Esophageal cancer tumorspheres involve cancer stem-like populations with elevated aldehyde dehydrogenase enzymatic activity

GONG ZHANG[1*], LEI MA[2*], YOU-KE XIE[1*], XIAO-BO MIAO[1] and CHUAN JIN[2]

[1]Cancer Research Institute of Southern Medical University, Guangzhou 510515; [2]Internal Medical Department of Guangzhou Medical University Cancer Institute and Hospital, Guangzhou 510182, P.R. China

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Abstract. Cancer stem cells (CSCs) form spheres *in vitro* in serum-free suspension culture. Sphere formation is particularly useful to enrich the potential CSC subpopulations as a functional approach. Few reports are currently available on tumorspheres in esophageal cancer (EC). The present study focused on evaluating the cancer stem-like properties and analyzing the difference between spheroid and adherent cells of the Eca109 human EC cell line. Immunofluorescence and immunoblotting analysis revealed that EC tumorspheres expressed the stem cell markers Nanog and Oct4 more highly, but showed a decreased expression of the differentiation marker CK5/6. The spheroids were chemoresistant to cisplatin compared to the adherent cells (32.5 vs. 135.8 μM in IC_{50}). Side population cells increased in tumorspheres compared to adherent cells (0.7 vs. 5.6%). A marked upregulation of drug-resistant genes (ABCG2 and MDR1) was observed in sphere-forming cells. We compared the profiles of adherent and spheroid cells by microarrays and obtained one representative differentially expressed gene, aldehyde dehydrogenase (ALDH). We also verified that the cancer stem-like cells of EC contained a high ALDH enzymatic activity. ALDH-positive cells were enriched by 11- to 12-fold in spheroids, compared to adherent cells (2.5 vs. 28.6%). Immunofluorescence and immunoblotting analysis also revealed a higher expression of ALDH in EC tumorspheres. In conclusion, our study verified that sphere-forming culturing can be utilized to demonstrate the putative esophageal CSCs, and identified a potential esophageal CSC surface marker, ALDH.

Correspondence to: Dr Chuan Jin, Internal Medical Department of Guangzhou Medical University Cancer Institute and Hospital, Luhu Street, Guangzhou 510182, P.R. China
E-mail: jinchuan361@163.com

*Contributed equally

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Introduction

Esophageal cancer (EC) is a highly malignant neoplasm. The 5-year survival rate of patients is only 10% (1). Advanced EC is one of the most refractory cancers and is associated with poor outcome. Conventional chemotherapy and radiotherapy are widely used for EC. However, more than 40% of EC cases eventually result in recurrence and patients succumb to chemotherapy- and radiotherapy-resistant disease (2). Mounting evidence suggests that small populations of cells within tumors, known as cancer stem cells (CSCs), contribute to tumor maintenance and progression and are intrinsically resistant to therapies (3). CSCs have the ability to recreate the full phenotypic heterogeneity of the parent tumor (4). These cells express distinct surface markers allowing for reproducible and differential purification. Several stem cell markers, such as Nanog and Oct4, have been used successfully to identify CSCs in normal and tumor tissue (5). In addition, side population (SP) cells found in various types of cancer have been reported to exhibit CSC characteristics (6).

The anchorage-independent tumorsphere culture of stem cells was instrumental in the study of adult CSCs (7-9). Sphere formation is particularly useful to enrich the potential CSC subpopulations as a functional approach (10,11). CSCs form spheres *in vitro* in serum-free suspension culture. In the suspension culture, tumorsphere-forming cells failed to express cytokeratins (CK), but were found to express stem cell markers (12). Thus, the suspension culture system is thought to maintain CSCs in their undifferentiated state, facilitating their enrichment.

However, few reports are currently available regarding tumorspheres in EC. Therefore, the aim of the present study was to enrich and identify EC cell subsets with CSC properties. The tumorsphere of EC is considered to be a valuable model for the further study of both CSCs and chemoresistance. To select esophageal CSC markers, we performed comparative global gene expression analyses between adherent and spheroid cells. We compared profiles of adherent and spheroid cells and obtained one representative differentially expressed gene, aldehyde dehydrogenase (ALDH). We also verified that the cancer stem-like cells of EC contained a high ALDH enzymatic activity.

