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ORIGINAL ARTICLE

Expressions of putative cancer stem cell markers ABCB1, ABCG2, and CD133 are correlated with the degree of differentiation of gastric cancer

Yang Jiang · Yan He · Hui Li · Hui-Ning Li · Lei Zhang · Wei Hu · Ya-Meng Sun · Fu-Lai Chen · Xiao-Ming Jin

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Abstract
Background The present study was carried out to determine whether a quantitative relationship exists between the expressions of 3 cancer stem cell (CSC) markers and the degree of differentiation of gastric cancer.
Methods The expressions of 3 putative CSC markers, ABCB1, ABCG2, and CD133, were detected in 90 human gastric adenocarcinoma cases by immunofluorescence assay. The differentiation statuses of 3 gastric cancer cell lines (the undifferentiated gastric cancer cell line HGC-27, the poorly differentiated gastric cancer cell line BGC-823, and the moderately-poorly differentiated gastric adenocarcinoma cell line SGC-7901) were observed and compared by performing the 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyl tetrazolium bromide (MTT) assay. Gastric xenotransplant cancers in nude mice were constructed to compare the malignancy of the 3 variously differentiated gastric cancer cell lines. The expressions of the 3 putative CSC markers were also detected in the 3 gastric cancer cell lines in vitro by flow cytometric analysis and in the 3 gastric xenotransplant cancers in vivo by immunofluorescence staining.
Results The expressions of ABCB1, ABCG2, and CD133 were generally correlated with the degree of differentiation of the gastric cancers. In the human gastric adenocarcinomas and in the cancer cell lines, the expressions of ABCB1, ABCG2, and CD133 increased with the increases in the malignancy grades of the gastric cancers. In the human gastric adenocarcinomas, poorly differentiated adenocarcinoma expressed more ABCB1, ABCG2, and CD133 than well-differentiated adenocarcinoma. In addition, the expressions of ABCB1 and CD133 were higher in the diffuse type than in the intestinal type of human gastric cancers. The undifferentiated cell line HGC-27 expressed more putative CSC markers than the moderately-poorly differentiated cell line SGC-7901. Similar results were observed in the xenotransplant tumors that arose from the 3 gastric cancer cell lines.
Conclusions The expressions of the CSC markers ABCB1, ABCG2, and CD133 differed in the gastric cancers with various degrees of differentiation, with poorly differentiated gastric cancer expressing relatively more CSC markers.

Keywords Cancer stem cell marker · Gastric cancer · Differentiation

Y. Jiang and Y. He contributed equally to this article.

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Y. Jiang · Y. He · H.-N. Li · L. Zhang · W. Hu · Y.-M. Sun · F.-L. Chen · X.-M. Jin (✉)
Department of Pathology, Harbin Medical University, 157 Xuefu Road, Nangang District, Harbin 150081, Heilongjiang, People's Republic of China
e-mail: jinxm55@yahoo.com.cn

H. Li
Department of Gastroenterology of the Second Affiliated Hospital, Harbin Medical University, Harbin, People's Republic of China

Introduction

Stem cells are defined as cells with significant potential for self-renewal and multi-lineage differentiation through asymmetric cell divisions [1]. According to the hypothesis of tumor origin, it is aberrant stem cells derived from

normal tissue-specific stem cells that can initiate cancer. Similar to the normal stem cell, tumor-initiated stem cells possess the property of self-renewal and can generate progenitor cancer cells. However, in contrast to normal stem cells, mutation-derived cancer stem cells (CSCs) display deregulated pathways of self-renewal and differentiation resulting in the production of the bulk of solid tumors. As a result of asymmetric divisions, cancer cells are not homogeneous. This suggests that a small population of CSCs exist which are highly tumorigenic [2].

To test whether the small population of cancer cells alone is a tumor-initiator, different sub-populations of human cancer cells have been isolated and injected into non-obese diabetic/severe combined immune-deficient (NOD/SCID) mice. For example, purified leukemia bone marrow stem cells were shown to initiate leukemia when injected into NOD/SCID mice, while other populations of leukemia cells failed to induce the disease [3, 4].

These findings provide substantial support for the CSC hypothesis; that is, there are small populations of cells which could be more tumorigenic than "common" cancer cells. Moreover, the CSC hypothesis also offers an attractive model of carcinogenesis and helps to explain clinical manifestations such as tumor aggressiveness, resistance to therapeutic agents, and tumor recurrence, and this may have major ramifications for cancer research and treatment. Although identifiable markers specific to stem cells are still indispensable, the different features that exist between CSCs and other cancer cells, such as differences in the resistance to therapeutic agents, different signal pathways, and different proteins associated with the differentiation of stem cells have served as the basis for a considerable amount of research to distinguish CSCs and common cancer cells [5]. With the advent of CSC research, numerous candidate markers were discovered. Among the putative CSC markers [6], 3 were applied in the present study: ATP-binding cassette C subfamily member 1 (ABCB1, also known as multiple drug-resistance protein 1, MDR1), ATP-binding cassette G subfamily member 2 (ABCG2, also known as breast cancer-resistance protein 1, BCRP1), and CD133 (human prominin-1).

To our knowledge, there is still a lack of evidence for the relationship of the expressions of the CSC markers ABCB1, ABCG2, CD133, and gastric cancer histological types. We detected the expressions of these 3 CSC markers in 90 human gastric adenocarcinoma cases. We also used three cultured gastric cancer cell lines and a nude mouse model in which we implanted cancer cells, and we studied the proliferative and invasive properties of these various cancer cell populations. By flow cytometry and immunohistochemistry, we detected the expressions of the 3 putative CSC markers in all the gastric cell lines in vitro and in vivo. Elucidation of the relationship of CSC markers with variously differentiated gastric cancers will be significant for clinical treatment and prognosis.

