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Research Article

Establishment of an Intracranial Xenograft Model from Colorectal Cancer in Irradiated Mice

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ABSTRACT

Colorectal cancer (CRC) is the most common type of gastrointestinal cancer metastasizing to the brain. In addition, patients with brain metastasis from CRC have low mean survival time. Preclinical studies play a crucial role in understanding histopathological characteristics of brain tumors and the discovery of anticancer agents. To conduct preclinical studies pertaining to brain metastasis, mouse models are often based on brain-tropic cancer cell lines or spontaneous incidence in orthotropic mouse models, genetically engineered mouse models or patient-derived xenografts. These models could recapitulate metastatic processes and genetic mutations in brain metastasis, but have particular drawbacks pertaining to low yield, prolonged time and concurrent metastases in other organs. Moreover, in xenograft models, genetically immunodeficient mice are often employed because of their long-term immunodeficiency, but they still have some certain constraints. In this study, we examined the ability of the human colorectal cancer cell line HCT116 to grow into intracranial tumors in BALB/c mice immunosuppressed by irradiation. In the irradiated group, 5/5 mice had intracranial tumors with the median tumor volume reaching 4.68x10^6 µm3 after a 7-day follow-up. The presence of colorectal tumors in the mouse brains was confirmed by histopathology. The results showed that irradiation at the dose of 3Gy x 2 caused immunodeficiency in healthy BALB/c mice and HCT116 cells could initiate tumors intracranially in BALB/c mice immunosuppressed by irradiation with a high take rate. BALB/c mice can be used for xenograft models via immunosuppression by irradiation. In addition, the human colorectal cancer cell line HCT116 shows the potential ability to form brain tumors in research animals.

Keywords:
HCT116; Brain tumors; BALB/c mice; Irradiation

1. INTRODUCTION

Colorectal cancer (CRC) is the third most common malignancy in the world[1] and the most common type of gastrointestinal cancer metastasizing to the brain with the incidence rate ranging from 0.1% to 11.5%. Mean survival time in brain metastasis from CRC remains low, ranging from 2 to 9.6 months. Risk factors of brain metastasis from CRC in patients include lung metastasis from CRC and *KRAS* mutations[2]. Brain metastases from CRC are distant-stage colorectal tumors with high malignancy and common symptoms include headache, motor disturbance, mental change, nausea or vomiting, seizure, aphagia, or visual disturbance according to the

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functional brain area involved3. Treatment for brain metastasis from CRC mainly depends on radiotherapy, surgical resection, chemotherapy, or a combination of the latter[2].

Animal models have been widely used in research for the screening of drug candidates, establishing the efficacy of anticancer agents and studying their effects on key hallmarks of cancer, including angiogenesis, invasiveness and necrosis[4]… In preclinical studies, mice are usually utilized to establish animal research models[5]. For xenograft models, the use of immunodeficient mice is necessary to reduce rejection responses because the presence of immune cells can hamper the proliferation of cancer cells *in vivo*[6]. In such studies, genetically immunodeficient mice (BALB/c nude mice, NOD/SCID mice, NOG mice or NIH-3 nude mice…) are usually employed because of their life-long immunodeficiency, therefore, they could bear foreign xenografts for scientists to explore anticancer effects of novel agents *in vivo* or to study tumor growth and metastasis *in vivo* in the long term[7,8,9]. However, the use of genetically immunodeficient mice has several disadvantages: high cost, unavailability, retarded growth, high mortality rate, and requirements for transportation and maintenance[7,9,10]. Therefore, there have been several studies on xenograft models using immunocompetent mice that are immunosuppressed by different methods, such as total body irradiation or immunosuppressive drugs (cyclosporine, ketoconazole cyclophosphamide…)[11,12,13,14]. In the study on xenografts of Ewing sarcoma and colon carcinoma, Floersheim *et al.* reported that immunosuppressed mice seemed to allow adequate tumor growth for short-term experiments with better animal survival than nude mice[12].

