Histol Histopathol (2016) 31: 299-306

http://www.hh.um.es

Histology and Histopathology

From Cell Biology to Tissue Engineering

Clinical significance of stem cell marker CD133 expression in colorectal cancer

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Summary. Objective: CD133, a glycoprotein, is expressed in different types of human stem cells and tumor cells. Detection of altered CD133 expression in colorectal cancer tissues could be useful as a marker for the prediction of colorectal tumorigenesis, progression, and prognosis. Methods: A total of 19 fresh and 145 paraffin-embedded tissue specimens from colorectal cancer patients were obtained for detection of CD133 expression using flow cytometry and immunohistochemistry, respectively. The tumorigenic capacity of tumor cells from 19 patients was assessed in nude mice. Association of CD133 expression was then analyzed for clinical significance. Results: The percentage of $CD133^-$ positive ($CD133^+$) tumor cell population ranged between 0.84% and16.75% (mean ratio=7.15%) of tumor cells in the 19 freshly isolated tissue samples. CD133 expression in tumor cells was associated with tumor lymph node metastasis (9.81% vs. 3.22%; p=0.013) and poor tumor differentiation (8.32% vs. 5.07%; p=0.043). In the 145 paraffin-embedded samples, $CD133^+$ colorectal cancer was also associated with local recurrence of tumorigenesis (p=0.035) and distant metastasis (p=0.017), while patients with over 5% $CD133^+$ tumor cells exhibited a decreased survival rate (p=0.001). Multivariate COX analysis showed that the depth of tumor invasion, histology, stages, lymph node metastasis, and CD133 expression were all independent prognosis factors for colorectal cancer (p=0.032, 0.011, 0.001, 0.002, and 0.030, respectively). Furthermore, as few as 5,000 $CD133^+$ colorectal cancer HCT116 cells were sufficient to form tumor xenografts, whereas $1x10^5$ $CD133^-$ tumor cells failed to develop tumor xenografts in nude mice. Conclusions: CD133 expression is a useful biomarker for prediction of colorectal cancer progression and survival of patients.

Key words: Colorectal cancer, Cancer stem cells, Tumorigenesis, CD133, Prognosis

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DOI: 10.14670/HH-11-676

Introduction

Colorectal cancer is one of most significant malignancies worldwide, accounting in 2008 for over 1.2 million new cancer cases and 608,700 cancer-related deaths (Jemal et al., 2011). Risk factors for colorectal cancer include high-fat diet, consumption of smoked foods, red meat, tobacco and alcohol products, physical inactivity, and obesity. In contrast, consumption of high fiber foods, fruits and vegetables is thought to reduce the risk of colorectal cancer. Moreover, persons with a family history of colorectal cancer, inflammatory bowel disease, or adenomatous polyps have an increased risk of developing colorectal cancer (Jemal et al., 2011).

Advances in basic research and clinical management have resulted in early detection, prevention, and improved therapeutic options for patients with colorectal cancer. These major advances, including surgical procedures, chemotherapy, radiotherapy and immunotherapy have markedly improved the survival rate of colorectal cancer patients. However, there are still many patients who die of the disease because of complications related to tumor recurrence and metastasis, or resistance to chemoradiotherapy. Although these complications are thought to be due to the presence of cancer stem cells,

further investigation of the underlying mechanism(s) of colorectal cancer recurrence and metastasis will aid in the development of novel strategies to effectively control this disease (Lapidot et al., 1994; Bonnet and Dick, 1997; Jin et al., 2004).

