Table 1. Patient characteristics

Gender	
Male	13
Female	14
Age (y)	
Mean	46.2
Range	17–78
Karnofsky index (%)	
Median	90
Range	80–90
Site	
Nasal and paranasal	11
Maxillary bone	8
Mandibular bone	2
Skull base	2
Parapharyngeal space	1
Temporal	1
Frontal bone	1
Parotid gland	1
Histology	
Osteosarcoma	9
Malignant fibrous Histiocytoma	5
Hemangioperisarcoma	3
Myxoid fibrous sarcoma	2
Leiomyosarcoma	2
Chondrosarcoma	2
PNET	1
Fibrosarcoma	1
Small round cell sarcoma	1
Spindle cell sarcoma	1
Gross tumor volume	
≥100 mL	14
<100 mL	13
Histopathological grade (UICC-2002)	
1–2 (low grade)	16
3–4 (high grade)	10
Unknown	1

Abbreviations: PNET, primitive neuroectodermal tumor; UICC, Union for International Cancer Control.

European Organization for Research and Treatment of Cancer scoring system or Late Effects in Normal Tissues Subjective, Objective, Management, and Analytic scales scoring system.

The secondary end point was to compare the efficacy with previous results obtained with 57.6 or 64.0 GyE for 14 patients with head and neck sarcoma. The mean age of the 14 patients was 34.4 years. The histologic breakdown was six osteosarcomas, three rhabdomyosarcomas, two liposarcomas, one malignant fibrous histiocytoma, one fibrosarcoma, and one chondrosarcoma.

Statistics

Survival estimates were calculated using the Kaplan-Meier method, and the statistical differences were evaluated by the log-rank test. Statistical significance was defined as a value of $p < 0.05$. All analyses were performed using the SPSS 11.0 software package (SPSS Inc., Chicago, IL).

Table 2. Acute reactions of normal tissues (National Cancer Institute-Common Toxicity Criteria, version 2.0)

	Grade 0	Grade 1	Grade 2	Grade 3	≥Grade 4
Skin	2	19	6	0	0
Mucous membrane	1	8	17	1	0

Table 3. Late reactions of normal tissues (Radiation Therapy Oncology Group/European Organization for Research and Treatment of Cancer or Late Effects in Normal Tissues Subjective, Objective, Management, and Analytic scales)

	Grade 0	Grade 1	Grade 2	Grade 3	≥Grade 4
Skin	20	6	0	0	0
Mucous membrane	17	9	0	0	0
Brain	20	5	1	0	0
Eye	24	0	1	0	1
Bone	20	1	1	4	0

RESULTS

The characteristics of the 27 patients recruited for this prospective study are summarized in Table 1. No patients were lost for follow-up. The median observation period was 37.0 months (range, 4.1-73.0 months) for all patients and 40.0 months (range, 23.2-73.0 months) for the 18 surviving patients.

Reactions of normal tissues

Regarding acute reactions, a Grade 3 mucosal reaction was observed in 1 patient. No Grade 4 mucosal acute reaction and Grade 3 or higher skin acute reaction was observed in any of the patients (Table 2).

Late reactions of normal tissues were evaluated in 26 patients because 1 patient died 4 months after C-ion RT from multiple lung metastases. Visual loss was observed in one eye of 1 patient whose optic nerve was entirely involved by the tumor. Severe pain in the maxillary bone (Grade 3) from sequestrum formation was observed in 4 patients. Grade 1 late reactions were most frequently observed for the skin, mucosal membrane, and brain (Table 3).

Survival and local control

The 3-year and 5-year local control rates were 91.8% (95% confidence interval [CI] = 81.0–100%) and 80.4%

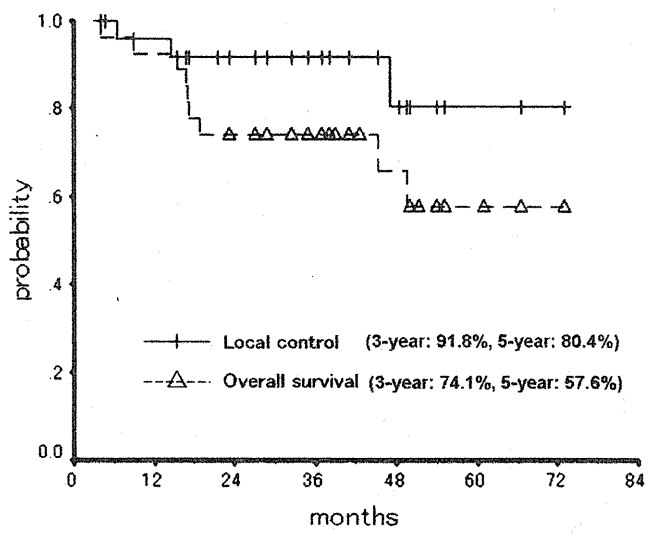

Fig. 2. Local control rate and overall survival rate for all patients enrolled in the present study ($n = 27$).

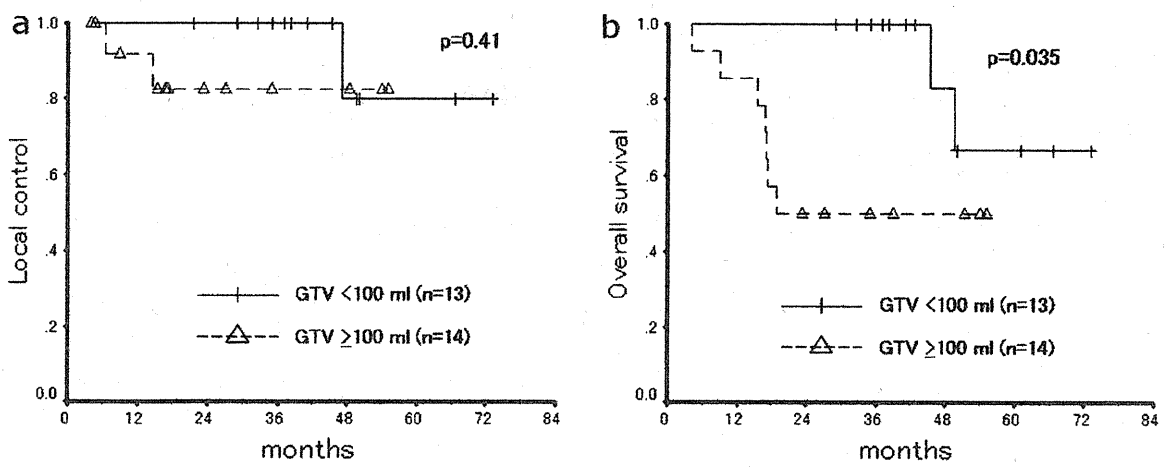

Fig. 3. Local control rate (a) and overall survival rate (b) with 70.4 GyE according to tumor volume.

(95% CI = 57.3–100%), respectively. Three patients had local recurrences. The 3- and 5-year overall survival rates were 74.1% (95% CI = 57.5–90.6%) and 57.6% (95% CI = 33.7–81.4%), respectively (Figure 2).

Although there was no significant difference in the local control rate between the patients with large GTV (≥100 mL, n = 14) and those with small GTV (<100 mL, n = 13), the overall survival rate for patients with large GTV (3-year: 50.0% [95% CI = 23.8–76.2%], 5-year: 50.0% [95% CI = 23.8–76.2%]) was significantly lower than that for patients with small GTV (3-year: 100%, 5-year: 66.7% [95% CI = 28.9–100%]; p = 0.035, Fig. 3).

There were no significant differences in local control and overall survival between patients with low-grade sarcomas (3-year local control: 86.7% [95% CI = 69.4–100%], 3-year overall survival: 75.0% [95% CI = 53.8–96.2]) and those with high-grade sarcomas (3-year local control: 100%, 3-year overall survival: 80.0% [95% CI = 55.2–100%]; p = 0.22 and p = 0.63, respectively).

The 3-year local control rate and overall survival rate for patients treated with 57.6 or 64.0 GyE/16 fr (n = 14, median observation period = 18.7 months) in the previous study were 23.6% (95% CI = 0.3–46.8%) and 42.9% (95% CI = 16.9–68.8%), respectively. The local control rate in the present study with 70.4 GyE was significantly higher than that with 57.6 or 64.0 GyE (3-year, 91.8% vs. 23.6%, $p < 0.0001$; Fig. 4a). Furthermore, the overall survival rate with 70.4 GyE tended to be higher than that with 57.6 or 64.0 GyE (3-year, 74.1% vs. 42.9%, p = 0.09; Fig. 4b).

The 3-year local control rate and overall survival rate for patients with osteosarcoma in the present study (n = 9, median observation period = 18.8 months) were 85.7% (95% CI = 59.8–100%) and 44.4% (95% CI = 12.0–76.9%), respectively. The local control rate for patients with osteosarcoma treated with 70.4 GyE was significantly higher than that for patients treated with 57.6 or 64.0 GyE (n = 6, median observation period = 13.0 months; 3-year, 85.7% vs. 0%, p = 0.0039; Fig. 5a). The overall survival rate with 70.4 GyE tended to be higher than that in our previous study with 57.6 or 64.0 GyE (3-year, 44.4% vs. 16.7%, p = 0.142; Fig. 5b).

DISCUSSION

The purpose of the present prospective study was to evaluate the safety of C-ion RT with 70.4 GyE/16 fr. In a previous dose-escalation study on C-ion RT of head-and-neck tumors including sarcoma (19), Grade 3 acute reactions of normal

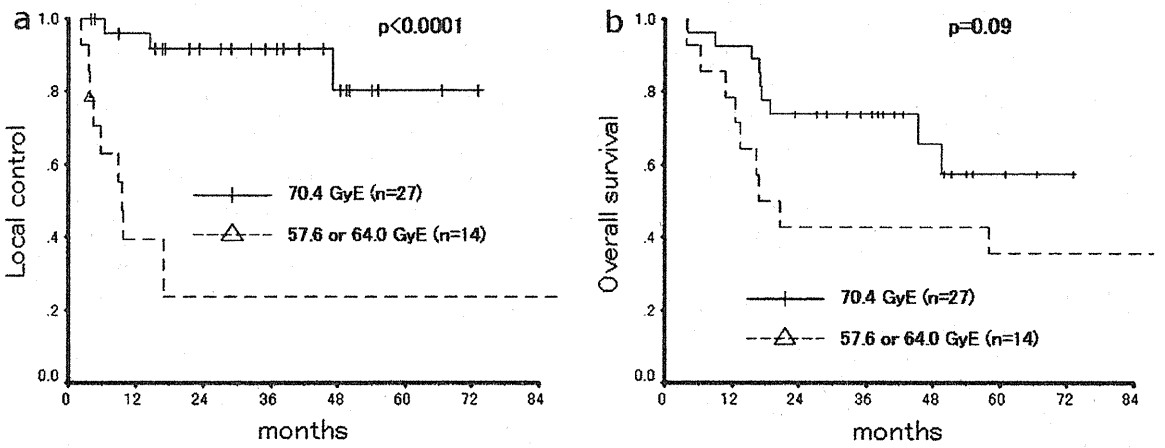

Fig. 4. Local control rate (a) and overall survival rate (b) according to prescribed dose.

