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Original Article

Study on like-stem characteristics of tumor sphere cells in human gastric cancer line HGC-27

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Abstract: Stem-like cancer cells are called cancer stem cells (CSCs) or tumor stem cells (TSCs). Methods for sorting CSCs are mainly based on the marker (CD133+/CD44+) or side population cells. However, CD133+/CD44+ cells or side population cells are very rare or even undetectable. In the present study, the tumor sphere of human gastric cancer (HGC) cell line HGC-27 was used for CSCs enrichment, and stem-like characteristics were verified by Hoechst 33342 staining technology, cell growth rate assays, sphere differentiation assay, clone formation, chemotherapy resistance study and tumor formation in an animal model. Our results demonstrated that the tumor sphere cells of HGC-27 cell line could be used to enrich CSCs, which may contribute to human gastric cancer stem cell biology research.

Keywords: Cancer stem cells, tumor sphere, gastric cancer, stem-like characteristics

Introduction

Human gastric cancer (HGC) is associated with multiple factors, including living condition, genetics, viral infection, and environment [1, 2]. Although advances in radiation and chemotherapy have improved the prognosis of individuals with HGC, the prognosis is still unsatisfactory as a result of therapeutic resistance and metastasis. Thus, studying cancer stem cells (CSCs) of HGC is significant to better understand its origin and progression. The concept of CSCs was introduced years ago to explain tumor cell heterogeneity, and recent studies suggested that CSCs may be responsible for tumorigenesis and contribute to resistance to cancer therapy. Recently, CSCs were isolated from several human tumors, including leukemia, breast cancer [3], brain tumors [4], prostate cancer [5], and ovarian cancer [6]. CSCs are more important than other tumor cells because they are capable of self-renewing, differentiating, and maintaining tumor growth and heterogeneity, playing an important role in both tumorigenesis and therapeutics. However, research has been hampered due to lacking good isolation method of CSCs. Fortunately, analysis of the hematopoietic system has shown that bone marrow stem cells contain a subpopulation that effluxes the DNA binding dye, Hoechst 33342, out of the cell membrane. These cells are called side population (SP) cells, which are shown to have stem cell characteristics and enrich the stem cell population [7-10]. It is now possible to obtain CSCs-like SP cells using a fluorescence-activated cell sorting (FACS) technique based on Hoechst 33342 efflux. In 1992, Reynolds and Weiss [11] demonstrated that adult mammalian brain contained cells giving rise to neurosphere clones. The culture method they used has been employed to isolate and characterize adult stem cells. Concurrent studies have confirmed that sphere culture systems can be used just as well to separate CSCs from many human cancers and cancer cell lines [12-18]. These studies have suggested that the CSCs can be enriched in the tumor spheres when cultured in serum-free medium supplemented with proper mitogens, such as EGF and BFGF. If it is also the case for HGC, it may be a convenient way to procure enough number of CSCs. We therefore investigated the stem-like characteristics of tumor sphere cells of human gastric cancer cell line HGC-27.