Cancer stem cells are a small number of tumor cells that have the ability to generate daughter tumor cells that self-renew and maintain tumor phenotypes (Lapidot et al., 1994; Bonnet and Dick, 1997; Jin et al., 2004). Cancer stem cells were successfully isolated and identified in some solid tumors, such as breast cancer (Al-Hajj et al., 2003), brain cancer (Singh et al., 2004), rectal cancer (O'Brien et al., 2007; Ricci-Vitiani et al., 2007), prostate cancer (Richardson et al., 2004; Collins et al., 2005), or lung cancer (Kim et al., 2005). Cancer stem cells are responsible for tumor resistance to chemotherapy and radiation therapy, as well as for recurrence and metastasis (Lapidot et al., 1994; Bonnet and Dick, 1997; Jin et al., 2004). Nevertheless, the exact identity of colorectal cancer stem cells in colorectal cancer tissues remains to be defined (Lapidot et al., 1994; Bonnet and Dick, 1997; Jin et al., 2004). O'Brien et al. showed that all colorectal cancer stem cells were $CD133^+$ and that the colorectal cancer cells of $CD133^+$ subpopulation had a strong tumorigenic ability (O'Brien et al., 2007). Indeed, CD133 is a glycoprotein that is expressed in different types of human stem cells, including hematopoietic cells, endothelial progenitor cells, neuronal and glial cells, mammary glands, and digestive tract and tumor stem cells (such as glioblastoma and colorectal cancer) (Horn et al., 1999; Corbeil et al., 2000; Sanai et al., 2003; Shmelkov et al., 2005; Singh et al., 2005; Mizrak et al., 2008). Therefore, in this study, we examined changes inCD133 expression in colorectal cancer tissues. We then confirmed the tumorigenic potential of $CD133^+$ colorectal cells in nude mice. Our study advances the development of CD133 as a marker for prediction of colorectal tumorigenesis, progression, and prognosis and for future target therapy of colorectal cancer patients.

Materials and Methods

Study population

In the present study, we prospectively recruited 19 patients with histologically confirmed colorectal cancer from the Surgical Oncology Service of China Medical University. Patients were recruited in the time period between October 2012 and September 2013 for flow cytometry identification of $CD133^+$ tumor cells. These patients did not receive any adjuvant therapy, such as chemotherapy or radiotherapy before undergoing surgery. We then respectively obtained 145 cases of colorectal cancer tissue specimens that covered dates between January 2005 and December 2008 for immunohistochemical analysis of CD133 expression. The 145 patients from whom these samples were obtained met the following inclusion criteria. They had: 1) surgically resected tumor; 2) more than 10 pathologically examined lymph nodes; 3) available medical records; 4) documented informed consent; and 5) follow-up data. This study was approved by our institutional ethics and all patients consented to this study.

Flow cytometry analysis of $CD133^+$ tumor cells

Fresh colorectal cancer tissue (1 cm^3 per specimen) was obtained from the operating room and placed in a sterile specimen tube containing Gibco® RPMI 1640 medium and then transported to the laboratory on ice within 15 min. Next, the tissue specimens were cut into 1 mm^3 pieces, which were subsequently digested with collagenase III (Sigma, St Louis, MO, USA) in dissociation flasks at 37°C for 1 h and then filtered through a 45-μm nylon mesh and washed twice with phosphate buffered saline (PBS). After that, the cells were stained with Trypan blue to identify and remove dead cells, and cell numbers were counted under an inverted microscope. Cells were subsequently transferred to a 5-ml tube, washed twice with HBSS with 2% heat-inactivated calf serum (HICS) 5 min each by centrifuging at 1,000 rpm, re-suspended in 100 μl ($1x10^6$ cells) of HBSS with 2% HICS. A 5 μl volume of Sandoglobin solution (1 mg/ml) was added into the cell solution and incubated on ice for 10 min and further incubated with anti-CD2, -CD3 -CD10, -CD16, -CD18, and -CD31 antibodies (BD PharMigen, San Diego, USA) for 20 min on ice and subsequently incubated with a secondary antibody for 20 min and with 1 μl of streptavidin, conjugated with the indicated fluorescent dye, for an additional 20 min. These cell samples were subjected to flow cytometry using a FACSVantage machine (Facscalibur, BD, San Jose, CA, USA). Cells were routinely sorted twice, and reanalyzed for $CD133^+$ cell purity, which typically was >95%. Dead cells were eliminated by using the viability dye 7AAD and $CD133^+/CD326^+$ tumor cells were selected by using anti-CD133 and CD326 antibodies. Similarly, we also sorted colorectal cancer HCT116 cells using flow cytometry.

