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**Title**

Monitoring tumor proliferative response to radiotherapy using  $^{18}\text{F}$ -fluorothymidine in human head and neck cancer xenograft in comparison with Ki-67

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## **Abstract**

*Objective* Although radiotherapy is an important treatment strategy for head and neck cancers, it induces tumor repopulation which adversely affects therapeutic outcome. In this regard, fractionated radiotherapy is widely applied to prevent tumor repopulation. Evaluation of tumor proliferative activity using  $^{18}\text{F}$ -fluorothymidine (FLT), a noninvasive marker of tumor proliferation, may be useful for determining the optimal timing of and dose in the repetitive irradiation. Thus, to assess the potentials of FLT, we evaluated the sequential changes in intratumoral proliferative activity in head and neck cancer xenografts (FaDu) using FLT.

*Methods* FaDu tumor xenografts were established in nude mice and assigned to control and two radiation-treated groups (10 and 20 Gy). Tumor volume was measured daily.  $^3\text{H}$ -FLT was injected intravenously 2 hrs before sacrifice. Mice were sacrificed 6, 24, 48 hrs, and 7 days after the radiation treatment. Intratumoral  $^3\text{H}$ -FLT level was visually and quantitatively assessed by autoradiography. Ki-67 immunohistochemistry (IHC) was performed.

*Results* In radiation-treated mice, the tumor growth was significantly suppressed compared with the control group, but the tumor volume in these mice gradually increased with time. In the visual assessment, intratumoral  $^3\text{H}$ -FLT level diffusely decreased 6 hrs after the radiation treatment and then gradually increased with time, whereas no apparent changes were observed in Ki-67 IHC. Six hours after the radiation treatment at 10 and 20 Gy, the intratumoral  $^3\text{H}$ -FLT level markedly decreased to 45 and 40% of the control, respectively ( $P < 0.0001$ , vs control), and

then gradually increased with time. In each radiation-treated group, the  $^3\text{H}$ -FLT levels at 48 hrs and on day 7 were significantly higher than that at 6 hrs. The Intratumoral  $^3\text{H}$ -FLT levels in both treated groups were 68 and 60% at 24 hrs ( $P < 0.001$ ), 71 and 77% at 48 hrs ( $P < 0.001$ ), and 83 and 81% on day 7 ( $P = \text{NS}$ ) compared with the control group.

*Conclusion* Intratumoral FLT uptake level markedly decreased at 6 hrs and then gradually increased with time. Sequential evaluation of intratumoral proliferative activity using FLT can be beneficial for determining the optimal timing of and dose in repetitive irradiation of head and neck cancer.

**Key words:**  $^{18}\text{F}$ -fluorothymidine; Radiotherapy; Tumor proliferation; Ki-67 labeling index; Head and neck cancer xenograft

## **Introduction**

Radiotherapy has long been used for curative or palliative management of many cancers. Over the past decades, the preferred treatment strategies for squamous cell carcinoma of the head and neck have gradually shifted from surgery to organ-preservation approaches such as radiotherapy with or without chemotherapy. Despite the large variety of treatment methods available for the management of advanced head and neck carcinomas, still these tumors remain highly challenging owing to their aggressiveness and complex anatomical location [1, 2]. Among the treatment challenges associated with head and neck carcinomas, tumor cell repopulation is an important indicator of tumor aggressiveness and resistance to various types of treatment. As a response to radiotherapy, squamous cell carcinomas of the head and neck show accelerated repopulation of clonogenic tumor cells during the course of treatment, which adversely affects treatment outcome and leads to locoregional treatment failure [3, 4, 5]. Accordingly, altered radiation fractionation schedules and strategies are widely applied to overcome the repopulation [6]. The response of a tumor to radiotherapy or chemoradiotherapy greatly depends on tumor biology and micro-environmental characteristics. Because of variations in biological behavior, tumors often respond differently to the same treatment [7, 8]. Considering this known heterogenic biological tissue response among individual patients, evaluation of tumor proliferative activity in response to radiotherapy is useful for radiotherapy planning, that is,

determining the optimal timing of and dose in the fraction radiotherapy schedule at which the undesirable side effects of radiotherapy and unnecessary cost are reduced.

Tumor proliferation is a hallmark of the cancer phenotype and is one of the useful markers for evaluating the therapeutic effect and prognosis after the therapy in clinical oncology [9-12]. Histological analysis of Ki-67 labeling index is a gold standard of tumor proliferation [13]. However, Ki-67 labeling index can only be used to evaluate the tumor proliferation in biopsy samples or excised tumor tissues. Thus, a noninvasive method to evaluate tumor proliferation is necessary.  $^{18}\text{F}$ -fluorothymidine (FLT) PET which reflects thymidine kinase 1 (TK1) activity is one of the noninvasive methods of detecting tumor proliferation [14-18]. TK1 activity is extremely sensitive to ionizing radiation, and the changes in FLT uptake level directly reflect the biological effect of radiation therapy [19, 20]. Several studies have shown that tumor proliferation level decreases after radiotherapy, as detected by FLT PET [11, 15, 18, 21]. As for the head and neck carcinomas, Molthoff et al. [21] demonstrated that FLT is useful for predicting the tumor response and regrowth in HNX-OE xenografts. They demonstrated the relationship between in vivo tracer uptake (FLT and FDG) and tumor growth curve after “repetitive irradiation” (fractionated radiotherapy), and found the predictive value of the PET tracer uptake on tumor growth. However, they performed histopathological analysis of Ki-67 only at late stages (38 and 36 days after radiotherapy) in their study. They also performed another study to determine the radiosensitivity of HNX-OE xenografts using the tumor growth curve after

different single doses in radiotherapy. However, analysis of sequential changes in tumor proliferative activity at an early stage after radiotherapy using FLT and Ki-67 was not performed. Evaluation of sequential changes in tumor proliferative activity is useful for determining the optimal timing of and dose in the “repetitive irradiation”. Accordingly, in our study, we evaluated the sequential changes in intratumoral proliferative activity at an early stage after single-dose radiotherapy in human head and neck cancer xenografts, using FLT and compared the results with those of evaluation using another proliferative marker, Ki-67.

