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Research Article

Rapid Isolation of Gastric Adenocarcinoma Cancer Stem Cells as a Target for Autologous Dendritic Cell-Based Immunotherapy

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Background: Gastric cancer (GC) is a malignancy cause associated with a high death rate in the world. Cancer stem cells (CSCs) are a rare immortal subpopulation of cells within tumors with characteristics of the ability to self-renew, initiate tumor, and differentiate into defined progenies as well as and high resistance to conventional therapies.
Objectives: Despite the use of surgery and chemotherapy for GC therapy, there are no efficient therapeutic protocols for it to date. Therefore, rapid isolation of CSCs in order to therapeutic targets, especially immunotherapy is very important.
Materials and Methods: Cancerous cell suspension isolated from patients with GC was cultured in the serum-free medium containing EGF, bFGF, LIF, and heparin under non-adherent culture conditions to generate spheres. Expression of mRNA level stemness transcription factors (OCT4, SOX2, SALL4, and Cripto-1), CD44 variable isoforms (CD44s, CD44v3, CD44v6, CD44V8-10) of spheroid-forming single cells compared with gastric normal tissue cells using real time PCR and molecules of CD44, CD54, and EpCAM as gastric CSC markers, and stemness factor Oct4 using flow cytometry, as well as tumorgenicity using subcutaneous injection of sphere-forming cells to nude mice were investigated.
Results: Few cancerous cells isolated from patients with GC were able to generate three-dimensional spheroid colonies in the serum-free medium containing EGF, bFGF, LIF, and heparin under non-adherent culture conditions, and form xenograft tumors in immunodeficient nude mice after subcutaneous injection. Spheroid-forming single cells upregulated stemness transcription factors OCT4, SOX2, SALL4, and Cripto-1 that are associated with pluripotency and self-renewal and CD44 isoforms (CD44s, CD44v3, CD44v6, CD44V8-10) compared with gastric normal tissue cells. Finally, molecules of CD44, CD54, and EpCAM as gastric CSC markers and stemness factor Oct4 were expressed in sphere-forming cells.
Conclusion: We suggested that the sphere formation and tumorigenicity assays are two procedures, leading to the rapid isolation of cancer cells with certain stem-like properties in order to target CSCs using autologous dendritic cell therapy, especially in patients with advanced disease.

Keywords: CSC Markers; Gastric cancer; Sphere; Sphere-forming cells; Tumorigenicity

1. Background

Gastric cancer (GC) kills 700000 people a year, and that's why known as the fourth most common malignancy in the world (1, 2). Patients with advanced GC have the low five-year survival rate (20-30%) and high recurrence risk (3). The most common types of available conventional treatments against GC are surgery as the critical curative therapy and chemotherapy and radiotherapy as the neoadjuvant therapies. However, novel therapeutic strategies owing to a lack of effective

treatment protocols are critically needed (4, 5). Cancer stem cells (CSCs) or cancer-initiating cells (CICs) are a small subpopulation of cancer cells with unlimited self-regeneration and tumor-initiating ability. Besides, CSCs are responsible for tumor development, invasion, metastasis, and high resistance to conventional therapies (6-9). According to CSCs hypothesis, tumors, like adult tissues, are generated from cells that exhibit self-renewal capacity and differentiate into tissue cells (10). Therefore, targeting CSCs may completely treat cancer (11-14). $CD8^+$ T cells, natural killer (NK) cells, cytokine-induced killer (CIK) cells, oncolytic virotherapy (OVT), and dendritic cell (DC)-based vaccines as immunotherapy have used for targeting CSCs (15). The efficiency and effectiveness of dendritic cells (DCs) -based therapies, especially DCs loaded with CSC lysate, and CSC RNA-pulsed DCs has been demonstrated *in vitro* and *in vivo* in various cancers (16-19). Therefore, the isolation and confirmation of CSCs are critical. Studies of adult stem cells first described a sphere-forming assay as a functional approach (20). Assessment of stemness features of CSC subpopulations and efficient enrichment of CSCs using this method extensively used during the last two decades (21-25).

