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Preliminary screening and identification of stem cell-like sphere clones in a gallbladder cancer cell line GBC-SD*

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Abstract: This paper aims to screen and identify sphere clone cells with characteristics similar to cancer stem cells in human gallbladder cancer cell line GBC-SD. GBC-SD cells were cultured in a serum-free culture medium with different concentrations of the chemotherapeutic drug cisplatin for generating sphere clones. The mRNA expressions of stem cell-related genes CD133, OCT-4, Nanog, and drug resistance genes ABCG2 and MDR-1 in sphere clones were detected by quantitative real-time polymerase chain reaction (PCR). Stem cell markers were also analyzed by flow cytometry and immunofluorescent staining. Different amounts of sphere clones were injected into nude mice to test their abilities to form tumors. Sphere clones were formed in serum-free culture medium containing cisplatin (30 μmol/L). Flow cytometry results demonstrated that the sphere clones expressed high levels of stem cell markers $CD133^+$ (97.6%) and $CD44^+$ (77.9%) and low levels of $CD24^+$ (2.3%). These clones also overexpressed the drug resistance genes ABCG2 and MDR-1. Quantitative real-time PCR showed that sphere clones expressed stem cell genes Nanog and OCT-4 284 and 266 times, respectively, more than those in the original GBC-SD cells. Immunofluorescent staining showed that sphere clones overexpressed OCT-4, Nanog, and SOX-2, and low expressed MUC1 and vimentin. Tumor formation experiments showed that 1×10^3 sphere clone cells could induce much larger tumors in nude mice than 1×10^5 GBC-SD cells. In conclusion, sphere clones of gallbladder cancer with stem cell-like characteristics can be obtained using suspension cultures of GBC-SD cells in serum-free culture medium containing cisplatin.

Key words: Gallbladder cancer, Stem cell gene, Sphere clone, Suspension culture
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1 Introduction

Cancer stem cells (CSCs), which are undifferentiated cancer cells, are present in various types of cancers and exhibit characteristics of self-renewal and multi-differentiation. They play an important role in tumor formation and associate closely to patient's prognosis. Only a small portion of cells in the tumors exhibit stem cell-like properties and play decisive roles in tumor occurrence and progression (Clarke *et al*., 2006). However, it has been demonstrated that they be isolated by several ways including surface marker sorting (Al-Hajj *et al*., 2003; Singh *et al*., 2004), side-population cells selecting (Hadnagy *et al*., 2006; Moserle *et al*., 2010), and mammosphere cultures (Ponti *et al*., 2005; Yu *et al*., 2008). Most CSCs form clones when nurtured in low adhesive vessels (Chumsri and Burger, 2008; Kobayashi *et al*., 2008; Gilbert and Ross, 2009), and further generate sphere clones in the present of growth factors such as epidermal growth factor (EGF). Therefore the sphere clone formation assay has been widely used for the separation and identification of CSCs (Ponti *et al*., 2005; Yu *et al*., 2008).

The separation of gallbladder CSCs has hitherto not been reported. In the present study, we isolated sphere clones with characters similar to gallbladder CSCs using serum-free medium containing cisplatin.

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This study for the first time established the method and characterization toward the separation and identification of gallbladder CSCs.

2 Materials and methods

2.1 Materials

Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA, USA). EGF, basic fibroblast growth factor (bFGF), Noggin, leukocyte inhibitory factor (LIF), and cisplatin were purchased from Sigma (St. Louis, MO, USA). Fluorescence quantitative polymerase chain reaction (PCR) kit and reverse transcription kit were purchased from Toyobo (Osaka, Japan). The flow sorter was obtained from BD Biosciences (San Jose, CA, USA). The fluorescence microscopy and digital image photography systems were purchased from Olympus (Tokyo, Japan). GBC-SD cell lines were provided by the Qilu Medical College (Jinan, Shandong Province, China). Nude mice were purchased from Shanghai Experimental Animal Center of the Chinese Academy of Sciences, China. Four-week old female mice were used in the study. All the experimental protocols involving live animals were reviewed and approved by Shanghai Medical Experimental Animal Care Commission.