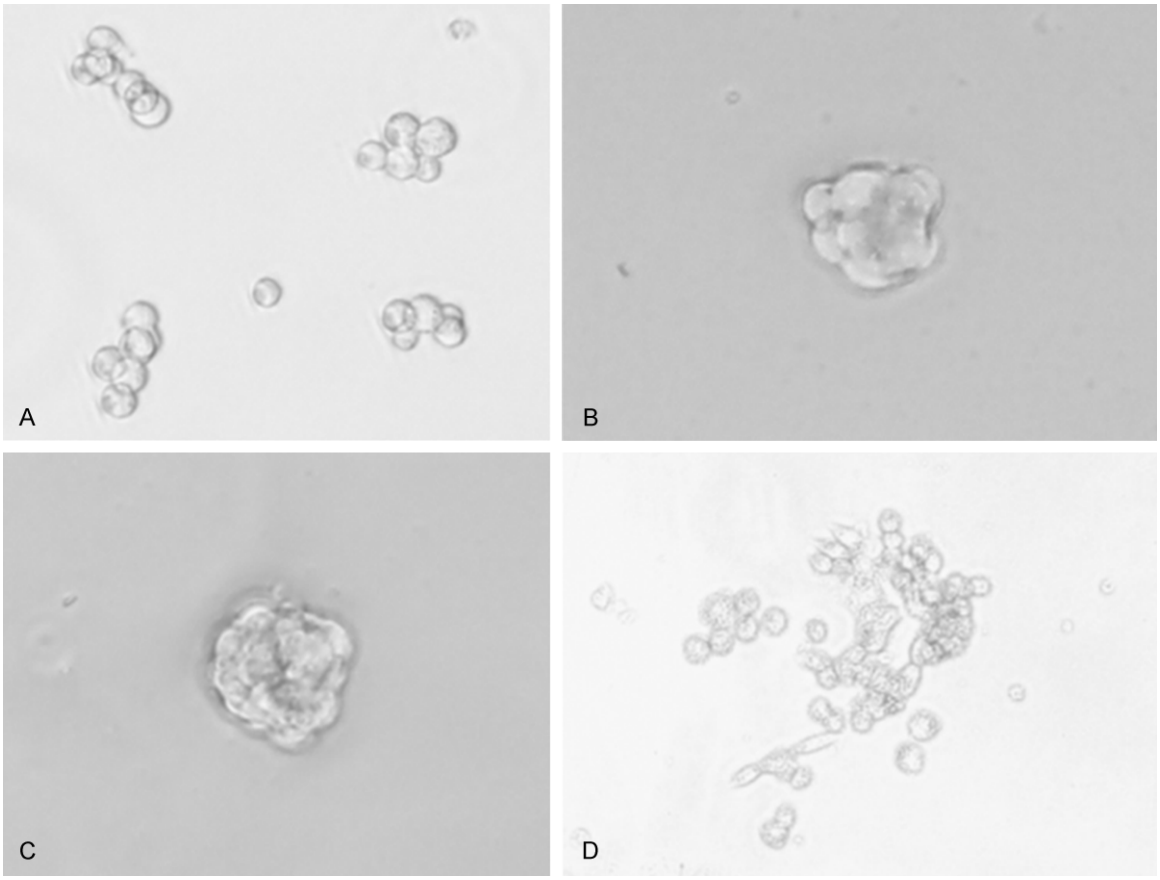

Figure 1. Formation and differentiation of HGC-27 tumor sphere cells. A. The HGC-27 cells were cultured in an incubator filled with 5% CO_2 at a temperature of 37°C. The cells were maintained in serum-supplemented medium, SSM. To induce tumor spheres formation, SSM was removed; then, the cells were dissociated by 0.25% trypsin for 3 minutes at a temperature of 37°C. The enzymatically dissociated single cells were plated at 2000 cells/well in serum-free medium, SFM in 6-well culture dishes. Two days after plating, spherical clusters were formed. B. One week after plating, tumor spheres were formed. C. Two weeks after plating, tumor spheres became bigger and pyknotic. D. To induce tumor sphere cells differentiation, the HGC-27 tumor spheres were plated in SSM again after two weeks in SFM. These sphere cells differentiated, adhered to the plastic and showed the typical morphologic features of differentiated cells.

Materials and methods

Materials

The epidermal growth factor (EGF), basic fibroblast growth factor (BFGF), trypsin and Hoechst 33342 dye were purchased from Sigma-Aldrich; the fetal bovine serum was obtained from Invitrogen, and the Dulbecco modified eagle medium (DMEM) and DMEM-F12 (1:1) were purchased from Hyclone; MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl 2H-tetrazolium bromide) was obtained from Promega, and nude mice were purchased from the animal institute of the Chinese Academy of Medical Science and Peking Union Medical College. The HGC-27 cells were purchased from Institute of Biochemistry and Cell Biology.

