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Isolation and characterization of cancer stem cells from primary head and neck squamous cell carcinoma tumors

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Summary

Drug resistance remains a significant problem in the treatment of patients with head and neck squamous cell carcinoma (HNSCC). Recent reports showed that a sub-population of highly tumorigenic cells (cancer stem cells) is uniquely resistant to chemotherapy, and suggesting that these cells play an important role in the relapse of HNSCC. The development of methods for the isolation and culture of cancer stem cells is a key step to enable studies exploring the mechanisms underlying the role of these cells in chemoresistance. Here, we describe a method to isolate cancer stem cells from primary head and neck tumors and for the generation of orospheres, *i.e.* the culture of these cells in suspension in ultra-low attachment plates.

Keywords

Head and neck squamous cell carcinoma; Cancer stem cells; Chemoresistance; Self-renewal; Multipotency; Orospheres

1. Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide, with approximately 500,000 cases diagnosed annually (1). In the past, treatment of HNSCC was primarily limited to upfront surgical resection when technically feasible. Owing to the high morbidity often associated with radical surgery, most patients today receive radiation combined with chemotherapy as front line treatment for locally advanced HNSCC. Chemotherapy alone is implemented for the treatment of distant metastatic disease,

but most patients do not exhibit substantial response. A major difficulty in treating HNSCC is the frequency of recurrent disease, with 20–40% of patients developing loco-regional recurrence and 5–20% developing distant metastatic disease at 2 years (2). Chemotherapy for HNSCC typically involves the use of platinum, taxane, or pyrimidine analog agents (3). While these agents are effective at indiscriminately debulking tumor cells, recent data report that a subpopulation of cells, called cancer stem cells (CSCs), are resistant and survive these therapies (4–6). Indeed, mounting evidence suggests that chemotherapy enriches the cancer stem cell population (6,7). Notably, the mechanisms exploited by CSCs to resist chemotherapy are largely unknown. Therefore, the development of methods for the isolation and characterization of CSCs from primary tumors is a critical enabling step to improve the mechanistic understanding of the processes mediating chemoresistance, and for the development of therapeutic strategies to overcome this resistance.

The most widely used method to study CSCs is the sphere assay. The sphere assays were originally used for culturing normal neuronal stem cells (8). Since the initial discovery that culturing cells in ultra-low attachment plates and serum-free (or low serum) conditions enhance maintains their undifferentiated state, the assay evolved as an important tool to study not only normal stem cells but also CSCs. Multiple reports show that CSCs are anoikis-resistant and able to grow in suspension as spheres, as for example the breast CSCs (9). Spheres from head and neck tumors are called orospheres (10,11). The orosphere assay is useful to evaluate the stemness, self-renewal and tumorigenicity of CSCs, but also to study processes involved in the chemoresistance of these cells to drugs (6).

This chapter will describe in detail the method for isolation of CSCs from HNSCC and for their culture in suspension as orospheres. There is ongoing discussion about the ideal markers to identify head and neck CSCs. Here, we have used the combination of ALDH activity and CD44 expression to sort these cells from primary tumors (12,13). The selection of markers can certainly be adapted to the specific tumor of interest. Nevertheless, the overall principles of the assay described here might be germane to other tumor types.

2. Materials

2.1 Tumor digestion

1. Supplemented media (*see* NOTE 1).
2. Sterile petri dish.
3. Sterile razor.
4. Tumor digestion reagents:
 1. Collagenase/hyaluronidase solution (STEMCELL Technologies, Vancouver, BC, Canada).
 2. Miltenyi Biotech human tumor dissociation kit (Miltenyi Biotech, San Diego, CA, USA).
5. 40 μ m nylon cell strainer for 50 mL conical tube.
6. Serum: serum neutralizes the digestion process.

7. Ammonium-Chloride-Potassium (ACK) lysing buffer (Gibco Life Technologies, Grand Island, NY, USA) (*see* NOTE 2).

2.2 Fluorescence activated cell sorting

1. 1xPBS or 1xPBS with 2% fetal bovine serum (staining buffer).
2. 5 mL round bottom flow cytometry tubes.
3. 4-Diethylaminobenzaldehyde (DEAB) (STEMCELL Technologies, Vancouver, BC, Canada).
4. Activated ALDEFLUOR™ (STEMCELL Technologies, Vancouver, BC, Canada).
5. APC CD44 and APC isotype IgG (BD Pharmingen, Franklin Lakes, NJ, USA).
6. Lineage markers: PE-Cy5 CD2, CD3, CD10, CD16, CD18 (BD Pharmingen, Franklin Lakes, NJ, USA).
7. 7-aminoactinomycin (7-AAD) (BD Pharmingen).
8. Aluminum foil.

