<table>
<thead>
<tr>
<th>Title</th>
<th>Genetic effects of x-ray and carbon ion irradiation in head and neck carcinoma cell lines</th>
</tr>
</thead>
<tbody>
<tr>
<td>Author(s)</td>
<td>Yamamoto, N; Ikeda, C; Yakushiji, T; Nomura, T; Katakura, A; Shibahara, T; Mizoe, JE</td>
</tr>
<tr>
<td>Journal</td>
<td>Bulletin of Tokyo Dental College, 48(4): 177-185</td>
</tr>
<tr>
<td>URL</td>
<td><a href="http://hdl.handle.net/10130/418">http://hdl.handle.net/10130/418</a></td>
</tr>
</tbody>
</table>
Genetic Effects of X-Ray and Carbon Ion Irradiation in Head and Neck Carcinoma Cell Lines

Nobuharu Yamamoto, Chihaya Ikeda, Takashi Yakushiji, Takeshi Nomura, Akira Katakura, Takahiko Shibahara and Jun-etsu Mizoe*

Department of Oral and Maxillofacial Surgery, Tokyo Dental College, 1-2-2 Masago, Mihama-ku, Chiba 261-8502, Japan
* Hospital, Research Center for Charged Particle Therapy National Institute of Radiological Sciences, 4-9-1 Anagawa, Inage-ku, Chiba 263-8555, Japan

Received 1 June 2007/Accepted for publication 17 December, 2007

Abstract

The effects of X-ray and carbon ion irradiation on DNA and genes in head and neck carcinoma cells were examined. Four head and neck cancer cell lines (squamous cell carcinoma, salivary gland cancer, malignant melanoma, normal keratinocyte) were treated with 1, 4, and 7 GyE of carbon ion, or 1, 4, and 8 Gy of X-ray, respectively. DNA and RNA in the treated cells were extracted and purified. PCR-LOH (polymerase chain reaction-loss of heterozygosity) analysis with 6 microsatellite regions on chromosome 17 was performed to determine DNA structural damage, and then microarray analysis was performed to reveal changes in gene expression. PCR-LOH analysis detected high LOH in cells treated by radiation, indicating that most of the damage by X-ray occurred in the target region on one of the homologous chromosomes. However, carbon ion caused homodeletion, which means deletion of the counterparts in both homologous chromosomes.

Key words: Head and neck carcinoma—Loss of heterozygosity (LOH)—X-ray irradiation—Carbon ion irradiation

Introduction

Head and neck cancer is one of the most common malignancies worldwide. In the Far East Asia and India, in particular, the incidence is much higher, with up to 40% of malignancies occurring in the head and neck regions.

Human cancers result from the accumulation of genetic alterations at specific chromosomal regions, involving a multistep process, and much evidence indicates that there are a number of tumor suppressor genes (TSGs) involved in carcinogenesis. On the other hand, the treatment of head and neck tumor is very difficult, because this region is involved in many important functions such as articulation, mastication, and swallowing. These functions are closely connected with the patient's personality and self-confidence. Carbon ion radiotherapy, one of the new conservative radiotherapies, is focused on from this point
view. Therefore, it is important to investigate the mechanism of the effects of carbon ions on DNA structure and gene expression. Although conventional X-ray treatment is an effective modality for a wide variety of human cancers, in certain cases it continues to provide poor results.

To obtain an improved therapeutic effect, dose escalation is essential, but this 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. These high LET-charged particles can severely damage the tumor, with fewer effects on normal tissue. Beam modulation by bolus absorbers and collimator blocks allows 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 surrounding normal tissues.

Carbon ion beams emit high LET radiation characterized by higher relative biological 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 Radiological Sciences (NIRS), Chiba, Japan, since 1994\(^{16,27,29,40}\). Carbon ions were selected for clinical trials, because they have the biologic characteristics of high LET, with 78 KeV/\(\mu\)m 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 \(^{3,16,27,29,33,36}\). Preliminary results of phase II clinical trials have shown extremely favorable therapeutic results in the treatment of head and neck cancers (including oral cancers) that were otherwise intractable with conventional photon radiation\(^{16,27}\). As stated above, radiotherapy with heavy charged particles is significantly effective in the therapy of head and neck cancers. However, severe adverse effects such as refractory ulceration at the adjacent normal tissues have also been reported. A suitable treatment strategy is certainly necessary to reduce injury to surrounding normal tissues.

