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Influence of CD133$^+$ expression on patients' survival and resistance of CD133$^+$ cells to anti-tumor reagents in gastric cancer

De-Hu Chen[#], Rui-Qi Lu[#], Xiao-Chun Ni, Ju-Gang Wu, Shou-Lian Wang, Bo-Jian Jiang, Ji-Wei Yu[*]

Department of General Surgery, Third People's Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, PR China

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ABSTRACT

Objective: To investigate the influence of CD133$^+$ expression on patients' survival and resistance of CD133$^+$ cells to anti-tumor agents in gastric cancer (GC).

Methods: Influence of CD133 expression on prognosis was analyzed employing samples from patients with GC. GC cell lines were utilized to separate CD133$^+$ and CD133$^-$ subpopulations by immunomagnetic separation and to analyze the biological features of two subpopulations *in vitro* and *in vivo*, especially in resistant to anti-tumor reagents and its apoptotic mechanism.

Results: The lower CD133$^+$ group showed a significantly better survival compared with the higher CD133$^+$ group. The highest content of CD133$^+$ subpopulations for KATO-III cells had stronger proliferative ability than CD133$^-$ subpopulations. A single CD133$^+$ cell was capable of generating new cell colony and the tumorigenicity rate in nude mice was 100% for CD133$^+$ clonal spheres or for CD133$^+$ cells, but 0% for CD133$^-$ cells. Furthermore, the higher expression levels of Oct-4, Sox-2, Musashi-1 and ABCG2 in CD133$^+$ clonal spheres were identified compared with CD133$^+$ cells or CD133$^-$ cells. Under the treatment of anti-tumor reagents, CD133$^+$ cells had lower suppression rates compared with CD133$^-$ cells while lower level of Bcl-2 and higher level of Bax were found in CD133$^+$ cells compared with CD133$^-$ cells.

Conclusions: The patients with lower CD133$^+$ expression had a better survival. Enriched CD133$^+$ cells in clonal sphere shared the ability to be self-renewable, proliferative, tumorigenic and resistant to anti-tumor agents as probably regulated by Bcl-2 and Bax.

1. Introduction

Tumor invasion and metastasis is considered to be the primary cause of death for patients with gastric cancer (GC). GC frequently recurs and metastasizes following the seemingly successful initial treatment, with a recurrence rate of approximately 40% within 2–3 years of surgical treatment. Furthermore, it has a five-year survival rate of only 25% [1,2].

It is conventionally believed that tumorigenesis results from the homogeneous multiplication of all tumor cells. Primary tumor cells enter into clonal proliferation due to the continuous mutations in genetically susceptible key genes regulating cell growth [3]. However, accumulating evidences reveal that tumorigenesis results from cell differentiation disorder involving multiple genes and multiple steps in a heterogeneous progression [4]. It is hypothesized that malignant tumors are hierarchically composed of multiple heterogeneous cell subpopulations. Among these subpopulations, tumor initiating cells (TICs) are a specific subpopulation expressing single or multiple cell surface markers, which can initiate and construct tumor histological phenotype. Additionally, TICs determine the drug resistance and the tumor invasiveness/metastasis [5]. CD34$^+$/CD38$^+$ cell subpopulation has been demonstrated to duplicate a leukemia animal model in immunodeficient severe combined immunodeficiency mice, which suggests the long life span and

*Corresponding author: Ji-Wei Yu, PhD, MD, Department of General Surgery, Third People's Hospital, Shanghai Jiao Tong University School of Medicine, No 280, Mohe Road, Shanghai, 201900, China.
Tel/Fax: +86 021 56691101
E-mail: jenniferyu919@126.com

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[#]These authors contributed equally to this work.

self-renewal capacity of TICs. It is, therefore, rational that human myeloblastic leukemia derives from stem cells [6]. TICs with $CD133^+$ expression have been subsequently identified in various solid tumors, such as breast cancer, brain tumor, prostate cancer, malignant melanoma, colon cancer, liver cancer, pancreatic cancer and head and neck squamous cell carcinoma, though $CD133^+$ cancerous cells have not been classified as cancer stem cells of stomach until now [7–14].

It has been possible to enrich and characterize TICs through the specific cell surface markers. However, the GC TICs are yet to be isolated successfully due to the limitation in current separation technique and biomarkers. Smith *et al*. reported that the gastrointestinal tumor cells expressed CD133 receptors were antagonized by the anti-CD133 antibodies in combination with chemotherapeutic agents and suppressed the GC xenograft growth in nude mice [15]. This finding implies that $CD133^+$ cells probably possess the tumorigenic potential of TICs. Furthermore, our previous study showed that CD133 was related to chemoresistance in GC cells [16].

In this study, more clinical specimens were included to quantify the CD133 expression in primary lesion of GC tissue in relation to prognosis, which was not deluded in the CD133 protein level in our preliminary study [17]. To obtain a larger amount of $CD133^+$ cells for further steps of biological, tumorigenic and resistant to anti-tumor investigations, we also examined the percentage of $CD133^+$ cell subpopulation among four kinds of GC cell lines varying in cell differentiation by flow cytometry and attempted to enrich $CD133^+$ cells through immunomagnetic separation combined with serum-free culture (*i.e.* floating culture). Due to the highest percentage of $CD133^+$ cells in KATO-III cell lines among these four GC cell lines, the enriched $CD133^+$ cells from KATO-III were applied for further evaluations regarding proliferative potential, colony-forming capacity, tumorigenic capability *in vivo*, the mRNA expressions of relative stem cell markers and resistance to anti-tumor drug including the suppression rate of cell growth and mRNA expressions of Bcl-2 and Bax regulating apoptosis. These findings from such investigations are beneficial to advance our knowledge about $CD133^+$ cells specificities probably like TICs to some extent and resistant mechanism of anti-tumor drug regarding GC $CD133^+$ cells.