2. Objective

The main aim of the study was rapid isolation of CSCs using the sphere-forming assay (SFA) and CSCs confirmation by tumorigenicity assay in patients with GC.

3. Materials and Methods

3.1. Isolation and Culture of Gastric Cancer Cells

Fresh tumor specimens (0.3-4 mL) obtained from GC patients (**Table 1**) were disaggregated into single cell suspension in DMEM/F12 medium (Biosera, France) containing collagenase type I (Gibco, USD) (300 $U.mL^{-1}$), penicillin (Biosera) (500 $U.mL^{-1}$), streptomycin (Biosera) (500 $mg.mL^{-1}$), and amphotericin B (Biosera) (1.25 $mg.mL^{-1}$) at 37 °C for 2 hr. The cells (1×10^5 cell. mL^{-1}) were cultivated in epidermal growth factor (EGF) 20 $ng.mL^{-1}$ (Gibco), basic fibroblast growth factor (bFGF) 10 $ng.mL^{-1}$ (Gibco), leukemia inhibitory factor (LIF) 10 $ng.mL^{-1}$ (ProSpec, Israel), heparin 4 $\mu g.mL^{-1}$ (Sigma-Aldrich, Germany), B-27 supplement 2% (Gibco), penicillin 100 $U.mL^{-1}$, and streptomycin 100 $\mu g.mL^{-1}$, and HEPES 8 mM (Biosera) in DMEM/F12 medium in T-25 ultra-low attachment flask (Corning, USA) at 37 °C for 1-2 months in a humidified 5% CO_2 incubator to generate spheres.

3.2. In Vitro Tumorsphere Formation Assay

After centrifuging and dissociating the primary gastrospheres, the resulting single cells (5×10^4 cells. mL^{-1}) were cultured in serum-free DMEM/F12 medium containing EGF, bFGF, LIF, heparin, B-27 supplement, penicillin, streptomycin, and HEPES at 37 °C for two weeks to produce spheres. Then, the spheres were passaged every two weeks for at least three months.

3.3. RNA Extraction, cDNA Synthesis, and Quantitative Real-time PCR

Total RNA was extracted from sixth spherical colonies and gastric normal tissue cells using mRNA Isolation Kit (Roche Applied Science, Penzberg, Germany) according to the manufacturer's protocol. cDNA was synthesized using Easy cDNA Synthesis Kit (Pars Tous Biotechnology, Mashhad, Iran). Quantitative real-time PCR was performed with the fluorescent dye SYBR Green/ROX Master Mix and primers as listed in **Table 2** on an Mx3000P QPCR System (Stratagene, CA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene was amplified as the endogenous control gene for normalization. The expression of genes relative to gene GAPDH was calculated as $2^{-\Delta\Delta Ct}$.

3.4. Detecting Gastric CSCs Surface Markers CD44, CD54, and EpCAM, and Stemness Marker Oct4 by Flowcytometry

Sixth spherical colonies were dissociated by TrypLE™ Select 1X (Gibco) and passed through a 40 µm cell strainer. The spheroid-derived single cells suspended in PBS stained

Table 1. Clinicopathological characteristics of patients.

Case	Sex	Age	Site	Size (cm^3)	Grade	Stage
1	Man	52	Non-cardia	6	Poor	T3N0M0
2	Man	59	Cardia	7.5	Moderate	T3N1M0
3	Man	71	Non-cardia	6	Poor	T4aN3aM0

