

Assessment of Boron Neutron Capture Therapy on Three Dimensional Model of Patient-Derived Cancer-Associated Fibroblasts and Oral Cancer

IZUMI HARA

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Abstract. Radiotherapy is widely used for management of oral cancer across both early and advanced disease stages, either as a definitive treatment or as part of multimodal therapy; however, its clinical application is often limited by radiation-induced toxicity to surrounding normal tissues. Boron neutron capture therapy (BNCT) is a targeted radiotherapy modality that selectively induces high-linear energy transfer (LET) radiation within tumor cells, enabling localized tumor control while sparing normal tissues. Despite its potential for high tumor selectivity, the effects of BNCT within a cancer-associated fibroblast (CAF)-rich tumor microenvironment (TME) remain poorly understood. In this study, we established a three-dimensional (3D) oral cancer model consisting of an oral cancer cell layer overlying a type I collagen stromal layer containing either patient-derived normal oral fibroblasts (NOFs) or CAFs (3D cancer NOFs and 3D cancer CAFs models, respectively). We compared the biological responses of these models to X-ray irradiation and BNCT. Both models showed time-dependent tumor stratification and invasion, with more pronounced invasion in the CAF-containing model. X-ray irradiation significantly reduced tumor area, depth of invasion, and cancer cell survival in the 3D cancer NOFs model, whereas the 3D cancer CAFs model showed limited reduction in tumor burden despite decreased CAF numbers, suggesting that the presence of CAFs confers resistance to X-ray treatment. BNCT induced thinning of the cancer cell layer, significant decreases in tumor area, invasion, and cancer cell survival in both models, while preserving NOFs in the 3D cancer NOFs model and markedly depleting CAFs in the 3D cancer CAFs model. These findings indicate that CAFs can confer resistance to X-ray irradiation, whereas BNCT maintains antitumor efficacy even in CAF-rich TMEs. Our 3D oral cancer model thus represents a useful preclinical platform for evaluating TME-oriented radiotherapy strategies and supports BNCT as a promising option for CAF-rich, poor-prognosis oral cancers.

Introduction

Oral cancer accounts for approximately 2.5% of all new cancer cases (1), and more than 90% of cases are classified as oral squamous cell carcinoma (OSCC) (2). OSCC is relatively sensitive to radiotherapy and chemotherapy (3); therefore, multidisciplinary treatment combining surgical resection, radiotherapy, and chemotherapy is widely employed. Radiotherapy plays a pivotal role across a broad range of disease stages—from early to advanced—either as a definitive treatment or as a component of multimodal therapy. X-ray irradiation remains the most commonly

used radiotherapeutic modality in clinical practice; however, it frequently induces adverse effects on surrounding normal oral tissues—such as mucositis, xerostomia, and dysgeusia—which markedly impair patients' quality of life and limit dose escalation for tumor control (4, 5). Therefore, the development of radiotherapeutic strategies with higher tumor selectivity is essential.

Boron neutron capture therapy (BNCT) is a particle radiotherapy that selectively destroys cancer cells through a nuclear reaction between boron-10 (^{10}B) accumulated in tumor cells and externally applied neutrons (6, 7). Tumor selectivity is achieved by preferential accumulation of boron-containing compounds—such as boronophenylalanine (BPA)—in cancer cells via upregulated amino acid transporters (e.g., L-type amino acid transporter 1 (LAT1)), resulting in higher intracellular boron concentrations in tumor tissue compared with surrounding normal tissues (8). Upon neutron irradiation, the $^{10}\text{B}(\text{n},\alpha)^7\text{Li}$ reaction generates high-linear energy transfer (LET) α -particles and lithium recoil nuclei with a very short path length (approximately 5–9 μm), confining cytotoxic effects to boron-loaded cells and their immediate vicinity (9). Owing to its ability to deliver high-precision and minimally invasive treatment while sparing normal tissues, BNCT has gained increasing clinical importance and is now approved in Japan for the treatment of unresectable, locally advanced, or recurrent head and neck cancers (10).

In recent years, cancer treatment has shifted its focus from tumor cells alone to the surrounding environment that plays a crucial role in tumor progression, known as the tumor microenvironment (TME). TME is a dynamic network composed of cellular components—including cancer cells, cancer-associated fibroblasts (CAFs), and immune cells—as well as extracellular matrix (ECM) and secreted factors (11). Among these components, CAFs are known to play essential roles in tumor progression, invasion, angiogenesis, and therapeutic resistance—including radioresistance (12–14). Although CAFs represent a potential therapeutic target, their substantial heterogeneity often limits the effectiveness of therapies directed solely at CAFs (15). Thus, treatment strategies that consider both cancer cells and stromal components are required (16).

For in vitro evaluation of radiotherapy effects, clonogenic assays have traditionally been applied; however, they are not suitable for cell types with limited colony-forming ability (17). Additionally, two-dimensional (2D) cultures poorly recapitulate key TME features—such as cell-cell interactions, oxygen and nutrient gradients, and ECM

architecture—resulting in insufficient representation of in vivo radiosensitivity (18, 19).

To overcome these limitations, three-dimensional (3D) culture models that better replicate tumor architecture and stromal organization have emerged as promising preclinical platforms for evaluating cancer–stroma interactions and treatment responses (19, 20). These models allow the formation of physiologically relevant characteristics, including spatial multicellular arrangement and hypoxic niches, providing a more accurate assessment of radiation responses (21, 22).

