Bull Tokyo Dent Coll (2007) 48(4): 177–185

Original Article

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[2)].

Human cancers result from the accumulation of genetic alterations at specific chromosomal regions, involving a multistep process[19,30,31)], 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[4,6,14,15,17,23]. 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/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[3,16,27,28,33,36]. 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 analysis[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 ~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.

Table 1 Sequence of primers used for PCR-LOH analysis

Markers	Locations	Size of PCR products (bp)	Sequence of primers
D17S261	17p12-11.1	157–171	5′-CAGGTTCTGTCATAGGACTA-3′ 5′-TTCTGGAAACCTACTCCTGA-3′
D17S1176	17p13.1	95–109	5′-ACTTCATATACATATCACGTGC-3′ 5′-TCAATGGAGAATTACGATAGTG-3′
TP53	17p13.1	103	5′-TTGCCTCTTTCCTAGCACTG-3′ 5′-CCAAGACTTAGTACCTGAAG-3′
D17S250	17q11.2-12	151–169	5′-GGAAGAATCAAATAGACAAT-3′ 5′-GCTGGCCATATATATATTTAAACC-3′
D17S1320	17q21	180	5′-ACTTTCCAGAAAATCTCTGCTC-3′ 5′-CCACGTCTTTTCTGTGTTCC-3′
D17S1329	17q21	170	5′-GACTCTGAAGGTAAAGAGCAA-3′ 5′-CTCCCCTGCCTTGGGAGTAG-3′

Briefly, a 290-MeV/nucleon carbon ion beam with 6-cm SOBP was used through on 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 48 h 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[25,26]. 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[21]. 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

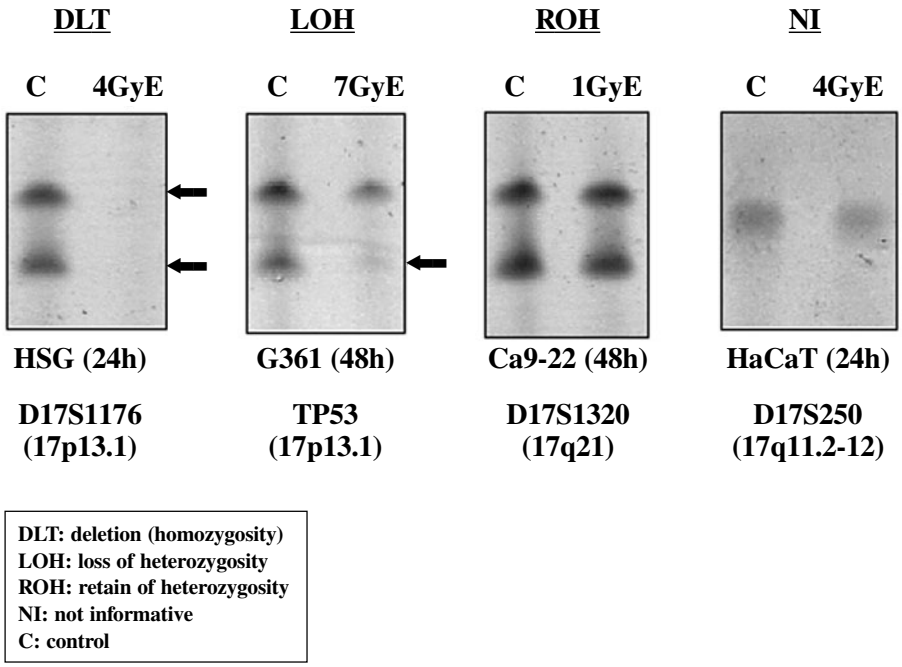

Fig. 1 Typical patterns of electrophoresis
Microsatellite polymorphism analysis in cell lines. Carbon ion irradiated-doses are shown at top, and locus symbols at bottom. Paired control (C) and tumor (T) cell lines demonstrating deletion of both alleles (DLT), loss of upper allele (LOH), retained heterozygosity (ROH) and not-informative (NI), respectively.

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.