Although several studies have focused on the biologic effects of carbon ions, 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 used widely for comprehensive gene expression analyses\(^ {7,9,39}\). 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 present study, DNA structural mutations were examined by PCR-LOH (polymerase chain reaction-loss of heterozygosity) analysis. The effects of carbon ions on carcinoma cells are discussed in comparison with X-ray.

**Materials and Methods**

1. **Cell line and cell culture conditions**

The following head and neck carcinoma-derived cell lines were used for this study: Ca9-22 (derived from oral squamous cell carcinoma: OSCC), HSG (from salivary gland tumor), G361 (from malignant melanoma), and HaCaT (from normal human squamous cells) (Human Science Research Resources Bank, Osaka, Japan). All cell lines were grown in RPMI-1640 medium supplemented with 10% 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 \(\sim\)90%.

2. **Radiation treatment**

The cell lines were treated with different doses (1, 4, and 8 Gy) of X-ray and also with different doses (1, 4, and 7 GyE) of carbon ion beam. All procedures of X-ray and carbon ion irradiation were carried out at the NIRS.
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). Structural damage was determined using DNA extracted at 1, 24, and 48h after irradiation.

3. Clonogenic survival assay of Ca9-22

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 non-irradiated cells. These experiments were performed once.

4. DNA preparation

Genomic DNAs were isolated by the standard method using phenol-chloroform extraction and refined, washed and precipitated with ethanol. The concentrations of extracted DNA were estimated by spectrophotometric method and kept frozen at −80°C. From each DNA sample, 50 ng/μl was used as a template for the PCR amplification procedure.

5. DNA analysis on microsatellite loci

We selected 6 highly informative microsatellite markers (D17S261, D17S1176, TP53, D17S250, D17S1320, and D17S1329) on chromosome 17 (Table 1). All primers were obtained from Research Genetics (Huntsville, AL). PCR amplification was performed in a total reaction volume of 20 μl, as described previously. Each PCR reaction mixture contained 250 ng sample DNA, 20 pmol each primer, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3.0 mM MgCl$_2$, 2 mM dNTP, and 0.5 unit Taq DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT). PCR was performed with 26 to 30 cycles of denaturation at 94°C for 1 min, annealing at 52 to 58°C for 1 min, and extension at 72°C for 1 min using a DNA Thermal Cycler (Perkin-Elmer Cetus, Norwalk, CT). After dilution with an adequate volume of formamide-dye mixture (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol), the PCR products were heat-denatured (98°C, 5 min.), chilled on ice, and electrophoresed on 6% urea-formamide-polyacrylamide gel at 3W for 2 to 3 h, depending on fragment size. Silver staining of the gels was performed using the DNA Silver Staining Kit (Amersham Pharmacia Biotech AB, Sweden). To ensure reproducibility in each case with LOH or microsatellite instability (MSI), all tests were performed under the

<table>
<thead>
<tr>
<th>Markers</th>
<th>Locations</th>
<th>Size of PCR products (bp)</th>
<th>Sequence of primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>D17S261</td>
<td>17p12-11.1</td>
<td>157–171</td>
<td>5'-CAGGTTCTGTCA TACAGGACTA-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5'-TTCTGGGAACCTACTCTCGA-3'</td>
</tr>
<tr>
<td>D17S1176</td>
<td>17p13.1</td>
<td>95–109</td>
<td>5'-AGTTCATACATACATACGTGC-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5'-TCAATGGAGAATTACGATAG-3'</td>
</tr>
<tr>
<td>TP53</td>
<td>17p13.1</td>
<td>103</td>
<td>5'-TTGCTCTTCTTCGACTG-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5'-CCAAGACTTATGACCTGAG-3'</td>
</tr>
<tr>
<td>D17S250</td>
<td>17q11.2-12</td>
<td>151–169</td>
<td>5'-GGAGAAATCAATAAGCATAAT3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5'-GCTGGGCTATATATATATTAAAC-3'</td>
</tr>
<tr>
<td>D17S1320</td>
<td>17q21</td>
<td>180</td>
<td>5'-ACCTTCCAGAGAAATCTCTGTCG-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5'-CCACGTTCTTCTGTGTCC-3'</td>
</tr>
<tr>
<td>D17S1329</td>
<td>17q21</td>
<td>170</td>
<td>5'-GACTCTGAAGGTAAAAGGCAA-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5'-CTGCCCTCGCTTGGGAGTAG-3'</td>
</tr>
</tbody>
</table>
same conditions.