2.2 Drug-resistant gallbladder cancer GBC-SD cell culture and screening

The GBC-SD cells were cultured in DMEM medium containing 10% (v/v) FBS at 37 °C with 5% CO_2. Fresh medium was changed daily. When 85% cells were adhered, the medium was replaced, and the cells were cultured for 48 h. When cells reached 95% confluences in the exponential growth phase, they were used for subsequent experiments. Cisplatin (30 μmol/L) was added to the culture medium, and the cells were cultured for another 24 h. Cell culture media were removed along with unattached cells and cells were cultured in the same medium with cisplatin for 48 h. Then, cells were collected with 0.25% (v/v) trypsin-EDTA, and the cell pellets were suspended in a serum-free DMEM medium (CSC culture medium) containing 10 ng/ml EGF, 10 ng/ml bFGF, 10 ng/ml Noggin, 1000 U/ml LIF, and 20 μmol/L cisplatin to prevent cells from adhering and differentiating.

2.3 Tumorigenic cell preparation from nude mice and sphere clone culture

Cisplatin-screening GBC cells were injected into nude mice. Tumors were formed in four weeks and were removed. The excessive tissues around the tumors were trimmed, and the tumor tissues were cut into pieces. Trypsin-EDTA (0.02%–0.25%) was repeatedly used to digest the tumor tissue for preparing single cell suspensions. The single cell suspensions were collected and cultured in the serum-free DMEM medium to obtain sphere clone cultures at 37 °C in a 5% CO_2 incubator. Half of the medium was replaced every two days. Cell growth was monitored, and adherent cells were discarded. The suspended cells or cell clusters were collected and diluted to single cell suspensions, and then re-inoculated in serum-free medium for continuous passages.

2.4 Screening of suspension cells and sphere clones by cisplatin treatment

Cisplatin was added to the serum-free medium at different concentrations ranging from 10 to 80 μmol/L. Cell survival was estimated 48 h later to determine the optimal cisplatin concentration. The cell suspensions and spheres were then cultured in medium containing the optimal cisplatin concentration.

2.5 Stem cell-associated gene expression profile in sphere clones by real-time PCR

Real-time PCR was used to examine the mRNA expressions of ABCG2, CD133, MDR-1, OCT-4, and Nanog genes. Total RNA was isolated from the sphere clones. Real-time PCR was performed in two steps. The amplification proportion of the target fragment was calculated by a relative quantitative method. 18S RNA was used as an internal reference. The annealing temperature was maintained at 60 °C, and 45 cycles of amplification were performed. The primers for quantitative PCR were designed using the DNAstar software (Table 1).

2.6 Detections of CSC markers OCT-4 and SOX-2 and cell differentiation markers MUC1 and vimentin by immunofluorescent analysis

Sphere clone cells were fixed in a 4% (v/v) paraformaldehyde solution at room temperature for 20 min, and then washed twice with 1× phosphate

Table 1 Primers for fluorescence quantitative PCR designed by using the DNAstar program

Gene	Primer pair sequence
18S RNA	5′-CCGCCGCGTCTTCAACCCCTACAC-3′ 5′-CCATCCCTGCCGGCATCATACTG-3′
ABCG2	5′-CTGAGATCCTGAGCCTTTGG-3′ 5′-AAGCCATTGGTGTTTCCTTG-3′
CD133	5′-TGGATGCAGAACTTGACAA-3′ 5′-ATACCTGCTACGACAGTCGTG-3′
MDR-1	5′-CCTGGCAGCTGGAAGACAAAT-3′ 5′-TCCTCCAAATGCAATCACAG-3′
OCT-4	5′-GGCCCGAAAGAGAAAGCG-3′ 5′-ACCCAGCAGCCTCAAAATCCTC-3′
MUC1	5′-GCACTCACCATAGCACG-3′ 5′-GGCCAGAGTCAATTGTAC-3′
Vimentin	5′-GGCTCAGATTCAGGAACAGC-3′ 5′-GCTTCAACGGCAAAGTTCTC-3′
GAPDH	5′-GGTGAAGGTCGGTGTGAACGGA-3′ 5′-TGTTAGTGGGGTCTCGCTCCTG-3′
SOX-2	5′-GCCGAGTGGAAACTTTTGTC-3′ 5′-GTTCATGTGCGCGTAACTGT-3′
Nanog	5′-GGGCCTGAAGAAAACTATCCA-3′ 5′-TGCTATTCTTCGGCCAGTTGT-3′