Methods

Gastric adenocarcinoma samples

A total of 90 gastric adenocarcinoma tissue samples were obtained from the surgery department of the Second Affiliated Hospital of Harbin Medical University and the Fourth Affiliated Hospital of Harbin Medical University. The tissues were obtained from 68 men and 22 women (mean age 55.8 $\pm$ 11.9 years) between 2007 and 2009. All the gastric adenocarcinomas were graded according to the classification of the Japanese Gastric Cancer Association [7] and the Lauren classification. Each fresh tumor tissue sample was preserved in liquid nitrogen and tissue blocks were kept frozen at -70°C until processed into frozen sections for fluorescence immunohistochemistry assay. This study was granted Institutional Review Board approval by Harbin Medical University Medical Center and received ethical approval from the Ethics Committee. All subjects provided their written informed consent and were assured of their anonymity and the confidentiality of the data obtained.

Cell culture

We used 3 human gastric cancer cell lines in this study: HGC-27 (HGC), BGC-823 (BGC), and SGC-7901 (SGC). All of the cell lines were purchased from the Cell Resource Center of Institutes for Biological Science, Shanghai, China. HGC and BGC were cultured in RPMI 1640 with 2 mmol/L glutamine, 10% fetal calf serum (Invitrogen, Carlsbad, CA, USA) and antibiotics. SGC was maintained in DMEM medium supplemented with 10% fetal calf serum, glutamine, and antibiotics.

Mice and orthotopic human gastric xenotransplant cancers

Three-week-old female BALB/c nude mice ($n = 24$) were purchased from the Shanghai Laboratory Animal Center (Shanghai, China). The construction of orthotopic gastric xenotransplant cancers in nude mice followed the method in the study we had described before [8]. In total, 3 mice received subcutaneous injections of gastric cancer cells to raise xenograft tumors; the other 21 mice were randomly divided into 3 groups of 7 mice each to receive orthotopic xenotransplant tumors. All animals, except for the mice that died during the culture, were sacrificed 75 days after tumor transplantation. The tumors were harvested and fixed in formalin, and paraffin sections were cut (SM2000; Leica, Wetzlar, Germany) and stained with H&E. Unfixed

frozen sections were cut (CM1100; Leica, Wetzlar, Germany) for fluorescence immunohistochemistry. Because all of the tumor tissues were implanted in the tunica serosa of the stomach, tumor invasion depth was evaluated as follows: if the tumor cells appeared in the stomach mucosa, the case would be counted as score 4; similarly, the score was 3 for lamina muscularis mucosae or submucosa, score 2 for tunica muscularis, and score 1 for tunica serosa. The volume and invasive depth of the transplanted tumors were evaluated, as well as the survival time of the mice transplanted with the gastric cancer cells.

Cell proliferation assay

Cell growth was determined by the 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyl tetrazolium bromide (MTT) assay. Cancer cells were plated at 5×10^3 cells per well in 96-well microtiter plates. After incubation for 1–4 days, 200 µl MTT (5 mg/L phosphate-buffered saline [PBS]; Sigma-Aldrich, St. Louis, MO, USA) was added to each well and the plates were incubated for 4 h. The absorbance was recorded on a microplate reader at a wavelength of 490 nm.

Flow cytometric analysis

Cell cultures were treated with 0.25% trypsin–ethylene diaminetetraacetic acid solution for 1–2 min and washed three times in 4°C PBS. The harvested cells were maintained in a solution containing 0.1% fetal calf serum and 0.1% NaN_3. Primary antibodies were added and the cells were incubated for 1 h at 4°C, then washed three times with PBS at 4°C. The cells were incubated with the secondary antibodies for 30 min at room temperature in the dark. Finally, the cells were observed by flow cytometry (BD FACSCalibur, Cell Quest 5.2.1; Becton–Dickinson, Franklin Lakes, NJ, USA). Immunoglobulin G (IgG) isotypes were used as negative controls. Each cell line was assayed in triplicate. The primary antibodies used in the present study were: mouse monoclonal anti-p-glycoprotein (ABCB1, clone F4; Sigma-Aldrich, St. Louis, MO, USA), mouse monoclonal anti-breast cancer-resistance protein (ABCG2, clone BXP-21; Sigma-Aldrich, St. Louis, MO, USA), rabbit polyclonal antibody to CD133-stem cell marker (ab19898; Abcam, Cambridge, MA, USA). The secondary antibodies were: fluorescein isothiocyanate (FITC)-conjugated horse anti-mouse IgG, FITC-conjugated goat anti-rabbit IgG, and FITC-conjugated rabbit anti-goat IgG (all from Vector Laboratories, Burlingame, CA, USA).

Immunofluorescence staining of 3 CSC markers in human gastric adenocarcinomas and 3 xenotransplant tumors

For the human gastric adenocarcinoma samples, frozen sections from each case were fixed with acetone, and nonspecific binding was blocked by 15-min incubation with secondary antibody animal serum. The primary antibodies of ABCB1 (1:50), ABCG2 (1:40), and CD133 (1:50) (all antibodies were the same as those that were used in the flow cytometric analysis) were incubated with the sections overnight at 4°C, followed by 3 washes with PBS. The sections were incubated with either FITC-conjugated horse anti-mouse, goat anti-rabbit, or rabbit anti-goat antibodies (1:200, Vector Laboratories) for 1 h at room temperature (RT). After being washed with PBS, the specimens were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories) and observed at 200× magnification with a biological research microscope (E-800; Nikon Eclipse, Tokyo, Japan). No positive staining was seen when omission of the primary antibody served as the negative control. The staining was scored semiquantitatively as the strongest (4, strong staining in more than 50% of cancer cells); moderately strong (3, strong staining in more than 20% and less than 50% of cancer cells); weak-strong (2, strong staining in less than 20% of cancer cells); weak (1, diffuse weak staining); and negative (0, no staining) [9].

For the xenotransplant tumors that arose from the 3 gastric cancer cell lines, three tumor samples for each group were studied. All the steps followed the protocol of the experiment with the human gastric cancer, except for the dilution of the primary antibodies: ABCB1 (1:100), ABCG2 (1:50), and CD133 (1:50). For each sample, 10 random fields were examined at 400× magnification to calculate the ratio of positive cells. Quantification was performed by two independent observers.