In brain metastasis, the brain environment has unique characteristics: before colonization in the brain, metastatic cancer cells have to cope with distinct metabolism, extracellular matrix (full of tenascin, laminin and glycosaminoglycans… instead of fibronectin and collagen in other systemic organs) and interact with various tissue-resident cell types (microglia, oligodendrocytes, astrocytes and neurons…)[3,15]. In addition, the blood-brain barrier (BBB) formed by a complex system of endothelial cells, astroglia, pericytes, with continuous tight junctions that prevent the passage of most circulating cells and even many therapeutic agents[3,15]. In the situation of brain tumors, the BBB becomes more permeable to circulating tumor cells, but most systemic therapeutic agents are still hampered to cross the BBB[3,15]. Therefore, it is crucial to establish preclinical models that faithfully recapitulate key characteristics of the brain microenvironment, so animal models using subcutaneous injection of cancer cells may not be relevant. There are several methods to establish an intracranial tumor model based on mice in preparation for the research of brain metastasis. Firstly, a brain metastatic cell line should be established by injecting cancer cells (from humans or rodents) into the arterial circulation of mice and there may be a few cells following the blood stream to penetrate into the brain. Subsequently, these cancer cells will be recovered from the brain, propagated *in vitro* and re-implanted into mice arterially. By repeating this *in vivo* selection process, some studies have established brain-tropic cancer cell lines that are aggressively metastasizing derivatives in the animal brains[16,17]. Then, these brain metastatic cancer cell lines can be used to xenograft animal models via systemic inoculation or intracranial inoculation, however, this approach is rather time-consuming and animals often succumb to the primary tumor burden or concurrent metastases in other organs[18]. In addition, an animal model of brain metastasis can be established from orthotopically implanted models or from genetically engineered animal models which spontaneously develop brain tumors[19,20,21]. Moreover, patient-derived xenografts were also utilized to establish animal models of brain metastasis via orthotopic[22], systemic[23] or intracranial[24] implantation of patient-derived tissue or cells into animals. In these studies, cells undergoing *in vivo* selection are likely to recapitulate key characteristics of brain metastasis in humans. However, these models are time-consuming, show high mortality rates due to the burden of primary tumors and concurrent metastases in other organs, and have low yield, therefore resulting in a large number of animals involved to increase the incidence of brain metastasis in mice[19,20,].

The lack of animal research models regarding brain tumors from CRC makes preclinical studies difficult to assess. In addition, the use of genetically immunodeficient mice for xenograft models also has some disadvantages. Besides, the human colorectal cancer cell line HCT116 was demonstrated to have high potential for organ metastasis[25,26]. Therefore, in the present study, we examined the ability of the human colorectal cancer cell line HCT116 to grow into brain tumors in BALB/c mice immunosuppressed by irradiation.

2. MATERIALS AND METHODS

2.1. Cell line, culture condition and cell preparation

The colorectal cancer cell line HCT116 was obtained from the American Type Culture Collection (ATCC; Virginia, USA). Cells were cultured in the DMEM medium (Cytiva, Massachusetts, USA) containing 10% fetal bovine serum (FBS) (Cytiva, Massachusetts, USA) and 1% Penicillin-Streptomycin (Sigma-Aldrich, Missouri, USA) in an atmosphere of 5% CO2/95% air at 37^{o}C.

Cells, from sub-confluent cultures, were harvested by trypsinization and centrifugation. Cells were then washed with PBS (Solarbio, Germany) twice before resuspending in DMEM media at the concentration of $2x10^5$ cells/µL.