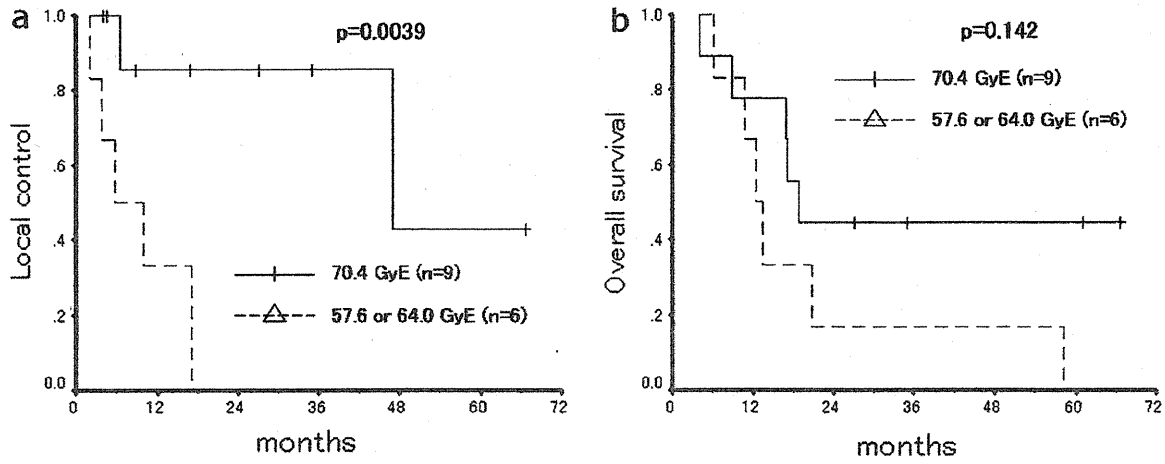

Fig. 5. Local control rate (a) and overall survival rate (b) in patients with osteosarcoma according to prescribed dose.

tissues with 64.0 GyE/16 fr occurred in 67% of the patients. In the present study, the incidence of Grade 3 acute reactions was lower despite delivery of a higher dose. This decrease in severe toxicity was most likely due to the fact that, compared with the previous study, a multifield irradiation technique was more frequently used, resulting in improved conformity of the dose distribution and lowering of facial skin doses.

However, late reactions of normal tissues of Grade 3 or higher were observed in 23.1% of the patients in the present study. This is high in comparison to rates in other reports of 65 GyE high-linear energy transfer particle radiation therapies for head-and-neck cancers. In the present study, severe maxillary pain occurred in 4 patients, in whom the maxillary bone had been invaded or surrounded by the tumor. The patients complained of pain in bone that had been irradiated with more than 70% of the prescribed dose in C-ion RT. All of the patients with severe maxillary pain underwent sequestrectomy, resulting in resolution of severe pain and considerable recovery of quality of life. Under similar circumstances, 1 patient lost his right vision 10 months after C-ion RT. In this patient, almost the entire length of the right optic nerve was irradiated with the full irradiation dose (70.4 GyE) because the optic nerve was largely surrounded by the tumor. Therefore, such severe late reactions of normal tissues were considered unavoidable in these patients. Other major late complications were less common, possibly because exposure of normal tissues was reduced with the use of accurate imaging/positioning methods and a high-tech irradiation system. Guadagnolo *et al.* reported that 6 of their 27 patients who received postoperative conventional radiation therapy with 50–66 Gy for osteosarcoma of the head and neck had severe/moderate complications (20). The present results suggest that C-ion RT (even with 70.4 GyE) would not cause severe reactions of normal tissues with acceptable toxicity in comparison to conventional radiation therapy.

It has been reported that local control rates in patients with gross sarcomas of the head and neck treated by surgical resection with or without radiotherapy and chemotherapy are less than 70% (12, 21–24) (Table 4). The present study showed that C-ion RT with 70.4 GyE/16 fr was tolerable and had a substantial antitumor effect against bone and soft-tissue sarcomas of the adult head and neck. All of the patients in this study had advanced gross lesions in the head and neck that were unsuitable for curative surgical resection. The 3-year local control rate was higher than 90% despite the advanced stage of the sarcomas. The local control rate was better than that in past studies using all types of radiation therapy, including other particle beams (21–25) (Table 5). The local control rate with 70.4 GyE was also

Table 4. Comparisons of overall survival and local control of sarcomas of the adult head and neck

Institution (year)	Histology	Treatment	*n*	MOP (mo)	5-year LC (%)	5-year OS (%)
MSCMCC (12) (1970–2001)	Soft-tissue sarcoma	Surgery ± X-ray ± chemo	112	139	45	35
RMH (21) (1944–1988)	Soft-tissue sarcoma	Surgery ± X-ray ± chemo	103	50	47	50
MGH (22) (1972–1993)	Soft-tissue sarcoma	Surgery ± X-ray ± chemo	46	50	69	74
UCSF (23) (1961–1993)	Soft-tissue sarcoma	Surgery ± X-ray ± Chemo	65	64	66	56
NCI (24) (1985–1996)	Osteosarcoma	Surgery ± X-ray ± chemo	496	—	—	59.7
NIRS (current study) (2001–2008)	Bone and soft-tissue sarcoma	Carbon ion RT	27	37.0	80.4	57.6

Abbreviations: LC = 5-year local control rate; MOP = median observation period; MSCMCC = M. Sklodowska-Curie Memorial Cancer Center; NCI = national cancer institute; NIRS = National Institute of Radiological Sciences; OS = 5-year overall survival; RMH = Royal Marsden Hospital; UCSF = university of california san francisco.

Table 5. Comparisons of overall survival and local control of unresectable sarcomas of the adult head and neck

Institution (year)	Histology	Treatment	*n*	MOP (months)	5-year LC (%)	5-year OS (%)
RMH (21) (1944–1988)	Soft-tissue sarcoma	X-ray ± chemo	17	50	21	36
MGH (22) (1972–1993)	Soft-tissue sarcoma	X-ray ± chemo	14	50	55	63
UCSF (23) (1961–1993)	soft tissue sarcoma	X-ray ± chemo	5	64	0	9
NCI (24) (1985–1996)	osteosarcoma	X-ray ± chemo	71	—	—	21.7
LBL (25) (1977–1992)	bone and soft tissue sarcoma	Helium ion radiotherapy	19	51	58	71
NIRS (current study) (2001–2008)	bone and soft tissue sarcoma	Carbon ion radiotherapy	27	37.0	80.4	57.6

Abbreviations: LC = 5-year local control rate; MOP = median observation period; MGH = Massachusetts General Hospital; NCI = national cancer institute; NIRS = National Institute of Radiological Sciences; OS = 5-year overall survival; RMH = Royal Marsden Hospital; UCSF = university of california san francisco; LBL, Lawrence Berkeley Laboratory

significantly better than that in our previous study with 57.6 or 64.0 GyE. These results indicate that C-ion RT with 70.4 GyE has a significant benefit for patients with sarcoma of the head and neck. An increase in dose by only 10–20% significantly improved local control of sarcomas of the head and neck. This is consistent with results reported by Kamada *et al.* for C-ion RT performed in patients with sarcoma in the trunk of the body (26). The results might indicate that there is a rapid rise in the tumor control probability (TCP) curve of sarcoma in C-ion RT around 70 GyE. Furthermore, the overall survival with 70.4 GyE tended to be better than that with 57.6 or 64.0 GyE, although the difference was not statistically significant ($p = 0.10$). This was probably affected by distant metastasis after C-ion RT, especially in patients with large gross tumors. However, in cases of sarcoma, it has been suggested that not only distant metastasis but also local control were related to overall survival (20, 27). It remains possible that the good local control in the present study significantly improve overall survival, although more long-term observation is required. Possible reasons for the inconsistency in overall survival rates in the present study and past studies are variations in histology (grade), number of patients and treatment method, especially chemotherapy.

Osteosarcoma was the most common histologic type in the present study. Osteosarcoma is a highly malignant bone tumor but is rare in the craniofacial area. The local control rate in 9 patients with unresectable osteosarcoma in the present study was significantly better than that in 6 patients treated with 57.6 or 64.0 GyE/16 fr in our previous study. The 3-year overall survival rate was 44.4%, whereas reported 3-year survival rates for patients with unresectable osteosarcoma of the head and neck are 30% or less (24, 28). The overall survival rate in the present study was also superior to that in past studies using conventional therapy and superior to the results of our previous study with 57.6 or 64.0 GyE/16 fr.

Several studies have demonstrated that it might be necessary to use adjuvant chemotherapy for bone and soft-tissue sarcomas to suppress distant metastasis and to extend the survival of patients. Randomized clinical trials have shown that neoadjuvant or adjuvant chemotherapy is effective for preventing relapse in patients with non-metastatic osteosarcoma in the extremities (29–31). Although a large meta-analysis showed no benefit of chemotherapy for patients with osteosarcoma of the head and neck, it demonstrated that incorporation of chemotherapy into the treatment regimen for patients with high-grade tumors may improve survival (10). In addition, a retrospective analysis showed a trend of better survival in patients with high-grade osteosarcoma of the mandible and maxilla who received adjuvant chemotherapy (24). The usefulness of chemotherapy in patients with osteosarcoma of the head and neck is still a matter of debate. Several studies have demonstrated that adjuvant chemotherapy could improve prognosis for patients with soft-tissue sarcoma. However, the necessity of systematic chemotherapy is also still controversial. Although chemotherapy was not performed in the present study, it is possible that chemotherapy with C-ion RT can further improve the prognosis of sarcomas of the head and neck.

In conclusion, C-ion RT with 70.4 GyE/16 fr for bone and soft-tissue sarcoma of the adult head and neck appears to be effective with acceptable toxicities in comparison to conventional RT and C-ion RT with lower doses.

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Clinical Investigation

Experience with Carbon Ion Radiotherapy for WHO Grade 2 Diffuse Astrocytomas

Azusa Hasegawa, D.D.S., Ph.D.,* Jun-etsu Mizoe, M.D., Ph.D.,* Hirohiko Tsujii, M.D., Ph.D.,* Tadashi Kamada, M.D., Ph.D.,* Keiichi Jingu, M.D., Ph.D.,* Yasuo Iwadate, M.D., Ph.D.,† Youichi Nakazato, M.D., Ph.D.,‡ Masao Matsutani, M.D., Ph.D.,§ and Kintomo Takakura, M.D., Ph.D.¶, for the Organizing Committee of the Central Nervous System Tumor Working Group

**Research Center for Charged Particle Therapy Hospital, National Institute of Radiological Sciences, Chiba, Japan; †Department of Neurological Surgery, Chiba University Graduate School of Medicine, Chiba, Japan; ‡Department of Human Pathology, Gunma University Graduate School of Medicine, Gunma, Japan; §Department of Neurological Surgery, Saitama Medical University, Saitama, Japan; and ¶Department of Neurological Surgery, Tokyo Women's Medical University, Tokyo, Japan*

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Summary

Between October 1994 and February 2002, 14 patients with diffuse astrocytoma were enrolled in a phase I/II clinical trial of carbon ion radiotherapy. Carbon ion radiotherapy was administered in 24 fractions over 6 weeks. The carbon ion dose was escalated from 50.4 Gy equivalent (GyE) to 55.2 GyE. Patients were divided into two groups according to their carbon ion doses: a low-dose group in 9 patients and a high-dose group in 5 patients. High-dose group showed significant improvement in PFS

Purpose: To assess outcomes of carbon ion radiotherapy for diffuse astrocytomas in adults.
Methods and Materials: Between October 1994 and February 2002, 14 patients with diffuse astrocytoma, identified as eligible for carbon ion radiotherapy, were enrolled in a phase I/II clinical trial. Carbon ion radiotherapy was administered in 24 fractions over 6 weeks. The normal tissue morbidity was monitored carefully, and the carbon ion dose was escalated from 50.4 Gy equivalent (GyE) to 55.2 GyE. Patients were divided into two groups according to their carbon ion doses: a low-dose group in which 2 patients were irradiated with 46.2 GyE and 7 patients were irradiated with 50.4 GyE, and a high-dose group in which 5 patients were irradiated with 55.2 GyE.
Results: Toxicities were within acceptable limits, and none of the patients developed Grade 3 or higher acute or late reactions. The median progression-free survival (PFS) time was 18 months for the low-dose group and 91 months for the high-dose group ($p = 0.0030$). The median overall survival (OS) time was 28 months for the low-dose group and not reached for the high-dose group ($p = 0.0208$).
Conclusion: High-dose group patients showed significant improvement in PFS and OS rates compared to those in the low-dose group, and both dose groups showed acceptable toxicity.

