RESEARCH ARTICLE

Growth, Clonability, and Radiation Resistance of Esophageal Carcinoma-derived Stem-like Cells

Jian-Cheng Li[1&], Di Liu[2&], Yan Yang[1], Xiao-Ying Wang[1], Ding-Long Pan[1], Zi-Dan Qiu[1], Ying Su[3], Jian-Ji Pan[3]

Abstract

Objective: To separate/enrich tumor stem-like cells from the human esophageal carcinoma cell line OE-19 by using serum-free suspension culture and to identify their biological characteristics and radiation resistance. Methods: OE-19 cells were cultivated using adherent and suspension culture methods. The tumor stem-like phenotype of CD44 expression was detected using flow cytometry. We examined growth characteristics, cloning capacity in soft agar, and radiation resistance of 2 groups of cells. Results: Suspended cells in serum-free medium formed spheres that were enriched for CD44 expression. CD44 was expressed in 62.5% of suspended cells, but only in 11.7% of adherent cells. The suspended cells had greater capacity for proliferation and colony formation in soft agar than the adherent cells. When the suspended and adherent cells were irradiated at 5 Gy, 10 Gy, or 15 Gy, the proportion of CD44+ suspended cells strongly and weakly positive for CD44 was 77.8%, 66.5%, 57.5%; and 21.7%, 31.6%, 41.4%, respectively. In contrast, the proportion of CD44+ adherent cells strongly positive for CD44 was 18.9%, 14.%, and 9.95%, respectively. When the irradiation dose was increased to 30 Gy, the survival of the suspended and adherent cells was significantly reduced, and viable CD44+ cells were not detected. Conclusion: Suspended cell spheres generated from OE-19 esophageal carcinoma cells in serum-free stem medium are enriched in tumor stem-like cells. CD44 may be a marker for these cells.

Keywords: Esophageal carcinoma - tumor stem-like cells - CD44

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Introduction

Esophageal cancer is one of the most common malignancies throughout the world, and its incidence has evident regional characteristics. China has a high incidence of esophageal cancer, and the annual incidence and deaths in China account for 52.5% and 41.8% of those in the world. Local recurrence and distant metastasis are the main reasons for failure of esophageal cancer treatment. Recent studies have suggested that tumor stem-like cells are responsible for metastatic potential and possess innate resistance mechanisms against chemotherapy and radiation-induced cancer cell death. After their initial detection in leukemia , tumor stem-like cells have been widely discovered in solid tumors, including breast , prostate, glioma , oophoroma, and colon (Cheng et al., 2010). However, these cells have remained elusive in esophageal cancer. In this study, we attempted to cultivate cell spheres enriched in esophageal adenocarcinoma tumor stem-like cells using a serum-free culture method, with the goal of analyzing biological characteristics to build a foundation for further study of esophageal adenocarcinoma tumor stem-like cells.

Materials and Methods

Cell lines, Reagents and Equipment

The human esophageal adenocarcinoma cell line OE-19 was obtained from the Chinese Academy of Sciences Type Culture Collection cell bank. RPMI-1640 medium and DMEM-F12 medium were supplied by Hyclone. Calf serum was purchased from Hangzhou Sijiqing. Trypsin and dimethyl sulfoxide DMSO were purchased from Sigma. Agarose was purchased from Beijing Bangding Biotechnology Company. Trypan Blue dye was purchased from Huamei Biological Engineering Co., Ltd. Basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) were purchased from (RD). Insulin and B27 were purchased from Invitrogen. FITC-labeled mouse anti-human CD44 antibody and FITC-labeled mouse anti-human IgG1 isotype control antibody were purchased from Biolegend Company. DK-8D electric heated water tank was purchased from Hysen letter Instrument Co., Ltd. Clean benches were purchased from Xun Haibo Industrial Co., Ltd. Medical Equipment Factory. CO_2 incubator was Sanyo. A low-temperature freezer (−80°C) was purchased from Reveco. Nikon TE200 inverted microscope was

*[1]Provincial Clinical College of Fujian Medical University, [3]Provincial Clinical College of Fujian Medical University, Fujian Provincial Tumor Hospital, Fuzhou, Fujian, [2]Department of Oncology, Sichuan Provincial People's Hospital, Chengdu, Sichuan, China [&]Equal contributors *For correspondence: Jianchengli6@126.com*

purchased from Nikon. The flow cytometer was purchased from Beckman. The Libra electronic analysis system was purchased from Mettler. The linear accelerator was an Elekta Compact.

