healthy from laboratory data was enrolled among donors (N = 30). Their median age was 50 years (range: 40–70 years).

Magnetic labeling

Fifty milliliters of anti-coagulated blood were centrifuged with 400*g* for 35 min. Buffy coat were collected into 50-ml conical tubes. For magnetic labeling, the cells were first permeabilized using CellPerm Solution (40 ml) from the Carcinoma Cell Enrichment Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) and incubated for 5 min at room temperature. Fixation was done using 5 ml of Cell Fix Solution for 30 min at room temperature. The cells were then washed twice in dilution buffer. Two hundred microliters of FcR Blocking Reagent (Miltenyi Biotec) was used for the prevention of unspecific binding. Two hundred microliters of anti-cytokeratin (7/8) Micro Bead was used for magnetic labeling. The magnetic labeling was performed at room temperature for 45 min. Finally, the cells were washed once in cell stain solution and resuspended in 1 ml of dilution buffer for magnetic cell separation.

Magnetic separation of cytokeratin 7/8-positive tumor cells

For magnetic separation, the labeled cells were passed through MS+ (Miltenyi Biotec) separation columns that had been equilibrated with dilution buffer. The negative cells were washed off the column with 3 ml of dilution buffer. The retained cells were eluted from the column outside the magnetic field by pipetting 1 ml of dilution buffer onto the column and the cellular suspension was filtered through a mesh with 30-μm diameter holes.

Immuncytochemical labeling and microscopic analysis

Cells were magnetically separated as described above, and the enriched cell fraction was spun down on slides using a cytocentrifuge. Slides were air-dried overnight at room temperature. Fast Red TR/Naphthol AS-MX Substrate Solution was added to cell spots for 15 min in humidity chamber at 25°C. Slides were then stained with dilute Meyer's hemalum solution 1:2 in 100 mM of Tris–HCl, pH 8.2, washed twice for 1 min in double distilled water, and coverslipped using glycerin plus PBS (1:1).

Results

Tumor tissues of 21 patients with primary colon cancer, together with matched adjacent normal tissues, were collected; 11 patients had also liver metastasis. Clinical pathological characteristics and biological specimens of each patient are summarized in Table 1. Fresh tissues (Fig. 1A) were processed and after dissection, viable cells were obtained from 17 out of 18 tumor tissues, 11 out of 18 normal mucosa, and 11 out of 11 liver metastasis (Table 2). We evaluated the growth potential of these primary cells under serum-free conditions. First generation of colon spheres (Fig. 1B) were successfully cultured in 11 out of 17 samples from tumors, in 9 out of 11 from metastasis and in 6 out of 11 from adjacent normal tissues (Table 2). To assess whether the ability to form spheres was maintained during the time of culture, cells were expanded and after 5 months (Table 2) 5 out of 17 samples from tumor tissues, 6 out of 11 from liver metastasis, and 2 out of 11 from non-

TABLE 1. Summary of patient population, tumor sample information, and phenotype characteristics

				Tumor					Liver metastasis					Normal tumor adjacent tissue				
Sample	Age/sex	Tumor stage	Grade	Vitality	Spheres	CSCs	CD133	CD44	Vitality	Spheres	CSCs	CD133	CD44	Vitality	Sphere	SCs	CD133	CD44
1	66/M	T2N0M0	2	+	+	−	−	+						+	+	−	−	−
2	70/M	T2N0M0	2	+	−	−	−	+						+	+	−	−	−
3	64/M	T3N0M0	2	+	−	−	−	+						−				
4	81/F	T3N2M0	3	+	−	−	−	+						+	+	+	−	+
5	68/M	T3N0M0	3	+	−	−	−	−						+	−	−	−	−
6	68/M	T4N2M0	3	+	−	−	−	−						+	−	−	−	−
7	62/M	T2N0M0	2	+	+	−	−	−						+	+	−	−	+
8	42/M	T3N1M0	3	+	+	−	+	+						+	−	−	−	+
9	82/M	T4N2M0	3	+	+	+	+	−						−				
10	70/M	T3N1M0	3	+	+	+	+	−						+	−	−	−	+
11	63/F	T4N1M1	3	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+
12	52/M	T3N2M1	2	+	+	+	+	+	+	+	+	+	+	−				
13	87/F	T4N2M1	2	+	+	+	+	+	+	+	+	+	+	−				
14	73/M	T3N2M1	3	−	+	+	+	+	+	−								
15	67/M	T4N2M1	2	+	+	−	+	+	+	+	+	+	+	+	+	−	−	+
16	72/M	T3N2M1	3	+	+	−	+	+	+	+	−	+	+	−				
17	61/F	T3N2M1	2	+	+	−	+	+	+	−	−	+	+	−				
18	63/F	T4N0M1	3	+	−	−	+	+	+	+	−	+	+	+	−	−	−	−
19	66/F	T4N2M1	3						+	+	−	−	−					
20	64/F	T3N1M1	2						+	−	−	+	+					
21	74/M	T3N1M1	3						+	+	+	+	+					

