Comparison of the Radiobiological Effect of Carbon Ion Beam Therapy and Conventional Radiation Therapy on Cervical Cancer

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Carbon beam therapy/Uterine cervix/p53/Ki-67/p27.

Little clinical evidence has been provided to show the minimization of radiation resistance of tumors using high linear energy transfer radiation. We therefore investigated the radiobiological and molecular pathological aspects of carbon beam therapy. A total of 27 patients with squamous cell carcinoma (SCC) of the cervix were treated using a carbon beam and 50 control patients with SCC of the cervix using a photon beam. The expression of Ki-67, p53, and p27 proteins before radiotherapy and 5 and 15 days after therapy initiation were investigated using immunohistochemistry. Similar changes were observed in Ki-67 labeling index (LI) and p53 LI during carbon and photon beam therapies. However, for carbon beam therapy, the mean p27 LI significantly decreased from 25.2% before treatment to 18.6% on the 5th day after treatment initiation, followed by a significant increase to 36.1% on the 15th day. In contrast, for photon beam therapy, the p27 LI consistently decreased from the initial 19.9% to 13.7% on the 15th day. Histological effects were observably stronger under carbon than photon beam therapy, though no statistically significant difference was observed (p = 0.07 on the 5th day and p = 0.10 on the 15th day). The changes in p27 LI under carbon beam therapy were significantly different from those under photon beam therapy, which suggests important molecular differences in the radio-biological response between therapies. Further investigation is required to elucidate the clinical relevance of these putative changes and optimize the relative biological effectiveness of carbon beam to X-ray.

INTRODUCTION

Compared to conventional low linear energy transfer (LET) radiation therapy, which uses photons, high LET radiation therapy, which uses neutrons and heavy charged particles, shows advantageous dose distribution and radiobiological effects, namely a decreased oxygen enhancement ratio and capacity for sublethal and potentially lethal repair of damage in cancer cells, as well as reduced cell cycle-dependent radiosensitivity.¹ Recently, we reported that carbon ion beam therapy in a clinical setting was equally effective in hypoxic and oxic tumors.² To date, however, little clinical evidence has been available to clearly demonstrate the difference between high LET and conventional photon beam therapy.

We have previously shown histological and molecular pathological evidence of biological changes during radiation therapy. Ki-67 staining during the early period of cervical cancer radiation therapy revealed a transient increase in growth fraction,³ which was suggested to be due to recruitment of G0 cells into the cell cycle. Radiation was also shown to increase the expression of p53, a tumor-suppressor which controls cell cycle transition from G1 to S phase.⁴ In contrast, radiation was reported to decrease the expression of p27/Kip1, which plays an important role in regulating transition from G1 to S phase by binding to and inhibiting the cyclin E cyclin-dependent kinase (CDK) complex.⁵ Given these findings on conventional radiation therapy, a better understanding of tumor cell kinetics would be facilitated by investigating the cervical cancer cell cycle during high LET treatment. In particular, cellular kinetic information from various human tumors during high LET radiation therapy is of potential value.

In June 1994, cancer treatment by heavy charged particle radiation therapy was initiated at the National Institute of
Radiological Sciences (NIRS: Chiba, Japan) using carbon ions generated by a heavy-ion medical accelerator (HIMAC, Chiba, Japan). 6) Between June 1995 and February 1998, we carried out phase I and II clinical trials for advanced uterine cervix cancer (protocol 9403), and have since reported the preliminary results. 7)

Here, to clarify the cell kinetics of specific tumor tissues, we investigated various clinical histological and immunohistochemical parameters in tumor cells during carbon beam therapy, including the relative changes in Ki-67, p27, and p53 labeling indexes (LI). Results were compared with those with conventional radiation therapy.

