Isolation and characterization of CD133+ cell population within human primary and metastatic colon cancer

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Abstract. – *Background.* "Cancer stem cells" (CSC) have been identified as a minority of cancer cells responsible for tumor initiation, maintenance and spreading. Although a universal marker for CSC has not yet been identified, CD133 has been proposed as the hallmark of CSC in colon cancer. The aim of our study was to assess the presence of a CD133+ cell fraction in samples of colon cancer and liver metastasis from colon cancer and evaluate their potential as tumor-initiating cells.

Methods. Tissue samples from 17 colon cancers and 8 liver metastasis were fragmented and digested using collagenase. Cell suspensions were characterized by flow cytometry using anti-CD133, CD45 and CD31 antibodies. CD133+ cells were also isolated by magnetic cell sorting and their tumor-initiating potential was assessed versus the remaining CD133- fraction by soft-agar assay.

Results. Our results confirmed the existence of a subset of CD133+ tumor cells within human colon cancers. Interestingly, CD133+ cells were detectable in liver metastasis at a higher percentage when compared to primary tumors. Soft-agar assay showed that CD133+ cell fraction was able to induce larger and more numerous colonies than CD133-cells.

Conclusion. Our findings data that the CD133+ colon cancer cells might play an important role in both primary tumors as well as in metastatic lesions thus warranting further studies on the role(s) of this subset of cells in the metastatic process.

Key Words:

Colon cancer, Cancer stem cells, CD133 cell population, Liver metastatic colon cancer.

Introduction

Colon cancer is one of the most common type of cancers and a leading cause of morbidity and mortality in developed countries[1]. It originates from epithelial cells lining the gastrointestinal tract, which undergo sequential mutations in specific DNA sequences that disrupt normal mechanisms of proliferation and self-renewal. Inborn genetic aberrations, tobacco smoking, environmental carcinogens and chronic inflammatory states, as well as several others unknown factors, drive the transition from healthy colonic epithelia to increasingly dysplastic adenoma and finally to colorectal cancer[2]. Despite recent advances on the molecular mechanisms responsible for colon tumorigenesis, current anticancer treatments are often unable to eradicate the disease. A better understanding of the factors involved in the initiation, progression and metastasis of colon cancer is mandatory in order to develop novel and more efficient diagnostic and therapeutic strategies.

Recent studies have demonstrated that in several type of human tumors, only a minority of cancer cells appear to be "tumor-initiating" and possess a metastatic phenotype. Therefore, the "stochastic" theory for the cellular origin of cancer, based upon the assumption that all cancer cells are equally malignant and able to give rise to tumors, has been abandoned in favor of

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the "hierarchical" theory. The latter assumes that tumors are hierarchically organized and that only a rare subpopulation of undifferentiated cells at the apex of this hierarchy have the unique biological properties necessary for tumor initiation, maintenance and spreading. Given the similarities between tumor-initiating cells and normal stem cells (SC), the tumor-initiating cells have been termed "cancer stem cells" (CSC)[3-5]. Biologically distinct populations of CSC have been identified in cancers within the hematological malignancies[6] and in most solid tumors, including colon cancer[7,8]. The origin of CSC is still unclear but the discovery of stem cells in the majority of normal tissues, including colon crypts, support the hypothesis that normal SC might represent a possible target for tumorigenic mutations, due to both their long life and their capacity of self-renewal, and therefore might originate CSC[9].

Although, a universal marker for cancer stem cells has not been identified, several studies have suggested that the CSC fraction within a variety of human cancers, including colon and liver cancers, may be identified by the expression of the CD133 surface marker[7,8,10,11]. Human CD133 (also known as prominin1in rodens) is a 5-transmembrane glycoprotein of 865 amino acids with a total molecular weight of 120 kDa. The specific CD133 function remains still unclear, but its expression has been shown to characterize normal as well as CSC in human tissues[12]. Thus, CD133 has been used to isolate and characterize CSC from several tumors, including colon cancers. Indeed, it has been recently reported that expression of the cell surface marker CD133 identifies a subpopulation of cells within human colon cancers. These cells shows specific features of CSC, being able to initiate tumor growth and to reproduce human colon cancers in immunodeficient mice[7,8].

The present study was aimed to verify the presence of CD133+ cells in a subset of primary and metastatic human colon cancers and to acquire more information on the biology of these cells.

