Evaluation of various cryoablation protocols in multiple cell lines using quantitative biomarkers of Bioluminescence imaging: an *in vitro* study

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Abstract

Background & Aims: To investigate whether the optimal treatment protocols of cryoablation differ among various cell lines utilizing bioluminescence imaging (BLI).

Methods: Eight luciferase-expressing cancer cell lines originated from various organs were used in this study, including lung, renal, liver, breast, colon, prostate, and skin. These cell lines were uniformly seeded in 96-well plates. The cell lines were then divided into four groups: Group 0 (G0), as a control group, and three treatment groups (G1-G3). G1-G3 were subjected to cryoablation at temperatures of -45°C, -60°C, and -80°C, respectively, with varying treatment cycles (single, double, or triple cycle, respectively). Each freeze–thaw cycle consisted of ten minutes of freezing followed by five minutes of thawing. Finally, the average radiance of the cells was assessed using BLI.

Results: Cryoablation at "-60°C and -80°C" showed a significant decrease in average radiance, even after a single cycle of treatment in all cell lines. The average radiance of cells treated with -45°C was substantially lower than that of untreated cell lines except melanoma.

Conclusion: We found that -45°C successfully eradicated all cancer cells across multiple cycles except melanoma. The most effective cryoablation protocol for all cell lines was observed at temperatures of -60°C or lower.

Key words: cryoablation, freeze–thaw cycles, multiple cycles, freezing temperature, bioluminescence imaging

Introduction

Cryoablation stands out from numerous ablation techniques, such as radiofrequency ablation (RFA), microwave, high-intensity focused ultrasound, laser interstitial thermotherapy, microwave thermotherapy, and radiosurgery, due to its several distinct advantages. Among them, cryoablation can be performed under local anesthesia, which alleviates patient discomfort and subsequently shortens recovery periods[1]. Moreover, live monitoring during the treatment process is facilitated by the formation of an 'ice-ball', which can be easily visualized via computed tomography (CT), magnetic resonance imaging (MRI) or ultrasonography[2,3]. Finally, cryoablation provides a diverse range of probe options for tumors in the 1-10 cm range, thereby offering greater flexibility than other ablation methods[4,5].

The tissue injury from cryoablation has been known for many years as it occurs from two main mechanisms. First mechanism, or the direct injury is caused by ice crystal formation in intracellular or extracellular space after freezing. Ice crystal formation in intracellular space is the most important factor for cellular death. Ice crystals eliminate water from biological system by osmotic pressure which in turn produces host deleterious effect. The second mechanism is caused by microvascular stasis after the thawing stage, as the tissue congests and becomes edematous. This mechanism is commonly seen in *in vivo* environment. From the molecular perspective, apoptosis is the main mechanism of cellular death after cryoablation[6].

Although cryoablation holds several distinct advantages, establishing optimal treatment protocols can be a complex process since the protocols may be influenced by numerous factors, such as the temperature applied, the duration of freezing, and the number of freezing cycles[7,8]. Previous studies suggests that a longer freezing duration and slower thawing may be the main factors that disrupt cells, reducing cell survival[6,9]. Although a single-cycle treatment typically

necessitates cooling to -40°C or lower to ensure sufficient cancer cell destruction[10], repeating treatments at higher temperatures may also prove effective[11]. Based on the findings from the previous report by Yang WL et al., the tolerance to cryo-injury at different temperatures varies among different human colorectal cancer cell lines[12]. At -10°C, the order of tolerance from least to greatest was KM12C, HCT116, KM12SM, and HT29. However, the order of tolerance changed to KM12C, HT29, HCT116, and KM12SM at -20°C[12]. These results indicate that the ideal protocol for cryoablation may need to be tailored to the specific tumor cell type, as different cell lines respond differently to cryo-injury at different temperatures. However, minimal research has been conducted to ascertain the variation in cell-killing effects among different tumor types and whether the optimal treatment protocols differ correspondingly.

Bioluminescence imaging (BLI) is a widely used molecular imaging technique that captures photons emitted as bioactive signals from a tissue or cells during a catalytic luciferin-luciferase reaction[13,14]. The technique has been utilized in the preclinical evaluation of the therapeutic effects of anticancer treatments on tissue or cells. It allows the quantification of viable cells in tumors following treatment using various orthotopic and xenograft models. BLI is a cost-efficient method that provides relatively high detection sensitivity compared with other imaging processes[15].

The aim of this study was to investigate whether the optimal treatment protocols of cryoablation differ among various cell lines utilizing the BLI technique.