Keywords: Carbon ion radiotherapy, Diffuse astrocytomas, Dose escalation study, High-LET radiotherapy, Phase I/II clinical trial

Reprint requests to: Jun-etsu Mizoe, M.D., Ph.D., Research Center for Charged Particle Therapy, NIRS, Anagawa 4-9-1, Inage-ku, 263-8555 Chiba-city, Japan. Tel: 81-43-206-3360; Fax: 81-43-256-6506; E-mail: junetsumizoe@gmail.com

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and OS rates compared to those in the low-dose group. Toxicities were within acceptable limits. None of the patients developed a Grade 1 or higher acute brain reaction (RTOG), and a Grade 3 or higher late brain reaction (RTOG/ EORTC).

Introduction

Diffuse astrocytomas, classified as World Health Organization Grade 2, are generally slow-growing brain tumors. Although surgery is the treatment of choice, radical resection of the tumor is very difficult in terms of the preservation of brain function, even if patients have long survival times. However, after partial or subtotal resection, the long-term prognosis is often poor because of malignant transformation. Therefore, adjuvant therapy is administered to prevent malignant transformation. Generally, tumor cells that remain after surgery are treated with photon radiotherapy; however, the therapy must incur minimal damage to the normal brain tissue and other adjacent normal structures, such as the optic nerve, eyeball, brainstem, and others. The close proximity of normal tissue is therefore a limiting factor for photon radiotherapy, often making it impossible to deliver an adequate radiation dose to the tumor site.

In 1993, the Heavy Ion Medical Accelerator in Chiba (HIMAC) was constructed at the National Institute of Radiological Sciences (NIRS) as a part of a comprehensive 10-year strategy for cancer control in Japan (1). The HIMAC produces a high-linear energy transfer (LET) charged-particle beam of carbon ions with sufficient intensity and has been used in human trials since June 1994. High-LET charged particle therapy, such as that with fast neutrons and heavy ions, has greater biological effectiveness than low-LET radiotherapy, such as that with photons and protons. In addition, charged particles, such as carbon ions, have excellent dose-localizing properties compared with fast neutrons and photons. Furthermore, a charged particle beam's maximum depth of range can be adjusted by varying the energy. In carbon ion radiotherapy, the treatment beam lines are equipped with a pair of wobbler magnets, beam scatterers, ridge filters, multileaf collimators, and a compensation bolus to make the treatment volume conform to the target volume (2, 3). The resulting isodose distribution can be adjusted for the target volume with a high dose of irradiation to the tumor and a minimized dose of irradiation to the surrounding normal tissues. In this context, carbon ion radiotherapy is a promising option for the effective treatment of intractable brain tumors, owing to its specific properties of high relative biological effectiveness (RBE) and favorable dose distribution of charged particles.

From October 1994 to February 2002, phase I/II dose escalation studies of carbon ion radiotherapy were performed with patients with brain tumors including diffuse astrocytomas, malignant gliomas (4), and metastatic brain tumors. The purpose of this study was to estimate the outcomes of 14 diffuse astrocytomas in adults.

Methods and Materials

Patients and study design

Eligibility criteria for this clinical trial required histologically proven astrocytoma, ages between 18 and 80 years old, a Karnofsky performance status (KPS) of 60% or more, neurological function status (NFS) of Grade I or II, absence of anticancer chemotherapy within the previous 2 weeks, survival expectancy of 6 months or more, and absence of meningeal dissemination. Only patients with diffuse astrocytoma were included. Twopathologists, one from our institution and one from each of the hospitals where the biopsy orsurgery was performed, confirmed histology. All patients provided written informed consent before enrollment. The NIRS Ethical Committee on Human Clinical Research approved this study in 1993.

Acute reactions were classified according to the Radiation Therapy Oncology Group (RTOG) scoring system; maximum reactions were observed within 3 months after carbon ion radiotherapy. Late reactions were classified according to the RTOG/European Organization for Research and Treatment of Cancer (EORTC) scoring system. Magnetic resonance imaging (MRI) findings were graded according to an analytical category based on MRI data for the brain in the Late Effects Normal Tissues scoring system: Subjective, Objective, Management, and Analytic (LENT-SOMA) tables (5). All patients were followed with multiple MRIs performed every 2 months in the first year, every 3 months in the second year, and every 6 months after 2 years.

Carbon ion radiotherapy

Patients were positioned in customized cradles (Moldcare; Alcare, Tokyo, Japan) and immobilized with a low-temperature thermoplastic device (Shellfitter; Kuraray Co., Ltd., Osaka, Japan). A set of computed tomography (CT) images obtained at intervals of 3 or 5 mm was acquired for treatment planning. Three-dimensional treatment planning was performed with Heavy Ion Plan software (M. Endo, National Institute of Radiological Sciences, Chiba, Japan) (6). The gross tumor volume was determined with MRI that included the high-signal area of the T_2-weighted images, corresponding to a minimum 5-mm margin as the clinical target volume (CTV). The CTV and normal structures were delineated on the CT images to permit dose-volume histogram analysis. A margin of 3 to 6 mm was usually added to the CTV to obtain the final planning target volume. The radiation oncologist and the neuroradiologist decided the gross tumor volume that was confirmed by MRI.

Table 1 Patient and treatment characteristics and outcome

Patient	Sex	Age (yrs)	KPS (%)	NF	Tumor location	ESR	CTV (ml)	C-ion dose (GyE)	Outcome after C-ion	TTP (mo)	Salvage	MT	Outcome	F/UT (mo)
1	F	48	100	1	Occipital/parietal	PR	62.3	50.4	Recurrence	15.5	OP	AA	Death	56.2
2	M	32	100	1	CC	Bx	56.7	50.4	Recurrence	24.6	OP	GB	Death	28.5
3	M	18	70	2	BG	Bx	20.8	46.2	Recurrence	21.4	Chemo	Unproven	Death	23.1
4	M	41	90	1	Temporal	GTR	51.5	50.4	Recurrence	9.9	OP	AA	Death	16.6
5	F	26	90	1	Frontal	PR	102.9	50.4	Progression	58.0	OP	AA	Alive	152.4
6	M	30	70	2	Frontal/temporal	Lobectomy	208.8	50.4	Recurrence	1.6	None	Unproven	Death	9.5
7	M	22	90	1	Frontal/temporal	Recurrence	135.9	50.4	Recurrence	33.2	None	Unproven	Death	53.4
8	F	40	70	2	Frontal/temporal	PR	333.9	46.2	Recurrence	74.4	RT	Unproven	Death	87.8
9	F	28	90	1	BG	Bx	45.2	50.4	Recurrence	3.9	Chemo	Unproven	Death	13.3
10	M	29	90	1	Frontal	Bx	206.1	55.2	Recurrence	91.4	OP	None	Alive	119.6
11	M	43	90	1	Frontal	PR	150.7	55.2	Recurrence	35.9	OP	GB	Death	40.5
12	M	46	90	1	Occipital/parietal	PR	46.9	55.2	NED	94.5	None	None	Alive	94.5
13	M	33	60	2	Cerebellum	PR	53.9	55.2	NED	91.3	None	None	Alive	91.3
14	F	66	90	1	Frontal	Bx	29.6	55.2	NED	87.9	None	None	Alive	87.9

Abbreviations: KPS = Karnofsky performance status; NF = neurologic function; CC = corpus callosum; BG = basal ganglia; ESR = extent of surgical resection; PR = partial resection; Bx = biopsy only; GTR = gross total resection; CTV = clinical target volume; C-ion = carbon ion; NED = no evidence of disease; TTP = time to progression; MT = malignant transformation; AA = anaplastic astrocytoma; GB = glioblastoma; OP = operation; Chemo = chemotherapy; RT = photon radiotherapy; F/UT = follow-up time (months).

Carbon ion dose was expressed in terms of photon equivalent dose (Gray equivalent dose [GyE]), which was defined as the physical dose multiplied by the RBE of carbon ions. Carbon ions have the biological characteristics of high-LET with 78 KeV/μ at the distal part of the spread of the Bragg peak (SOBP), where the RBE of carbon ions was assumed to be 3.0 (2, 3). The biological flatness of the SOBP was normalized by the survival fraction of human salivary gland tumor cells.

Carbon ion radiotherapy with a total dose of 50.4 GyE was administered initially in 24 fractions over 6 weeks. Normal tissue morbidity was monitored carefully for at least 1 year after carbon ion radiotherapy, and the carbon ion dose was escalated by 10% when there was no Grade ≥3 severe normal tissue morbidity.

Data analysis

Progression-free survival (PFS) rates and overall survival (OS) rates were calculated by the Kaplan-Meier method using StatView software (version 5.0; SAS Institute Inc.Cary, NC). Time to recurrence was measured from the first day of carbon ion radiotherapy. A log-rank test was used to evaluate the prognostic factors in various clinical factors (age, gender, KPS, NFS, tumor site, tumor volume) and therapeutic factors (extent of surgery, prescribed tumor dose). Results of acute and normal tissue reactions were evaluated by a nonparametric Wilcoxon rank-sum test. A p value of less than 0.05 was considered statistically significant.

Results

Patients

Between October 1994 and February 2002, 15 patients were enrolled. One female patient, whose planned dose was 50.4 GyE, experienced a convulsion during carbon ion radiotherapy. MRI findings showed tumor progression, and carbon ion radiotherapy was stopped at 29.9 GyE in 13 fractions. She continued her treatment with photon radiotherapy and concomitant chemotherapy. Because her carbon ion dose was approximately 59% of the scheduled dose (50.4 GyE), she was excluded from the trial according to the protocol's evaluation criteria that the dose must be more than 90% of the scheduled total dose to be eligible for analysis. Consequently, 14 patients were evaluated.

Carbon ion radiotherapy for 2 patients in the 50.4-GyE group was concluded at 46.2 GyE in 22 fractions by the physician's decision to avoid high-dose irradiation to the basal ganglion area for deep-seated and large tumors. These patients were included in the evaluation.