In this study, we established a 3D oral cancer model consisting of a cancer cell layer overlying a fibroblast-embedded stromal layer using patient-derived CAFs. Using this model, we aimed to elucidate the effects of BNCT on both cancer cells and CAFs and to explore the potential of TME-oriented radiotherapy strategies for oral cancer.

Materials and methods

Cell culture. Human tongue squamous cell carcinoma cell line HSC-4 (TIMS(JCRB) Bank, JCRB0624), patient-derived normal oral fibroblasts (NOFs), and cancer-associated fibroblasts (CAFs) were used in this study. NOFs and CAFs were provided by Niigata University for the purpose of the present research (Niigata University Ethical Committee Approval #2022-0300) (23). The isolation, characterization, and primary culturing of NOFs and CAFs were performed as previously described (23). Both NOFs and CAFs were derived from the same OSCC patient, with NOFs isolated from tumor-free margins and CAFs isolated from tumor-involved regions. All cells were cultured in α -MEM (Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum (FBS; Corning, NY, USA) in a humidified atmosphere containing 5% CO₂ at 37°C.

Fabrication of a 3D oral cancer model using patient-derived fibroblasts. A two-layered 3D oral cancer model, consisting of a stromal layer with NOFs or CAFs and an upper cancer cell layer, was constructed with modifications from a previously reported protocol (24). First, 250 μ L of type I collagen matrix was prepared by mixing Cellmatrix Type I-A (Nitta Gelatin, Osaka, Japan), 5 $\times$ concentrated DMEM (Nitta Gelatin, Osaka, Japan), and reconstitution buffer (Nitta Gelatin, Osaka, Japan) at a ratio of 7:2:1, and then added into a 12-well cell culture insert (Thincert deep-well plate; Greiner Bio, Kremsmünster, Austria). Subsequently, 750 μ L of the same collagen mixture containing 2×10^5 NOFs or CAFs was overlaid to generate the stromal layer. After adding 5 mL of medium to each well and 0.5 mL inside the insert, the gels were cultured under submerged conditions for 7 days. Medium was changed every two days. On day 2, the stromal layer was gently detached from the insert wall with a scalpel to promote spontaneous contraction. After 7 days, 2.0×10^5 HSC-4 cells suspended in 10 μ L medium were seeded onto the surface of the contracted stromal layer and cultured for an additional 7 days under submerged conditions. On day 14, the 3D oral cancer model was raised at an air–liquid interface by reducing the medium volume

to 4.2 mL. The culture continued to promote epithelial stratification for another 7 days. The number of days since the start of the air–liquid interface culture is denoted as post-air–liquid interface culture day (PD), and day 14 is equal to PD 0. Models prepared with NOFs and CAFs were referred to as the 3D cancer NOFs model and 3D cancer CAFs model, respectively.

X-ray irradiation on 3D oral cancer model. X-ray irradiation was performed on PD 2 using an MX-80 Labo cabinet irradiator (mediXtec Corporation, Chiba, Japan) at 80 kV and 1.25 mA. Irradiation doses of 0, 5, 10, and 20 Gy were delivered at a dose rate of 26.40 Gy/h. After the irradiation, the 3D oral cancer models were cultured for another 5 days at the air–liquid interface, with refreshing the medium every two days.

Evaluation of boron uptake in the 3D oral cancer models. BNCT treatment planning was based on previous methodology with minor modifications (25). Because BNCT efficacy depends on ¹⁰B accumulation and incident neutron flux, boron concentration in the 3D models was first quantified. The boron drug Borofalan (STEBORONINE, Stella Pharma, Osaka, Japan) was added to culture medium at a volume ratio of 1:10 for 24 hours prior to sampling. The collected samples were heated after being treated with HNO₃ and measured by an inductively coupled plasma-mass spectrometer (ICP-MS, Agilent7500cx, Agilent Technologies, Tokyo, Japan).

Neutron irradiation on 3D oral cancer model. Based on the measured boron concentration, the irradiation time was adjusted so that radiation dose to the cancer cell layer was equivalent to that of X-ray irradiation. Borofalan was added to the medium 24 hours before irradiation at a volume ratio of 1:10. Neutron irradiation was performed on PD 2 at the Kyoto University Research Reactor (KUR) using heavy-water neutron irradiation equipment at 1 MW reactor power (26). Neutron fluence was measured by radioactivation analysis using gold foil (27). After irradiation, the models were cultured for 5 additional days with medium changes every two days.

Histological analysis. After 21 days of total culture, the models were fixed in 4% paraformaldehyde and paraffin-embedded. Sections of 5 μ m thickness were stained with Masson's trichrome (MT) staining. Images were acquired at 10 $\times$ magnification using an all-in-one fluorescence microscope (BZ-X800; Keyence, Osaka, Japan). Collagen-rich light-blue regions were defined as the stromal layer, while dark-purple epithelial-like regions above the stromal layer were defined as the cancer cell layer. Tumor area ratio was calculated as the percentage of tumor area in the total cross-sectional area using ImageJ software (ver. 1.54; NIH, MD, USA), based on a modified protocol (28). Depth of invasion (DOI) was defined as the vertical distance from the tumor surface to the deepest invasive point and measured at three hotspot regions per section. Hotspots were defined as regions with relatively high cancer cell density. Numbers of cancer cells and fibroblasts were counted using ImageJ. Based on our previous findings (28), fibroblasts were identified by their

smaller nuclear and cellular size relative to cancer cells. The relative survival rate was defined as the ratio of the number of cells in irradiated groups to that in non-irradiated controls, calculated separately for cancer cells and fibroblasts in both models. All histological analysis was performed in a blinded manner.