Statistical analysis

All data are presented as mean values ± standard error of mean (SEM). Comparisons between groups were evaluated by Student's *t*-test, analysis of variance (ANOVA) test, or the Mann–Whitney *U*-test; $P < 0.05$ was considered significant.

Results

The expressions of the putative CSC markers ABCB1, ABCG2, and CD133 increased with increases in the degree of differentiation in the human gastric adenocarcinomas

The immunostaining results of the CSC markers ABCB1, ABCG2, and CD133 in human gastric cancers are shown in Fig. 1 and Table 1 (more details could be seen in supplementary material). ABCB1 was expressed both in the cytoplasm and in the membrane of the gastric cancer cells,

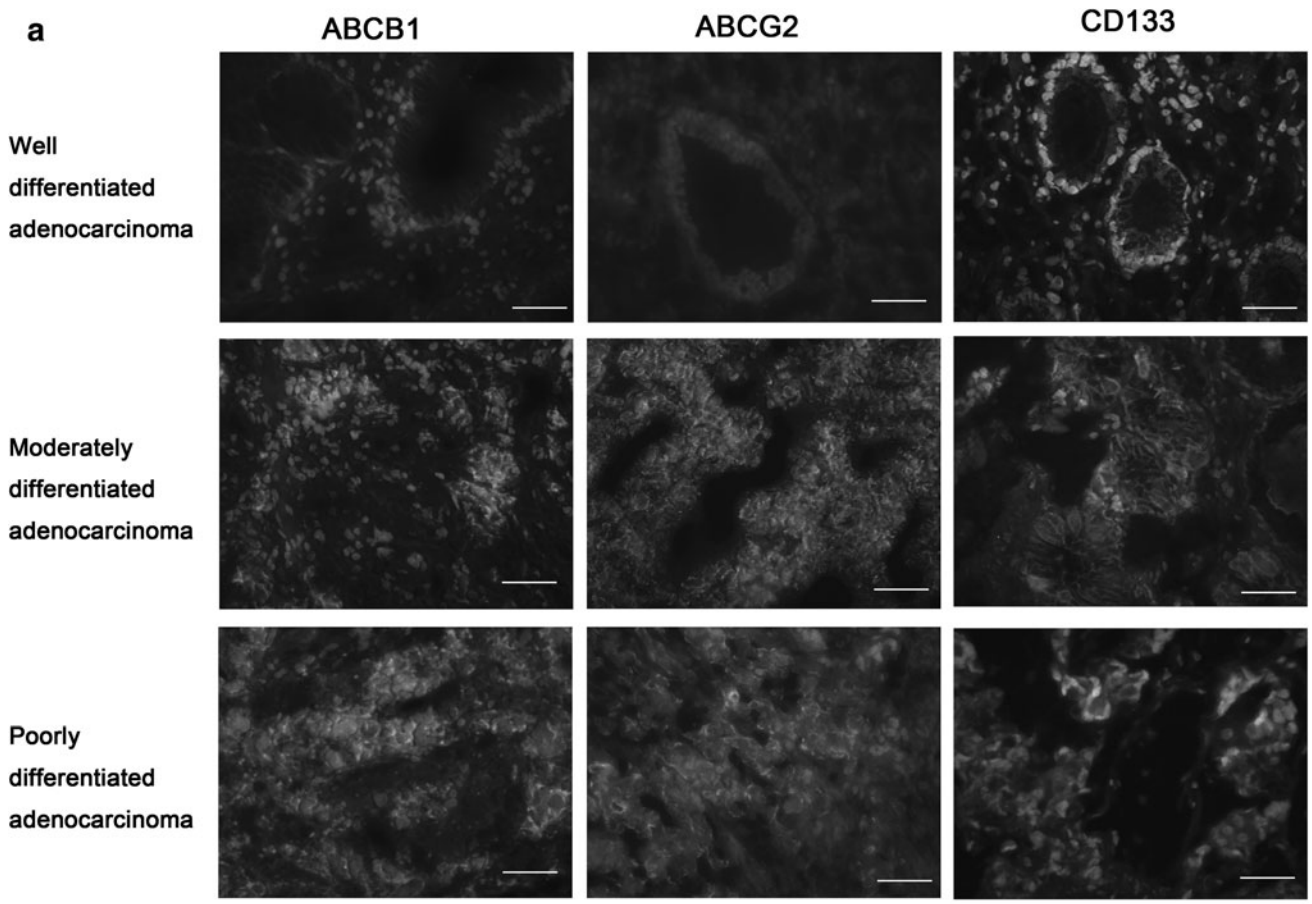

Fig. 1 **a** Representative immunofluorescence micrographs showing expressions of the cancer stem cell (CSC) markers, ABCB1, ABCG2, and CD133 (fluorescein isothiocyanate [FITC] labeling) in 3 variously differentiated gastric adenocarcinomas. The nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). ×400, *bar* 50 μm. **b** *Bar graphs* show comparison of the immunohistochemistry scores of the 3 CSC markers expressed in the variously differentiated gastric cancers. $^{**}P < 0.01$ by Mann–Whitney *U*-test. **c** *Bar graphs* show comparison of the immunohistochemistry scores of the 3 CSC markers in diffuse and intestinal types of gastric cancers. $^{**}P < 0.01$, $^{*}P < 0.05$ by Mann–Whitney *U*-test

while CD133 was mainly expressed in the membrane of the gastric cancer cells (Fig. 1a). Cancer stem cell (CSC) marker immunohistoreactivities were evaluated by using the semiquantitative scoring method mentioned in the "Methods" section. A study of CSC marker expression in human gastric cancer in relation to patient age, gender, tumor differentiation, Lauren classification, and invasion depth was performed (Table 1). The data showed that the

