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Role of cancer stem cell in radioresistant head and neck cancer

Young Min Park [a,b], Sei Young Lee [c], Suk Won Park [d], Se-Heon Kim [b,*]

[a] Department of Otorhinolaryngology, Bundang Jesaeng Hospital, Deajin Medical Center, Seongnam, Gyeonggi, Republic of Korea
[b] Department of Otorhinolaryngology, Yonsei University College of Medicine, Seoul, Republic of Korea
[c] Department of Otolaryngology, Chung-Ang University College of Medicine, Republic of Korea
[d] Department of Radiation Oncology, Chung-Ang University College of Medicine, Republic of Korea

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ABSTRACT

Objective: Radioresistance is the main determinant of treatment outcome in head and neck cancer. The aim of this study was to establish radioresistant head and neck cancer cell lines and isolate cancer stem cells from them to investigate the role of cancer stem cells in radioresistant head and neck cancer.

Methods: To induce radioresistant cell lines, radiation was delivered to SCC15, SCC25, and QLL1 cells with an accumulated dosage of 60 Gy over 30 cycles of irradiation. After a total of 60 Gy of irradiation, the radioresistance of irradiated cancer cells was verified by MTT assay. The radioresistant cells were cultured in serum-free medium in ultra-low-attachment culture flasks to induce sphere-forming cells. Then, sphere-forming cells were analyzed using Western blotting to identify the expression of stem cell markers, such as Nanog and Sox-2.

Results: The MTT assay of cell viability showed more radioresistance in the irradiated cancer cell lines than in the non-irradiated cancer cell lines. Sphere-forming cells were identified in all three cancer cell lines 3–5 days after serum deprivation. All sphere-forming cells from the three cancer cell lines expressed stem cell markers. Sphere-forming cells showed more radioresistance than monolayer cells after irradiation by colony forming assay.

Conclusion: Cancer stem cells seem to play an important role in the radioresistance of head and neck cancer. Further research is required to find a precise mechanism of radioresistance related to cancer stem cells.

1. Introduction

The main treatment modalities for head and neck squamous cell carcinoma (HNSCC) are surgery, radiotherapy (RT), and chemotherapy. Among these methods, RT can be used as monotherapy for the treatment of early-stage HNSCC, and it can be applied as an adjunct treatment for advanced-stage HNSCC. Although RT is highly standardized and performed using the same protocol in all patients with the same location and volume of tumor, some patients with HNSCC show a good response to RT, while other patients show treatment failure due to tumor radioresistance. Because radioresistant HNSCC is usually resistant to other treatment modalities, including chemotherapy, the final treatment outcomes of these patients are very poor. If the degree of response to RT prior to treatment can be predicted, other treatment modalities without cross-resistance such as surgery could be performed initially, and RT could be omitted in cases in which radioresistance is highly expected. In previous studies, several genes related to radioresistance were identified using molecular biology techniques [1–4]. Akervall et al. proposed c-MET and YAP-1 as biomarkers to predict the radioresistance of HNSCC [5]. However, no reliable biomarkers

* Corresponding author at: Department of Otorhinolaryngology, Yonsei University College of Medicine, 50 Yonsei-ro, Seodaemun-gu, Seoul 120-752, Republic of Korea. Tel.: +82 10 8899 0456; fax: +82 2 393 0580.
E-mail address: shkimmd@yuhs.ac (S.-H. Kim).

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have yet been established that could be used clinically to predict radioresistance. To develop a reliable biomarker of radioresistance in patients with HNSCC, the mechanisms of radioresistance should be investigated more precisely.

Recently, there has been growing evidence that cancer comprises heterogeneous cells showing distinct proliferation and differentiation capabilities in solid cancers and hematopoietic cancers. Among these heterogeneous cells, a small subpopulation of cells, referred as cancer stem cells (CSCs), shows self-renewal and tumor-initiating capabilities. Regarding radioresistance, CSCs have unique characteristics such as a relatively slower proliferation rate and lower tumor-initiating capability, compared to differentiated cancer cells. Thus, it can be assumed that CSCs themselves or specific characteristics of CSCs might play a role in the radioresistance of solid cancers. Bertrand et al. insisted that CSCs play an important role in the occurrence of radioresistance in patients with HNSCC and that this radioresistance is associated with an increase in specific cell cycles of CSCs [6]. However, there have been few studies regarding the role of CSCs in the acquired radioresistance of HNSCC.