Table 2. Primer sequences for the amplification of target genes and GAPDH

Genes	Primer sequence 5' -> 3'
CD44s	For: TCCAACACCTCCCAGTATGACA Rev: GGCAGGTCTGTGACTGATGTACA
CD44v3	For: GCAGGCTGGGAGCCAAAT Rev: GAGGTGTCTGTCTCTTTCATCTTCATT
CD44v6	For: GGAACAGTGGTTTGGCAACAG Rev: TTGGGTGTTTGGCGATATCC
CD44v8-10	For: TCCCTGCTACCAATATGGACTC Rev: ACTCTGCTGCGTTGTCATTG
OCT4	For: GCAGCGACTATGCACAACGA Rev: CCAGAGTGGTGACGGAGACA
SOX2	For: AACAGCCCGGACCGCGTCAA Rev: TCGCAGCCGCTTAGCCTCGT
SALL4	For: TGCAGCAGTTGGTGGAGAAC Rev: TCGGTGGCAAATGAGACATTC
Cripto-1	For: GGGATACAGCACAGTAAGGAG Rev: ACGGTGGTAGTTCTGGAGTC
GAPDH	For: GGAAGGTGAAGGTCGGAGTCA Rev: GTCATTGATGGCAACAATATCCACT

with FITC anti-mouse/human CD44 (Bio Legend, USA), PE anti-human CD54 (Bio Legend), FITC anti-human EpCAM (Bio Legend), PE anti-human oct-4 antibodies (Bio Legend) (After permeabilization of cells with 0.1% saponin in PBS) and all the isotype controls (FITC-IgG1 (Bio Legend), PE-IgG1 (Bio Legend)) for 15-20 minutes on ice in the dark. After washing the labeled cells with PBS, the fluorescence intensity of individual cells was measured by a flow cytometer (BD FACSCalibur, Franklin Lakes, NJ). Finally, data was processed by the FlowJo software.

3.5. In Vivo Tumorigenicity Experiment

After the approval of the experimental procedures by the Institutional Animal Care and Use Committee of Mashhad University of Medical Sciences, for the evaluation of tumor formation in animal experiments, sphere-forming cells (1×10^6 cells) dissociated from sixth spherical colonies in serum-free DMEM/F12 medium/Matrigel (Sigma-Aldrich) (1:1) were subcutaneously injected into the right flank of three male athymic nude mice (C57BL/6 strain), 4-6 weeks, using 100 µL micro syringe in North Research Center, Pasteur Institute of Iran and maintained for four weeks.

4. Results

4.1. Gastro Spheres Were Formed from Tumor Tissue of GC Patients.

Few cancer cells obtained from tumor tissues can form floating three-dimensional spheroid colonies, tumorspheres, in the serum-free medium under low-adherent conditions (22). After culturing cell suspension obtained from tumor specimens of GC patients, gastrospheres were generated after one month (**Fig. 1 A-C, G-I**).

4.2. Sphere-Forming Cells Could Generate Tumors pheres.

To confirm the self-renewal ability of spheroid-forming single cells, we recultured them in CSC conditioned culture medium after enzymatically dissociating and mechanically disrupting them to form new nonadherent anchorage-independent spheres and then passaged them five times for at least three months. The results exhibited that sphere-forming cells dissociated from sixth spherical colonies retained self-renewal capacity (**Fig. 1 D-F, K-M**).

4.3. Gastro Spheres Overexpress Genes Related to CD44 Splice Variants and Stemness.

The expression at the mRNA level of 8 genes described as CD44 splice variants (CD44s, CD44v3, CD44v6, CD44v8-10) and pluripotency (OCT4, SOX2, SALL4, Cripto-1) was analyzed in tumor spheres. Sixth spherical colonies showed higher expression of listed genes (**Table 2**) than gastric normal tissue cells (**Fig. 2**).