Statistical analysis. Comparisons between PD0 and PD2, PD4, and PD7, as well as between pre- and post-irradiation

conditions, were performed using an unpaired t-test. Differences among multiple groups were analyzed using one-way ANOVA followed by Tukey's post-hoc test. Statistical significance was set at $p < 0.05$. Unless otherwise indicated, data are presented as mean $\pm$ standard deviation (SD).

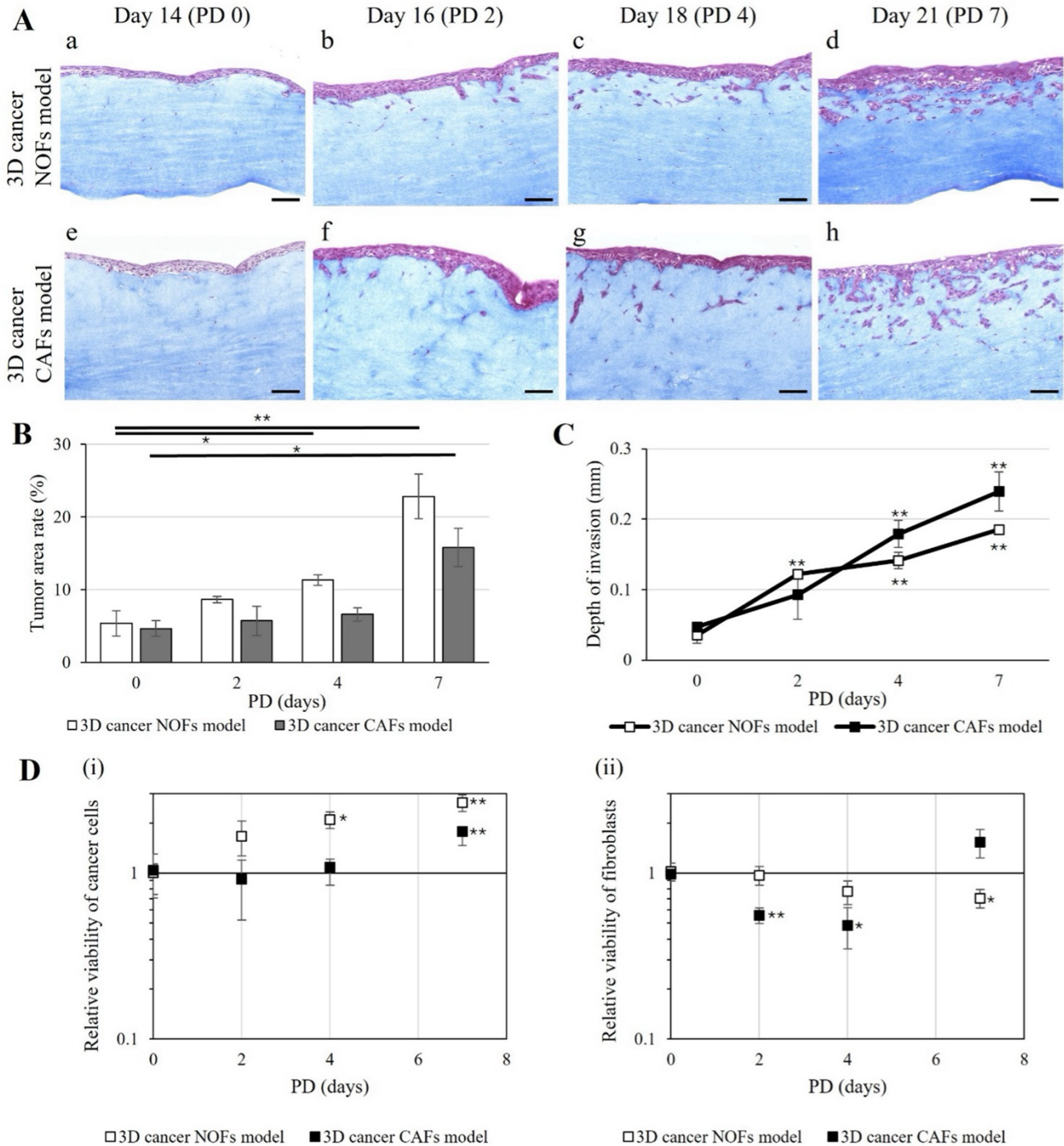


Figure 1. Histological analysis of the 3D oral cancer models.

(A) Representative histological images of the 3D cancer NOFs model (a-d) and the 3D cancer CAFs model (e-h) at PD 0 (a,e), PD 2 (b,f), PD 4 (c,g), and PD 7 (d,h). MT staining. Scale bar: 100 μ m.

(B) Comparison of tumor area ratio at PD 0, 2, 4, and 7.

(C) Comparison of DOI at PD 0, 2, 4, and 7.

(D) Relative viability after the air-liquid interface culture. (i) Cancer cell viability and (ii) fibroblast viability.