Immunohistochemistry

Colorectal tissue specimens were fixed in formalin and embedded into paraffin blocks, and 4 μm-thick sections were prepared on glass slides precoated with 3-aminopropyl triethoxysilane for immunohistochemistry. Tissue samples were stained with hematoxylin and eosin to determine histological type and grade of tumors. For immunohistochemistry, we followed a previous study with minor modifications (Al-Rawi et al., 2004). Briefly, the sections were de-waxed in xylene and rehydrated in a series of gradient alcohol and washed with PBS. After that, the sections were blocked in 3% H_2O_2 for 30 min and incubated with normal serum for 30 min and then incubated with the primary antibody for 60 min (all at

room temperature). Next, sections were washed three times then incubated with the biotinylated secondary antibody (Multilink swine anti-goat/mouse/rabbit immunoglobulin, Dako Inc. Carpinteria, CA, USA) for 30 min and subsequently with an avidin-biotin complex at a dilution of 1:1000 (Vector Laboratories Ltd., city, CA, USA) for an additional 30 min at room temperature. The immunoreactive products were visualized by catalysis of 3, 3-diaminobenzidine (DAB) with horseradish peroxidase in the presence of H_2O_2. The sections were then counterstained in Gill's Hematoxylin and dehydrated in ascending grades of methanol before clearing in xylene, and mounting with a coverslip.

Stained tissue sections were reviewed and scored under a light microscope. Cells were considered as $CD133^+$ if their cytoplasm or membrane was stained with brown particles. We utilized the Maeda method, i.e., at low magnification (× 40 to × 200) to find the highest area with the nest CD133-positive cancer cell ratio for ten selected fields (Maeda et al., 2008). We then counted 1000 tumor cells for $CD133^+$ cells and calculated the ratio of $CD133^+$ cells (100 cells were counted per area) using a high-power microscope lens (× 400). The proportion of $CD133^+$ cells in tumor tissue was divided into two levels: <5% vs. ≥5% of $CD133^+$ expression.

Nude mouse xenograft assay

A total of 40 mice (4-8 week old NOD/SCID immunodeficient mice) were randomly divided into 2 groups that received: 1) 5,000 $CD133^+$ colon cancer HCT116 cells or 2) $1x10^6$ CD133-negative HCT116 cells. The flow cytometry-sorted $CD133^+$ and other cell populations were suspended in 1640/Matrigel (1:1 volume) and then subcutaneously injected into the flank region of the nude mice. The mice were then observed twice a week over a period of 56 days for tumor xenograft formation and growth.

Statistical analyses

Data were analyzed using SPSS13.0 statistical software (SPSS, Chicago, IL, USA). The student t-test and chi-square test were performed to generate p value for association of $CD133^+$ levels with clinicopathological data form the patients. Kaplan-Meier curves and the Log rank method were used to analyze survival of patients stratified by $CD133^+$ and a COX model was used to analyze prognosis factors.

Results

Association of ratio of $CD133^+$ cells with biological behaviors of colorectal cancer

We first analyzed CD133 expression in 19 freshly isolated tissue specimens using flow cytometry and found that CD133 protein was expressed in 0.84% - 16.75 % of tumor cells in each case, with an average of 7.15%. $CD133^+$ was associated with tumor lymph node metastasis (9.81% vs. 3.22%, p=0.013; Fig. 1 and Table 1) and tumor differentiation (8.32% vs. 5.07%, p=0.043;

Table 1. Association of CD133 expression with the biological behaviors of colorectal cancer patients.

	Cases (n=19)	Mean (7.15 ± 6.50 %)	p value
Age (yrs.)			0.341
<60	5	6.82	
≥60	14	7.91	
Sex			0.587
Male	13	7.39	
Female	6	6.24	
Depth of invasion			0.395
T2	7	6.73	
T3	12	7.59	
Dukes stage			0.137
B	12	7.67	
C	7	6.46	
Histological grade			0.043
I/II	15	5.07	
III	4	8.32	
Lymphovasular invasion			0.013
Yes	7	9.81	
No	12	3.22	

Table 2. Association of CD133 expression with clinicopathological features of colorectal cancer (n=145).