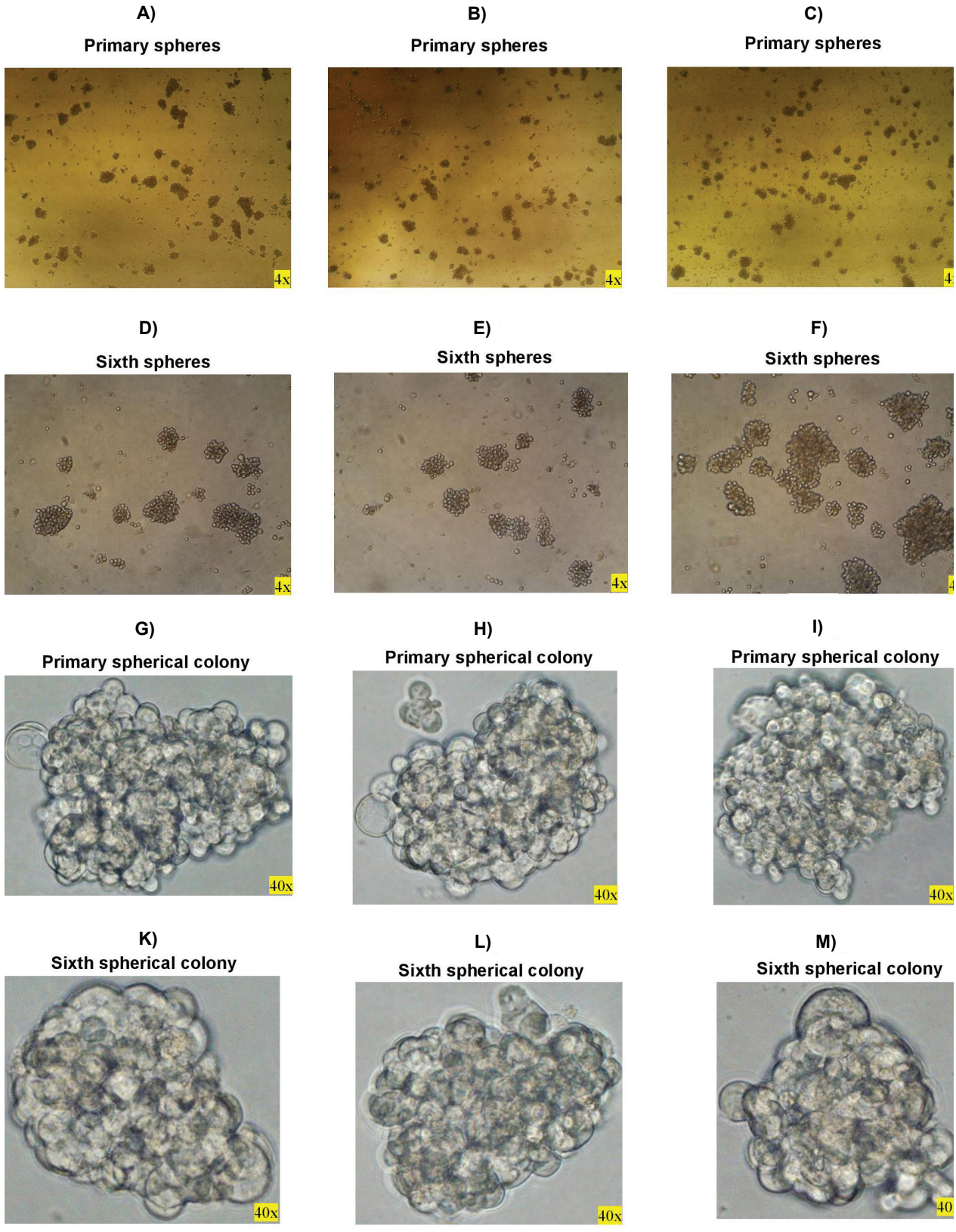

Figure 1. Formation of gastrospheres in the serum-free medium under low-adherent conditions. Tumor spheres generated from cancer cells of patient 1 (a, g), patient 2 (b, h), patient 3 (c, i). (Original magnification 4×, 40×). Spheroid-forming single cells of patients formed spherical colonies (Patient 1; d, k, patient 2; e, l; patient 3; f, m) and maintained the self-renewing capacity after the 5th Passage. (Original magnification 4×, 40×)