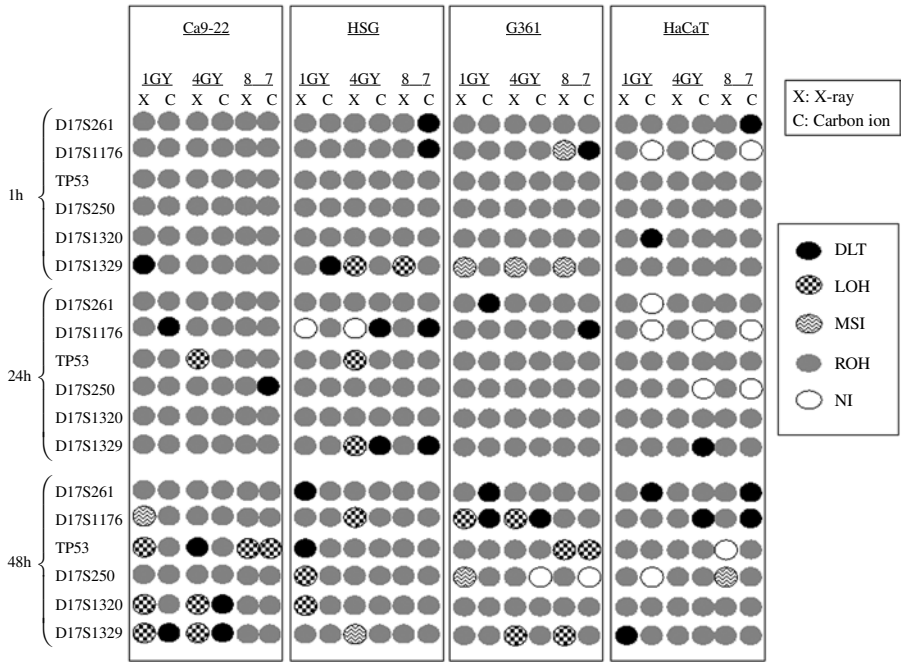

Fig. 2 Deletion mapping of chromosome 17 in 4 head and neck cancer cell lines
Doses and beams are shown at top and locus symbols and DNA-extracted times on left.

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.

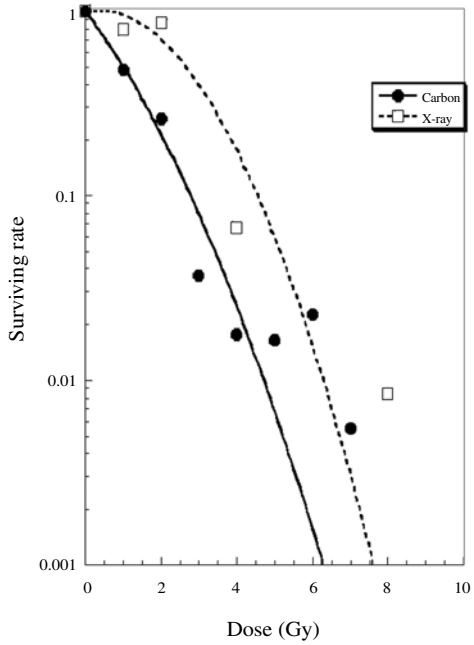

Fig. 3 Survival curves of Ca9-22 cells exposed to carbon ion beams or X-rays
Each point represents value of one experiment.