buffered saline (PBS) for 10 min each. The cells were subsequently blocked in 1× PBS buffer containing 0.2% (v/v) Triton-100 and 5% (v/v) normal goat serum at 32 °C for 1 h, followed by 5 min washing with PBST (1× PBS containing 0.2% Triton-100) for three times. The primary antibody was added, and the cells were incubated at 32 °C for 1 h, washed three times with PBST, and then labeled with FITC- or TRITC-labeled secondary antibody and Hoechst 33342. After incubation in the dark at 32 °C for 30 min, the cells were washed three times with PBST under dark conditions and photographed under a fluorescent microscope.

2.7 Detections of CD133, CD24, and CD44 expressions by flow cytometry and Hoechst 33342 exclusion test

The cells were labeled with anti-CD133, anti-CD24, and anti-CD44 monoclonal antibodies (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). After labeling, the cells were washed with PBS one time and subsequently stained with FITC- or PE-labeled rabbit anti-mouse IgG4 for 30 min in the dark. The cells were analyzed on a flow cytometer after three washes with PBS. Cell suspension was treated with Hoechst 33342 at a final concentration of 1 μg/ml. After incubation at 37 °C for approximately 7–10 min, the cells were centrifuged at 1000 r/min for 5 min. Flow cytometric analysis was then performed. Blue fluorescence was obtained due to the presence of Hoechst 33342, which is excited by ultraviolet (UV) fluorescence, which was produced using a krypton laser. The excitation wavelength of Hoechst 33342 is 352 nm, and its emission wavelength is in the range of 400–500 nm. The blue fluorescence topographic map was analyzed.

2.8 Tumor formation from gallbladder cancer sphere clones

Sphere clones were collected by centrifugation at 100×*g* for 10 min and cells were resuspended in PBS and counted. The cells were divided into two groups: sphere clone cells group and GBC-SD cells group. Five nude mice were used in each experimental group. The cell suspension (100 μl) was injected subcutaneously at four points in each mouse with different cell numbers from 1×10^5, 1×10^4, 1×10^3, to 1×10^2 cells. Tumors were formed in two months after injection. Tumor sizes were monitored and measured weekly.

2.9 Statistical analysis

The SPSS 10.0 statistical package was used for analysis. The Mann-Whitney test (rank sum test) was used for two independent samples.

3 Results

3.1 In vivo screening of drug-resistant GBC-SD cells

Cultured GBC-SD cells were injected into nude mice (Fig. 1a). Tumors were growing during the eight-week experimental period (Figs. 1b and 1c). The tumor volume in mice injected with 0.1 μmol cisplatin was significantly lower than that in control mice injected with 0.1 μmol PBS (Fig. 1d).

3.2 Induction of sphere clones in CSC culture medium and cisplatin

Tumors from mice treated with cisplatin were removed and subsequently cultured in a serum-free DMEM medium containing several growth factors (10 ng/ml EGF, 10 ng/ml bFGF, 10 ng/ml Noggin, and 1000 U/ml LIF; Fig. 2a). Two days later, some cells began to grow in suspension (Fig. 2b). One week

later, cells in suspension started to form clusters gradually (Fig. 2c). Two weeks after in vitro culture, cells exhibited definite amplification phenotypes with an increased cell number (Fig. 2d).