Methods

Adherent culture, suspension culture, and subculture of OE-19 cells: OE-19 cells were cultured in RPMI-1640 medium containing 10% fetal calf serum under 5% CO_2 at 37°C. The cells were digested using 0.25% trypsin and then subcultured. For adherent culture, OE-19 cells were re-suspended in serum-free medium (DMEM-F12 medium containing 10 ng/ml bFGF, 20 ng/ml EGF, 10 μl/ml B27, and 5 μg/ml insulin) in a single-cell suspension and then cultured in 6-well plates. Each well was filled with 3 ml of medium and the cells were incubated under 5% CO_2 at 37°C. For suspension cultures, suspended cell spheres were collected and digested by trypsin, and then subcultured.

Expression of CD44 in suspended cells and adherent cells: OE-19 cells were subcultured for 6 passages. Adherent cells and suspended cell spheres were collected during the logarithmic growth phase, digested with 0.25% trypsin, and centrifuged to form a single-cell suspension. The cells were washed 2 times in PBS, and the cell concentration was adjusted to 10^6 cells/ml. Each type of cell culture was divided into 2 tubes, with 1 ml per tube. FITC Mouse Anti-Human CD44 antibody (10 μl) and FITC Mouse IgG1 Isotype Control CD44 antibody (5 μl) were added and incubated in the dark for 30 minutes at 4°C. The cells were then centrifuged at 2000 rpm for 3 minutes. The supernatant was removed, and cells were suspended in 2 ml of PBS for testing.

Cell growth curves: The cell growth curves were determined by cell counting at a regular time every day. The adherent cells and suspended cell spheres were digested into single cells using 0.25% trypsin. Live cells counted according to Trypan blue staining, and the cells were seeded in 6-well culture plates (10^6/well) and incubated at 37°C under 5% CO_2 for 8 days. Every day, 3 plates were selected for digestion and counting of cells, and the mean was used to draw cell growth curves.

Colony formation assays in soft agar: Adherent growing cells and suspended cell spheres were digested using 0.25% trypsin to generate a single-cell suspension at a density of 105 cells/ml; then, 100 μl was removed from each sample and placed in 2 tubes containing 9 ml of pre-warmed RPMI-1640 culture medium containing 15% fetal bovine serum. After mixing, 1 ml of 3% agar was added, followed by additional mixing. Cells were then seeded in 6-well plates, with 1,000 cells seeded in each well in in 1 ml of medium. The culture plate was shaken to ensure that the cells were uniformly dispersed, and then the cells were incubated at 37°C under 5% CO_2 for 2 weeks. MTT liquid (0.5 ml) was added to each well of the 6-well plates and incubated for 30 min at 37°C, and the number of cells was counted for more than 50 clones. Cells were imaged using an optical camera.

Radiation resistance of suspended and adherent cells: Adherent cells and suspended cell spheres were collected during the logarithmic growth phase. Each type of cell culture was divided into 3 groups and then placed into 25-ml flasks. Synthetic pigskin (1 cm) was placed in the bottom of the flasks for colloidal compensation, and the flasks were then exposed to 5 Gy, 10 Gy, and 15 Gy of X-ray for 1 min every other day for a total of 3 times. The expression of CD44 and the survival ratio of the cells were determined by flow cytometry on the first, second, and third day after irradiation, and cell growth curves were drawn. The irradiation parameters were 6MVX, skin-distance 100 cm, actual dose rate 325 MU/min, radiation field 40 × 40 cm^2, gantry 180°, and radiation from the bottom up.

Statistical analysis: Statistical analysis was conducted using SPSS PASW Statistics 18.0 software. Data are reported as the mean ± SD from at least 3 independent tests. The significance of differences was analyzed using Student's t-test. $P < 0.05$ was considered to indicate a statistically significant result.