* $p < 0.05$, ** $p < 0.01$, unpaired t-tests, compared to PD 0. (N=3)

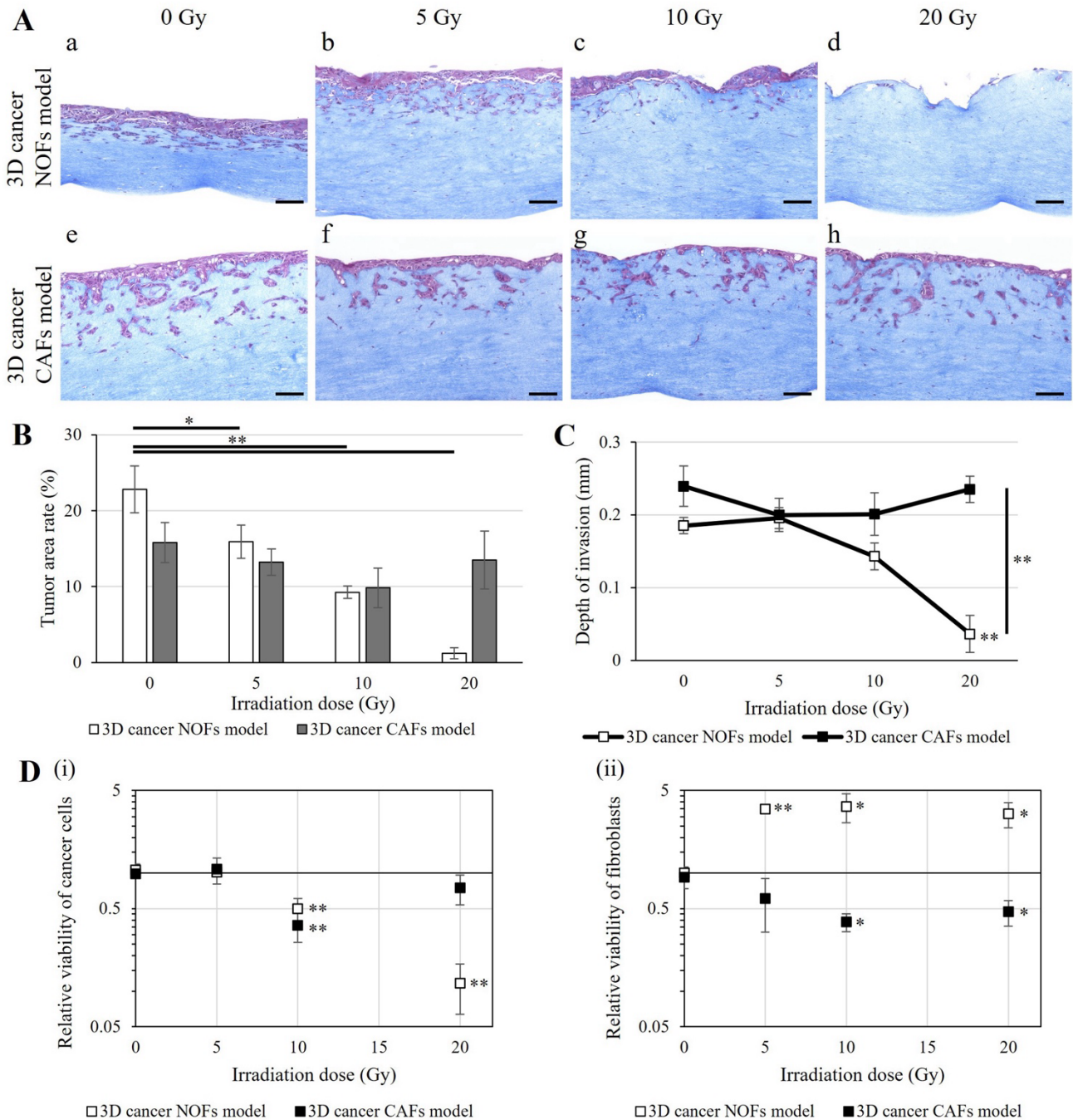


Figure 2. Histological analysis of the 3D oral cancer models after X-ray irradiation.

(A) Representative histological images of the 3D cancer NOFs model (a-d) and the 3D cancer CAFs model (e-h) five days after irradiation.

Doses: 0 (a,e), 5 (b,f), 10 (c,g), and 20 (d,h) Gy. MT staining. Scale bar: 100 μ m.

(B) Comparison of tumor area ratio after X-ray irradiation (0, 5, 10, 20 Gy).

(C) Comparison of DOI following X-ray irradiation (0, 5, 10, 20 Gy).

(D) Relative viability after the air-liquid interface culture. (i) Cancer cell viability and (ii) fibroblast viability.

* $p < 0.05$, ** $p < 0.01$, unpaired t-tests, compared to 0 Gy. (N=3)

Results

Establishment and histological characterization of the 3D oral cancer models. The 3D cancer NOFs and 3D cancer CAFs models were fabricated for histological evaluation. Progressive invasive tumor growth was observed over time (Fig. 1A). At PD 0, the cancer cell layer consisted of only a few layers, whereas stratification and invasion into the stromal layer became evident from PD 2 onward. Tumor area ratio increased progressively in both models (Fig. 1B).