Materials and methods

Cells and culture conditions. The Eca109 human esophageal cancer cell line was purchased from the Shanghai Cell Biology Institute of the Chinese Academy of Sciences, China. The cells were cultured in DMEM medium (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone) and 100 U/ml penicillin/streptomycin (Gibco, Langley, OK, USA). Cultures were maintained in a humidified incubator at 37˚C in 5% CO_2 air atmosphere.

Tumorsphere culture and differentiation. Cells (1,000 cells/ml) were cultured in suspension in serum-free Ham's F-12 medium (Gibco), supplemented with B27 (1:50; Gibco), 20 ng/ml EGF (Invitrogen, Grand Island, NY, USA) and 20 ng/ml FGF (Invitrogen). To propagate spheres *in vitro*, spheres were collected by gentle centrifugation, dissociated to single cells and then cultured to generate tumorspheres of the next generation. To guide the differentiation of spheres *in vitro*, spheroids were cultured in DMEM supplemented with 10% FBS without growth factors.

Immunofluorescent staining. For immunofluorescent staining, adherent or semi-differentiated spheroid cells were grown on the surface of cover slides. Spheroid staining was performed in 96-well microplates. The cells were fixed with 4% paraformaldehyde. Following rehydration in PBS, cells were incubated with respective primary antibodies at 37˚C for 45 min. Mouse anti-Nanog, Oct4, CK5/6 and ALDH1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used as primary antibodies. Slides or spheroids were then washed with PBS for 15 min and secondary antibodies were incubated at 37˚C for 45 min. Alexa594-conjugated goat anti-mouse IgG (against anti-Nanog, Oct4 and CK5/6; Invitrogen) or FITC-conjugated goat anti-mouse IgG (against anti-ALDH1; Invitrogen) were used as secondary antibodies. The nuclei were stained with DAPI. Sections were examined with confocal microscopy (Olympus-FV1000, Japan).

Immunoblotting. Total protein was extracted from spheroid or adherent Eca109 cells using cell lysis buffer. Proteins were run in 10% SDS-PAGE and transferred on a PVDF sheet. The blots were incubated for 1-2 h in blocking solution (5% skimmed milk in Tris-buffer), and then for 1 h using the following primary antibodies: mouse anti-Nanog, Oct4, CK5/6, ABCG2, MDR1, ALDH1 and GAPDH (Santa Cruz). The sheet was then incubated for 1 h with HRP-conjugated secondary antibodies (Invitrogen) against mouse immunoglobulins. The bands were visualized using the ECL-Plus detection system (Bio-Rad, Hercules, CA, USA).

Drug sensitivity assay to antitumor drug. Cells obtained from adherent or spheroid Eca109 cells were seeded in 96-well microplates at a density of 3,000 cells/well. The cells were treated with increasing concentrations of cisplatin (Sigma-Aldrich, St. Louis, MO, USA) as indicated by the manufacturer's instructions. MTT assay was performed to determine the cell viability following exposure to cisplatin for 72 h. The number of living cells was directly proportional to the absorbance at 490 nm.

Hoechst staining and SP cell assay. Cells obtained from adherent or spheroid Eca109 cells were suspended in DMEM/2% FBS at $1x10^6$ cells/ml and stained with Hoechst-33342 dye (5 μg/ml; Sigma-Aldrich) for 90 min at 37˚C. Following this incubation, cells were washed with ice-cold PBS and stained with propidium iodide (1 μg/ml; Sigma-Aldrich) to label and exclude dead cells. The cells were maintained at 4˚C for the flow cytometric analysis and for sorting of the SP fraction using a FACSAria flow cytometer (BD Biosciences, San Jose, CA, USA).

RNA isolation and microarray analysis. Eca109 spheroids were filtered by a cell strainer (40 μm; BD Biosciences). Spheroids with a diameter of >40 μm were selected. Total RNA was extracted separately from adherent and spheroid Eca109 cells using TRIzol reagent (Invitrogen), according to the manufacturer's instructions. RNA was subjected to GeneChip_ expression array analysis with two-cycle target labeling (implemented by CapitalBio Corp., Beijing, China). Briefly, cDNA was synthesized from total RNA using T7-Oligo (dT) primers, and biotinylated cRNA was synthesized using cDNA. Labeled cRNA (2 μg) was hybridized to the 22K Human Genome Array. The array image was scanned and analyzed using LuxScan 10KA.