## **Materials and Methods**

### ***Tumor xenograft model and irradiation***

Nine-week-old male BALB/c athymic nude mice (supplied by Japan SLC, Inc., Hamamatsu, Japan) were used in these experiments. All experimental protocols were completely approved by the Laboratory Animal Care and Use Committee of Hokkaido University. A human head and neck cancer xenograft model was established using the human head and neck cancer cell line FaDu (American Type Culture Collection, Manassas, VA USA), which is an established human hypopharyngeal squamous cell carcinoma and grows as an undifferentiated carcinoma in nude mice. The FaDu cells were maintained in Eagle’s minimum essential medium (Invitrogen Life Technologies, Inc., Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), penicillin-streptomycin, amino acid, and 0.03% glutamine. The cells were incubated in an



atmosphere of 5% CO<sub>2</sub> and 95% air at 37°C. The FaDu cells (5x10<sup>6</sup> cells/0.1 ml) were inoculated subcutaneously into the right flank of each mouse. All animal manipulations were performed using sterile techniques.

Twelve to thirteen days after inoculation (when the tumors reached 10-12 mm in diameter), the mice were randomly assigned to three groups: one non-radiation-treated control and two radiation-treated (10 and 20 Gy) groups (n=24 for each group) (Fig. 1). Data were collected at four time points for each group (6, 24, and 48 hrs, and 7 days from irradiation to sacrifice; n=6 for each time point). A tumor growth curve was derived from the animals assigned to day 7 in each group. Tumor size was measured using a caliper everyday from the start of radiation treatment, and tumor volume was calculated using the following formula:  $\pi/6 \times \text{larger diameter} \times (\text{smaller diameter})^2$ .

In the radiation-treated groups, mice were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg body weight) and positioned on the table of the radiation chamber. A mouse was covered with a cotton sheet to prevent hypothermia during radiation treatment. A lead shielding block was used to protect the non target parts of the body of the mouse from radiation. A round hole (12-15 mm in diameter) was made in the lead shield and the tumor of each mouse was exposed properly to the irradiation field. Tumors were irradiated at a single dose of 10 and 20 Gy using an X-ray generator (MBR- 1520R-3; Hitachi, Tokyo, Japan) at a dose rate

of 1.69 Gy/min (150 kV, 20 mA, 2 mm Al filter). In the non-radiation-treated control groups, the mice were also anesthetized by intraperitoneal injection of pentobarbital, as a sham operation.

***FLT autoradiography, Ki-67 immunohistochemistry and H&E staining***

[Methyl- $^3\text{H}$  (N)]-3'-fluoro 3'-deoxythymidine ( $^3\text{H}$ -FLT) (specific activity, 74–370 GBq/mmol) was purchased from Moravek Biochemicals Inc., CA. Mice were injected with 0.185 MBq of  $^3\text{H}$ -FLT in the tail vein. Two hours after  $^3\text{H}$ -FLT injection, these mice were sacrificed and tumors were immediately excised. Each excised tumor tissue was then sectioned at 2 to 3 mm thickness to maximize the division surface of the obtained slice, embedded in Tissue-Tek medium (Sakura Finetechnical Co., Ltd., Tokyo, Japan), and frozen in isopentane/dry ice. The frozen specimens were cut into cryosections, with one 10- $\mu\text{m}$  thick cryosection used for autoradiography (ARG) and two adjacent 5- $\mu\text{m}$  thick cryosections for Ki-67 immunohistochemistry (IHC) and hematoxylin and eosin (H&E) staining, using a CM3050-Cryostat (Leica Microsystems, Wetzlar, Germany) at  $-20^\circ\text{C}$ . The 10- $\mu\text{m}$  thick cryosections were placed in a phosphor image plate cassette, together with a set of calibrated standards [23], and the cassette was subjected to ARG for 5 weeks to detect the distribution of  $^3\text{H}$ -FLT. ARG images were analyzed using a computerized imaging analysis system (FLA 7000 Bio-Imaging Analyzer; Fuji Photo Film Co., Ltd., Tokyo, Japan).

One of the adjacent 5- $\mu$ m thick sections was immunohistochemically stained for Ki-67 to assess tumor proliferation. Briefly, after rehydration and antigen retrieval, endogenous peroxidase activity was blocked by incubating with methanol containing 0.3% hydrogen peroxide. Thereafter, sections were incubated with a monoclonal rabbit anti-human Ki-67 antibody (clone SP6) (Thermo Fisher Scientific, CA). The bound antibody was visualized using the avidin/biotin conjugate immunoperoxidase procedure with a histofine SAB-PO kit (Nichirei, Tokyo, Japan) and 3, 3'-diaminobenzidine tetrahydrochloride. The slides were counterstained with Mayer's hematoxylin solution (Wako, Osaka, Japan). Images of the tumor sections stained by the anti-Ki-67 antibody were captured under a microscope (Biozero BZ-8000; Keyence Co., Osaka, Japan) and converted to black and white using Image J. The other adjacent 5- $\mu$ m thick section was stained with H&E.