6. Assessment of LOH and MSI

LOH in the tumor DNA samples was assessed by scanning densitometry and analyzed with National Institute of Health (NIH) software (Image version 1.62, Dr. W. Rasband, NIH, Bethesda, MD, USA). The intensities of the signals in tumor DNA were compared with those of the corresponding normal DNA. A reduction in signal intensity of more than 50% was required for LOH. Commonly deleted regions were defined by considering the loci most frequently showing LOH, together with multiple interstitial deletions. Microsatellite instability (MSI) for DNA samples was also assessed as positive in cases with additional bands in the tumor sample that were not observed in the corresponding normal sample or in cases with a band shift in the tumor sample that contrasted with those of the corresponding normal bands.

Results

1. Analysis of allelic loss

Structural DNA changes occurring on chromosome 17 after X-ray and carbon ion irradiation of cell lines derived from malignant tumors in head and neck were analyzed using PCR-LOH assay. Typical results of electrophoresis are shown in Fig. 1. Deletion (DLT), LOH, ROH, and NI signify homodeletion, heterodeletion, retention of heterozygosity, and not informative, respectively. A deletion map was created covering both kinds of beam (X-ray and carbon ion), 3 different doses (1 Gy/GyE, 4 Gy/GyE, 8 Gy/7 GyE), 4 cell lines, and 3 different DNA-extracted times (1, 24, and 48h after irradiation) (Fig. 2). PCR-LOH analysis revealed high LOH, such as in Ca9-22, HSG and G361, when they were treated with X-ray. However, in normal keratinocyte cell line, HaCaT, only two cases of DNA mutations (DLT or MSI) were found.
In contrast, after carbon ion irradiation, DLT occurred at many region regardless of type of cell line. However, LOH was detected at only one locus.

2. Survival rates

The survival rates for Ca9-22 cell exposed to carbon ion beams or X-rays are shown in Fig. 3. Each curve represents one experiment. In Ca9-22 cells, there was a significant difference in survival curves for carbon ion beams and X-rays. The survival curve for Ca9-22 cells irradiated with carbon ion beams showed a steep curve, whereas X-ray-irradiated Ca9-22 cells showed a gentle curve.

Discussion

Radiotherapy, an inevitable component of modern cancer management, is a major treatment modality that can potentially provide a cure for patients with OSCC\(^{34}\). The success or
failure of radiotherapy can be affected by the radiosensitivity of the tumor target and the limits imposed on treatment by the radiosensitivity of normal tissues. Recently, several studies using microarrays technique have successfully identified and classified a set of human genes that are radiosensitive to X-ray irradiation\(^{1,8,10,12,18,32}\).

Modern curative radiotherapy requires higher doses to tumors and minimal irradiation to the surrounding normal tissues. Carbon ions produce increased density of local energy deposition with high LET components, resulting in radiobiologic advantages. It is an area of active investigation to elucidate the mechanisms underlying the increased biologic effectiveness of dense irradiation. Several studies have evaluated the correlation between tumor responses to carbon ion irradiation and the expression status of known genes\(^{11,13,38,41}\).

Irradiation with high LET carbon ion beams caused glioma cells with either the wild-type or mutant \(p^53\) gene to fail to proliferate and apoptosis, more effectively than X-rays\(^{15}\). In addition, the effects of carbon ion beams are reduced by \(G_1\) arrest, which is independent of \(p21\) expression\(^{15}\). To date, no report has focused on the gene expression profiles of head and neck carcinoma cells exposed to X-ray and carbon ion beam irradiation simultaneously.