2. Materials and methods

2.1. Clinical specimens, cells and reagents

Primary lesions of GC and paired peri-cancer gastric tissue (at a place of $\geq$5 cm far from tumor margin as non-cancerous tissue identified by pathological examination) were harvested from histologically documented GC patients who underwent definitive surgery at our hospital from January 2009 to December 2010. None of these patients accepted any preoperative chemotherapy or radiotherapy. Chemotherapeutic treatment of 5-fluorouracil (5-FU), cisplatin and leucovorin was applied 6 times as a protocol of 3 weeks for each patient after surgery. All patients were followed up for 1–37 months until February 2012. This study was approved by ethical committee of our hospital before its start and preoperative informed consent was obtained from each patient registered in this study in accordance with institutional guidance.

The cell line KATO-III, SGC-7901, AGS and MKN-45 were purchased from American Type Culture Collection. The Roswell Park Memorial Institute 1640 medium was purchased from Gibco, US. Human epidermal growth factor (hEGF) and human basic fibroblast growth factor (bFGF) were purchased from PeproTech, US. The CD133 separation kit, mouse anti-human CD133 monoclonal antibody and CD133-PE flow antibody were purchased from Miltenyi, Germany. Horseradish peroxidase-labeled goat-anti-mouse secondary antibody was purchased from Jackson, US. The target primers were purchased from Sangon Biotech (Shanghai) Co., Ltd., Shanghai, China. The RT-PCR assay kit was purchased from Takara, Japan. The ABC kit was purchased from Santa Cruz, US and the CCK-8 assay kit was purchased from Cayman, US.

2.2. Semi-quantitative RT-PCR

The total RNA was extracted using Trizol. RT-PCR assays were performed as previously reported [16]. The experiments were duplicated and independently run in triplicates.

2.3. Western blot assay

The western blot assay was performed as previously reported [16]. The experiments were performed in duplicates and independently repeated in triplicates.

2.4. Immunohistochemical assay

Immunohistochemical staining was performed using the strept avidin–biotin complex method. Peri-cancer tissues from the same patient with GC were used as negative control. Phosphate buffer saline (PBS) was used as blank control in replacement of primary antibodies and the positive samples supplied by the vendor were used as positive controls [16].

2.5. Cell culture and flow cytometry

KATO-III cells were cultured in ATCC containing 20% fetal bovine serum. The SGC7901, AGS and MKN-45 cells were cultured in Roswell Park Memorial Institute 1640 containing 10% fetal bovine serum. All cells lines were maintained in a humidified atmosphere at 37 °C with 5% CO_2. They were rinsed in PBS and were suspended again to a density of 1×10^5 cells/mL and then 300 μL of this cell suspension was incubated with FcR blocking reagent (100 μL) and CD133-PE antibody (100 μL) for 30 min at 4 °C in darkness. The immunoglobulin G-PE antibody was used as negative control. The cell suspension (500 μL) was rinsed twice with PBS to determine the percentage of $CD133^+$ cell subpopulation with flow cytometry (Becton Dickinson, US) [18].

2.6. Immunomagnetic separation (IMS)

KATO-III cells were harvested at a density of 1×10^7 cells/mL. The cell suspension (300 μL) was separated using MiniMACS as instructed by the vendor [19]. The $CD133^+$ and CD133-negative ($CD133^-$) cells were resuspended in serum-free ATCC media supplemented with 20 ng/mL hEGF and 10 ng/mL bFGF. Flow cytometry was used to determine the percentages of $CD133^+$ cell subpopulations prior to and following the separation.

2.7. Cell counting kit-8 (CCK-8) assay

Following the separation, $CD133^+$ and $CD133^-$ cells were resuspended in serum-free media supplemented with 20 ng/mL hEGF and 10 ng/mL bFGF at a density of 1×10^4 cells/mL. The cells were seeded in 96-well plates at a volume of 200 μL/well. The cells were incubated with CCK-8 for 24 h and then the absorbance at 450 nm was measured to calculate cell growth rates.

2.8. Colony-forming assay

The $CD133^+$ and $CD133^-$ cells were cloned through the limiting dilution as previously reported [20]. The cells were resuspended to a density of 500 cells/mL and were seeded in 96-well plates at a volume of approximately 2 μL/well. Under an inverted phase-contrast microscope (Olympus, Japan), the wells containing single cell were supplemented with 200 μL of ATCC serum-free media with 20 ng/mL hEGF and 10 ng/mL bFGF. The wells containing the clone cultures were appropriately labeled and the cells were dissociated, collected and passaged onto 24-well plates when the cells almost reached the confluency.