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

Table 2 Genetic expressions in the carbon-irradiated OSCC cell line

Functions	Genes
Gene expression	*ACTB, ADRB2, AKAP12, BRF2, CLK1, COTL1, EMP1,*
Cancer	*FST, H3F3B, INHBA, INHBB, IRF1, JUN, KLF2,*
Cell growth and proliferation	*MAPK3, MAPK8, MYC, ODC1, POLR2A, POLR2F,*
Cell death	*POLR2L, PTHLH, PTN, SFRS12, SFRS2, SFRS6,*
Cell compromise	*SNAPC1, SNAPC2, SNAPC3, SNAPC4, SPN, SRPK1,*
DNA replication	*TBP, TFRC, VIL2, ATP2B1, BCAR1, BCAR3, CASP3,*
Recombination and repair	*CLTC, CXCL2, CXCL3, DACH1, EHD1, FGF5, IGF1R,*
Carbohydrate metabolism	*IGF2, IL18, IL8RB, INSR, IRS1, IRS2, ITPR1, JAK2,*
Cell morphology	*NEDD4, NEDD9, NPM1, NRG1, NUP98, PTGS2,*
Cellular movement	*PTPN12, RAPGEF2, RELA, SCN2A1, SNAP29, SOCS1,*
Cell cycle	*STAT1, SYNCRIP, TNFAIP3, CBLB, CSF1R, DTR,*
Cell development	*DUSP4, EIF3S1, EIF3S3, EIF3S6, EIF3S7, EIF3S8,*
Immune and lymphatic system development and function	*EIF3S9, GLIPR1, GRB2, IL11, IL11RA, IL6ST, JAK1,*
Hematologic system development and function	*MAPK14, MAPK3, MYOD1, NONO, NP, PML, PTPRE,*
Protein synthesis	*SARA1, SFPQ, SPHK1, SPRY2, TNFAIP3, TOP1, TP53,*
	TRAF2, TYK2, VAV1

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 *p53* 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

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 4 h 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

1) Achary MP, Jaggernauth W, Gross E, Alfieri A, Klinger HP, Vikram B (2000) Cell lines from the same cervical carcinoma but with different radiosensitivities exhibit different cDNA microarray patterns of gene expression. Cytogenet Cell Genet 91:39–43.
2) Ah-See KW, Cooke TG, Pickford IR, Soutar D, Balmain A (1994) An allelotype of squamous cell carcinoma of the head and neck using microsatellite markers. Cancer Res 54:1617–1621.
3) Asakawa I, Yoshimura H, Takahashi A, Ohnishi K, Nakagawa H, Ota I, Furusawa Y, Tamamoto T, Ohishi H, Ohnishi T (2002) Radiation-induced growth inhibition in transplanted human tongue carcinomas with different p53 gene status. Anticancer Res 22:2037–2043.
4) Blakely EA, Kronenberg A (1998) Heavy-ion radiobiology: new approaches to delineate mechanisms underlying enhanced biological effectiveness. Radiat Res 150:126–145.
5) Christiansen H, Saile B, Neubauer-Saile K, Tippelt S, Rave-Frank M, Hermann RM, Dudas J, Hess CF, Schmidberger H, Ramadori G (2004) Irradiation leads to susceptibility of hepatocytes to TNF-alpha mediated apoptosis. Radiother Oncol 72:291–296.
6) Demizu Y, Kagawa K, Ejima Y, Nishimura H, Sasaki R, Soejima T, Yanou T, Shimizu M, Furusawa Y, Hishikawa Y, Sugimura K (2004) Cell biological basis for combination radiotherapy using heavy-ion beams and high-energy X-rays. Radiother Oncol 71:207–211.
7) Francioso F, Carinci F, Tosi L, Scapoli L, Pezzetti F, Passerella E, Evangelisti R, Pastore A, Pelucchi S, Piattelli A, Rubini C, Fioroni M, Carinci P, Volinia S (2002) Identification of differentially expressed genes in human salivary gland tumors by DNA microarrays. Mol Cancer Ther 1:533–538.
8) Fukuda K, Sakakura C, Miyagawa K, Kuriu Y, Kin S, Nakase Y, Hagiwara A, Mitsufuji S, Okazaki Y, Hayashizaki Y, Yamagishi H (2004) Differential gene expression profiles of radioresistant oesophageal cancer cell lines established by continuous fractionated irradiation. Br J Cancer 91:1543–1550.
9) Golub TR, Slonim DK, Tamayo P, Huard C, Gaasenbeek M, Mesirov JP, Coller H, Loh ML, Downing JR (1999) Molecular classification of cancer: class discovery and class prediction by gene expression monitoring. Science 286: 531–537.
10) Guo WF, Lin RX, Huang J, Zhou Z, Yang J, Guo GZ, Wang SQ (2005) Identification of differentially expressed genes contributing to radioresistance in lung cancer cells using microarray analysis. Radiat Res 164:27–35.
11) Hei TK, Zhao YL, Roy D, Piao CQ, Calaf G, Hall EJ (2001) Molecular alterations in tumorigenic human bronchial and breast epithelial cells induced by high LET radiation. Adv Space Res 27:411–419.
12) Hellman B, Brodin D, Anderson M, Dahlman-Wright K, Isacsson U, Brattstrom D, Bergqvist M (2005) Radiation-induced DNA-damage and gene expression profiles in human lung cancer cells with different radiosensitivity. Exp Oncol 27:102–107.
13) Higuchi Y, Nelson GA, Vazquez M, Laskowitz