Gene expression profiling using high-density microarrays is an excellent tool to identify novel candidate biomarkers in human cancers associated with regulation of important cancer-related cellular events, such as cell growth regulation and apoptosis. Indeed, several studies have successfully used microarrays to identify and classify a set of human genes in response to ionizing radiation\(^{8,10,12,18}\). To highlight gene expression changes in OSCC cells exposed to carbon ion beams, we used a high-throughput gene chip containing 54,675 oligonucleotide-based probe sets to analyze change in gene expression after carbon ion irradiation. It has been demonstrated that gene expressions are dramatically changed between 1 to 72h after irradiation\(^{5,22,24,35,37}\). In particular, changes in gene expression profiles at 3 or 4h postirradiation have been identified in keratinocytes\(^{20}\) and in umbilical vein endothelial cells\(^{25}\).

In the current study, structural DNA changes occurring on chromosome 17 after X-ray and carbon ion irradiation of cell populations derived from malignant tumors in the head and neck were analyzed using PCR-LOH assay. After X-ray irradiation, a larger amount

<table>
<thead>
<tr>
<th>Functions</th>
<th>Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTB, ADRB2, AKAP12, BRF2, CLK1, COTL1, EMP1, FST, HIF3B, INHBA, INHBB, IRF1, JUN, KLF2, MAPK3, MAPK8, MYC, ODC1, POLR2A, POLR2F, POLR2L, PTHHL1, PTN, SFRS12, SFRS2, SFRS6, SNAPC1, SNAPC2, SNAPC3, SNAPC4, SPN, SRPK1, TBP, TFRC, VHL, A20B1, BCA1, BCA3, CASP3, CLTC, CXCL2, CXCL3, DACH1, EHD1, EGF3, IGF1R, IGFB, IL18, IL8RB, INSR, IRS1, IRS2, ITPR1, JAK2, NEDD4, NEDD9, NPM1, NR1G1, NUP98, PTGS2, PTPN12, RAPGEP2, RELA, SCN2A1, SNAP29, SOCS1, STAT1, SYNCRIP, TNFAIP3, CBLB, CSF1R, DTR, DUSP4, EIF3S1, EIF3S3, EIF3S6, EIF3S7, EIF3S8, EIF3S9, GLIPR1, GRB2, IL11, IL11RA, IL6ST, JAK1, MAPK14, MAPK3, MYO1D1, NONO, NP, PML, PTPRE, SARA1, SFPQ, SPHK1, SPRY2, TNFAIP3, TOP1, TP53, TRAF2, TYK2, VAV1</td>
<td></td>
</tr>
</tbody>
</table>
of LOH was detected rather than DLT. At high doses, however, it was found that LOH tended to decrease. In addition, after carbon ion irradiation, LOH was detected only in one location, whereas all other DNA impairments were marked by the presence of DLT. These results indicated that most of the damage by X-ray occurred in the target region on one of the homologous chromosomes in carcinoma cells. Carbon ion beam caused homo-deletion (DLT), which means deletion of the counterparts in both homologous chromosomes.

We selected the time point of 4h to monitor the early response of OSCC cells to irradiation, and identified 98 genes that were modulated by carbon ion irradiation at all doses in each of the OSCC-derived cell lines, Ca9-22 by using microarray analysis.

In conclusion, this comprehensive gene expression analysis provided an interesting approach to effectively identifying candidate genes involved in cellular radioresistance. These genes may help to disclose the molecular mechanisms of radioresistance in head and neck carcinoma, and could serve as radiotherapeutic molecular markers for choice of the appropriate radiotherapy in this disease.

Acknowledgements

This work was supported by a Research Grant from the Ministry of Education, Science and Culture, Japan (No. 15592135).

References

13) Higuchi Y, Nelson GA, Vazquez M, Laskowitz


Reprint requests to:
Prof. Takahiko Shibahara
Department of Oral and Maxillofacial Surgery,
Tokyo Dental College,
1-2-2 Masago, Mihama-ku,
Chiba 261-8502, Japan
E-mail: shibahara@tdc.ac.jp