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The role of cancer stem cell in acquiring radioresistance of head and neck cancer

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The role of cancer stem cell in acquiring radioresistance of head and neck cancer

Directed by Professor Se-Heon Kim

The Doctoral Dissertation
submitted to the Department of Medicine,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree
of Doctor of Philosophy

Young Min Park

June 2015

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Young Min Park, M.D.

April 2015

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ABSTRACT

The role of cancer stem cell in acquiring radioresistance of head and neck cancer

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Purpose: Radioresistance is one of the main determinants of the treatment outcome in head and neck cancer. The aim of this study was to establish radioresistant head and neck cancer cell lines and separate 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, irradiation was delivered to SCC15, SCC25, and QLL1 cells with an accumulated dosage of 60 Gy over 30 cycles of irradiation. Radioresistance of irradiated cancer cells were verified by MTT assay. Radioresistant cells were cultured in serum-free media in ultra-low–attachment culture flasks to induce sphere-forming cells. Cancer stem cell characteristics of sphere-forming cells were verified by western blot for CD-44, Oct-4, Nanog, Sox-2, and CD-133. 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 to 5 days after serum deprivation. All sphere-forming cells from the three different cancer cell lines expressed stem cell markers; however, they showed different levels of expression. Sphere-forming cells showed more radioresistance than monolayer cells after irradiation by colony forming assay. Inhibition of Src in sphere-forming cells showed reduced radioresistance, low expression of cancer stem cell markers, and less invasion and migration in radioresistant head and neck cancer.

Conclusion: Cancer stem cells seem to be the cause of the radioresistant properties of radioresistant head and neck cancer. Src may also play a key role in maintaining radioresistance, and it may be used in target therapy for radioresistant head and neck cancer.

Key words : head and neck cancer, cancer stem cell, radioresistance

The role of cancer stem cell in acquiring radioresistance of head and neck cancer

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I. INTRODUCTION

The main treatment modalities for head and neck squamous cell carcinoma (HNSCC) are surgery, radiotherapy (RT), and chemotherapy. Among these methods, RT can used as a 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 the radioresistance of their tumor. 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. (5) proposed c-MET and YAP-1 as biomarkers to predict the radioresistance of HNSCC. However, no reliable biomarkers 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 than 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. (6) 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. 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 of HNSCC and aimed to elucidate the molecular mechanism of radioresistance among CSCs.

II. MATERIALS AND METHODS

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, MA, 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. Establishment of radioresistant HNSCC cell lines

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

3. Sphere-forming cell culture using serum-depravation culture

Irradiated HNSCC cells were placed under stem cell suspension culture conditions, consisted 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 the ultra-low attachment culture flask. As spheres (>20 lm diameter) appeared in suspension culture conditions, they were harvested.

4. Cell viability assay (MTT) and colony forming assay

To investigate whether irradiated HNSCC cell lines acquire radioresistance or not and compare radioresistance between monolayer cells and sphere-forming cells, cell viability assay (MTT) and colony forming assay (CFA) were done. The cell viability was examined by MTT assay at 0, 3, 6, 9 and 12 days after delivering 5G irradiation for confirming radioresistance and 3 days after delivering 8G irradiation for comparing 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 37℃ for 2 h and washed once with 100μl 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 100ul of Dimethyl sulfoxide (DMSO, Sigma-Aldrich) for 15 min at room temperature with strong agitate. The absorbance was finally determined at 570 nm using a micro plate reader (Molecular Devices).

CFA also was done for comparing radioresistance between monolayer

cells and sphere-forming cells. Two sets of 2,000 monolayer cells and sphere-forming cells were plated on 60 mm culture flask each and one set was irradiated with 8G radiation on the next day and the other was not irradiated. The number of colony between non-irradiated cells and irradiated cells was compared.

5. Western blot

After 3days 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 deoxy cholate (SDC) and 0.2 mM phynylmethylsulfonylfluofide (PMSF)) was added, and then collected. 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 centrifuge. 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 an antibody specific for the following proteins: β-actin (1:5,000 dilution; Santa Cruz Biotechnology, USA); Oct-4 (1:1000 dilution; Cell Signaling, USA); Nanog (1:1000 dilution; Cell Signaling, USA); Sox-2 (1:1000 dilution; Cell Signaling, USA); CD44 (1:1000 dilution; Cell Signaling, USA); Src (1:1000 dilution; Cell Signaling, USA). Detection of bound antibody on each blot was assessed with horseradish peroxidase-conjugated secondary antibody visualized by enhanced chemiluminescence (ECL; Western blot detection kit; Amersham Pharmacia Biotech, USA).