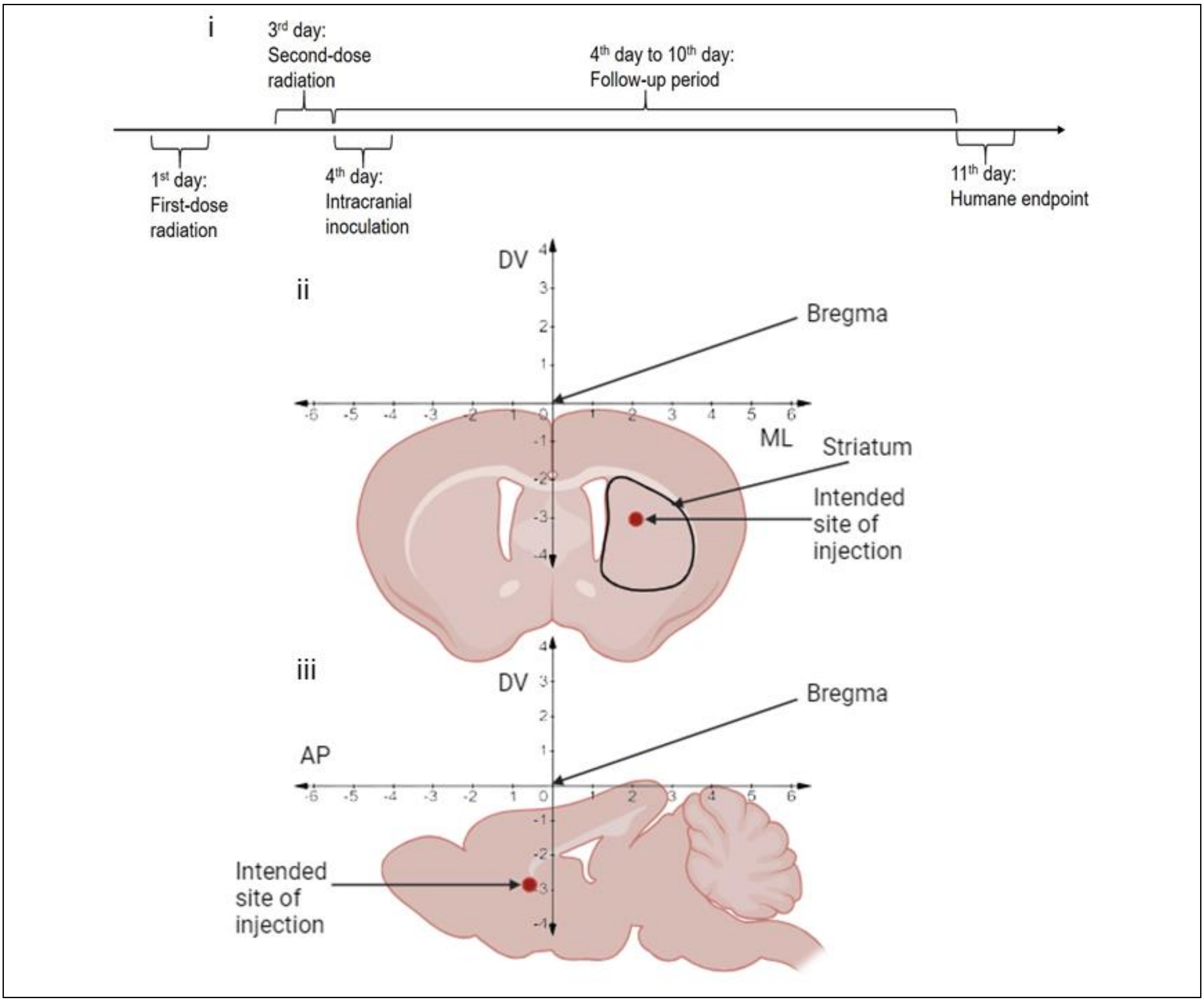

Figure 1. Timeline of the mouse model and coordinates for intracranial injection of cancer cells. (i) Timeline of immunosuppression by irradiation, intracranial inoculation and humane endpoints for mice in the experiment. (ii) The coronal view and (iii) the sagittal view of the mouse brain using the coordinates provided available on the website: https://labs.gaidi.ca/mouse-brain-atlas/. Coordinates for the expected injection site (red dot): 2.0 mm lateral (ML = 2.0 mm), 0.5 mm anterior (AP = 0.5 mm) and 3.0 mm deep (DV = 3.0 mm) to the bregma. The striatum of each hemisphere is the brain parenchyma surrounded by black line. (AP: anteroposterior, ML: mediolateral and DV: dorsoventral).

2.2 Animals

BALB/c mice (BioLASCO, Taiwan) were kept in different cages at the Laboratory Animal Research Center, Vietnam Military Medical University. The caring condition was 60 ± 5% in relative humidity and 27 ± 2°C in temperature.