Clinicopathological features	n	$CD133^+$ cells		p value
		≥5% (n=52)	<5% (n=93)	
Age (yrs.)				0.364
<60	68	27	41	
≥60	77	25	52	
Sex			0.138	
Male	83	34	49	
Female	62	18	44	
Depth of invasion				0.408
Tis	5	1	4	
T1	4	0	4	
T2	12	4	8	
T3	121	46	75	
T4	3	1	2	
Histological grade				0.015
I	17	3	14	
II	86	27	59	
III	42	22	20	
Dukes stage				0.203
A	9	1	8	
B	59	17	42	
C	72	31	41	
D	5	3	2	
Lymphovasular invasion				0.027
Yes	77	34	43	
No	68	18	50	
Local recurrence				0.035
Yes	26	14	12	
No	119	38	81	
Distant metastasis				0.017
Yes	18	11	7	
No	127	41	86	

Table 1).

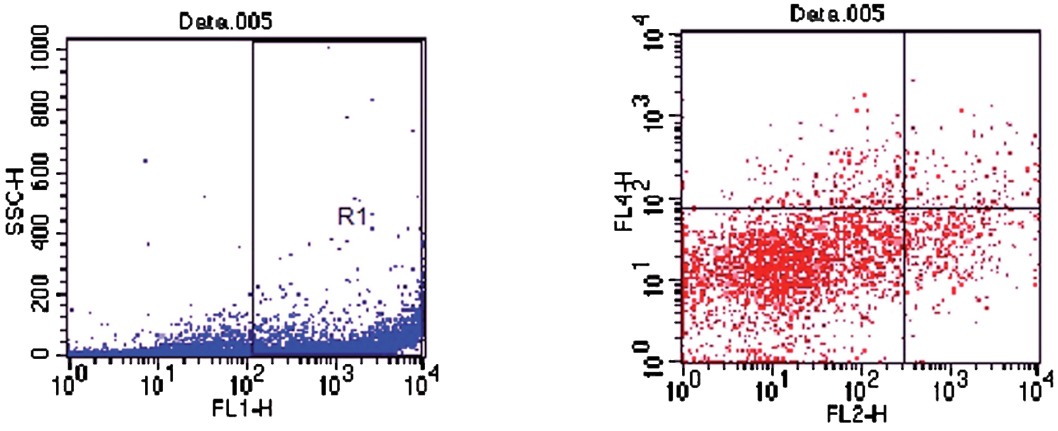
CD133⁺ status associated with clinicopathological data from colorectal cancer patients

We then confirmed the data on 145 colorectal cancer tissue specimens using immunohistochemistry and found that CD133 expression was expressed in the cell membrane and cytoplasm of more than 5% of tumor cells in 52 cases (35.86%) and in 93 (64.14%) specimens with less than 5% of tumor cell positivity (Fig. 2A,B).

CD133 expression was significantly associated with the presence of tumor lymph node metastasis and differentiation grade (Table 2). Moreover, CD133⁺ tumors had a high occurrence rate of the local recurrence and distant metastasis (P=0.035 and 0.017, respectively; Table 2). However, CD133 expression was not associated with age, gender, depth of tumor invasion or tumor stage (Table 2).

Association of CD133 expression with prognosis of colorectal cancer

Of the 145 colorectal cancer patients who were followed, 138 had complete records, resulting in a CCP follow-up rate of 95.17%, with an average follow-up time of 51.71 months. Tumors composed of ≥5% CD133⁺ tumor cells were associated with poorer postoperative survival compared to those composed of <5% CD133⁺ tumor cells (p=0.001, Fig. 3A). However, well differentiated colorectal cancer cases with ≥5% CD133⁺ tumor cells vs. <5% CD133⁺ tumor cells had no survival difference (p=0.153). In contrast, poorly differentiated colorectal cancer with ≥5% CD133⁺ tumor cells vs. <5% CD133⁺ tumor cells had significantly worse survival (p=0.004). Moreover, non-lymph node metastasized colorectal cancer with ≥5% CD133⁺ tumor cells vs. <5 % CD133⁺ tumor cells had a worse survival rate (p=0.001). Similarly, lymph node metastasized



Quadrant Statistics

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Tube:
Acquisition Date: 29-Apr-10
Gated Events: 3770
X Parameter: FL2-H (Log)
Quad Location: 316, 72