The mean follow-up period was 62 months (range, 10–152 months) for surviving patients. The total dose was escalated from 50.4 GyE to 55.2 GyE. Fourteen patients were divided into two groups: a low-dose group, in which 9 patients were irradiated with a total dose of 46.2 (2 patients) or 50.4 (7 patients) GyE, and a high-dose group, in which 5 patients were irradiated with a total dose of 55.2 GyE. Table 1 shows patient characteristics, treatment specifications, and outcomes. There were 9 males and 5 females, 18 to 66 years of age, with a median age of 32.5 years old. Tumors were located in the frontal lobe in 4 patients; the frontal and temporal lobes in 3 patients; the occipital and parietal lobes in 2 patients; the basal ganglia in 2 patients; the temporal lobe in 1 patient; the corpus callosum in 1 patient; and the cerebellum in 1 patient. Surgical intervention prior to carbon ion radiotherapy consisted of gross total resection for 1 patient, partial resection for 6 patients, and biopsy for 5 patients. The CTV ranged from 20.8 ml to 333.9 ml (median, 59.5 ml). There were two portals for 8 patients, three portals for 4 patients, five portals for 1 patient, and six portals for 1 patient.

Toxicity

Acute skin reactions were minor: an RTOG Grade 2 reaction occurred in only 2 patients (14%), and Grade 1 or lower reactions occurred in the remaining patients. No patient had a Grade 1 or higher acute brain reaction. There were no significant differences

Table 2 Late morbidity by RTOG/EORTC grading

Anatomic site	Patient group	No. of patients with late morbidity grade (%)				p value
		0	1	2	3	
Skin	Low dose (n = 9)	8 (89)	1 (11)	0	0	0.5510
	High dose (n = 5)	5 (100)	0	0	0	
Brain	Low dose (n = 7)*	2 (29)	5 (71)	0	0	0.0650
	High dose (n = 5)	0	3 (60)	2 (40)	0	

* Estimates could not be made for 2 patients because of local recurrence within 3 months after carbon ion radiotherapy.

Table 3 Late brain morbidity by LENT-SOMA grading

Patient group	No. of patients showing maximum reaction (%)				p value
	Grade 0	Grade 1	Grade 2	Grade 3	
Low dose (n = 7)*	2 (29)	1 (14)	2 (29)	2 (29)	0.2404
High dose (n = 5)	0	1 (20)	2 (40)	2 (40)	

* Estimates could not be made for 2 patients because of local recurrence within 3 months after carbon ion radiotherapy.

in acute skin and brain reactions between the low-dose and high-dose groups (p = 0.5998 and p = 1.000, respectively).

Late skin reactions were also minor in nature, and only 1 patient in the low-dose group developed a (RTOG/EORTC) Grade 1 skin reaction. There was no occurrence ofGrade 2 or higher brain reaction in the low-dose group (Table 2). Two (40%) of the 5 patients in the high-dose group developed a (RTOG/EORTC) Grade 2 brain reaction. Although there were no statistically significant differences in late skin and brain reactions between the low-dose and the high-dose groups, the brain reactions in the high-dose group tended to be of a higher grade than those in the low-dose group (p = 0.0650).

Late brain reactions graded by LENT-SOMA tables based on the MRI findings are listed in Table 3. Although 2 of the 7 patients (29%) in the low-dose group and 2 of the 5 patients (40%) in the high-dose group developed a Grade 3 brain reaction, the reactions of all patients regressed to Grade 1 after a period of no treatment or after treatment with a short course of steroids. There was no significant difference in late brain reactions by LENT-SOMA criteria between the low-dose and high-dose groups.

Table 4 Progression-free survival according to clinical and treatment prognostic factors

Characteristic	5-year PFS (%)	p value
Age (years)		
<32.5	25.0	0.4088
>32.5	50.0	
Gender		
Male	33.3	0.8829
Female	40.0	
Neurologic function		
1 (able to work)	30.0	0.9047
2 (able to be at home)	50.0	
KPS (%)		
≧90	30.0	0.9047
<90	50.0	
Extent of surgical resection		
Gross total/partial	37.5	0.8052
Biopsy	33.3	
Clinical target volume (ml)		
>59.5	28.6	0.4155
<59.5	42.9	
Location		
Frontal/temporal	37.5	0.8688
Others	33.3	
C−ion dose		
Low-dose group	11.1	0.0030
High-dose group	80.0	

Abbreviations: PFS = progression free survival; KPS = Karnofsky performance status.

PFS rates

The 5-year PFS rate was 36% (standard error of the mean [SEM] = 13%) for all patients, 11% (SEM = 11%) for the low-dose group, and 80% (SEM = 18%) for the high-dose group. Factor analyses established the fact that only the carbon ion dose was associated with significantly longer PFS (Table 4) and that the difference between the two doses was statistically significant (p = 0.0030) (Fig. 1a). Median PFS times were 33 months for all patients, 18 months for the low-dose group patients, and 91 months for the high-dose group patients. Salvage operation, palliative radiotherapy, and chemotherapy for patients with recurrent tumors.

Eight of the 9 patients in the low-dose group and 2 of the 5 patients in the high-dose group showed local relapse. The other patient in the low-dose group had a marginal recurrence within 5 mm from an edge of the CTV (Table 1, Patient 5). Six of the 11 patients with tumor recurrence were treated by surgical resection. Of these, 5 patients were diagnosed with malignant transformation on the basis of the histological findings (3 patients with anaplastic astrocytoma and 2 patients with glioblastoma). Of these 5 patients, 2 patients were given chemotherapy, and 1 patient was given palliative treatment with gamma-knife radiotherapy; 2 patients did not receive any salvage treatment.

OS rates

The 5-year and 10-year OS rates were 43% (SEM = 13%) and 36% (SEM = 13%), respectively, for all patients; 22% (SEM = 14%)

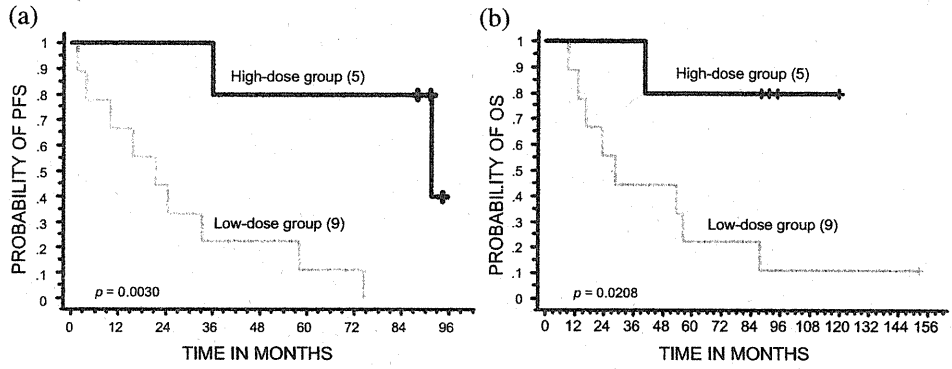

Fig. Kaplan-Meier curves show PFS (a) and OS (b) according to carbon ion dose.

and 11% (SEM = 11%), respectively, for the low-dose group; and 80% (SEM = 18%) and 80% (SEM = 18%), respectively, for the high-dose group. Results of the prognostic factor analyses for OS in the 14 patients are shown in Table 5, and only carbon ion dose showed statistical significance. The difference between the two dose curves was statistically significant ($p = 0.0208$) (Fig. 1b). The median survival timewas 53.4 months for all patients, 8 months for the low-dose group, and was not reached for the high-dose group.

Discussion

Diffuse astrocytomas are generally known as slow-growing tumors that constitute approximately 40% of all glial neoplasms.

Table 5 Overall survival according to clinical and treatment prognostic factors

Characteristic	5-year OS (%)	10-year OS (%)	*p* value
Age (years)			
<32.5	37.5	37.5	0.7523
>32.5	50.0		
Gender			
Male	33.3	33.3	0.5602
Female	60.0	40.0	
Neurologic function			
1 (Able to work)	40.0	40.0	0.6091
2 (Able to be at home)	50.0		
KPS (%)			
≧90	40.0	40.0	0.6091
<90	50.0		
Extent of surgical resection			
Gross total/partial	50.0	37.5	0.7247
Biopsy	33.3	33.3	
Clinical target volume (ml)			
>59.5	42.9	28.6	0.9839
<59.5	42.9		
Location			
Frontal/temporal	50.0	37.5	0.8015
Others	33.3		
Carbon ion dose			
Low-dose group	22.2	11.1	0.0208
High-dose group	80.0	80.0	

Abbreviations: OS = overall survival; KPS = Karnofsky performance status.

These tumors may often undergo malignant transformation (7–9). Watanabe *et al.* (8) indicated that *p*53 gene mutations were detected in 79% of postoperative recurrent low-grade astrocytomas that had progressed to anaplastic astrocytoma or glioblastoma. Also, van den Bent *et al.* (9) reported that the histological confirmation of recurrence showed high-grade tumors in 66% (no early radiotherapy group) and 72% (early radiotherapy group) of patients with low-grade glioma. Therefore, radiotherapy is often administered as adjuvant therapy after surgery.

The 5-year PFS rate for patients who undergo postoperative radiotherapy is approximately 50% (10–12), while the 5-year OS rate ranges from 50% to 70% (11–15). Several reports have indicated that the survival rate decreases with the extent of resection (10, 16–18). Hanzely *et al.* (11) evaluated the benefits of radiotherapy by comparing the outcomes of early postoperative radiotherapy with those of delayed radiotherapy and found that the 5-year PFS rate was 52.2% for patients who underwent early postoperative radiotherapy and 39.5% for those who did not. They also stated that the PFS rate with early postoperative radiotherapy with subtotal resection was higher than that with extensive resection. Shaw *et al.* (18) indicated that postoperative radiotherapy with subtotal resection was associated with a higher survival rate than biopsy alone. In our study, the 5-year PFS and OS rates were 36% and 43%, respectively, in all patients; 22% and 11%, respectively, in the low-dose group; and 80% and 80%, respectively, in the high-dose group. Although our study was small, the prescribed tumor dose was identified as the only prognostic factor for PFS and OS with carbon ion radiotherapy. Given the fact that almost all patients in our study underwent partial resection or biopsy, the results of this study, especially those of the high-dose group, may be better than those obtained with photon radiotherapy.

With regard to the timing of radiotherapy for postoperative diffuse astrocytomas, several reports have noted significantly improved survival with early radiotherapy (13, 18–20). Shaw (21) and Hanzely *et al.* (11) reported an advantage of early postoperative radiotherapy for low-grade astrocytomas. In contrast, others have found no survival benefit with early postoperative radiotherapy (10, 17, 22, 23). Several physicians have reported that the potential long-term benefits of radiotherapy observed overlong follow-up periods might be offset by treatment-related brain toxicity (16, 24) and have recommended a "wait and see" policy, especially for young patients. Results of the EORTC randomized trial for the long-term efficacy of early versus that of delayed radiotherapy for low-grade astrocytomas demonstrated no differences in OS times, although the time to tumor progression was longer with early radiotherapy (9, 25). However, Klein *et al.* (26) indicated that seizures impaired quality of life and that the

use of antiepileptic agents had a negative impact on cognitive function. The 2005 EORTC report (9) stated that early radiotherapy improved seizure control and extended the time to tumor progression. Therefore, early radiotherapy can help these patients maintain a good quality of life until late tumor recurrence. Furthermore, Surma-aho *et al.* (27) reported that patients who underwent postoperative radiotherapy for low-grade gliomas developed long-term leukoencephalopathy and cognitive dysfunction that resulted in a decreased capacity to carry out daily routine activities. The most appropriate timing for optimal treatment of postoperative diffuse astrocytomas remains controversial, because some patients have exhibited long survival times even in the absence of adjuvant radiotherapy.

