論　文　内　容　要　旨

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

（無血清培養系を用いた扁平上皮癌細胞株からの放射線耐性細胞の樹立とその機能解析）

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Introduction

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. It has been considered that cancer cells are functionally heterogeneous that undergo not only proliferation but also differentiation and maturation to a certain degree and contain a small population of cancer stem cells (CSCs). It seems logical that cures of cancer can be achieved only if the CSCs population is eliminated.

Several treatment modalities such as operation, radiation, and chemo therapy have been reported to be effective in treating many kinds of cancer including OSCC. Among them, radiation therapy (RT) plays a major role in the management of OSCC. 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. On the other hand, RT using low dose rate (LDR) radiation has been introduced in the treatment of prostate and oral cancers.

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 (10μg/ml), transferrin (5μg/ml), 2-aminoethanol (10μM), sodium selenite (10nM), 2-mercaptoethanol (10μM), and oleic acid conjugated with fatty acid-free bovine serum albumin (9.4μg/ml)).

All cell lines were irradiated weekly at a dose of 2.2Gy/day, 4days/week with a low dose rate (LDR) irradiation system (RM1000, Chugai Technos, Japan), or at 5Gy/5.75min, twice a week with a high dose rate (HDR) system (Gamma cell 40 Exactor, Best Theratronics, Canada) in serum-free defined culture. After irradiating 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. After 14 days of culture, the colonies were stained with Giemsa, and counted.

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 prime surface (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 ($0.25 x10^6$ -$1x10^6$ 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. In DNA microarray data, *IGF2* and *krt13* are highly expressed in RR-strains compare to WT among and were chosen for further investigation. Theirs high expression in RR-strains in RNA level and protein level were confirmed by RT-qPCR analysis, western blot and immunohistochemical staining of nude mice tumors. Silencing of those genes in RR-strains by siRNA were also conducted to confirm their relations with radiation resistance. The function of the *IGF2* and *krt13* in RR-strains were studied by silencing with siRNA and overexpression by generation of stable transfectant krt13-A431 cell for checking their radiation sensitivity, pluripotent stem cell marker expression, growth in monolayer and sphere forming ability.

Results

By using LDR and HDR system, RR-strains from A431-WT and NA-WT cells designated A431-LDR, NA-LDR, A431-HDR, and NA-HDR, were successfully isolated in serum-free defined culture. The D_{37} value of A431-LDR, A431-HDR, A431-WT, NA-LDR, NA-HDR, and NA-WT was 5Gy, 3.7Gy, 2.3Gy, 7.5Gy, 5.5Gy and 4.6Gy, respectively. These cells exhibited higher expression of cancer stem cell marker such as CD133, higher sphere formation and higher migration abilities compare to those of WT cell lines. LDR-RR cells showed significant higher expression of pluripotent stem cell marker *Nanog* than in WT and HDR-RR cells. In addition, the RR cells exhibited higher tumor forming ability compared to WT cells in nude mice xenograft.

LDR radiation can generate higher radiation resistance of cancer cells, higher expression of Nanog, higher migration ability and tumor forming ability than in HDR system.

DNA microarray analysis revealed over 500 genes were overexpressed in RR-strains compared to WT cells. Among them, *IGF2 and krt13* genes were high expression in RR-strains compare to WT cells. RT-qPCR analysis further confirmed that both RR-strains overexpressed *IGF2* and *krt13*. In addition, pathway analysis of the DNA microarray analysis revealed that various pathways, such as MAPK, JAK/STAT signaling pathway, apoptosis pathway, TGF-β signaling pathway and cytokine-cytokine receptor interaction were activated in RR-strains. Gene Ontology analysis of microarray analysis also showed that the genes involved in keratinization, inflammatory response, wound healing and response to cytokine stimulus were enriched.

Silencing of *IGF2* by siRNA in RR-strains made them sensitive to radiation compare to RR-strains transduced with negative control siRNA (NC). When silencing *krt13* with siRNA-*krt13*, A431 RR-strains showed higher radiation sensitivity than negative control cells.

Silencing of *IGF2* in A431-LDR exhibited lower expression of *krt13*, *Nanog* and *Oct4* in mRNA level. WT-A431 cells overexpressed *krt13* showed higher cellular ability, such as proliferation, sphere formation, migration and exhibited elevated expression of *IGF2*, *Nanog* and *Oct4* in mRNA level than in control cells.

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.