### ***Quantitative analysis of $^3\text{H}$ -FLT by ARG and Ki-67 by IHC***

For the quantitative evaluation of  $^3\text{H}$ -FLT radioactivity, regions of interest (ROIs) were placed to cover the entire tumor tissue on each ARG image with reference to the H&E sections. The radioactivity in each ROI was shown by photostimulated luminescence per unit area, PSL/mm<sup>2</sup> (PSL = a.D.t: a = constant; D = radioactivity exposed on the imaging plate; t = exposed time); then the count of PSL/mm<sup>2</sup> from tumor sections was recorded, calculated and converted to the percentage injected dose per gram (%ID/g) of tissue using the activity of the standards, with

the assumption that tissue density is  $1 \text{ g/cm}^3$  after normalization of tumor with body weight ( $\% \text{ID/g} \times \text{kg}$ ) [22, 23]. For the quantitative analysis of tumor proliferation, the Ki-67 labeling index, that is, the percentage of the number of Ki-67 positive nuclei to the total number of nuclei was used. The Ki-67 positive nuclei and total nuclei were counted under a microscope field ( $\times 400$  objective magnification,  $0.644 \text{ mm}^2$  per field) using Image J. Twelve fields per section were randomly analyzed excluding peripheral connective tissue and central necrotic tissue.

### ***Statistical analysis***

All statistical analyses were performed using Stat View version 5.0 (SAS Institute, Inc.). All values are expressed as means  $\pm$  SD (standard deviation). One-factor repeated measures analysis of variance (ANOVA) was carried out to compare tumor volume among the non-radiation-treated control and radiation-treated (10 and 20 Gy) groups (Fig. 2). A two-way factorial ANOVA was performed to compare intratumoral  $^3\text{H}$ -FLT uptake level (Fig. 4a) and the percentage of Ki-67 labeling index (Fig. 5a) with time among the three groups. One-way ANOVA followed by a Bonferroni post-hoc test was used to assess the significance of difference among the three groups at each time point and among the time points in each group for tumor volume (Fig. 2) and intratumoral  $^3\text{H}$ -FLT uptake level (Fig. 4a, 4b). A  $P$ -value of  $< 0.05$  was considered significant.

## Results

### *Tumor growth curve*

The tumor growth curves are shown in Fig. 2. In the non-radiation-treated control group, tumor volume increased with time. Compared with the control group, the tumor growth was significantly suppressed in mice treated with 10- and 20-Gy radiation, but the tumor volume in these mice gradually increased with time. A significant difference in tumor volume was observed between the non- radiation-treated control and radiation-treated groups on day 7 (\*  $P < 0.001$  for both Control vs Treat-10 Gy and Control vs Treat-20 Gy), whereas no statistically significant difference was observed between the two radiation-treated groups. No significant differences in tumor volume were noted among the three groups until day 6.

### *Adjacent image comparison of $^3\text{H}$ -FLT ARG and Ki-67 IHC*

Figure 3 shows the representative images of  $^3\text{H}$ -FLT ARG and Ki-67 IHC. In the non-radiation-treated control group,  $^3\text{H}$ -FLT ARG showed high intratumoral  $^3\text{H}$ -FLT uptake levels at all time points. Intratumoral  $^3\text{H}$ -FLT uptake level diffusely decreased at 6 hrs, and then gradually increased with time 24 and 48 hrs, and day 7 after radiation treatment with 10 and 20 Gy (Fig. 3). Distributions of Ki-67 positive nuclei at 6, 24, and 48 hrs and on day 7 were visually similar in

non-radiation-treated control and radiation-treated groups (Fig. 3). H&E staining showed a significant increase in necrotic area on day 7 in the control group.

### ***Quantitative analysis of $^3\text{H}$ -FLT by ARG and Ki-67 by IHC***

Figure 4 shows the quantitative analysis of intratumoral  $^3\text{H}$ -FLT uptake level. In the non-radiation-control group, the  $^3\text{H}$ -FLT uptake level slightly increased with time, but the differences were not significant. Six hours after the radiation treatment with 10 and 20 Gy, intratumoral  $^3\text{H}$ -FLT uptake level significantly decreased to 45 and 40%, compared with that in the non-radiation-treated control groups ( $P < 0.0001$ ). Thereafter, a gradual increase in intratumoral  $^3\text{H}$ -FLT uptake level was observed with time in both radiation-treated groups, and the  $^3\text{H}$ -FLT uptake levels at 48 hrs and on day 7 were significantly higher than that at 6 hrs in the corresponding radiation-treated group (Fig. 4b). The intratumoral  $^3\text{H}$ -FLT uptake levels were 68 and 60% at 24 hrs ( $P < 0.001$  for both), 71 and 77% at 48 hrs ( $P < 0.001$  for both), and 83 and 81% on day 7 ( $P = \text{NS}$  for both) in the mice treated with 10 and 20 Gy, respectively, compared with the non-radiation-treated control mice (Fig. 4a). The  $^3\text{H}$ -FLT uptake levels in the tumors were  $4.45 \pm 0.69$ ,  $2.01 \pm 0.30$ , and  $1.79 \pm 0.59$  [ $(\% \text{ID/g}) \times \text{kg}$ ] at 6 hrs,  $4.15 \pm 0.48$ ,  $2.81 \pm 0.50$ , and  $2.52 \pm 0.84$  [ $(\% \text{ID/g}) \times \text{kg}$ ] at 24 hrs,  $4.94 \pm 0.81$ ,  $3.53 \pm 0.83$ , and  $3.81 \pm 0.46$  [ $(\% \text{ID/g}) \times \text{kg}$ ] at 48 hrs, and  $6.00 \pm 1.41$ ,  $5.05 \pm 0.47$ , and  $4.90 \pm 0.95$  [ $(\% \text{ID/g}) \times \text{kg}$ ] on day 7 in the non-radiation-treated control and the 10-Gy and 20-Gy radiation-treated groups, respectively (Fig. 4).