**MATERIALS AND METHODS**

**Treatment facility and beam quality**

The design parameters of HIMAC, the first heavy ion accelerator in the world to be dedicated solely to medical treatment, are based on the requirements of radiological medicine. 8) The characteristic dose penetration profile of carbon beams is known as "Bragg's peak". To generate a biologically equivalent dose distribution for treatment planning, mono-energy from a Bragg peak is spread out over a number of degrees during radiation therapy. Particle radiation doses are expressed in terms biologically equivalent to X-ray megavoltage by using the relative biologic effectiveness (RBE) value of an acute reaction to calculate the Gray (Gy) equivalent radiation dose (GyE), which has been previously described. 9) Based on previous experimental data and clinical results from fast neutron therapy at the NIRS, clinical RBE was set at 3.0. 5)

**Patient characteristics**

**Patients treated with carbon ion beam**

Between June 1995 and July 1998, 27 patients with squamous cell carcinoma were treated with a carbon ion beam in a phase I/II study, details of which have been previously reported. 7) All 27 patients were eligible for this study. Ages ranged from 36 to 72 years, with a mean ± standard deviation (SD) and median age of 56 ± 9 and 54 years, respectively. A total of 19 and 8 patients had stage IIIB and IVA carcinoma, respectively, in addition to bladder invasion, with the exception of one patient with rectal invasion. A total of 14 patients had squamous cell carcinoma of the large cell non-keratinizing type, 3 of the small cell non-keratinizing type, and 10 of the keratinizing type (Table 1). Informed consent was obtained from patients and their families after explanation of disease status, treatment methods and tumor biopsy measurements by the treating physicians.

**Patients treated with a photon beam**

A total of 50 consecutive patients with stage IIIB and IVA squamous cell carcinoma of the cervix who received photon beam therapy alone at the NIRS between 1988 and 1993 were employed as controls. Ages ranged from 37 to 81 years, with a mean ± SD and median age of 64 ± 12 and 65.5 years, respectively. A total of 47 and 3 patients had stage IIIB and IVA carcinoma, respectively. A total of 30 patients had squamous cell carcinoma of the large cell non-keratinizing type, 9 of the small cell non-keratinizing type, and 11 of the keratinizing type (Table 1). After explanation of the study procedure, informed consent was obtained from all 50 patients.

Clinical staging and histological classification were based on the criteria of the International Federation of Gynecology and Obstetrics (London) 8) and the World Health Organization (Geneva). 9) Study approval was obtained by the NIRS institutional review board.

**Specimens**

Tumor biopsies were performed when possible before treatment and on the 5th and 15th day after therapy initiation (described as the "5th day" and "15th day", respectively). For carbon ion beam therapy, 27, 22 and 12 specimens from patients were obtained for immunostaining before therapy and on the 5th and 15th day, respectively. Biopsy was declined by 5 patients on the 5th day and 15 patients on the 15th day due to a deterioration in physical condition caused by the acute adverse effects of radiation therapy. For photon beam therapy, 50, 50 and 32 specimens were obtained before therapy and on the 5th and 15th day, respectively. Biopsy was declined by 18 patients on the 15th day for the reasons described above. All cancer specimens excised from cervical tumors were fixed in 10% formaldehyde for approximately 24 hours and embedded in paraffin.

**Carbon ion beam therapy**

Carbon ion beam energy ranged from 350 MeV to 400 MeV, with 24 treatment fractions given over six weeks at four fractions per week. To cover cervical tumor and pelvic lymph node chains, anteroposterior and posteroanterior ports were used for 16 fractions over four weeks. For the boost dose, an additional eight fractions over two weeks were given by lateral opposing ports. Because this phase I and II study was carried out in a dose-escalating fashion, treatment

**Table 1.** Patient characteristics

<table>
<thead>
<tr>
<th></th>
<th>Carbon ion beam</th>
<th>Photon beam</th>
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</thead>
<tbody>
<tr>
<td>The number of patients</td>
<td>27</td>
<td>50</td>
</tr>
<tr>
<td>Stage IIIB / IVA</td>
<td>19 / 8</td>
<td>47 / 3</td>
</tr>
<tr>
<td>Age (mean / median)</td>
<td>56 / 54</td>
<td>64 / 65.5</td>
</tr>
<tr>
<td>Histological subtype</td>
<td>10 / 14 / 3</td>
<td>11 / 30 / 9</td>
</tr>
</tbody>
</table>