HGC-27 cell tumor spheres Culture and differentiation

The HGC-27 cells were cultured in an incubator filled with 5% CO_2 at a temperature of 37°C. The cells were maintained in serum-supplemented medium, SSM (DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin G, and 100 μg/ml streptomycin, 1 mmol/ml L-glutamine). To induce tumor spheres formation, SSM was removed, and then, the cells were dissociated by 0.25% trypsin for 3 minutes at a temperature of 37°C. The enzymatically dissociated single cells were plated at 2000 cells/well in serum-free medium, SFM (DMEM-F12 (1:1) supplemented with 10 ng/ml basic fibroblast growth factor (bFGF), 20 ng/ml epidermal growth factor (EGF), 100 units/ml

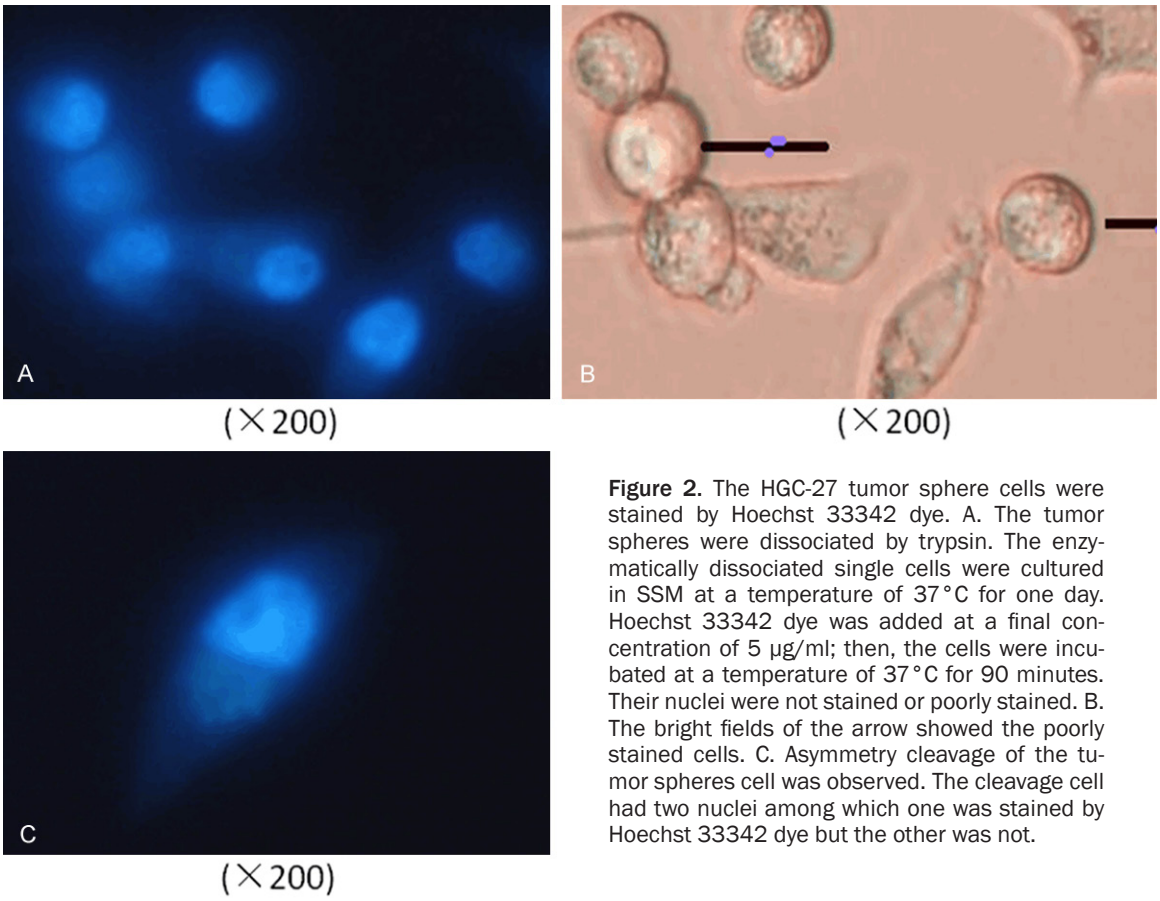

Figure 2. The HGC-27 tumor sphere cells were stained by Hoechst 33342 dye. A. The tumor spheres were dissociated by trypsin. The enzymatically dissociated single cells were cultured in SSM at a temperature of 37°C for one day. Hoechst 33342 dye was added at a final concentration of 5 μg/ml; then, the cells were incubated at a temperature of 37°C for 90 minutes. Their nuclei were not stained or poorly stained. B. The bright fields of the arrow showed the poorly stained cells. C. Asymmetry cleavage of the tumor spheres cell was observed. The cleavage cell had two nuclei among which one was stained by Hoechst 33342 dye but the other was not.

penicillin G, and 100 μg/ml streptomycin, 1 mmol/ml L-glutamine and without fetal bovine serum) in 6-well culture dishes. To induce tumor sphere cells differentiation, the HGC-27 tumor spheres were plated in SSM again after two weeks in SFM.

Hoechst 33342 staining

The HGC-27 tumor spheres were dissociated by trypsin. The enzymatically dissociated single cells were cultured in SSM at a temperature of 37°C for one day. Hoechst 33342 dye was added at a final concentration of 5 μg/ml, and then, the cells were incubated at a temperature of 37°C for 90 minutes. After incubation, the cells were washed gently twice with PBS for microscopic viewing and capturing.