Results

Suspension cultures of OE-19 cells

Adherent cells were grown in serum-containing medium (Figure 1A). Suspension-cultured OE-19 cells showed adherent growth on the second day, and some cells grew in suspension on the third day (Figure 1B). The amount of suspended cells then gradually increased. At 1 week, a visible sphere of suspended cells had formed (Figure 1C). The suspended cell spheres were largest at 2 weeks (Figure 1D). When suspended cell spheres were dissociated into single cells, the suspended new cells could still form new spheres in serum-free medium; suspended cells were subcultured in this manner.

Expression of CD44 in suspended cells and adherent growing cells

Flow cytometry analysis showed CD44 was expressed in 11.74% of adherently cultured of OE-19 cells (Figure 2A), while CD44 was expressed by 62.46% of suspended

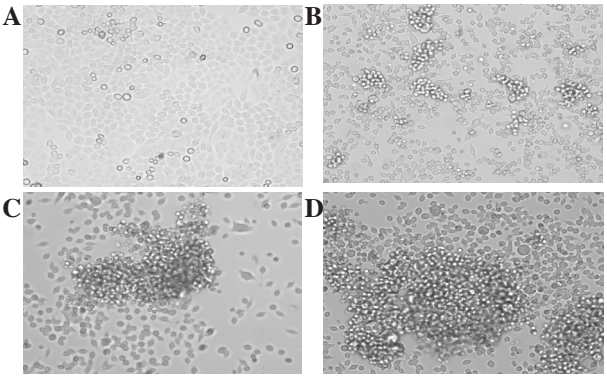
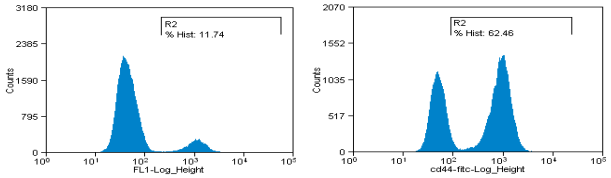


Figure 1. Adherent Cells in the Serum-containing Medium (A); Suspended Cell Spheres Cultured in Serum-free Medium for 3 Days (B); Suspended Cell Spheres Cultured in Serum-free Medium for 1 Week (C); Suspension Cell Spheres Cultured in Serum-free Medium for 2 Weeks (D)



A B

Figure 2. Expression of CD44 in 11.74% of Adherent Cells (A); Expression of CD44 in 62.46% of Suspended Cells (B)

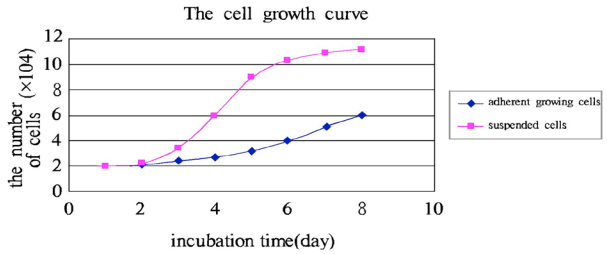
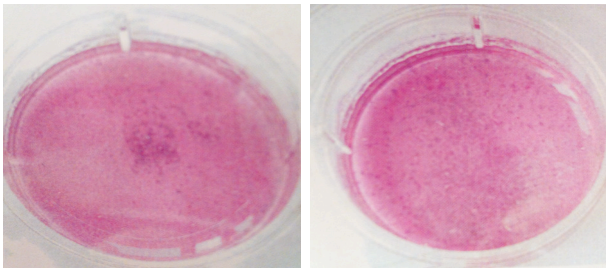


Figure 3. Cell Growth Curves of Suspended Cells and Adherent Cells



A B

Figure 4. Colony Formation of Adherent Cells in Soft Agar (A); Colony Formation of Suspended Cells in Soft Agar (B)

Table 1. Colony Formation of Suspended Cells and Adherent Cells in in Soft Agar (mean ± SD, n = 3)

Group	Suspended cells	Adherent cells	P-value
Number of clones	135±9	63±7	0.023

cells. Thus, CD44 may be a marker of stem-like cells in esophageal carcinoma (Figure 2B).

