# 腫瘍細胞選択的粒子線治療「ホウ素中性子捕捉療法」と抗血管新生薬による症候性脳放射線壊死の治療

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# Cell-selective Particle Radiation, Boron Neutron Capture Therapy and Treatment of Symptomatic Radiation Necrosis in the Brain by Anti-angiogenic Agent

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Boron neutron capture therapy (BNCT) has been advocated as a novel particle radiation therapy for malignant tumors that targets tumor cells biologically. Since 2002, we have applied this unique radiotherapy for 133 malignant gliomas and malignant meningiomas at our institution. In addition, we recently applied anti-angiogenic agents aggressively for intractable symptomatic radiation necrosis in the brain.

Here is our latest comprehensive data regarding these unique treatments, including those I presented at the 32nd annual meeting of the Japanese Neurosurgical Congress, along with some new findings.

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***Key words***: bevacizumab, boron neutron capture therapy (BNCT), positron emission tomography (PET), radiation necrosis

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## はじめに

Boron neutron capture therapy（以下 BNCT）は原理上腫瘍に対する細胞選択的照射が可能な唯一の放射線治療法である．ホウ素（$^{10}$B）化合物を投与し，その後，熱中性子もしくは熱外中性子を照射する．ホウ素化合物自体には細胞毒性はなく，また中性子の殺細胞効果もきわめて小さいが，ホウ素同位体$^{10}$B 原子核は中性子を捕獲し，きわめて線エネルギー付与（粒子が 1 $\mu$m 運動する間に周囲に付与するエネルギー：keV/$\mu$m）の高いヘリウム原子核（$\alpha$粒子）とリチウム反跳核をそれぞれ，9 $\mu$m と 4 $\mu$m という，細胞 1 個に相当する距離に放出し，その細胞を破壊する細胞選択的な粒子線治療ともいえる（Fig. 1）[3)]．すなわち殺細胞効果はホウ素中性子捕獲反応の生じた細胞に限局され，隣接する細胞には影響を及ぼさない．そこで，ホウ素化合物を腫瘍に選択的に集積できれば，腫瘍選択的な細胞破壊が可能となる．

本稿では，まず BNCT 時にその適応決定，線量 simulation に用いる F-BPA-PET を紹介し，次いで悪性神経膠腫に対する治療効果，悪性髄膜腫に対する治療効果，F-BPA-PET による治療効果の判定や放射線壊死，pseudoprogression の鑑別を紹介する．さらには原子炉に代わる新規中性子源として開発してきた小型加速器による治験を紹介する．最後に高線量放射線治療の宿命ともいえ

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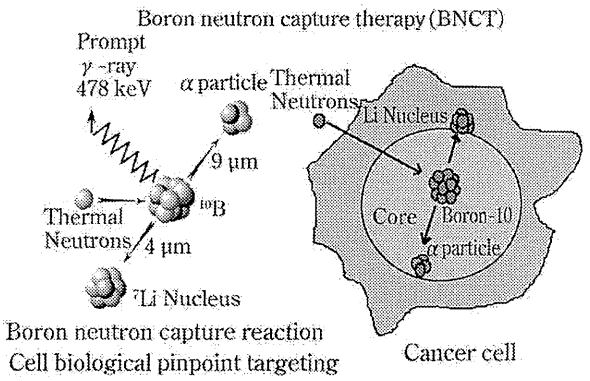


**Fig. 1 Principle of BNCT**

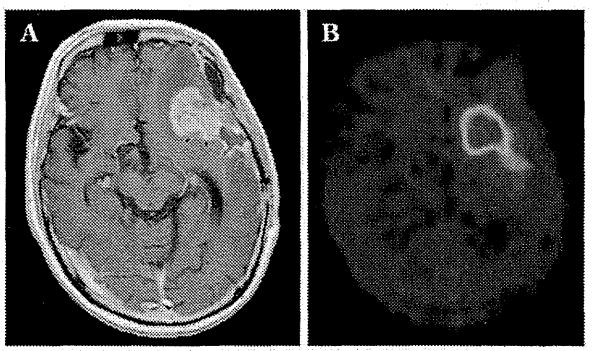


**Fig. 2 Typical F-BPA-PET findings in glioblastoma multiforme (GBM)**
A: T1-Gd enhanced MRI revealed a left frontal mass. B: F-BPA-PET imaging showed marked BPA accumulation not only in the enhanced area but also in the surrounding brain. The lesion/normal brain ratio of the tracer uptake in this case was 7.1.

る，脳放射線壊死に対する抗血管新生薬による治療法とその薬事申請への行程を紹介する．

## F-BPA-PET

BNCT における治療用化合物 BPA (boronophenylalanine) を用いた PET 検査を中性子照射に先立って行う．BPA は文字通りホウ素化した phenylalanine であり，腫瘍において亢進したアミノ酸代謝を利用し，腫瘍内に能動的かつ選択的に集積される．フッ素ラベルした BPA をトレーサとして利用することにより，PET により腫瘍内および脳内 BPA 濃度が推測され，治療の適応決定および照射線量が simulation できる．**Fig. 2** に BPA-PET による BPA の取り込みを示す．この症例では左前頭葉部腫瘍は反対側正常脳に比べて，7.1 倍のトレーサの集積を示している．この PET が示す情報は大きく，2 つの情報が存在する．1 つは 7.1 倍という数字は，同一部位に腫瘍細胞と正常細胞が存在すれば（浸潤部領域にそのような situation が想像できる），腫瘍細胞は正常細胞の 7.1 倍の粒子線を吸収することを示す．もう 1 つの情報は，造影 MRI に比較して，その外側にもトレーサの集積を認めることより，造影域より外側に浸潤している細胞にも targeting できていることを物語っている[10]．

## 悪性神経膠腫に対する BNCT の効果

悪性黒色腫とともに最も初期より BNCT が適応されてきた疾患が悪性神経膠腫であった．われわれは再発悪性神経膠腫に BNCT を適応し，すべての症例で画像上顕著な効果を認めた[7)10)]．また，新規診断膠芽腫にも積極的に BNCT を適応し，化学療法なしに良好な成績を収めている[8]．この経験をもとに，現在 BNCT 後に追加 X 線外照射および temozolomide を併用した，新規診断膠芽腫に対する多施設共同研究を厚生労働科学研究費のサポートをいただき，展開中である．

初発膠芽腫に対する BNCT の効果および問題点を示す症例を **Fig. 3** として提示する．左側脳室三角部近傍の膠芽腫である．手術による部分摘出の後，BNCT を施行した．BNCT 施行前，施行 8 カ月後の頭部 MRI および脊髄 MRI を A，B，C として提示している．BNCT は良好な局所制御を示しているが，脊髄髄腔内播種をきたし，この症例を亡くしている．われわれの BNCT の経験では，このように局所制御は比較的良好であるが，およそ半分の症例は髄腔内播種で亡くしている．今後の課題と考えている．

すでに放射線治療歴を有する再発悪性神経膠腫に対しても，本治療法は細胞選択性を有するので，積極的な照射を行ってきた．再発神経膠腫に対する RPA 分類を用いて[2]，BNCT の成績と既存治療法の成績を比較すると，予後不良群でその生存期間中央値を有意に延長している[13]．しかしながら，たとえば **Fig. 2** に示した症例では，病変と正常脳のトレーサの集積比が 7.1 倍と高値を示すが，逆に考えると正常脳は腫瘍の 1/7.1 の粒子線を被曝する．再発例の場合にはすでに許容線量限界に近い放射線治療が施行されているので，BNCT といえども，脳放射線壊死が問題となる．この点に関しては後述の抗血管新生療法を展開している．

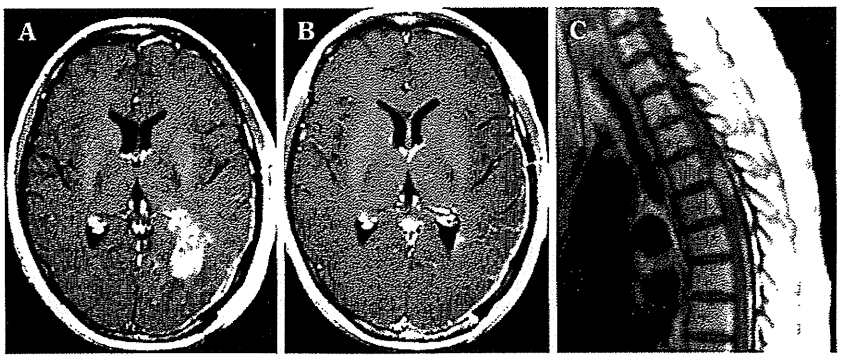


Fig. 3 Periodic Gd-enhanced MRI findings of a GBM case treated by BNCT. A newly diagnosed GBM case in which the left trigonal lesion was treated by BNCT.
A: Brain MRI, prior to BNCT. B: Brain MRI, 8 months after BNCT. C: Spinal MRI, 8 months after BNCT, showing CSF dissemination of the lesion at the spinal cord.