Healthy BALB/c mice (weighed 20-25g), from 6- to 8- weeks olds, were divided into 2 groups (irradiated and control, n = 10 per group). Mice in the irradiated group were immunosuppressed by X-irradiation at the dose of 3 Gy twice on the 1st and 3rd days (Figure 1i).

2.3 Total WBC, neutrophil and lymphocyte counting

From half of the mice in each group, mouse blood samples were collected by the retro-orbital bleeding technique into EDTA 2-mL test tubes (HTM, Vietnam) on the 4th day and 10th day to evaluate the efficacy of immunosuppression. Total WBC, neutrophil and lymphocyte counting was then performed in an automated hematology analyzer (Sysmex, Japan).

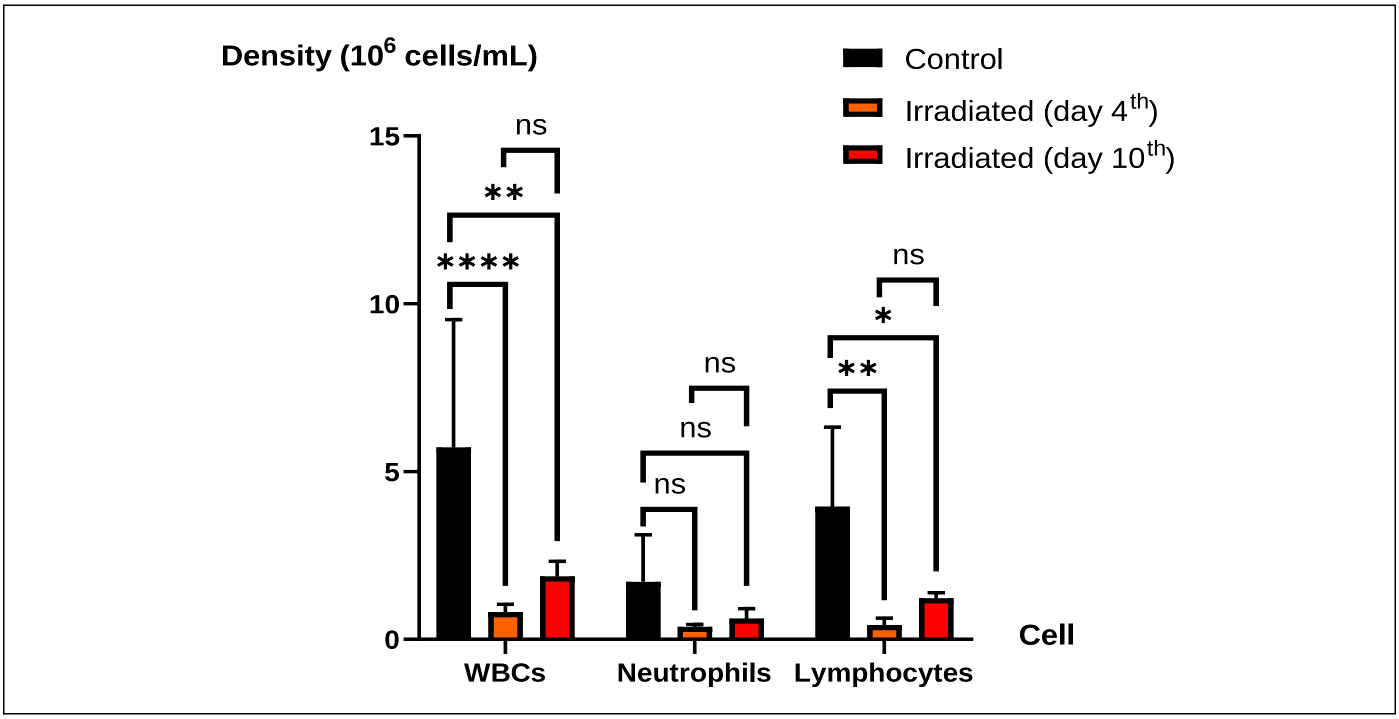

Figure 2. The comparison of density (10^6 cells/mL) of total white blood cells, neutrophils and lymphocytes between the control group and the irradiated group (4 and 10 days after the first irradiation). Data was analyzed by two-way ANOVA test and post-hoc Tukey test. Results were presented as mean ± SD (n = 5 per group). (*P < 0.05, **P<0.01 and ns is not significant).