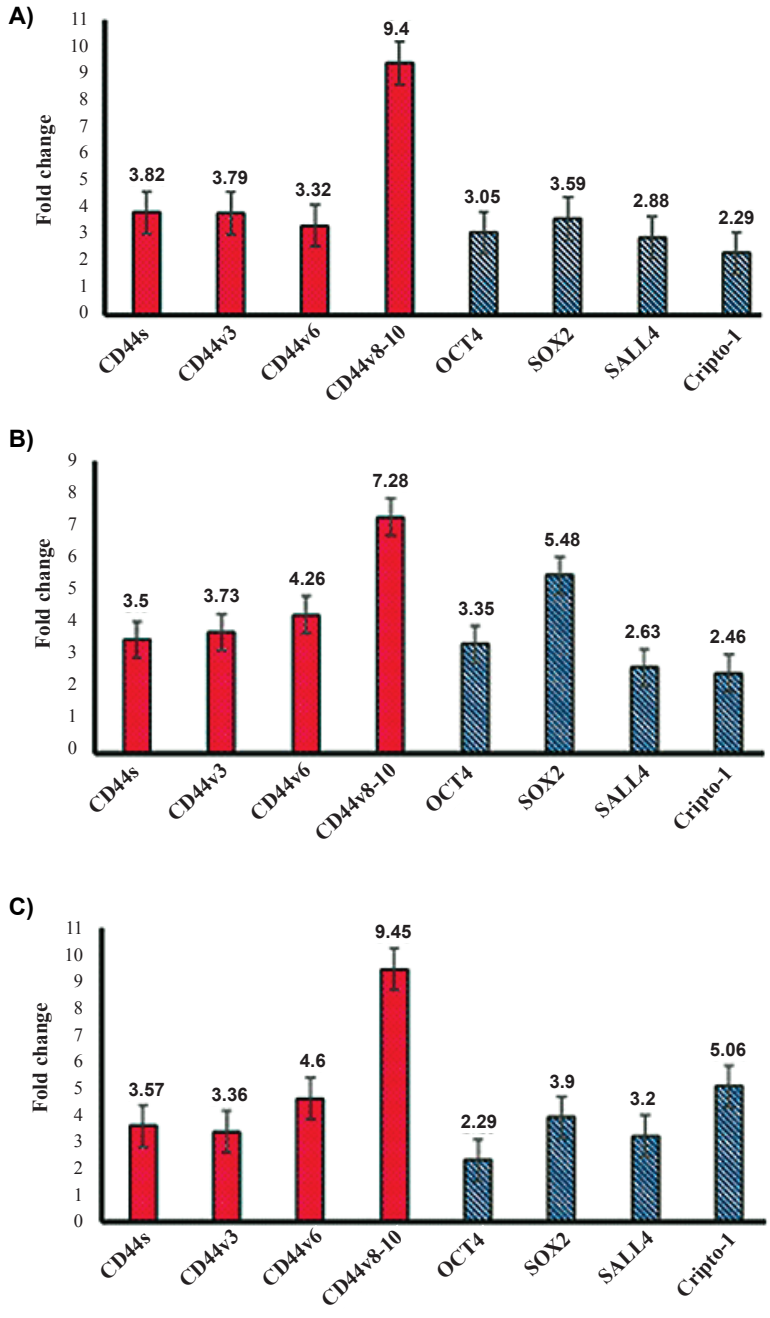

Figure 2. Analysis of the expression of steınness and CD44 splice variants-related genes in spheroid-forming single cells dissociated from sixth spherical colonies. **A)** patient 1. **B)** patient 2. **C)** patient 3.

4.4. Gastro Spheres Expressed Cellular Surface Markers of CD44, CD54, and EpCAM, and Stemness Marker oct4.

The identity of CSC surface markers (CD44, CD54, EpCAM) and stemness marker oct-4 of spheroid-forming single cells dissociated from sixth spherical colonies was determined using flow cytometry. We showed that these cells expressed the mentioned markers (**Fig. 3** and **Table 3**).