Materials and Methods

Tissue Collection

Tissue specimens including cancer and normal surrounding tissues were obtained from patients undergoing surgery for primary (n = 17) or metastatic to the liver (n = 8) colon cancers at the Department of Surgical Sciences, Catholic University of Rome upon patients' written consent and approval by the Catholic University Ethical Committee. Patients age ranged from 24 to 81 years (mean = 61.4). Tissues were collected and processed for CSC isolation and histopathological analysis.

Tissue Disaggregation

Tissue specimens were collected in serum free-DMEM medium containing 25 units/ml of penicillin and 25 μg/ml of streptomycin (Sigma, St Louis, MO) and were kept at 4°C during the transfer to the laboratory, where samples were mechanically fragmented and digested by 0.2% collagenase I (Sigma) at 37°C on a shaker for 90 min. Cell suspensions were filtered through cell streiners (100 μM). Contaminating red bloods cells were removed by incubation with ammonium chloride solution for 5 min at 4°C.

Flow Cytometry

To minimize loss of cell viability, all experiments were performed on fresh cell suspension prepared shortly before flow cytometry. Recovered cells were characterized using the following antibodies: Phycoerytrin(PE)-conjugated anti-CD133/1, PE-conjugated anti-CD133/2 (Miltenyi Biotech, Bergish Gladback, Germany), Fluorescein isothiocynate (FITC)-conjugated anti-CD45 and FITC-conjugated anti-CD31 (Beckton Dikinson, Mountain View, CA, USA). Conjugated isotype-matched monoclonal antibodies were used to establish monoclonal background fluorescence. After 30 min incubation, cells were washed and analyzed by flow cytometry using BD FACSCalibur system (Beckton Dikinson, Mountain View, CA, USA).

Magnetic cells Isolation

CD133+ and CD133- cells was isolated using CD133 Cell Isolation Kit (Miltenyi Biotech, Bergish Gladback, Germany). Briefly, cells will be labeled with micro beads CD133/1-conjugated, then sorted by the manufacturer's isolation kit. The purity of the isolated cells was evaluated by flow cytometry using PE-conjugated anti-CD133/2.

Soft Agar Colony Formation Assay

Purified CD133+ and CD133- cells were suspended in soft agar in 6-well culture plates at a density of 5000 cells/well and cultured for 2 weeks. At the end, colonies were counted under the microscope.

Reverse Transcription-Polymerase Chain Reaction

Total RNA was isolated from tissues homogenized (3 colon cancers, 3 metastasis, 3 normal colons and 3 normal livers) using RNeasy Miny Kit (Quiagen, Hilden, Germany). The RNA concentration of each sample was quantified by spectrophotometry (Beckman Coulter DU800, Fullerton, CA, USA) and its purity was assessed by the absorbance ratio 260/280 nm. RNA integrity was examined by agarose gel electrophoresis. One microgram of total RNA was reverse transcribed using the SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's guidelines. cDNA products were amplified by 30 cycles of PCR using 5 pmole of specific primers for CD133/1 and GAPDH. Gene specific primers were designed using OLIGO software. The sequence of both the forward and reverse primers and the expected sized of the PCR-amplified DNA are listed in Table I. The polymerase chain reaction products were analyzed on 2% agarose gel with ethidium bromide.

Immunohistochemistry

Immunohistochemistry was performed on frozen tissues. After peroxidase inhibition with 3% H_2O_2 for 20 min, the section were incubated with 20% normal goat serum for 30 min. Then, the slides were incubated at room temperature with the monoclonal mouse anti-human CD133-1 antibody (Miltenyi Biotech, Bergish Gladback, Germany). The reaction was visualized using DAB Substrate Chromogen (Dako, Milan, Italy).

Statistical Methods

Statistical analysis was performed by applying the independent t test using the Statistical Package for the Social Sciences (SPSS) version 13.0 (Chicago, Inc., USA). Significance was determined when probability (P) values were <0.05 and all tests were two-sided.

Table I. Primers used for RT-PCR.

