Thesis

Establishment and characterization of radiation resistant strains from squamous cell carcinoma cell lines in serum-free defined culture

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Summary

Squamous cell carcinoma (SCC), including oral squamous cell carcinoma (OSCC), has been increasing in the world and being the most common cancer in South-East Asian countries and being at high rate of recurrence.

Radiation therapy (RT) plays a major role in the management of OSCC among various cancer therapies. Despite therapeutic and technological advances such as ionizing radiation, gamma rays and charged particles to kill cancer cells through DNA damage directly or indirectly caused by free ion radicals, some patients will have persistence of irradiated tumor or develop locoregional failure, resulting in significant morbidity and mortality.

RT using high dose rate (HDR) radiation has been widely used as an effective modality to treat human cancer by various types of modern delivering techniques but not low dose rate (LDR) radiation.

Therefore, to elucidate the cellular and molecular mechanisms involved in radiation resistance of cancer cells upon radiation therapy using HDR or LDR might be worthwhile to develop effective therapies to circumvent radiation resistance. In this work, mechanisms of radio-resistance to RT in squamous cell carcinomas including oral SCC and strategies used to overcome this resistance were studied. To study that, it might be useful to establish radiation resistant-cancer cells in *in vitro*. Although there have been several reports of isolating radiation resistant cancer cells, and their use to elucidate cellular and molecular mechanisms in radiation resistance, these

radiation resistant (RR) SCC cells were isolated under serum-supplemented culture condition. Serum-supplemented medium contains a lot of undefined or unknown proteins, factors, and lipids which may exhibit unknown biological effects on cancer cells *in vitro*. Thus, serum-free defined medium can show exact biological characteristic of the cells.

In this study, first I have tried to isolate and establish RR-SCC strains from SCC and OSCC cell lines in serum-free defined culture, and then characterized their cellular and molecular properties, and defined functional genes involved in radiation resistance.

Materials and methods

Two SCC cell lines, A431 derived from vulvar SCC, and NA/HO-1-N-1 from OSCC were used in this study. The cells were cultured in serum-free DF6F medium (1:1 mixture of Dulbecco's Modified Eagle Media and Ham-F12 medium supplemented with insulin (10 μg/ml), transferrin (5 μg/ml), 2-aminoethanol (10 μM), sodium selenite (10 nM), 2-mercaptoethanol (10 μM), and oleic acid conjugated with fatty acid-free bovine serum albumin (9.4 μg/ml)).

All cell lines were irradiated weekly at a dose of 2.2Gy/day, 4 days/week with a low dose rate (LDR) irradiation system (RM1000, Chugai Technos, Japan), or at 5Gy/5.75 mins, twice a week with a high dose rate (HDR) system (Gamma cell 40 Exactor, Best Theratronics, Canada) in serum-free defined culture. After irradiating under 60Gy as a whole dose by LDR and HDR irradiation system, we have isolated 4 RR sub-strains from 2 SCC cell lines. To confirm the radiation resistance of these RR-strains, colony survival assay was performed as follow. The wild type (WT) and RR strains were irradiated at a dose of 0Gy, 2Gy, 4Gy, 6Gy, and 8Gy, respectively, and examined radiation resistance by colony survival assay.

To clarify the biological properties of these cells, several cellular abilities such as growth in monolayer culture, sphere formation in suspension culture, and migration in Boyden-chamber

method were examined in serum-free culture. For the growth assay, the cells were seeded at 10^4cells/well in 24-well plate and counted cell numbers every day by the Coulter Counter. For sphere formation assay, the cells were seeded in 35mm low-attachment dish at 10^3cells/dish, and then sphere numbers were counted on day 5. For migration assay, the cells were seeded at $5x10^4$cells/well in 24-well collagen coated chemotaxicell well in DF medium supplemented with 0.1% BSA and stained with Giemsa for counting number of migrated cells/mm^2. The ratio of CD133 positive cell in each cell line was examined by flowcytometry as cancer stem cell marker.

Total RNAs extracted and purified from all cell lines, were used for Real Time-quantitative PCR (RT-qPCR), and DNA microarray analysis. Expression of pluripotent stem cell markers *Nanog*, *Oct4* and *Sox2* in WT and RR-strains in WT and RR-strains was examined with RT-qPCR.

To study the tumor forming ability in nude mouse, WT and RR-strains ($0.25 x10^6$ -$5x10^6$ cells/100μl DF) were injected subcutaneously to the dorsal back skin of nude mice (BALB/c-nu/nu), and tumor size was measured every week. Then the tumors were excised, weighted, fixed in 4% paraformaldehyde for 24hr, and embedded in paraffin for H&E and immuno-histochemical staining.

DNA microarray analysis of all cells was conducted for further investigation. Overexpressed genes were investigated by RT-qPCR, Western blot and colony survival formation after silencing with siRNA. Transfection of high expressed gene into WT cells was conducted to clarify its molecular characterization.

Results

The current study has demonstrated that:

1. Radiation resistant cell strains could be successfully isolated from NA OSCC cell line, and A431, a vulvar squamous cell carcinoma cell line with LDR and HDR system in serum-free defined culture.
2. Radiation resistant strains exhibited higher sphere formation and migration abilities, also have several characteristics of aggressive cancer cell compare to those of parental cell lines. Radiation resistant strains can promote higher tumor formation ability compare to parental cells in nude mice xenograft.
3. LDR radiation can generate higher radiation resistance of cancer cells, higher expression of Nanog, higher migration ability and tumor forming ability than HDR system.
4. RR-cells exhibited higher expression of *IGF2* and *krt13*. Silencing *IGF2* and *krt13* decreased survival rate of RR-cells upon radiation exposure. Thus, *IGF2* and *krt13* might be good target genes to overcome radiation resistance and an indicator for radiation sensitivity.
5. Overexpression of *krt13* in A431 cell exhibited higher proliferation rate, sphere formation, migration ability, higher *Nanog*, *Oct4* and higher survival rate suggesting that *krt13* is an important gene in radiation resistance.
6. These radiation resistant cells might be very useful not only to study mechanisms of radiation resistance, but also to develop novel cancer treatment modality to circumvent radiation resistance of cancer.

As a conclusion, this study clearly demonstrated that radiation resistant SCC cells can lead to cancer recurrence after radiation therapy, having higher population of cancer stem cells and high tumorigenicity through overexpressing various genes such as *IGF2* and *krt13*.

Establishment of radiation resistant cancer cell model in serum-free defined culture and its use would be powerful tools to elucidate the mechanism of acquisition of radiation resistance and could potentially be targeted for the development of novel diagnosis and therapeutic modalities.