Table 1 Expression of 3 CSC markers in gastric adenocarcinomas in relation to age, gender, malignancy grade, Lauren classification, invasion depth, and metastasis

	Immunohistoreactive scores and *P* value						
	n	ABCB1	*P*	ABCG2	*P*	CD133	*P*
Age (years)							
≤55	46	2.07 ± 0.02	0.61	1.72 ± 0.03	0.27	2 ± 0.03	0.56
>55	44	2.15 ± 0.02		1.43 ± 0.03		2.16 ± 0.03	
Gender							
Male	68	1.56 ± 0.02	0.85	2.09 ± 0.02	0.8	1.1 ± 0.01	0.25
Female	22	1.64 ± 0.07		2.05 ± 0.05		0.82 ± 0.04	
Invasion depth							
Mucosa	4	1.25 ± 0.19	0.8	2 ± 0.19	0.36	1.13 ± 0.06	0.09
Submucosa	2	1.5 ± 0.43		1.75 ± 0.32		0.5 ± 0.15	
Tunica muscularis	18	1.43 ± 0.09		2.43 ± 0.08		1.57 ± 0.11	
Tunica serosa	66	1.65 ± 0.02		2 ± 0.02		0.92 ± 0.01	
Tumor grade							
Well-differentiated	25	1.04 ± 0.05	0.065[a]	1.44 ± 0.04	0.08[a]	0.44 ± 0.03	0.131[a]
Moderately differentiated	28	1.64 ± 0.04	0.337[b]	2.04 ± 0.04	0.072[b]	0.71 ± 0.03	**0[b]**
Poorly differentiated	37	1.97 ± 0.03	**0.005[c]**	2.54 ± 0.03	**0.001[c]**	1.7 ± 0.02	**0[c]**
Lauren type							
Intestinal type	60	1.35 ± 0.02	**0.005**	1.93 ± 0.02	0.103	0.88 ± 0.02	**0.014**
Diffuse type	30	2.13 ± 0.05		2.37 ± 0.04		1.37 ± 0.03	
Metastasis							
No	17	1.88 ± 0.07	0.247	2.24 ± 0.07	0.549	1.18 ± 0.06	0.383
Yes	73	1.55 ± 0.02		2.04 ± 0.02		1 ± 0.01	

Values represent means ± SEM. Test used: Mann–Whitney *U*-test

Bold fonts show significant difference

CSC cancer stem cell

[a] Mean comparison of well-differentiated adenocarcinoma group and moderately differentiated adenocarcinoma group

[b] Mean comparison of moderately differentiated adenocarcinoma group and poorly differentiated adenocarcinoma group

[c] Mean comparison of poorly differentiated adenocarcinoma group and well-differentiated group

expression of ABCB1 was higher in poorly differentiated adenocarcinomas than in well-differentiated gastric cancers (1.97 ± 0.03 vs 1.04 ± 0.05). There was more ABCG2 expression in poorly differentiated cancers than in well-differentiated cancers (2.54 ± 0.03 vs 1.44 ± 0.04). The poorly differentiated cancers expressed more CD133 than the moderately differentiated and the well-differentiated cancers (1.7 ± 0.02 vs 0.7 ± 0.03 and 0.44 ± 0.03, respectively, Fig. 1b; Table 1). The diffuse-type cancers expressed more ABCB1 than the intestinal type (2.13 ± 0.05 vs 1.35 ± 0.02, $P < 0.01$), while the expression of ABCB2 showed no significant difference between these types (2.37 ± 0.04 vs 1.93 ± 0.02). There was more CD133 expression in diffuse-type cancers than in intestinal-type cancers (1.37 ± 0.03 vs 0.88 ± 0.02, $P < 0.05$, Fig. 1c; Table 1). However, no significant differences in the expression of the 3 CSC markers was found in relation to age, sex, invasion depth, or metastasis (Table 1).

HGC and BGC exhibited a more malignant inclination than SGC in vitro and in vivo

To determine differences in the 3 gastric cancer cell lines in vitro, analysis of proliferation in the 3 cell lines was conducted under the same conditions for each cell line by performing the MTT assay. Generally, the HGC cells' growth peak appeared on the third day of culture. In contrast, SGC cells multiplied slowly during the first 3 days in culture. The growth peak of BGC cells occurred on day 3 in culture. Statistical analysis indicated that the proliferation rate of SGC cells was slower than that of the other two cell lines (by Student's *t*-test, $P < 0.05$). No statistically significant difference was found between HGC and BGC (Fig. 2a).

To compare differences in the xenotransplant tumors, gastric cancer cells were orthotopically transplanted into the gastric serosa of nude mice. We then evaluated metastasis, tumor volumes, mean survival times of the