Cell growth rate

The enzymatically dissociatedHGC-27 tumor sphere cells were diluted to a density of about $1/4\times10^4$ cells/ml with SSM, and then, 200 μl/well diluted cell suspension was plated to 96-well culture dishes. During 6 days, the absorbance of the cells was measured using the MTT method, and the cell growth curve was plotted according to the data. Data represented were the means of three independent experiments.

Clone formation

The HGC-27 cells and HGC-27 tumor spheres were enzymatically dissociated as described above. The dissociated cells were diluted to a density of about 5 cells/ml with SSM, and then, the 200 μL/well diluted cell suspension was plated to 96-well culture dishes. The wells with 1 cell were observed every day. After two weeks, the clone number was counted. The clone formation efficiency (CFE) was the ratio of the clone number to the planted cell number. Data represented were the means of three independent experiments.

Chemotherapy resistance studies

The enzymatically dissociated HGC-27 cell and HGC-27 tumor spheres cell were plated in 96-well culture dishes with 1×10^4 cell/well respectively. Chemotherapeutic agents, cisplatin and

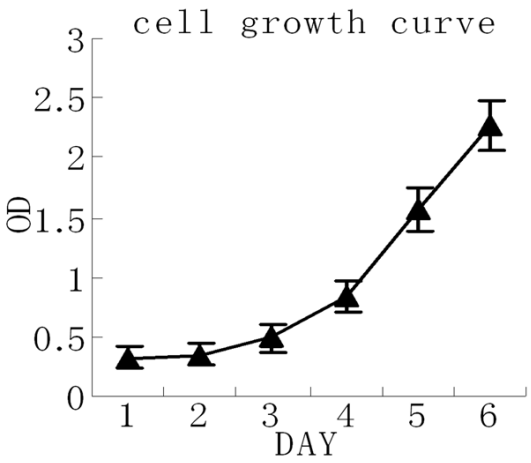

Figure 3. The cell growth curve of HGC-27 tumor sphere cells within 6 days after replated in SSM. The enzymatically dissociated HGC-27 tumor sphere cells were diluted to a density of about $1/4\times10^4$ cells/ml with SSM; then, 200 μl/well diluted cell suspension was plated to 96-well culture dishes. During the 6 days, the absorbance of the cells was measured using the MTT method, and the cell growth curve was plotted according to the data. Data represented were the means of three independent experiments. The result showed that the cell growth rate was lower in the first two days and higher in the last two days.