Abbreviations: K: keratinizing type, LCNK: large cell non-keratinizing type, SCNK: small cell non-keratinizing type.
was initiated with a fractionated dose of 2.2 GyE and increased by 0.2 GyE per step to 2.4 GyE, 2.6 GyE, 2.8 GyE, and 3.0 GyE. The irradiated dose during the first and third week therefore ranged from 8.8 to 12 GyE and 26.4 to 36 GyE, respectively.17

Photon beam therapy
Control patients were treated with a combination of external and high dose rate intracavitary irradiation, the details of which have been previously reported.10 Patients received external whole pelvic irradiation at 1.8 Gy per fraction five times per week for a total dose of 30.6 Gy in 17 fractions during 3.5 weeks. This implies that when biopsy was carried out on the 15th day, intracavitary irradiation had not yet been performed. The irradiated dose during the first and third week was therefore 9.0 Gy and 27.0 Gy, respectively. Then, central shielding pelvic field at a dose of 2 Gy per fraction five times per week was performed for a total dose of 20 Gy in 10 fractions. In addition to central shielding irradiation, patients received intracavitary irradiation using a remote afterloading system with either a 60Co or 192Ir source. Patients were subjected to a single weekly session for four weeks with a fraction dose of 5.5–6.0 Gy at Point A for a total dose ranging from 22 to 24 Gy.

Histological examination
Morphological characteristics were investigated using sectioned specimens by conventional hematoxylin and eosin staining. Histological changes induced by radiation were classified by grade using the Oboshi-Shimosato classification system.11 Classification criteria are shown in Table 2.

Immunohistochemical analysis
Tissue sections were cut and placed on silane-coated micro slides, then deparaffinized and dehydrated. To unmask antigens, slides were heated at more than 95°C for 20 minutes in 0.01 M citrate buffer, pH 6, using a Microwave Processor (H2800; Energy Beam Sciences Inc., Agawam, MA, USA). Following unmasking, sections were allowed to cool at room temperature for 1 hour. Endogenous peroxidases were blocked with 3% hydrogen peroxide for 10 minutes and sections were washed three times with phosphate-buffered saline (PBS; Gibco, Invitrogen Corporation, Scotland, U.K.), followed by overnight incubation at 4°C with either anti-Ki-67 (MIB-1; Immunotech International, Marseilles, France), anti-p53 (DO7, 1:100; DAKO, Glostrup, Denmark), or anti-p27 monoclonal antibodies (4D10, 1:40; Novocastra, Newcastle, U.K.). Sections were then incubated for 30 minutes with labeled-polymer-conjugated second antibody (Envision kit; DAKO, Carpinteria, CA, USA). After incubation, sections were washed three times with PBS and development with 3,3’-diaminobenzidine tetrahydrochloride (DAKO, Carpinteria, CA, USA) for 2 to 5 minutes at room temperature and hematoxylin counterstaining. Controls were obtained by incubating sections with PBS instead of antiserum. Cells were considered positive for Ki-67, p53, and p27 staining when cancerous and exhibiting nuclear staining for Ki-67, p53, and p27 proteins, respectively (Fig. 1a–c). Ki-67, p53, and p27 LI were calculated as percentages (± standard error) of Ki-67-, p53-, and p27-positive cancer cells by counting more than 1000 cancer cells in three specimen fields at a × 200 microscopic magnification.

Statistical analysis
Differences in mean values of Ki-67, p53, or p27 LIs before radiation therapy and after the first and third week of radiation therapy were determined using Student’s t-test. Differences in histological examination were determined using the Mann-Whitney U test. Values were considered significant when p < 0.05.

RESULTS

Histological change
Figure 2 shows the histological changes in tumor tissues treated with carbon and conventional photon beam therapies.
according to the Oboshi–Shimosato classification system. On the 5th day, histological changes with carbon beam therapy were apparent, whereas those with photon therapy were minimal. Grade 0, 1, 2a and 2b histological effects from carbon beam therapy were observed in 0, 9, 10 and 2 specimens, respectively, whereas those from photon beam therapy were observed in 4, 27, 18 and 1 specimens, respectively. Although no significant difference was observed between therapies (p = 0.07), histological effects appeared stronger in carbon than photon beam therapy. On the 15th day, grade 1, 2a, 2b and 3 histological effects from carbon beam therapy were observed in 0, 3, 7 and 2 specimens, respectively, whereas those from photon beam therapy were observed in 1, 13, 18 and 0 specimens, respectively. Although without statistical significance (p = 0.10), histological effects were noticeably stronger in carbon than photon beam therapy.