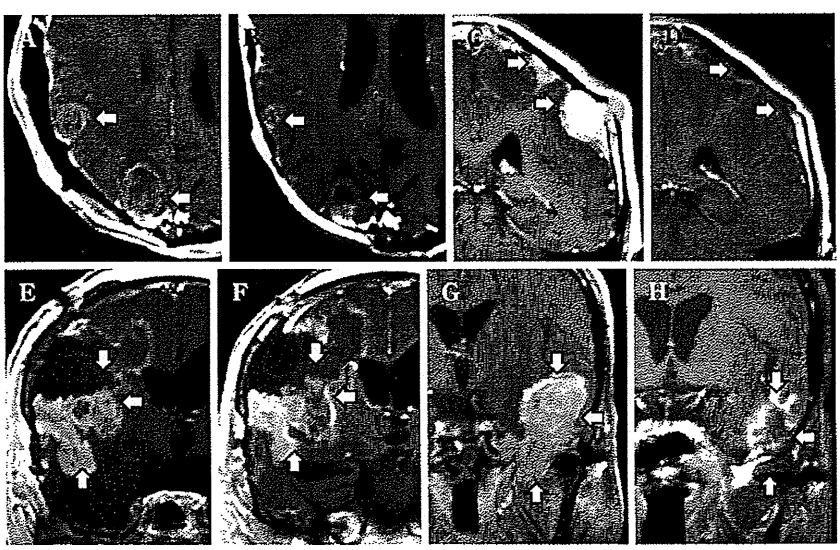


Fig. 4 Typical MRI changes of malignant meningiomas treated by BNCT
A, B: Prior to BNCT and 3 months after BNCT of a anaplastic meningioma.
C, D: Prior to BNCT and 4 months after BNCT of a anaplastic meningioma.
E, F: Prior to BNCT and 5 months after BNCT of an anaplastic meningioma.
G, H: Prior to BNCT and 4 months after BNCT of a rhabdoid meningioma.

## 高グレード髄膜腫に対する BNCT の効果

高グレード髄膜腫（high grade meningioma：HGM）は手術，定位放射線治療を行っても，その予後は悪い[6)18)]．ことに WHO grade 3 に属する anaplastic meningioma の予後は悪い．われわれは世界に先駆けて，これら HGM に対しても積極的に BNCT を適応してきた[11)19)]．Fig. 4 に anaplastic meningioma 3 例，rhabdoid meningioma 1 例の BNCT 前後の MRI を示す．この 4 例以外でもすべての症例で画像上顕著な腫瘍縮小効果を経験している．われわれの施設に紹介をいただく症例はすべて，複数回の手術，X 線外照射，定位放射線治療等が施行され，それでも制御不能な治療不応症例であるが，良好な局所制御を認めている．2011 年 9 月までに治療を終え，その後 1 年以上の経過を観察しえた 20 例では，局所再発は 4 例で認めたのみであるが，多くの症例を BNCT 後に失っ

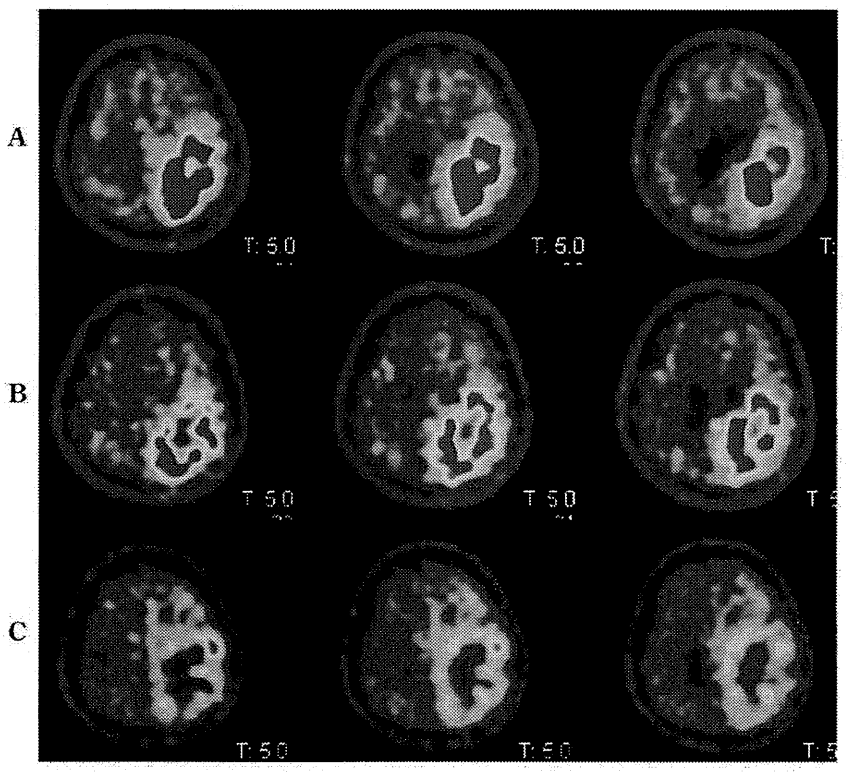


**Fig. 5 Periodic change of F-BPA-PET imaging of a recurrent anaplastic astrocytoma treated by BNCT**
A : Prior to BNCT, the lesion/normal brain ratio was 7.0.
B : 3 months after BNCT, the lesion/normal brain ratio was 3.5.
C : 9 months after BNCT, the lesion/normal brain ratio was 2.5.

ている．このうちの7例は照射野外再発，6例が全身転移によるものであり，3例が髄腔内播種による難治性水頭症による頭蓋内圧亢進により失っており，この疾患のコントロールの困難さを痛感している（論文印刷中）．これらはいずれも疾患が治療時にはすでに進行していることに起因しており，これを回避するには初期の再発時にBNCTを行う以外に有効な手立てはないと考えている．

## F-BPA-PETによる治療効果判定

Fig. 5 に再発神経膠腫（anaplastic astrocytoma）のBNCT前，BNCT 3カ月後，9カ月後のF-BPA-PETを示す．BNCT 3カ月後に本PETを施行した理由は若干の浮腫，造影域の拡大を認めたため，腫瘍のprogressionかpseudoprogressionかの判断を行うために施行したものであり，このPETにより病変/正常脳比の低下を確認してpseudoprogressionと判断し，経過を観察している．9カ月後のPETではさらにこの比が低下を示しており，良好な治療効果を確認している．本症例は前述の再発神経膠腫に対するRPA分類ではクラス3に分類され，再発時治療後の生存期間中央値は文献上，わずか3.8カ月と報告されている．本症例は，本稿準備時すでにBNCT後11カ月が経過しているが再発の徴候は認めていない．われわれは本PETを腫瘍再発と脳放射線壊死の鑑別[9]やpseudoprogressionとtrue progressionの鑑別に用いている[12]．

## 加速器中性子源によるBNCT

上述のBNCTはすべて原子炉からの中性子を用いたBNCTの成績である．原子炉を用いる限り，またホウ素化合物も医薬品としてのGMPグレードを開発しない限り，BNCTは医療としては認知されない．数年前にわれわれが厚生労働省に本治療を当時の高度先進医療に申請した折から，この点は指摘されており，大きな宿題であった．この要求に応えるため，某製薬メーカーと医療機器開発メーカーの主導により，加速器中性子源とGMPグレードBPAの開発が行われ，著者が治験責任医師とな

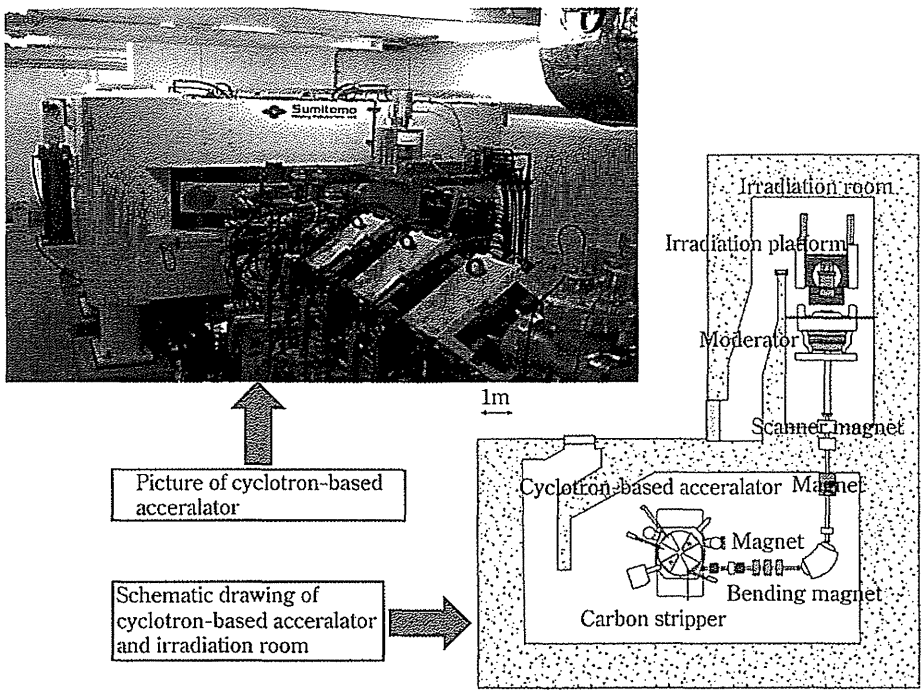


**Fig. 6** Photograph of the cyclotron-based accelerator for neutron generation and a schematic drawing of the total irradiation room

り，再発悪性神経膠腫を対象として，第 1 相臨床試験（治験）を開始している．Fig. 6 にサイクロトロン型小型加速器中性子源（実写）とその治療室の見取り図を提示する．原子炉に比してはるかに小型化された加速器の実寸が見て取れる．本治験が成功すれば院内 BNCT が可能となり，薬事承認を目指して臨床試験を遂行中である．

## 症候性脳放射線壊死に対するベバシズマブの静脈内投与による治療

BNCT や強度変調放射線治療あるいは定位放射線治療等の高線量放射線治療は着実に，頭蓋内悪性腫瘍の治療成績を向上させている[5)8)17)20)]．一方でこれら高線量，高精度放射線治療の適応により，症候性脳放射線壊死が問題となっている．症候性脳放射線壊死に対してはステロイドホルモン等が経験的に投与されてはいるが，有効な治療法は確立されていない．