Furthermore, tumor invasion into the stroma was observed, with mean DOI values of 0.19 mm in the 3D cancer NOFs model and 0.24 mm in the 3D cancer CAFs model at PD 7 (Fig. 1C). Relative cell survival rates were plotted on a logarithmic scale, normalized to the median value at PD 0 (Fig. 1D). The relative survival rate of cancer cells increased over time in the 3D cancer NOFs model, whereas minimal change was observed until PD 4 in the 3D cancer CAFs model, followed by a slight increase at PD 7 (Fig. 1D(i)). Fibroblast survival decreased until PD 4 in both

Irradiation time	Type of model	Boron concentration (ppm)	Thermal Neutron Fluence (cm ²)	Total dose (Gy-eq)
0 minute	3D cancer NOFs	53.91 ± 4.14	0	0
	3D cancer CAFs	56.14 ± 6.24	0	0
20 minutes	3D cancer NOFs	53.91 ± 4.14	2.21 × 10 ¹²	8.71 ± 0.67
	3D cancer CAFs	56.14 ± 6.24	2.21 × 10 ¹²	9.07 ± 1.01
40 minutes	3D cancer NOFs	53.91 ± 4.14	4.48 × 10 ¹²	17.66 ± 1.36
	3D cancer CAFs	56.14 ± 6.24	4.48 × 10 ¹²	18.38 ± 2.04

Table 1. Evaluation of boron concentration in the 3D oral cancer models and total absorbed dose of the 3D models after neutron irradiation for 20 or 40 minutes.

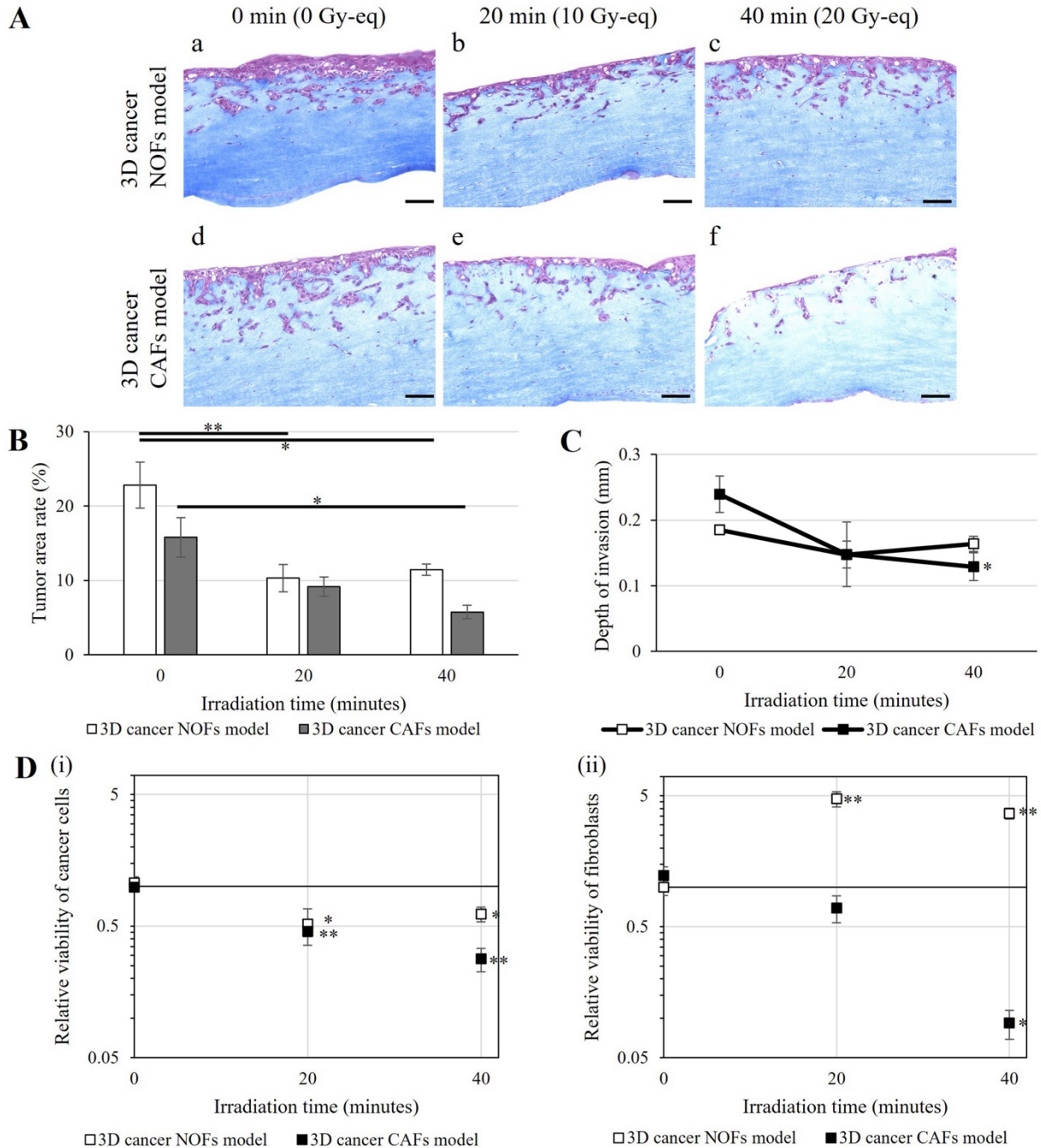


Figure 3. Histological analysis of the 3D oral cancer models after BNCT.