With regard to brain toxicity resulting from carbon ion radiotherapy, a late Grade 2 brain reaction, scored on the basis of RTOG/EORTC, was detected in 2 patients (40%) in the high-dose group, and no other adverse reactions were observed. Furthermore, almost all late brain reactions were minor according to the LENT-SOMA tables, based on the MRI findings. Although 4 patients in the two groups developed a Grade 3 reaction, the reaction of all patients regressed spontaneously to Grade 1or after treatment with a short course of steroids. In our previous study, Kishimoto *et al.* (28) reported that carbon ion radiotherapy for head and neck tumors could induce brain injury. Among the 281 patients analyzed in that study, 40 patients developed radiation-induced brain injury. One-third of the patients who developed radiation-induced necrosis showed improvement in the necrotic lesions. No unexpected or severe brain damage was noted, because accurate localization of carbon ion radiotherapy was achieved, and the irradiated volume of the normal brain was relatively small. Although we did not perform a systematic assessment of quality of life in our study, no serious toxicity related to carbon ion radiotherapy was observed during the follow-up period.

Dose escalation was carried out in two steps (50.4 and 55.2 GyE) in this phase I/II clinical trial. There was a clear improvement in PFS (80%; SEM = 18%) and OS (80%; SEM = 18%) in the high-dose group (55.2 GyE) compared with those in the low-dose group. Late brain morbidity showed a tendency toward a higher grade with the higher dose. Therefore, dose fractionation of carbon ion radiotherapy by 55.2 GyE in 24 fractions over 6 weeks was considered optimal for treating diffuse astrocytomas. In addition, stereotactic radiotherapy, gamma knife radiotherapy, and proton radiotherapy have shown improvements in treatment outcomes and alleviation of radiation toxicity (29–33). Based on these findings, we have decided not to proceed with the next dose escalation for diffuse astrocytomas.

Conclusions

In this phase I/II clinical trial of carbon ion radiotherapy for diffuse astrocytomas, we observed a clear improvement in PFS and OS in the high-dose group (55.2 GyE in 24 fractions over 6 weeks) compared with those in the low-dose group, without severe morbidity of normal tissues.

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Tumor and Stem Cell Biology

Cancer Research

Effects of Carbon Ion Beam on Putative Colon Cancer Stem Cells and Its Comparison with X-rays

Xing Cui[1], Kazuhiko Oonishi[2,4], Hirohiko Tsujii[2], Takeshi Yasuda[3], Yoshitaka Matsumoto[1], Yoshiya Furusawa[1], Makoto Akashi[4], Tadashi Kamada[2], and Ryuichi Okayasu[1]

Abstract

Although carbon ion therapy facilities are expensive, the biological effects of carbon ion beam treatment may be better against cancer (and cancer stem cells) than the effects of a photon beam. To investigate whether a carbon ion beam may have a biological advantage over X-rays by targeting cancer stem–like cells, human colon cancer cells were used *in vitro* and *in vivo*. The *in vitro* relative biological effectiveness (RBE) values of a carbon ion beam relative to X-rays at the D10 values were from 1.63 to 1.74. Cancer stem–like $CD133^+$, $CD44^+/ESA^+$ cells had a greater ability for colony and spheroid formation, as well as *in vivo* tumorigenicity compared with the $CD133^-$, $CD44^-/ESA^-$ cells. FACS (fluorescence-activated cell sorting) data showed that cancer stem–like cells were more highly enriched after irradiation with X-rays than carbon ion at doses that produced the same level of biological efficacy. A colony assay for cancer stem–like cells showed that RBE values calculated by the D10 levels were from 2.05 to 2.28 for the carbon ion beam relative to X-rays. The *in vivo* xenotransplant assay showed an RBE of 3.05 to 3.25, calculated from the slope of the dose–response curve for tumor growth suppression. Carbon ion irradiation with 15 Gy induced more severe xenograft tumor cell cavitation and fibrosis without significant enhancement of cells with putative cancer stem cell markers, CD133, ESA, and CD44, compared with 30 Gy X-rays, and marker positive cells were significantly decreased following 30 Gy carbon ion irradiation. Taken together, carbon ion beam therapy may have an advantage over photon beam therapy by improved targeting of putative colon cancer stem–like cells. *Cancer Res; 71(10); 3676–87. ©2011 AACR.*

Introduction

Colorectal cancer is currently the most common gastrointestinal malignancy and remains the third most common cancer and second leading cause of cancer-related deaths in developed countries (1). Although surgical resection has been the first choice for treatment of colorectal cancer, half of patients still suffer recurrence, presumably because of disseminated micrometastases present at the time of surgery (2). Radiation therapy is the most effective nonsurgical intervention for cancer treatment, but most cancers also invariably recur after radiation therapy. Therefore, determination of the mechanisms of recurrence and radioresistance in these tumors and development of powerful therapeutics could lead to advances in the treatment of cancer.

Authors' Affiliations: [1]Heavy-Ion Radiobiology Research Group, [2]Research Center Hospital for Charged Particle Therapy, and [3]Department of Radiation Emergency Medicine, Research Center for Radiation Emergency Medicine, National Institute of Radiological Sciences (NIRS); [4]Department of Medicine and Clinical Oncology, Graduate School of Medicine, Chiba University, Chiba, Japan

Corresponding Author: Xing Cui, Heavy-Ion Radiobiology Research Group, Research Center for Charged Particle Therapy, National Institute of Radiological Sciences, 4-9-1 Anagawa Inage-ku, Chiba, Chiba 263-8555, Japan. Phone: 043-206-3231; Fax: 043-206-4149; E-mail: saisei@nirs.go.jp

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The Heavy-Ion Medical Accelerator in Chiba (HIMAC) is the first heavy-ion accelerator specially dedicated to medicine in the world and has now become the world's leading heavy-ion cancer treatment facility (3,4). Although heavy-ion beam facilities are large scale and hugely expensive, several new heavy-ion therapy facilities, such as CNAO (Italy), PTC Marburg (Germany), SAGA HIMAT (Japan), ETOILE (France), Shanghai Particle Therapy Hospital (China), and Mayo Clinic (USA), are under construction or in planning worldwide. A one-third smaller and cheaper carbon ion radiotherapy facility based on the highly advanced technology was designed and constructed in Gunma University in Japan, and clinical trial was successfully initiated in March 2010. High linear energy transfer (LET) particle therapy has various advantages because of the production of spread out Bragg's peaks (SOBP), which cover tumors with biologically equivalent dose distributions. Therefore, high LET heavy-ion therapy has several potential advantages over low LET photon therapy such as increased relative biological effect, reduced oxygen enhancement ratio, decreased cell-cycle–dependent radiosensitivity, and induced complex DNA damage that is not easily repaired (5,6). Over the past decades, HIMAC has been successful in treating more than 5,000 cases of various human cancers and achieved promising clinical outcomes for many radioresistant tumor types, including recurrent colorectal cancer, hepatocellular carcinoma, chondroma, and sarcoma (7–10).

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Recently, cancer stem cells have been identified in a growing number of solid tumors, which are typically recognized by virtue of the expression of cell surface markers; most of them are transmembrane glycoproteins, such as CD133, CD44, and EpCAM (ESA; refs. 11,12). Cancer stem cells have the ability to generate tumors that recapitulate the original tumor when xenotransplanted into animals, whereas the remaining non-cancer stem cell tumor bulk most often cannot (13–18). The cancer stem cells that populated the original tumor may have resistance to the treatments to repopulate the recurrent tumor even after the bulk of the tumor has been removed by resection or chemoradiation therapy (19–22). Therefore, the key point in curatively treating cancer is how to effectively eradicate those minor cancer stem cells in the bulk of the tumor (23–25). Because heavy-ion radiotherapy has potential advantages in treating many human radioresistant cancers, we hypothesize that heavy-ion irradiation may effectively target these cancer stem–like cells. To the best of our knowledge, our study is the first to explore whether heavy-ion irradiation may have advantages over X-rays in targeting human colon cancer stem–like cells.

Materials and Methods

Cell lines

The colon adenocarcinoma cell lines HCT116 and SW480 were purchased from American Type Culture Collection. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% with heat-inactivated 5% (v/v) fetal calf serum (FCS; Beit-Ha'Emek), 100 unit/mL penicillin, and 100 μg/mL streptomycin (Invitrogen) in a 37°C with 5% CO_2 in air. The medium was changed every other day.

Colony and spheroid formation assay

A clonogenic survival assay was conducted as described previously (6). In brief, the appropriate plating density was designed to produce 20 to 40 surviving colonies in each 6-cm dish or T25 flask. After incubation for 14 days, the colonies were fixed and stained with 0.3% methylene blue in ethanol, and colonies containing more than 50 cells were counted as survivors. At least, 3 parallel samples were scored in 3 to 5 repetitions conducted for each irradiation condition.

For assays of clonogenicity and ability to grow as "tumor spheres" in suspension, HCT116 and SW480 cells were sorted to obtain populations of $CD133^+$ and $CD133^-$, $CD44^+/ESA^+$ and $CD44^-/ESA^-$cells by BD FACSAria (Becton Dickinson). For the clonogenicity assay and spheroid assay, the sorted $CD133^+$ and $CD133^-$, $CD44^+/ESA^+$ and $CD44^-/ESA^-$ cell subpopulations were then resuspended in a cell density of 500 or 2,000 cells/mL and were plated triplicate in a 6-cm dish or 96-well plates precoated with a layer of 1% agarose and left to grow for 1 to 2 weeks. To quantify the number of colonies and sphere formations as well as spheroid formation rates, each positive or negative stem-like cell was applied to 12 wells, the rate of spheres per well calculated, and the data are presented as percentage of the wells that contained spheres.

In vivo tumorigenic assays

Immediately after sorting, aliquots of the particular cell populations were counted and cell viability was determined using a conventional trypan blue test. The sorted cells were centrifuged at 300 × *g* for 5 minutes at 4°C, suspended in serum-free medium (DMEM with 1% penicillin/streptomycin), then various numbers of $CD133^+/CD133^-$, $CD44^+/ESA^+$, and $CD44^-/ESA^-$ cells ranging from 1×10^3 to 2×10^5 cells are injected subcutaneously into the hind legs of 6- to 8-week-old male NOD/SCID (severe combined immunodeficient mice)mice. The animals were sacrificed at the indicated time intervals (4–10 weeks) when tumor nodules were identified on their body surfaces. Tissues were fixed in formaldehyde and examined histologically. All experiments involving the use of animals were carried out in accordance with NIRS institutional animal welfare guidelines. NOD/SCID mice (Charles River Laboratories) were maintained under defined conditions at the NIRS Animal Facility.

Tumor growth delay assays

BALB/cAJcl-*nu/nu* male mice (5-week-old) were purchased from CLEA Japan, Inc. Mice were provided with water and food *ad libitum* and were housed at 5 animals per cage. All surgical procedures and care administered to the animals were in accordance with the NIRS Animal Care and Use Committee. Tumors were established by subcutaneous inoculation of 8×10^5 HCT116 cells into the right leg of the mouse. Tumor growth was monitored every 3 days by measuring 2 perpendicular diameters. Tumor volume was calculated according to the formula: $0.52 \times a \times b^2$, where a and b are the largest and smallest diameters, respectively. The tumor growth delay (TGD) of xenograft tumors after treatment with X-rays or carbon-ion was estimated at the tumor volume of 500 mm^3. The relative biological effectiveness (RBE) of carbon-ions at the middle of a 6-cm SOBP relative to 200 keV X-rays was calculated by KaleidaGraph software.