2.9. RT-PCR of stem cell-associated markers

The sphere clone cells in $CD133^+$ subset group and $CD133^-$ subset group were collected for RT-PCR as previously reported [17,21]. The primer sequences were presented in Table 1. The experiments were duplicated and independently repeated in triplicates.

2.10. Drug resistance testing and cell apoptosis-associated gene profiling

Selected $CD133^+$ and $CD133^-$ cells were resuspended in serum-free ATCC media supplemented with 20 ng/mL hEGF and 10 ng/mL bFGF at a density of 1×10^4 cells/mL. The cells were seeded in 96-well plates at a volume of 200 μL per well. Fluorouracil (5-FU, 5–20 mmol/L), cisplatin (5–20 mmol/L) and etoposide (VP-16, 0.1–0.4 mmol/L) were supplemented in triplicates for either concentration of each agent. Following 24 h treatment, CCK-8 assay was performed to reflect the number of viable cells and further cell growth suppression rates for each chemotherapeutic agent. The cell growth suppression rate = $[(A_{blank} - A_{experiment} - A_{zeroing})/(A_{control} - A_{zeroing})] \times 100\%$ [21,22]. The cells were seeded in 6-well plates (2 mL/well) and were treated with 10 mmol/L 5-FU, 10 mmol/L cisplatin and 0.2 mmol/L VP-16 for 24 h. The total RNA was extracted to analyze the apoptosis-associated genes.

2.11. Subcutaneous tumor as xenotransplantation in nude mice

Six-week-old male nude athymic BALB/c mice were used to examine tumorigenicity. Sphere clone cells from single $CD133^+$ cell, $CD133^+$ cells, $CD133^-$ cells and unselected cells all of which were isolated from KATO-III cells were injected into the left axillary subcutaneous tissues of nude mice ($n = 5$ per group) (1×10^4 cells in 0.2 mL volume). The outcome indicators were consisted of the survival rate of mice, the growth rate, the volume and the mass of xenograft tumors. All animal work was carried out according to institutional guidelines.

2.12. Statistical analysis

SPSS 16.0 (SPSS Inc, USA) was used for the statistical analysis. Quantitative data were expressed as mean ± SD and analyzed by One-way ANOVA or student's *t*-test. The ratio data were expressed as percentage and compared using the Fisher's exact probability test. The survival curves were plotted using the Kaplan–Meier method and compared using the Log-rank test. Statistical significance was defined as $P < 0.05$.

3. Results

3.1. CD133 expression in primary lesion of GC in relation to patients' survival

Based on the immunohistochemistry results, the CD133 positivity rates were 66% (33/50) in primary lesion of GC and

Table 1
Primers sequences and primary functions of stem cell-associated genes.

Genes	Primers		Product size (bp)	Main functions
Sox-2	Upstream	5′-CAAGATGGCCCAGGAGAACC-3′	518	Transcription factor in early embryonic development
	Downstream	5′-GCTGCGAGTAGGACATGCTGTA-3′		
Nanog	Upstream	5′-CAGCTGTGTGTACTCAATGATAGATTT-3′	179	Transcription factor in early embryonic development
	Downstream	5′-ACACCATTGCTATTCTTCGGCCAGTTG-3′		
Oct-4	Upstream	5′-GGCGTTCTCTTTGGAAAGGTGTTC-3′	577	Transcription factor in early embryonic development
	Downstream	5′-CAAAGCTCCAGGTTCTCTTG-3′		
Musashi-1	Upstream	5′-TAATTCCTGTCCAGCAGTCTC-3′	317	Stem cell/progenitor cell inhibitive transcription factor
	Downstream	5′-GAACCATCCCGTCCTGTATCAT-3′		
EGFR	Upstream	5′-TAACAAGCTCACGCAGTTGG-3′	202	Epidermal growth factor receptor
	Downstream	5′-GCCCTTCGCACTTCTTACAC-3′		
ABCG2	Upstream	5′-GCGACCTGCCAATTTCAAAT-3′	311	Multiple drug resistance-associated factor in tumor cells
	Downstream	5′-AGCCAGTTGTAGGCTCATCCA-3′		
Bcl-2	Upstream	5′-TTGGATCAGGGAGTTGGAAG-3′	295	Anti-apoptotic factor
	Downstream	5′-TGTCCCTACCAACCAGAAGG-3′		
BAX	Upstream	5′-AAGAAGCTGAGCGAGTGT-3′	265	Pro-apoptotic factor
	Downstream	5′-GGAGGAAGTCCAATGTC-3′		
GAPDH	Upstream	5′-ACGGATTTGGTCGTATTGGGCG-3′	197	Internal control
	Downstream	5′-CTCCTGGA AGATGGTGATGG-3′		

10% (5/50) in paired peri-cancer tissues ($P < 0.001$). The $CD133^+$ particles were rarely observed in paired peri-cancer tissues but were mainly observed on the membrane of tumor cell in primary lesion of GC (Figure 1A,B). The CD133 mRNA was found to be increased in GC tissue compared with the paired peri-cancer tissue ($P < 0.001$) (Figure 1C,D). Similarly, the CD133 protein was demonstrated to be elevated in GC tissue compared with the paired peri-cancer tissue ($P < 0.001$) (Figure 1E,F).