DT, Slater JM, Pearlstein RD (2002) Apolipoprotein E expression and behavioral toxicity of high charge, high energy (HZE) particle radiation. J Radiat Res 43:219–224.
14) Hofman-Huther H, Scholz M, Rave-Frank M, Virsik-Kopp P (2004) Induction of reproductive cell death and chromosome aberrations in radioresistant tumour cells by carbon ions. Int J Radiat Biol 80:423–435.
15) Iwadate Y, Mizoe J, Osaka Y, Yamaura A, Tsujii H (2001) High linear energy transfer carbon radiation effectively kills cultured glioma cells with either mutant or wild-type p53. Int J Radiat Oncol Biol Phys 50:803–808.
16) Kamada T, Tsujii H, Tsuji H, Yanagi T, Mizoe JE, Miyamoto T, Kato H, Yamada S, Morita S, Yoshikawa K, Kandatsu S, Takeishi A; Working Group for the Bone and Soft Tissue Sarcomas (2002) Efficacy and safety of carbon ion radiotherapy in bone and soft tissue sarcomas. J Clin Oncol 20:4466–4471.
17) Kanai T, Endo M, Minohara S, Miyahara N, Koyama-ito H, Tomura H, Matsufuji N, Futami Y, Fukumura A, Hiraoka T, Furusawa Y, Ando K, Suzuki M, Soga F, Kawachi K (1999) Biophysical characteristics of HIMAC clinical irradiation system for heavy-ion radiation therapy. Int J Radiat Oncol Biol Phys 44:201–210.
18) Kitahara O, Katagiri T, Tsunoda T, Harima Y, Nakamura Y (2002) Classification of sensitivity or resistance of cervical cancers to ionizing radiation according to expression profiles of 62 genes selected by cDNA microarray analysis. Neoplasia 4:295–303.
19) Knudson AG (1993) Antioncogenes and human cancer. Proc Natl Acad Sci USA 90:10914–10921.
20) Koike M, Shiomi T, Koike A (2005) Identification of Skin injury-related genes induced by ionizing radiation in human keratinocytes using cDNA microarray. J Radiat Res 46:173–184.
21) Komiya A, Suzuki H, Aida S, Yatani R, Shimazaki J (1995) Mutational analysis of CDKN2 (CDK4I/MTS1) gene in tissues and cell lines of human prostate cancer. Jpn J Cancer Res 86:622–625.
22) Koshikawa T, Uematsu N, Iijima A, Katagiri T, Uchida K (2005) Alterations of DNA copy number and expression in genes involved in cell cycle regulation and apoptosis signal pathways in gamma-radiation-sensitive SX9 cells and -resistant SR-1 cells. Radiat Res 163:374–383.
23) Kramer M, Weyrather WK, Scholz M (2003) The increased biological effectiveness of heavy charged particles: from radiobiology to treatment planning. Technol Cancer Res Treat 2: 427–436.
24) Kruse JJ, te Poele JA, Velds A, Kerkhoven RM, Boersma LJ, Russell NS, Stewart FA (2004) Identification of differentially expressed genes in mouse kidney after irradiation using microarray analysis. Radiat Res 161:28–38.
25) Lanza V, Pretazzoli V, Olivieri G, Pascarella G, Panconesi A, Negri R (2005) Transcriptional response of human umbilical vein endothelial cells to low doses of ionizing radiation. J Radiat Res 46:265–276.
26) Maniatis T, Fritsch EF, Sambrook J (1982) Molecular Cloning; A Laboratory Manual edition 1, pp. 280–281, Cold Spring Harbor Laboratory Press, New York.
27) Miyamoto T, Yamamoto N, Nishimura H, Koto M, Tsujii H, Mizoe JE, Kamada T, Kato H, Yamada S, Morita S, Yoshikawa K, Kandatsu S, Fujisawa T (2003) Carbon ion radiotherapy for stage I non-small cell lung cancer. Radiother Oncol 66:127–140.
28) Mizoe J, Tsujii H, Kamada T, Matsuoka Y, Tsuji H, Osaka Y, Hasegawa A, Yamamoto N, Ebihara S, Konno A; Working Group for Head-And-Neck Cancer (2004) Dose escalation study of carbon ion radiotherapy for locally advanced head-and-neck cancer. Int J Radiat Oncol Biol Phys 60:358–364.
29) Nakano T, Suzuki M, Abe A, Suzuki Y, Morita S, Mizoe J, Sato S, Miyamoto T, Kamada T, Kato H, Tsujii H (1999) The phase I/II clinical study of carbon ion therapy for cancer of the uterine cervix. Cancer J Sci Am 5:362–369.
30) Nowell PC (1993) Foundation in cancer research. Chromosomes, and cancer: the evolution of an idea. Adv Cancer Res 62:1–17.
31) Nowell PC, Croce CM (1986) Chromosomes, genes, and cancer. Am J Pathol 125:8–15.
32) Ogawa K, Utsunomiya T, Mimori K, Tanaka F, Haraguchi N, Inoue H, Murayama S, Mori M (2006) Differential gene expression profiles of radioresistant pancreatic cancer cell lines established by fractionated irradiation. Int J Oncol 28:705–713.
33) Oohira G, Yamada S, Ochiai T, Matsubara H, Okazumi S, Ando K, Tsujii H, Hiwasa T, Shimada H (2004) Growth suppression of esophageal squamous cell carcinoma induced by heavy carbon-ion beams combined with p53 gene transfer. Int J Oncol 25:563–569.
34) Palme CE, Gullane PJ, Gilbert RW (2004) Current treatment options in squamous cell carcinoma of the oral cavity. Surg Oncol Clin N Am 13:47–70.
35) Rube CE, Wilfert F, Uthe D, Schmid KW, Knoop R, Willich N, Schuck A, Rube C (2002) Modulation of radiation-induced tumour necrosis factor alpha (TNF-alpha) expression