In the present study, we aimed to establish radioresistant HNSCC cell lines under conditions that closely resemble a real clinical setting and separate CSCs from these cell lines using serum-deprivation culture. We also investigated the role of CSCs in acquired radioresistance in HNSCC.

2. Materials and methods

2.1. Cell lines and culture

QLL1 cells (a squamous cell carcinoma cell line originating from metastatic lymph nodes in oral cancer) were a generous gift from Dr. J. Shah (Memorial Sloan-Kettering Cancer Center, New York, NY, USA). The SCC15 and SCC25 cell lines (squamous cell carcinoma cell lines originating from the tongue) were purchased from the American Type Culture Collection (Rockville, MD, USA). Cells were cultured in minimum essential medium (MEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, penicillin (50 mg/ml), and streptomycin (50 mg/ml).

2.2. Establishment of radioresistant HNSCC cell lines

Cells were cultured in T75 flasks until they were 80% confluent and a 2 Gy dose of radiation was delivered at room temperature with a linear accelerator (21iX, Varian). Afterward, a 2 Gy dose of radiation was delivered repetitively when cells were grown to 80% confluence after previous irradiation until a cumulative dose of 60 Gy was reached.

2.3. Sphere-forming cell culture using serum-deprivation culture

Irradiated HNSCC cells were placed under stem cell suspension culture conditions, consisting of serum-free DMEM/F12 medium supplemented with N2 (GIBCO), B27 (GIBCO), human recombinant epidermal growth factor (EGF; 20 ng/ml, R&D Systems), and human basic fibroblast growth factor (bFGF; 20 ng/ml, R&D Systems) in an ultra-low attachment culture flask. As spheres (>20 lm diameter) appeared in suspension culture conditions, they were harvested.

2.4. Cell viability assay and colony forming assay

To investigate whether irradiated HNSCC cell lines acquire radioresistance and compare radioresistance between monolayer cells induced in serum-containing differentiated conditions and sphere-forming cells induced in serum-deprived undifferentiated conditions, a cell viability assay (MTT) and colony forming assay (CFA) were performed. The cell viability was examined by MTT assay at 0, 3, 6, 9 and 12 days after delivering 5 Gy irradiation to confirm radioresistance and 3 days after delivering 8 Gy irradiation to compare radioresistance between monolayer cells and sphere-forming cells. After 0.5 mg/ml thiazolyl blue tetrazolium bromide (MTT, Sigma-Aldrich) was added to each well, cells were incubated at 37 °C for 2 h and washed once with 10 μl of phosphate-buffered saline (PBS, BIOWEST, Nuaillé, France) at former plate over a flowing the wall and washing the cell over a shaking the plate softly and suction. The formed crystals were solubilized in 100 μl of dimethyl sulfoxide (DMSO, Sigma-Aldrich) for 15 min at room temperature with strong agitation. The absorbance was determined at 570 nm using a micro plate reader (Molecular Devices).

CFA also was performed to compare radioresistance between monolayer cells induced in serum-containing differentiated conditions and sphere-forming cells induced in serum-deprived undifferentiated conditions. Two sets of 2000 monolayer cells and sphere-forming cells were plated on 60 mm culture flasks; one set was irradiated with 8 Gy radiation the next day and the other was not irradiated. The number of colony was compared between non-irradiated cells and irradiated cells.

2.5. Western blotting

After sphere formation, the cells were washed twice with ice-cold PBS. Lysis buffer (RIPA buffer containing 1% TX-100, 20 mM Tris, pH 7.5, 100 mM NaCl, 1 mM Na3VO4, 40 mM NaF, 5 mM ethylene glycol-bis (beta-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), 0.2% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate (SDC), and 0.2 mM phenylmethylsulfonyl fluoride (PMSF)) was added. The cell extracts were centrifuged for 10 min at 15,800 × *g* and the resulting supernatant (RIPA lysate) was used for Western blotting. Sphere-forming cells were used as whole cell lysate without centrifuging. Protein concentration was determined by a BCA protein assay (Pierce Chemical Co., USA). Thirty micrograms of total cellular protein were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to nitrocellulose membranes. Blots were probed with antibodies specific for the following proteins: β-actin (1:5000 dilution; Santa Cruz Biotechnology, USA), Nanog (1:1000 dilution; Cell Signaling, USA), and Sox-2 (1:1000 dilution; Cell Signaling, USA). Detection of bound antibody on