Table 1. The clone formation efficiency of the HGC-27 cells and the HGC-27 tumor sphere cells

Cell types	Planted cell number	Clone number	CFE	Mean
HGC-27 cell	93	0	0	
	89	1	1.11%	0.77%
	104	2	1.19%	
Sphere cell	97	9	9.27%	
	112	10	8.93%	8.71%*
	101	8	7.92%	

Note: Compared to HGC-27 cell, *P<0.05.

etoposide, were added at the following final concentrations: 15 μg/mL, 30 μg/mL, 45 μg/mL. Cell viability was evaluated at 24 hours and 48 hours after treatment with MTT. Data represented were the means of three independent experiments.

Tumor formation in an animal model

The HGC-27 cell and HGC-27 tumor spheres were enzymatically dissociated for single cell suspensions. The cells were suspended in 200 μL PBS and kept at a temperature of 4°C until subcutaneous injection at the neck back of 5-week-old female nude mice. 1×10^3, 1×10^4, 1×10^5, 5×10^6, and 1×10^7 cells suspended in PBS were injected into each mouse, respectively. The mice were monitored twice weekly for palpable tumor formation and photographed. Eight weeks after injection, all mice were killed by cervical dislocation, and then the presence of tumor was confirmed by necropsy.

Statistical analysis

Statistical analysis was performed using SPSS16.0 software. Count data were analyzed by chi-square test, and measurement data by independent samples t-test. P<0.05 was considered as statistically significant.

Results

The HGC-27 cells formed spheres and HGC-27 tumor spheres cell differentiated

In this study, the HGC-27 cells were plated to SFM at 2000 cells/well in 6-well culture dishes. The formation of tumour spheres was observed under an inverted light microscope. Two days after plating, spherical clusters were formed. One week after plating, tumor spheres were formed. Two weeks after plating, tumor spheres became bigger and pyknotic. Conversely, after replated in SSM, these sphere cells differentiated, adhered to the plastic and acquired the typical morphologic features of differentiated cells (shown in **Figure 1**).

Some of the HGC-27 tumor spheres cell had the capability of excluding hoechst 33342 dye and showed asymmetry cleavage

The dissociated HGC-27 tumor sphere cells were stained by Hoechst 33342 dye. The results showed that some were resistant to Hoechst 33342 dye and the nuclei were not stained or poorly stained. In addition, asymmetric cleavage of the tumor spheres cells was observed (shown in **Figure 2**). The cleavaging cell had two nuclei, among which one was stained by Hoechst 33342 dye but the other was not.

The HGC-27 tumor sphere cell growth curve

MTT method was used to determine the growth rate of the enzymatically dissociated HGC-27 tumor sphere cells from the 2nd day after plated in SSM. The result showed that the cell