Aldefluor assay by FACS. The ALDEFLUOR kit (StemCell Technologies, Durham, NC, USA) was used to analyze the population with a high ALDH enzymatic activity. Cells obtained from adherent or spheroid Eca109 cells were suspended in ALDEFLUOR assay buffer containing ALDH substrate and incubated during 40 min at 37˚C. As a negative control, for each sample of cells an aliquot was treated with 50 mmol/l diethylaminobenzaldehyde (DEAB), a specific ALDH inhibitor. FACS was performed using a FACSAria flow cytometer (BD Biosciences).

Statistical analyses. Data were analyzed using statistics soft SPSS 13.0 and were shown as the means ± SD. $P<0.05$ were considered statistically significant.

Results

Esophageal cancer tumorsphere contains cells with cancer stem-like properties. Ponti *et al* first reported that breast CSC properties could be propagated *in vitro* as non-adherent mammospheres under serum-free culture conditions (13). In the present study, we attempted to enrich the CSC population from the Eca109 EC cell line. To observe the differentiation of the tumorspheres, spheres were cultivated in serum-driven culture. After 48 h of culture, floating undifferentiated cells attached to the plastic, gradually migrating from tumorspheres and differentiating into adherent cells (Fig. 1A). We detected two typical CSC markers, Nanog and Oct4, that were spheroid-cultured under differentiation conditions by immunofluorescence. In addition, the expression of the marker which indicates EC surface epithelium, CK5/6, was also observed. As shown in Fig. 1B, Nanog and Oct4 were expressed in the center of the semi-differentiated spheroids. However, a markedly decreased expression was observed at the edge of the semi-differentiated spheroids. Inversely, CK5/6 expression was almost absent in

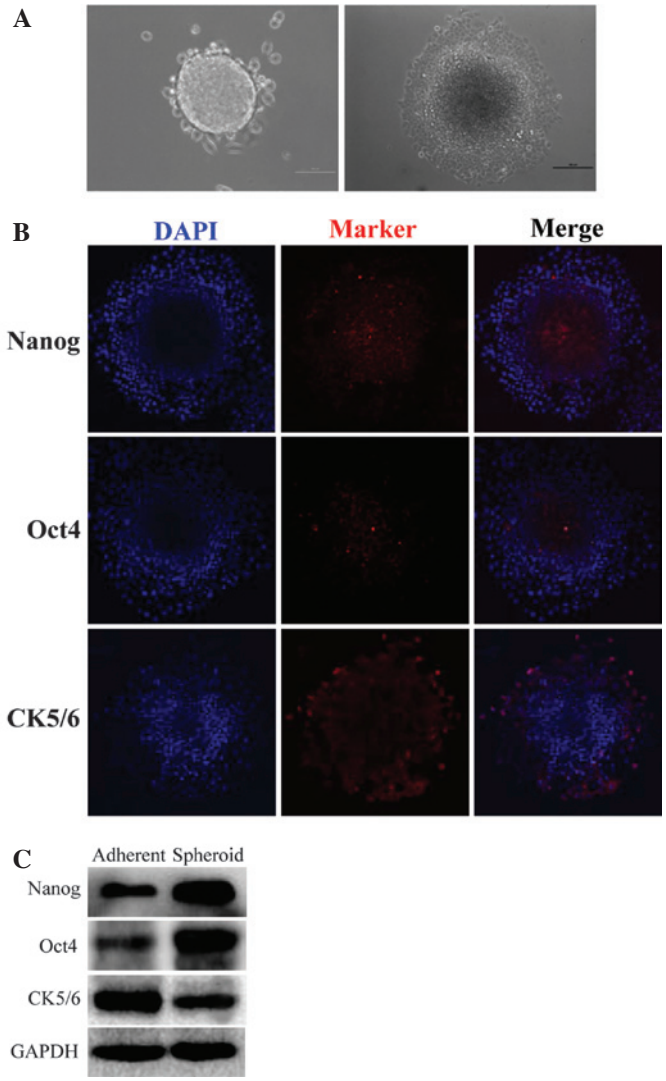

Figure 1. (A) Microscopical analysis of Eca109 EC tumorsphere cultivated in serum-free (left panel) and differentiation conditions for 48 h (right panel). Scale bars, 100 μm. (B) Spheroids of Eca109 cells were cultured under serum-driven cultures for 48 h. The indicated antibodies were analyzed by immunofluorescence using a confocal laser scanning microscope. (C) Expression levels of Nanog, Oct4 and CK5/6 in the adherent and spheroid Eca109 cells examined by immunoblotting are shown.