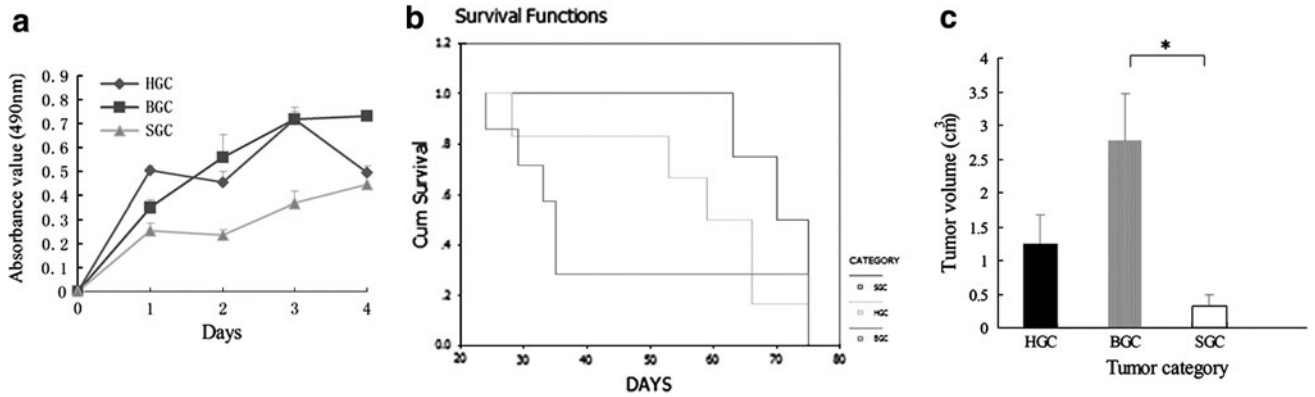

Fig. 2 Comparisons of cell proliferation and outcomes in the transplanted tumors of 3 gastric cancer cell lines. **a** Growth curves of the 3 cancer cell lines. Data are expressed as means ± SEM. The rate of proliferation of SGC was slower than that of the other cell lines. **b** Survival days of tumor-bearing nude mice. **c** Comparison of transplant tumor volumes of the 3 cancer cell lines. Data are expressed as means ± SEM. Although the volume of SGC was smaller than that of the other 2 cancer cell lines, only the volume of BGC was significantly different from the volume of SGC by unpaired Student's *t*-test. $*P < 0.05$

Table 2 Descriptive statistics in tumor-bearing nude mice

Groups	Mice	Successful transplantation (%)	Metastasis (%)	Mean survival days
HGC	7	6/7 (89)	1/6 (17)	57
BGC	7	7/7 (100)	0	43
SGC	7	4/7 (57)	0	70

HGC undifferentiated gastric cancer cell line, *BGC* poorly differentiated gastric cancer cell line, *SGC* moderately-poorly differentiated adenocarcinoma cell line

tumor-bearing mice, and the depth of tumor invasion. Of the 21 mice that received tumor cell transplants, 7 of 7 mice showed tumors that arose from BGC cancer cells, and tumors arose in 6 of 7 mice that were transplanted with HGC cells. For the recipients of SGC cells, 4 of 7 mice developed tumors in their stomach wall (Table 2). All mice were kept under sterile conditions for 75 days after surgery. After surgery, in the HGC group, the tumor-bearing mice survived for 28, 53, 59, 66, 66, and 75 days (mean 57.83 days). The survival days of the BGC recipient mice were 24, 29, 33, 35, 35, 75, and 75 days (mean 43.71 days). Four mice with SGC tumor cells survived for 63, 70, 75, and 75 days (mean 70.75 days). Although the mice with SGC tumor cells lived for a longer time than the other groups, there were no statistically significant differences between the survival times for the mice with the 3 different cancer cell lines (by log-rank test of survival analysis; Fig. 2b). The tumor volumes differed dramatically between individual mice; the mean volume of the BGC tumors (2.775 ± 0.689 cm^3) was significantly greater than the mean SGC tumor volume (0.317 ± 0.175 cm^3) ($P < 0.05$) (Fig. 2c).

Morphological and histopathological views of the subcutaneously implanted tumors from the 3 cancer cell lines showed various degrees of differentiation (Fig. 3a, b). HGC cancers showed the clustering of relatively small cancer cells with hyperchromatic nuclei and an obvious nucleolus. Parenchymal cells were abundant and stromal structures were rare in the HGC tumors, and no glands had formed. Anarchic multiple spindles and necrosis were frequent in the HGC cancers. In the BGC cancers, cellular atypia was similar to that in the HGC cancers; however, constituents that resembled a basal lamina appeared between the cancer cells. With respect to the SGC cancers, some cells had arranged themselves into structures that resembled glands. Mitoses were less frequent in SGC cancer cells than in the other 2 types of cancer cells. The volumes of the HGC tumors were smaller than those of the BGC tumors, and this may be attributed in part to the necrosis that was frequently found in the HGC tumors.

The tumor invasive depth scores analyzed by the Mann–Whitney *U*-test indicated that the invasiveness of the SGC cells (1.5 ± 0.38) was less than that of the HGC (2.83 ± 0.16) and BGC (3 ± 0.14) cells (Fig. 3c, d; Table 3, $P < 0.05$).

To avoid confusion caused by cancer cells spreading directly to local organs, we considered metastasis only when cancer cells were found in extraabdominal organs such as the lungs and brain. Only one instance of pulmonary metastasis was found in the HGC group.

Expressions of the 3 putative CSC markers exhibited differences in the 3 gastric cancer cell lines in vitro and in vivo

The expressions of ABCB1, ABCG2, and CD133 in the 3 gastric cancer cell lines were measured by flow cytometric analysis in vitro (Fig. 4a) and by immunostaining in the 3 xenotransplant tumors in vivo (Fig. 5a). In vitro, HGC cells, an undifferentiated gastric cancer cell line, manifested the