4.5. Spheroid-Forming Single Cells Exhibited High Tumorigenicity In Vivo.

The sphere-forming cells dissociated from sixth spherical colonies were subcutaneously inoculated into immunocompromised (nude) mice for confirmation of tumorigenicity *in vivo*. We found that these cells could initiate tumor growth and form tumors in mice after four weeks (**Fig. 4**).

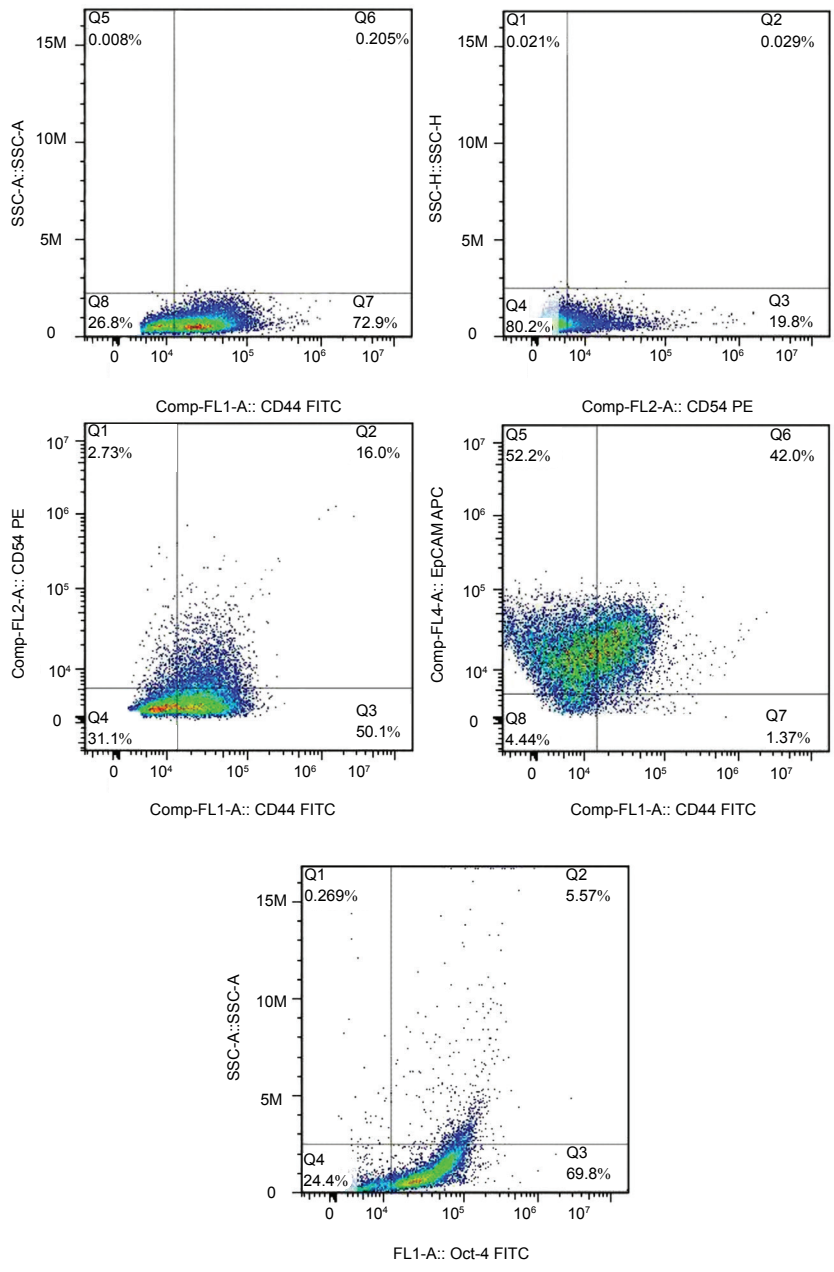

Figure. 3 Flow cytometry expression analysis of surface markers CD44, CD54, and EpCAM, and stemness marker oct4 in sphere-forming cells dissociated from sixth spherical colonies**.**

Table 3. Percentage of expression of surface markers and stemness marker of parental sphere-forming cells isolated from patients.