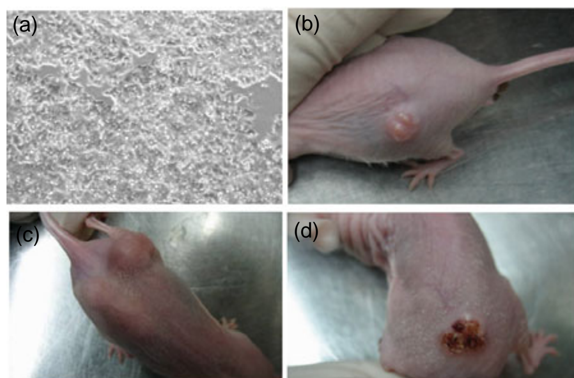

Fig. 1 Culturing of gallbladder cancer GBC-SD cells and in vivo screening of drug-resistant cells
(a) Normal culture of drug-resistant GBC-SD cell line; (b) Image of a nude mouse at three weeks after receiving 5×10^5 GBC-SD cells; (c) Image of a nude mouse at eight weeks after receiving 5×10^5 GBC-SD cells; (d) Image of a nude mouse at four weeks after receiving 5×10^5 GBC-SD cells. Cisplatin (0.1 μmol) was injected every week into the mouse subcutaneously

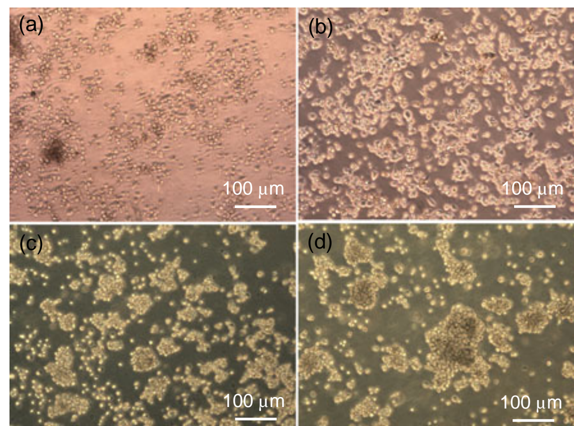

Fig. 2 Sphere clone induction in cancer stem cell culture medium
(a) Cell preparation from tumors from Fig. 1d (48 h after culture); (b) Cells cultured for 48 h in stem cell culture media; (c) Cells cultured for one week in stem cell culture media; (d) Cells cultured for two weeks in stem cell culture media

3.3 Formation of sphere clones with increased expressions of stem cell markers and drug-resistant genes

Two weeks after cultured with 30 μmol/L cisplatin, some cells underwent apoptosis, whereas survived cells were healthy and grew in tighter clusters (Fig. 3a). Four weeks later, sphere clones were observed (Fig. 3b). In high cisplatin concentrations groups (60–80 μmol/L), cells failed to proliferate and apoptosis occurred gradually (Figs. 3c and 3d). Quantitative real-time PCR analysis revealed that the expressions of Nanog, OCT-4, and CD133 in sphere clones were 284-, 266-, and 187-fold, respectively, higher than those in GBC-SD cells. The expressions of MDR-1 and ABCG2 also increased by 150-fold (Fig. 4). Flow cytometry analysis of sphere clones for stem cell markers revealed that 97.6% of the sphere clones were $CD133^+$, 2.3% were $CD24^+$, and 77.9% were $CD44^+$ (Fig. 5).

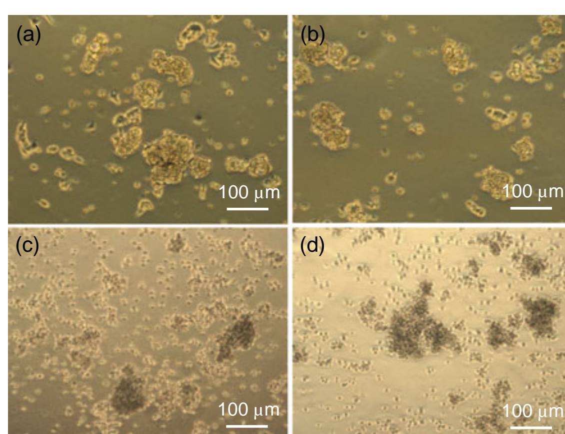

Fig. 3 Formation of drug-resistant sphere clones
(a) Two weeks after coculture with 30 μmol/L cisplatin, some cells underwent apoptosis. The surviving cells were healthy and grew in tight clusters. (b) After four weeks in 30 μmol/L cisplatin, the cells exhibited the typical morphology of sphere clones. (c, d) After two weeks in 60 (c) or 80 (d) μmol/L cisplatin, apoptosis gradually occurred in the cells