2.4 Intracranial implantation

The surgical procedure was conducted on the 4th day. Mice (the other 5 mice in each group) were anesthetized intraperitoneally with Ketamin® (Rotexmedica, Germany) at the dose of 162.5 mg/kg body weight and their head fur was removed by hair-removing cream. After each mouse were unresponsive to toe poking, a skin incision was made in the head.

Then, a 1-mm hole was made at 2 mm lateral and 0.5 mm anterior to the bregma (Figure 1ii and 1iii).

Cell suspension (10^6 cells/5µL/mouse) was aspirated by a 5 µL glass microsyringe (Shanghai Heqi Glassware, China) and injected slowly into the mouse brain through the hole at 3 mm deep (Figure 1ii and 1iii). Next, the incision was sutured and mice were detected for survival and their body weight changes (compared with their weight before intracranial inoculation) in the following 7 days.

2.5 Specimen processing and histopathological staining

On the 10th day or when each mouse was dead, brains were collected, fixed in 10% neutral buffered formalin (Leica Biosystems, Germany) and embedded in paraffine (Leica Biosystems, Germany). Next, formalin-fixed and paraffin-embedded (FFPE) specimens were cut into 5-µm-thick layers by a microtome (Leica Biosystems, Germany) and stained with hematoxylin and eosin (H&E) (Leica Biosystems, Germany). Tumor sizes were measured with ImageJ ver. 2.0 (NIH and LOCI, USA) in length (*l*) and width (w) and tumor volumes were calculated according to the following formula:

$$V = \frac{l}{2}\, x\, w^2$$

2.6 Data analysis

Statistical analysis was conducted using GraphPad Prism ver. 8.4 (GraphPad Software, Inc., USA). Three or more groups were compared by two-way ANOVA test and post-hoc Sidak’s multiple comparisons test. Student’s t test or Mann-Whitney test was performed to compare the differences between two groups. Data was shown as mean ± SD or median ± IQR (interquartile range) depending on its normal distribution and $P < 0.05$ was considered significantly different.

3. RESULTS

3.1. Irradiation causes immunosuppression in BALB/c mice.

As shown in Figure 2, healthy BALB/c mice exposed to X ray at the dose of 3Gy each time on the 1st and 3rd days of the experiment showed marked reductions in the density of total white blood cells and lymphocytes in peripheral blood when tested on the 4th day ($P < 0.05$). The implications of irradiation for the

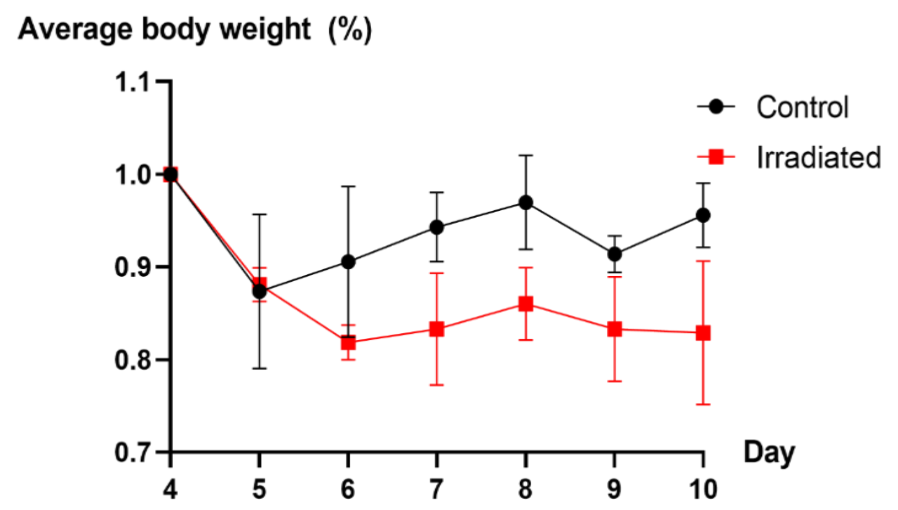

Figure 3. Changes in the average mouse weight (%) (mean ± SD) between the control and irradiated groups in 7 days after intracranial inoculation (n = 5 per group).

immune system in BALB/c mice lasted up to the endpoint of the experiment (on the 10th day, $P < 0.05$). The number of neutrophils tended to decrease in mice after exposure to the full-dose radiation, however, there was no significant difference in comparison with mice in the control group.

