Experimental Cell Research ■ (■■■■) ■■■–■■■

Contents lists available at ScienceDirect

Experimental Cell Research

journal homepage: www.elsevier.com/locate/yexcr

Research Article

Transforming growth factor-beta1 promotes the migration and invasion of sphere-forming stem-like cell subpopulations in esophageal cancer

Dongli Yue [a,b], Zhen Zhang [a,b], Jieyao Li [a,b], Xinfeng Chen [a,b], Yu Ping [a,c], Shasha Liu [a,c], Xiaojuan Shi [a,b], Lifeng Li [a,b], Liping Wang [b], Lan Huang [a], Bin Zhang [a,d], Yan Sun [b,e], Yi Zhang [a,b,c,f,*]

[a] Biotherapy Center, the First Affiliated Hospital of Zhengzhou University, No. 1 Jianshe Road, Zhengzhou 450052, Henan, PR China
[b] Department of Oncology, the First Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, PR China
[c] School of Life Sciences, Zhengzhou University, Zhengzhou 450000, PR China
[d] Robert H. Lurie Comprehensive Cancer Center, Department of Medicine-Division of Hematology/Oncology, Northwestern University Feinberg School of Medicine, Chicago, IL 60611, USA
[e] Department of Medical Oncology, Cancer Hospital, Chinese Academy of Medical Sciences, PR China
[f] Institute of Clinical-Medicine, the First Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, Henan, PR China

ARTICLE INFO

Article history:
Received 27 February 2015
Received in revised form
12 June 2015
Accepted 13 June 2015

Keywords:
Cancer stem cells (CSCs)
Sphere-forming cells
Ultra low attachment surface culture
Esophageal cancer
TGF-β1

ABSTRACT

Esophageal cancer is one of the most lethal solid malignancies. Mounting evidence demonstrates that cancer stem cells (CSCs) are able to cause tumor initiation, metastasis and responsible for chemotherapy and radiotherapy failures. As CSCs are thought to be the main reason of therapeutic failure, these cells must be effectively targeted to elicit long-lasting therapeutic responses. We aimed to enrich and identify the esophageal cancer cell subpopulation with stem-like properties and help to develop new target therapy strategies for CSCs. Here, we found esophageal cancer cells KYSE70 and TE1 could form spheres in ultra low attachment surface culture and be serially passaged. Sphere-forming cells could re-differentiate and acquire morphology comparable to parental cells, when return to adherent culture. The sphere-forming cells possessed the key criteria that define CSCs: persistent self-renewal, overexpression of stemness genes (*SOX2, ALDH1A1 and KLF4*), reduced expression of differentiation marker *CK4*, chemoresistance, strong invasion and enhanced tumorigenic potential. SB525334, transforming growth factor-beta 1(TGF-β1) inhibitor, significantly inhibited migration and invasion of sphere-forming stem-like cells and had no effect on sphere-forming ability. In conclusion, esophageal cancer sphere-forming cells from KYSE70 and TE1 cultured in ultra low attachment surface possess cancer stem cell properties, providing a model for CSCs targeted therapy. TGF-β1 promotes the migration and invasion of sphere-forming stem-like cells, which may guide future studies on therapeutic strategies targeting these cells.

1. Introduction

Esophageal cancer is the fifth and eighth most frequent cause of cancer-related death in male and female worldwide respectively, with a 5-year survival rate of 26.2% due to late diagnosis, rapid growth and metastasis [1]. Despite therapeutic advances in the treatment of esophageal carcinoma, more than 40% of esophageal carcinoma cases still result in recurrence [2]. One of the main causes of treatment failure is the emergence of resistant cancer cells after therapy, followed by relapse of the cancer. This phenomenon can be explained by cancer stem cells (CSCs) theory. The existence of CSCs were first identified and characterized in patients with acute myeloid leukemia [3,4]. Since the identification of leukemic stem cells, CSCs have been found to reside in a number of other malignancies, including breast [5,6], brain [7,8], lung [9], prostate [10], colon [11,12], melanoma [13], and pancreatic cancers [14]. Cancer stem cells (CSCs) are a limited number of cancer cells with a self-renewal potential and multilineage differentiation capacity and play a dominant role in tumor initiation, metastasis, immune evasion and resistance to current therapies leading to recurrence. CSCs exhibit self-renewal by activating developmental pathways including Wnt, Hedgehog (Hh), and Notch and by aberrant expression of stem-related genes such as *BMI1, OCT3/4, SOX2, KLF4* and *NANOG* that participate in their maintenance [15]. As CSCs are thought to be the main reason of therapeutic failure, these cells must be effectively targeted to elicit long-lasting therapeutic responses.