Name	Sequence
GAPDH-for	5'-GGCTCTCCAGAACATCATCC-3'
GAPDH-rev	5'-TGTCATCATATTTGGCAGGT-3'
CD133-for	5'-TTGGCTCAGACTGGTAAATCCC-3'
CD133-rev	5'-ATAGGAAGGACTCGTTGCTGGT-3'

Results

CD133+ Cells are Present Within Colon Tumor and Metastasis Vith Variable Frequency

First, we investigated whether CD133+ cancer cells could be found within colon cancers and hepatic metastasis samples. To quantify the percentage of CD133+ cells in tumor samples with respect to normal tissues harvested from the same patient, we analyzed by flow cytometry the cell suspensions derived from dissociation of both neoplastic and normal surrounding tissues. The percentage of CD133+ cells was consistently higher in tumor samples when compared to healthy tissues in the majority of samples analyzed. The percentages of CD133+ cells ranged from 0% to 3.3% (median value: 0.32%) in normal colons and were significantly higher in the corresponding colon cancers (range 0.1% to 20,.44; median = 2%) ($p<0.01$). Similarly, CD133 expression was significantly higher in liver metastasis (median value: 14.7%, range from 6% to 26.5%) compared to the surrounding healthy liver tissue (median value: 0.12%, range from 0% to 2.1%) ($p<0.02$). It is noteworthy that CD133+ fraction detected in metastasis was significantly higher when compared to the value obtained in primary colon cancers ($p<0.01$) (Table II). Then, we analyzed the CD133+ populations for the presence of hematopoietic and endothelial contamination. In colon tumors and metastasis only 0.2% of CD133+ cells were positive for the pan-hematopoietic marker CD45 and less then 0.5% were positive for endothelial progenitors marker CD31+. Instead, the large majority (98%) of CD133+ cells within normal samples were positive for CD45 or CD31 (Figure 1).

Soft Agar Colony Formation Assay

To determine whether CD133+ tumor cells were more tumorigenic than their CD133-counterparts, we compared their anchorage-independent growth using the soft agar colony formation assay. Indeed, anchorage-independent growth is a hallmark of tumor cells. First, we purified CD133+ and CD133- cells derived from dissociation of colon tumors and metastasis specimens by magnetic cell sorting. The purity of CD133+ and CD133- cells populations was generally higher than 90%, as revealed by post-sorting flow cytometry analysis. Purified CD133+ and CD133- cells were suspended in

Table II. Case description of primitive colon cancer (A) and liver metastasis (B) samples.

| A | | | | |
Case	Age/Sex	Site	Dukes/Astler-Coller	CD133 expression (%)
1	58/F	Right	D	10.50
2	72/F	Right	C3	0.15
3	73/M	Right	C2	12.50
4	60/F	Left	B2	19.50
5	72/M	Left	C2	0.20
6	57/F	Sigma	C2	0.97
7	45/M	Sigma	C3	0.27
8	77/F	Right	B2	6
9	55/F	Sigma	C3	20.44
10	54/M	Right	D	0.19
11	65/F	Right	C2	2
12	61/M	Sigma	D	2.52
13	55/F	Right	D	6.40
14	72/M	Left	B2	0.82
15	67/M	Sigma	C3	0.20
16	71/M	Left	B2	0.10
17	69/M	Left	B2	19.43

| B | | | | |
Case	Age/Sex	Site		CD133 expression (%)
1	69/F	Liver		14.70
2	37/F	Liver		16.40
3	66/M	Liver		7.20
4	61/M	Liver		6.00
5	24/F	Liver		10.00
6	65/M	Liver		8.15
7	72/F	Liver		26.50
8	58/F	Liver		15.12

soft agar and colonies were counted after two weeks. As shown in Figure 2, CD133+ cells displayed a significantly higher ability to form colonies in soft agar which were also larger in size than the ones obtained from CD133- cells. No difference in colony formation ability was observed between CD133+ cells isolated from primary colon cancers and metastasis (Figure 2B).

RT-PCR Studies

We performed semi-quantitative RT-PCR analysis of CD133 expression in 6 tumor samples (3 colon cancers and 3 metastasis) and 6 normal tissues (3 normal colons and 3 normal livers). The band, corresponding to the CD133, was clearly detectable in colon cancer and metastasis, while the band was hardly detectable in normal colon and normal liver (Figure 3A). According to FACS results, the expression of CD133 was higher in metastasis compared to primitive colon cancers (Figure 3B).

