

Tumorigenicity of Esophageal Cancer Stem Cells (ECSCs) in nude mouse xenograft model

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Abstract

Background and objectives: Modeling cancer in vivo is a very important tool to investigate cancer pathogenesis and molecular mechanisms involved in cancer progression. Laboratory mice are the most common animal used for rebuilding human cancer in vivo. Cancer stem cells (CSCs) are the main reason of failure in cancer therapy because of tumor relapse and metastasis. Isolation of cancer stem cells helps us to study their function and behavior. In the current study we separate cancer stem-like cells using sphere formation assay then investigate their tumorigenicity in xenograft tumor model.

Methods: YM1 cancer cells were cultured in serum-free media (SFM) in low adherent culture dishes for enrichment of cancer stem cells. The resulting spheres containing cancer stem-like cells were dissociated into single cells and were injected into the dorsal flank of B6 nude mice.

Results: A few days after injection, subcutaneous tumors formed. The growth curves of the resulting tumors were plotted using their weekly recorded lengths. The tumors' volume and weight were measured. The size of resulting tumors was appropriate to the number of cells injected. Pathological analysis confirmed esophageal origin of the resulting tumors.

Conclusion: Using laboratory mice models is a practical modeling system that provides us investigation of human tumors pathogenesis in vivo.

Keywords: Cancer stem cell, ESCC, Xenograft mouse model

Introduction

Esophageal cancer is the 8th most common type of cancer with two main histological subtypes: esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (1, 2). ESCC is the major subtype which comprises %80 of cases in the world (3). More than 50% of patients with esophageal cancer present with cancer metastasis and 5-year-survival rate of patients with ESCC is only 15% to 25% (4, 5). Although human tumor cell lines have increased our knowledge about cancer pathogenesis studying these cell lines in vitro has some drawbacks that originate from the different environments which cells are exposed to in vitro and in vivo. In human body the tumor cells growth and maintenance are affected by different types of stromal cells. The cells are in a three dimensional structure with vascular and immune systems which are in direct communication with them. Furthermore the tumor cells in vitro are subjected to genetic alteration due to multiple passages. These all lead to changes in tumorigenicity mechanisms in cultured tumor cells which for example cause different drug efficacy results between the treated cells in vitro and the patients (6). Modeling tumorigenesis in laboratory animals allows us to study tumors in vivo and makes it possible to isolate the tumors in all stages and study them pathologically, biochemically and genetically. Animal models are important preclinical tools to evaluate and test new cancer therapies (7). Several types of animals are used in cancer research including zebrafish (8), mice (9), rats (10), rabbits (11), and monkeys (12) which among them mice are the most commonly used animals. To make laboratory animal models transplantation of tumor cells into animals

and production of genetically engineered mice are mostly used (7). In xenograft transplantation assay human tumor cells are injected into murine host with a compromised immune system (e.g. Nude mice) to prevent human cells rejection (13).

Cancer stem cells (CSCs) are a small subpopulation of cancer cells within the bulk of the tumor. These cells are first recognized in leukemia (14) and after that isolated from solid tumors such as breast (15), brain (16), colon (17), ovary (18) and pancreas (19). CSCs are thought to be the origin of most human tumor types and the main reason of treatment resistance, tumor relapse, and metastasis (20). Owing to the absence of unique cell surface markers and distinct morphology, CSCs are studied based on their functionality (21). In sphere formation assay, which is a marker independent method, tumor cells are cultured in serum-free medium on low adherent plates. These circumstances exert particular stress on cells. The normal differentiated cells, which must adhere to a matrix to survive, die but cancer stem-like cells persist, divide and form three dimensional spheres (22). In the current study, we aimed to monitor cancer stem cells tumorigenicity using xenograft mouse models.

Materials and Methods

Cell line and culture protocol

We used YM1 esophageal cancer cells developed from a 50-year-old Iranian woman (Turkmen race) with esophageal squamous cell carcinoma (ESCC) (23). We cultured cells in DMEM-F12 (GIBCO) medium which was supplemented with 10% fetal bovine serum (FBS) (GIBCO) and 100 units/ml penicillin and 100 µg/ml streptomycin

(BIOIDEA). The adherent cells were passaged and maintained in CO₂ incubator at 37 °C. After a few passages the adherent cells were cultured in DMEM-F12 medium supplemented with 5% FBS to become adapted to serum-free medium used in sphere formation assay.