脳放射線壊死組織の手術摘出標本の検討から，放射線壊死における脳浮腫の原因が壊死巣周囲の脆弱な血管新生であり，さらにその原因が血管内皮増殖因子（vascular endothelial growth factor：VEGF）の過剰産生にあることをわれわれは解明した[16)]．そこでこの知見をもとに，抗 VEGF 抗体製剤であるベバシズマブを症候性脳放射線壊

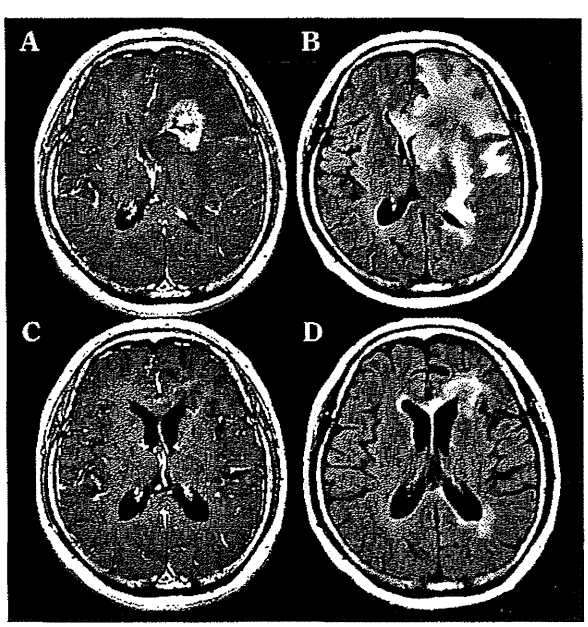


**Fig. 7** Case of radiation necrosis successfully treated by bevacizumab. Radiation necrosis was due to repetitive SRSs for a metastatic brain tumor of uterus cancer.
A：Pre-treatment T1-Gd MRI. B：Pre-treatment FLAIR MRI. C：Post-treatment T1-Gd MRI. D：Post-treatment FLAIR MRI. A drastic decrease of Gd-enhancement and brain edema was observed by 6 cycles of bevacizumab treatment.

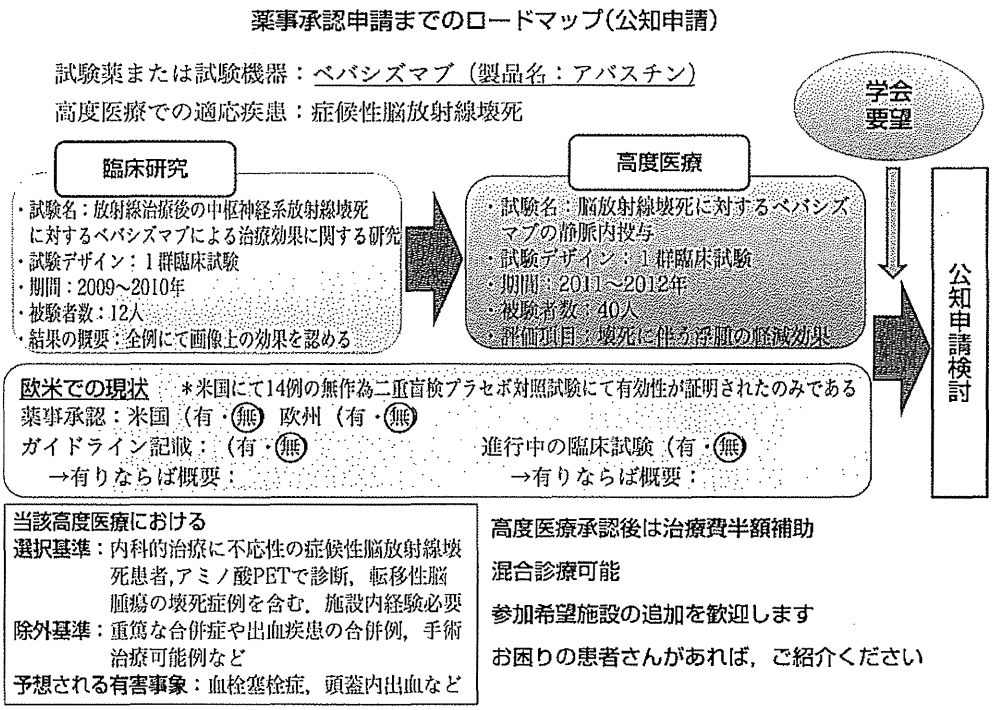


**Fig. 8 Roadmap for obtaining permission for on-label use of bevacizumab for symptomatic radiation necrosis in the brain**

死の症例に投与したところ，原因となる腫瘍の組織型や用いた放射線の種類を問わず，投与症例全例で顕著な脳浮腫および造影領域の縮小を認めた[4]．ここで問題となるのは放射線治療後に生じる脳浮腫の増悪が，腫瘍再発によるものかそれとも脳放射線壊死によるものかの鑑別を要する点である．われわれは上述の F-BPA-PET により，その鑑別を行っている[4)14)]．

これらの経験をもとに，厚生労働省に高度医療（第3項先進医療）として「症候性脳放射線壊死に対する核医学的診断とベバシズマブの静脈内投与による治療」を申請し，厚生労働科研費のサポートもいただき，2011年4月1日より40症例を登録予定数として全国16施設による多施設共同臨床研究を展開している[14]．本臨床試験ではより多くの施設の参画を期待して，その診断には BPA-PET のほか，Met-PET も利用可としており，その詳細は拙稿をご参照いただきたい[14]．われわれの経験症例を Fig. 7 に示す．

53歳女性．子宮体癌に対して全摘出術を受けた．その直後より脳内に多発性転移巣を認め，複数回の定位放射線治療を受けている．左前頭葉の病変には2度の定位照射が施行され，2度目の照射の2カ月後より，頭痛，失語症を発症し，ステロイドホルモンの投与によっても軽快せず，当科に紹介された．多発性の転移巣はいずれも BPA-PET で活動性を認めず，ことに左前頭葉の病変は病変/正常脳比 2.1 と算出され，われわれの定めた脳放射線壊死の基準値を満たし，ベバシズマブの投与を biweekly，5 mg/kg で行った．数回投与により症状は改善し，6回投与後の MRI では造影域，浮腫とも顕著に軽快している．

本稿準備中の2103年1月25日に予定症例数の40例の登録を終え，順調に臨床試験は進行している．臨床試験の成績をもとにした，われわれの描いている薬事承認を目指したロードマップを Fig. 8 に示す．症候性脳放射線壊死は重篤な機能予後および生命予後をきたす疾患ではあるが，母集団となる患者総数はそれほど多くなく，治験は組みにくい．そこで治験というプロセスを踏まずに薬事承認を目指すシステムとして，高度医療に思い至った次第である．本臨床試験にて万人が認めうる優れた成績を治めることができれば，各種学会からの学会要望を添えて，公知申請を行い，厚生労働省に薬事申請を認可していただくという戦略を厚生労働省の担当官との間で構築した．よっておよそ2年後に，日本定位放射線治療学会，日本脳神経外科学会，日本放射線腫瘍学会，日本核医学会からの学会要望がいただけるよう，臨床試

験を行っている次第である[14]．

最後に再発悪性神経膠腫に対して，BNCT を行うと，しばしば pseudoprogression に遭遇する[12]．この pseudoprogression は intensive treatment の証として認識されているが[1]，多くの場合無症候であり，ステロイドホルモンの投与で対応可能であることが多い．最近われわれは再発悪性神経膠腫に対しての BNCT 後に symptomatic pseudoprogression となった症例に，ベバシズマブを投与したところ，劇的な改善を経験しており，本治療の新たな展開と考えている[15]．Pseudoprogression と脳放射線壊死との間に明確な線を引くことは難しい．一般には pseudoprogression は脳放射線壊死と比べて，画像上の悪化に比して症状は軽く，かつ治療から発症までの期間が短いことが特徴と理解されている．文献 15 で紹介した症例は治療から画像上の増悪までの期間が短く，PET 上も壊死の診断はできず，symptomatic pseudoprogression と判断した症例である．ベバシズマブの治療効果を考えると，今後再発症例の BNCT 後には本治療を適応すべき症例も増加すると考える．

**謝　辞**

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要　旨

腫瘍細胞選択的粒子線治療「ホウ素中性子捕捉療法」と
抗血管新生薬による症候性脳放射線壊死の治療

宮武　伸一

悪性腫瘍に対する新規放射線（粒子線）治療法として，ホウ素中性子捕捉療法（boron neutron capture therapy：BNCT）が提唱されている．われわれは2002年より本治療法をのべ133例に及ぶ悪性神経膠腫と悪性髄膜腫に適応してきた．また最近，症候性脳放射線壊死に対する抗血管新生療法を積極的に展開している．本論文では，第32回日本脳神経外科コングレス総会「グリオーマ 新しい時代の到来」において発表した上記内容に若干の加筆を行い，ここに発表した．

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RADIATION ONCOLOGY

CASE REPORT Open Access

# Boron neutron capture therapy with bevacizumab may prolong the survival of recurrent malignant glioma patients: four cases

Shin-Ichi Miyatake[1*], Shinji Kawabata[1], Ryo Hiramatsu[1], Motomasa Furuse[1], Toshihiko Kuroiwa[1] and Minoru Suzuki[2]

**Abstract**

**Background and importance:** Recurrent malignant gliomas (RMGs) are very difficult to control, and no standard treatments have been established for them. We performed boron neutron capture therapy (BNCT) for patients with RMG. BNCT enables high-dose particle radiation to be applied selectively to tumor cells. However, RMG cases generally receive nearly 60 Gy X-ray irradiation prior to re-irradiation by BNCT. Therefore, even with tumor-selective particle radiation BNCT, radiation necrosis in the brain and symptomatic pseudoprogression may develop. In four of our recent patients with RMG after BNCT, we applied the anti-VEGF antibody bevacizumab to treat two pathological entities. This approach appeared to prolong survival. Here we present the case reports of these four consecutive patients with RMG and discuss the novel use of bevacizumab in this context.