6. Lentiviral shRNA transfection

For stable shRNA transfection, MISSION® shRNA bacterial glycerol stocks containing four different constructs targeting Src were purchased from Sigma-Aldrich (St. Louis, MO). Plasmids were packaged with MISSION®

Lentiviral Packaging Mix (St. Louis, MO) and transfected using Lipofectamine 2000 transfection reagent (Life Technologies, Grand Island, NY) into HEK293T cells (ATCC, Manassas, VA). After transfection, media was changed and lentivirus was collected virus 48 hours later and filtered through 0.45 mm filters. Cells were infected with lentivirus in the presence of polybrene (8 g/ml). After forty-eight hours, cells were treated with 1 mg/mL of puromycin to select resistant clones. MISSION® shRNA Control Transduction Particles containing non-targeting shRNA were used as a control.

7. Human xenograft model

Male athymic nu/nu mice weighing approximately 20 g at 4–5 weeks of age were obtained from SLC (Shizoka, Japan). All mice were maintained in a laminar airflow cabinet under specific pathogen-free conditions. All facilities were approved by the Association of Assessment and Accreditation of Laboratory Animal Care, and all animal experiments were conducted under the institutional guidelines established for the Animal Core Facility at our institute. Xenograft tumors were implanted in the abdomens of 5- to 6-week-old male nude mice by subcutaneous injection of 1 × 107 scramble shRNA radioresistant SCC25 cells or Src shRNA radioresistant SCC25 cells in 100 ul of Matrigel (BD Biosciences, San Jose, CA, USA) and phosphate-buffered saline (PBS) half-and-half. When tumors reached a volume of 150–180 mm3, mice were randomly assigned to one of four groups to receive scramble shRNA with no treatment or radiotherapy and Src shRNA with no treatment or radiotherapy. The first day of irradiation was designated as day 1 and Irradiation was delivered on days 1, 8, and 15 with 1G dose. Tumor volume was calculated as 0.523 LW2, where L is length and W is width. Tumor responses to irradiation were compared by use of the Mann–Whitney test over a period of 3 weeks.

III. RESULTS

1. Acquisition of radioresistance in the irradiated HNSCC cell lines

After delivering 5 Gy radiation to the three 60 Gy irradiated and three nonirradiated HNSCC cell lines, both cell line groups showed a decrease of viable cancer cells during 12 days, but cytotoxic effects were significantly more severe in the nonirradiated cancer cells than the irradiated cancer cells in all three OSCC cell lines (Fig. 1). Accordingly, we confirmed that the all irradiated HNSCC cell lines obtained radioresistance after previous 60 Gy irradiation.

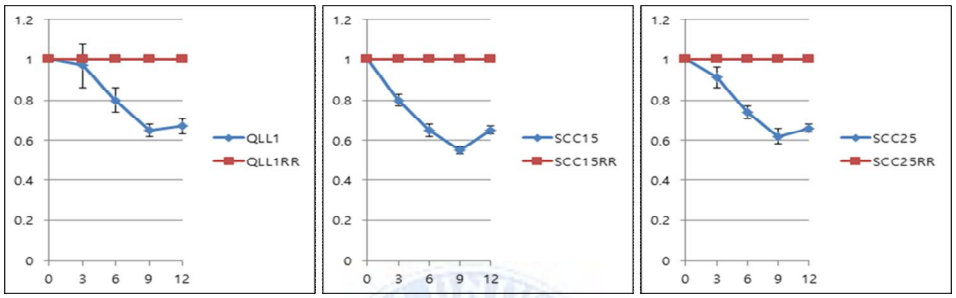


Figure 1. MTT assay was compared between 60 Gy irradiated HNSCC cell lines and nonirradiated HNSCC cell lines after delivering 5 Gy radiation to each group.