(A) Representative histological images of the 3D cancer NOFs model (a-c) and the 3D cancer CAFs model (d-f) five days after irradiation.

Irradiation time: 0 (a,d), 10 (b,e), and 20 (c,f) minutes. MT staining. Scale bar: 100 μ m.

(B) Comparison of tumor area ratio after neutron irradiation (0, 10, 20 minutes).

(C) Comparison of DOI following neutron irradiation (0, 10, 20 minutes).

(D) Relative viability after the air-liquid interface culture. (i) Cancer cell viability and (ii) fibroblast viability.

* $p < 0.05$, ** $p < 0.01$, unpaired t-tests, compared to 0 Gy. (N=3)

models; however, fibroblast survival increased at PD 7 in the 3D cancer CAFs model (Fig. 1D(ii)).

Effects of X-ray irradiation on the 3D oral cancer models. Following X-ray irradiation at 0, 5, 10, and 20 Gy, the 3D cancer NOFs and CAFs models were collected on PD 7 (five days post-irradiation) for histological evaluation. At PD 7, tumors in the 3D cancer CAFs model remained multilayered across all dose groups, whereas in the 3D cancer NOFs model the cancer cell layer became progressively thinner with increasing dose and largely lost its multilayered architecture at 20 Gy (Fig. 2A). In the 3D cancer NOFs model, X-ray irradiation significantly reduced the tumor area in a dose-dependent manner (Fig. 2B). In contrast, the tumor area in the 3D cancer CAFs model showed no significant changes following irradiation. DOI exhibited a dose-dependent decreasing trend in the 3D cancer NOFs model, with a significant reduction observed at 20 Gy, whereas the 3D cancer CAFs model did not exhibit irradiation-induced suppression of invasion (Fig. 2C). Notably, DOI at 20 Gy was significantly greater in the CAF-containing model compared with the NOFs model ($p < 0.01$), suggesting reduced X-ray responsiveness in CAF-rich tumor microenvironments. The relative survival rate of OSCC cells decreased to approximately 10% following 20 Gy irradiation in the 3D cancer NOFs model (Fig. 2D(i)). In contrast, although a significant reduction in cancer cell survival was observed at 10 Gy in the 3D cancer CAFs model, no further decrease occurred at 20 Gy. For fibroblasts, X-ray irradiation had no significant effect on NOFs, whereas CAFs exhibited dose-dependent radiosensitivity at ≥ 10 Gy (Fig. 2D(ii)).

Boron uptake in the 3D oral cancer models. Boron concentrations measured at PD 2 (24 hours following exposure to boron-containing medium at PD 1) were 53.91 ± 4.14 ppm in the 3D cancer NOFs model and 56.14 ± 6.24 ppm in the 3D cancer CAFs model, with no significant difference between models (Table 1).

Effects of BNCT on the 3D oral cancer models. Based on the measured boron concentrations, neutron irradiation times were calculated to deliver tumor doses of 10 and 20 Gy-Eq (gray-equivalent). The Gy-Eq dose represents the biologically weighted dose calculated by applying appropriate RBE and CBE factors to each absorbed dose component. To achieve these planned tumor doses, neutron exposure times of 20 and 40 min were required, respectively. The resulting absorbed doses were 8.71 ± 0.60 Gy-Eq and 17.66 ± 1.36 Gy-Eq in the 3D cancer NOFs model, and 9.07 ± 1.01 Gy-Eq and 18.38 ± 2.04 Gy-Eq in the 3D cancer CAFs model—consistent with dose planning (Table 1). Similar to X-ray outcomes, thinning of the cancer cell layer was observed after BNCT (Fig. 3A). Tumor area significantly decreased in a time-dependent manner in the 3D cancer NOFs model. Additionally, in the 3D cancer CAFs model, tumor area was significantly reduced following 40-min irradiation ($p < 0.05$) (Fig. 3B). DOI was reduced by 40% in the CAF-containing tumors after 40-min irradiation ($p < 0.05$) (Fig. 3C). The relative survival of OSCC cells significantly decreased in both models (Fig. 3D(i)). Fibroblast survival increased in the

NOF-containing tumors, whereas CAF survival decreased following BNCT (Fig. 3D(ii)).

Comparison between X-ray irradiation and BNCT. Both irradiation modalities significantly reduced cancer cell numbers in the 3D cancer NOFs model (Fig. 4A). In the 3D cancer CAFs model, BNCT exerted a stronger effect on OSCC cells than 20 Gy X-ray irradiation. Conversely, in the 3D cancer NOFs model, X-ray irradiation demonstrated greater tumor suppression than BNCT. Fibroblast counts remained unchanged in the 3D cancer NOFs model under both irradiation conditions. However, in the 3D cancer CAFs model, fibroblast numbers significantly decreased after irradiation, with BNCT showing a more pronounced effect at 20 Gy-Eq (Fig. 4B).