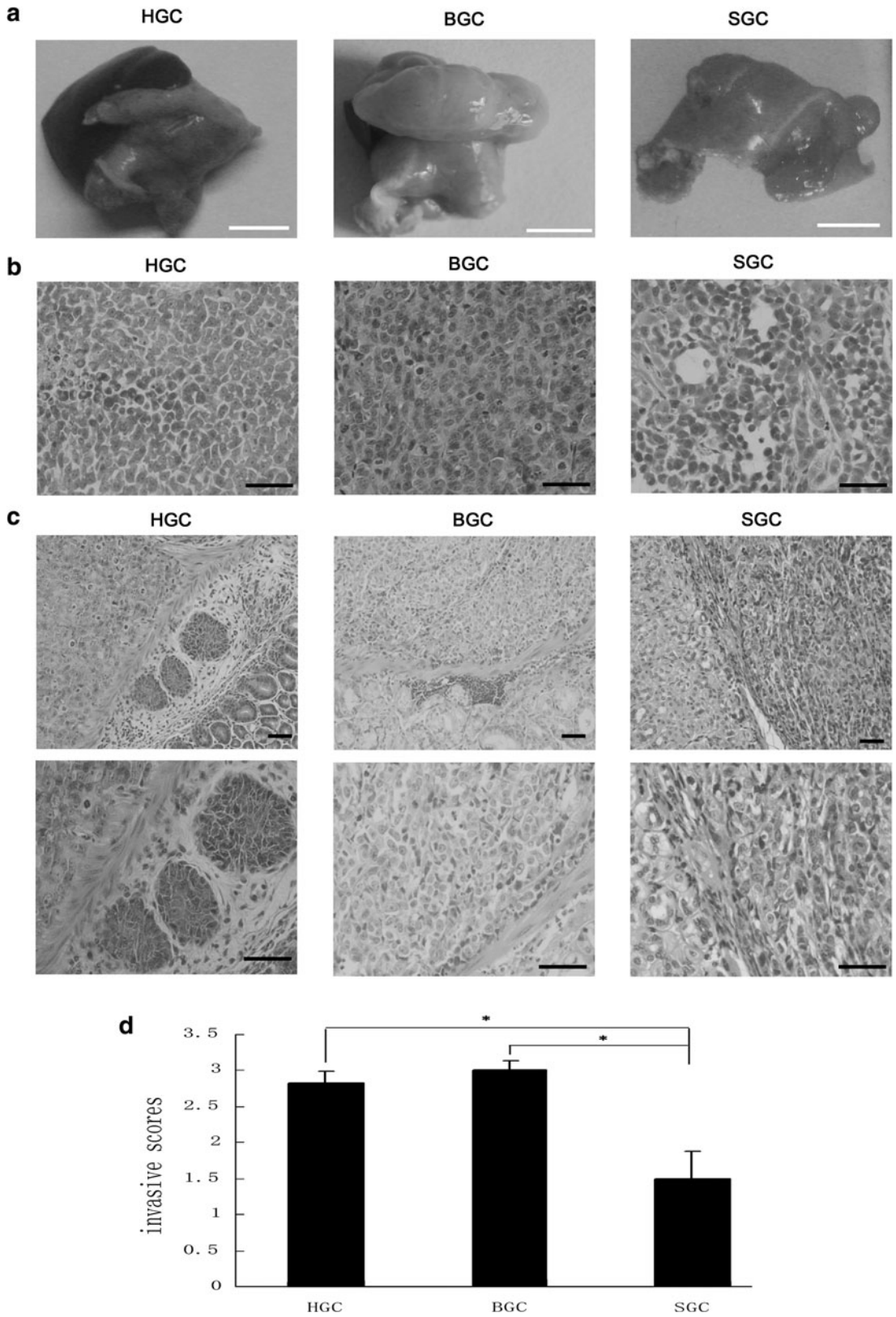

Fig. 3 Histopathology of the 3 gastric cancer cell line implants in nude mice. **a** Morphology of the 3 transplant tumors. *Bars* 5 mm. **b** Representative histological photomicrographs of 3 kinds of subcutaneous tumors. ×400, *bars* 50 μm. **c** Representative photomicrographs of 3 gastric wall xenograft tumors. *Upper panel* ×200, *bars* 50 μm. *Lower panel* ×400, *bars* 50 μm. **b**, **c** Stained with H&E. **d** *Bar graph* shows invasive depths of 3 xenotransplant tumors. $^{*}P < 0.05$

Table 3 Scores for gastric invasion depth in transplant tumor-bearing nude mice

	HGC	BGC	SGC[a]
Mucosa	1	2	
Lamina muscularis mucosae	1		
Submucosa	3	4	
Tunica muscularis			1
Tunica serosa	1	1	3
Total	6	7	4
Invasive scores (means ± SEM)	2.83 ± 0.16	3 ± 0.14	1.5 ± 0.38

[a] The invasion depth of SGC was lower than that of HGC and BGC, $P < 0.05$ (Mann–Whitney U test)

highest labeling percentages for ABCB1 (14.34 ± 2.1%), ABCG2 (11.75 ± 2.8%), and CD133 (30.33 ± 5.22%) compared with BGC (3.47 ± 0.19% for ABCB1, 0.93 ± 0.1% for ABCG2, and 3.06 ± 0.33% for CD133) and SGC (1.41 ± 0.16% for ABCB1, 1.66 ± 0.35% for ABCG2, and 3.01 ± 0.64% for CD133;all above $P < 0.05$, Fig. 4b).

The expression of the CSC markers in the xenotransplant tumor tissues of nude mice was determined by an immunostaining method, in which the positive cells were counted and their number was divided by the total cell number in the tissue sections. The percentage of positive cells showed that ABCB1 expression in the HGC xenotransplant tumors was higher (32.9 ± 0.54%) than that in the BGC (20.3 ± 0.89%) and SGC (20.1 ± 0.46%) xenotransplant tumors. The HGC tumors also expressed more ABCG2 (3.6 ± 0.15%) than the SGC tumors (2.06 ± 0.08%). For CD133, the percentage of positive cells in the HGC tumors (4.39 ± 0.3%) was higher than that in the BGC tumors (1.47 ± 0.11%) and SGC tumors (0.71 ± 0.06%). No other significant differences were found (all above $P < 0.05$; Fig. 5b).

Discussion

In the present study, we found that the expression of 3 putative CSC markers, ABCB1, ABCG2, and CD133, was significantly increased along with increases in the grades of malignancy of gastric cancer. In general, the more poorly differentiated gastric cancers expressed more CSC markers, whether in human samples or in established gastric cancer cell lines and their xenotransplanted tumors. We propose that this finding suggests that malignant gastric cancer may contain more CSCs, which causes the cancers to remain in a poorly differentiated status, so that they manifest more aberrant biological behavior. Studies have shown that the histological types of gastric cancer are related to the stage of cancers, and therefore the degree of differentiation is indirectly associated with the survival of patients [10]. Although the ultimate phenotypic marker that characterizes CSCs is still unclear, the CSC model has substantial ramifications for determining a cancer's origin, its resistance to therapeutic agents, and its likelihood of recurrence [11]. Many investigators have identified several markers that allow the isolation of small numbers of uncommon cancer cells from solid cancers. These subpopulations of cancer cells have demonstrated properties of survival and proliferation that were proven by assays of tumorigenesis in nude mice [12]. In the present study, we assumed that the expressions of putative markers were related to the degree of differentiation of cancers.