Case	CD44	CD54	DLL4	EpCAM	CD44CD54	CD44DLL4	CD54DLL4	CD44CD54DLL4	CD44EpCAM	Oct4
1	65.2	23.7	11.63	58.1	20.52	10	3.43	8.76	53.62	75
2	72.9	19.8	9.75	52.2	16	7.44	2.92	7.24	42	69.8
3	78.32	20.19	13.74	63.6	17.5	12.8	2.37	9.93	57	81

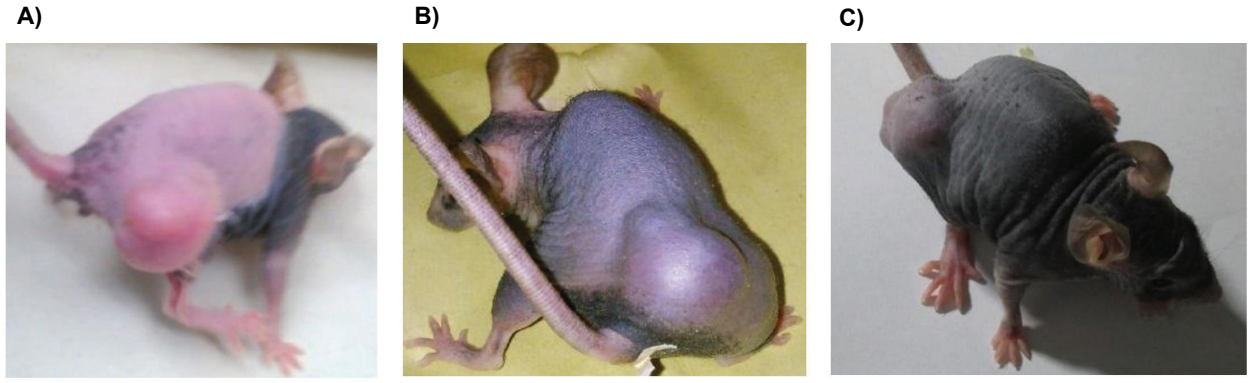

Figure 4. Tumor formation in nude mice injected with spheroid-forming single cells dissociated from sixth spherical colonies. **A)** patient 1 **B)** patient 2 **C)** patient 3