3.2. Tumors grow remarkably in mice immune-suppressed by irradiation.

Surgical procedures were performed to inject 10^6 HCT116 cells intracranially into the striatum in each mouse. All the mice showed recovery after implantation. Mice in the control group were all alive until the endpoint of the experiment. However, the survival rate of the irradiated group was just 3/5 as there were two mice dying consecutively on the 7th and 8th days of the experiments (Table 1). In addition, mice in the control group showed a quick recovery in average body weight while the irradiated group had a dramatic decrease in the percentage of average mouse weight (up to nearly 20%) after the inoculation (Fig. 3), suggesting the progression of malignant tumors *in vivo* in immunosuppressed mice.

When each mouse died or until the endpoint of the experiment, mouse brains were collected, processed into FFPE specimens and stained in H&E. The presence of malignant tumors was confirmed by histopathology. In the images of H&E-stained samples, tumors showed hypercellular cells with atypical nuclei, coarse chromatin and active mitosis (Figure 5i and 5ii), which are characteristics of malignant cells.

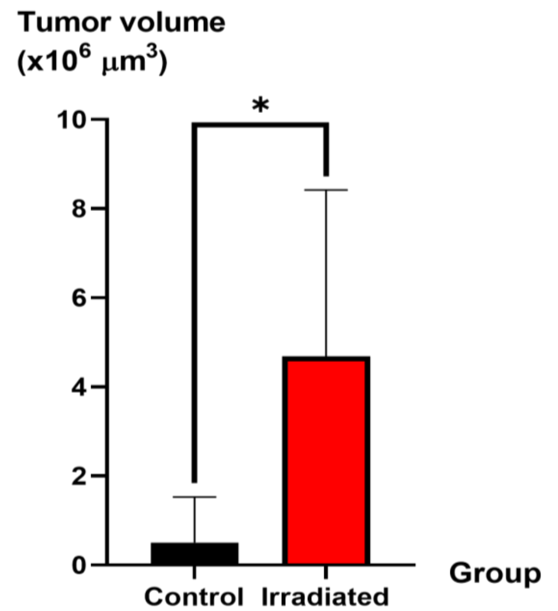

Figure 4. The comparison of tumor volumes between the control group and the irradiated group (n = 5 per group). Data was analyzed by Mann-Whitney test and results were shown as median ± IQR. (*P<0.05).