**Immunohistochemical changes**

Results showed that cancer cells positive for Ki-67, p53, and p27 proteins demonstrated intranuclear reactivity.

Figure 3 shows the Ki-67 LI changes during both carbon and photon beam therapies. Under carbon beam therapy, Ki-67 LI levels significantly increased from the initial 30.6 ± 3.1% to 58.7 ± 3.2% (p < 0.001) on the 5th day but decreased to 33.7 ± 7.0% on the 15th day (p = 0.001), whereas under photon therapy, levels increased from 36.8 ± 2.1% to 54.7 ± 2.1% on the 5th day (p < 0.001) but decreased to 26.8 ± 3.4% on the 15th day (p < 0.001).

Figure 4 shows the p53 LI changes during both treatments. Under carbon beam therapy, p53 LI levels slightly increased from 8.6 ± 2.2% to 12.9 ± 2.6% on the 5th day (p = 0.20) and continued to increase to 29.6 ± 5.4% on the 15th day (p = 0.004), which were significantly higher than before treatment (p = 0.013). Under photon beam therapy, p53 LI levels increased from 11.2 ± 2.0% to 15.7 ± 2.8% on the 5th day.
Fig. 3. Ki-67-labeling indices during carbon ion and photon beam therapy. The indices of carbon and photon beam therapies show similar patterns. Error bars indicate standard error.

Fig. 4. p53-labeling indices during carbon ion and photon beam therapy. The indices of carbon and photon beam therapies show similar patterns. Error bars indicate standard error.

day (p < 0.01) and continued to increase to 22.9 ± 3.8% on the 15th day (p = 0.01).

Figure 5 shows the p27 LI changes during both treatments. Unlike changes in the p53 index, under carbon beam therapy p27 LI levels decreased from 25.2 ± 4.0% to 18.0 ± 3.4% on the 5th day (p = 0.19) but increased to 36.1 ± 5.9% on the 15th day (p = 0.008), whereas under photon beam therapy, levels decreased from 19.9 ± 2.5% to 18.1 ± 2.4% on the 5th day (p = 0.56) and continued to decrease to 13.7 ± 3.3% on the 15th day (p = 0.31), without statistical significance [please check]. These results show that p27 LI levels on the 15th day were significantly higher with carbon than photon beam therapy (p = 0.001).

Fig. 5. p27-labeling indices during carbon ion and photon beam therapy. In contrast to carbon ion beam therapy, the p27-LI from photon beam therapy continued to decrease on the 5th day after treatment initiation. The p27-LI level on the 15th day of carbon beam therapy was significantly higher than that of photon beam therapy (p = 0.001). Error bars indicate standard error.

DISCUSSION

The present study is the first trial to use heavy charged particle therapy for cervical cancer. Results showed more satisfactory short-term survival and local control rates for this considerably advanced stage of disease than our previously reported rates of response. 1) In the previous clinical study, despite the usual absence of observed macroscopic tumor degeneration during conventional photon beam therapy, we observed the beginning of gross cervical tumor degeneration in the first week of carbon beam therapy. The present histological and molecular pathological study therefore sought to confirm the earlier radiation response to high LET beam therapy than to conventional photon beam therapy, as well as to determine any biological differences in radiation effects at the molecular level.

Results showed no significant difference in Ki-67 LI changes between carbon and photon beam therapies. The increase in Ki-67 growth fraction within one week of irradiation is attributed to the irradiation-induced recruitment of quiescent tumor cells. 2) The recruitment of G0 cells into the cell cycle may therefore occur in similar proportions during both treatments. Flow cytometry analysis performed on human tumors has provided evidence of G2 blockade and radiation-induced cell cycle redistribution, 3) as well as prognostic association between aneuploid cell populations and cell cycle parameters. 4) Zhou et al. reported a more frequent G2 blockade induced by carbon than photon beams, and according to the period of cell survival days, RBE of carbon ion beam to gamma ray of 3.5.