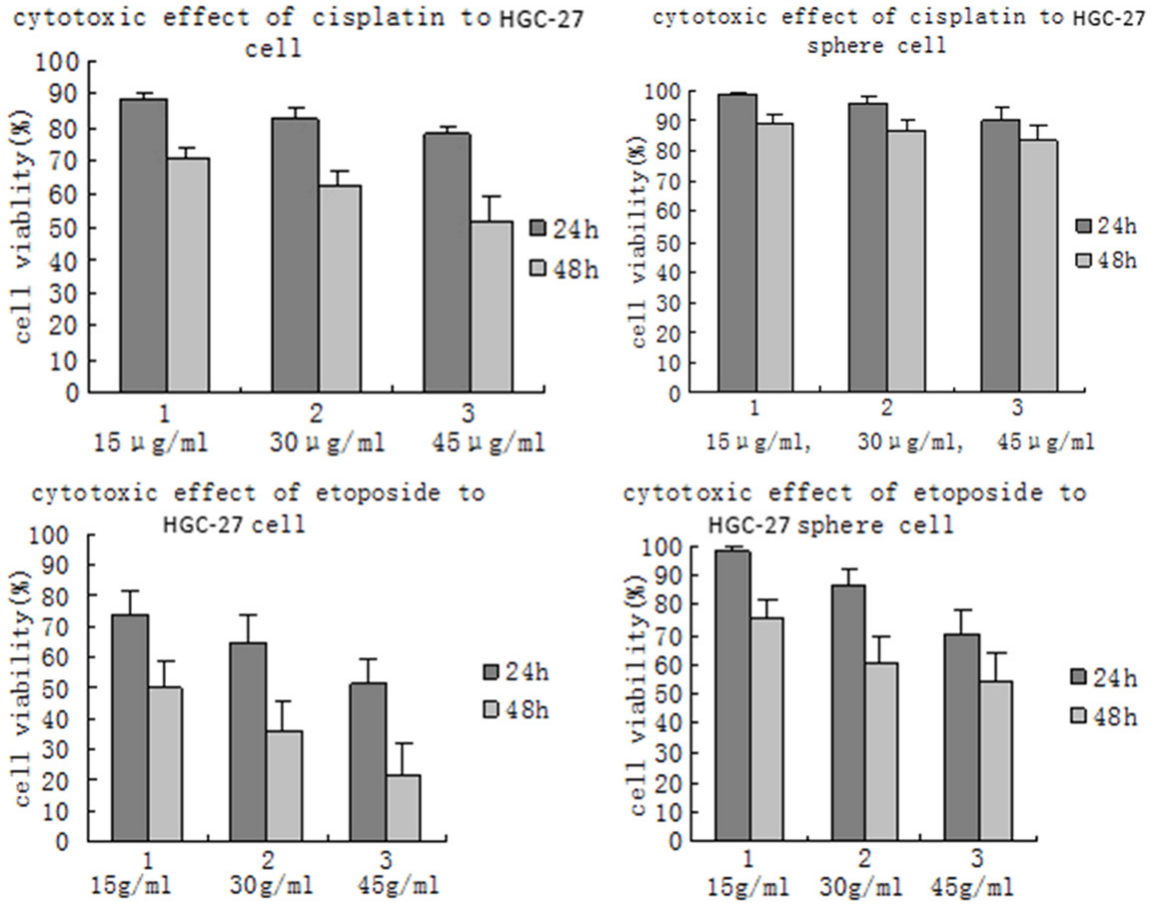

Figure 4. The viability of chemotherapy-treated HGC-27 cells and HGC-27 tumor sphere cells. The enzymatically dissociated HGC-27 cell and HGC-27 tumor spheres cell were plated in 96-well culture dishes with 1×10^4 cell/well respectively. Chemotherapeutic agents, cisplatin and etoposide, were added at the following final concentrations: 15 μg/ml, 30 μg/ml, 45 μg/ml. Cell viability was evaluated at 24 hours and 48 hours after treatment with MTT. Data represented were the means of three independent experiments. The result showed that cisplatin and etoposide displayed lower cytotoxic activity on HGC-27 tumor sphere cells than HGC-27 cells. The cytotoxic activity of etoposide was greater than cisplatin. The cytotoxic activity of cisplatin and etoposide increased gradually along with the increase of chemotherapic agent concentration.

growth rate was lower in the first two days and higher in the last two days (shown in **Figure 3**).

The clone formation capability of HGC-27 cells and HGC-27 tumor sphere cells

In order to identify whether HGC-27 cells and HGC-27 tumor sphere cells could form clone in 96-well culture dishes, 100 HGC-27 cells or HGC-27 tumor sphere cells were seeded into two 96-well culture dishes respectively. The clone number was counted after two weeks and it was found that the cloning forming rates of the HGC-27 tumor sphere cells were higher than those of HGC-27 cells. There was significant significance between the two groups ($P< 0.01$) (shown in **Table 1**). Data represented were the means of three independent experiments.