For the survival analysis, the average value (0.6205 ± 0.1119) of CD133 protein expression for GC tissue with the range of 0.471–0.924 was set as the cut-off. The CD133 expression was 0.542 ± 0.046 for the lower positivity expression group ($n = 25$) and 0.728 ± 0.093 for the higher positivity expression group ($n = 25$). The Kaplan–Meier survival analysis showed that the lower $CD133^+$ expression group had a significantly better survival compared with the higher $CD133^+$ expression group ($P = 0.014$) (Figure 1G).

3.2. Enrichment and characterization of $CD133^+$ subpopulation in human GC cells

The percentage of $CD133^+$ cell subpopulation was (28.0 ± 2.0)% for KATO-III cells (Figure 2A), (16.5 ± 1.5)% for SGC-7901 cells, (6.0 ± 2.0)% for AGS cells and (4.0 ± 2.0)% for MKN-45 cells by flow cytometry before IMS and floating culture. KATO-III cells were selected to enrich for further experiments. The percentage of $CD133^+$ cell subpopulation was (91.0 ± 3.0)% (Figure 2B) for $CD133^+$ selection group and (17.0 ± 4.0)% for $CD133^-$ selection group following the IMS. After 1 week in serum-free culture, the enriched level of $CD133^+$ cells reached (95.0 ± 2.0)% (Figure 2C) and

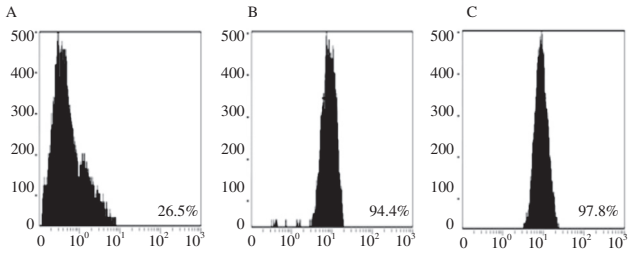

Figure 2. Representative graphs of flow cytometry and percentages of $CD133^+$ subpopulations in KATO-III cells before and after the immunomagnetic selection.
A: Unselected KATO-III cells; B: $CD133^+$ cells just following the immunomagnetic selection; C: $CD133^+$ cells at 1 week of serum-free culture following the immunomagnetic selection.

(14.0 ± 6.0)% for $CD133^+$ and $CD133^-$ selection groups respectively. In contrast, to exclude the false influence of $CD133^+$ antibody, the isotype-immunoglobulin G labeled in random control group exhibited $CD133^+$ cell subpopulation percentages of (1.5 ± 0.5)% and (1.3 ± 0.4)% following the IMS and then at 1 week after serum-free culture respectively.

3.3. In vitro characterization of $CD133^+$ cells

3.3.1. Clonal sphere-forming capacity

Following the limited dilution, single $CD133^+$ cells (Figure 3A) proliferated into 2–3 cell clones on Day 3–4 (Figure 3B), a small clone sphere with decade cells on Day 5–6 (Figure 3C) and into larger clone spheres containing several decades cells on Day 8–9 onwards (Figure 3D). In contrast, the clonal spheres could not be identified from the floating culture of single $CD133^-$ cells after the limited dilution.