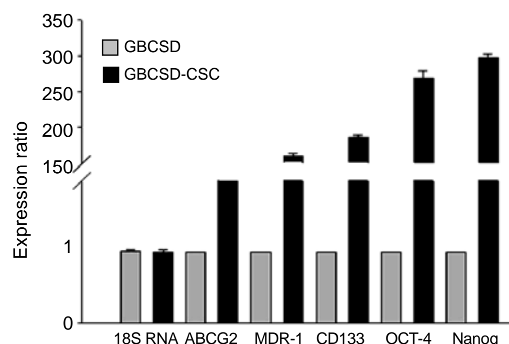

Fig. 4 Expressions of ABCG2, CD133, MDR-1, OCT-4, and Nanog in normal cultured GBC-SD cells and drug-resistant sphere clones detected by real-time fluorescence quantitative PCR
Data were normalized using 18S RNA as the internal reference

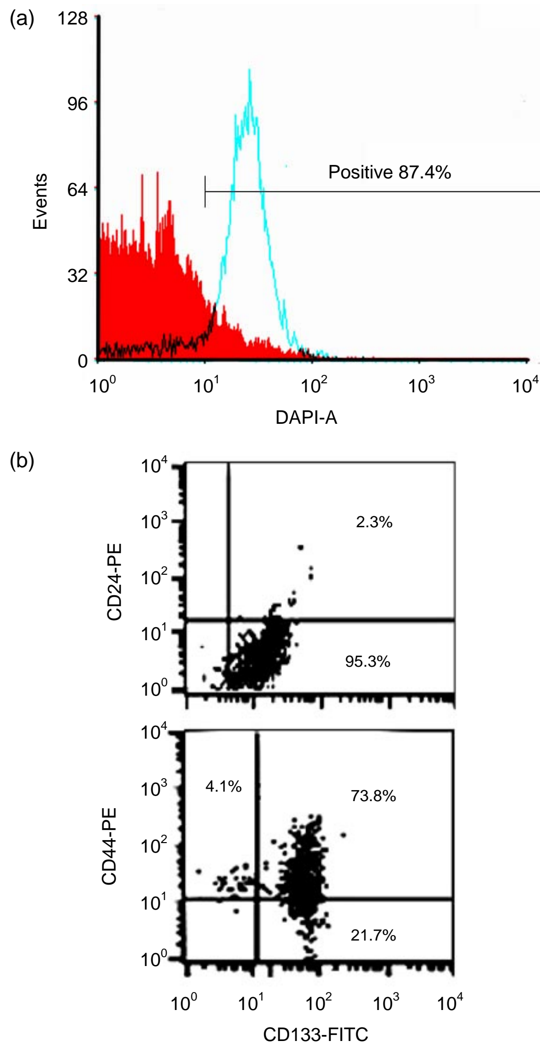

Fig. 5 Stem cell markers of sphere clones detected by flow cytometry and immunofluorescence

(a) The exclusion rate of Hochest 33342 was 12.6%. (b) Sphere clones expressed CD133$^+$ and CD44$^+$, but not CD24$^+$. Among the clones, 97.6% cells were CD133$^+$, 2.3% were CD24$^+$, and 77.9% were CD44$^+$

3.4 Detection of sphere clone pluripotency and differentiation by immunofluorescent staining

In the serum-free culture medium, the sphere clones grew in suspension and expressed OCT-4 and SOX-2 (Fig. 6, in page 261). Semi-quantitative real-time PCR was used to characterize GBC-SD sphere clones and the results showed that the expressions of both MUC1 and vimentin were down-regulated in sphere clones compared with cells cultured in serum-containing medium (after adhesion). However, both OCT-4 and SOX-2 were overexpressed in sphere clones. In contrast, adherent gallbladder cancer cells cultured with serum-containing media overexpressed MUC1 and vimentin but expressed weakly both OCT-4 and SOX-2 (Figs. 7 and 8).