Sphere formation assay

The adherent cells were detached and washed with phosphate-buffered saline (PBS) and were suspended in low adherent 6mm Petri dishes (Labtron) containing 3 ml serum-free medium consists of DMEM/F12 , 100 IU/ml penicillin, 100 µg/ml streptomycin(BIOIDEA), 20 ng/ml human recombinant epidermal growth factor (hrEGF), 20 ng/ml human recombinant basic fibroblast growth factor (hrbFGF)(ROYAN) and 2% B27 supplement (GIBCO) at a density of 50000 cells/ml. The Petri dishes were maintained in CO₂ incubator at 37 °C. After the formation of the spheres, they should be passaged. Otherwise, their diameter exceeds 60 µm and central cells die from the lack of nutrients and peripheral cells will differentiate. The spheres were collected by slow centrifugation and dissociated with trypsin-EDTA and pipetage. Then the single cells were centrifuged to eliminate the enzyme and cultured in serum-free medium again.

Xenograft transplantation assay

After three passages, the resulting spheres were dissociated using trypsin and washed with PBS. Then the specific number of single cells in a final volume of 300 µl of PBS/ Matrigel mixture (1:1 volume) was injected subcutaneously into the dorsal flank of 4-week-old B6 nude mice (Table1). After detection of first tumors, their sizes were

measured once a week using the caliper and their volumes were measured using $\pi/6 \times L \times W \times H$ formula (24). 48 days after injection mice were sacrificed and the tumors were removed and weighed by a digital balance.

Table1. The number of cancer stem cells used for subcutaneous injection into three nude mice

	Mouse 1	Mouse 2	Mouse 3
The number of cells in 150 µl PBS	4×10 ⁶ cells	4×10 ⁵ cells	4×10 ⁴ cells
Matrigel	150 µl	150 µl	150 µl

Results

We used the sphere formation assay to isolate cancer stem cells (Figure1).

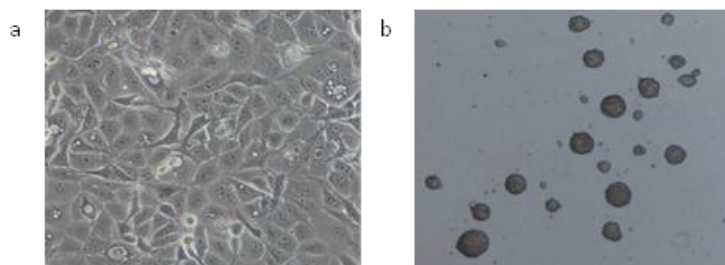


Figure1: **a** The adherent cells (20×10) **b** spheres (10×10). The adherent cells were passaged in serum free medium on low adherent dishes and three dimensional spheres formed.

Then we used in vivo xenograft transplantation assay to investigate the ability of these cancer stem cells to form subcutaneous tumors in nude mice. The Length (the longest dimension), width (the distance perpendicular to and in the same plane as the length) and height (diameter of the tumor perpendicular to the length and width) of each tumor was measured with caliper once a week (Figure2, Table2).



Figure2. Illustration of the method used to measure the tumor size. The tumor length, width and height were measured once a week using a caliper.

The tumor growth curve of each tumor was plotted with Excel software using the recorded length of them. In these growth curves the horizontal axis is based on day and the vertical axis is based on tumor length in cm (Figure3).

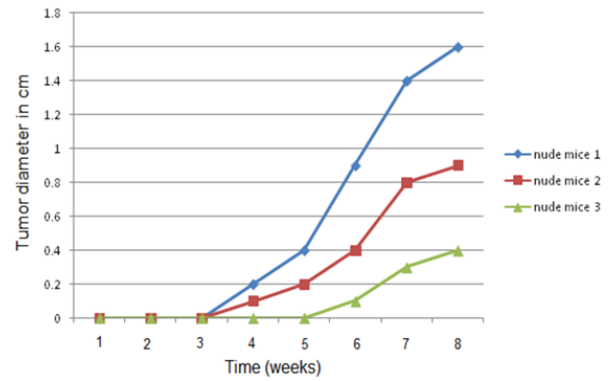


Figure3. The tumors growth curves. The tumor growth curve of each tumor was plotted using the recorded length of the tumors

We measured the size of the three tumors before and after dissection and calculated their volumes with the $\pi/6 \times L \times W \times H$ formula (Figure4, Table3).