Irradiation

Cells or mice were irradiated with carbon-ion beams accelerated by the HIMAC at NIRS. The details concerning the beam characteristics of the carbon-ion beams, biological irradiation procedures, and dosimetry have been described elsewhere (3, 4). Briefly, the initial energy of the carbon-ion beams was 290 MeV/n, 50KeV/μm, 6-cm, SOBP. The energy of heavy-ion beams at the irradiation site was obtained by comparing the calculated and measured depth–dose distribution. As a reference, mice were also irradiated with conventional 200 kV_p X-rays (Pantac HF-320S; Shimadzu Co.) at NIRS. Cells were irradiated with 2, 4, 6, or 8 Gy of X-rays or 1, 2, 4, or 6 Gy carbon ions. Transplanted xenograft tumors were irradiated with various doses of X-rays (15, 30, and 60 Gy) or carbon-ions (5, 15, and 30 Gy).

FACS analysis

FACS (fluorescence-activated cell sorting) analysis for the cells or xenograft tumors irradiated with X-rays or carbon ion was conducted with BD FACSAria according to the manufacturer's protocol (Becton Dickinson). For *in vitro* analysis,

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the cells were prepared and labeled with conjugated anti-human CD133⁻PE (phycoerythrin; Miltenyi Biotec), CD44⁻FITC (Miltenyi Biotec), and ESA-PE (Miltenyi Biotec). Isotype-matched immunoglobulin served as control. Cells were incubated for 20 minutes at each step and were washed with 2% FCS/PBS between steps. The percentage of $CD133^+$, $CD44^+$, and ESA^+ present was assessed after correction for the percentage of cells reactive with an isotype control. For *in vivo* analysis, tumors were minced with scissors under sterile conditions, rinsed with HBSS and incubated for 2 hours at 37°C in serum-free DMEM supplemented with 200 units/mL collagenase type II and type IV (Sigma-Aldrich), 120 μg/L penicillin, and 100 μg/mL streptomycin. Cells were further disaggregated by pipetting and serial filtration through cell dissociation sieves (size: 40 and 80 meshes; Sigma-Aldrich). Contaminating erythrocytes were lysed by incubation in ammonium chloride. Single-cell suspensions were assessed with BD FACSAria in the same way as *in vitro* cell analysis.

Gross morphology and histopathology

Gross morphologic changes were followed up to 12 weeks after a single fraction of X-ray or carbon-ion radiation. At selected time points, tumors were excised and histopathologic examinations were conducted. Xenograft tumors from different groups were fixed in 10% neutral formalin and processed in paraffin-embedded sections followed by sectioning (4 μm) onto slides. Sections were stained with hematoxylin and eosin (H&E) and assessed microscopically.

Immunohistochemistry

Immunohistochemical staining was conducted with the Elite ABC Kit (Vector Laboratories) according to the manufacturer's protocol (26, 27). In brief, sections cut from formalin-fixed, paraffin-embedded tissue blocks were deparaffinized and rehydrated through a graded series of ethanol and incubated in 0.3% hydrogen peroxidase in methanol to block endogenous peroxidase action. For antigen retrieval, sections were placed in boiling 10 mmol/L citrate buffer (pH 6.0). The slides were preincubated with normal horse serum (1:50 dilution; Vector Laboratories) to diminish nonspecific binding of the secondary antibody and then incubated overnight at 4°C with anti-CD133 (AC133, human monoclonal; Miltenyi Biotec; 1:200 dilution), anti-CD44 (mouse monoclonal; BD Transduction Labs; 1:100 dilution), and anti-ESA (human monoclonal; Miltenyi Biotec; 1:200 dilution). Slides were then rinsed and incubated with universal secondary antibody containing anti-mouse/anti-goat IgG (Vectastain ABC Elite kit; Vector Laboratories) for 30 minutes, developed with diaminobenzadine (Vectastain ABC Elite kit; Vector Laboratories) for 10 minutes, and counterstained with hematoxylin for 2 minutes. Ten fields were selected and expression was evaluated in 1,000 tumor cells with high power (200×) microscopy. As a negative control, the sections were stained without primary antibodies to monitor the background staining level. Cytoplasmic and/or membrane staining were considered to indicate specific CD133, CD44, and ESA immunoreactivity. Each slide was assessed for the intensity of immunostaining, background, and percentage of cells expressing the target protein.

Western blotting

Western blot analyses were conducted as previously described (26). Cells or tumor tissues were lysed using the Pierce Nuclear and Cytoplasmic Extraction Reagent Kit (Pierce). Samples were normalized for protein concentration using the Pierce BCA protein assay; 50 μg of each cytoplasmic or nuclear extract sample was analyzed by SDS/5% PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane. Membranes were blocked with 2% ECL Advance Blocking Agent in TBS Tween 20 for 1 hour and probed overnight with monoclonal antibodies specific for CD133, ESA, and CD44. β-Actin was used as a loading control. Subsequently, membranes were incubated with rabbit anti-mouse or anti-rabbit horseradish peroxidase–conjugated secondary antibody (Sigma-Aldrich). Blots were visualized by ECL Advanced Kit (GE Healthcare Life Sciences) and quantitated using ImageQuant LAS 4000 Mini Biomolecular Imager. Densitometric analysis was obtained with Fujifilm Multi Gauge Software (version 3.0).

Statistical analysis

One-way ANOVA and Bonferroni multiple comparison tests were used when mean differences between the groups were evaluated by StatView software (SAS Institute, Inc.). For all comparisons, values of $P < 0.05$ were defined as significant.

Results

Survival fraction by carbon-ion versus X-ray irradiation

The HCT116 and SW480 cells were irradiated with carbon-ion or X-rays up to 6 Gy, and their survival fraction was measured from the colony formation. Figure 1 shows dose–response curves for cell killing effects on the 2 human colon cancer cell lines irradiated with X-rays or 50 keV/μm SOBP carbon ion beams. The surviving fractions for the HCT116 and SW480 irradiated with X-rays and carbon ions decreased exponentially with increasing doses. On the basis of these survival curves, the RBE values calculated by the D10, which is determined as the dose (Gy) required to reduce the surviving fraction to 10%, relative to X-rays, is about 1.63 to 1.74 for carbon-ion beams.

Determination of cancer stem–like cell properties of $CD133^+$ and $CD44^+/ESA^+$ cells

Having isolated the $CD133^+$, $CD44^+/ESA^+$ cells from the HCT116 and SW480 cells, we next determined their cancer stem–like cell properties. $CD133^+$, $CD44^+/ESA^+$ cells have higher clonal and spheroid formation capacities *in vitro* and robust tumorigenicity in xenograft model. When equal numbers of 500 cells were plated in a dish, $CD133^+$, $CD44^+/ESA^+$ colorectal cancer cells from HCT116 formed 64 ± 10 and 87 ± 6 clones, whereas $CD133^-$ or $CD44^-/ESA^-$ cancer cells formed only 20 ± 6 and 22 ± 3 clones ($P < 0.01$; Fig. 2A). These data showed that $CD133^+$ or $CD44^+/ESA^+$ colorectal cancer cells had much greater clonal formation capacities than that of $CD133^-$ or $CD44^-/ESA^-$ cancer cells. To investigate the ability to form spheroid bodies, isolated $CD133^+$, $CD44^+/ESA^+$, and $CD133^-$, $CD44^-/ESA^-$ cells were cultured in 96-well plates precoated with a layer of 1% agarose. After being in culture for

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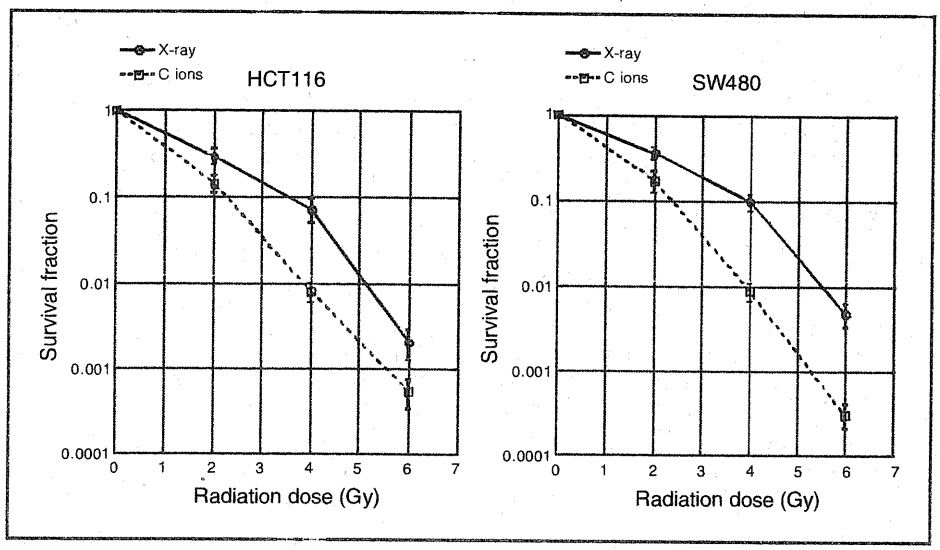

Figure 1. Survival curves of HCT116 and SW480 cells plated immediately after X-ray or carbon ion (C ion) irradiation. The graphs show the mean and standard error calculated from 3 independent experiments.

1 week, $CD133^+$, $CD44^+/ESA^+$ aggregated and formed spheroid bodies (Fig. 2B). The ability to form spheroid bodies in $CD44^+/ESA^+$ was significantly higher than that in $CD133^-$, $CD44^-/ESA^-$ ($P < 0.01$; Fig. 2B). Figure 2C shows representative positive cells for CD133, CD44, and ESA markers.