**Clinical presentation:** Four patients with RMGs were treated with BNCT at our institutes. Upon the referral for BNCT, they were assessed as belonging to the recursive partitioning analysis (RPA) class 3 (n = 3 patients) or RPA class 4 (n = 1 patient) (the RPA classification for RMG was advocated by Carson et al. in 2007). The estimated median survival times for RPA classes 3 and 4 were 3.8 and 10.8 months, respectively, after some treatment at the recurrence. We applied BNCT for these four patients and administered bevacizumab when the lesions were considered radiation necrosis or symptomatic pseudoprogression. The class 3 patients survived after the BNCT for 14, 16.5 and > 23 months, and the class 4 patient survived > 26 months, with favorable improvements in clinical symptoms.

**Conclusion:** BNCT with the addition of bevacizumab for radiation necrosis or symptomatic pseudoprogression improved the clinical symptoms and prolonged the survival in RMG patients.

**Keywords:** Bevacizumab, Boron neutron capture therapy, Recurrent malignant glioma

## Background

The prognosis of recurrent malignant gliomas (RMGs) is poor, and no standard treatment has been established [1]. Since 2002 at our institute, we have been applying a form of tumor-selective particle radiation, boron neutron capture therapy (BNCT), for RMGs and observed favorable survival outcomes [2,3]. BNCT is a biochemically targeted radiotherapy based on the nuclear capture and fission reactions that occur when non-radioactive boron-10, which is a constituent of natural elemental boron, is irradiated with low-energy thermal neutrons to yield high-linear-energy transfer alpha particles and recoiling lithium-7 nuclei. These particles are released within a very short range such as 9 μm, and therefore the cytotoxic effects are confined within boron-10-containing cells [4].

Boron-10-containing compounds can be accumulated selectively in tumor cells by several mechanisms. For example, boronophenylalanine (BPA) is selectively and preferentially accumulated in tumor cells via the augmented metabolism of amino acids compared to normal cells. Even with this novel and selective particle radiation technique, radiation damage — chiefly radiation necrosis (RN) and symptomatic pseudoprogression (psPD) — often occurs [5,6]. The radiation damage is especially likely in RMG cases, because full-dose X-ray treatment (XRT) is generally part of the treatment history in such cases.

Bevacizumab (BV), an anti-vascular endothelial growth factor (VEGF) antibody, has recently been used for the

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treatment of symptomatic RN [7,8]. Based on our analysis of human RN surgical specimens, we previously demonstrated that the edema in RN is caused by the overexpression of VEGF in reactive astrocytes [9]. Following this determination, we used BV in an attempt to control the symptomatic RN and the symptomatic psPD encountered after BNCT for RMGs [5,7]. Here we present a case series report of our last four consecutive cases of RMG treated with BNCT and BV, with >18-month observation periods. All four patients had RMGs after primary treatment with XRT and chemotherapy consisting chiefly of temozolomide (TMZ). The patients' profiles and survival data are listed in Table 1. Three of the patients were classified as recursive partitioning analysis (RPA) (advocated by Carson et al. in 2007 [1]) class 3 and one was classified as RPA class 4.

## Case presentation

### Case 1

A 44-year-old male's craniotomy showed anaplastic astrocytoma. He received standard chemoradiotherapy (XRT 60 Gy with TMZ). Unfortunately the lesion recurred with aggravation of aphasia and right hemiparesis, which forced him to retire from his job. The Karnofsky performance status (KPS) was 70%, and he was classified as RPA class 3. The patient was then referred to our institute for BNCT. Upon referral, MRI showed a slightly enhanced lesion with mild perifocal edema (Figure 1). A simultaneous fluorine-18-labeled BPA positron emission tomography (F-BPA-PET) image showed marked tracer uptake in the left parietofrontal region (Figure 1), with a 6.0 lesion/normal (L/N) brain ratio of the tracer, indicating that the lesion was a highly malignant tumor. BNCT was applied for this patient according to our recent protocol for RMGs and meningiomas [10]. Briefly, only BPA was administered over a 2-hr period (200 mg/kg/hr) just prior to and during the neutron irradiation (100 mg/kg/hr). The neutron irradiation time was decided based on a simulation not to exceed 12.0 Gy-Eq (Gray-equivalent) for the peak brain dose. The 10-B concentration in the blood during the neutron irradiation was 23.0 parts per million (ppm). By BNCT, the maximum brain dose, maximum tumor dose, and minimum tumor dose were estimated as 11.4, 118, and 36.1 Gy-Eq, respectively. Here, "Gy-Eq" corresponds to the biologically equivalent X-ray dose that would have equivalent effects on tumors and on the normal brain. The dose estimation was performed by the measurement of blood boron concentration and F-BPA-PET data prior to neutron irradiation as described elsewhere [2,6,10].

After the BNCT, an MRI showed gradual enlargement of both perifocal edema and contrast enhancement, whereas sequential F-BPA-PET showed a favorable decrease of tracer uptake (Figure 1, lower panel). F-BPA-PET was originally developed to estimate the absorbed dose in BNCT, as described above [2,11,12]. The background uptake of the tracer F-BPA is very low compared to that of fluorodeoxy-glucose and even compared to that of methionine as a tracer. Thereafter, RN and psPD have been differentially diagnosed from tumor progression by F-BPA-PET [6,13]. Ten months after the BNCT, the patient's KPS worsened to 60%, and so we administered BV 5 mg/kg biweekly, three times. Just prior to the BV administration, F-BPA-PET showed a more decreased L/N ratio, which indicated that the aggravation shown by MRI was RN and not a recurrence of the tumor. After the BV treatment, MRI showed improvement of the perilesional edema and a decrease in contrast enhancement. The BV treatment stabilized the patient's symptoms for 6 months but then his symptoms recurred, prompting us to perform a re-challenge with BV another three times. The patient is now stable and doing well, 23 months after the BNCT (Table 1).

### Case 2

A 41-year-old man underwent surgery for his right parietal glioblastoma (GBM) with subtotal excision. Standard treatment with XRT and TMZ was performed, but the tumor recurred 5 months after the surgery. Upon referral for BNCT, the patient's KPS was assessed as 90% and he was classified as RPA class 4. MRI showed a definitively enhanced lesion with moderate perifocal edema (Figure 2). A simultaneous F-BPA-PET image showed marked tracer uptake in the right parietal region with a 3.8 L/N ratio of the tracer, indicating that the lesion was a recurrent malignant tumor and not psPD (Figure 2,

**Table 1 The background of the four patients with recurrent malignant glioma (RMG)**

| Case No. | Age | Sex | Hist. | RPA class | Irradiated dose (Gy-Eq) | | | BV cycles | PsPD or RN | Survival (Months from BNCT) |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Brain (Max) | Tumor (Max) | Tumor (Mini) | (Months from BNCT) | | |
| 1 | 43 | M | AA | 3 | 11.4 | 118 | 36.1 | 3 (11 M) | RN | 23 M, alive |
| 2 | 41 | M | GBM | 4 | 12.1 | 88.5 | 36.6 | 4 (14 M) | RN | 26 M, alive |
| 3 | 60 | M | AA | 3 | 10.8 | 110 | 82.3 | 6 (4 M) | PsPD | 16.5 M |
| 4 | 34 | F | AOA | 3 | 11.5 | 71.6 | 30.1 | 6 (2 M) | PsPD | 14 M |

Hist, histology; RPA, recursive portioning analysis; BV, Bevacizumab; PsPD, pseudoprogression; RN, radiation necrosis; BNCT, boron neutron capture therapy.

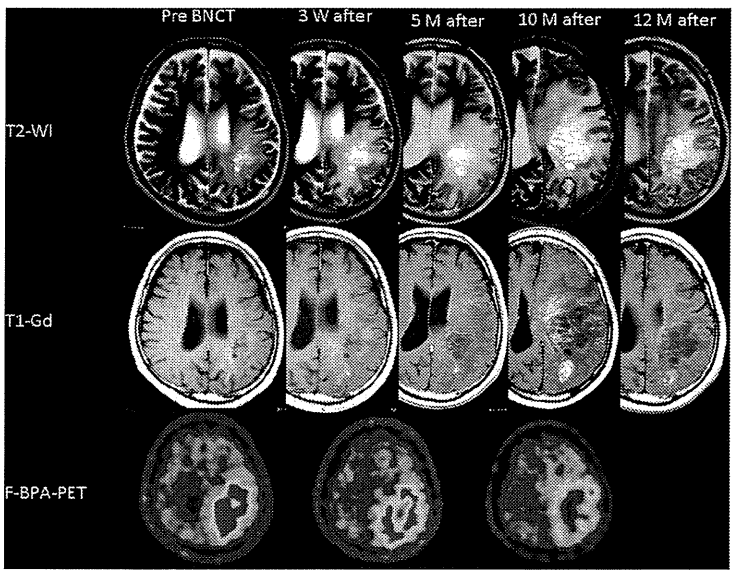


**Figure 1 Sequential change of T2-weighted MRI (upper column), Gd-enhanced T1-weighted MRI (middle column) and F-BPA-PET (lower column) of Case 1, a 44-year-old male.** The timing of the MRIs is depicted above the MRIs. F-BPA-PET images were taken just before the BNCT and at 1 month and 10 months after the BNCT. These PET images show the gradual decrease of the tracer uptake as a promising effect of the BNCT. BV was started 10 months after the BNCT, and the MRI showed marked improvement of both perifocal edema and contrast enhancements by BV treatment.