2. Comparing radioresistance between each HNSCC cell lines

We compared the degree of radioresistance between each three HNSCC cell lines. After 60Gy irradiation was delivered, each three HNSCCs were irradiated with 2Gy, 4Gy, and 8Gy irradiation. MTT assay and CFA were done for comparing degree of radioresistance and cytotoxic effect was more severe along with the increase of radiation dose. There was no significant difference between three HNSCC cell lines. (Fig. 2)

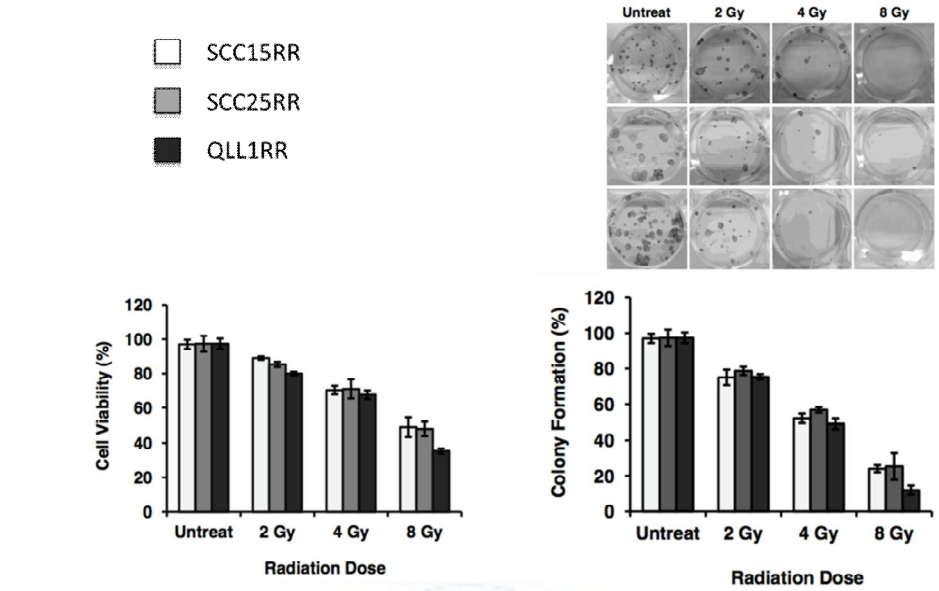


Figure 2. There was no significant difference regarding degree of radioresistance between three radioresistant HNSCC cell lines.

3. Sphere formation from radioresistant HNSCC cell lines

We attempted to employ stem cell suspension culture conditions to establish sphere-forming cells using single-dissociated tumor cells derived from three radioresistant HNSCC cell lines. All cell lines showed sphere-like clusters within 3days after serum depravation. All spheres had a well-defined circular shape and the size of spheres grew beyond 10 lm in diameter. (Fig. 3)

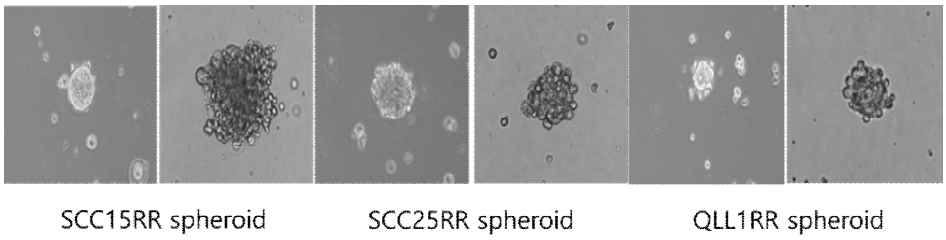


Figure 3. Sphere-forming cells. Cells from radioresistant HNSCC cell lines generate non-adherent spherical colonies 3days after seeding in serum-free condition with defined growth factors (EGF and bFGF). Magnification: 100x.

4. Stem cell marker expression of sphere-forming cell derived from radioresistant HNSCC cells

We performed Western blot to determine whether sphere-forming cells expressed stem cell markers (CD44, Oct4, Sox2, and Nanog) in serum-depraved undifferentiated and serum-containing differentiated conditions. We found that the protein expression levels of CD44, Sox2 and nanog were significantly higher in sphere-forming cells, compared to differentiated counterparts. (Fig. 4) These results suggest that stem cell suspension culture condition imparts radioresistant HNSCC derived spheres-forming cells to possess stem cell feature.