F, female; M, male.

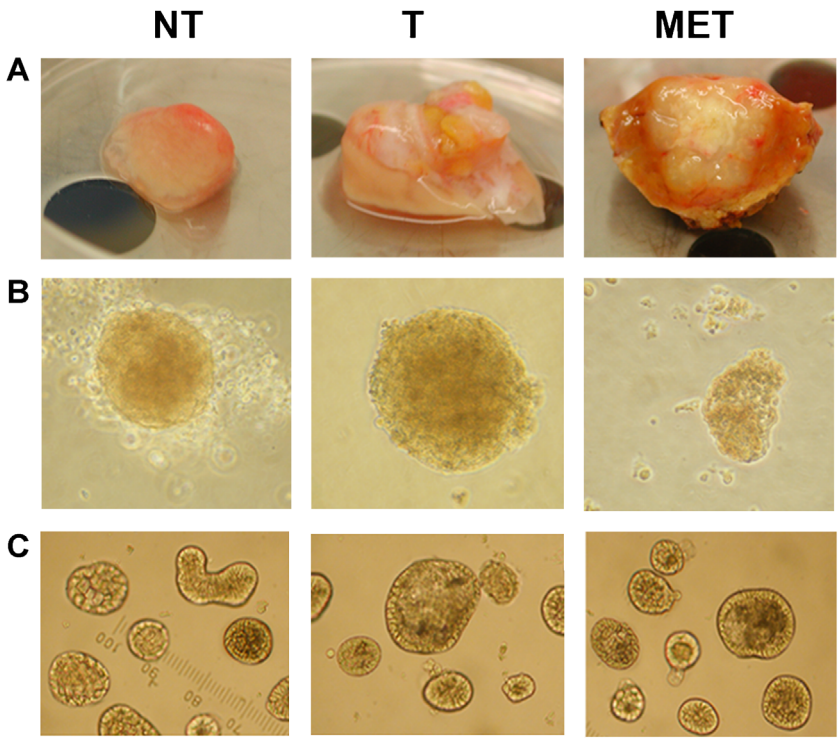

Fig. 1. A: Fresh tissues of primary colon cancer (T), matched adjacent normal tissues (NT), and liver metastasis (MET). B: Phase contrast photo of first and (C) third generation colon spheres growth under serum-free conditions (40×).

tumor tissues were able to form spheroids of second and third generation (Fig. 1C).

The presence of $CD44^+$ cells and $CD133^+$ cells was evaluated at two isolation steps (Table 2): on the cell suspensions obtained 1 week after sample processing, representative of naïve samples (Fig. 2A), and in clones obtained after 5 months of cell selection (Fig. 3). CD44 was detectable in 13 out of 17 samples from primary tumors, in 6 out of 11 from normal adjacent mucosa, and in 10 out of 11 from metastasis. All non-tumor specimens were $CD133^-$, whereas 11 out of 17 tumor and 10 out of 11 metastasis samples were $CD133^+$. $CD133^+$ cells were significantly higher in the tumor compartment than in the metastatic compartment, and higher in liver metastasis than in non-tumor tissue (21.6 ± 1.8 vs. 5.4 ± 1.09 vs. 0.56 ± 0.3). The $CD44^+$ cells were significantly higher in the liver metastasis than tumor and non-tumor compartment (12.6 ± 1.19 vs. 10.7 ± 1.37 vs. 9.18 ± 2.2). Then, we confirmed CD44 and CD133 expression levels in the clones obtained after 5 months of cells selection (Fig. 3) and we found that 100% of the CSCs isolated from the non-tumor

