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BIOLOGY CONTRIBUTION

ENHANCEMENT OF *SPHK1 IN VITRO* BY CARBON ION IRRADIATION IN ORAL SQUAMOUS CELL CARCINOMA

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Purpose: The purpose of this study was to assess the gene expression changes in oral squamous cell carcinoma (OSCC) cells after carbon ion irradiation.

Methods and Materials: Three OSCC cell lines (HSC2, Ca9-22, and HSC3) were irradiated with accelerated carbon ion beams or X-rays using three different doses. The cellular sensitivities were determined by clonogenic survival assay. To identify genes the expression of which is influenced by carbon ion irradiation in a dose-dependent manner, we performed Affymetrix GeneChip analysis with HG-U133 plus 2.0 arrays containing 54,675 probe sets. The identified genes were analyzed using the Ingenuity Pathway Analysis Tool to investigate the functional network and gene ontology. Changes in mRNA expression in the genes were assessed by real-time reverse transcriptase–polymerase chain reaction.

Results: We identified 98 genes with expression levels that were altered significantly at least twofold in each of the three carbon-irradiated OSCC cell lines at all dose points compared with nonirradiated control cells. Among these, *SPHK1*, the expression of which was significantly upregulated by carbon ion irradiation, was modulated little by X-rays. The function of *SPHK1* related to cellular growth and proliferation had the highest p value (p = 9.25e-7 to 2.19e-2). Real-time reverse transcriptase–polymerase chain reaction analysis showed significantly elevated *SPHK1* expression levels after carbon ion irradiation ($p < 0.05$), consistent with microarray data. Clonogenic survival assay indicated that carbon ion irradiation could induce cell death in Ca9-22 cells more effectively than X-rays.

Conclusions: Our findings suggest that *SPHK1* helps to elucidate the molecular mechanisms and processes underlying the biologic response to carbon ion beams in OSCC. © 2006 Elsevier Inc.

Oral squamous cell carcinoma, Carbon ion irradiation, Microarray, Pathway analysis, *SPHK1*.

INTRODUCTION

Oral squamous cell carcinoma (OSCC) remains a significant health problem and requires a multidisciplinary treatment (1). Surgery alone or in combination with adjuvant radiotherapy for more advanced lesions is the standard of care. Surgical resection is considered the treatment of choice, but complete resection is exceptional for locally advanced OSCC. Postoperative irradiation is generally accepted as an adjunct to surgery in a multimodal therapy approach. After complete and marginal resection, adjuvant radiation therapy improves locoregional control and overall survival rates. Although conventional X-ray treatment is an effective modality for a wide variety of human cancers, in certain cases it continues to yield poor results.

To achieve an improved therapeutic effect, dose escalation is essential, but it increases the risk of oral toxicity. High linear energy transfer (LET) radiotherapy with heavy ions, such as neon and carbon ions, provides superb biologic effects and has excellent dose-localizing properties (2–7). These high LET-charged particles can severely damage the tumor with fewer effects on normal tissue. Beam modula-

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tion by bolus absorbers and collimator blocks can construct precise beam penetration and sharp lateral edges in three dimensions. The resulting isodose distribution can be made to conform closely to the target volume, allowing a high dose to the tumor with minimal irradiation of the surrounding normal tissues.

Carbon ion beams emit high LET radiation characterized by higher relative biologic effectiveness (RBE) than low LET radiation such as X-rays. The efficacy of carbon ion therapy has been demonstrated in clinical trials at the National Institute of Radiologic Sciences, Chiba, Japan, since 1994 (8–11). Carbon ions were selected for clinical trials because they have the biologic characteristics of high LET with 78 KeV/μm at the distal end of the spread-out Bragg peak (SOBP) and because they show good dose-localizing properties compared with heavier ions. These advantages have been shown in various cancers (8, 9, 12–15). However, severe adverse effects such as refractory ulceration at the adjacent normal tissues have also been reported. A suitable treatment strategy is certainly a necessity to reduce injury of surrounding normal tissues.

Although several studies have focused on the biologic effects of carbon ion, few have attempted to understand the molecular basis of carbon ion therapy. There is an urgent need to elucidate the molecular mechanisms and processes underlying carbon ion irradiation. In recent years, a cDNA microarray system has been widely used for comprehensive gene expression analysis (16–18). The emerging technology of high-density cDNA microarray provides the ability to analyze comparatively the mRNA expression of thousands of genes in parallel.