Cell growth curves of suspended cells and adherent cells

The cell growth curves of suspended cells as shown in Figure 3 demonstrate an "S" shape where the initial incubation period is days 1–3, the period of slow growth days 3–4, and the logarithmic growth phase is days 4–6, after which the number of cells reaches a plateau at days 7–8. In contrast, the cell growth curve of adherent cells is relatively flat without an evident "S" shape. The numbers of cells in the 2 groups were analyzed by paired t test; the number of cells in the 2 groups was $(7.45 \pm 4.03) \times 10^4$ and $(4.31 \pm 1.53) \times 10^4$. The difference was significant ($P < 0.05$).

Colony formation capacity in soft agar

When suspended esophageal cancer cells were cultured in soft agar medium for 2 weeks, most of the cells formed clear cell clones. Each clone comprised 40–60 cells. Adherently cultured cells formed significantly fewer clones ($P < 0.05$) (Figure 4; Table 1).

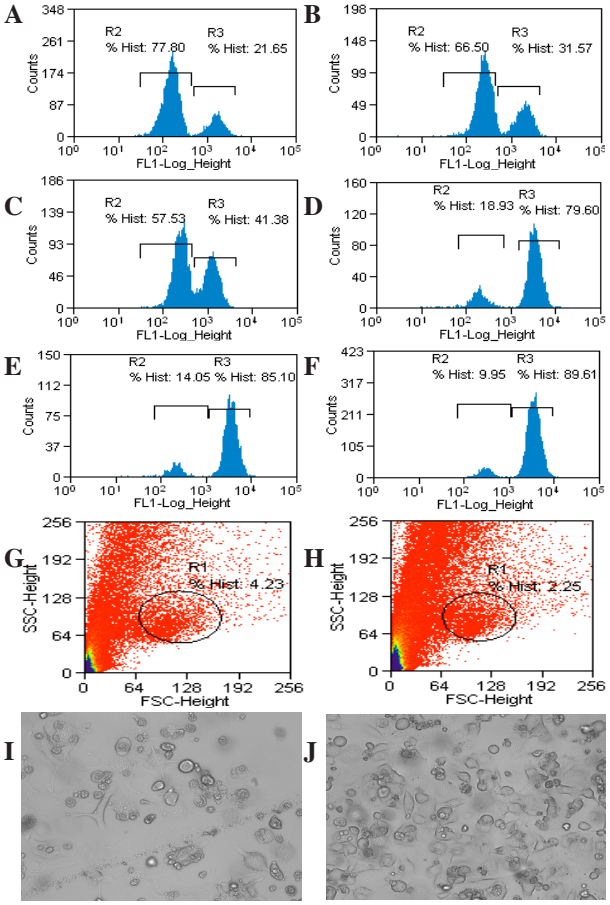


Figure 5. Expression of CD44 in Suspended Cells Irradiated with 5 (A); Expression of CD44 in suspended cells irradiated with 10 Gy (B); Expression of CD44 in Suspended Cells Irradiated with 15 Gy (C); Expression of CD44 in Adherent Cells Irradiated with 5 Gy (D); Expression of CD44 in Adherent Cells Irradiated with 10 Gy (E); Expression of CD44 in Adherent Cells Irradiated with 15 Gy (F); The Survival Rate of Suspended Cells after Irradiation with 30 Gy (G); The Survival Rate of Adherent Growing Cells after Irradiation with 30 Gy (H); Adherent Cells Irradiated by 30 Gy (10 × 10) (I); Suspended Cells Irradiated with 30 Gy (10 × 10) (J)

Changes in cell growth and expression of CD44 after exposure to different doses of radiation

The suspended and adherent cells were exposed to different doses of radiation (Figure 5). When the suspended cells and adherent cells were irradiated at 5 Gy, 10 Gy, and 15 Gy, the proportion of suspended CD44+ cells strongly and weakly positive for CD44 was 77.8%, 66.5%, 57.53%; and 21.65%, 31.57%, 41.38%, respectively (Figure5 A-C). In contrast, the proportion of adherent CD44+ cells strongly positive for CD44 was 18.93%, 14.05%, and 9.95%, respectively (Figure 5 D-F). When the irradiation dose was increased to 30 Gy, the survival of the suspended and adherent cells was significantly reduced (Figure 5 G-H). Electron microscopy revealed changes in cell morphology (Figure 5 I-J). Flow cytometry indicated a survival rate of approximately 2.25% for the adherent cells, whereas the survival rate of suspended cells in spheres was 4.23%, and CD44+ viable cells were not detected. After irradiation, the number