Materials and Methods

Cell lines

Eight types of luciferase-expressing cancer cell lines were employed in this *in vitro* study (Table 1). All luciferase-expressing cell lines were purchased from the Japanese Collection of Research Bioresources Cell Bank (Tokyo, Japan) or Riken Bioresource Research Center (Ibaraki, Japan). All tumor cell lines stably expressed the luciferase gene as a reporter. This study has been approved by the research ethics committee at Gunma University Graduate School of Medicine.

Initially, colon (Col-26-luc) cancer cells were cultivated at six distinct densities (1×10^4, 5×10^4, 1×10^5, 5×10^5, 1×10^6, and 5×10^6 cells per $0.32cm^2$ area) to determine the suitable cell seeding density. Subsequently, BLI was implemented to ascertain the correlation between cell density and average radiance to check the correlation between cell density and average radiance. Based on the average radiance rate of BLI and optimal signal intensity, each cancer cell line was seeded at a density of $5x10^6$ per well in fifteen 96-well plates with 0.2 ml of medium, in accordance with the cell culture guidelines provided by Thermo Fisher Scientific Inc., Waltham, U.S.A. The cells were suspended in medium containing 10% fetal bovine serum (Wako Chemical, Osaka, Japan), 1% antibiotic (penicillin–streptomycin, Wako Chemical, Osaka, Japan), Dulbecco's phosphate buffered saline (Sigma–Aldrich, Japan LLC, Tokyo, Japan) and other required substances. Trypsin/0.53 mM ethylenediaminetetraacetic acid in Hanks balanced salt solution (Wako Chemical) was used for cell passage. For imaging

evaluation, D-luciferin sodium salt (FUJIFILM Wako Pure Chemical Co., Ltd, Osaka, Japan) and PBS were used, maintaining a final concentration of 150 µg/ml (D-luc solution).

Cryoablation technique

When the confluence had reached over 80%, the cells were divided into three separate groups depending on the respective treatment cycle. Group 1 (n=192) received one treatment cycle, Group 2 (n=192) received two treatment cycles, and Group 3 (n=192) received three treatment cycles. A control group (n=64) received no treatment. The cryoablation procedure was performed after aspirating the medium from each well plate. From each treatment group, 24 culture wells per cell line were placed in different freezers that were adjusted to temperatures of -45°C (Nihon Freezer, NF140C, Tokyo, Japan), -60°C (Nihon Freezer, NF140C, Tokyo, Japan) or -80°C (Ultra-low freezer, PHC Holdings Corporation, Tokyo, Japan). The protocol for one cryoablation cycle comprises 10 minutes of freezing, followed by a 5-minute thawing process in a cell incubator with a temperature of 37°C and 5% CO_2 (**Fig. 1**).

Imaging protocol

For imaging in this study, the IVIS® Lumina III *In Vivo* Imaging System (Perkin Elmer Inc., WA) was employed. Following each treatment, 0.075 µL of D-Luc solution was added to each well to generate the BLI signal. After an incubation period of two minutes, BLI was captured at two-minute intervals for a total of twenty minutes, with each exposure lasting ten seconds. The fields of view encompassed an area of 25x25 cm^2 utilizing 500 nm excitation and 600 nm emission.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 10.0.0 for Windows (GraphPad Software, San Diego, CA). The ratio of average radiance was calculated as "value of -xx℃/value of G0x100%". One-way ANOVA followed by Holm-Šídák's multiple comparisons test was performed for the evaluation of treatment effects between all groups. A *p* value of less than 0.05 (*) or 0.01 (**) was considered statistically significant.

Results

Correlation between cell density and average radiance

A strong positive curvilinear relationship was observed between the density of cancer cells and the average radiance of BLI, with a correlation coefficient of 0.96 ($p<0.01$ **Fig. 2**).

Comparison of cell-killing effect of single-cycle treatment

At -45°C, all but melanoma (B16F10-luc) cell lines showed a significant decrease in radiance when compared to the control group, indicating that the cell-killing effect was good but not sufficient as there were some residual viable cells on BLI. Meanwhile, after a single cycle of treatment at -45°C, the melanoma (B16F10-luc) cell line showed a relatively higher average radiance than the non-treated group. At -60°C and -80°C, all cell lines showed complete extinction ($p<0.01$; **Fig. 3**).

Comparison of cell-killing effect of multicycle treatment

All cell lines treated with a double or triple cycle treatment at -45°C demonstrated a significantly lower average radiance when compared to the untreated cells (p<0.01). BLI radiance was evidently low in all cancer cell lines after double and triple cycles at -60°C and 80°C (p<0.01, **Fig. 4,5**).