3.5 Ability to differentiate into fibroblasts of sphere clone cells

Adherent sphere clone cells could present some morphology of fibroblast. We used immunofluorescence to label the sphere clone cells with the fibroblast marker vimentin, and found they were positive, suggesting that the sphere clone cells had the ability to differentiate into fibroblasts (Fig. 9).

3.6 Tumor formation from gallbladder cancer sphere clones

Sphere clones cells with different cell numbers (1×10^3, 1×10^4, and 1×10^5) were subcutaneously injected into the nude mice to test their abilities to form tumors. Tumor formation was observed at eight weeks after cell injection. Tumors can be observed in all groups of spheres clone cells. However, only 1×10^5 GBC-SD cells can form tumors, which are significantly smaller than the tumors produced by spheres clone cells (Fig. 10).

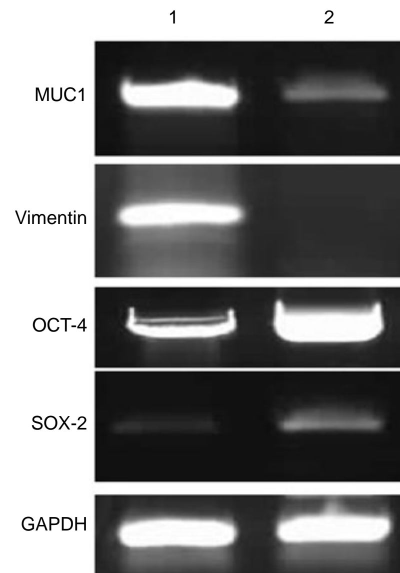

Fig. 7 Real-time PCR determinations of OCT-4, SOX-2, MUC1, and vimentin expressions in sphere clones and cells cultured in serum-containing medium

Lane 1: cells adherent after culture in serum-containing medium; Lane 2: cell clones from serum-free culture medium

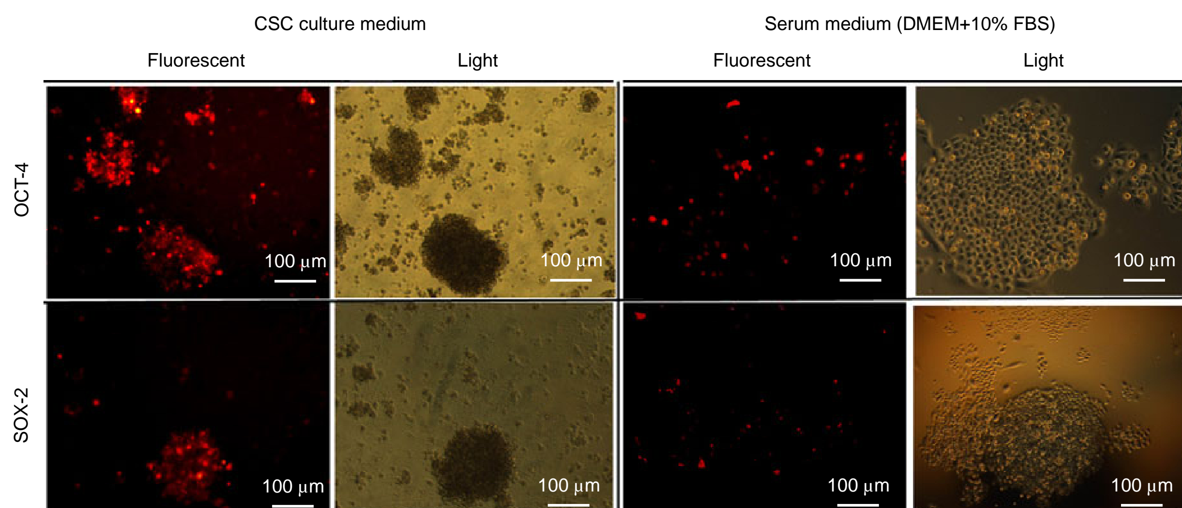

Fig. 6 Immunofluorescent staining to detect OCT-4 and SOX-2 expressions in sphere clones and cells cultured in serum-containing medium
Photographs were taken under fluorescent and light microscopes