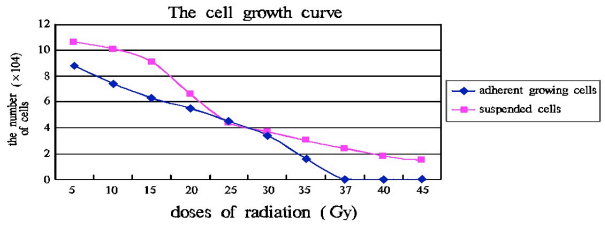


Figure 6. Cell Growth Curves of Suspended Cells and Adherent Cells after Exposure to Different Doses of Radiation

of suspended and adherent cells decreased. When the irradiation dose increased to 30 Gy, the number of adherent cells further decreased until all cells were dead, whereas after irradiation of suspended cells at 45 Gy, some cells were still alive (Figure 6).

Discussion

Suspension culture of OE-19 cells in serum-free stem medium: Tumor stem-like cells or tumor-initiating cells are defined as a subset of cells in tumors that have the capability to self-renew and differentiate. Thus, these cells can either initiate or maintain a tumor. Lapidot et al. (1994) reported human acute myeloid leukemia stem cells with the CD34+/CD38– phenotype in the first report on malignant tumor stem cells. In recent years, the existence of cancer stem cells in other tissues has been explored. Tumor stem cells (including stem-like cells and tumor-initiating cells) have been widely discovered in solid tumors, including glioma (Singh et al., 2003; Singh et al., 2003; Singh et al., 2004; Rahman et al., 2009), breast cancer (Al-Hajj et al., 2003; Ponti et al., 2005), medulloepithelioma (Taylor et al., 2005), skin cancer (Perez-Losada et al., 2003), and prostate cancer (Richardson et al., 2004; Collins et al., 2005), retinoblastoma (Seigel et al., 2005), pancreatic cancer (Li et al., 2007), and hepatoblastoma (Hayashi et al., 2011). Animal models of human tumors have been shown to contain gastric cancer stem cells (Houghton et al., 2004) and lung cancer stem cells (Noh et al., 2009). Isolation of cancer stem cells from tumor cell lines also has been conducted. Kondo et al. (2004), Setoguchi et al. (2004), and Patrawala et al. (2005) have found cancer stem cells in many tumor cell lines using the SP method. In addition, some studies have shown that many tumor cells have stem cell characteristics based on CD133 expression. Atsushi et al. (2006), Jun et al. (2007), Monika et al. (2007), and Elena et al. (2007) found cancer stem-like cells that expressed CD133 in hepatoma cell lines, prostate cancer-derived epithelial cell lines, pancreatic cancer cell lines, and melanoma cell lines, respectively. These studies indicate that tumor stem cells exist among cells cultured from most tumor types.

Okumttra et al. (2003) discovered a group of heterogeneous cells in esophageal cancer cells. Tumor stem cells play an important role in tumor recurrence and metastasis. However, isolation of esophageal cancer stem cells has remained difficult. Two methods are often used to isolate cancer stem cells: phenotypic marker screening and Hoechst 33342 dye exclusion (side population analysis, SP) (Smith et al., 2009). Marker screening is difficult because the molecular characteristics of cancer stem cells are not clear, and the screening of a large number of known markers is not feasible. However, toxicity limits the further application of the Hoechst 33342 method.