To determine the efficacy of tumor initiation from cells with or without CD133, CD44, and ESA markers, we carried out limiting dilution experiments. A variable number of human cells, ranging from 1,000 to 2,000,000, were injected into NOD/SCID mice to test their xenotumor abilities. As few as 1000

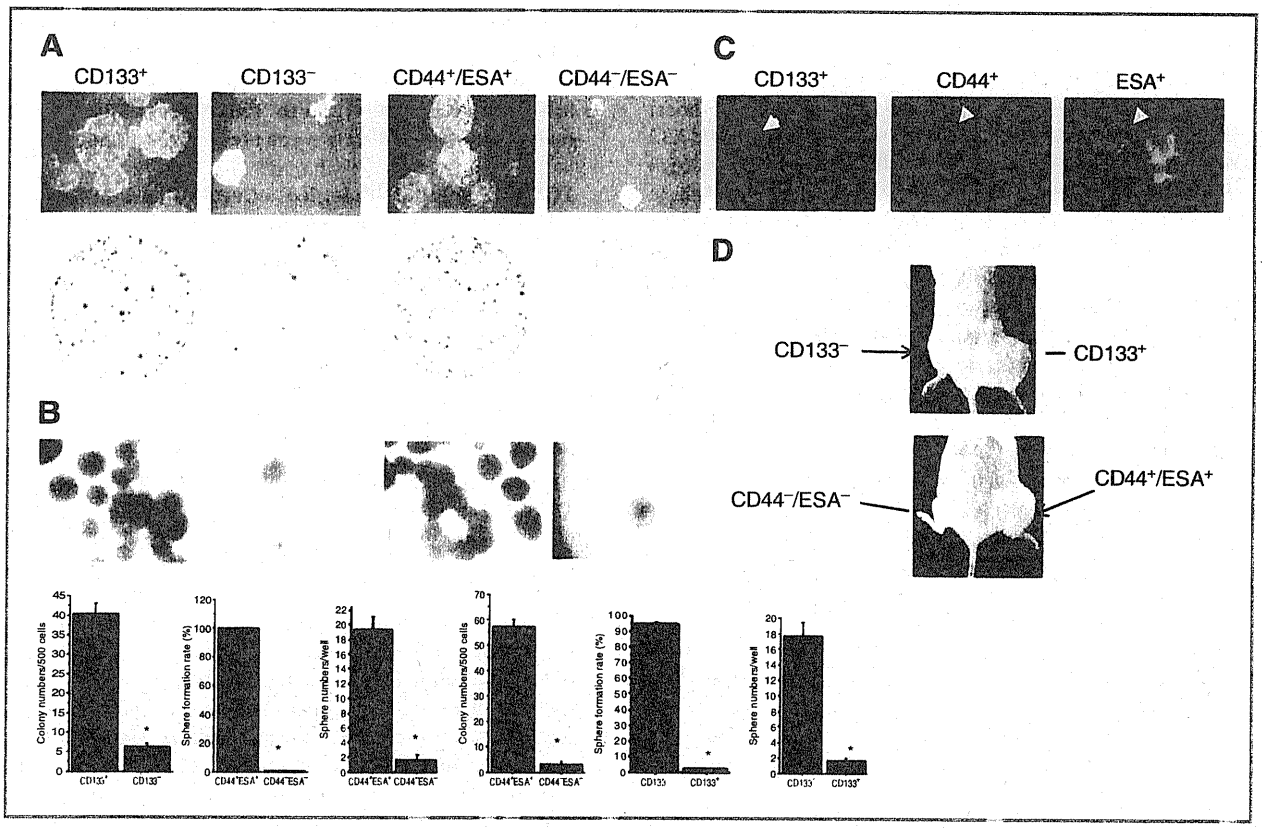

Figure 2. Colony, spheroid, and tumor formation of cancer stem—like and noncancer stem—like cells delivered from HCT116 cells. Colony (A) and spheroid formation (B) of $CD133^+$, $CD133^-$, $CD44^+/ESA^+$, and $CD44^-/ESA^-$ cells after being in culture for 1 to 2 weeks. C, representative photographs of positive cancer stem—like HCT116 cells. D, tumorigenicity of $CD133^+$, $CD133^-$, $CD44^+/ESA^+$, and $CD44^-/ESA^-$ cells after subcutaneous injection into the hind legs of NOD/SCID mice. *, $P < 0.01$, compared with colony or sphere formed from $CD133^+$ or $CD44^+/ESA^+$ cells.

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Table 1. Tumor formation ability of sorted HCT116 colorectal cancer cells using surface markers (number of tumors formed/number of injections)

Groups	2×10^5	2×10^4	1×10^4	5×10^3	1×10^3
Unsorted	6/6	3/6	1/6	0/6	0/0
$CD133^+$	0/0	6/6	5/6	3/5	3/5
$CD133^-$	0/0	2/6	0/6	0/5	0/5
P		0.001	0.001	0.001	0.001
$CD44^+/ESA^+$	0/0	5/5	5/5	4/5	3/5
$CD44^-/ESA^-$	0/0	1/5	0/5	0/5	0/0
P		0.001	0.001	0.001	0.001

NOTE: $P < 0.01$ compared with results from marker-negative cells.

$CD133^+$, or $CD44^+/ESA^+$ cells were sufficient to generate a tumor within 6 to 10 weeks after implantation, whereas the number of $CD133^-$, $CD44^-/ESA^-$ cells that had a similar capacity was more than 20,000 (Table 1). These data showed that the tumorigenicity of $CD133^+$, $CD44^+/ESA^+$ colorectal cancer cells was much higher than $CD133^-$ or $CD44^-/ESA^-$ cells (Fig. 2D and Table 1). Combining these results, our data suggested that $CD133^+$, $CD44^+/ESA^+$ cells isolated from HCT116 and SW480 cells present the characteristics of cancer stem–like cells.

Changes in proportion of cancer stem–like cells *in vitro* by carbon-ion versus X-ray irradiation

In vitro FACS analyses showed that the percentage of cancer stem–like cells which were positive for CD133, CD44, and ESA were more significantly increased after 48 or 72 hours X-ray than carbon ion irradiation. The percentage of $CD133^+$ cells in unirradiated HCT116 cells was about 3%, and it was increased more than 3-fold after irradiation with 2 Gy X-rays and further increased 5-fold after 4 Gy X-ray irradiation. In comparison, $CD133^+$ cells were increased only 2-fold by 1 Gy carbon ion and 3-fold by 2 Gy carbon ion irradiation for which the doses induced equivalent effects by X-ray. The percentage of $CD44^+$ cells was increased by more than 1.5-fold after irradiation with 2 Gy X-rays and further increased 2.5-fold after 4 Gy X-ray irradiation. In contrast, $CD44^+$ cells were unchanged by 1Gy carbon ion and decreased 0.5-fold by 2 Gy carbon ion irradiation. Interestingly, the percentage of ESA^+ cells did not change by either 2 or 4 Gy but increased 2-fold by 6 Gy X-rays irradiation. In contrast, ESA^+ cells were unchanged after carbon ion irradiation even by the dose up to 4 Gy (Fig. 3A). The proportion of $CD133^+/CD44^+$ and $ESA^+/CD44^+$ cells were enriched 2- to 3-fold by 2 or 4 Gy X-rays, whereas those of double positive cells were decreased or unchanged by 1 or 2 Gy carbon ion irradiation (Fig. 3A).

Clonogenic assays of cancer stem–like cells *in vitro* by carbon-ion versus X-ray irradiation

Clonogenic assays were conducted to determine the different radiosensitivity of cancer stem–like cells between carbon ion and X-ray irradiation. Figure 3B shows dose–response curves for cell killing effects on cancer stem–like $CD133^+$, $CD44^+/ESA^+$ cells and noncancer stem–like cells sorted from HCT116 or SW480 cells. The results showed that the surviving fractions for cancer stem–like $CD133^+$, $CD44^+/ESA^+$ cells are significantly higher than noncancer stem–like $CD133^-$, $CD44^-/ESA^-$ cells after exposure to either X-rays or carbon ion beams, suggesting that cancer stem–like cells show resistance to both X-rays and carbon ions We have also determined the resistance of $CD133^+$, $CD44^+/ESA^+$ cells to chemotherapy (data not shown). The surviving fractions for the cancer stem–like cells sorted from the 2 cell lines after irradiation with X-rays and carbon ions decreased exponentially with increasing doses. On the basis of these survival curves, the RBE values calculated at the D10 level for cancer stem–like cells were calculated to be about 2.05 to 2.28, whereas RBE values for noncancer stem–like cells were about 1.22 to 1.44.

TGD and relative biological effects of carbon-ion relative to X-ray irradiation

Transplanted xenograft tumors grow fast without any treatment and the tumor volume became more than 400 mm^3 after being subcutaneously implanted into mice for 3 weeks. Treatment with X-rays (30 Gy) effectively suppressed tumor growth and reduced the tumor size and volume about 10%, but the tumor rapidly regrew after 4 weeks and to double in volume after another 4 weeks (Fig. 4A). In contrast, treatment with carbon-ion (30 Gy) radiation increased tumor size at the first week and then gradually decreased. The tumor was reduced to the same size as prior to radiation after 4 weeks and actually became less than half in volume after 8 weeks, and finally disappeared after 12 weeks without any regrowth and relapse (Fig. 4A). To determine the tumor growth control possibility by carbon-ion radiation, the xenograft tumors were also treated with various doses. Carbon-ion irradiation with 15 Gy can suppress tumor growth without significant initial increase in tumor size and volume but regrew after 7 to 8 weeks. As expected, treatment with 5 Gy carbon-ion or 15 Gy X-ray failed to control tumor growth anymore; however, the tumor was completely regressed without regrowth with 60 Gy X-ray irradiation in the 12-week follow-up (data not shown). Xenotransplanted tumor control possibility by carbon-ion and X-ray radiation at various doses is summarized in Table 2.

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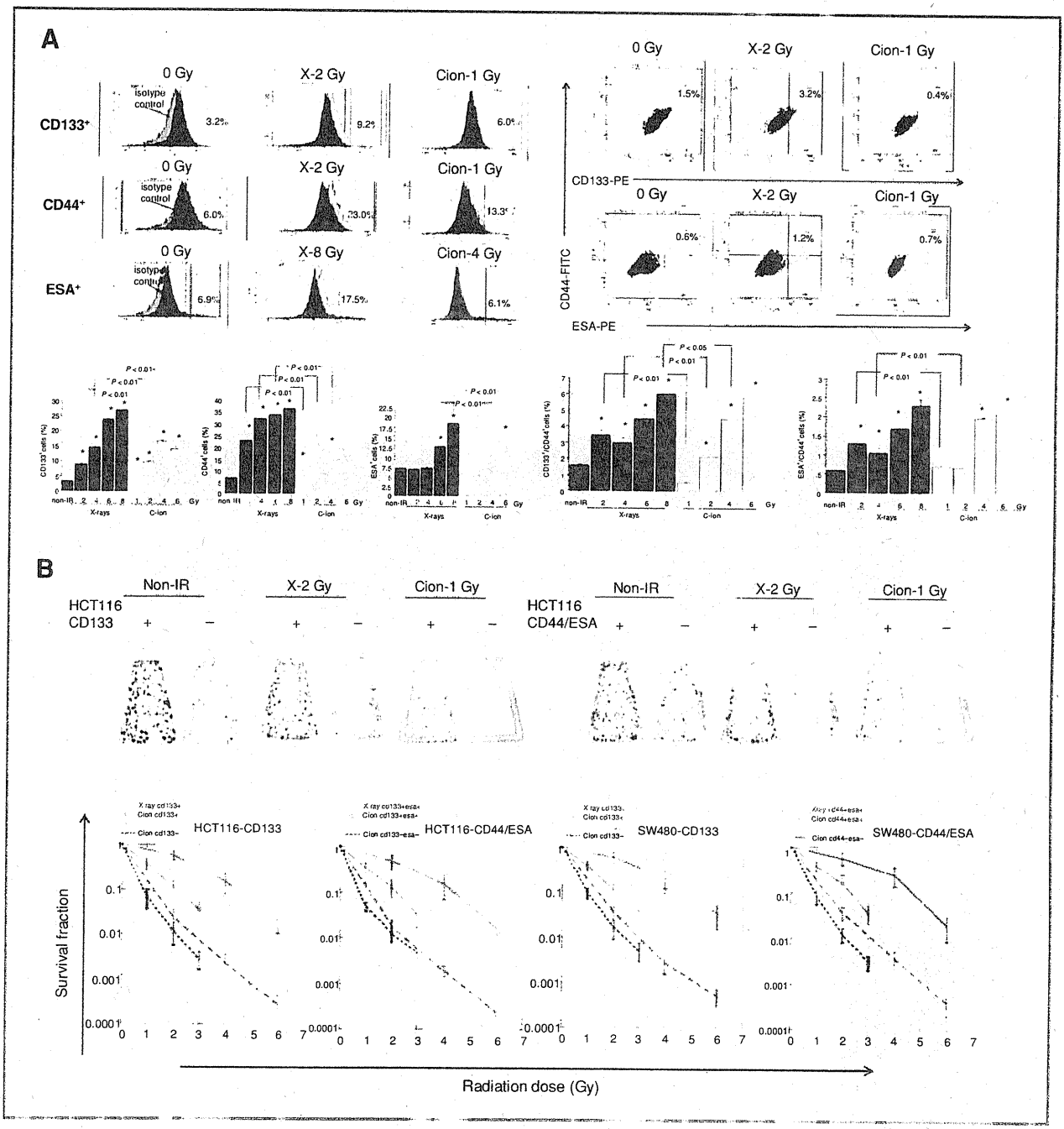

Figure 3. A, percentage changes of $CD133^+$, $CD44^+$, ESA^+, $CD133^+/CD44^+$, and $CD44^+/ESA^+$ cells by FACS analysis 48 or 72 hours after irradiation with X-rays or carbon ions in HCT116 cells. *, $P < 0.01$, compared with unirradiated cells. B, survival curves of cancer stem–like cells and noncancer stem–like cells sorted from HCT116 and SW480 cells after irradiation with X-rays or carbon ion. Representative photos of colony formation from $CD133^+$, $CD133^-$, $CD44^+/ESA^+$, and $CD44^-/ESA^-$ cells after irradiation with X-ray or carbon ions are displayed. The graphs show the mean and standard error calculated from 3 independent experiments.