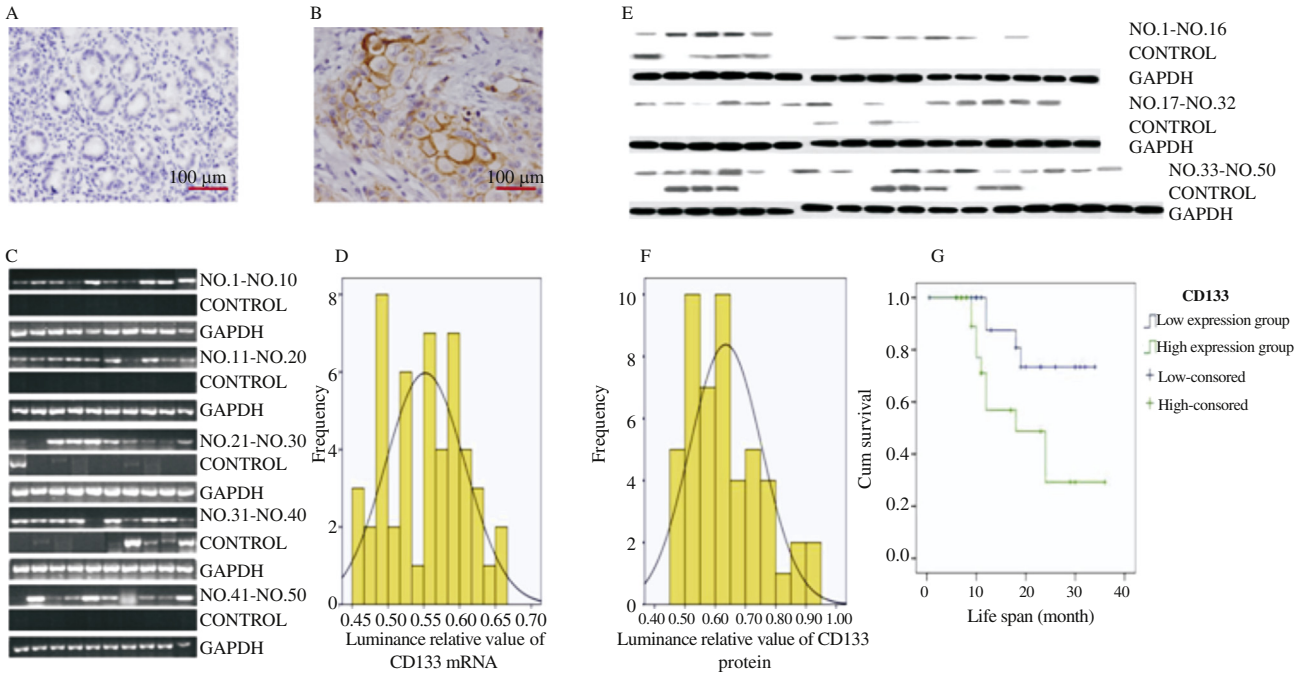

Figure 1. Expression of CD133 in primary lesion of GC and survival curves.
A: Negative expression in peri-cancer tissue by immunohistochemistry; B: Positive expression in primary lesion of GC by immunohistochemistry; C: Electrophoresis bands of CD133 mRNA in primary lesion ($n = 50$), in pared peri-cancerous tissues (control) ($n = 50$) and glyceraldehyde-3-phosphate dehydrogenase (GPADH) mRNA was used as internal control; D: Bar charts of CD133 mRNA of primary lesion; E: Electrophoresis bands of CD133 protein in primary lesion ($n = 50$), in pared peri-cancerous tissues ($n = 50$) and GAPDH protein was used as internal control; F: Bar charts of CD133 protein of primary lesion; G: Survival curves in higher $CD133^+$ expression group and in lower $CD133^+$ expression group.

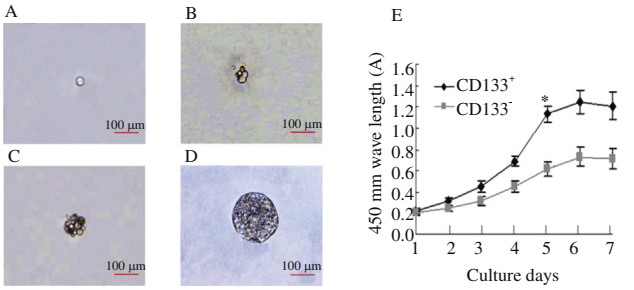

Figure 3. Clonal sphere formation of single CD133$^+$ cell after limited dilution and growth curves of CD133$^+$ cells and CD133$^-$ cells.
E: Comparison of the proliferation capacity of CD133$^+$ cells and CD133$^-$ cells by CCK-8 assay. $P < 0.05$, CD133$^+$ group *vs.* CD133$^-$ group.

3.3.2. CCK-8 assay

CD133$^+$ cells exhibited a significantly higher proliferative rate compared with CD133$^-$ cells (Figure 3E). Meanwhile, the population doubling time (21 ± 3) h for CD133$^+$ cells was significantly shorter than that for CD133$^-$ cells [(40 ± 8) h, $P = 0.001$].

3.3.3. Detection of mRNA regarding to stem cell-associated genes

RT-PCR assay indicated that the mRNA expressions of Sox-2, Oct-4, Musashi-1 and ABCG2 in CD133$^+$ clonal cell spheres were significantly higher than those in CD133$^+$ cells and CD133$^-$ cells respectively (Figure 4A). And the mRNA expression of Sox-2, Oct-4, Musashi-1 and ABCG2 in CD133$^+$ cells group were not significantly different from those in CD133$^-$ cells group (Figure 4A). However, the mRNA expression of Nanog and EGFR did not differ significantly among CD133$^+$ clonal cell spheres, CD133$^+$ cells and CD133$^-$ cells (Figure 4A).

3.4. In vivo tumorigenicity

The forming rates of xenograft tumor were 100% for clonal cells, 100% for CD133$^+$ cells and 80% for unselected cells. However, xenograft tumor couldn't be formed following the inoculation of CD133$^-$ cells. At 4 weeks following the inoculation, clonal cells group and CD133$^+$ cells group had significantly larger average volume and heavier average mass of xenograft tumor compared with those in the unselected cells group (Table 2).

Table 2
Tumorigenic capacity of xenografts-inoculated tumor.

Groups (n = 5)	Tumorigenic rate	Mean volume (mm^3)	P value	Mean mass (mg)	P value
Clonal spheres	100%	718.5 ± 183.3	0.001*	524.4 ± 101.8	0.002*
CD133$^+$ cells	100%	631.5 ± 243.2	0.005*	405.6 ± 126.3	0.008*
Unselected cells	80%	160.8 ± 135.8		116.7 ± 103.0	
CD133$^-$ cells	0%	N/A	N/A	N/A	N/A

N/A: Means not applicable; *: Compared with that in the group of unselected cells.

The xenograft tumors exhibited a round or oval shape with a pale as a macroscopic observation. The tumor tissue had more parenchymal cells but fewer interstitial cells in hematoxylin-eosine (H&E) section under microscopy. Tumor cells exhibited a cluster-band pattern with sparse necrotic foci. The tumor cells had a larger cell volume and abundant cytoplasms. Also, their cell nuclei had a larger volume and exhibited a polymorphic pattern (Figure 5A). Immunohistochemistry against CD133 showed that the positive granules were mainly located in cell membrane and cytoplasms (Figure 5B).

