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

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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

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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.