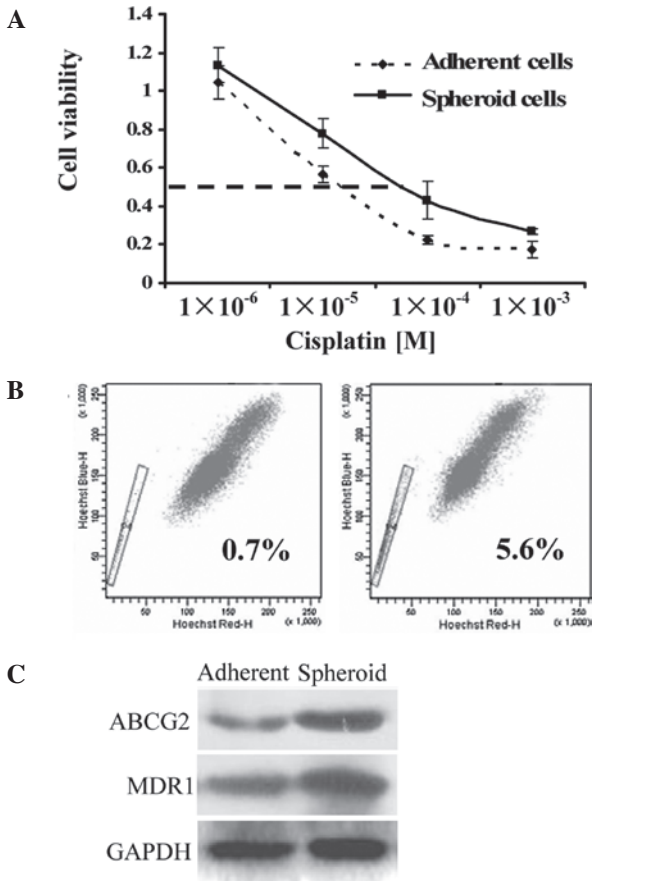

Figure 2. (A) Dose-response curves of cells obtained from adherent and spheroid Eca109 cells following 72 h of treatment with cisplatin. Bars are the standard error (n=3). (B) Comparative FACS analysis of SP cells of adherent (left panel, 0.7%) and spheroid (right panel, 5.6%) Eca109 cells. (C) Expression levels of ABCG2 and MDR1 in the adherent and spheroid Eca109 cells examined by immunoblotting.

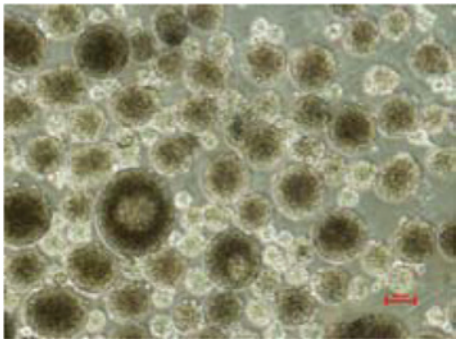

Figure 3. Image of spheroids with a diameter of >40 μm which were selected to perform the microarray analysis. Scale bars, 100 μm.

the center of the semi-differentiated spheroids, but was markedly expressed at the edge of the semi-differentiated spheroids.

Cancer stem-like properties were confirmed at the protein level in EC spheroids by immunoblotting. As expected, cancer cells cultured in the serum-free medium caused a CSC marker shift in the cells, including a marked upregulation of the CSC markers Nanog and Oct4, and the downregulation of the epithelium marker CK5/6 (Fig. 1C). The results indicated that EC tumorspheres demonstrated an increased expression of stem cell markers.