Quantitative analysis of Ki-67 by IHC is shown in Fig. 5. No significant differences were observed in the percentage of Ki-67 index with time and among the three groups. The Ki-67 labeling indices in tumor tissues were  $61.6 \pm 21.1$ ,  $53.3 \pm 20.8$ , and  $54.2 \pm 21.1\%$  at 6 hrs,  $58.5 \pm 11.1$ ,  $56.0 \pm 10.1$ , and  $52.4 \pm 16.4\%$  at 24 hrs,  $60.2 \pm 19.8$ ,  $58.53 \pm 12.1$ , and  $58.0 \pm 15.7\%$  at 48 hrs, and  $71.9 \pm 12.4$ ,  $65.9 \pm 5.0$ , and  $64.0 \pm 8.0\%$  on day 7 in the non- radiation-treated control and the 10-Gy and 20-Gy radiation-treated groups, respectively (Fig. 5a, 5b).

## Discussion

The major findings of this study are that intratumoral  $^3\text{H}$ -FLT uptake level significantly decreased at 6 hrs and then gradually increased with time in the radiation-treated groups (Fig. 3a, 4a, 4b), although the tumor proliferation marker, the Ki-67 labeling index, did not show significant changes among the non-radiation-treated control and radiation-treated groups (Fig. 5a, 5b). In the radiation-treated groups, tumor growth was suppressed compared with that in the non-radiation-treated control group, but tumor volume gradually increased with time (Fig. 2). These findings indicate that  $^3\text{H}$ -FLT can be used to sensitively evaluate intratumoral proliferative activity at an early stage after a single dose in the radiotherapy of head and neck xenografts.

In our study, the intratumoral  $^3\text{H}$ -FLT uptake level significantly decreased at early time points (6 hrs) and then gradually but significantly increased with time. As FLT is a substrate of TK and FLT uptake positively correlates with cell growth and TK1 activity, the rapid decrease in

FLT uptake level observed may be due to a rapid decrease in TK1 activity after radiation treatment [15, 19, 24]. Fowler et al. suggested that as soon as a proportion of cells have been killed after radiotherapy, the size of the tumor cord shrinks, indicating that enhanced nutrition is available to all surviving cells, which leads to the repopulation of surviving cells [25]. Surviving cells after radiotherapy change to an aggressive phenotype [21] and head and neck cancer shows a higher tendency of repopulation after radiotherapy than the other cancer. After radiotherapy, the strategy to deal with damaged DNA can be divided into three components in eukaryotes: recognition of the injured DNA; a period of damage assessment; and the implementation of the appropriate response, namely, DNA repair and redistribution of cells among the cell-cycle phases or cell death [26, 27]. DNA repair and redistribution of cells among the cell cycle might lead to cellular proliferation, which may increase TK1 activity [24] and may be reflected by the increase in intratumoral FLT uptake level with time after a single dose of radiotherapy in our study.

Reoxygenation and repopulation may occur after the repair of potentially lethal/sublethal radiation damage and redistribution of cells among the cell-cycle phases; these processes are denoted as the “four R’s” of radiotherapy [27, 28]. To determine the optimal timing of and dose in repetitive irradiation, it is important to detect tumor repopulation stimulated by radiation. It is difficult, however, to specify the exact timing for DNA repair and repopulation, because the DNA repair timing and the start of repopulation depending on the tissue type and cell cycle phase.



Wither et al. [28] showed that after radiotherapy, DNA repair occurs earlier and the repair rate is related to the proliferative activity of the tissue. Highly proliferative tissues manifest a response within hours, days, or weeks. Head and neck cancers are very aggressive and show a higher tendency of repopulation after radiotherapy than other cancers [3]. Thus, our data may indicate the early repair of radiation-induced damage followed by repopulation.

Ki-67 is a nuclear protein that is expressed in cycling cells but not in quiescent cells. Therefore, it represents a measure of tumor growth fraction, and Ki-67 labeling index is considered as a gold standard for measuring tumor cell proliferation. Ionizing radiation damages the DNA of proliferating tumor cells resulting in the inhibition of tumor growth [29]. However, on the basis of the previous reports, the role of Ki-67 after radiotherapy is controversial. Valente et al. [30] showed that the Ki-67 index decreased after 10 Gy radiotherapy, which provides an independent variable of responsiveness to radiotherapy in oral squamous cell carcinoma. Takahashi et al. [10] showed the transitional increase in Ki-67 index after a single dose in radiotherapy, which is useful for predicting the tumor response to radiotherapy. Koutsimpelas et al. [29] observed no significant difference in Ki-67 index between the treated and control groups 7 days after 6 Gy radiation treatment, but the Ki-67 index was significantly higher in the radiation-treated human hypopharynx squamous cell carcinoma than in the control group on day 70. Thus, the changes in Ki-67 expression level should be reevaluated in head and neck cancer after radiotherapy. In our study, we found the suppression of tumor growth after radiation

treatment compared with the non-radiation-treated control group. However, there were no significant changes in Ki-67 index among the non-radiation-treated control and radiation-treated groups in our study. These findings are partly consistent with the results obtained by Koutsimpelas et al. [29]. The sensitivity of cells to radiation varies widely depending on which phase the cells are in during radiation. Cells in the G2 and M phases are about three times more sensitive than cells in the S phase. Following irradiation, the mammalian cell cycle halts in the G1 and/or G2M phase and is delayed in the S phase [15, 31]. A stopped or delayed mitotic division due to the G2M phase arrest and a prolonged cycling time may lead to suppression of tumor growth. Ki-67 is expressed during the late G1, S, G2, and M phases of the cell cycle. Radiotherapy causes G2M phase arrest and the arrested cells during the cell cycle also contain Ki-67 even though they are not actively proliferating [32], which may explain the absence of changes in Ki-67 index in the tumor after radiotherapy.