in the lung tissue by pentoxifylline. Radiother Oncol 64:177–187.
36) Schulz-Ertner D, Nikoghosyan A, Thilmann C, Haberer T, Jakel O, Karger C, Kraft G, Wannenmacher M (2004) Results of carbon ion radiotherapy in 152 patients. Int J Radiat Oncol Biol Phys 58:631–640.
37) Tachiiri S, Katagiri T, Tsunoda T, Oya N, Hiraoka M, Nakamura Y (2006) Analysis of gene-expression profiles after gamma irradiation of normal human fibroblasts. Int J Radiat Oncol Biol Phys 64:272–279.
38) Takahashi A, Ohnishi K, Tsuji K, Matsumoto H, Aoki H, Wang X, Tamamoto T, Yukawa O, Furusawa Y, Ejima Y, Tachibana A, Ohnishi T (2000) W AF1 accumulation by carbon-ion beam and alpha-particle irradiation in human glioblastoma cultured cells. Int J Radiat Biol 76:335–341.
39) Toruner GA, Ulger C, Alkan M, Galante AT, Rinaggio J, Wilk R, Tian B, Soteropoulos P, Hameed MR, Schwalb MN, Dermody JJ (2004) Association between gene expression profile and tumor invasion in oral squamous cell carcinoma. Cancer Genet Cytogenet 154:27–35.
40) Tsujii H, Mizoe J, Kamada T, Baba M, Kato S, Kato H, Tsujii H, Yamada S, Yasuda S, Ohno T, Yanagi T, Hasegawa A, Sugawara T, Ezawa H, Kandatsu S, Yoshikawa K, Kishimoto R, Miyamoto T (2004) Overview of clinical experiences on carbon ion radiotherapy at NIRS. Radiother Oncol 73:41–49.
41) Zhao Y, Shao G, Piao CQ, Berenguer J, Hei TK (2004) Down-regulation of Betaig-h3 gene is involved in the tumorigenesis in human bronchial epithelial cells induced by heavy-ion radiation. Radiat Res 162:655–659.

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