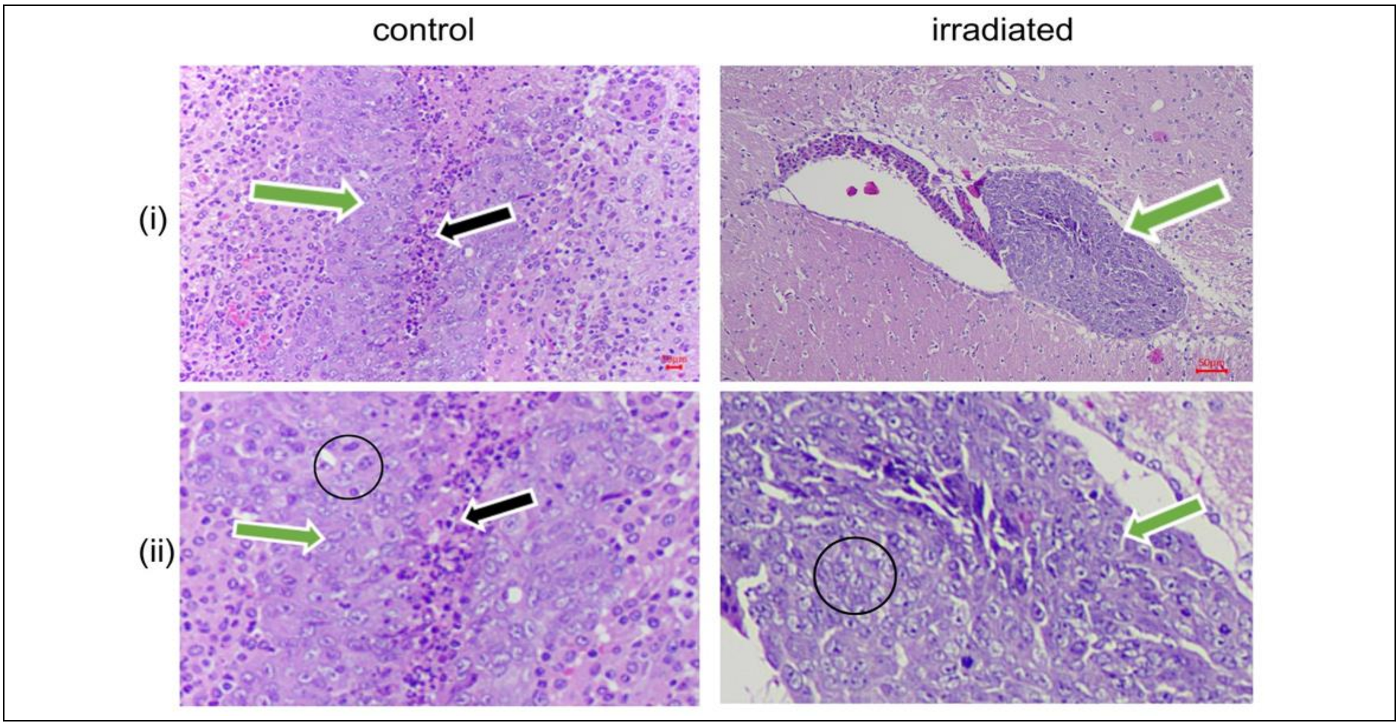

Figure 5. Brain tumors grow in vivo in BALB/c mice after intracranial implantation of HCT116 cells. (i) images of H&E-stained specimens in the control and the irradiated groups under 20x microscope objective; (ii) Tumors grow in vivo with actively mitotic cells. Green arrow: tumor cells having atypical nuclei, coarse chromatin and hypercellularity; black arrow: white blood cells; black circle: tumor cells showing active mitosis.

Regarding engraftment rate, all the mice in the irradiated group had tumors developed intracranially while just 3 out of 5 mice in the control group showed tumors in the brains (Table 1). By comparison, the median tumor volume in the irradiated group was found to be 4.68×10^6 μm^3, higher than that of the control group (0.09×10^6 μm^3), and the difference was statistically significant ($P < 0.05$) (Figure 4).

4. DISCUSSION

Animal models have tremendous importance in the research of cancer. They are useful tools to study histopathological characteristics of cancer and to test anticancer activities of novel agents[27,28]. Because of some disadvantages of genetically immunodeficient mice, there have been studies regarding the use of mice immunosuppressed by different methods to produce xenograft models. In rejection response, tumors from a different species are rejected predominantly by cellular immune response which is initiated by CD4+ T cells[6]. In this study, we developed a xenograft model using healthy BALB/c mice immunosuppressed by total body irradiation and the condition of immunodeficiency in mice in our research was consistent with other studies[11,29]. In either the irradiated group or the control group, 5 out of 10 mice were only used to take blood samples for immunodeficiency assessment while the other 5 mice in each group were injected with cancer cells because mice are vulnerable to death after blood collection. By counting total WBCs, neutrophils and lymphocytes, our study showed remarkable reductions of these cells in peripheral blood though a decrease in neutrophils was not statistically significant. The deficiency in lymphocytes reflected a decrease in T-cell-mediated rejection response. Moreover, the immunosuppression lasted throughout the course of our experiment, and the irradiation was conducted before intracranial inoculation of cancer cells, so it is likely to reduce risks of potential pharmacological interactions with anticancer agents if tested. Amini et al. used a regimen of cyclosporine A, ketoconazole and cyclophosphamide to suppress the immune system of BALB/c mice and their mouse models successfully induced subcutaneous tumors with significant size[14]. However, it is noteworthy that these agents may be administered daily throughout the course of experiments and some models still showed poor take rate of xenograft or marginal tumor growth[12,30]. It may be because these agents show anticancer effects on cancer cells[31,32]. Regarding mouse weight change, we found out that mouse weight in the irradiated group decreased by approximately 20%, suggesting the progression of cancer. It is of note that this drop in body weight may impact the way of outcome interpretation if body weight change is chosen as a criterion for toxicity or antitumor activity of a tested drug in preclinical studies[33].