Immunohistochemistry

We performed immunohistochemical analysis on tissue sections from 10 cases (3 colon cancers, 3 metastasis, 2 normal colons and 2 normal livers), to determine the anatomical location of CD133+ cells. All samples analyzed showed similar results: a small number of CD133+ cells were detectable in all tumor specimens and were scattered throughout the tumor sections, whereas CD133+ expressing cells were barely detectable in normal tissues (Figure 4).

Discussion

Tumorigenesis involve a variety of molecular events which are not yet completely understood. Thus, cellular origin of human cancers is still controversial and the mechanisms responsible for the complexity and heterogeneity of cancers remain to be defined[13,14]. Several studies have suggested that human cancer can be considered
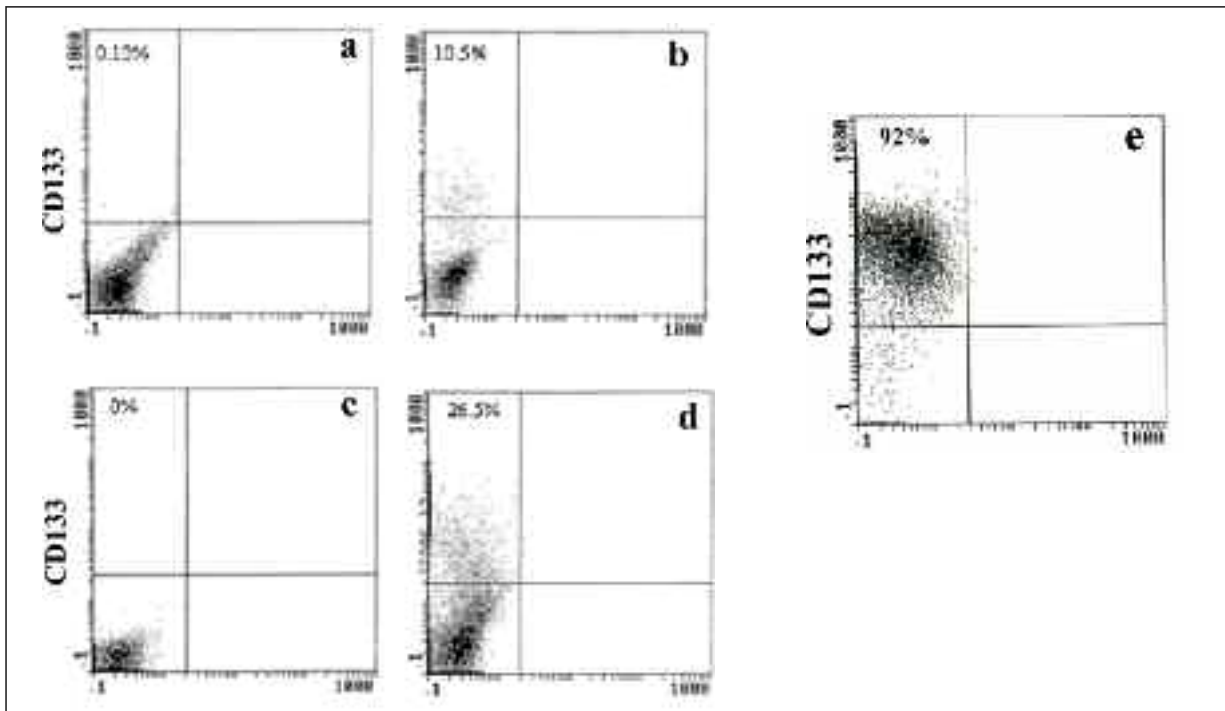
Figure 1. Flow cytometric analysis of CD133 expression in freshly isolated cells from representative cases of primary colon cancer tissue *(B)* and normal surrounding tissue *(A)* and from a liver metastasis *(D)* and normal surrounding liver tissue *(C)*. CD133 expression in colon cancer cells after magnetic cell purification is shown in *(E)*.

as a stem cell disease. Thus, according to the "cancer stem cell" model, tumors are viewed as complex tissues in which abnormal growth is driven by a minority of cancer stem cells which exhibit characteristics similar to normal stem cells, including expression of the protein telomerase and self-renewal capacity. Cancer stem cells have the ability to function as cancer-initiating cells when transplanted into immunodeficient mice giving rise to an heterogeneous populations of cancer cells reminiscent of the same morphological and antigenic features of the original tumor[4,7,8,15]. Cancer stem cells are characterized by a distinctive profile of surface markers