3.5. The gene profiles regarding apoptosis and growth inhibition associated with resistance to anti-tumor drug

3.5.1. Among the apoptosis-associated gene profiles

Unselected cells, CD133$^+$ and CD133$^-$ cells did not significantly differ in the mRNA expressions of Bcl-2 and Bax prior to the treatment. At 12 h following the treatment with anti-tumor agents, the Bcl-2 mRNA expression decreased, but the Bax mRNA expression increased significantly in unselected cells, CD133$^+$ cells and CD133$^-$ cells compared with those in the untreated control cells. Among treated cells, the Bcl-2 mRNA expression was significantly higher in CD133$^+$ cells compared with that in CD133$^-$ cells. Whereas the Bax mRNA expression was significantly lower in CD133$^+$ cells compared with that in CD133$^-$ cells (Figure 4B).

3.5.2. Tumor cell growth inhibition

The treated KATO-III cells exhibited growth suppression in the presence of chemotherapeutic agents of various concentrations compared with untreated control cells. At 12 h

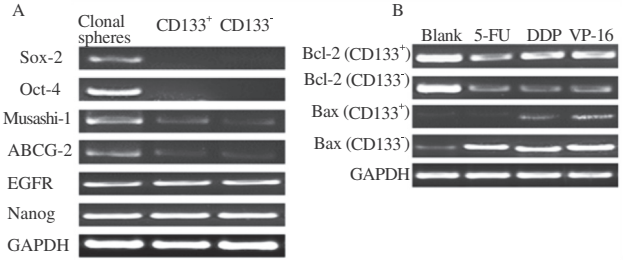

Figure 4. Representative mRNA expressions of ABCG2, EGFR, stem cell markers and apoptosis-associated genes.
A: Representative mRNA expression of ABCG2, EGFR, stem cell markers in clonal spheres, CD133$^+$ cells and CD133$^-$ cells; B: Representative mRNA expressions of apoptosis-associated genes in CD133$^+$ cell subpopulation and CD133$^-$ cell subpopulation. Blank group: Without treatment of anti-tumor agent. GPADH mRNA was used as internal control.

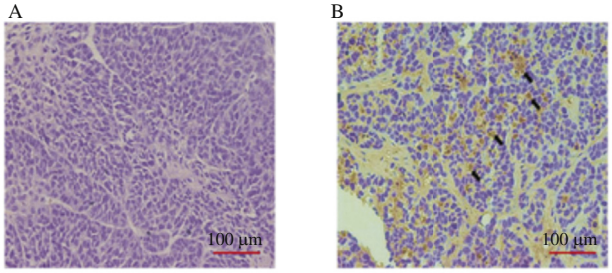

Figure 5. Microscopic observations with H&E staining and CD133 immunohistochemical staining on xenogeneic tumor by transplantation of CD133$^+$ cells in nude mice.
A: H&E staining profiles of the xenograft tumor; B: CD133$^+$ profiles in the xenograft tumor by immunohistochemical staining; Black arrow: CD133$^+$ cells.

following the treatment with 5-FU, cisplatin and VP-16, $CD133^+$ and $CD133^-$ cells exhibited an apoptosis-associated morphological alteration. At 48 h following the treatment, the number of apoptotic cells increased. Within the concentration range of 5–20 mmol/L for 5-FU and cisplatin and 0.1–0.4 mmol/L for VP-16, the number of apoptotic cells increased with the concentration elevated. Furthermore, $CD133^+$ and $CD133^-$ cells exhibited a similarly cellule morphology.

With the elevated doses of FU, cisplatin or VP-16, the growth inhibition rate of either $CD133^+$ cells or $CD133^-$ cells gradually increased while $CD133^+$ cells exhibited a significantly lower growth inhibition rate compared with $CD133^-$ cells at 12 h following the treatment of anti-tumor agents (Table 3).

Table 3
Growth inhibition rates of $CD133^+$ cells and $CD133^-$ cells by CCK-8 assessment under the treatment of 5-FU, cisplatin or VP-16. Mean ± SD, $n = 4$.

Anti-tumor agents	Concentrations	$CD133^+$	$CD133^-$	P value
5-FU	5	30.56 ± 1.99	32.81 ± 2.67	0.045
(mmol/L)	10	33.48 ± 2.32	45.87 ± 3.58	0.049
	15	67.52 ± 4.51	78.97 ± 2.88	0.045
	20	88.60 ± 1.95	95.73 ± 2.12	0.046
Cisplatin	5	45.89 ± 3.64	50.21 ± 3.22	0.043
(mmol/L)	10	60.83 ± 2.60	73.36 ± 1.72	0.049
	15	75.19 ± 2.22	82.29 ± 2.01	0.048
	20	81.20 ± 1.18	90.46 ± 1.89	0.049
VP-16	100	37.21 ± 3.80	35.55 ± 3.23	0.048
(μmol/L)	200	40.59 ± 5.82	43.02 ± 4.36	0.051
	300	59.33 ± 2.02	70.21 ± 2.35	0.042
	400	78.49 ± 3.22	89.32 ± 3.54	0.047

4. Discussion

The investigation into gastric mucosal epithelial cytokinetics, precancerous lesion, gastric microcancer and morphologically variable GC cells raises the doubt over the previous theory regarding the pathogenesis of GC. The histological origin of GC is still unclear. It is likely to derive from a single cell, namely, primitive stem cell, located in the granular isthmus [23]. Primitive stem cells are multipotent, whilst pro-cancerous factors and tumorigenicity process vary greatly. Therefore, GC exhibits a highly variable morphology and biological behavior.