It is of interest to compare the present findings with those of monitoring using  $^{18}\text{F}$ -FDG.  $^{18}\text{F}$ -FDG is a widely used PET tracer for diagnosis, staging, and restaging of a wide variety of tumors by reflecting glucose metabolism. However,  $^{18}\text{F}$ -FDG highly accumulates in inflammatory lesions [33] and radiotherapy causes acute inflammation. Therefore,  $^{18}\text{F}$ -FDG PET is often not applicable to monitoring the early tumor responses to radiotherapy [15, 31]. On the other hand, several studies demonstrated that FLT-PET can noninvasively predict early tumor responses to radiotherapy [15, 18, 21]. Radiotherapy causes cellular DNA damage leading to

decreased intracellular proliferation, which can noninvasively be detected by FLT PET [15, 18]. Moreover, in our study, early tumor response (6 hrs) to radiotherapy was detected in term of the significant decrease in intratumoral  $^3\text{H}$ -FLT uptake level. Thereafter, a gradual increase in intratumoral  $^3\text{H}$ -FLT uptake level was detected, which may reflect the tumor repopulation. Indeed, tumor growth was suppressed in the radiation-treated groups, but tumor volume gradually increased with time. Thus FLT would be a potential tracer for detecting the early tumor response and tumor repopulation following radiotherapy.

In conclusion, intratumoral FLT uptake level significantly decreased at an early time point and then gradually increased with time, reflecting intratumoral proliferative activity after a single dose in the radiotherapy of head and neck cancer xenografts. These findings suggest that sequential evaluation of intratumoral proliferative activity using FLT can be beneficial for determining the precise radiotherapy planning, that is, determining the optimal timing of and dose in repetitive irradiation with modern radiotherapy modalities for human head and neck cancers.

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Conflict of interest No potential conflicts of interest were disclosed.

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## Figure legends

**Fig. 1** Experimental protocol of this study.

**Fig. 2** Growth curves of tumors in non-radiation-treated control and radiation-treated (10 and 20 Gy) mice. One-factor repeated measures ANOVA showed significant changes in tumor growth curves among the three groups ( $F = 94.03$ ;  $P < 0.0001$ ). There was a significant interaction between tumor volume and radiation dose ( $F = 11.24$ ;  $P < 0.0001$ ). One-way ANOVA followed by the Bonferroni post-hoc test showed significant differences in tumor volume between the non-radiation-treated control and radiation-treated groups on day 7 (\*  $P < 0.001$  for both Control vs Treat-10 Gy and Control vs Treat-20 Gy). No significant differences were observed between the two radiation-treated groups. Data are expressed as mean  $\pm$  SD. Control, non-radiation-treated control group. Treat-10 Gy, group treated with radiation at 10 Gy. Treat-20 Gy, group treated with radiation at 20 Gy.

**Fig. 3** (a) Representative images of  $^3\text{H}$ -FLT ARG (left), immunohistochemical staining of Ki-67 (middle) and H&E staining (right). (b) Representative magnified images of selected area of Ki-

67 IHC (left) and H&E staining (right). Control, non-radiation-treated control group. Tt-10 Gy, group treated with radiation at 10 Gy. Tt-20 Gy, group treated with radiation at 20 Gy.

**Fig. 4** Quantitative analysis of intratumoral  $^3\text{H}$ -FLT uptake level by ARG. (a) Two-way factorial ANOVA showed significant changes in intratumoral  $^3\text{H}$ -FLT uptake level with time ( $F = 43.03$ ;  $P < 0.0001$ ) and among the groups ( $F = 38.97$ ;  $P < 0.0001$ ). The interaction between the effects of radiation dose and time point was not significant ( $F = 1.9$ ;  $P = 0.85$ ). One-way ANOVA followed by the Bonferroni post-hoc test showed significant changes in intratumoral  $^3\text{H}$ -FLT uptake level among the three groups 6 hrs (\*\*  $P < 0.0001$ ), 24 hrs (\*  $P < 0.001$ ), and 48 hrs (\*  $P < 0.001$ ) after the radiation treatment. (b) Significant changes in intratumoral  $^3\text{H}$ -FLT uptake level were observed at various time points; 6 vs 48 hrs ( $\dagger P < 0.001$ ) and 6 vs day 7 ( $\dagger\dagger P < 0.0001$ ) for the groups treated at 10 and 20 Gy. Control, non-radiation-treated control group. Treat-10 Gy, group treated with radiation at 10 Gy. Treat-20 Gy, group treated with radiation at 20 Gy.

**Fig. 5** Quantitative analysis of Ki-67 labeling index. (a, b) No significant differences were observed in Ki-67 index among the three groups ( $F = 1.02$ ;  $P = 0.36$ ) at various time points ( $F = 2.1$ ;  $P = 0.10$ ) and their interactions ( $F = 0.08$ ;  $P = 0.99$ ). Control, non-radiation-treated control

group. Treat-10 Gy, group treated with radiation at 10 Gy. Treat-20 Gy, group treated with radiation at 20 Gy.

### ***Supplemental Materials and methods***

[Methyl-<sup>3</sup>H (N)]-3'-fluoro 3'-deoxythymidine (<sup>3</sup>H-FLT) (specific activity, 74–370 GBq/mmol) was purchased from Moravek Biochemicals Inc., CA. Mice were injected with 0.185 MBq of <sup>3</sup>H-FLT in the tail vein. Two hours after the <sup>3</sup>H-FLT injection, the mice were sacrificed, and tumors were immediately excised. Each excised tumor was then sectioned to obtain two adjacent slices. One slice was embedded in Tissue-Tek medium (Sakura Finetechnical Co., Ltd., Tokyo, Japan) and frozen in isopentane/dry ice for ARG. The radioactivity of <sup>3</sup>H-FLT was determined using a liquid scintillation counter (LSC) using the other slice. Tracer uptake level in the tissue was expressed as the percentage of injected dose (ID) per gram of tissue after being normalized to the animal's weight [(%ID/g) × kg].