Figure 2. *A,* Representative examples of soft agar colony-formation assay. Purified CD133+ and CD133- cells, derived from dissociation of primary colon tumors and metastasis specimens by magnetic cell sorting, were plated at a density of 5000 cells/well on soft agar and colonies were counted after two weeks. *B,* Data represent the mean ± standard deviation of 2 separate experiments with triplicate wells per condition.
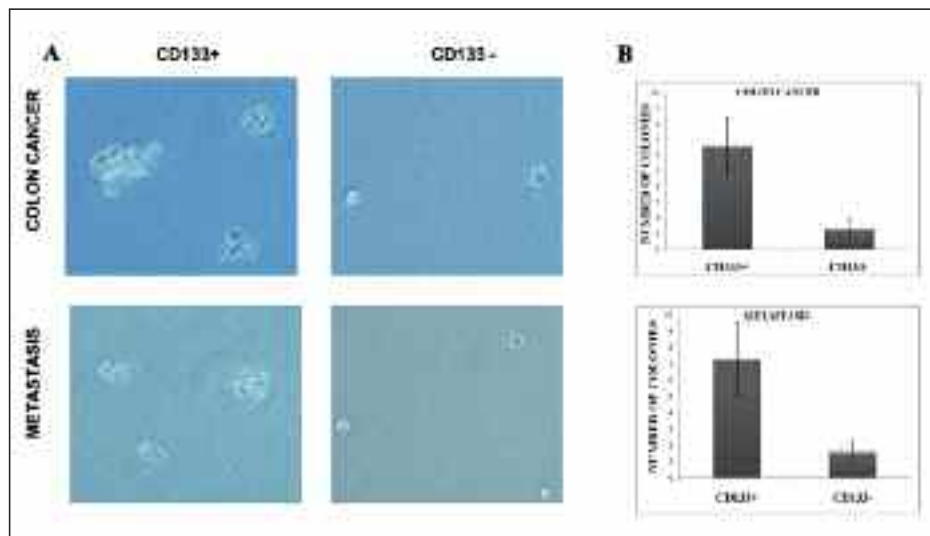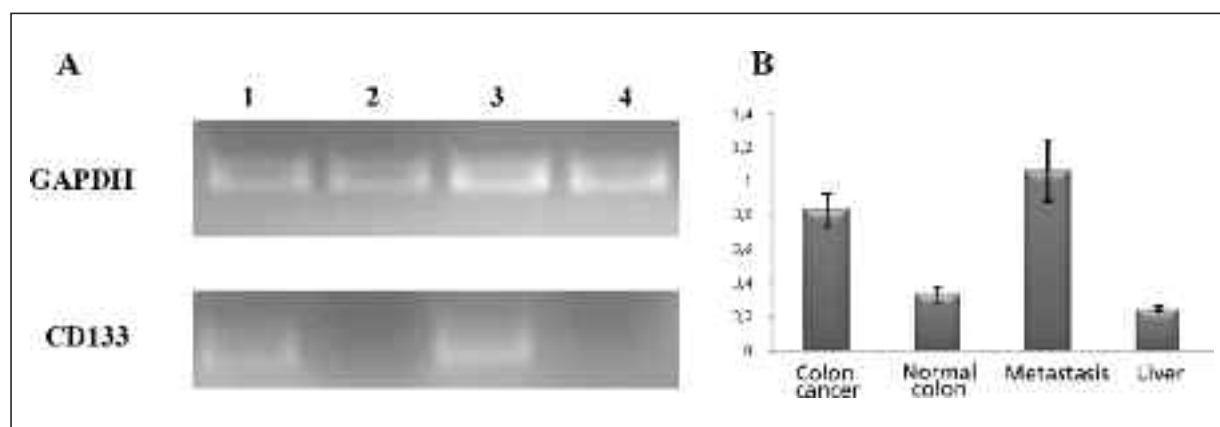
Figure 3. *A,*, A representative example of semiquantitative RT-PCR analysis of CD133 expression in colon cancer (lane 1), normal colon tissue (lane 2), liver metastasis (lane 3) and normal liver (lane 4). Expression of GAPDH as control of RT-PCR efficiency is shown in the lower panel. *B,* Densitometric values corresponding to the intensity of the CD133 band in the indicated samples. Values represent the mean of three experiments and are expressed as ratio CD133/GAPDH.
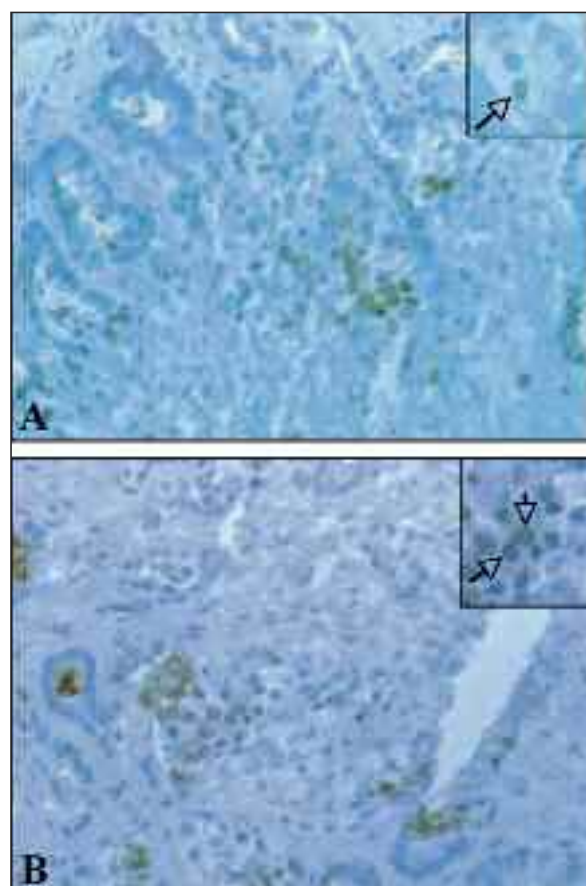