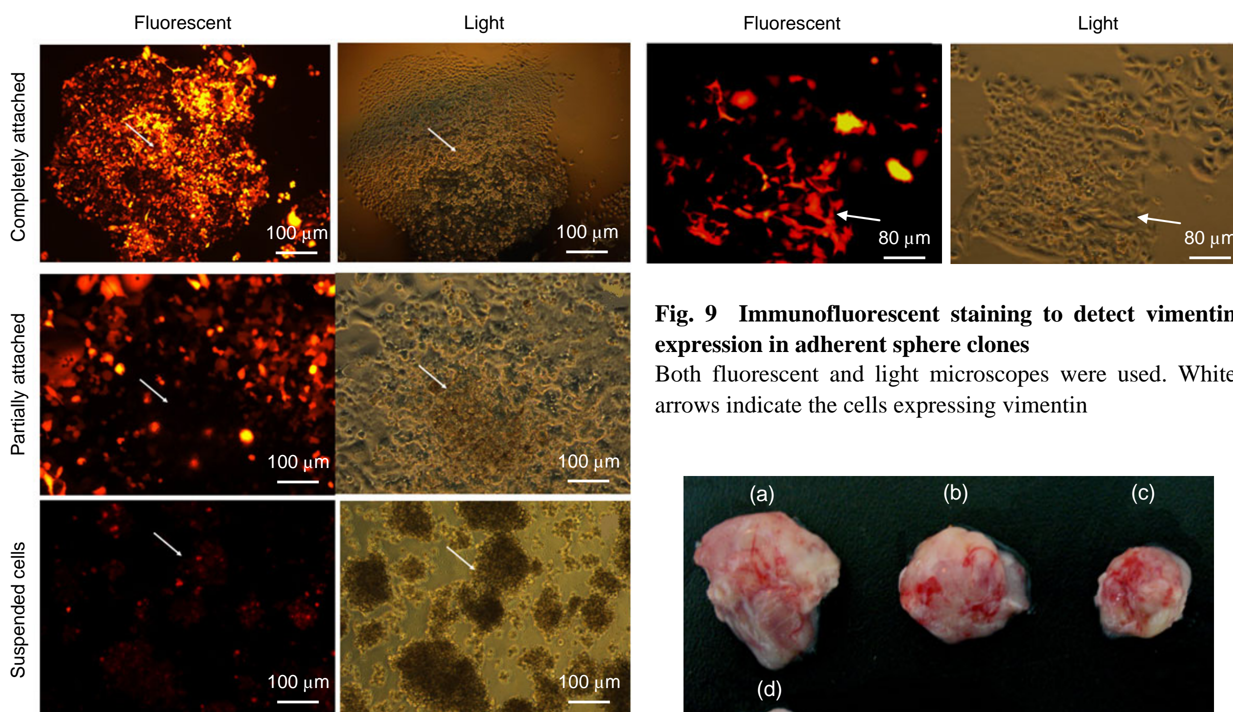

Fig. 8 Immunofluorescent detection of MUC1 expression in different states of spheres clones
MUC1 expression was correlated with cell adhesion. High MUC1 expression was observed in completely adhered cells, followed by partially adhered cells, but negligible in cell suspensions. Photographs were taken under both fluorescent and light microscopes. White arrows indicate the cells expressing MUC1

Fig. 9 Immunofluorescent staining to detect vimentin expression in adherent sphere clones
Both fluorescent and light microscopes were used. White arrows indicate the cells expressing vimentin

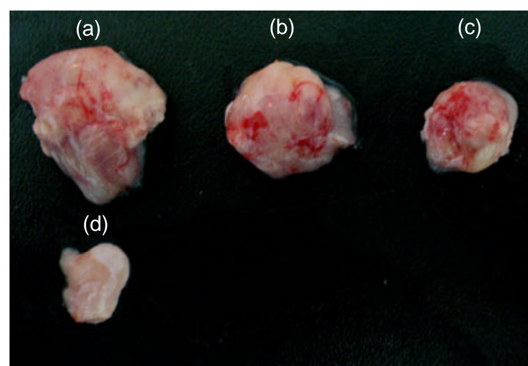

Fig. 10 Tumor formation derived from 1×10^5 (a), 1×10^4 (b), and 1×10^3 (c) sphere clone cells, and 1×10^5 GBC-SD cells (d), eight weeks after subcutaneous injection into the nude mice