2.3 Orosphere culture

1. Sphere media: DMEM/F12 medium (500 mL) (Gibco Life Technologies, Grand Island, NY, USA), fibroblast growth factor (FGF) (20 ng/mL), epithelial growth factor (EGF) (20 ng/mL), N2 supplement (Gibco Life Technologies, Grand Island, NY, USA) (*see* NOTE 3).
2. 6-well low-attachment plate (Corning, Corning, NY, USA).
3. 0.05% trypsin/Ethylenediaminetetraacetic acid (EDTA) (Gibco Life Technologies, Grand Island, NY, USA).
4. Trypsin neutralizing solution (Lonza, Walkersville, MD, USA).

Methods

3.1 Preparation for digestion

1. Place tumor in supplemented media.
2. Pour off the media and add fresh supplemented media. Centrifuge at 130 g-force for 5 minutes at 4°C. Wash the tumor 3–4 times (*see* NOTE 4).
3. Place tumor with 5 mL of supplemented media on a petri dish.
4. Cut the tumor into small pieces (approximately 4 mm × 4 mm in size) with sterile razor blade (*see* Fig. 1a) (*see* NOTE 5).
5. Collect minced tumor fragments into a 50 mL conical tube. Add 25–30 mL base media without supplements. Centrifuge at 130 g-force for 5 minutes at 4°C.

3.2 Tumor digestion

1. Collagenase/hyaluronidase method
 1. Decant media and place the tumor fragments on a new sterile petri dish. Add 6–10 mL of 1x collagenase/hyaluronidase solution.
 2. Mix the tumor fragments and digestion solution by pipetting several times with 25 mL pipette. Incubate in 37°C for 15 min.
 3. Take out the petri dish from the incubator. Pipet the mixture 2–3 times to mechanically digest the tumor. Repeat step 2 and 3 2 more times (*see* NOTE 6).
 4. Prepare a 50 mL tube with 5 mL serum and place 40 µm nylon mesh on top of the tube. Filter the tumor digestion mix and collect cell suspension. Collect and filter remaining cell/fragment mixture with 5 mL of supplemented media (*see* NOTE 7). Centrifuge at 130 g-force for 5 minutes at 4°C.
 5. Decant media. Add 1–5 mL of ACK lysing buffer. Incubate in room temperature for 1 minute. Centrifuge at 130 g-force for 5 minutes at 4°C.
 6. Decant ACK lysing buffer and resuspend the cell pellet in PBS (*see* NOTE 8).
2. GentleMACS method
 1. Prepare enzyme cocktail mix with RPMI in the appropriate tube for GentleMACS.
 2. Transfer minced tissue fragments into the enzyme cocktail mix. Close the tube tightly (*see* Fig. 1b).
 3. Mechanically dissociate tumors using GentleMACS homogenizer. Select appropriate tumor dissociation program.
 4. Incubate in 37°C for 30 min on a shaker or rotator.
 5. Repeat step 3 and 4.
 6. Repeat step 3 (*see* Fig. 1c).
 7. Transfer the digested tumor to 50 mL tube with 40 µm cell strainer placed on top. Use equal volume of supplemented medium to collect residual tumor cells and undigested tissues. Collect digested cell suspension by filtering with the cell strainer.
 8. Centrifuge at 130 g-force for 5 minutes at 4°C.
 9. Decant media. Add 1–5 mL of ACK lysing buffer. Incubate in room temperature for 1 minute. Centrifuge at 130 g-force for 5 minutes at 4°C.

10. Decant ACK lysing buffer and resuspend the cell pellet in PBS (*see* NOTE 8).

3.3 Isolation of cancer stem cells

1. Prepare and label tubes.
 - Unstained
 - 7-AAD
 - Lineage
 - APC IgG
 - APC CD44
 - DEAB
 - ALDH
 - Sample: T1, T2, T3, etc.
2. Count the cells recovered from tumor digestion. Add 1×10^5 cells to each control tube except DEAB tube. Add 1×10^6 cells to the sample tube.
3. Resuspend cells in 1 mL PBS or staining buffer.
4. Add 1 μ L of DEAB reagent to the control DEAB tube (*see* NOTE 9). Add 1 μ L of ALDEFLUOR™ reagent control ALDH tube. Mix by pipetting once and transfer 500 μ L of cells to control DEAB tube (*see* NOTE 10). Mix well.
5. Add 5 μ L of ALDEFLUOR™ reagent to each sample tube. Mix well. Protect from light by covering the tubes with aluminum foil. Incubate in 37°C for 30–45 minutes.
6. Centrifuge at 130 g-force for 5 minutes at 4°C. Remove PBS or staining buffer from all the tubes. Resuspend cells in 1 mL PBS or staining buffer.
7. Add 1 μ L of APC isotype IgG to control IgG APC tube. Add 1 μ L of APC CD44 antibody to control APC tube. Add 5 μ L APC CD44 to all the sample tubes.
8. Add 1 μ L of CD2, CD3, CD10, CD16 and CD18 to control Lineage tube. Add 5 μ L of the same antibodies to the sample tubes.
9. Protect from light by covering the tubes with aluminum foil. Incubate in 4°C for 30 minutes.
10. Add 1 mL of PBS to all of the tubes. Centrifuge at 130 g-force for 5 minutes at 4°C.
11. Aspirate PBS and resuspend cells with 1 mL PBS. Centrifuge at 130 g-force for 5 minutes at 4°C.
12. Aspirate PBS. Add 200 μ L PBS to all the control tubes except control 7AAD tube.