lower panel). He was treated with BNCT, with the same protocol as Case 1. The boron-10 concentration in the blood during the neutron irradiation was 30.2 ppm. By BNCT, the maximum brain dose, maximum tumor dose, and minimum tumor dose were estimated as 12.1, 88.5, and 36.6 Gy-Eq, respectively. One week after the BNCT, a contrast-enhanced T1-weighted MRI showed a marked shrinkage of the mass, and that at 3 months later showed slight enlargement of the enhanced lesion, which was presumed to be psPD. Periodic MRIs showed

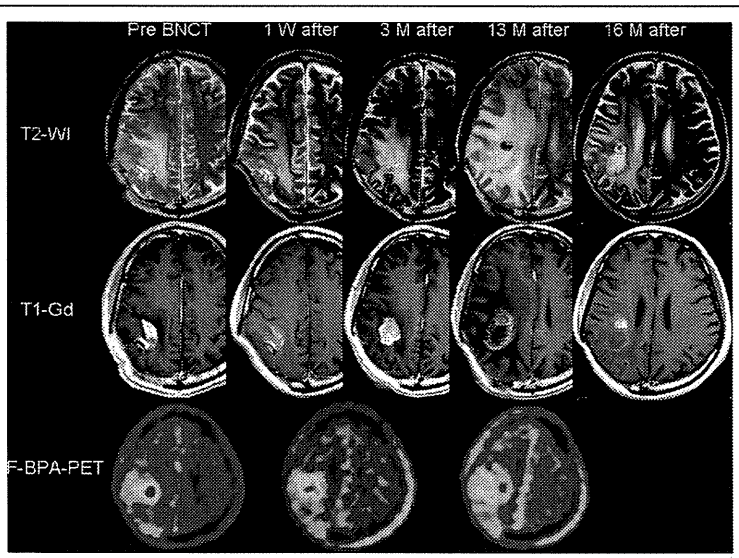


**Figure 2 Sequential change of T2-weighted MRI (upper column), Gd-enhanced T1-weighted MRI (middle column) and F-BPA-PET (lower column) of Case 2, a 41-year-old man.** The timing of the MRI is depicted above the MRI. F-BPA-PET images were taken just before the BNCT, 1 month after and 12 months after the BNCT. These PET images show the gradual decrease of the tracer uptake as a promising effect of BNCT. BV was started 13 months after the BNCT, and an MRI showed a marked positive effect of the BV treatment on the perifocal edema and contrast enhancements.

gradual enlargement of both the enhanced lesion and perifocal edema, whereas F-BPA-PET showed a gradual decrease of the tracer uptake. The final L/N ratio, 1 year after BNCT, was 2.3. This L/N ratio and the MRI 13 months after the BNCT suggested that the lesion was RN.

The patient was not able to continue his work as a cook, and we decided to begin intravenous BV treatment biweekly (5 mg/kg). After four treatments, MRI showed marked improvement in the perifocal edema and left hemiparesis. The patient is now doing well and has resumed his work as a cook, 26 months after the BNCT, without tumor progression or recurrence of the RN.

### Case 3

A 56-year-old male experienced speech disturbance and mild right hemiparesis. First he received a craniotomy with a diagnosis of gemistocytic astrocytoma, followed by fractionated XRT (total 50 Gy) and repetitive chemotherapy with nitrosourea. Three years later, a recurrent lesion appeared with Gd enhancement on MRI. Re-craniotomy revealed GBM histologically. After surgery, the enhanced lesion gradually grew and the patient's sensory aphasia worsened despite the repeated administration of TMZ. He was referred to our institute for BNCT. Upon his referral, he was assessed as RPA class 3. The boron-10 concentration in the blood during the neutron irradiation was 30.0 ppm. Using BNCT, the maximum brain dose, maximum tumor dose, and minimum tumor dose were estimated as 10.8, 110, and 82.3 Gy-Eq, respectively, as shown in Table 1. His right hemiparesis and aphasia gradually worsened after the BNCT, even with an escalating dose of corticosteroids. Four months after the BNCT, a follow-up MRI and F-BPA-PET suggested that the lesion was symptomatic psPD, not tumor progression. The patient was successfully treated with BV, as we recently reported, along with the periodic changes of the neuroimages and the detailed clinical course [5]. We lost this patient to local tumor progression 16.5 months after the BNCT.

### Case 4

A 27-year-old female manifested left hemiparesis. A right frontal enhanced mass was removed gross/totally, and the histological diagnosis was anaplastic oligo-astrocytoma. She received fractionated XRT (total 72 Gy) and repetitive chemotherapy with nitrosourea. The lesion recurred and re-craniotomy was performed 4 years later, with the same pathological diagnosis. This was followed by TMZ chemotherapy. Unfortunately, a recurrence was confirmed by MRI and she was referred to us for BNCT. The boron-10 concentration in the blood during the neutron irradiation was 21.4 ppm. By BNCT, the maximum brain dose, maximum tumor dose, and minimum tumor dose were 11.5, 71.6, and 30.1 Gy-Eq, respectively (Table 1). After the BNCT, her hemiparesis gradually became aggravated despite an increased dose of corticosteroids. MRI taken 2 months after the BNCT showed an enlarged enhanced lesion with increased perilesional edema. We judged this aggravation as symptomatic psPD. We started BV treatment for her. The patient was bedridden just prior to the BV treatment, but after two BV treatments her hemiparesis improved markedly and she could walk. Her neuroimages and clinical symptoms showed marked improvement, as we reported previously [5]. Unfortunately we lost her because of tumor extension to the opposite hemisphere 14 months after the BNCT.

The neuroimages, including F-BPA-PET scans of Cases 3 and 4, were published elsewhere [5] and thus are not included in this brief report.

## Discussion

In comparison with many Phase I and II trials for RMG [1], BNCT showed a marked survival benefit for RMG in our previous study, in which BV was not used [3]. Briefly, BNCT resulted in median survival times (MSTs) (months and 95% confidence intervals) as follows: for all RPA classes (Classes 1–7), 10.8 (7.3–12.8) (n = 22), and in the poor-prognosis group (RPA class 3 + 7), 9.1(4.4–11.0) (n = 11). In a meta-analysis reported in the Journal of Clinical Oncology [1], the MSTs in all RPA classes and in the poor-prognosis group (RPA class 3 + 7) were 7.0 (6.2–8.0) (n = 310) and 4.4 (3.6–5.4) (n = 129), respectively. These data showed the superiority of BNCT for RMGs, especially in poor-prognosis groups. In comparison, our previous data showed MSTs of RPA class 3 and 4 as 7.3 and 12.0 months, respectively, although the number of the patients was quite limited: 4 cases in class 3 and 3 cases in class 4 [3].

In our recent patients undergoing BNCT for RMGs, we have begun to treat RN or symptomatic psPD aggressively by administering BV. We applied intravenous BV treatment for four recent RMG patients treated with BNCT at our institute and in whom we encountered RN or symptomatic psPD; these cases are reported here. Three of these four patients were classified as RPA class 3 and one as class 4 (Table 1). The estimated survival time of class 3 patients is 3.8 months and that of class 4 patients is 10.8 months [1]. Our three class 3 patients survived for 14, 16.5, and > 23 months, and the class 4 patient has survived for over 26 months.

At a glance, BNCT with BV seemed to prolong the survival of RMGs strikingly in comparison not only with Carson's data set but also with our previous BNCT data. Although of course no definitive conclusion can be drawn from such a small number of cases.

In our limited experience, there is no obvious histological difference between RN and psPD [6]. The center part of each pathology is characterized as histological necrosis, and

marked angiogenesis is observed in the boundary of the necrotic core and normal brain tissue [9]. Clinically, most psPD occurs at a relatively early stage after intensive treatments and is self-limiting without severe sequelae [14]. In most cases, psPD improves over time without intensive treatments. On the other hand, RN often shows severe symptoms and occurs at least a half year after radiotherapy. Thereafter, symptomatic psPD is especially difficult to distinguish from RN. In Table 1, we distinguish them only from the duration of the symptomatic onset after BNCT.

We have described herein the use of BV for RN or psPD after BNCT. BV was approved for the treatment of RMGs as an anticancer agent [15,16], and several trials of re-irradiation using XRT or hypo-fractionated stereotactic radiotherapy in combination with BV just before radiotherapy for RMGs have recently been conducted, with favorable preliminary safety and response results [17-19]. The authors of those reports described the role of BV not only as an anticancer agent but also for normalizing the perfusion pressure and oxygenation effects during irradiation. BV may also prevent RN and symptomatic psPD after re-irradiation.

We are now planning a prospective clinical trial of BNCT using BV immediately after neutron irradiation for RMG patients with poor prognosis (class 3 + 7). We are also conducting a clinical trial of BNCT for RMGs using a small accelerator in-hospital, instead of an atomic reactor. We hope to determine whether accelerator-based BNCT with BV could be used as a standard treatment for RMGs.

## Conclusion

BNCT with the addition of BV for radiation necrosis or symptomatic pseudoprogression improved the clinical symptoms and might prolong the survival of RMG patients.

## Consent

Written informed consent was obtained from the patient for the publication of this report and any accompanying images.

### Abbreviations

BNCT: Boron neutron capture therapy; BPA: Boronophenylalanine; BV: Bevacizumab; GBM: Glioblastoma; Gy-Eq: Gray-equivalent; KPS: Karnofsky performance status; L/N: Lesion/normal; PET: Positron emission tomography; ppm: parts per million; psPD: pseudoprogression; RMG: Recurrent malignant gliomas; RN: Radiation necrosis; RPA: Recursive portioning analysis; TMZ: Temozolomide; XRT: X-ray treatment.