Discussion

CAFs are key regulators of the TME, which comprises cancer cells, stromal cells, and an ECM rich in collagen. CAFs promote cancer cell proliferation, invasion, and metastasis through direct cell–cell contact and the secretion of various cytokines and chemokines. In head and neck, lung, and colorectal cancers, CAF infiltration and activation correlate with poor prognosis (29). Moreover, CAFs play a central role in remodeling stromal collagen and shaping tissue microarchitecture (30). Given that collagen provides both mechanical and biochemical support for tumor invasion, alterations in collagen organization and matrix stiffness critically influence tumor progression patterns (31). Based on this background, we established a 3D oral cancer model by co-culturing patient-derived CAFs and a human OSCC cell line within type I collagen gels, and investigated the impact of CAFs on radiotherapy responses from a histomorphological perspective. We found that seeding CAFs into the stromal compartment led to a marked time-dependent increase in cancer cell number and depth of invasion, enabling the construction of a highly aggressive 3D oral cancer model (Fig. 1). These findings are consistent with previous reports that CAFs promote tumor growth and invasion via cell–cell contact and soluble factors (32), and they support the concept that collagen-based ECM remodeling is a major driving force of tumor formation and invasion (33, 34). Collectively, this study is novel in that it established a 3D oral cancer model incorporating patient-derived CAFs and NOFs and evaluated the effects of BNCT, providing important evidence that it may serve as a translational research model for assessing radiotherapeutic responses in the context of the TME.

In the field of head and neck oncology, there is growing emphasis on minimally invasive treatments that maintain tumor control while reducing normal tissue toxicity, and various novel radiotherapy modalities are being developed (35). Among them, high-LET radiation such as α -particles offers potent cytotoxicity with relatively limited damage to surrounding normal tissues, as it induces dense ionization tracks that cause direct and irreparable DNA damage in tumor cells, and BNCT is a representative example. In BNCT, tumor-selective accumulation of ^{10}B followed by irradiation with thermal neutrons generates high-LET α -particles and ^7Li recoil nuclei, delivering highly localized

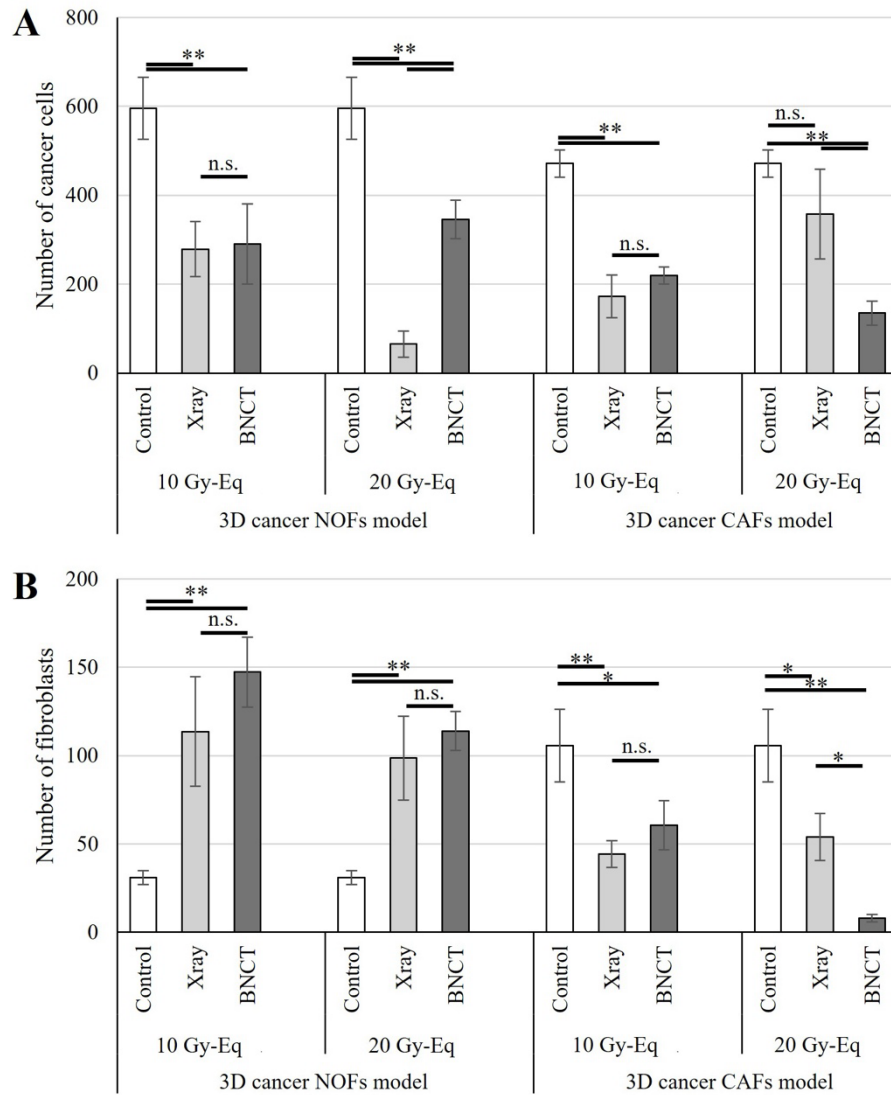


Figure 4. Comparison of treatment effects between X-ray irradiation and BNCT.

(A) Comparison of cancer cell counts after X-ray or BNCT (10 or 20 Gy-Eq) in the 3D cancer NOFs and 3D cancer CAFs models.