13. Prepare 7-AAD solution by adding 5 μ L 7-AAD for each 1 mL of PBS. Add 200 μ L 7-AAD solution to control 7-AAD tube. Resuspend sample tubes with 500 μ L 7-AAD solution.
14. Sort cancer stem cells by fluorescence activated cell sorting (FACS) (*see* Fig. 2) (*see* NOTE 11).

3.4 Orosphere assay

1. Seed sorted cancer stem cells in 6-well low-attachment plate. 5,000 sorted cells are plated in each well of low-attachment plate (*see* NOTE 12). Add 1.5 mL of sphere media in each well (*see* NOTE 13).
2. Feed spheres every 3–4 days by adding 300 μ L of sphere media to each well.
3. Count spheres on day 3 and 7 (*see* NOTE 14) (*see* Fig. 3).
4. On day 7, collect spheres in 15 mL tube. Centrifuge at 130 g-force for 5 minutes at 4°C.
5. Wash with PBS. Centrifuge at 130 g-force for 5 minutes at 4°C. Remove supernatant.
6. Add 1 mL 0.05% trypsin/EDTA. Incubate for 5–10 minutes in room temperature. Mechanically dissociate the spheres by pipetting up and down every 2 minutes until spheres are invisible.
7. Add 1 mL trypsin neutralizing solution and mix. Centrifuge at 130 g-force for 5 minutes at 4°C.
8. Count the cells.
9. Seed sorted cells in low-attachment plate. 5,000 sorted cells are plated in each well of low-attachment plate. Add 1.5 mL of sphere media in each well (*see* NOTE 13).
10. Feed spheres every 3–4 days by adding 300 μ L of sphere media to each well. Count spheres on day 10 and 14.

4. Notes

- 4.1 Depending on the tumor type, add necessary supplements and growth factors to keep tumor fragment. The media should include antibiotics (e.g. amphotericin B, AAA, nystatin, etc.) to prevent bacterial/fungal contamination.
- 4.2 ACK lysis buffer eliminates red blood cells from the digested cell pellet.
- 4.3 CSCs from different tumor type require different supplements needed for sphere formation. Here, we describe the supplements needed for orosphere assay.
- 4.4 Washing the tumor prevents potential contamination during cancer stem cell culture following isolation of cancer stem cells.

- 4.5 Mincing the tumor into smaller pieces is important for good cell recovery from digestion. Too much cutting results in unhealthy cells after digestion, and too little cutting results in poor tumor digestion.
- 4.6 As the tumor fragments are digested, the solution will become opaque, and the tumor fragments will be able to pass through 10 mL pipette.
- 4.7 Incompletely digested tumor fragments can be stored for future use. Re-suspend the chunks in freeze media that contains 5% DMSO and 5% serum and move gradually to liquid nitrogen for storage.
- 4.8 Serum containing staining buffer will enhance the cell viability during the staining procedure.
- 4.9 After adding DEAB to the tube, close the lid so the solution will not evaporate.
- 4.10 Transfer the cell suspension as soon as possible to minimize the background ALDEFLUOR™ signal.
- 4.11 CSCs are 7-AAD negative, Lineage negative, ALDEFLUOR™ positive, CD44 positive cells.
- 4.12 Cell density can affect the number and the quality of orosphere. Optimize the number of cells plated in each well.
- 4.13 Add chemotherapeutic reagents to the sphere media to study the chemoresistance of CSCs.
- 4.14 Orospheres are considered as non-adherent colony with at least 25 cells.

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Figure 1.
Tumor digestion steps. A. Cut tumors into small fragments. B. Before tumor digestion. C.
After tumor digestion. Notice the digestion media turned opaque after the digestion process.

Figure 2.
Representative fluorescence activated cell sorting scheme from a primary head and neck squamous cell CSC.

Figure 3.
Examples of orospheres. Scale bar = 100 μ m.