### Competing interests

There is no conflict of interest to disclose for any of the authors.

### Authors' contributions

S-IM conceived of the study and participated in the follow-up of patients. SK, RH, and MS applied BNCT in the atomic reactor. MF followed the patients with bevacizumab. TK selected the patients for BNCT. All authors read and approved the final manuscript.

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# High linear-energy-transfer radiation can overcome radioresistance of glioma stem-like cells to low linear-energy-transfer radiation

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Ionizing radiation is applied as the standard treatment for glioblastoma multiforme (GBM). However, radiotherapy remains merely palliative, not curative, because of the existence of glioma stem cells (GSCs), which are regarded as highly radioresistant to low linear-energy-transfer (LET) photons. Here we analyzed whether or not high-LET particles can overcome the radioresistance of GSCs. Glioma stem-like cells (GSLCs) were induced from the GBM cell line A172 in stem cell culture medium. The phenotypes of GSLCs and wild-type cells were confirmed using stem cell markers. These cells were irradiated with $^{60}$Co gamma rays or reactor neutron beams. Under neutron-beam irradiation, high-LET proton particles can be produced through elastic scattering or nitrogen capture reaction. Radiosensitivity was assessed by a colony-forming assay, and the DNA double-strand breaks (DSBs) were assessed by a histone gamma-H2AX focus detection assay. In stem cell culture medium, GSLCs could form neurosphere-like cells and express neural stem cell markers (Sox2 and Musashi) abundantly in comparison with their parental cells. GSLCs were significantly more radioresistant to gamma rays than their parental cells, but neutron beams overcame this resistance. There were significantly fewer gamma-H2AX foci in the A172 GSLCs 24 h after irradiation with gamma rays than in their parental cultured cells, while there was no apparent difference following neutron-beam irradiation. High-LET radiation can overcome the radioresistance of GSLCs by producing unrepairable DNA DSBs. High-LET radiation therapy might have the potential to overcome GBM's resistance to X-rays in a clinical setting.

**Keywords:** glioblastoma multiforme; glioma stem cells; linear energy transfer; neutron beams; gamma rays

## INTRODUCTION

Radiation therapy with surgery and chemotherapy is the standard treatment for glioblastoma multiforme (GBM) [1]. However, the prognosis of patients with GBM has not improved in recent decades, and almost half of GBM patients do not survive the first year after diagnosis. Thus, another, more promising therapy for GBM is needed. Recently, some reports have shown the presence of glioma stem cells (GSCs) in malignant gliomas [2–4]. These cells are highly resistant to radiotherapy because of their enhanced checkpoint response to radiation [5]. Other studies have shown that GSCs express high levels of sirtuin family genes (especially the SirT1 gene) and that these upregulations are relevant to radiosensitivity because they modulate apoptotic activity in response to irradiation to GSCs [6]. As a result, GSCs are now known to play important roles in tumor progression and relapse after radiotherapy and chemotherapy, and new therapeutic strategies targeting GSCs should be developed to treat patients with GBM. In the previous reports, radioresistance of GSCs was studied in a subpopulation with a specific phenotype. In these studies, it was difficult to use appropriate control cells for the GSCs. Therefore, we induced glioma stem-like cells (GSLCs) in which the phenotypes of GSCs

were enriched, and used the wild-type GBM cells as controls in this study.

On the other hand, we have applied boron neutron capture therapy (BNCT) for malignant brain tumors, including GBM [7–9]. This is a unique tumor-selective particle radiotherapy using neutron irradiation, especially thermal neutron irradiation. Boron-10 ($^{10}$B) releases alpha ($^{4}$He) and $^{7}$Li particles through $^{10}$B (n,α)$^{7}$Li reaction. The key players in the anti-tumor effects of BNCT are these high linear-energy-transfer (LET) particles. With BNCT, good results have already been achieved for patients with newly diagnosed GBM and recurrent malignant glioma [9, 10], although the numbers of such cases in clinical trials have been limited.

So far, the radioresistance of GSCs has been examined mainly in terms of low-LET radiation such as X-rays or gamma rays. Therefore, we hypothesized that high-LET radiation could overcome the radioresistance of GSCs. In fact, a previous study showed that high-LET radiation was more effective than low-LET radiation for promoting DNA damage [11]. Here, we employed a reactor neutron-beam irradiation system that produces high-LET proton particles through elastic scattering and nitrogen capture reaction. We analyzed the usefulness of high-LET radiation for overcoming the radioresistance to low-LET radiation in GSCs using GSLCs, as well as the ability of these cells to recover from radiation-induced DNA damage by a gamma-H2AX assay.

## MATERIALS AND METHODS

### Cell culture

The human GBM cell line A172 was purchased from American Type Culture Collection (Manassas, VA) and cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (FBS) with penicillin and streptomycin at 37°C in an atmosphere of 5% $CO_2$. GSLCs were induced from A172 cells in serum-free medium (SFM) as described previously [12]. The SFM was composed of DMEM/F12 (Sigma-Aldrich, St Louis, MO), 20 ng/ml basic fibroblast growth factor (Peprotech, Rocky Hill, NJ), 20 ng/ml epidermal growth factor (Peprotech), 2 μg/ml heparin (Sigma-Aldrich), and B27 supplement (50×; Life Technology/Invitrogen).

### Western blot analysis

Cells were cultured for 7 d in each culture medium. Protein samples were prepared with 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. Immune complexes were formed by incubation with the stem cell markers CD133 (Cell Signal Technology, Danvers, MA), Sox2 (Cell Signal Technology), and Musashi (Cell Signal Technology) overnight at 4°C. As a control for the housekeeping gene products, Ku70 (Thermo Scientific, Waltham, MA) was employed. Blots were washed and incubated for 1 h with horseradish peroxidase-conjugated anti-mouse and anti-rabbit secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoreactive protein bands were detected by using an enhanced chemoluminescence Advance Western Blotting Detection Kit (GE Health Care, Buckinghamshire, UK), and Image Reader LAS-1000 Pro ver. 2.5 (Fuji Photo Film, Tokyo, Japan).

### Fluorescence-activated cell sorting analysis

Cells were cultured for 7–14 d in each culture medium. Cells were collected and incubated with anti-CD133 antibody (Bioss, Woburn, MA) for 1 h at 37°C. After washing, the cells were incubated with Alexa Fluor 647-labeled anti-rabbit secondary antibody for 30 min at 37°C, then analyzed by fluorescence-activated cell sorting (FACS) using a BD FACS Aria Cell Sorter (BD Bioscience, San Jose, CA).

### Gamma-ray and neutron-beam irradiation

Two sets of A172 cells, one cultured with serum-containing medium (DMEM + 10% FBS) and the other cultured with SFM, were trypsinized, and single-cell suspensions were placed into a Teflon tube and irradiated at room temperature by neutron beams or gamma rays.

At the Heavy Water Column of the Kyoto University Research Reactor (KUR), neutron-beam irradiation was performed at a power of 1 MW. The neutron fluence was measured from the radioactivation of gold foil. Contaminating gamma rays, including secondary gamma rays, were measured with thermoluminescence dosimeter (TLD) powder. The TLD used was beryllium oxide (BeO) enclosed in a quartz glass capsule. BeO itself is sensitive to thermal neutrons [13]. The average neutron fluxes were $1.0 \times 10^{9}$ n/cm$^{2}$/s for the thermal neutron range (less than 0.6 keV), $1.6 \times 10^{8}$ n/cm$^{2}$/s for the epithermal neutron range (0.6–10 keV), and $9.4 \times 10^{6}$ n/cm$^{2}$/s for the fast neutron range (more than 10 keV). The total absorbed doses resulting from fast, epithermal, and thermal neutron-beam irradiation were calculated as the sum of the absorbed doses attributed primarily to $^{1}$H(n,n)$^{1}$H, $^{14}$N(n,p)$^{14}$C, and contaminating gamma rays. The dose-converting coefficients and details of the calculation method have been described previously [14, 15].

Gamma-ray irradiation was applied using a $^{60}$Co gamma-ray irradiator at a dose rate of 1.3 Gy/min.

### Colony-forming assay

Cell survival was defined using a colony-forming assay. The irradiated cells were seeded into 100 mm dishes at various densities depending on the physical dose that cells received, and cultured in a serum-containing medium. After 13–15 d, the colonies were stained with methylene blue. A cell cluster containing at least 50 cells was considered a single colony. The surviving fraction was calculated as the number of colonies of treated cells divided by that for the control cells. The $D_{10}$ values were derived by linear quadratic model analysis

for cell survival curves. The relative biological effectiveness (RBE) for neutron beams was obtained as the ratio of the mean value of $D_{10}$ to that of gamma rays.

### Gamma-H2AX focus assay

A Gamma-H2AX focus assay was performed to detect DNA double-strand breaks (DSBs) [16]. Cells were poured onto 22 × 22 mm coverslips in 35 mm dishes filled with medium and placed in an incubator for the stated repair time after irradiation. Briefly, cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS), permeabilized for 10 min on ice in 0.5% Triton X-100 in PBS, washed thoroughly with PBS, and then blocked for 1 h with 3% skim milk in PBS. The coverslips were then incubated with an antibody against histone H2AX phosphorylated on serine 139 (Upstate Biotechnology, Lake Placid, NY) for 2 h at 37°C. After incubation with primary antibody, the cells were washed with PBS, and Alexa Fluor 488-labeled anti-mouse IgG secondary antibodies (Invitrogen) were added. The coverslips were incubated for 1 h at 37°C, washed with PBS, and sealed onto glass slides with 0.05 ml PBS containing 10% glycerol (Wako, Osaka, Japan) and 20 μg/ml DAPI (4'6-diamidino-2-phenylindole; Invitrogen). The cells were examined using a Keyence fluorescence microscope (Keyence, Osaka, Japan), and the green intensity of the phosphor-H2AX signal on digitized images was automatically analyzed using the software package Dynamic Cell Count (Keyence). Using this software package, the numbers and sizes of foci exhibiting high-intensity staining with gamma-H2AX (green) in each type of A172 cell population were determined in more than 100 areas per condition.