Table 1. Survival rate and engraftment rate of mice in the control group and the irradiated group at the endpoint of the experiment.

Group	Survival rate	Engraftment rate
Control (n=5)	5/5	3/5
Irradiated (n=5)	3/5	5/5

However, in this study, 2 out of 5 mice in the irradiated group died before the humane endpoint while all the mice in the control group were alive throughout the follow-up. In addition, we figured out that mice in the control group had low take rate (3/5) with minimal tumor development (median tumor volume = $0.09\text{x}10^6$ μm^3) and they were still immunocompetent. Meanwhile, mice in the irradiated group had higher engraftment rate (5/5) with significant tumor size (median tumor volume = $4.68\text{x}10^6$ μm^3 and maximum tumor size = $8.93\text{x}10^6$ μm^3) and were more vulnerable to some pathogens in the environment because of severe immunodeficiency. Perhaps the progression of malignant tumors and vulnerability to pathogens caused premature deaths. To improve survival rate, it might be necessary to use autoclaved water and antibiotics judiciously during experiments[14]. In the irradiated group, the take rate was as high as that in other xenografts using immune-suppressed animals or nude mice[30,34,35]. Histologically, the brain tumors based on HCT116 cells were found to be well-demarcated instead of diffusely infiltrating into the brain parenchyma and seemed not to show angiogenesis. In comparison with tumors in the irradiated group, tumors in the control group were found to be largely infiltrated and surrounded by clusters of white blood cells, suggesting the presence of rejection response against injected cancer cells in immunocompetent mice.

In the light of recent findings, we know that primary tumor cells have to undergo different dynamic steps of *in vivo* metastatic cascade before having the ability to colonize the brain. This process includes invasion, detachment from primary tumors, intra-vasation, escape from immune attacks, extravasation and adaptation to the new microenvironment in metastatic organs[15]. It is recommended to use brain-tropic cancer cells to establish mouse models of brain metastasis. However, depending on different objectives, researchers may omit the *in vivo* selection. Kita et al. used the colorectal cancer cell line KM12SM which exhibits a high potential for brain metastasis after internal carotid artery inoculation[36], or Seehawer et al. also injected Kmt2c and Kmt2d-knockout breast cancer cells into mice using mammary fat pad and intracardiac injections to identify drivers that lead to tropism and adaptation of cancer cells to the brain microenvironment[37]. In this study, we used the human colorectal cancer cell line HCT116, which has been demonstrated to have the ability of distant metastasis, especially in liver and lungs[25,26]. HCT116 cells were also reported to be induced in genes, such as *VEGFA* and *VEGFR2* to be more brain-tropic[38]. Hence, this approach to mouse models of brain tumors can be adopted by focusing on some particular genes related to brain metastasis, which may be more effective than the process of *in vivo* selection of a brain metastatic cell line in some situations. Taken together, a xenograft model of brain tumors from CRC can be approached with the following procedures:

1) Subculturing colorectal cancer cells and immunosuppressing healthy mice by irradiation (recommended dose: 3 Gy x 2).
2) Injecting cell suspension of 10^6 cells/5 μL/mouse at 2 mm lateral, 0.5 mm anterior and 3 mm deep from the bregma.
3) Histopathological staining and evaluating.

5. CONCLUSION

BALB/c mice can be used for xenograft models via immunosuppression by irradiation. In addition, the human colorectal cancer cell line HCT116 shows the potential ability to form brain tumors in research animals.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Ethics approval

This research was reviewed and approved by the the Animal Ethics Committee of Vietnam Military Medical University (VMMU, 2020), Hanoi, Vietnam.

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