The superfamily of ATP binding cassette (ABC) proteins is the most useful of the putative CSC markers [13] because of the property these proteins have of expelling fluorochrome. ABC transporter proteins are also associated with multidrug resistance and are located in the plasma membranes of cells and in the membranes of cellular organelles [14]. Numerous experimental studies have revealed that the expression of ABCB1 and ABCG2 is high in "side population" cancer cells, and this feature was commonly considered to be associated with CSCs [15]. Surowiak et al. [9] detected the expression of ABCB1 in breast cancers, with results showing that breast cancer cases of grade 3 had a higher overall immunoreactivity score than cases of grade 2. The work of Zen et al. [16] indicated that the expression of ABCG2 in human hepatocellular carcinoma was greater than that in dysplastic nodules. Our results support these findings. We found that the expressions of ABCB1 and ABCG2 were higher in the poorly differentiated human gastric adenocarcinomas and in the undifferentiated gastric cancer cell line HGC, compared with the expressions in the relatively well-differentiated gastric adenocarcinomas and gastric cancer cell lines. In addition, the expression of ABCB1 was greater in the diffuse type of gastric cancer than in the intestinal type of gastric cancer.

CD133 is commonly found on stem and progenitor cells of various tissues [17]. Recent studies show that, compared with unsorted cancer cells, the subpopulation of $CD133^+$ cancer cells contains a higher concentration of progenitor cancer cells [18]. Furthermore, according to research conducted on human oligodendroglial tumors [19] and astrocytomas [20], the quantity of CD133 was higher in poorly differentiated/diffuse-type cancers than in well-differentiated/intestinal-type cancers. Although we found that the quantity of CD133 expression varied, our results demonstrated that the CD133 staining was strongest in poorly differentiated human gastric cancers and in the HGC cell line compared with their components (BGC and SGC cell lines in present study), a finding which could support the concept that the population of poorly differentiated cancer cells may contain more stem cells.

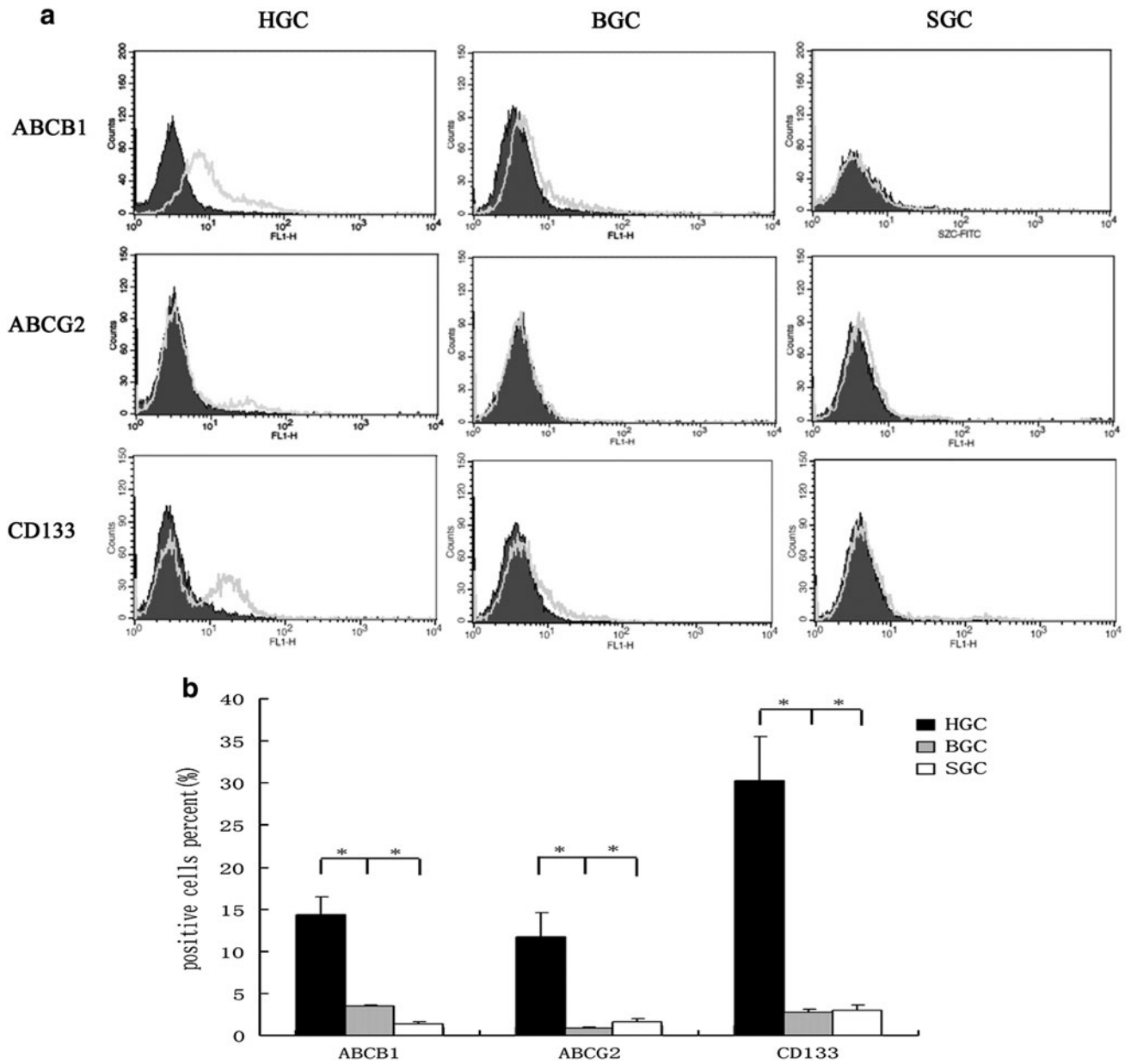

Fig. 4 Flow cytometry data for the expressions of ABCB1, ABCG2, and CD133 in the 3 cancer cell lines in vitro. **a** Representative flow cytometry analysis results. **b** *Bar graphs* show quantitative analysis of positive cell percentages. $*P < 0.05$, by analysis of variance (ANOVA) test

The malignancy grade of cancer, especially in gastrointestinal tract cancer, is an important factor in patient survival. However, we did not discover any novel predictive factors in the present study; this was partly due to the parameters that we examined; namely, the relationships that we found to exist in the histology classifications, and expressions of CSC markers, not in the important points which directly correlate with the prognosis of gastric patients, such as lymph node metastasis. Further investigations of other CSC markers are necessary in larger numbers of gastric cancer cases.