EC tumorspheres exhibit an increased expression of ABC-transporter and resistance to chemotherapeutic drugs. To examine whether EC tumorspheres possess a hypothesized chemoresistant phenotype of the CSCs, we assessed the sensitivity of the sphere-forming cells and the differentiated cells to drugs commonly used in chemotherapy. The EC tumor cells from the spheroids exhibited an increased IC_{50} (half maximal inhibitory concentration) value (4- to 5-fold; 32.5 vs. 135.8 μM) to cisplatin compared to the control adherent cells (Fig. 2A). Tumor cells resistant to chemotherapy occur in part due to the overexpression of ATP-binding cassette multidrug-resistance gene1 (MDR1) (14) and ATP-binding cassette sub-family G member 2 (ABCG2) (15). This property correlates with the ability to expel dyes, defined as a flow cytometry SP (6). SP cells have also been reported to exhibit CSC characteristics (16). In our study, EC cells cultured in suspension cultures were found to contain an 8-fold increase in the proportion of SP

Table I. Differentially expressed genes (sphere vs. adherent).

ID	Gene symbol	Ratio	Gene description
12964	EREG	38.0760	Epiregulin precursor
5324	S100A2	36.6280	S100 calcium-binding protein A2
13689	IL1B	25.3600	Interleukin-1 β precursor
9469	DUSP6	19.1750	Dual specificity protein phosphatase 6
10355	TNC	17.8630	Tenascin precursor
13167	IGFBP7	16.8850	Insulin-like growth factor binding protein 7 precursor
4767	SAT	11.5940	Diamine acetyltransferase 1
7765	CLECSF2	11.5100	C-type lectin superfamily member 2
20212	EGR1	10.4540	Early growth response protein 1
4340	FOS	9.6953	Proto-oncogene protein c-fos
5981	KLK11	8.8023	Kallikrein 11 precursor
16753	FHL1	8.3500	Skeletal muscle LIM-protein 1
7978	MMP1	8.3340	Interstitial collagenase precursor
7871	HAS3	8.1108	Hyaluronan synthase 3
15869	TNFAIP3	7.3406	Tumor necrosis factor, α-induced protein 3
12711	COL17A1	7.2343	Collagen α 1 (XVII) chain
1415	IL1A	7.2178	Interleukin-1 α precursor
14111	SERPINB7	7.0842	Megsin
8895	GBP2	6.6438	Interferon-induced guanylate-binding protein 2
958	LTB	6.5935	Lymphotoxin-β
5021	**ALDH1A1**	6.5078	Aldehyde dehydrogenase 1 family, member A1
1813	TIMP1	6.5020	Metalloproteinase inhibitor 1 precursor
6918	SERPINB2	6.4078	Plasminogen activator inhibitor-2 precursor
7661	S100A4	6.3883	S100 calcium-binding protein A4
6163	GPR87	6.3117	Probable G protein-coupled receptor GPR8
6936	**ALDH1A3**	6.0678	Aldehyde dehydrogenase 6
2523	FST	6.0256	Follistatin precursor
5966	LAMC2	5.9743	Laminin γ-2 chain precursor
6517	BF	5.9716	Complement factor B precursor
1393	FGFBP1	5.8656	Fibroblast growth factor binding protein 1
5322	TSN1	5.6896	Tetraspanin 1
3284	PLAG1	5.6665	Pleiomorphic adenoma gene 1
3883	PHCA	5.4134	Alkaline phytoceramidase
6664	C10orf116	5.4024	Adipose most abundant gene transcript 2
1512	CXCL10	5.2340	Small inducible cytokine B10 precursor
3028	SNX8	5.1606	Sorting nexin 8
968	**ALDH3A1**	5.1533	Aldehyde dehydrogenase, dimeric NADP-preferring
1336	SEMA3A	5.0699	Semaphorin 3A precursor
17731	MA17	5.0025	17 kDa membrane associated protein

Bold indicates >5-fold difference in the expression of the gene involved.

cells compared to the adherent controls (0.7 vs. 5.6%; Fig. 2B). ABCG2 and MDR1 were also confirmed at the protein level by immunoblotting. The result indicated that ABCG2 and MDR1 were substantially increased in tumorspheres compared to the adherent cells (Fig. 2C).