Normal gastric epithelial stem cells or progenitor cells are primitive cells that are self-renewable and multiplicative as adult stem cells in some profiles. Gastric epithelial pluripotent stem cells differentiate into progenitor cells and further into various types of differentiated cells. This property plays an important role in gastric mucosal regeneration and homeostasis maintenance [24]. It is proposed in the theory of TICs that they may be originated from normal stem cells or pro-cancerous progenitor cells with cumulative genetic mutations [4]. TICs may differ from normal epithelial stem cells or progenitor cells in biological behavior and phenotype, but they are self-renewable, unlimitedly proliferative and multipotent [25]. As target cells with cumulative genetic mutations, TICs play an essential role in the very early stage of GC oncogenesis and are thought to cause abnormal proliferation, invasion and metastasis of GC [26].

Multiple cell markers are currently available for the identification of stem cells-like TICs. Various tumor precursor cells, especially of epithelial origin, highly express CD133, though tumor precursor cells in various tissues vary in phenotype [7–17]. The CD133 protein is located in the cell membrane projections and is involved in the formation of cell membrane topology. Meanwhile, the expression level of CD133 decreases rapidly along with the cell differentiation. Therefore, CD133 could be considered probably to be a molecular marker for the enrichment of stemness cells for further identification of them [27]. While CD133 is well documented as a pivotal TICs surface marker in current literature [28,29], TICs share identical surface markers with stemness cells from the perspective of histological origin.

Our previous study showed that $CD133^+$ cells existed in the tumor thrombi of primary GC vessels [30,31]. The expression level of CD133 mRNA has been revealed to be positively correlated with the lymph node metastasis but negatively associated with the expression level of Ki-67, a marker of tumor cell proliferation [16,30,31]. Therefore, it can be hypothesized that GC $CD133^+$ cell subpopulation could contain static primary and secondary TICs that may be more likely to be metastatic [32]. The upregulated CD133 expression is believed to be essential for the maintenance of gastrointestinal epithelial stem cells.

CD133 is found to be expressed on the cell surface as well as projections at the bottom of crypt, where it is thought to harbor stem cells [15]. Moreover, anti-CD133 antibody combined with chemotherapeutic agents antagonizes CD133 receptor and suppresses the *in vitro* proliferation of GC cells. Furthermore, it suppresses the xenograft tumor survival in nude mice [15]. Therefore, GC $CD133^+$ cells are potential tumorigenic TICs in some extent.

This investigation demonstrated that CD133 mRNA and protein were significantly higher in GC tissues compared with those in peri-cancer tissues of GC. Furthermore, among four GC cell lines, KATO-III cells had the highest percentage of $CD133^+$ cell subpopulation, which enabled the establishment of a high-purity $CD133^+$ cell subpopulation following their separation for further investigation of their biological features. IMS rendered the efficient enrichment of $CD133^+$ cell subpopulation from KATO-III cells. Following this separation, the percentage of $CD133^+$ cell subpopulation reached up to 90%. The selected cells exhibited no compromise in cell vitality. IMS overcame the technical drawbacks of flow cytometry, namely, cell contamination and reduced cell viability. The biological characteristics of GC cell lines with varying differentiation grade are yet to be studied regarding the percentage of $CD133^+$ cell subpopulation.

The propagation of TICs was the primary step for the investigation of TICs characteristics and was essential for the purification of TICs. Unlike conventional culture of passaged tumor cells, serum-free floating culture supplemented with EGF and bFGF was used to prevent tumor stem cells from differentiation in the process of culture and propagation. The bFGF is known to promote the asymmetric division of neural stem cells, which are self-renewable and responsive to EGF. In the presence of EGF, EGF-responsive stem cells are self-replicative to produce secondary bFGF-responsive neural stem cells through mitosis and asymmetric division [8,33,34]. Singh *et al*. isolated sphere cells from neuroepithelial tumor using serum-free culture media [8]. These cells were characteristic of tumor stem cells. Moreover, tumor stem cells have been identified in

colon and liver cancer [33,34]. Selected $CD133^+$ cells, more proliferative than $CD133^-$ cells, could be maintained, which suggested that differentiating tumor cells became apoptotic in the serum-free floating culture supplemented with growth factors. However, non-differentiable TICs maintained self-renewal and undifferentiation through asymmetric division. Following the supplementation of serum, $CD133^+$ cells decreased in number, emerged sparsely distributed, became adherent and became morphologically senescent. Moreover, $CD133^+$ cells turned from single cells into cell spheres containing dozens of cells within two weeks following further *in vitro* clonal cell culture, which showed that selected $CD133^+$ cell subpopulation might have stem cells-like self-renewal and clone-forming potential. The phenomenon of clonal cell spheres derived from single cell with $CD133^+$ demonstrated that the clonal cell spheres mainly consisted of $CD133^+$ cell subpopulation. These clonal cell spheres shared similar or even stronger proliferative potential compared with $CD133^+$ cell subpopulation.