Log Data Units: Linear Value
Patient ID:
Panel:
Gate: G1
Total Events: 10000
Y Parameter: FL4-H (Log)

Quad	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
UL	238	6.31	2.38	91.85	47.50	224.83	162.12
UR	136	3.61	1.36	2847.57	1746.69	248.84	174.47
LL	3019	80.08	30.19	37.16	12.65	18.43	12.64
LR	377	10.00	3.77	1554.52	1034.06	31.34	24.72

Fig. 1. Flow cytometric detection of CD133 expression in colorectal cancer tissues. The X axis indicates CD133 expression, whereas the Y axis indicates CD326 expression. The ratio of CD133⁺ tumor cells in lymph node metastasized colorectal cancer tissues was significantly higher than that in non-lymph node metastasized colorectal cancer tissues.

colorectal cancer with ≥5% CD133$^+$ tumor cells vs. <5% CD133$^+$ tumor cells had a worse survival rate (p=0.002; Fig. 3B).

We then performed a multivariate COX analysis and found that the depth of tumor invasion, histological grade, Dukes stage, lymph node metastasis, and CD133$^+$ were all independent prognostic factors for prognosis of colorectal cancer (p=0.032, 0.011, 0.001, 0.002, and 0.030, respectively; Table 3).

In vivo tumorigenicity of CD133$^+$ HCT116 cells in a nude mouse xenograft model

To confirm that CD133$^+$ cells are indeed the cancer stem cell population, we sorted colorectal cancer HCT116 cells using flow cytometry. We then inoculated 5,000 CD133$^+$ cells into nude mice. As a negative control, we subcutaneously injected 1x10^5 CD133$^-$ negative HCT116 cells into the flank region of nude mice. After a 6-10 week observation period, CD133$^+$ tumor cells had formed a larger tumor lesion in 2 of 4 mice (Fig. 4A), whereas CD133-negative HCT116 cells failed to form tumor lesions (0 of 4 mice). Finally, 1x10^6 unsorted HCT116 cells formed xenograft tumors in 2/4 nude mice (Fig. 4B).

Table 3. Multivariate COX regression analysis of independent prognostic factors for colorectal cancer.

Clinicopathological features	Risk ratio	95% CI	p value
Age	1.103	0.539-2.842	0.546
Sex	1.042	0.725-2.455	0.439
Depth of invasion	1.257	1.015-2.624	0.032
Histological grade	1.902	1.655-3.673	0.011
Dukes stage	3.416	2.112-7.591	0.001
Lymphovasular invasion	2.165	1.692-4.839	0.002
CD133$^+$ tumor cell ratio	1.678	1.094-2.941	0.030

Discussion

Cancer stem cells are a small group of tumor cells that have the ability to generate daughter tumor cells and to self-renew in a manner that helps maintain the tumor phenotype (Lapidot et al., 1994; Bonnet and Dick, 1997; Jin et al., 2004). To date, numerous studies have implicated cancer stem cells in tumor resistance to chemo- and radiation therapy, as well as tumor recurrence and metastasis (Stingl, 2006; Li et al., 2008). Indeed, identification of cancer stem cells has become a very important task for the research community, as it may lead to a better understanding of tumorigenesis and aid in the development of novel targets for treatment or early detection and prevention of human cancers. In the current study, we first analyzed the expression of a cancer stem cell marker (CD133) in colorectal cancer tissues for association with colorectal tumorigenesis, progression, and prognosis. We then assessed the role of CD133 in xenograft formation and growth in nude mice.