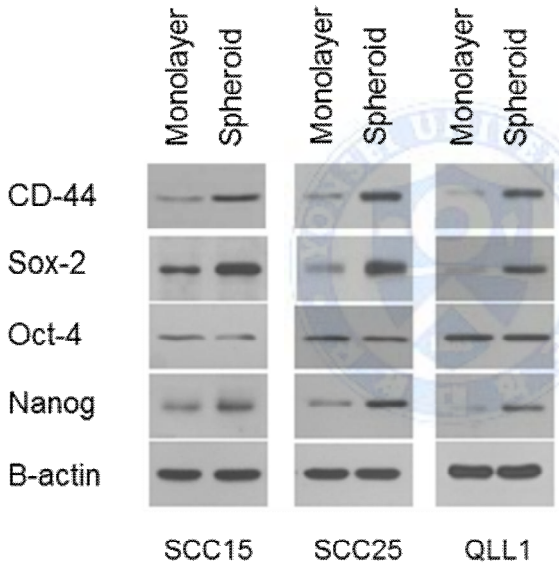


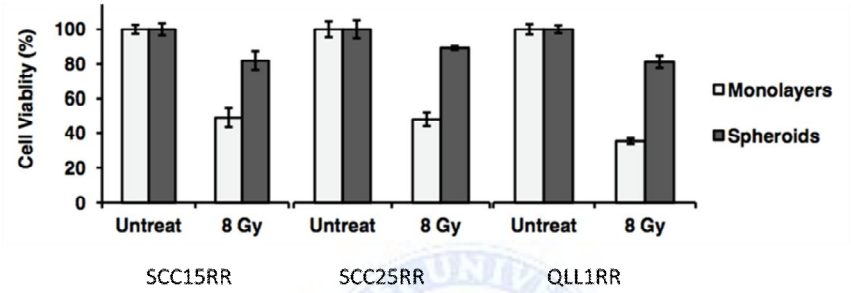
Figure 4. Stem cell marker expression in radioresistant HNSCC derived sphere-forming cells.

5. Role of cancer stem cell in radioresistance

We irradiate with 8Gy to serum-depraved undifferentiated sphere-forming cells (spheroid, cancer stem cell) and serum-containing differentiated cells (monolayered, control cells). After 8 Gy irradiation, MTT assay and CFA were done and there was a significant difference between cancer

stem cells and differentiated cells in cytotoxicity. (Fig. 5a and b) Cancer stem cells were more radioresistant compared to differentiated cells and it suggested that an acquired radioresistance of HNSCC might be derived from cancer stem cells itself or particular characteristics of cancer stem cells.

5a



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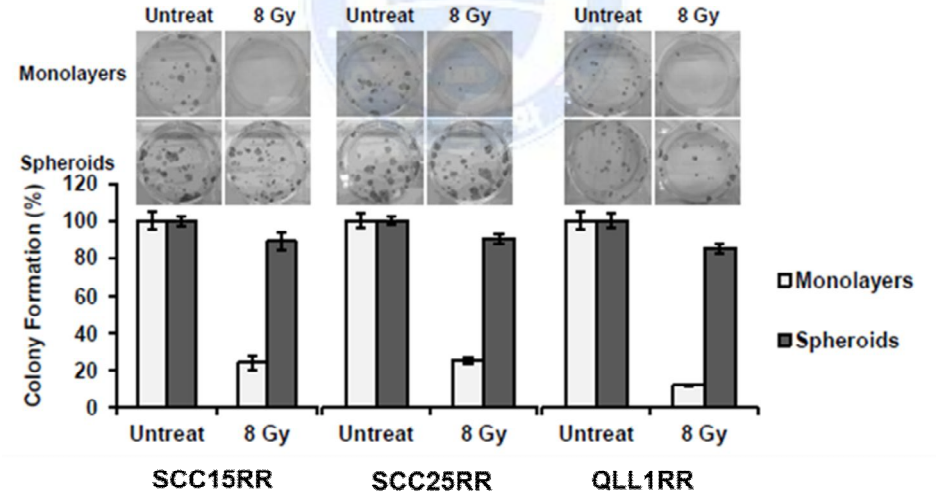
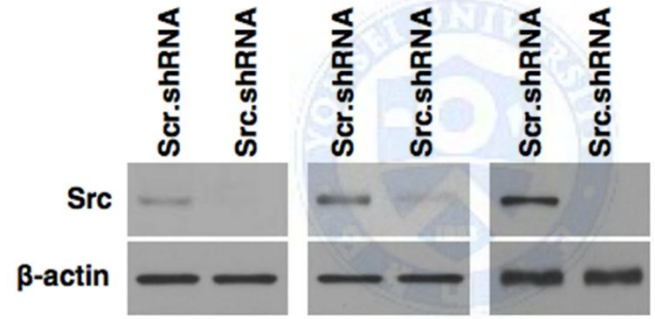


Figure 5. MTT assay and CFA after 8G irradiation delivered to cancer stem cells and differentiated cells. (a) MTT assay and (b) CFA showed significant cytotoxicity of differentiated cells and less severe cytotoxicity of cancer stem cells after 8 Gy irradiation.