TABLE 2. Summary of patients phenotype and functional characteristics

	NT	T	Liver metastasis
Number	18	18	11
Naive sample			
Vitality	11/18 (61%)	17/18 (94.4%)	11/11 (100%)
Expression markers/patients			
CD133	0/11 (0%)	11/17 (64.7%)	10/11 (90.9%)
CD44	6/11 (54.5%)	13/17(76.5%)	10/11 (90.9%)
Markers expression mean			
%CD133 positive cells	0.56 ± 0.3	21.6 ± 1.8	5.4 ± 1.09
%CD44 positive cells	9.18 ± 2.2	10.7 ± 1.37	12.6 ± 1.19
Sphere production (20 days)	6/11 (54.5%)	11/17 (64.7%)	9/11 (81.8%)
SCs isolated after 5 months cultured	2/11(18.2%)	5/17 (29.4%)	6/11 (54.5%)
SCs phenotype			
$CD133^+/CD44^+$	0	3/5 (60%)	6/6 (100%)
$CD133^+/CD44^-$	0	2/5 (40%)	0
$CD133^-/CD44^+$	2/2 (100%)	0	0
$CD133^-/CD44^-$	0	0	0
P-e sample			
$CD133^+/CD44^+$ cells/patients	0/18 (0%)	9/18 (50%)	10/11 (90.9)
% $CD133^+/CD44^+$ cells	1.3 ± 0.2	13.8 ± 1.1	8.5 ± 0.7

P-e, paraffin-embedded; SCs, stem cells.

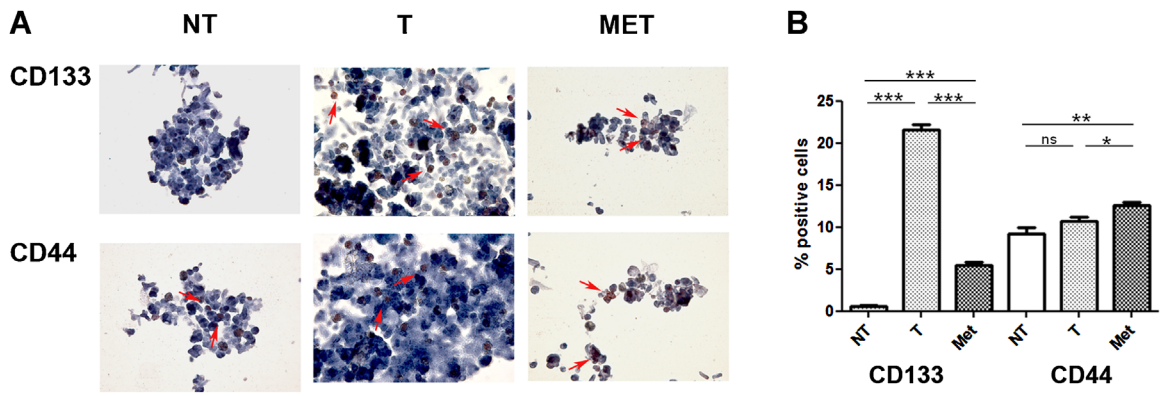

Fig. 2. CD44 and CD133 expression levels in tumor cells clusters (20×). CD44 and CD133 expression were evaluated. A,B: One week after samples processing in tumor cells clusters.

compartments were $CD133^{-}/CD44^{+}$; CSCs isolated from the tumor compartment were $CD133^{+}/CD44^{+}$ in 60% of cases and $CD133^{+}/CD44^{-}$ in 40%; CSCs from metastasis were all $CD44^{+}/CD133^{+}$.