Resistant to conventional chemotherapy of HGC-27 cells and HGC-27 tumor sphere cells

As shown in **Figure 4**, the cytotoxic effect of the chemotherapeutic agents, cisplatin and etoposide, was investigated, which are currently used in the clinical setting on gastric cancer. The result showed that cisplatin and etoposide displayed lower cytotoxic activity on HGC-27 tumor sphere cells than HGC-27 cells. The cytotoxic activity of etoposide was greater than cisplatin. The cytotoxic activity of cisplatin and etoposide increased gradually along with the increase of chemotherapeutic agent concentration.

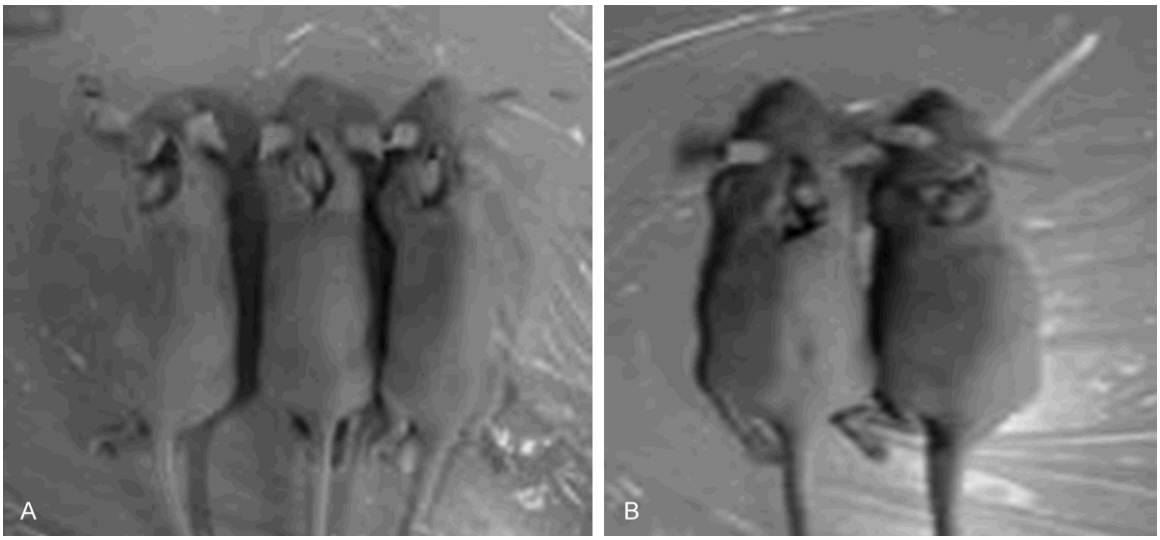

Figure 5. Tumorigenesis of HGC-27 cells and HGC-27 tumor sphere cells in nude mice. A. The HGC-27 cell and HGC-27 tumor spheres were enzymatically dissociated to obtain single cell suspensions. The cells were suspended in 200 μL PBS and kept at a temperature of 4°C until subcutaneous injection at the neckback of 5-week-old female nude mice. 1×10^3, 1×10^4, 1×10^5, 5×10^6, and 1×10^7 cells suspended in PBS were injected into each mouse respectively. The mice were monitored twice weekly for palpable tumor formation and photographed. Eight weeks after injection, all mice were killed by cervical dislocation; then, the presence of tumor was confirmed by necropsy. Tumorigenesis of 1×10^5, 1×10^6, and 1×10^7 HGC-27 tumor sphere cells in three mice. B. Tumorigenesis of 1×10^6 and 1×10^7 HGC-27 cells in two mice.