Serum-free suspension methodology is used to culture stem cells in vitro, mainly because it maintains the undifferentiated state of stem cells. Serum-free culture serves as a preliminary separation and amplification step for stem cells in vitro that is fast, simple, and practical. However, the stem cells must be passaged more than 6 times. In our experiments applying serum-free culture to enrich esophageal cancer stem cells, we found that these cells have more stringent requirements for serum-free culture. First, the adherent cells have to be washed more than twice (preferably 2–3 times) to achieve serum-free conditions. Second, there are stringent requirements regarding the growth factors used. B27 supplement contains various growth factors and is the ideal additive for serum-free culture, allowing cells to proliferate (a small sphere of cells had formed by the third day). In addition, we found that EGF and/or bFGF plays an important role in cell proliferation in serum-free culture. We observed that cells in long-term cultures do not proliferate and undergo apoptosis if EGF and/or bFGF are not added. Dontu et al. (2003) similarly found that EGF and/or bFGF stimulation induced proliferation in cell suspension cultures. However, serum-free culture is only a method to enrich stem cells. The cells obtained by this method are still heterogeneous cells. Additionally, serum-free culture limits the differentiation of cells to some extent, but does not completely prevent differentiation. In our experiments, we also found that suspended cell spheres should be cultured in serum-free medium for continuous passaging, as the reintroduction of serum-containing medium can result in adherent growth.

Phenotype of tumor stem-like cells in suspended cells spheres: CD44 is a widely distributed cell surface transmembrane protein that belongs to the adhesion molecule superfamily. By providing a specific link between cells and between cells and the matrix, CD44 plays an important role in tissue development, inflammation, wound healing, and many other pathophysiological processes. CD44 is closely related to cell motility, tumorigenesis, invasion, and metastasis, and it has received widespread attention in the study of gastrointestinal cancer. In recent years, many studies have found that cells expressing CD44 have the characteristics of cancer stem cells. For example, breast cancer stem cells have been characterized as ESA+CD44+ CD24–/low (Al-Hajj et al., 2003), prostate cancer stem cells as CD44+ahi1CD133+ (Collins et al., 2005), pancreatic cancer stem cells is ESA+ CD44+ CD24+(Li et al., 2007), and colorectal cancer stem cells as ESA+CD44+CD166+ (Dalerba et al., 2007). In this study, we probed CD44 expression in OE-19 esophageal cells. CD44 expression in suspended cell spheres was much higher than that in adherent cells; therefore, CD44 may be an esophageal tumor stem-like cell marker.

Suspension cell spheres have increased capacity for proliferation: Stem cells live in a particular environment called the stem cell niche. Under the control of the stem cell niche, stem cells are maintained in a balance between

self-renewal and differentiation in the G0 phase (Kordes et al., 2013; Pérez Mdel et al., 2013). Stem cell niches provide a microenvironment for stem cells to control proliferation and inhibit differentiation (Hanoun et al., 2013). One difference between tumor stem cells and normal stem cells is that tumor stem cells have unlimited self-proliferative capacity and disordered cell cycle regulation. Singh et al. (2004) found that the growth rate of CD133+ brain tumor stem cells was significantly higher than that of CD133– cells in vitro. In our study, a similar effect is demonstrated by the cell growth curves. Suspended cell spheres of OE-19 cells that were enriched in cancer stem cells grew much faster than adherent cells. The suspended cell spheres thus had a high proliferative capacity, in line with the characteristics of tumor stem-like cells.

Suspended cell spheres have self-renewal capacity: Self-renewal is an essential characteristic of stem cells, and at least daughter cell maintains the traits of the parent cell, whereas the other daughter cell may be directed toward differentiation and proliferation in a process called asymmetric division. Soft agar cloning experiments are typically used to detect the growth of non-adherent of tumor cells (Bentivegna et al., 2010; Toivanen et al., 2011). Not all tumor cells have the ability to form colonies in soft agar; colony formation of tumor cells is positively correlated with their tumorigenic ability (Freedman et al., 1974). According to the cancer stem cell theory, only tumor stem cells are capable of colony formation and tumorigenicity (Reya et al., 2001). We detected the self-renewal capacity of suspended cell spheres of OE-19 cells according to a method described in the literature. In our experiment, we found that the underlying concentration of the medium and the temperature of the soft agar play an important role in colony formation. In the soft agar cloning experiment, the number of clones formed from suspended cell spheres was higher than the number of clones formed from adherent cells ($P < 0.05$). Therefore, the suspended cell spheres had strong clone-forming ability, which is consistent with the notion of self-renewal of tumor stem cells.