### ***Supplemental results***

Supplemental Figure 1 shows the intratumoral <sup>3</sup>H-FLT uptake level determined using a liquid scintillation counter (LSC). In the non-radiation-control group, the <sup>3</sup>H-FLT uptake level slightly increased with time, but was not significantly different from those of the treated groups. Six hours after the radiation treatment at 10 and 20 Gy, intratumoral <sup>3</sup>H-FLT uptake level significantly decreased to 45 and 43% compared with that in the non-radiation-treated control group ( $P < 0.0001$ ). Thereafter, a gradual increase in intratumoral <sup>3</sup>H-FLT uptake level was observed in both radiation-treated groups, and the <sup>3</sup>H-FLT uptake levels at 48 hrs and on day 7

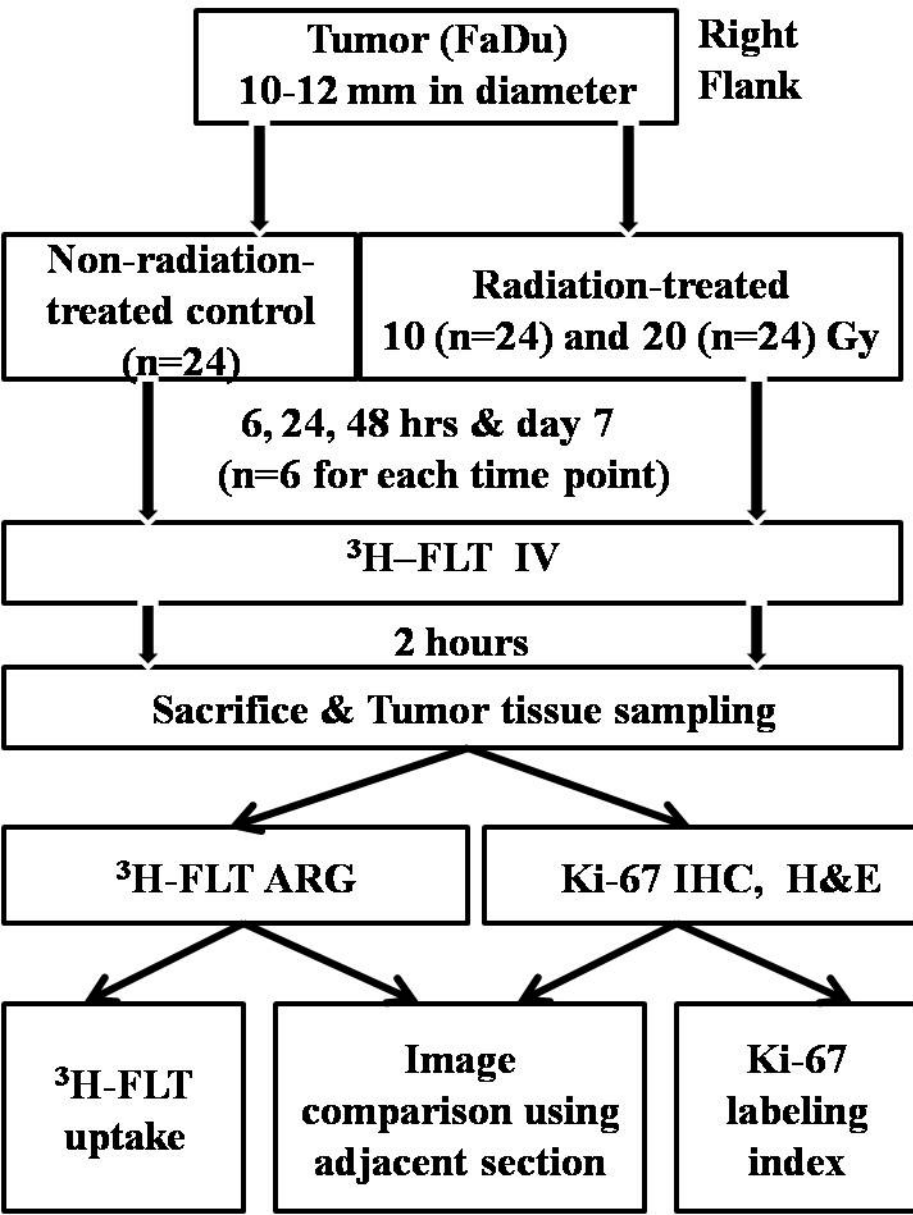
were significantly higher than that at 6 hrs in both radiation-treated groups (Suppl. Fig. 1b). The intratumoral  $^3\text{H}$ -FLT uptake levels were 66 and 57% at 24 hrs ( $P < 0.001$  for both), 65 and 70% at 48 hrs ( $P < 0.001$  for both), and 82 and 71% on day 7 ( $P = \text{NS}$  for both) in the mice treated with 10 and 20 Gy, respectively, compared with the non-radiation-treated control mice (Suppl. Fig. 1a). The  $^3\text{H}$ -FLT uptake levels in the tumors were  $0.117 \pm 0.19$ ,  $0.053 \pm 0.007$ , and  $0.050 \pm 0.016$  [(%ID/g)  $\times$  kg] at 6 hrs,  $0.116 \pm 0.015$ ,  $0.077 \pm 0.011$ , and  $0.066 \pm 0.022$  [(%ID/g)  $\times$  kg] at 24 hrs,  $0.128 \pm 0.021$ ,  $0.083 \pm 0.013$ , and  $0.090 \pm 0.015$  [(%ID/g)  $\times$  kg] at 48 hrs, and  $0.139 \pm 0.040$ ,  $0.114 \pm 0.022$ , and  $0.097 \pm 0.024$  [(%ID/g)  $\times$  kg] on day 7 in the non-radiation-treated control and the 10-Gy and 20-Gy radiation-treated groups, respectively.