Mounting evidence demonstrates that CSCs can be enriched and maintained by sphere formation in serum-free medium at low

* Corresponding author at: Biotherapy Center, the First Affiliated Hospital of Zhengzhou University, No. 1 Jianshe Road, Zhengzhou 450052, Henan, China. Fax: +86 371 66295320.
E-mail address: yizhang@zzu.edu.cn (Y. Zhang).

http://dx.doi.org/10.1016/j.yexcr.2015.06.007
0014-4827/

adherence [9,15–18]. These conditions favor growth of highly tumorigenic stem-like cells. In the suspension culture, sphere-forming cells were found to express stem cell markers, epithelial to mesenchymal transition (EMT) markers and aberrantly activate Wnt, Hedgehog and Notch signaling pathways, but failed to express differentiation markers [15,17,18]. For example, Justilien et al. demonstrated that lung cancer sphere cells exhibit stem-like properties because sphere cells displayed enhanced tumorigenic potential in vivo, expressed elevated mRNA for stem-related genes including *SOX2, OCT3/4, NANOG, ALDH1A1* and *CD133*, exhibited enhanced soft agar growth, and could redifferentiate and acquire morphology comparable to parental cells when returned to adherent culture [15]. Cao et al. reported that non-adherent tumor spheres from hepatoma cell lines cultured in stem cell conditioned medium possessed liver CSCs properties [16]. However, there are few reports currently available regarding tumor spheres in esophageal cancer. Accordingly, in the present study, we intended to enrich and identify the esophageal cancer cell subpopulation with stem-like properties.

2. Materials and methods

2.1. Cell lines and cell culture

Esophageal cancer cell lines KYSE70 and TE1 were all preserved in our laboratory and maintained as a monolayer in RMPI 1640 (Hyclone, USA) supplemented with 10% fetal bovine serum (Hyclone, USA), 100IU/ml of penicillin, and 100 μg/ml of streptomycin at 37 °C in a humidified 5% CO_2 incubator (Thermo, USA).

2.2. Sphere culture

Cells were cultured in serum-free DMEM/F12 medium (Invitrogen, USA) supplemented with 4 μg/mL heparin (Sigma, USA), B27 (1:50, GIBCO, USA), 20 ng/mL human recombinant epidermal growth factor (EGF) (Pepro Tech, USA), 20 ng/mL human recombinant basic fibroblast growth factor (bFGF) (Pepro Tech, USA), penicillin 100 IU/mL and streptomycin 100 μg/mL in Ultra Low Attachment Culture Flask (Corning, USA). Spheres were collected by gentle centrifugation (800 rpm) after 5–8 days, dissociated enzymatically (5 min in 0.05% trypsin, 0.53 mM EDTA-4Na; Invitrogen) and mechanically using a fire-polished Pasteur pipette as previously described [19], and then for experimental analysis or cultured to generate spheres of the next generation.

2.3. Sphere-forming assay

Serial sphere-forming capacity represents a valid surrogate for assessing CSCs self-renewal [19]. To investigate the ability to form cell spheres, 1000 cells were seeded in 24-well Ultra Low Attachment Plates(Corning, USA) in serum-free DMEM/F12 medium supplemented with heparin, B27, EGF and bFGF. After culturing for 7 days, the number of spheres was counted under a microscope (Leica, Germany). For secondary and tertiary sphere formation, the spheres were collected, dissociated into single cells and seeded in 24-well Ultra Low Attachment Plates.