The investigation of the mechanism of embryonic stem cell (ESC) self-renewal showed that Oct-4, Nanog and Sox-2 transcription factors formed a core regulatory ring controlling the transcription of 2260 mRNAs through modulating 16 major regulatory genes. These transcription factors contained an ESC-specific functional genome [35]. Selective knockdown of any gene located in this core regulatory ring resulted in the loss of ESC self-renewal but didn't disable their differentiation [36,37]. It has been reported that Msi-1 might be a surface marker of gastrointestinal epithelial stem cells and progenitor cells, which is assumed to maintain the undifferentiation of stem cells through the post-transcriptional translation [38,39]. The present study of ours may be the first report regarding the above mentioned stem cell markers in $CD133^+$ cell subpopulation from KATO-III cells. Meanwhile, both $CD133^+$ cell subpopulation and its clonal spheres expressed Msi-1 and EGFR. Additionally, $CD133^+$ clonal sphere cells expressed higher mRNA levels of Oct-4, Sox-2 and Musashi-1 compared with those in either $CD133^+$ cells or $CD133^-$ cell, which did not differ between $CD133^+$ cells and $CD133^-$ cells. All of these suggested that $CD133^+$ cell subpopulation might derive from stem cells of a single clone with colony-forming and replicative potential in GC cell lines *in vitro* and that $CD133^+$ clonal sphere cells might be classified as cancer stem cell while $CD133^+$ cells not existing in clonal sphere could not classified as cancer stem cell. There were not enough evidences demonstrating $CD133^+$ cells as cancer stem cells in this study, though $CD133^+$ cells not existing in clonal sphere shared some biological specificity like cancer stem cells *in vitro* and *in vivo*. This phenomenon indicated that some part of $CD133^+$ cells in clonal spheres being able to form clonal sphere possessed the ability of self-renewal and another part of them being able to differentiate relative matured cells with the loss of stem cell markers.

The tumorigenicity of TICs should be evaluated with regard to two aspects, *in vitro* clone-forming potential and *in vivo* xenograft tumor formation in immunodeficient animals [8]. This study determined the potent tumorigenicity of GC $CD133^+$ cells. Selected $CD133^+$ cells rendered the formation of xenograft tumor at the dose of 1×10^4 cells per animal, whereas $CD133^-$ cells could not form xenograft tumor. Further histological studies showed that $CD133^+$ cell subpopulation existed in the xenograft tumor similar to that in human primary GC tissue and that the patients' survival in lower $CD133^+$ protein expression group was better than that in higher $CD133^+$ protein expression group. Additionally, the analysis of xenograft tumor growth rate, volume and mass demonstrated that $CD133^+$ cells were more tumorigenic than unselected cells. These findings revealed that $CD133^+$ and $CD133^-$ cells differed in tumorigenicity with $CD133^+$ cells probably containing more TICs.

One of the problems regarding chemotherapy is that the untreated or recurrent tumors are primarily insensitive to antineoplastic or resistant to anti-tumor agents, which has preliminarily been investigated in the regulation of apoptosis-associated genes. This leads to many difficulties in our efforts to improve cancer patient survival. TICs usually remain static and intrinsically resistant though DNA self-repair and the action of ABC transport protein. Cross resistance emerges with anti-tumor agents with varying structure and mechanism of action [40,41]. Moreover, the resistance occurs in TICs and similar offspring cells through point mutation, genetic activation or gene amplification following the exposure to irradiation or procancerous factor [42].

The investigation of chemotherapy resistance in solid tumors associated with TICs shows that TICs are more resistant to chemotherapeutic agents than other tumor cells at rapid proliferation phase. TICs and offspring cells can proliferate in recurrent patients following chemotherapy or multi-drug resistant cell populations [43]. Additionally, multiple apoptosis-associated genes, such as Bcl-2, Bax, nuclear factor κB, mutant p53 and c-myc are all involved in tumor cell resistance [44–46].

Of chemotherapeutic agents, cell cycle specific agents refer to chemotherapeutic agents to which cells with specific phases (other than G_0-phase) are sensitive. Both 5-FU and VP-16 belong to cell cycle specific agents. The former agent acts on tumor cells at each phase of cell division, whereas the latter works on tumor cells at late S- or G_2-phase. As a cell cycle nonspecific agent, cisplatin targets at both proliferative and non-proliferative cell stages. The study on the resistance of KATO-III cells to these three agents in this investigation of ours showed that $CD133^+$ cell subpopulation had a significantly lower ability in the suppression rate of cell growth compared with that in $CD133^-$ cell subpopulation. Moreover, ABCG2, a resistance-associated gene, was expressed in $CD133^+$ cell subpopulation. Following the treatment with chemotherapeutic agents, anti-apoptotic Bcl-2 mRNA expression was down-regulated but pro-apoptotic Bax mRNA expression was up-regulated in $CD133^+$ cells.

In conclusion, the patients of GC with lower $CD133^+$ expression shared better survival in comparison with that in higher $CD133^+$ expression. $CD133^+$ cells, especially in $CD133^+$ clonal spheres with higher mRNA expressions of Oct-4 and Sox-2, shared stronger ability in proliferation, clone-forming potential, differentiable capability, tumorigenicity and resistant to chemotherapeutic agents probably regulated by Bcl-2 and Bax.

Conflict of interest statement

We declare that we have no conflict of interest.

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