Figure 4. Representative examples of immunohistochemical analysis of CD133 expression in a case of primary *(A)* and metastatic *(B)* colon cancers. Details of CD133 positive cells are shown in the insets. Original magnification × 200.

and they have been initially documented in leukemia and mieloma and subsequently identified in most of solid tumors[16-18].

Cancer stem cells 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 which usually characterize and complicate the course of the disease[19-21].

CD133 (also known as prominin-1 in rodents or AC133 in humans), a 120 kDa transmembrane and cell surface protein, has been shown to characterize normal and cancer stem cells in several human tissues, including the colonic mucosa. Thus, CD133 has been used to identify and isolate cancer initiating cells from human colon cancers 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[7,8]. However, the role played by these cells in the process of colon cancer metastasis, which is the most relevant clinical issue in the treatment of colon cancer patients, remains still unknown

Our results confirm the existence of a population of self renewing subset of CD133+ cells within human primary colon cancers. Our findings confirm that these cells, although present in a percentage significantly higher in tumors compared to normal tissue, represent only a small subset of cells (<20%) able to form colonies in soft agar *in vitro*, which correlates with the ability to form tumors *in vivo*. Using FACS analysis, we also demonstrated that these cells were negative for pan-hematopoietic (CD45) and endothelial (CD31) antigens thus confirming their epithelial origin. We also confirmed an increased expression of the CD133 mRNA in tumor compared to normal colon tissue and demonstrated by immunohistochemistry that cancer stem cells are scattered within the entire tumor mass.

As previously mentioned, an unresolved question is the role played by CD133+ cells in the metastatic process. To try to shed light on this problem, we also investigated the presence of CD133+ cells within human metastatic colon cancers. We found that CD133+ are detectable in liver metastasis from colon cancers at a higher percentage than that of primary tumors. We also demonstrated that CD133+ are barely detectable in normal liver tissue thus confirming that cells detected within metastatic tissue cannot be due to contamination of normal hepatic parenchimal cells. As for primary cancers, we demonstrated by FACS analysis that these cells were negative for CD45 and CD31 surface markers and confirmed an increased expression of the CD133 mRNA, which was even stronger than in primary colon cancer tissue.

Our results suggest that CD133+ colon cancer stem cells might play an important role in both primary tumors as well as in metastatic lesions. Further studies are required to investigate the role(s) of this subset of cells in the metastatic process and to develop novel therapies specifically targeting CD133+ cells, for the prevention and treatment of colon tumors and metastasis, with a great beneficial impact on the survival of colon cancer patients.

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