We also performed immunofluorescence analysis to determine the proportion of $CD133^{+}/CD44^{+}$ putative tumorigenic cells within each tumor, scanning for the presence of the FITC and TRITC staining in the same cell (Fig. 4). Immunohistochemical staining for CD133 (red, first column) and CD44 (green, second column) showed membrane localization of the two proteins in epithelial tumor cells localized at the base of the crypt (Fig. 4B–D), or in tumor cells scattered throughout the tumor sections (Fig. 4A). Small number of $CD133^{+}/CD44^{+}$ cells were observed only in primary tumors of patients who develop liver metastasis (Fig. 4D). Metastasis consists of cancer cells showing membrane CD133/CD44 accumulation (Fig. 4E).

We evaluated the difference in the presence of $CD44^{+}/CD133^{+}$ cells and found that are significantly higher in the tumor compartment than in the metastatic once and higher in liver metastasis than in non-tumor tissue (13.8 ± 1.1 vs. 8.5 ± 0.7 vs. 1.3 ± 0.2).

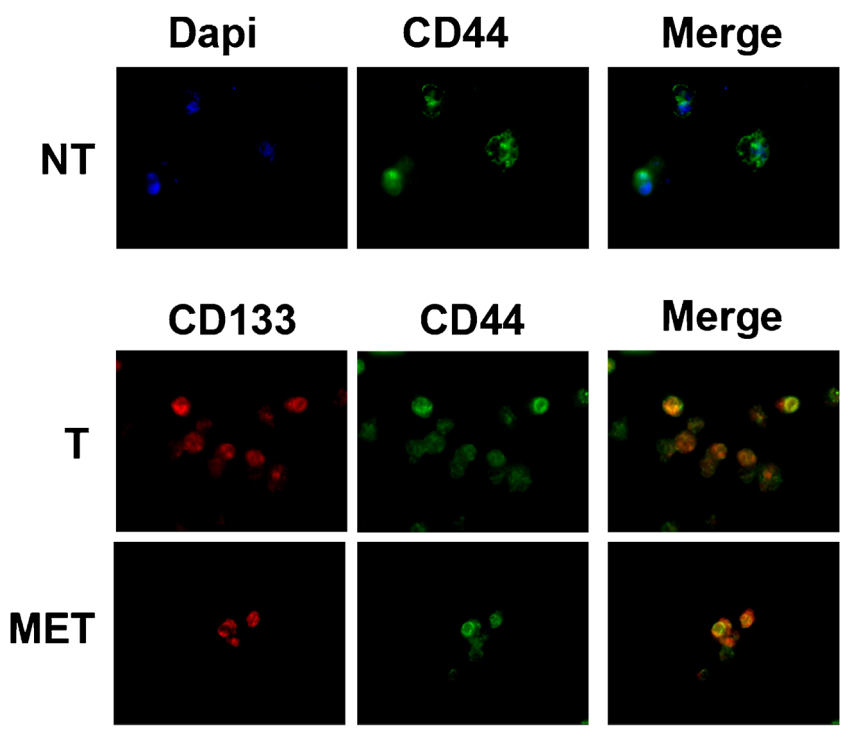

Fig. 3. Representative CD44 and CD133 staining in colon spheres growth for 5 months under serum-free conditions.