To calculate *in vivo* RBE values, the TGD of xenotransplanted tumors after treatment with 15 or 30 Gy of X-rays and 5 or 15 Gy carbon-ions were estimated. Due to the tumor bed effect, the extent of TGD determined from tumor growth curves is highly dependent on the end volume chosen. To minimize the influence of the tumor bed effect on the growth delay, we calculated by choosing a smaller size and essentially an earlier time for regrowth. The TGD was obtained according to the endpoint of the tumor regrown to a volume of about 500 mm^3. As shown in Table 3, TGD was 4 and 28 days for 15 and

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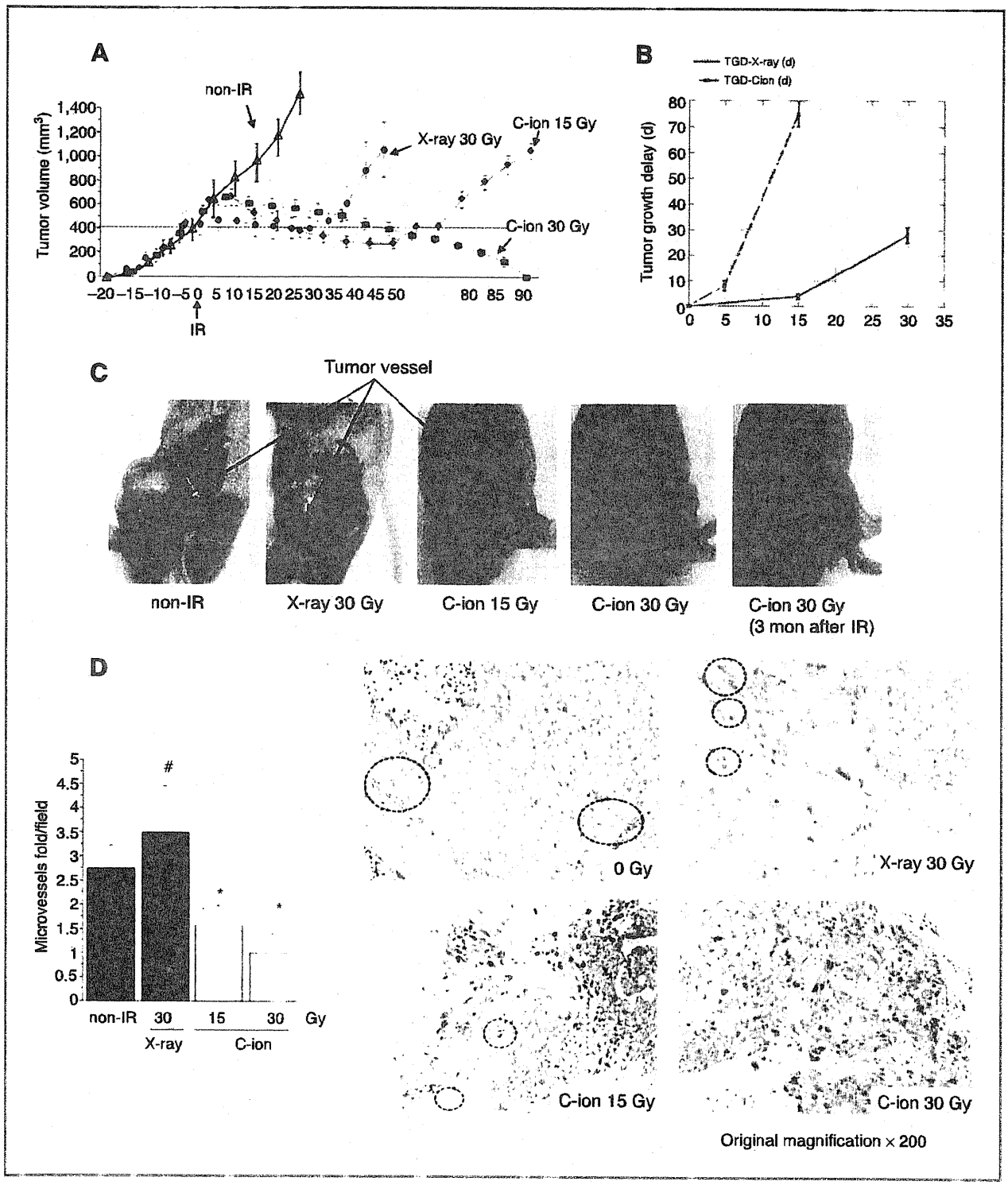

Figure 4. A, HCT116 xenograft tumor growth control by 30 Gy X-ray or 15 and 30 Gy carbon-ion irradiation. B, HCT116 xenograft TGD after treatment with X-rays or carbon-ion irradiation. Gross morphologic changes (C) and histopathologic features (D) of HCT116 xenograft tumors at 4 weeks after being treated with 30 Gy X-rays or 15 and 30 Gy carbon-ion irradiation. Arrows indicate microvessels in which red blood cells were seen. #, $P < 0.05$; *, $P < 0.01$ compared with that of control tumors.

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Table 2. Therapeutic efficacy of X-ray and carbon-ion irradiation in HCT116 colon cancer xenograft (12-week follow-up)

Group	Mice, *n*	Complete response	Partial response
Unirradiated	5	–	–
X-ray			
15Gy	5	0	5
30Gy	5	1	4
60Gy	5	5	0
Carbon-ion			
5Gy	5	0	5
15Gy	5	1	4
30Gy	5	5	0

30 Gy of X-rays but 10 and 76 days for 5 and 15 Gy of carbon-ion beams, respectively (Fig. 4B). On the basis of this TGD, the RBE values of 50 keV/μm carbon ion at the middle of a 6-cm SOBP relative to X-ray were calculated as 3.05 to 3.25 according to the formula analyzed by KaleidaGraph software (Fig. 4B and Table 3).

Gross morphologic and histopathologic changes after carbon-ion versus X-ray irradiation

Figure 4C illustrates gross tumor morphology before and after treatment with carbon-ion (15, 30 Gy) or X-ray (30 Gy) irradiation. Tumor-supplying vessels were very clearly seen in the unirradiated mice as well as in the X-ray irradiated mice, but markedly reduced in carbon-ion irradiated mice. It is further confirmed by microscopy observation that the microvessel counts were significantly reduced by carbon ion compared with X-ray irradiation (Fig. 4D). Histopathologic changes of xenograft tumors after irradiation with X-rays or carbon-ion for 4 weeks were examined by H&E staining. Histopathologic features showed that most of the tumor cells were not disrupted by 15 Gy X-rays or 5 Gy carbon ion irradiation (data not shown). At the same level of biological efficacy, 15 Gy carbon-ion irradiation predominantly induced colon cancer cell cavitations, fibrosis and the duct-like architecture was completely disrupted. In contrast, 30 Gy X-ray irradiation only partially destroyed colon cancer cells and the duct-like architecture still remained (Fig. 4D). It is clearly shown that almost all of the tumor cells were destroyed after irradiated with 30 Gy carbon-ions (Fig. 4D).

Expression of cancer stem–like cell marker CD133, CD44 and ESA after carbon-ion versus X-ray irradiation

The expression changes of cancer stem cell marker CD133, CD44, and ESA in the xenograft tumors at 4 weeks after irradiation with 30 Gy X-rays or carbon-ion (15, 30 Gy) were examined by immunohistochemical staining. It was shown that expression of both CD133 and CD44 was significantly suppressed by 15 Gy carbon-ion irradiation compared with unirradiated tumors, except ESA protein, but all 3 putative cancer stem cell proteins were remarkably inhibited after treatment with 30 Gy carbon-ion irradiation. In contrast, 30 Gy X-rays significantly increased expression of CD133, CD44, and ESA compared with those of unirradiated tumors (Fig. 5A). It was further confirmed by Western blotting analyses that expression levels of all CD133, CD44, and ESA proteins was significantly enhanced by 30 Gy X-rays compared with 15 Gy carbon-ion irradiation and all 3 proteins were predominantly reduced by 30 Gy carbon-ion irradiation (Fig. 5B).

Changes in proportion of cancer stem–like cells *in vivo* by carbon-ion versus X-ray irradiation

In vivo FACS analyses showed that the percentage of cancer stem–like cells which were positive for CD133, ESA were increased with 15 and/or 30 Gy but significantly decreased with 60 Gy X-rays irradiation after 1 month. In comparison, carbon-ion irradiation with 15 Gy did not change the percentage of these positive cancer stem–like cells, whereas irradiation with 30 Gy significantly reduced the percentage of positive cancer stem–like cells (Fig. 5C). It is not detectable in any of cancer stem–like cells by *in vivo* FACS analysis after the tumor irradiated with 30 Gy carbon ion or 60 Gy X-rays for 2 to 3 months.

Discussion

We found that the *in vitro* RBE values, calculated by the D10 relative to the X-rays, are about 1.63 to 1.74 for the 50-keV/μm SOBP carbon ion beam on HCT116 or SW480 cells in this study. RBE values are known to be dependent on LET, and our results are almost consistent with previous reports using HIMAC carbon-ion beams on various human cancer cell lines such as lung, pancreas, and brain tumors, which reported 1.06 to 1.33 for a 13-keV/μm-beam, 1.42 to 1.69 for a 50-keV/μm-beam, and 2.00 to 3.01 for a 77-keV/μm-beam (4, 28). Recent accumulating experimental evidence suggests that cancer stem cell content may differ among tumors and that a higher proportion of cancer stem cells is correlated with higher

Table 3. TGD and RBE values estimated in this study

X-ray		Carbon ion		RBE ± SE (95% CI)
15 Gy	30 Gy	5 Gy	15 Gy	
TGD ± SE (95% CI), d				
4 ± 1 (2–6)	28 ± 3 (24–33)	8 ± 2 (7–13)	76 ± 4 (71–82)	3.15 ± 0.16 (3.05–3.25)