The existence of CSCs provides new targets for cancer research and treatment. In our study, we detected the expression of three putative CSC markers in variously differentiated gastric adenocarcinomas and cancer cell lines, and predicted the correlation between cancer cell differentiation status and CSCs. The results implied that poorly differentiated gastric adenocarcinomas and gastric cancer cell lines expressed more putative CSC markers. We propose that the correlation of CSC markers with the degree of differentiation of gastric cancer reflects the difference of CSCs in gastric cancers of various

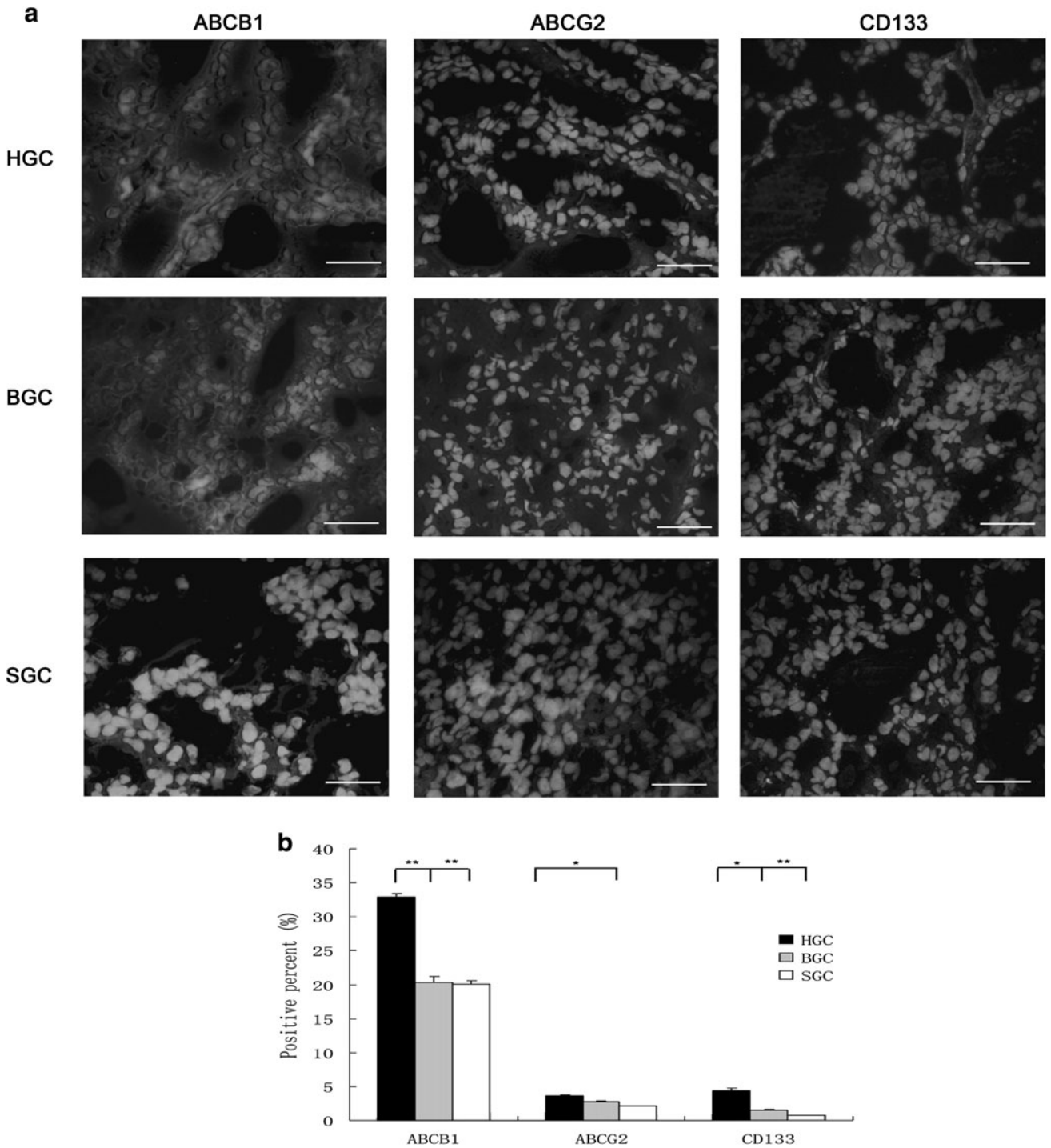

Fig. 5 The expressions of ABCB1, ABCG2, and CD133 in 3 xenograft gastric tumors. **a** Representative immunofluorescence micrographs showing expressions of ABCB1, ABCG2, and CD133 (FITC labeling) in 3 xenograft tumors. The nuclei were stained by DAPI. ×400, *bars* 50 μm. **b** Quantitative analysis of percentages of positive cells. Statistically significance differences were seen in the xenotransplants of the variously differentiated gastric cancer cells by ANOVA test. $*P < 0.05$, $**P < 0.01$

differentiations. Together, these data suggest that the existence of CSCs within gastric cancers might be the major biological difference between the poorly differentiated gastric cancers and the well-differentiated gastric cancers.

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