6. Inhibition of Src in the cancer stem cells

All three radioresistant HNSCC cell lines expressed Src and it was completely inhibited by shRNA for Src. (Fig. 6a) After inhibition of Src, we reattempt serum-depravation culture for sphere-formation with radioresistant HNSCC cell lines, but size of sphere originating from Src inhibited cancer cell lines was significantly decreased comparing to scramble. (Fig. 6b) As for the stem cell markers, Src inhibited sphere-forming cells showed the reduced expression of CD44 and Nanog comparing to scramble. (Fig. 6c) Accordingly, Src had a crucial role to maintain the characteristics of cancer stem cells and to induce sphere-forming cell in the serum-depraving culture of radioresistant HNSCC.

6a



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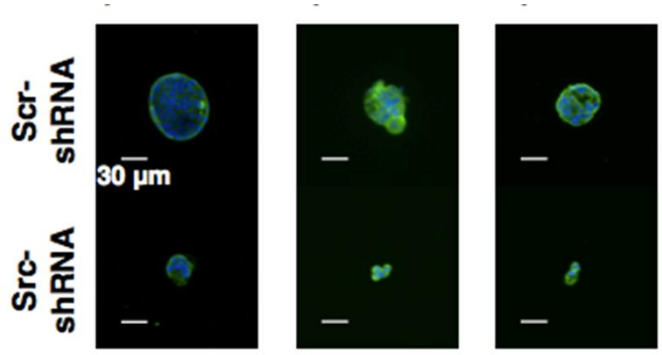

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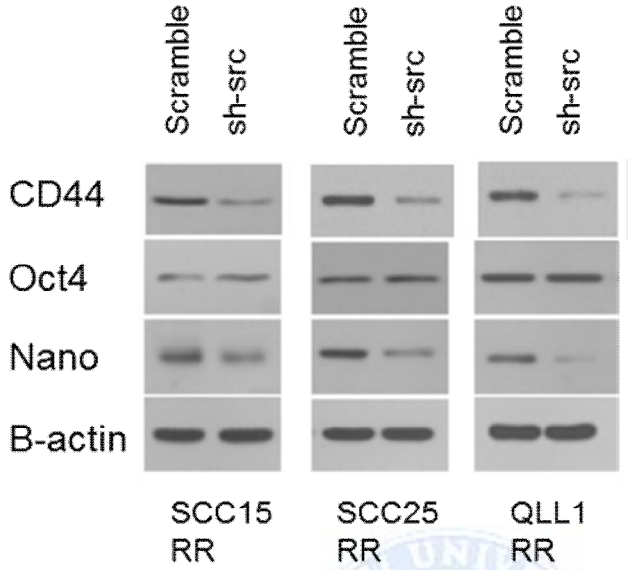


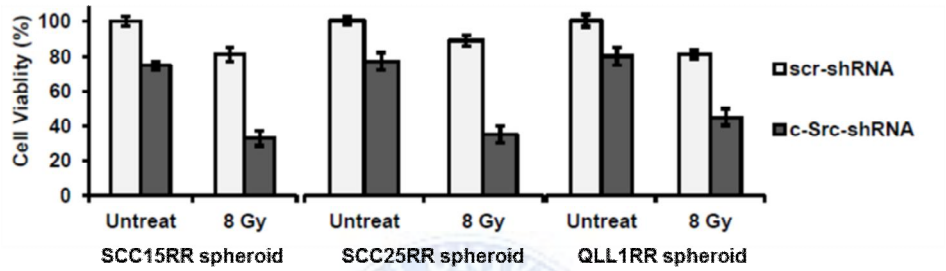
Figure 6. Inhibition of Src in the radioresistant HNSCC cell lines. (a) Src was expressed in all three radioresistant HNSCC cell lines and completely inhibited by shRNA for Src. (b) Spheres induced by Src inhibited radioresistant HNSCC were significantly smaller than Src expressing radioresistant HNSCC. (c) Expression of stem cell markers were reduced in the Src inhibited sphere-forming cells comparing to scramble.