**Supplemental Fig. 1** Quantitative analysis of intratumoral  $^3\text{H}$ -FLT uptake level using a liquid scintillation counter. (a) Two-way factorial ANOVA showed significant changes in intratumoral  $^3\text{H}$ -FLT uptake level with time ( $F = 45.17$ ;  $P < 0.0001$ ) and among the groups ( $F = 15.56$ ;  $P < 0.0001$ ). The interaction between the effects of radiation dose and time point was not significant ( $F = 1.3$ ;  $P = 0.24$ ). One-way ANOVA followed by the Bonferroni post-hoc test showed significant changes in intratumoral  $^3\text{H}$ -FLT uptake level among the three groups at 6 hrs (\*\*  $P < 0.0001$ ), 24 hrs (\*  $P < 0.001$ ), and 48 hrs (\*  $P < 0.001$ ) after the radiation treatment. (b) Significant changes in intratumoral  $^3\text{H}$ -FLT uptake level were observed at various time points; 6

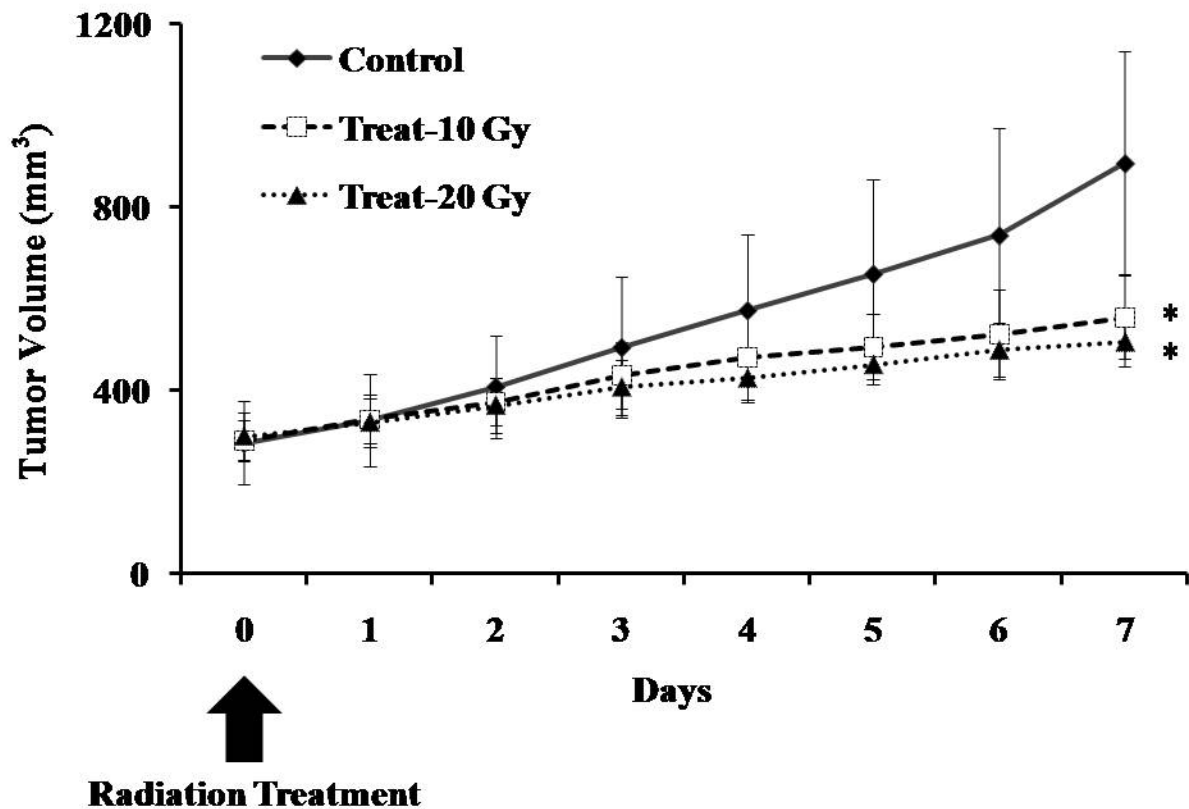
vs 48 hrs ( $\dagger P < 0.001$ ) and 6 vs day 7 ( $\dagger\dagger P < 0.0001$ ) for each group treated at 10 and 20 Gy.