In the current study, we performed microarray analysis using high-density Affymetrix U133 plus 2.0 GeneChip arrays (Affymetrix, Santa Clara, CA) to assess characteristic gene expression patterns of carbon-irradiated OSCC cells and to identify the carbon-induced genes that are not substantially altered by X-rays. The genes identified were used for network and gene ontology analysis.

METHODS AND MATERIALS

Cell line and culture conditions

The human OSCC-derived cell lines HSC2, Ca9-22, and HSC3 (Human Science Research Resources Bank, Osaka, Japan) were prepared for this study. Cells were maintained in Dulbecco's modified Eagle's medium F-12 HAM (Sigma Chemical Co., St. Louis, MO) and supplemented with 10% heat-inactivated fetal bovine serum and 50 units/mL penicillin and streptomycin. All cultures were grown at 37°C in a humidified atmosphere of 5% carbon dioxide for routine growth. Transfer to fresh medium was performed when confluence was ~90%.

Irradiation using carbon ion beams and X-rays

All procedures of carbon ion irradiation were carried out at the National Institute of Radiologic Sciences. Briefly, a 290-MeV/nucleon carbon ion beam with 6-cm SOBP was used through experimental port. Cells plated in 75 cm^2 plastic flasks (Corning Inc., Corning, NY) were irradiated at the distal end of the SOBP (LET = 75 keV/μm). Three irradiated dose points (1, 4, and 7 Gy) were set.

In addition, the cells grown in 10-cm tissue culture dishes were irradiated with one dose of radiation (2, 4, or 8 Gy) using X-ray irradiation equipment (MBR-1520R-3; Hitachi, Tokyo, Japan) at Chiba University at a source-to-target distance of 55 cm when the cells were 70% to 80% confluent.

Clonogenic survival assay

Cell survival was measured using a clonogenic survival assay. After exposure to various doses of either carbon ion beams or X-rays, cells were seeded into 60-mm tissue culture dishes and cultured for approximately 14 days to allow colonies to form. The colonies were stained with a solution of crystal violet (Sigma) and counted. The survival fraction at each dose was determined as a ratio of plating efficiencies for irradiated and nonirradiated cells. These experiments were performed once.

Isolation of RNA

Total RNA was extracted with Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA) from irradiated and nonirradiated cells 1, 2, 4, 8, and 12 h after irradiation, according to the manufacturer's instructions. The quality of total RNA was determined by Bioanalyzer (Agilent Technologies, Palo Alto, CA).

Hybridization of RNAs to oligonucleotide arrays and data analysis

For microarray analysis, 4 h after irradiation was selected as the time point to monitor the early response of OSCC cells to carbon ion or X-ray irradiation and to identify differentially expressed early genes after carbon ion or X-ray irradiation that mediate cellular events such as DNA repair and apoptosis. We used Human Genome U133 plus 2.0 GeneChip oligonucleotide arrays containing 11 pairs of matched/mismatched 25-mer oligonucleotide probes for each of ~38,500 transcripts of known genes. Carbon- or X-ray–irradiated HSC2, Ca9-22, and HSC3 cells were analyzed and compared with cells that were not irradiated. For hybridization, 20 μg of total RNA per sample was prepared according to the manufacturer's protocols (Affymetrix). Fragmented cRNA (15 μg of each) was hybridized to the Human Genome oligonucleotide arrays. Arrays were stained with phycoerythrin-streptavidin and the signal intensity was amplified by treatment with a biotin-conjugated antistreptavidin antibody, followed by a second staining using phycoerythrin-streptavidin. The arrays stained a second time were scanned using the Affymetrix GeneChip Scanner 3000. Expression data were analyzed using GeneChip Operating Software 1.1 (Affymetrix) and GeneSpring 6.1 (Silicon Genetics, Redwood City, CA).

Network and gene ontology analyses

The identified genes were used for network and gene ontology analyses. Gene accession numbers were imported into the Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, Mountain View, CA). The genes were categorized based on molecular functions using the software. The identified genes also were mapped to genetic networks in the IPA database and ranked by score. The score reflects the probability that a collection of genes equal to or greater than the number in a network could be achieved by chance alone. A score of 3 indicates that there is a 1/1,000 chance that the focus genes in a network are caused by random chance. Therefore, scores of $\geq$3 have a 99.9% confidence level of