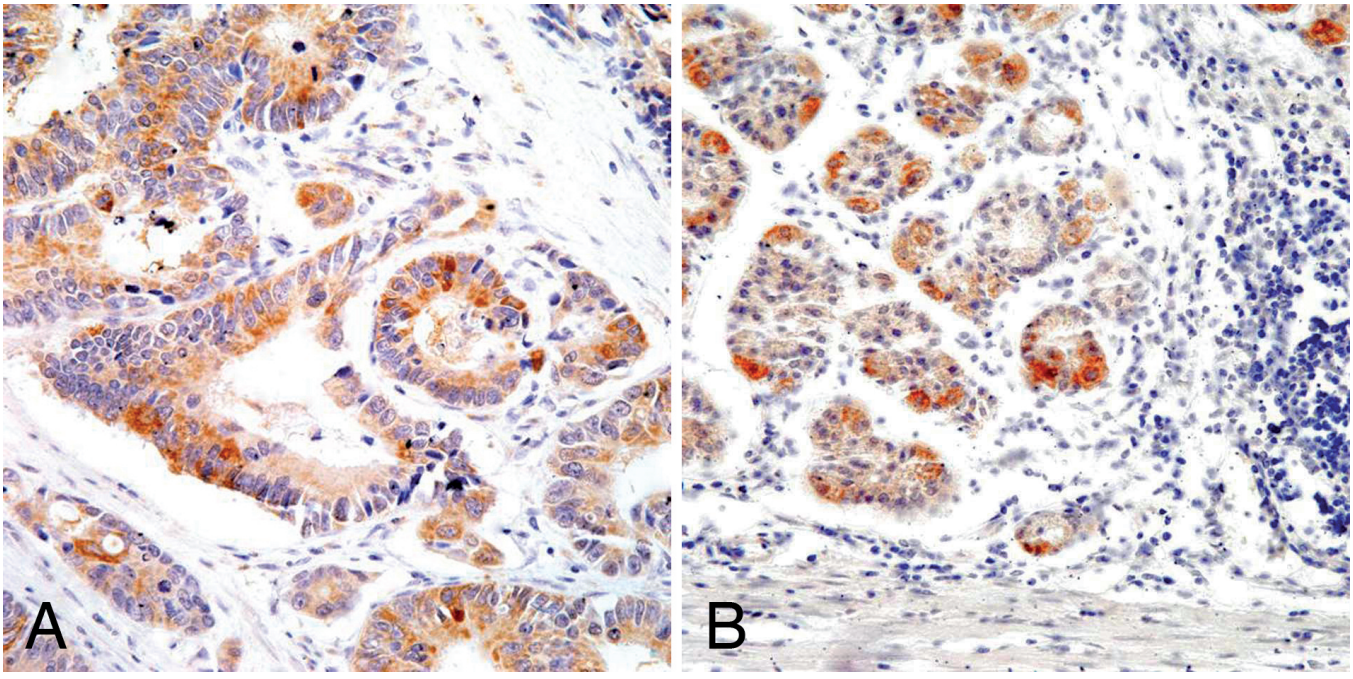

Fig. 2. Immunohistochemical detection of CD133 expression in colorectal cancer tissues. **A.** CD133$^+$ was expressed in more than 5% of tumor cells. **B.** CD133$^+$ was expressed in less than 5% of tumor cells. A, B, x 400

Our data indicated that CD133 expression in tumor cells was associated with tumor lymph node metastasis, poor differentiation, tumor local recurrence, and distant metastasis. The multivariate COX analysis showed that depth of tumor invasion, histology, stages, lymph node metastasis, and CD133 expression were all independent prognostic factors for colorectal cancer. Furthermore, $CD133^+$ colorectal cancer HCT116 cells had a greater potential to form tumor xenografts compared to $CD133^-$ tumor cells or even parental cancer cells in nude mice. Thus, detection of CD133 expression is a useful biomarker for prediction of colorectal cancer progression and survival of patients and indeed may be a novel target for control of colorectal cancer in the future.

Previous studies also investigated $CD133^+$ tumor cells in colorectal cancer tissues or cell lines. For example, Yang et al assessed the level of $CD133^+$ tumor cells and showed that different colorectal cancer cell