Tumorigenesis of HGC-27 cells and HGC-27 tumor sphere cells in nude mice

In this experiment, 1×10^3, 1×10^4, 1×10^5, 1×10^6 and 1×10^7 HGC-27 cells and HGC-27 tumor spheres cells suspended in 200 μL PBS were injected s.c. in neck back of each mouse, respectively. In the consecutive observation for eight weeks, it was found that the HGC-27 tumor sphere cells had stronger tumorigenic potential in nude mice than HGC-27 cells. The lowest amounts of HGC-27 cells and HGC-27 tumor spheres cells to induce tumorigenesis were 1×10^6 and 1×10^5, respectively (**Figure 5**).

Discussion

Although the 5-year survival rate of HGC has improved as a result of advances in radiation and chemotherapeutic strategies, the long-term prognosis is still poor [19, 20]. Thus, it is important to further elucidate the essence and origin of this cancer. It is well known that tumor is composed of heterogeneous cell types. Evidence suggests that tumor is a disease of stem cells, which, like a normal organ, contains a small population of cells with a high proliferative capacity, self-renewing potential, multi-differentiation ability, and resistance to chemotherapy and radiotherapy [21, 22]. All these characteristics are similar to normal adult stem cells and even embryonic stem cells. Consequently, this subpopulation of cells is named CSC or tumor stem cells (TSC) [23-25]. Recent advances in stem cell research have demonstrated that the CSCs can be enriched in the spheres when cultured in serum-free medium supplemented with proper mitogens, such as EGF and bFGF. To obtain cells with stem cell properties from HGC-27 cell lines, HGC-27 tumor sphere cells were enriched according to the procedures of Suzuki et al. [26] and Dontu et al. with some changes [27].

In this study, the formation of HGC-27 tumor spheres was observed under an inverted light microscope. Conversely, after replated in SSM, these sphere cells differentiated. The dissociated sphere cells were stained by Hoechst 33342 dye, and the result showed that some of the tumor cells excluded Hoechst 33342 dye with nuclei unstained (or poorly stained).

Self-renewal and differentiation are properties of stem cells that allow them to generate additional CSC and phenotypically diverse cancer cells with a limited proliferation potential. In vitro experiments revealed that HGC-27 tumor

sphere cells grew faster than HGC-27 cells and had a higher CFE. In addition, in vivo treatment of nude mice showed that 100000 tumor sphere cells were required to form the tumor, whereas at least 1000000 HGC-27 cells were necessary to form the tumor. Thus, the tumor formation ability of tumor sphere cells was 10 times higher than that of HGC-27 cells. Asymmetrical cell division is a particularly attractive mechanism because only one division is required for self-renewal and differentiation [28, 29]. In this study, we found asymmetrical cell division with Hoechst 33342 fluorescent dyeing. The cleavage cells had two nuclei, among which one was stained by Hoechst 33342 dye but the other was not. Perhaps they were cleavage CSC.

According to the CSC theory, not all cancer cells are created equally [30]. Tumors may have a built-in population of pluripotent cells that can survive chemotherapy and radiotherapy, and the resident CSC may repopulate the tumor even when the bulk of non-tumorigenic cells are killed [31]. The Hoechst 33342 exclusion ability conferred by ABC transporters forms the basis for the CSC, and many chemical drugs may be pumped out of cells in the same way. Because they are the primary treatment for cancer patients, we also conducted a drug sensitivity assay to investigate whether HGC-27 tumor sphere cells could resist treatment more readily than HGC-27 cells. In the drug sensitivity assay, the HGC-27 tumor sphere cells were more resistant to cisplatin and etoposide than HGC-27 cells [32, 33].

Our results supported that HGC-27 tumor sphere cells enriched from HGC-27 cell had many properties of stem cells, including unlimited proliferation potential, self-renewal, differentiation, resistance to chemotherapy, and strong tumor formation ability. These findings suggested that HGC-27 tumor sphere cells may be a novel field of tumor research, enhancing our understanding about tumor formation, maintenance andtreatment.

Disclosure of conflict of interest

None.

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