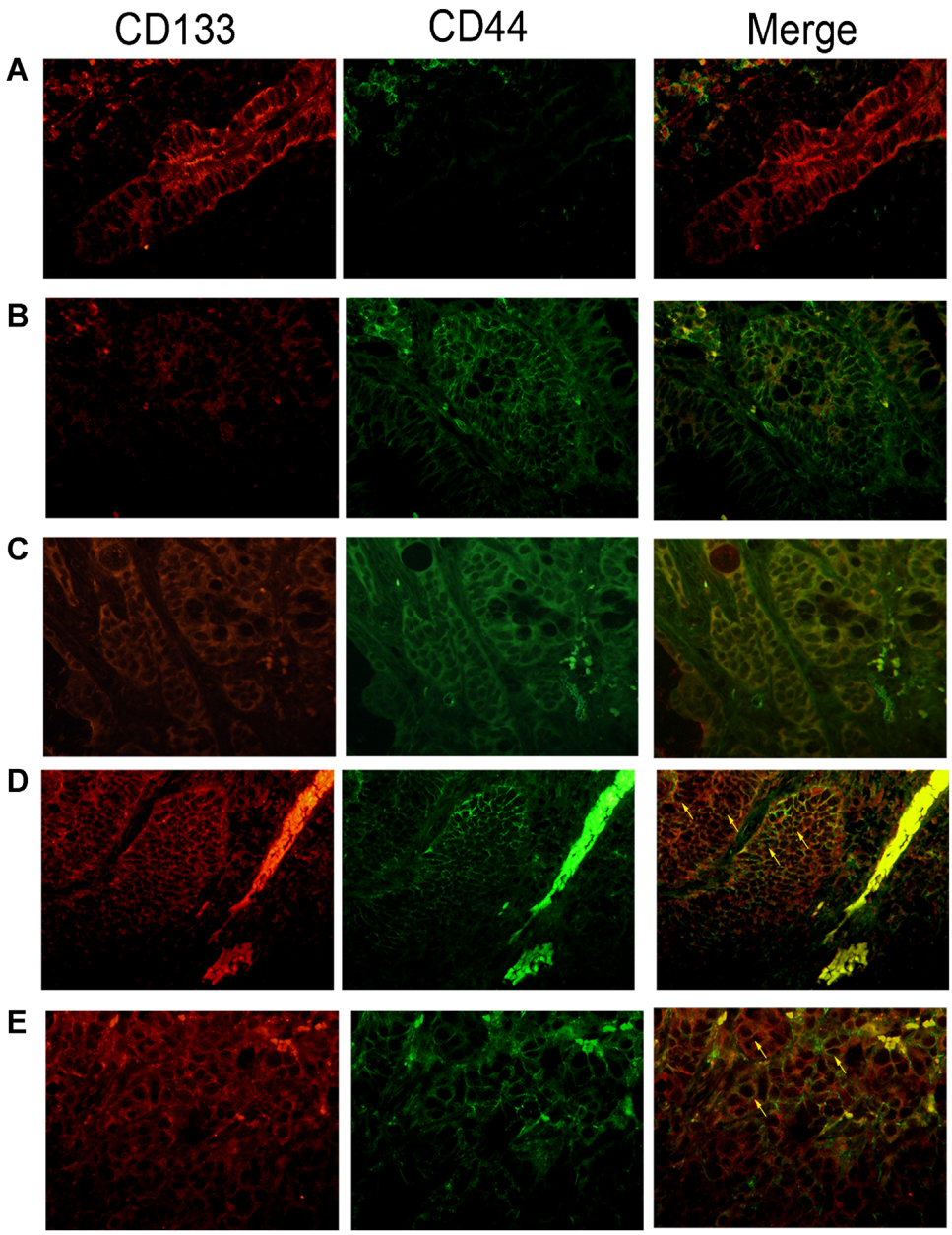

Fig. 4. Representative immunohistochemical staining for CD133 (red, first column) and CD44 (green, second column) in adjacent serial sections of human colon cancer formalin-fixed, paraffin-embedded specimens of primary (A–D) and metastatic (E) colon cancers. The primary tumors were $CD133^{+}/CD44^{-}$ (A), $CD133^{-}/CD44^{+}$ (B), $CD133^{-}/CD44^{-}$ (C), $CD133^{+}/CD44^{+}$ (D), respectively. Small number of $CD133^{+}/CD44^{+}$ cells were scattered throughout the tumor sections (arrow in D,E). C: $CD133^{-}/CD44^{-}$ tumor in which only a specific signals are visualized.

When investigating the difference between the tumor phenotype in the non-metastatic group (10 patients) and the metastatic group (eight patients), the colocalization analysis showed more $CD133^{+}/CD44^{+}$ cells in the metastatic group (100%) compared to the non-metastatic group (10%). $CD133^{-}/CD44^{+}$ cells were detected in 40% of the tumors, while a complete lack of both proteins was evident in 30% of tumors. The percentage of $CD133^{+}/CD44^{+}$ cells in the primary tumor and the corresponding liver metastasis in the metastasis group is presented in Table 3. We did not find a significant difference in the number of $CD133^{+}/CD44^{+}$ cells between the primary tumors and the corresponding liver metastasis (60.8 ± 27.99 vs. 50.3 ± 16.38; Mann Whitney P value >0.05), suggesting that they have similar cells of origin.