5. Discussion

Classical models of carcinogenesis include the stochastic and hierarchical models (26, 27). In the stochastic model, most tumor cells can increase extensively and contribute considerably to tumor maintenance. Furthermore, the random mutations and the subsequent clonal selections cause carcinogenesis. In the hierarchical model, a small subpopulation of cancer cells can propagate expansively and contribute to carcinogenesis. CSCs subsets in solid tumors as cancer-initiating cells strongly support the hierarchical model. Therefore, the isolation of CSCs possibly bears great therapeutic implications. There usually are three types of CSCs isolation methods. One is a side population (SP) that based on the efflux of the Hoechst 33342 dye or Rhodamine 123 by the ABCG family of membrane-associated transporters. However, utilization of this technique due to the low purity and specificity of isolated cells, and dye toxicity is limited (6). The second type is sorting based on the cell surface and intracellular markers using the fluorescence-activated cell sorting (FACS) and the magnetic-activated cell sorting (MACS). The limitations of this method include the small number and low viability of isolated cells, damage of surface markers after enzymatic treatment, and high cost (6). Markers $CD44^+$, $CD44V8\text{-}10^+$, $CD133^+$, $CD24^+$, $CD54^+$, $CD90^+$, $CD49f^+$, $CD71^+$, $EpCAM^+$, and $ALDH1^+$ (28) have been proposed to enrich gastric CSC fraction. However, their sensitivity and specificity for isolating, identifying, and characterizing these cells vary. The third approach is spheroid colony formation under nonadherent conditions with serum-free media. Sphere culture has many advantages over the previous two methods. For example, Takaishi *et al.* demonstrated that the percentage of cells with CD44 marker within MKN-45, MKN-74, N-87, and MKN-28 cells were different (MKN-45, MKN-74 cells: 94%, N-87 cells: 5%, MKN-28 cells:0%). $CD44^+$ cells could generate spheroid colonies. However, few $CD44^-$ cells were able to formed spheres (22). Other researchers have reached similar conclusions (29-31). Therefore, isolation of cells with sphere-forming capacity is more important than sorting cells based on markers. Here, we isolated spheroid colonies (colonospheres) from tumor tissue of patients with GC and confirmed self-renewal capacity *in vitro* and tumor-initiating ability *in vivo* of derived from colonospheres. The upregulation of pluripotency and self-renewal markers (OCT4, SOX2, SALL4, and Cripto-1) of spheroid-forming single cells compared with gastric normal tissue cells was also investigated. Interaction of Nanog and Oct-4/Sox2 associated with pluripotency and self-renewal of stem cell-like cancer cells and regulate their self-renewing properties and multilineage differentiation capacity (32). Tian *et al*. showed that downregulation of Sox2 with small interfering RNA (siRNA) diminished the generation of spheroid colonies and drug efflux, increased apoptosis rate in sphere-forming cells *in vitro*, and suppressed tumorigenicity *in vivo* (33). SALL4 plays a fundamental role in tumor growth, development, progression, invasion, and therapy resistance. SALL4 directly activated CD44 expression, resulting in the promotion of gastric cancer progression. Therefore, SALL4 knockdown repressed growth, proliferation, metastasis, and invasion of CSCs (34). Our results exhibited that sphere-forming

cells upregulated stemness factors OCT4, SOX2, SALL4, and Cripto-1 compared with gastric normal tissue cells. The ability of CSCs to form a tumor *in vivo*, in an immunodeficient mice, has become a standard to identify them, especially in solid tumors. Two immunodeficient mouse models, including nude and NOD/SCID mice which do not reject xenografts, are often used for analysis of the tumorigenicity. Theoretically, injection of a single CSC ought to be able to establish a tumor in a mouse model. Accordingly, the CSCs ability to form tumors at low densities is one important test for determining prospective CSC population (35-37). Self-renewal properties of CSCs were confirmed *in vivo* by generating tumor in mice. Therefore, it was concluded that sphere-forming cells from sixth spheres exhibited the ability of self-renewal *in vivo* tumorigenicity experiments.

Molecules of CD44, CD44V8-10, CD54, and EpCAM are gastric CSC markers. CD44 caused enhancement of proliferation and survival of CSCs, cancer metastasis facilitation and tumor relapse, and death of patients with GC (31). CD54 and CD44V8-10 play a pivotal role in promoting the release of CSCs to blood vessels and tumor initiation, respectively (31, 38). EpCAM as epithelial-specific antigen (ESA) in cancer cells is responsible for proliferation, metastasis, and invasion (39). Furthermore, overexpression of CD44v6 and CD44v3 is associated with the generation of metastatic lymph nodes from intestinal-type GC and carcinogenesis, and GC development, respectively (31, 40, 41). Here, we demonstrated that spheroid-forming single cells upregulated CD44 variable isoforms (CD44v3, CD44v6, and CD44V8-10) compared with gastric normal tissue cells and expressed gastric CSC markers CD44, CD54, and EpCAM.

6. Conclusion

Our data demonstrated that there are two fundamental properties for rapid isolation and confirmation of CSCs in patients with GC (16, 17, 42). One is the sphere-forming assay (SFA) to evaluate the existence and self-renewal capacity of CSCs, and the other is tumor-initiating ability. Therefore, CSCs could be targeted using autologous DC to treat GC, especially patients with advanced disease.

Conflicts of interest

The authors declare no conflicts of interest.

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