7. Change of radioresistance after Src inhibition in radioresistant HNCC

We compared degree of radioresistance between Src inhibited and Src intact radioresistant HNSCC in vitro and in vivo. MTT assay was done after delivering 8 Gy irradiation to Src inhibited and Src intact sphere-forming cells each. Src inhibited sphere-forming cells showed reduced radioresistance compared to Src intact sphere-forming cells. (Fig. 7a) Tumor growth in the human xenograft model was significantly suppressed in the Src inhibited radioresistant HNSCC cell lines than Src intact radioresistant HNSCC cell lines after irradiation. (Fig. 7b) Although Src inhibition alone or irradiation showed significantly reduced tumor growth each, but inhibition of Src combined with irradiation showed synergistic

effect on growth inhibition of radioresistant HNSCC. Accordingly, inhibition of Src in the radioresistant HNSCC seems to reduce radioresistance and increase radiosensitivity of radioresistant HNSCC.

7a



7b

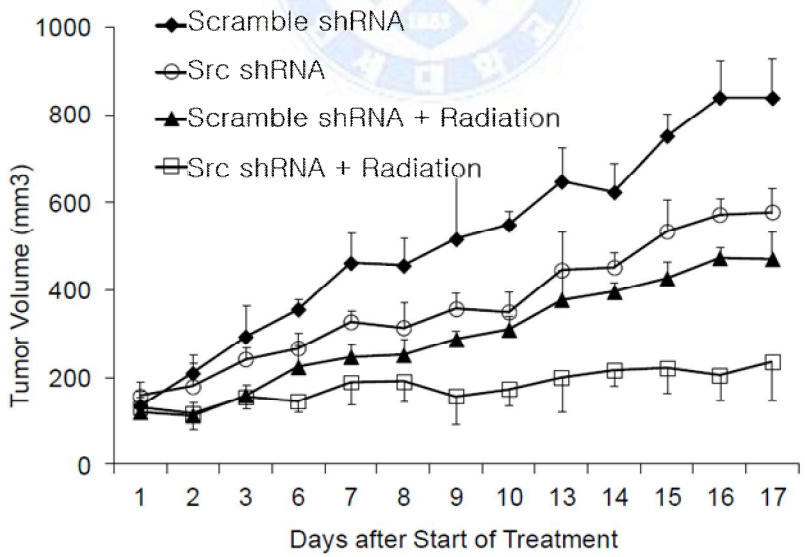


Figure 7. Change of radioresistance after Src inhibition in radioresistant HNCC. (a) MTT assay showed reduced radioresistance of Src inhibited sphere-forming

cells. (b) Tumor growth of Src inhibited radioresistant HNSCC after irradiation was significantly reduced compared to Src intact radioresistant HNSCC in the human xenograft model.

IV. DISCUSSION

RT with or without chemotherapy has been widely used as the main modality in organ-preserving strategies for the treatment of HNSCC. Accordingly, RT is considered one of the most important treatments for HNSCC, and resistance to RT is main cause of treatment failure in patients with HNSCC. The advantage of RT is that it is highly standardized; therefore, variation between treatment methods is minimal with the same tumor extent, and the overall treatment outcome of patients is expected to be similar with the same TNM stage. However, not all patients are successfully cured after RT, and the individual outcome of patients is not predictable prior to RT. Thus, significant numbers of patients undergo unnecessary RT and might lose the opportunity for another treatment option that could be effective for their tumor. This phenomenon occurs because the biology of each patient's tumor is different. If we can determine whether a tumor would respond well to RT before the initiation of treatment, the patient's therapy could be tailored according to the individual expected response, leading to improved survival rates of patients with HNSCC. Akervall et al. (5) reported that the overexpression of YAP-1 is related to radioresistance; therefore, prognostic factors could predict the cause-specific survival and recurrence-free survival of patients with HNSCC. Lee et al. (7) reported that increased expression and nuclear translocation of NM23-H1 was observed in radioresistant cell lines using cDNA array and proteomics. In addition, several investigators have performed studies related to biomarkers for predicting radioresistance. However, a reliable biomarker has not yet been established (8-15).