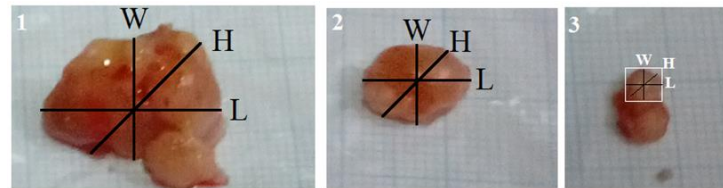


Figure4. The measurement of the tumors' sizes after dissection. To calculate the volumes of the tumors, their sizes were measured before and after dissection.

Table2. The recorded size of tumors measured once a week

Nude mouse	The number of injected cells	tumor size in 3 rd week	Tumor size in 4 th week	Tumor size in 5 th week	Tumor size in 6 th week	Tumor size before dissection
Mouse1	4×10^6	0.2 cm	0.4 cm	0.2 cm	1.4 cm	1.6 cm
Mouse2	4×10^5	0.1 cm	0.2 cm	0.4 cm	0.8 cm	0.9 cm
Mouse3	4×10^4	0	0	0.1 cm	0.3 cm	0.4 cm

Table3. The calculation of tumor volume.

Nude mouse	Tumor length(L) in the mouse body	Tumor volume in the mouse body $\pi/6 \times L \times W \times H$ (Tumor length(L) after dissection	Tumor volume after dissection $\pi/6 \times L \times W \times H$ (Tumor weight after dissection
Mouse1	1.6 cm	$\pi/6 \times 1.6 \times 1.2 \times 1.5 = 1.44$ cm	2.1 cm	$\pi/6 \times 2.1 \times 1.4 \times 1.8 = 2.65$ cm	1.9 gr
Mouse2	0.9 cm	$\pi/6 \times 0.9 \times 0.6 \times 0.8 = 0.22$ cm	1.0 cm	$\pi/6 \times 1.0 \times 0.7 \times 0.8 = 0.28$ cm	0.37 gr
Mouse3	0.4 cm	$\pi/6 \times 0.4 \times 0.2 \times 0.3 = 0.01$ cm	0.5 cm	$\pi/6 \times 0.5 \times 0.3 \times 0.4 = 0.03$ cm	0.1 gr

Discussion

The laboratory mouse is the most common animal used to model cancer in vivo. The mouse small size and simple maintenance in addition to the physiological similarities to human have made it a useful system to model cancer of humans (25, 26). The xenograft tumor in nude mice is a useful model to study human tumors. The growth curves give us special data about human tumor biology in vivo. Furthermore, xenograft tumors can be used to study the effect of multiple agents like hormones, antibodies and so on, on tumor growth (24).

In the present study, we monitor the ability of cancer stem cells, isolated with sphere formation assay, to form xenograft tumors in nude mice. We used three different cell dilutions which all made measurable tumors. The tumors in the first and the second mice were measurable three weeks after injection but the third mouse had a measurable tumor since 5th week. The size of tumors was appropriate to the number of cells injected and the minimum cell number required for tumor formation in our study was 4×10^4 cells. The Matrigel used in the mixture of injection leads the tumor cells concentrate in the injection site and prevents the cells from spreading around, so it results in the formation of a uniform tumor and makes it easy to measure the size of the tumor. It is

noted that Matrigel must be kept on ice at the time of injection because it transforms into a gel-like state in room temperature which makes it difficult to inject the cells. So it is better to prepare the cells in micro tubes after cell counting and inject them immediately after Matrigel addition to the mixture of cells in PBS.

Conclusion

Modeling human cancers using mouse models is a functional method which gives us the possibility to simulate and study human cancer in vivo. In our study YM1 cancer stem-like cells enriched by sphere formation assay were able to make subcutaneous tumors in nude mouse models.

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Declarations

Ethics approvals and consent to participate

This study was approved by the ethical committee of Golestan University of Medical Sciences.

Conflict of interest

There are no conflicts to declare

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