Peripheral blood from the eight metastatic colorectal cancer patients was collected and processed as described in methods and CTCs were isolated. We found in 6 out of 8 detectable population of CTCs ranging from 20 to 250 per 15 ml of blood (Fig. 5A). CTC retain three different types of cells or cell clumps, as follows: cytokeratin-positive single-standing tumor cells (Fig. 5B); cytokeratin-positive cell clumps containing at least two cells, consisting only of cytokeratin-positive cells (Fig. 5C); mixed cell clusters containing more than three cells, with at least one of them cytokeratin-positive (Fig. 5D). In this

TABLE 3. Percentage of $CD133^+/CD44^+$ cells in the primary tumor and the corresponding liver metastasis in patients with metastases

Sample	Tumor	Liver metastasis
11	8.3	36.1
12	64.5	35
16	100	70
14	84	50
15	23.5	43.3
16	60.8	58.3
17	72.2	73.3
18	28.1	47.2
19	73.5	69.2
20	45.6	85.4
21	40	50.3
Median	60.8	50.30
SD DEV	27.99	16.38

study, no cytokeratin-positive cells were found in the blood of healthy donors. We found 20% of cancer cells showing membrane CD133/CD44 accumulation; positive cells were not usually simple, rather they were doublets or clusters. The $CD113^+/CD44^+$ cells were attached to $CD133^-/CD44^-$ cells; they appeared either in doublets or in mixed clusters.

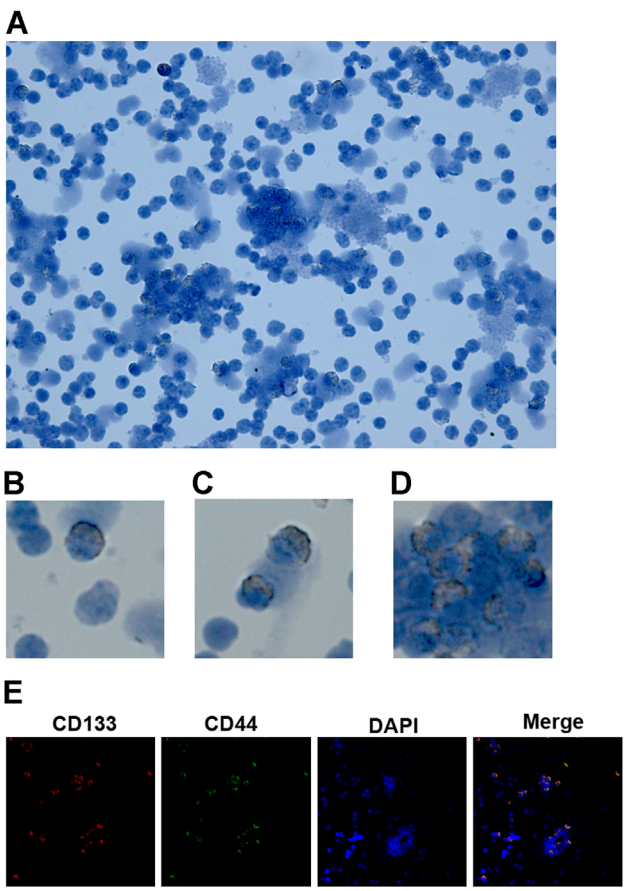

Fig. 5. A) Representative CTCs found in peripheral blood from metastatic colorectal cancer patients. Cells retain three different types of cells or cell clumps: B) cytokeratin-positive single-standing tumor cells; C) cytokeratin-positive cell clumps containing at least two cells, consisting only of cytokeratin-positive cells; D) mixed cell clusters containing more than three cells, with at least one of them cytokeratin-positive. E) Representative CTCs processed for immunofluorescence microscopy as described in the Materials and Methods. Cells were immunostained with an anti-CD44 mouse monoclonal antibody (green) and an anti-CD133 rabbit polyclonal antibody (red).