RT plays an important role in the treatment of HNSCC, but also has a disadvantage. An adaptive response sometimes appears in cancer cells during the process of treatment, and tumors showing an adaptive response tend to be more

resistant, aggressive, and invasive (16). An accurate mechanism should be precisely investigated to overcome the radioresistance of HNSCC. It was recently reported that the appearance of radioresistance was intimately associated with the characteristics of CSCs (17). CSCs were shown to be inherently resistant to various treatments such as RT or chemotherapy, and RT itself could induce genetic mutation and epigenetic alteration of CSCs, causing acquired resistance in CSCs (17). The intrinsic and acquired radioresistance of CSCs might be related to treatment failure and disease recurrence in patients with cancer (18).

In the current study, three radioresistant cancer cell lines were established under conditions similar to a real clinical setting. Spheroid cells were induced from these radioresistant cell lines using serum-deprivation culture, and we confirmed that the spheroid cells were CSCs using stem cell markers. Using MTT and colony formation assays, the radioresistance of CSCs and differentiated cancer cells was compared, and we observed that the radioresistance of CSCs was higher than that of differentiated cancer cells, confirming that CSCs play an important role in the expression and maintenance of radioresistance in HNSCC. We observed that the radioresistance of CSCs and spheroid formation under serum-deprivation culture were significantly reduced after Src inhibition. Accordingly, it was confirmed that Src kinase, which is known to be related to tumor invasion, progression, angiogenesis, and metastasis, is deeply involved in the maintenance and expression of radioresistance in CSCs. In our human xenograft model, the tumor volume decreased most notably in the group with inhibited Src and radiation among all groups. Therefore, we confirmed that Src plays an important role in the maintenance and expression of radioresistance in HNSCC. However, whether reduced radioresistance and tumor invasiveness after inhibiting Src in radioresistant CSCs are caused by reduced CSCs or inhibition of the radioresistance of CSCs has not yet been determined.

As mentioned above, Src plays a major role in the expression and maintenance of radioresistance of HNSCC CSCs. Thus, Src inhibitors such as dasatinib, bosutinib, and saracatinib can help to control the radioresistance observed in HNSCC. However, because these Src inhibitors are nonspecific and

affect other kinases, many side effects can occur with the use of these inhibitors (19-21). To overcome these limitations, further research will focus on the relationship between radioresistance and downstream effectors of Src to identify specific potential targets for targeted therapy.

V. CONCLUSION

Cancer stem cells seem to be the cause of the radioresistant properties of radioresistant head and neck cancer. Src may also play a key role in maintaining radioresistance, and it may be used in target therapy for radioresistant head and neck cancer.

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ABSTRACT(IN KOREAN)

두경부 암의 방사선 내성 발현에 있어서 종양 줄기 세포의 역할

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박 영 민

본 연구는 방사선 내성 두경부 암 세포주를 만들고 이로부터 종양줄기세포를 분리하여 방사선 내성 발현에 있어서 종양줄기세포의 역할을 규명하고자 한 연구이다. 실제 임상과 유사한 상황에서 방사선을 조사하여 방사선 내성 두경부 암 세포주 (SCC15, SCC25, QLL1)를 만들었고 MTT assay를 시행하여 이들 세포주들이 방사선 내성을 획득하였음을 확인하였다. 이들 세포주로부터 종양줄기세포를 분리하여 줄기세포인자 (CD44, Oct-4, Nanog, Sox-2, CD-133)의 발현을 western blot을 통하여 확인하였다. Monolayer cell들에 비하여 spheroid cell들이 두경부 암에서 관찰되는 방사선 내성의 발현에 중요한 역할을 맡고 있음을 colony-forming assay를 통하여 확인하였고, Src을 inhibition함으로써 이들에서 관찰되던 방사선 내성의 정도가 감소함을 확인하였으며 in vivo study를 통하여 Src의 억제가 방사선 내성 두경부 암 세포주의 방사선 내성을 감소시킬 수 있음을 확인하였다. 본 연구의 결과를 바탕으로 두경부 암에서 관찰되는 방사선 내성의 발현에 종양줄기세포가 중요한 역할을 맡고 있으며, 특히 Src이 이러한 방사선 내성을 유지 및 발현하는데 필수적임을 확인하였다. 향후 targeted therapy의 대상으로 추가적인 연구가 필요할 것으로 보인다.

핵심되는 말 : 두경부암, 종양줄기세포, 방사선 내성