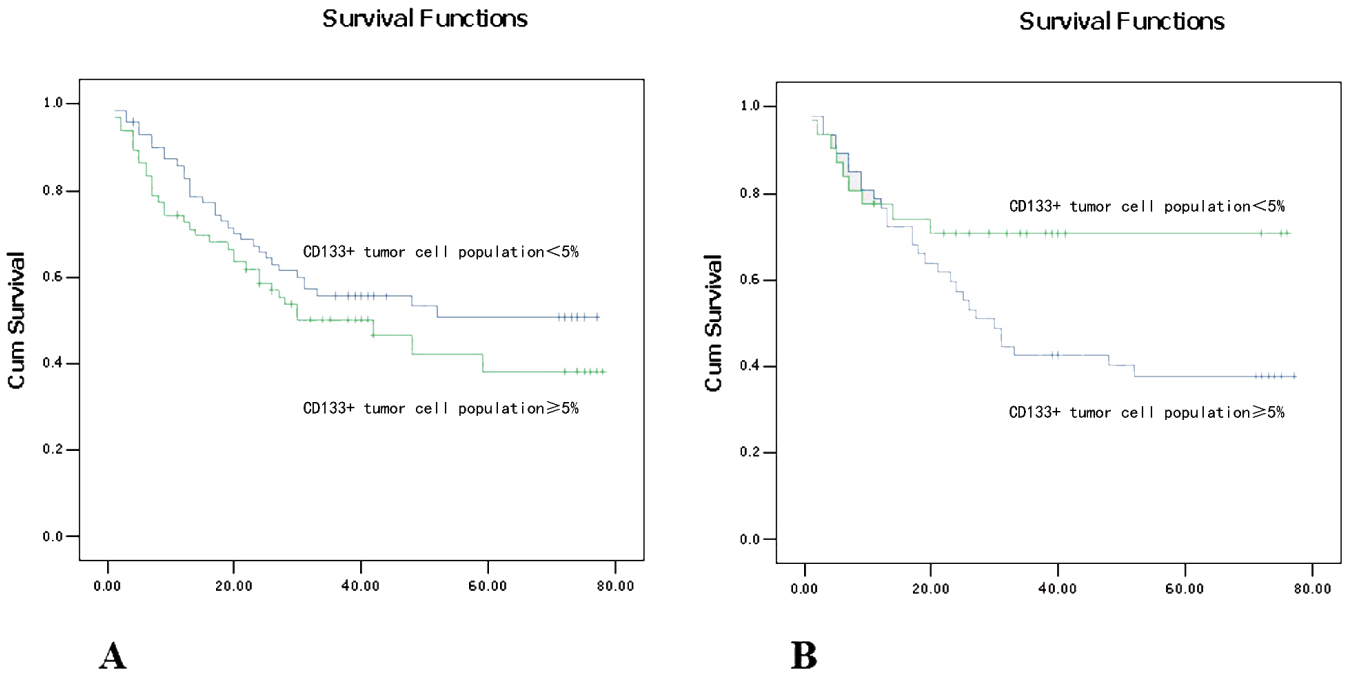

Fig. 3. Kaplan-Meier curves stratified by CD133 expression in colorectal cancer cells. **A.** Overall survival of patients stratified by $CD133^+$ tumor cell population (p=0.001,). **B.** Overall survival of patients stratified by $CD133^+$ tumor cell population and lymph node metastasis (p=0.002,).