2.4. RNA extraction, cDNA synthesis, and quantitative real time PCR

Total RNA was extracted from KYSE70 and TE1 cells by TRIzol reagent (Invitrogen, USA) and the first-strand cDNA was synthesized from 1 μg of total RNA using PrimeScript RT reagent Kit With gDNA Eraser (TaKaRa, Japan) as described earlier [20]. The cDNA was used as template. Quantitative real-time PCR (Q-PCR) was performed using FastStart Essential DNA Green Master (Roche, USA) and assessed by Agilent Mx3005P. GAPDH was used as an internal control. All Q-PCR reactions were performed in triplicate. The data was analyzed by $2^{\Delta\Delta Ct}$. Primers sequences for Q-PCR were shown in Table 1.

2.5. Transwell migration and invasion assay

Migration and invasion assays were performed using Transwell chambers. 2×10^4 cells were plated onto the top chamber with the non-coated membrane (migration) (24-well insert; 8 μm pore size; Corning, USA) or with matrigel-coated membrane (invasion) (24-well insert; 8 μm pore size; BD Biosciences). In both assays, cells were plated in 200 μl serum-free medium, and 600 μl full medium supplemented with 10% serum was used as a chemoattractant in the bottom chamber. After 24 h incubation at 37 °C, cells that did not migrate or invade through the pores were removed by a cotton-tipped swab. Cells on the lower surface of the membrane were fixed with 4% paraformaldehyde, stained with 0.5% crystal violet and counted under the microscope (Leika, USA) at × 200 magnification for 10 random fields. Both experiments were repeated triplicate independently.

2.6. Chemosensitivity assay

Cisplatin (DDP) from Sigma was dissolved according to the manufacturer's instructions. The parental cells and sphere-forming cells were seeded at 3000 cells/well in 96-well plates and treated with chemotherapeutic agents in quadruplicate. Cell viability was evaluated in CCK-8 assay (Biyuntian, China) after treatment with chemotherapeutic agents for 48 h, and the absorbance was measured at 450 nm using Multiskan Mk3 (Thermo, USA).The percentage of cell survival in treated cells was normalized with untreated controls.

After 24 h of treatment with DDP, cells were harvested and washed with ice-cold PBS twice. And then cells were suspended in the Annexin V-binding buffer to a final concentration of 1×10^6 cells/mL. Thereafter, cells were incubated with Alexa Fluor

Table 1
Primer sequences are shown for all genes tested.

Gene name	Sense sequence	Anti-sense sequence	Product size (bp)
GAPDH	GCACCGTCAAGGCTGAGAAC	TGGTGAAGACGCCAGTGGA	138
SOX2	TGAGCGCCCTGCAGTACAA	GCTGCGAGTAGGACATGCTGTAG	84
KLF4	ACACAAAGAGTTCCCATCTCAAG	GGTAGTGCCTGGTCAGTTCATC	123
ALDH1A1	CGCCAGACTTACCTGTCCTACT	TCAACATCCTCCTTATCTCCTTC	166
CK4	TACCACCACCCTGAACAAGAG	TGAAGTGAAGGAAGCACAGAGA	102
FIBRO	CAGTGGGAGACCTCGAGAAGA	GTCCCTCGGAACATCAGAAAC	169
NCAD	ACAGTGGCCACCTACAAAGG	CCGAGATGGGGTTGATAATG	201
VIM	GAGAACTTTGCCGTTGAAGC	GCTTCCTGTAGGTGGCAATC	163
TGF-β	GCCAGAGTGGTTATCTTTTGATG	AGTGTGTTATCCCTGCTGTCAC	120
SMAD4	CTCATGTGATCTATGCCCGTC	AGGTGATACAACTCGTTCGTAGT	146

Please cite this article as: D. Yue, et al., Transforming growth factor-beta1 promotes the migration and invasion of sphere-forming stem-like cell subpopulations in esophageal cancer, Exp Cell Res (2015), http://dx.doi.org/10.1016/j.yexcr.2015.06.007