Discussion

Cancer stem cell theory has profound implications in terms of cancer therapy. Indeed, current treatments are hardly able to completely eradicate cancer cells and are often complicated by the occurrence of tumor recurrence and/or metastasis. One hypothesis is that current chemotherapy attacks the bulk of cancer without affecting stem cells which can re-grow after treatment, and, eventually, develop the changes responsible for the occurrence of drug-resistance (Guzman et al., 2002; Guan et al., 2003; Dean et al., 2005).

Even though the precise contribution of $CD133^+/CD44^+$ TICs in mediating colon cancer metastasis in human is contradictory (Shmelkov et al., 2008), recently, it was demonstrated that $CD133^+/CD44^+$ cells are undifferentiated, endowed with extensive self-renewal and epithelial lineage differentiation capacity in vitro, more invasive in vitro and responsible solely for liver metastasis in vivo (Chen et al., 2011). In addition, recent evidence suggests that CD133 and CD44 proteins co-expression in colon cancer may be indicative for early liver metastases (Huang et al., 2011).

To try to shed light on this problem, cells isolated from fresh tissue specimens were cultured under non-adherent conditions to form colon spheres. We found that long-term, non-adherent culture conditions generate spheres of slowly proliferating cells; single cells, dissociated from spheres, express both CD44 and CD133. Our data emphasize that the marker CD133 is higher expressed in primary tumor cells than non-tumor cells or metastatic cells. We demonstrated the presence of cells also positive for CD44, in primary colon cancer tissue. 76.5% of tumor samples and 90.9% of liver metastases expressed CD44, while only 54.5% of non-tumor samples did. Indeed 64.7% of tumors and 90.9% of metastasis showed CD133 expression detectable whereas samples from normal tissues were all $CD133^-$, in agreement with literature data in which it has been stressed that CD133 expression was extremely infrequent in normal colon tissues compared to tumor tissues (Fang et al., 2010), and suggesting that the increased number of CD133 cells in cancer samples maybe a result of their oncogenic transformation. However, we found that the percentage of CD133 positive cells was significantly higher in the tumor compartment than in metastatic compartment (21.6 ± 1.8 vs. 5.4 ± 1.09) suggesting that CD133 expression only could be responsible for tumor growth. While the higher rate of $CD44^+$ cells in liver metastasis suggests its involvement in tumor metastasis.

These observations are enforced by those obtained dividing the non-metastatic patients from the metastatic ones and observing their tumor phenotype (Table 4): in the metastatic group all sample $CD133^+/CD44^+$, while in the non-metastatic group there was a prevalence of the $CD133^+/CD44^-$ phenotype.

CTC are believed to be directly involved in the biology of the metastatic process and recent observations support the hypothesis that expression of CD133 in the peripheral blood of

TABLE 4. Summary of primary tumor phenotype in the non-metastatic and metastatic group

	M0	M1
$CD133^+/CD44^+$	10% (1/10)	100% (8/8)
$CD133^-/CD44^-$	30% (3/10)	0
$CD133^+/CD44^-$	20% (2/10)	0
$CD133^-/CD44^+$	40% (4/10)	0

M0, non-metastatic group; M1, metastatic group.

patients affected with CRC might identify high-risk patients by detecting putative circulating CSCs that might be responsible for disease progression after apparently radical surgery (Pilati et al., 2012). We found detectable population of CTCs in 75% of metastatic colon cancer patients: 20% of CTCs showed membrane $CD133^{-}/CD44^{+}$ accumulation; positive cells were not usually simple, rather they were doublets or clusters. The $CD113^{+}/CD44^{+}$ cells were attached to $CD133^{-}/CD44^{-}$ cells; they appeared either in doublets or in mixed clusters. Our analysis supports the premises that CTCs represent a sampling of the phenotypic cell types present in the primary and metastatic tumor deposits, and the possibility that CTCs consist of a combination of cells with malignant potential, stem cell characteristics and actively migrating cells that may go on to form liver metastatic foci.

Collectively, these results suggest that $CD133^{+}$ colon cancer cells might play an important role in both primary tumors as well as in metastatic lesions but metastasis of the liver seems to be strictly related to the phenotype $CD133^{+}$ and $CD44^{+}$. Our data underline the importance of $CD133^{+}/CD44^{+}$ CSCs in liver metastasis thus warranting further studies on the role(s) of this subset of cells in the liver metastatic process.

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