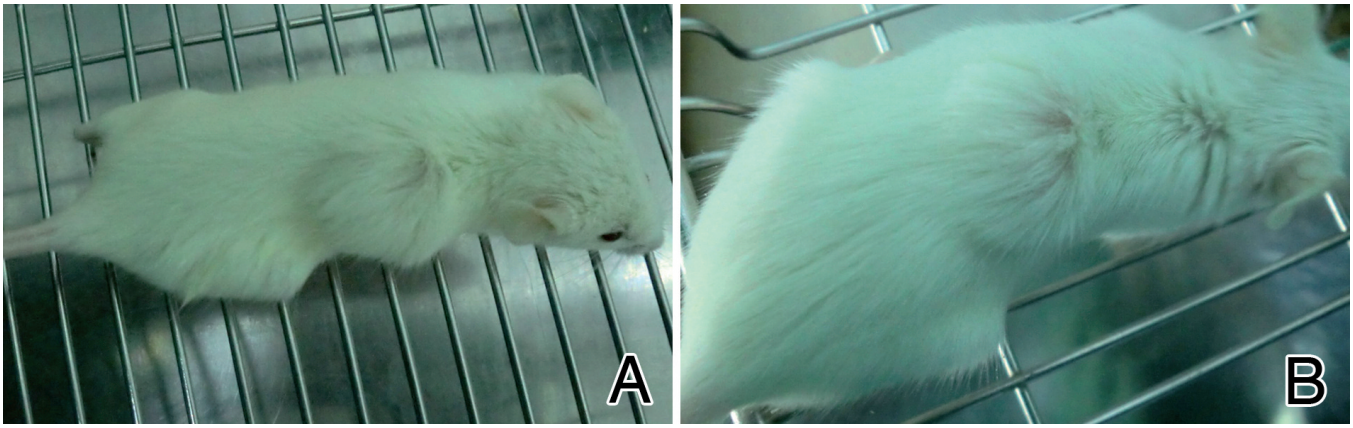

Fig. 4. The nude mouse xenograft assay. **A.** $CD133^+$ colorectal cancer HCT116 cells formed xenograft tumor in mice, whereas CD133-negative HCT116 failed to form tumor lesions. **B.** Unsorted colorectal cancer HCT116 cells formed xenograft tumors in mice.

lines have different levels of CD133 expression (Yang et al., 2011). Other studies showed that CD133 expression in colorectal cancer tissues was associated with clinical biological behaviors in colorectal cancer (Choi et al., 2009; Li et al., 2009). Li et al showed that high $CD133^+$ tumor cell ratio is an independent prognostic factor in colorectal cancer, although $CD133^+$ ratio of tumor cells was not associated with clinicopathological features of colorectal cancer (Li et al., 2009). Choi et al showed that CD133 expression was associated with male patients and colorectal cancer T stage (Choi et al., 2009). Our current findings are partially consistent and extend these earlier observations. In our present study, we used two sets of tissue samples with two different detection methodologies to assess CD133 expression in colorectal cancer tissues. We found that CD133 expression was associated with tumor lymph node metastasis, poor differentiation, tumor local recurrence, and distant metastasis. The multivariate COX analysis showed that CD133 expression was an independent prognostic factor for colorectal cancer. Our current study confirmed previous findings in other cancer types (Zhang et al., 2007; Beier et al., 2008; Song et al., 2008; Zeppernick et al., 2008). Of note, however, a report by Kojima et al showed no correlation between CD133 expression and colorectal cancer prognosis (Horst et al., 2008). This difference may be due to the lack of tumor stage and small sample size in that study (Horst et al., 2008).

Indeed, one of the methods used to identify cancer stem cells is to verify their biological characteristics including self-renewal, proliferation and differentiation (Jin et al., 2004; Kojima et al., 2008). In animal experiments, we were able to test the tumorigenicity of cancer stem cells or certain cancer stem cell marker-positive cells. In our current study, for example, as few as 5,000 $CD133^+$ colorectal cells could generate nude mouse tumor xenografts, whereas $1x10^5$ $CD133^-$ negative tumor cells failed to produce tumorigenesis. Similarly, O'Brien et al and Ricci-Vitiani et al showed that the $CD133^+$ subpopulation of colon cancer cells could produce tumor xenografts in nude mice, but the capability and efficiency of different tumor specimens in generating xenograft tumors was variable (O'Brien et al., 2007; Ricci-Vitiani et al., 2007). These data suggest that besides CD133, other proteins or markers may be needed to identify colorectal cancer stem cells. Furthermore, in an *in vitro* study, Li et al. demonstrated that colorectal cancer stem cells could generate more primitive progenitor cells in serum-free three-dimensional cultures and maintain undifferentiated state growth and self-renewal cell populations (Li et al., 2008). Thus, the search for novel cancer stem cell markers could further help identify and verify cancer stem cells (Shmelkov et al., 2008). Finally, our current study has a notable limitation that requires mention. Specifically, we generated nude mouse tumor xenografts using a colorectal cancer cell line, and not $CD133^+$ tumor cells from patients.

Acknowledgements. This study was supported in part by a grant from the China national natural science foundation (#30772100). We thank Medjaden bioscience limited for assisting in the preparation of this manuscript.

Conflict of interest statement. The authors declared that there is no conflict of interest in this work.

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Accpeted October 7, 2015