4 Discussion

Gallbladder cancer is the most common malignant tumor in the biliary system. The incidence of this disease ranks sixth among all human malignant tumors and its morbidity has increased gradually in recent years. Early invasion and metastasis to adjacent organs and insensitivity to chemotherapy lead to the poor prognosis of gallbladder cancer patients, and the five-year survival rate is below 5% (de Groen *et al*., 1999; Chen *et al*., 2007). Therefore, current research focuses on elucidating the pathogenesis of gallbladder cancer in order to develop effective treatment strategies.

CSCs have been reported to be separated and identified on the basis of surface markers. In this method, the CSCs were isolated firstly depending on the expression profiles of special markers. The abilities of these cells for self-renewal, differentiation, and tumorigenicity were then verified. However, this approach is lab intensive, especially in determining the separation markers. Furthermore, some markers are not specific. Therefore, pure cell population may not be isolated and clones are difficult to be formed. Recent studies have improved mammosphere-based methods via tumor chemotherapy in vivo before enrichment (Li *et al*., 2008). We obtained sphere clones of gallbladder cancer by using serum-free medium containing cisplatin. The isolated sphere clones were then examined for the following CSC characteristics: self-renewal capacity, pluripotency, chemotherapeutic drug resistance, and tumor formation by cells treated with low doses of the drug.

We isolated gallbladder cancer sphere clones from cells maintained in serum-free medium containing cisplatin. When LIF (differentiation suppressor) and Noggin (maintenance of stem cell character) were added to the serum-free medium, the clones could grow and form spheres, which exhibited CSC-like morphology. Quantitative real-time PCR analysis revealed that sphere clones overexpressed stem cell genes such as OCT-4, Nanog, and SOX-2, which are key players of the cell totipotency regulatory network and the material center for retaining the self-renewal property and regulating downstream genes associated with self-renewal, pluripotency, and differentiation. Flow cytometry analysis revealed that 97.6% of the sphere clone cells were $CD133^+$, 77.9% were $CD44^+$, whereas only 2.3% were $CD24^+$. In many solid tumors, such as prostate cancer tumors, hepatocellular carcinoma, and brain tumors, the surface adhesion molecule CD133 was identified as a specific marker for CSCs (Singh *et al*., 2004; Ma *et al*., 2008; Vander Griend *et al*., 2008). In breast cancer, $CD24^{low}$ or $CD44^+$ was recognized as surface markers for stem cells (Al-Hajj *et al*., 2003; Fillmore and Kuperwasser, 2007). The isolated gallbladder cancer sphere clones in the present study exhibited low expression levels of CD24 and high expression levels of CD44. This finding was consistent with previous study on the expression of surface markers by bile duct CSCs (Wang *et al*., 2009). Both quantitative real-time PCR analysis and immunofluorescent staining showed GBC cell clones grew in suspension and expressed abundantly OCT-4 and SOX-2 under CSC culture. However, under serum-containing medium (DMEM plus 10% FBS), the expressions of OCT-4 and SOX-2 (Fig. 6) rapidly reduced after cells adhered, whereas the expressions of cell differentiation markers MUC1 and vimentin increased. These results may indicate that the clones possessed distinct differentiation ability. Tumor fibroblasts play an important role in tumor development. Previous studies have been based on the hypothesis that tumor fibroblasts were recruited from the surrounding microvessels to the malignant tissues (Petersen *et al*., 2003; Puré, 2009). In serum-containing medium, sphere clones exhibited fibroblast morphology after adherence, and the cells could be labeled by the fibroblast marker vimentin (Fig. 9). Thus, sphere clones could differentiate into tumor fibroblasts. This finding would broaden the scope for future research on CSCs. In tumor formation experiments, 1×10^3 cells of sphere clones could induce subcutaneous tumors in nude mice, thereby indicating high tumorigenicity of sphere clones.

The present study determined that selected gallbladder cancer sphere clones exhibited certain CSC characteristics. This finding may enable the development of techniques and tools for further research on gallbladder CSCs.

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