The p53 gene on chromosome 17p is a tumor-suppressor gene which controls entry into the S phase of the cell

cycle.\(^5\) Mutation of this gene inactivates its tumor suppressor activity and is associated with tumor progression and behavior.\(^5\) Radiation increases p53 nuclear expression, followed by G1 arrest and subsequent apoptosis.\(^6\) The present study showed a significant increase in p53 LI levels on the 15th day under both carbon and photon beam therapies (p = 0.004). Compared to cells before radiation therapy, degenerated or swollen cancer cells on the 15th day of therapy were strongly positive for p53. We have previously reported the significant increase in mean p53 LI in tumors during radiation therapy,\(^7\) which implies that elevated p53 functions normally and repairs radiation-induced DNA damage. In the present study, the slightly greater increase in p53 index observed on the third week of carbon beam therapy suggests that carbon beams possibly cause greater DNA damage than photon beams. However, radiation therapy may also possibly directly affect p53-DNA and induce a more stable mutant p53 possessing a longer half-life, leading to a more intense immunohistochemical staining reaction.\(^7\)

The protein product from the p27/Kip1 gene on chromosome 12p associates with cyclin-Cdk complexes, thereby inhibiting their activities and consequently the G1 to S cell cycle transition.\(^5\) Many studies have reported the relationship between elevated expression of the putative p27 tumor suppressor gene and accurate prognosis of malignant tumors arising from systemic organs.\(^18-21\) The present study demonstrated that for carbon beam therapy, p27 LI levels decreased from the initial 25.2% to 18.6% on the 5th day but increased to 36.1% on the 15th day of treatment. In contrast, for photon therapy, the index similarly decreased from 19.9% to 18.1% on the 5th day but continued to decrease to 13.7% on the 15th day. In addition, these p27 protein-negative tumor cells were composed of degenerated or swollen cancer cells. Given findings by Sherr that p27\(^{kip}\) levels are elevated in quiescent cells,\(^20\) p27 LI changes induced by carbon beam therapy may reflect the accumulation of tumor cells in the G0 phase of the cell cycle, and may also correlate with cell senescence. The present study showed that the changes in p27 LI under carbon beam therapy were completely divergent from those in Ki-67 LI. Further, the p27 LI decrease at an early phase of carbon beam therapy may indicate tumor cell recruitment. A better assessment of the importance of changes in p27 expression, however, requires further study in a greater number of patients. In addition, at present the mechanism involved in the difference in p27 LI between carbon and photon beam therapies is not clearly understood.

Here, our results show that carbon beam therapy induces earlier macroscopic and microscopic tumor degeneration than photon beam therapy though there was no statistical significance. Masunaga et al. reported that the RBE value of 74 keV/\(\mu m\) carbon ion beam to gamma ray in quiescent tumor cells 12 hours after irradiation was 4.5.\(^22\) The RBE value of carbon ion beam to gamma ray may be larger than 3.0 because human cervical squamous cell carcinomas usually have a large fraction of quiescent cells. However, because histological changes are not only dependent on the response of tumor cells but also to that of normal surrounding tissue and other biological functions, such as blood flow and permeability of blood vessels, linking histological changes with molecular pathological markers is complex.

This study showed that no clear difference was observed between carbon and photon beam therapy based on Ki-67 LI and p53 LI, except for p27 LI. However, Higo et al. reported that the mRNA expression levels of SPHK1 were significantly upregulated by carbon beam in oral squamous cell carcinoma cell line.\(^23\) Further investigation is required to clarify the various forms of radiobiological behaviors, including the p27 LI changes reported here, between carbon and photon beam therapy.

REFERENCES


Received on October 19, 2007
Revision received on February 28, 2008
Accepted on April 1, 2008
J-STAGE Advance Publication Date: July 11, 2008