### Statistical analysis

Values are presented as means ± standard errors. Statistical analyses were performed using the unpaired, two-tailed Student's *t*-test. A significance level of $P < 0.05$ was used for all analyses. The data on cell survival were fitted to the linear-quadratic dose relationship.

## RESULTS

### Detection of stemness in GSLCs

Figure 1 shows the characteristics of the GSLCs. To induce GSLCs, we cultured the A172 cells in SFM, as described above. Seven days after culturing in SFM, these cells were form-floating, neurosphere-like spheroid cells (Fig. 1A). In the Western blotting analysis, we found that two neural stem cell markers, Sox2 and Musashi, were more highly expressed in the GSLCs than in the A172 cells cultured in serum-containing medium as control cells (CCs) (Fig. 1B). However, no apparent CD133 expression was detected in either GSLCs or CCs that were cultured for 7 d. Therefore, we changed the CD133-detection assay for FACS analysis by using several time-points. In the FACS analysis, the ratio of CD133-positive GSLCs increased by 9% after 14 d, whereas the ratio of CD133-positive CCs was unchanged (Fig. 1C). The FACS analysis confirmed marked positivity in the WERI-Rb-1 (WE) cells, a retinoblastoma cell line used as a control (data not shown).

### Radiosensitivity of GSLCs and CCs

The radiosensitivity of GSLCs was compared with that of CCs under gamma-ray or neutron-beam irradiation. Figure 2 shows the surviving fractions of A172 under the two culture conditions after gamma-ray or neutron-beam irradiation. After gamma-ray irradiation, GSLCs showed significantly greater radioresistance than CCs. On the other hand, after neutron-beam irradiation, there was no significant difference in the sensitivity between GSLCs and CCs. The $D_{10}$ values were calculated by linear regression analysis from the survival curves shown in Fig. 2, and the $D_{10}$ dose parameters for survival following irradiation and their RBEs are listed in Table 1. The $D_{10}$ value represents the radiation dose that produces a survival fraction of 10%. To examine the difference in radiosensitivity between GSLCs and CCs, we referred to the resistance ratio. This ratio was calculated from the $D_{10}$ dose of GSLCs per that of each respective CC by these two forms of irradiation. For example, under gamma-ray irradiation, the ratio of the $D_{10}$ dose of GSLCs to that of CCs was 3.98/3.02 = 1.318. On the other hand, under neutron-beam irradiation, the $D_{10}$ dose of GSLCs per that of CCs was 1.17/1.25 = 0.936. The resistance ratio of neutron beams was smaller than that of gamma rays. Consequently, neutron-beam irradiation overcame the resistance to gamma-ray irradiation in A172 GSLCs. In other words, these results suggested that A172 GSLCs, which were radioresistant to gamma rays, became sensitive to neutron beams.

### Persistence of gamma-H2AX foci following irradiation

Figure 3 shows representative images of each type of A172 cells at 24 h after each type of irradiation. The fluorescence intensity of gamma-H2AX foci produced by neutron beams was stronger than that produced by gamma rays in both GSLCs and CCs, under the same staining conditions and the same photographic exposure time (Fig. 3). At a glance, the foci in both CCs and GSLCs produced by neutrons seemed larger than those produced by gamma rays. Figure 4A and B show the change in the numbers of gamma-H2AX foci following 4 Gy of gamma-ray or neutron irradiation in GSLCs and CCs induced from A172 cells. There were significantly more gamma-H2AX foci per cell in CCs than in GSLCs 24 h after gamma-ray irradiation. However, after neutron-beam irradiation, there was no apparent difference between GSLCs and CCs in the number of gamma-H2AX foci. Figure 4C and D show the distribution histograms of the size of foci induced in GSLCs and CCs, respectively, and Fig. 4E shows the mean size of gamma-H2AX foci at 24 h post-irradiation,

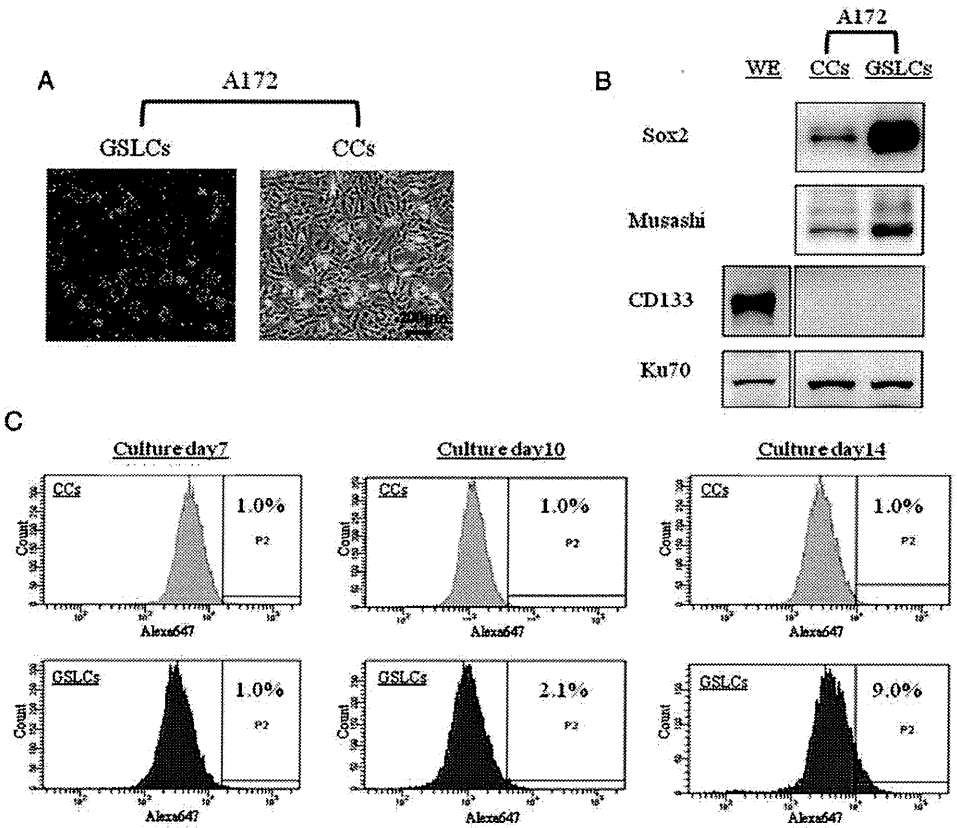


**Fig. 1.** Characteristics of the glioma stem-like cells. (**A**) The morphology of human glioma cell line A172 cultured for 7 d in serum-containing medium or serum-free medium. (**B**) The expression of typical stem cell marker proteins as examined by Western blot assays on Day 7 after culture. (**C**) The ratio of CD133-positive cells in FACS analysis; the number of days of culture is shown in each column, and the rate of CD-133-positive GSLCs was measured with a cutoff value obtained from the fluorescence intensity that occupied 1% by putative CD133-positive CCs in the total population. GSLCs = glioma stem-like cells; CCs: control cells; WE = WERI-Rb-1 (the retinoblastoma cell line used as a positive control for anti-CD133 Ab).

measured using the BZII image analysis system (Keyence). Figure 4C, D and E reveal definitively that neutron-beam irradiation induced larger gamma-H2AX foci than those observed after gamma-ray irradiation, not only in CCs but also in GSLCs of A172 cells. These results might suggest that DSBs were repaired more efficiently in GSLCs than in CCs following gamma-ray irradiation. In contrast, under neutron irradiation, the DNA DSBs were not repaired efficiently in either GSLCs or CCs.

## DISCUSSION

Research on GSCs has been conducted for many years, and GSCs have been found to contribute to the recurrence and resistance to therapy of malignant gliomas [2–6]. The difficulty of treating GBM may be attributed to the existence of GSCs in GBM, judging from the numerous published findings about GSCs.

In previous reports, GSCs were isolated from glioma tissues as spheres cultured in SFM containing stem-cell mitogens, epidermal growth factor and fibroblast growth factor, which is the same method used to isolate neural stem cells from brain tissue [2–4, 17]. Because of the lack of serum and the low plating density, most of the cells die, except those that divide in response to the stem-cell mitogens. The growth-factor-responsive cells proliferate to form floating clusters called neurospheres [18]. In this study, we induced GSLCs from cells of the human GBM line A172 using the same isolation-GSCs method as described previously [12]. In SFM containing the stem-cell mitogens, GSLCs were produced as neurosphere-like spheroid cells, and expressed neural stem cell markers such as Sox2 and Musashi (Fig. 1A and B) on Day 7 after induction. Actually, CD133 was hardly detected in Western blot analysis after 7 d of culture. Therefore, we performed FACS analyses and determined the ratio of CD133 positivity between GSLCs and CCs by kinetics study. The CD133-positive fraction in GSLCs