Gene expression profile analysis of EC spheroids based on microarray data. To clarify differential gene expression profiles between tumorsphere and the adherent cells of EC, microarray analysis was performed. A previous study has verified that the more serial passages in the spheroids, the more CSCs in spheroids (12). To ensure the reliability of microarray results, we achieved the 20th passage of EC spheroids. The spheroids were filtered by a cell strainer. Spheroids with a diameter of >40 μm were selected to perform the microarray analysis (Fig. 3). The mRNA expression profiles of the spheroid and adherent Eca109 cells were analyzed by human cDNA microarray. Among the 21,522 probes examined,

376 genes were upregulated (ratio >2.0) in the spheroid cells compared to the adherent cells, whereas 325 genes were downregulated in the spheroid cells. The upregulated genes were then assigned to a functional class using a gene ontology annotation tool by the Database for Annotation, Visualization and Integrated Discovery (Bioresource for array genes; http://david.abcc.ncifcrf.gov/). Based on their functions, the majority of these genes were classified into 'polymorphism', 'extracellular matrix', 'phosphoprotein', 'cell adhesion' and 'cell secretion' groups. In addition, we found 39 genes that showed a >5-fold upregulation in the spheroid cells compared to the adherent cells (Table I). Among these genes, three upregulated genes of the ALDH family exhibited a >5-fold difference in expression, including ALDH1A1, ALDH1A3 and ALDH3A1 (Table I, bold).

EC spheroids contain high ALDH enzymatic activity. In the different profiles, we found that three ALDH family-related genes were significantly upregulated in the tumorsphere. ALDH, which detoxifies intracellular aldehydes through oxidation, may have a role in the differentiation of stem cells through the oxidation of retinoic acid. (17) ALDH expression has been suggested as a potential functional marker for CSCs (18). To confirm this finding, we utilized the ALDEFLUOR assay to assess the size of the population with ALDH enzymatic activity in the Eca109 EC cell line. ALDEFLUOR-positive cells were enriched by 11- to 12-fold in spheroids, compared to the adherent cells (2.5 vs. 28.6%; Fig. 4A). The ALDH increase in the tumorsphere was further confirmed at the protein level by immunofluorescence and immunoblotting. Immunofluorescence indicated that ALDH1 expression was observed in the tumorspheres, but was markedly decreased in the adherent cells (Fig. 4B). Immunoblotting showed a similar result; ALDH1 was found to be upregulated in tumorspheres compared to the control adherent cells (Fig. 4C). These results suggest that ALDH1-positive cells represent the stem/progenitor population of EC.