(B) Comparison of fibroblast cell counts after X-ray or BNCT (10 or 20 Gy-Eq) in the 3D cancer NOFs and 3D cancer CAFs models.

n.s. (not significant), $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, one-way ANOVA, Tukey's test, compared to control (N = 3).

energy deposition on the order of a single cell diameter. Unlike conventional X-ray therapy, BNCT is characterized by low cell-cycle dependence and efficacy against hypoxic cells (36-38). These properties are expected to be advantageous for treating TME-driven radioresistant tumors. However, the direct effects of BNCT on TME components, including CAFs, as well as the responses mediated by tumor-stroma interactions, remain largely unclear. Thus, visualizing and evaluating CAF dynamics within a 3D model, as performed in the present study, is of substantial significance.

In the present study, we first assessed the response of the 3D models to X-ray irradiation. In the 3D cancer NOFs model, increasing X-ray dose led to a dose-dependent reduction in tumor area and cancer cell invasion. In contrast, in the 3D cancer CAFs model, although CAF numbers decreased after irradiation, no marked suppression of cancer cell number or invasion was

observed, suggesting that the presence of CAFs may attenuate the therapeutic efficacy of X-ray irradiation (Fig. 2). These observations are in line with clinical reports indicating that CAFs contribute to tumor regrowth and malignant progression after treatment and are associated with radiation-induced adverse events and poor prognosis (11, 39). Our findings support the notion that CAFs can act as a determinant of tumor resistance to conventional X-ray radiotherapy.

We next applied BNCT to the 3D oral cancer models. In the 3D cancer NOFs model, neutron dose escalation resulted in a reduction in cancer cell number and invasion, while NOF survival was largely preserved. This is consistent with clinical BNCT studies reporting relatively mild normal tissue toxicity (40) and suggests that our model can capture the normal-tissue-sparing characteristics of BNCT. Moreover, in the 3D cancer CAFs model, increasing neutron dose significantly reduced

cancer cell number, invasion, and CAF counts, indicating that BNCT may be effective even in tumors heavily infiltrated by CAFs, which are considered a poor prognostic factor (Fig. 3). The BNCT effects observed in this study are also concordant with previous organoid, animal, and clinical studies demonstrating the high tumor selectivity and strong tumor control capacity of BNCT (38, 41, 42).

Traditionally, 2D cell culture and animal models have been the mainstay of preclinical research. However, 2D cultures cannot adequately reproduce key TME elements such as cell-cell interactions, ECM organization, and oxygen gradients, often resulting in treatment responses that diverge from in vivo behavior (43). In addition, most animal models rely on immunodeficient mice that do not faithfully recapitulate human tumor stroma, making it difficult to accurately evaluate the influence of the human TME. In this context, the concept of “co-clinical trials,” which aims to enable direct comparison between preclinical and clinical data, has been proposed, and the importance of patient-derived 3D culture models and organoids as evaluation platforms is increasing (44). Our 3D oral cancer model, which reproduces CAF-dependent invasion and ECM remodeling using patient-derived CAFs and OSCC cells while allowing comparison of responses to X-ray irradiation and BNCT, may serve as a translational research model within such co-clinical trial frameworks.

Furthermore, the advent of immune checkpoint inhibitors has renewed interest in combination strategies with radiotherapy that may elicit abscopal effects (45). However, optimal dose fractionation, total dose, and treatment timing for combining immune checkpoint inhibitors with radiotherapy remain to be established, and there is a need to develop appropriate preclinical models. Because CAFs secrete inflammatory cytokines and immunosuppressive mediators and modulate the tumor immune microenvironment, applying BNCT to CAF-containing 3D models and simultaneously evaluating CAF responses and immune milieu alterations will be important for designing future combined radiotherapy-immunotherapy strategies.

This study has several limitations. First, we focused on early post-irradiation changes and did not sufficiently evaluate long-term tumor-stroma interactions such as CAF reactivation, phenotypic conversion of NOFs into CAF-like cells, or potential links to radiation-induced carcinogenesis (46, 47). Second, the present model does not fully reconstruct a multicellular TME that includes specific ECM compositions, immune cells, and vascular endothelial cells. Future studies should incorporate more physiologically relevant systems, such as 3D in vitro oral cancer models co-culturing four types of cells to mimic tumor-adjacent normal tissues (23) or oral cancer models with vascular networks (48), to analyze dose-response relationships under conditions closer to the in vivo environment. Third, because the current model is based on a single cancer cell line, it does not fully capture inter-tumoral heterogeneity; validation using multiple cell lines and patient-derived organoids will be necessary.

In conclusion, this study is one of the first to compare the responses of CAF-containing TMEs to X-ray irradiation and BNCT using a 3D oral cancer model. Our findings demonstrate that CAFs can attenuate the therapeutic

efficacy of X-ray irradiation, whereas BNCT retains its effectiveness in the presence of CAFs. These results suggest that BNCT may represent a promising therapeutic option for CAF-rich, poor-prognosis tumors, and that the establishment of 3D TME models as preclinical evaluation platforms will contribute to the development of personalized and minimally invasive radiotherapy strategies.

Conclusion

In this study, we established a 3D oral cancer model that recapitulates cancer cell proliferation and invasion by co-culturing patient-derived CAFs within a collagen-based stromal matrix and overlaying human oral squamous cell carcinoma cells. Our findings suggest that this 3D TME model represents a valuable research platform for elucidating tumor progression mechanisms and evaluating therapeutic responses in vitro.

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