Radiation resistance of suspended cell spheres: Despite the reduction of tumor recurrence by surgery, radiotherapy, and chemotherapy, the survival rate of patients with metastatic tumors has not improved, with aggressive cancer continuing to receive great attention from many cancer researchers (Rich et al., 2007). Using cell surface markers and allogeneic transplantation, cancer biologists have isolated subsets of tumor cells that have the characteristics of self-renewal and pluripotency; these cells are called cancer stem cells (CSC). CSCs have been shown to exhibit increased resistance to conventional chemo- and radio-therapies, and the repopulation of CSCs surviving after treatment may be the cause of treatment failure (Eyler et al., 2008). In our experiment, study the suspended cells and adherent cells were exposed to different doses of radiation. As the radiation dose increased, the proportion of suspended CD44+ cells strongly and weakly positive for CD44 decreased and increased, respectively. The proportion of adherent CD44+ cells strongly positive for CD44 also decreased. When the irradiation dose was increased to 30 Gy, the survival of the suspended and adherent cells was significantly reduced. Electron microscopy revealed morphological changes in the cells, and flow cytometry analysis revealed a survival rate of approximately 2.25% and 4.23% for adherent cells and cells in suspended cell spheres, respectively. CD44+ cells were not detected among the viable cells. Furthermore, when the irradiation dose was increased to 30 Gy, no adherent cells remained, whereas some suspended cells were still alive after irradiation at 45 Gy. Thus, the suspended cell spheres demonstrated radiation resistance.

In conclusions, we successfully cultured suspended cell spheres of the esophageal carcinoma cell line OE-19 in serum-free medium, and the spheres showed enriched tumor stem-like cells as indicated by CD44 expression, increased capacity for colony formation in soft agar, and radiation resistance. CD44 expression in the suspended cell spheres was much higher than that in adherent growing cells; therefore, CD44 may be a marker for esophageal tumor stem-like cells.

References

Al-Hajj M, Wicha MS, Benito-Hernandez A, et al (2003). Prospective identification of tumorigenic breast cancer cells. *Proc Natl Acad Sci U S A*, **100**, 3983-8.

Atsushi S, Masahito N, Hitomi A, et al (2006). Characterization of CD133 hepatocellular carcinoma cells as cancer stem/progenitor cells. *Biochem Biophys Res Commun*, **351**, 820-4.

Bentivegna A, Conconi D, Panzeri E (2010). Biological heterogeneity of putative bladder cancer stem-like cell populations from human bladder transitional cell carcinoma samples. *Cancer Sci*, **101**, 416-24.

Cheng L, Ramesh AV, Flesken-Nikitin A, et al (2010). Mouse models for cancer stem cell researchl. *Toxicol Patho*, **38**, 62-71.

Collins AT, Berry PA, Hyde C, et al (2005). Prospective identification of tumorigenic prostate cancer stem cells. *Cancer Res*, **65**, 10946-51.

Dalerba P, Dylla SJ (2007). Phenotypic characterization of human colorectal cancer stem cells. *Proc Natl Acad Sci U S A*, **104**, 10158-63.

Dontu G, Abdallah WM, Foley JM, et al (2003). In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells. *Genes Dev*, **17**, 1253-70.

Elena M, Floriana F, Enrico G, et al (2007). Melanoma contains CD133 and ABCG2 positive cells with enhanced tumourigenic potential. *Eur J Cancer*, **43**, 935-46.

Eyler CE, Rich JN (2008). Survival of the fittest: cancer stem cells in therapeutic resistance and angiogenesis. *J Clin Oncol*, **26**, 2839-45.

Freedman VH, Shin SI (1974). Cellular tumorigenicity in nude mice: correlation with cell growth in semi-solid medium. *Cell*, **3**, 355-9.

Hayashi S, Fujita K, Matsumoto S, et al (2011). Isolation and identification of cancer stem cells from a side population of a human hepatoblastoma cell line, HuH-6 clone-5. *Pediatr Surg Int*, **27**, 9-16.

Houghton J, Stoicov C, Nomura S, et al (2004). Gastric cancer originating from bone marrow-derived cells. *Science*, **306**, 1568-71.