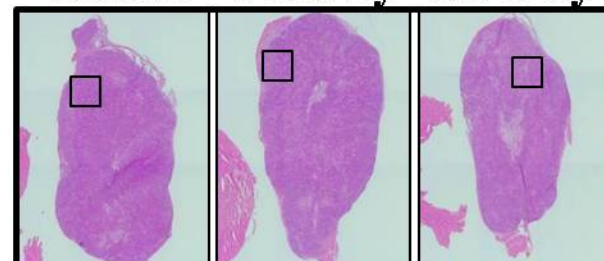
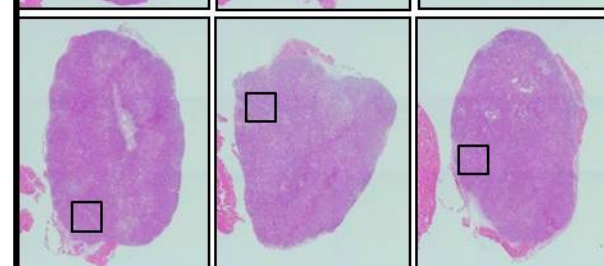
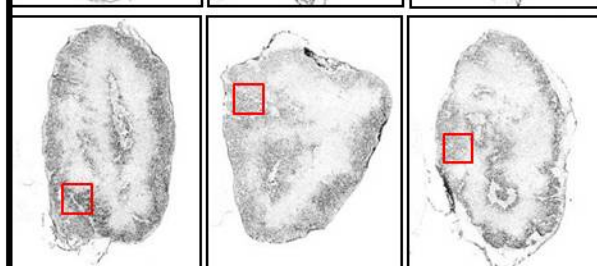
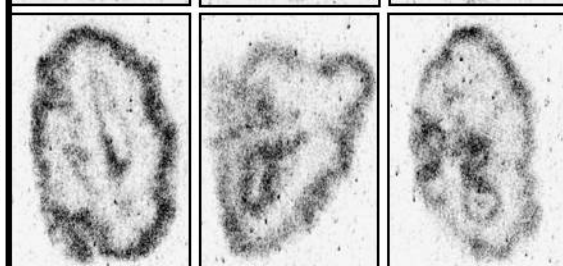
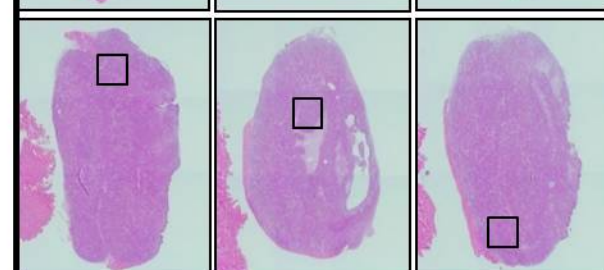
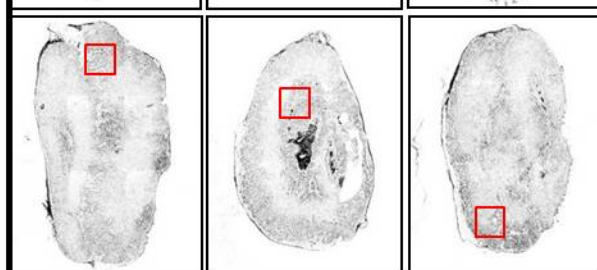
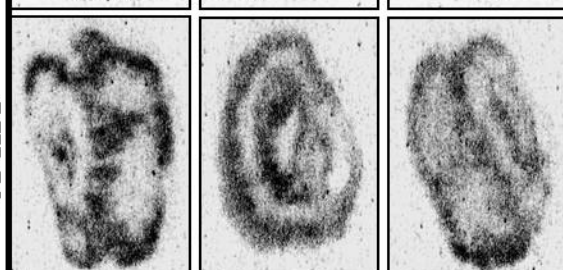
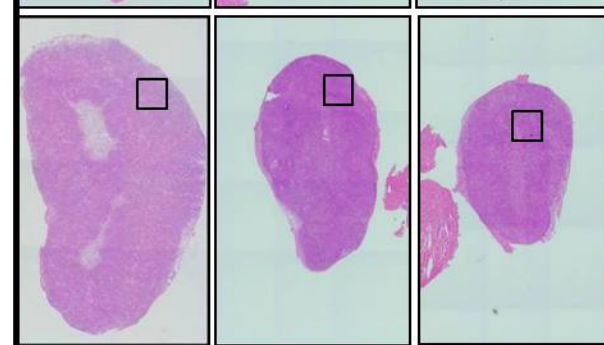
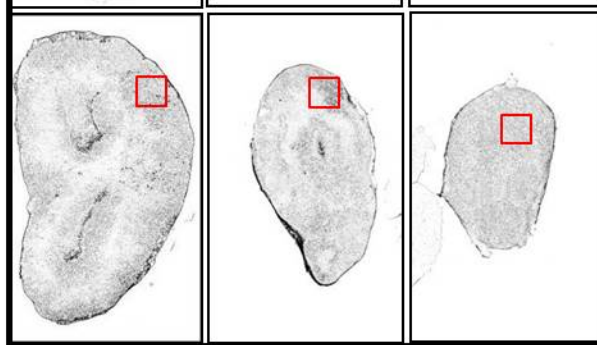
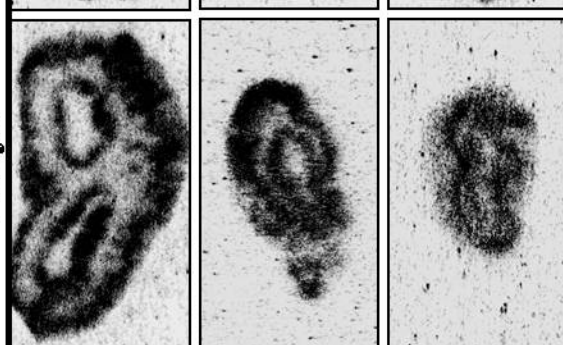
Control, non-radiation-treated control group. Treat-10 Gy, group treated with radiation at 10 Gy.

Treat-20 Gy, group treated with radiation at 20 Gy.







**$^3\text{H}$ -FLT ARG****Ki-67 IHC****H&E****Control Tt-10 Gy Tt-20 Gy****Control Tt-10 Gy Tt-20 Gy****Control Tt-10 Gy Tt-20 Gy****6 hrs****24 hrs****48 hrs****7 days****H****Low**



## Ki-67 IHC

**H&E**

## Control

### Tt-10 Gy

**Tt-20 Gy**

## Control

## Tt-10 Gy

## Tt-20 Gy

6 hrs

24 hrs

48 hrs

7 days

100  $\mu\text{m}$ 100  $\mu\text{m}$ 100  $\mu\text{m}$ 100  $\mu\text{m}$ 100  $\mu\text{m}$ 100  $\mu\text{m}$ 100  $\mu\text{m}$ 100  $\mu\text{m}$ 100  $\mu\text{m}$ 100  $\mu\text{m}$ 100  $\mu\text{m}$ 100  $\mu\text{m}$ 100  $\mu\text{m}$ 100  $\mu\text{m}$ 100  $\mu\text{m}$ 100  $\mu\text{m}$ 100  $\mu\text{m}$ 100  $\mu\text{m}$ 100  $\mu\text{m}$ 100  $\mu\text{m}$ 100  $\mu\text{m}$ 100  $\mu\text{m}$ 100  $\mu\text{m}$ 100  $\mu\text{m}$