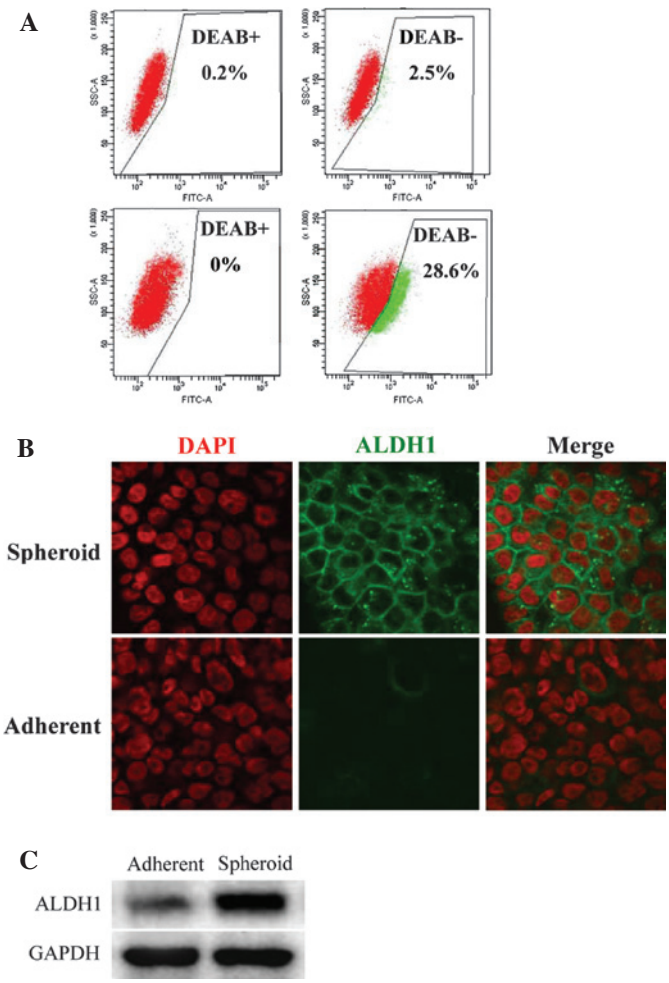

Figure 4. (A) Comparative FACS analysis of ALDH-positive cells of adherent (upper, 2.5%) and spheroid (bottom, 28.6%) Eca109 cells. (B) ALDH1 expression of spheroid and adherent Eca109 cells was analyzed by immunofluorescence using a confocal laser scanning microscope. (C) Expression levels of ALDH1 in the spheroid and adherent Eca109 cells examined by immunoblotting.

Discussion

Current therapies for EC eliminate most cells within a tumor. However, advanced EC still progresses to incurable, androgen-independent metastatic disease (19). According to the CSC hypothesis, current therapies fail to prevent cancer relapse and metastasis, since the small population of tumor stem cells is not susceptible to therapy (3). The tumorsphere, SP cells and drug-resistant cells have cancer stem-like properties. The SP technique is widely used to identify stem-like cells in cancer cells (20). SP cells derived from primary EC cells were more resistant to chemotherapeutic reagents and formed more colonies *in vitro* than non-SP cells; xenograft experiments revealed that SP cells were more tumorigenic *in vivo* (21). Drug resistance-related gene ABCG2 expression is an independent unfavorable prognostic factor in esophageal squamous cell carcinoma (22).

Previous studies have reported the application of sphere culture to isolate, enrich, maintain or expand potential CSC subpopulations from various types of cancer (23-27). It is generally agreed that, as with all stem cells, the tumorsphere-forming cells are capable of proliferation and self-renewal and possess higher tumorigenicity. To the best of our knowledge, few reports are available on the propagation of esophageal CSCs using sphere culture. In the present study, we provide a systematic investigation of sphere-propagating cells that are derived from the Eca109 EC cell line.

The hypothesis that our tumorspheres exhibitied stem-like properties was based on the following observations: i) Nanog and Oct4 were expressed in the undifferentiated spheroid cells, but the expression of CK5/6 was markedly decreased in undifferentiated spheroid cells; ii) spheroid cells contain more Nanog and Oct4 protein, but less CK5/6 protein than adherent cells; iii) tumorspheres exhibited an increased resistance to cisplatin; iv) spheroid cells had an increased prevalence of SP cells and v) the ABC-transporter protein was enriched in spheroid cells compared to adherent cells. Therefore, we suggest that the non-adherent tumorspheres cultured in serum-free conditions possess esophageal CSC properties. Thus, suspension culture may effectively be used to enrich esophageal CSCs.

To understand the mechanisms underlying the difference in spheroid and adherent cells in the Eca109 cell line, we performed gene chip analysis and found that three genes from the ALDH family were highly expressed in esophageal cancer

stem-like cells. This observation was further confirmed by immunoblotting. ALDHs are a superfamily of 17 intracellular enzymes that protect cells from the cytotoxic effects of peroxic aldehydes (28). Increased ALDH activity has also been found in stem cell populations in various types of cancer (18). ALDH activity may therefore provide a marker for normal and malignant stem as well as progenitor cells. Our study indicated that a high ALDH enzymatic activity is a function of EC tumorsphere. ALDH1 expression has been confirmed to associate with lymph node metastasis and poor survival in EC (29). Thus, we believe that ALDH is a putative CSC marker of EC.

In conclusion, our study outlines a condition for long-term culture of EC stem-like cells. This system is likely to be beneficial for the investigation of unique properties of EC stem-like cells in terms of their biology and their specific cell surface marker expression that distinguishes them from common EC cells. Regarding specific surface markers that are associated with stem-like cells, our current understanding is that ALDH is a potential esophageal CSC surface marker. Nevertheless, esophageal CSC cell surface markers remain to be identified. EC tumorsphere and our tumorsphere microarray analysis data provide a unique opportunity to find and identify such markers.

Acknowledgements

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