Jun M, Bungo F, Hongzhen Li, et al (2007). Identification of putative stem cell markers, CD133 and CXCR4, in Htert-immoralized primary nonmalignent and malignant tumor-

derived human prostate epithelial cell lines and in prostate cancer specimens. *Cancer Res*, **67**, 3153-61.
Kondo T, Setoguchi T, Taga T (2004). Persistence of a small subpopulation of cancer stem-like cells in the C6 glioma cell line. *Proc Natl Acad Sci U S A*, **101**, 781-6.
Kordes C, Häussinger D (2013). Hepatic stem cell niches. *J Clin Invest*, **123**, 1874-80.
Lapidot T, Sirard C, Vormoor J, et al (1994). A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. *Nature*, **367**, 645-8.
Li C, Heidt DG, Dalerba P, et al (2007). Identification of pancreatic cancer stem cells. *Cancer Res*, **63**, 1031-7.
Monika O, Patricia A, Ole A, et al (2007). Detection of tumor stem cell markers in pancreatic carcinoma cell lines. *Hepatobiliary Pancreat Dis Lin*, **6**, 92-7.
Noh MS, Jun BH, Kim S, et al (2009). Magnetic surface-enhanced Raman spectroscopic (M-SERS) dots for the identification of bronchioalveolar stem cells in normal and lung cancer mice. *Biomaterials*, **30**, 3915-25.
Okumttra T, Shimada Y, Imamura M, et al (2003). Neurotrophin receptor p75(NTR) characterizes human esophageal keratinceyte stem cells invitro. *Oneogene*, **22**, 4017-26.
Patrawala L,Calhoun T,Schneider-Broussard R, et al (2005). Side population is enriched in tumorigenic,stem-like cancer cells,whereas ABCG2+ and ABCG- cancer cells are similarly tumorigenic. *Cancer Res*, **65**, 6207-19.
Perez-Losada J, Balmain A (2003). Stem-cell hierarchy in skin cancer. *Nat Rev Cancer,* **3**, 434-43.
Pérez Mdel C, López A, Padilla P (2013). Space-time dynamics of Stem Cell Niches: a unified approach for Plants. *J Integr Bioinform*, **10**, 219.
Ponti D, Costa A, Zaffaroni N, et al (2005). Isolation and in vitro propagation of tumorigenic breast cancer cells with stem/progenitor cell properties. *Cancer Res*, **65**, 5506-11.
Reya T, Morrison SJ, Clarke MF, et al (2001). Stem cells, cancer and cancer stem cells. *Nature*, **414**, 105-11.
Rahman R, Heath R, Grundy R, et al (2009). Cellular immortality in brain tumor: an integration of the cancer stem cell paradigm. *Biochim Biophys Acta*, **1792**, 280-8.
Richardson GD, Robson CN, Lang SH, et al (2004). CD133, a novel marker for human prostatic epithelial stem cells. *J Cell Sci*, **117**, 3539-45.
Rich JN (2007). Cancer stem cells in radiation resistance. *Cancer Res*, **67**, 8980-4.
Singh SK, Clarke ID, Hide T, et al (2004). Cancer stem cell in nervous system tumors. *Oncogene*, **23**, 7267-73.
Singh SK, Clarke ID, Terasaki M, et al (2003). Identification of a cancer stem cell in human brain tumors. *Cancer Res*, **63**, 5821-8.
Singh SK, Hawkins C, Clarke ID, et al (2004). Identification of human brain tumor initiating cells. *Nature*, **432**, 396-401.
Seigel GM, Campbell LM, Narayan M, et al (2005). Cancer stem cell characteristics in retinoblastoma. *Mol Vis*, **11**, 729-37.
Setoguchi T, Taga T, Kondo T (2004). Cancer stem cells persist in many cancer cell lines. *Cell Cycle*, **3**, 414-5.
Smith PJ, Furon E, Wiltshire M, et al (2009). ABCG2-associated resistance to Hoechst 33342 and topotecan in a murine cell model with constitutive expression of side population characteristics. *Cytometry A*, **75**, 924-33.
Taylor MD, Poppleton H, Fuller C, et al (2005). Radial glia cells are candidate stem cells of ependymoma. *Cancer Cell*, **8**, 323-35.
Toivanen R, Berman DM, Wang H (2011). Brief report: a bioassay to identify primary human prostate cancer repopulating cells. *Stem Cell,* **29**, 1310-4.