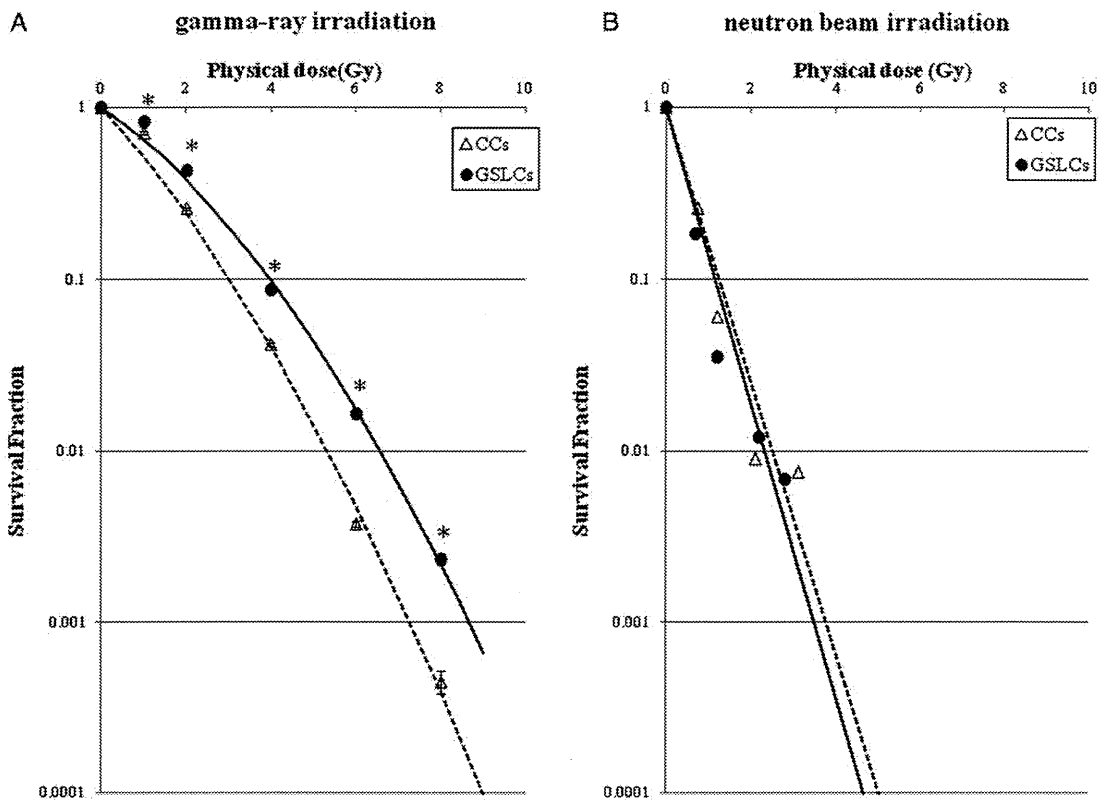


**Fig. 2.** Cell survival curves of GSLCs induced from A172 cells cultured with serum-free medium and CCs cultured with normal medium after gamma-ray (**A**), or neutron-beam irradiation (**B**). The data are fitted with a linear quadratic model. Bars represent the standard errors based on three independent experiments. $^*P < 0.05$ compared with the survival fraction of GSLCs and CCs. GSLCs = glioma stem-like cells; CCs = control cells.

**Table 1.** $D_{10}$ physical dose and RBE (relative biological effectiveness)

| | Irradiation | |
|---|---|---|
| **(CCs)** | **gamma rays** | **neutron beams** |
| $D_{10}$ physical dose | 3.02 | 1.25 |
| RBE[a] | | 2.42 |
| **(GSLCs)** | | |
| $D_{10}$ physical dose | 3.98 | 1.17 |
| RBE | | 3.40 |
| **Resistance ratio[b] (GSLCs/CCs)** | 1.318 | 0.936 |

[a]The ratio of the $D_{10}$ physical dose compared to that of gamma rays. [b]The ratio of the dose of radiation necessary to obtain the $D_{10}$ endpoint from GSLCs to that necessary in CCs. GSLCs = glioma stem-like cells, CCs = control cells, $D_{10}$ = the radiation dose that produces a surviving fraction of 10%.

increased gradually in comparison with that in CCs day by day, and on Day 14, 9% of GSLCs were CD133-positive, although many GSLCs were still negative for CD133. In addition, 30 d of induction culture resulted in a higher percentage of CD133-positive GSLCs—up to 21% (data not shown). We speculate that it took a long time for CD133-positive cells to be refined in the SFM, and thus there was an insufficient number of CD133-positive cells for detection by Western blot analysis on Day 7. Indeed, CD133 positivity in our GSLCs from A172 on Day 7 was still small in number, but other stemness markers increased compared with CCs, and CD133 is not always a good GSC marker [19–21]. In addition, GSLCs induced by this method showed the upregulation of ATP-binding cassette transporter G2 and increased chemo-resistance in comparison with CCs (data not shown and manuscript in preparation). Above all, these GSLCs from A172 were somewhat radioresistant for low-LET gamma rays. Thus, we judged that these GSLCs were adequate for our further experiments. In any event, GSLCs had some degree of stemness. Actually, we tried to induce GSLCs from three GBM lines. Among them, GSLC from A172 was most prominent with GSC phenotype and apparent radioresistance to low-LET γ-rays. Thereafter, we used GSLCs from A172 in the current studies. We assessed the radiosensitivity of GSLCs using colony-forming assay on

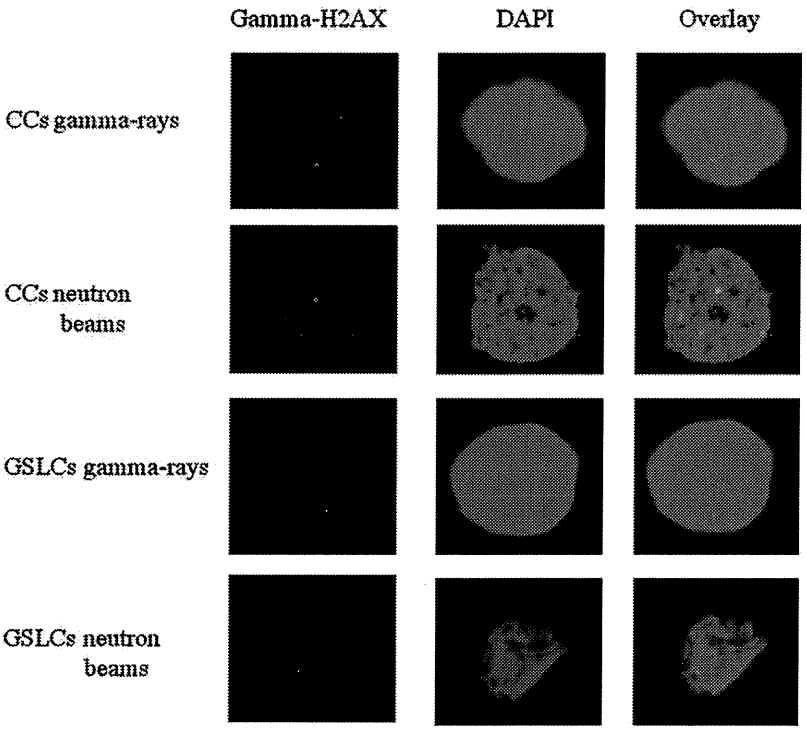


**Fig. 3.** Representative images of nuclear gamma-H2AX foci of CCs and GSLCs in A172. These cells were irradiated with different types of beams (total physical dose = 4 Gy) and fixed at 24 h post-irradiation for gamma-H2AX detection. DAPI = staining of nuclear DNA; Gamma-H2AX = staining of gamma-H2AX foci; GSLCs = glioma stem-like cells; CCs = control cells.

Day 7. Although, there might be a possibility of change in radiosensitivity associated with change of expression of CD133, especially in the later period of the induction of GSLCs, such as on Day 28, in the previous report [22], A172 CCs did not express CD 133, while radiation-induced GSLCs of A172 cells did express CD133. This is in accord with our experiment.

To evaluate the difference in radiosensitivity between GSLCs and CCs, we irradiated these cells with gamma rays or neutron beams, and found that the latter could overcome the radioresistance of GSLCs to gamma rays (Fig. 2 and Table 1). To obtain neutron beams, we used the Heavy Water Column of the KUR. These neutron beams consisted of fast, epithermal and thermal neutrons. Each neutron beam produced proton particles by elastic scattering ($^{1}H(n,n)^{1}H$) or nitrogen capture reaction ($^{14}N(n,p)^{14}C$) at irradiation, and these particles exhibited high-LET radiation. The LET of proton particles produced by the former reaction was about 50 keV/μm, and that produced by the latter reaction was about 35 keV/μm, whereas the gamma rays exhibited low-LET radiation. Therefore, it can be concluded that high-LET radiation can better overcome the radioresistance of GSLCs in comparison with low-LET irradiation. Ionizing radiation produces a broad spectrum of molecular lesions in DNA, including single-strand breaks, DSBs, and a great variety of base damages. DSBs are the most toxic form of DNA damage, because a single unrepaired DSB can lead to abnormal mitosis with losses of large fragments of DNA [23]. Further, it is generally accepted that high-LET radiation induces more serious DNA DSBs than low-LET radiation [11, 24]. In the current study, we demonstrated that high-LET radiation could damage GSLCs that were resistant to low-LET gamma rays. As previously described, GSCs have a large capacity to repair DSBs induced by low-LET radiation [5]. However, it was uncertain whether or not high-LET radiation could cause serious DSBs that were unrepairable, even in GSCs.

To clarify the response to DNA DSBs induced by gamma rays or neutron beams, we employed a gamma-H2AX assay. From a previous report, we judged the persistence of gamma-H2AX foci 24 h after treatment as unrepairable DSB [25]. GSLCs had a larger restoration capacity for DSBs than CCs after low-LET radiation, but could not repair DSBs sufficiently after high-LET radiation (Fig. 4A and B). Because reduced survival was accompanied by the persistence of DNA damage, as evidenced by the persistence of gamma-H2AX foci after irradiation [26], high-LET radiation could produce persistence of DSBs and induce fatal damage even