Comparison of cell-killing effect among therapeutic cycles

Among the treatment cycles, single cycle treatment showed significantly low radiance with a high cell killing effect in all cell lines at -60°C and -80°C (p<0.01), including most cell lines at -45°C. However, a single cycle of treatment showed poor efficacy at -45°C in the melanoma cell line.

In the double cycle at -45°C, there was significantly lower average radiance in colon (Col26-luc) and melanoma (B16F10-luc) cells when compared to the single cycle treatment (p<0.05, p<0.01; respectively, **Fig. 6**), while there was no significant change in all cell lines at -60°C and -80°C (**Fig. 7,8**). Triple cycle treatment yielded no significant difference when compared to double cycle treatment in all cell lines at -45°C, -60°C and -80°C (**Fig. 6-8**).

Discussion

According to the results of a single cycle of treatment, -60°C or -80°C was effective for all kinds of cell lines including melanoma (B16F10-luc). This might be attributed to the successful formation of lethal ice crystals in the cellular environment at low temperatures, a principal cause of cellular death[16]. At -45°C, the treatment effect was sufficient in all cell lines except melanoma. The melanoma cell line demonstrated substantial resistance to a single cycle

of cryoablation at -45°C, exhibiting a relatively higher average radiance than the non-treated group. This result could potentially be explained by certain aspects of cytokine release. Previous studies have shown that tumor cells can emit either proinflammatory mediators or immunosuppressive cytokines[17,18]. If immunosuppressive cytokines are released instead of proinflammatory mediators, it could stimulate the proliferation of regulatory T cells, possibly accelerating tumor growth and metastasis post cryoablation[19,20]. It is plausible that an insufficient freezing temperature may promote the release of immunosuppressive cytokines, thereby accelerating tumor growth and increasing the cell's average radiance. Therefore, the lethal temperature of cell death differed among tumor types, indicating that optimal treatment protocols need to be tailored to each tumor type. In contrast, some cell lines showed a positive response to the -45°C treatment. The results supported the report by Gage AA et al[21] and was in line with the theory of distinct lethal temperatures regarding cell line characteristics[22]. Some cell lines exhibited resistance to freezing and have a superior ability to proliferate and recovered at temperatures above -40°C[23]. However, the precise reasons for this discrepancy remain unclear.

Previous studies have reported that multiple cycles of freezing and thawing may be the key to enhancing the treatment effect[11,24]. According to these studies, cryoablation treatment with double cycle showed better cell-killing effect compared to single cycle. With the repetition of freeze–thaw cycles, the formation of intracellular ice and the area of necrosis may increase, resulting in an additional frozen area[21,25]. However, it was apparent that this result was not linear to the number of repetitions, as the double cycle and triple cycle of treatment yielded similar results. These findings align with a previous study, which reported no significant difference between double- and triple-cycle cryoablation[26].

BLI was used in our study to evaluate the treatment effect of cryoablation, as it shows the time-signal intensity curve (kinetic curve) from luciferin-luciferase activity by taking multiple measurements to define signal intensity. It provides a way to visually identify the period after substrate injection where peak emission and plateau occurs. According to the D-luc preparation standard for in vitro bioluminescent assays, we have acquired images up to 40 minutes and used the peak imaging time point for each cancer cell lines (10th to 20th minutes after D-luc injection), since the peak signal time differs depending on the cellular model.

Executing the cryoablation study in an *in vivo* environment is beneficial for accurately analyzing the results for further work in clinical practice. Although there are several studies providing enough information about cryoablation protocols, there may be differences between *in vitro* and *in vivo* studies. There was a previous study on cryoablation using BLI for treatment evaluation and diagnostic imaging both *in vitro* and *in vivo*[27]. In our study, posttreatment cellular death may have occurred via an indirect cellular injury, or the failure of microcirculation due to edema after thawing[28]. This, in turn needs to be compared to the results of a direct cellular injury mechanism by cryogenic necrosis after ice crystal formation in an *in vivo* environment. In cryosurgery development, *in vivo* investigations are the most important because tissue temperature is the main factor causing injury[28]. Recent studies[7,29] have mostly focused on monitoring and adjunctive therapy combined with cryoablation with major cancer cells of interest. Our study was focused on defining the differences in lethal temperature for each cancer cell type based on repeated freeze–thaw cycles.

Our study had several limitations. First, the cell culture media was not reintroduced to the cells during thawing with enough amount, which might have resulted in cell dehydration, a potential precursor to cell death in multiple cycle treatments. Second, we obtained BLI images

of cancer cells until 40 minutes posttreatment, which might be too early to evaluate apoptosisinduced cell death as it commonly occurs after 1-2 hours after cryoablation. Ideally, BLI images should have been acquired between 0.5 and 2 hours after treatment for more accurate evaluation. These factors should be considered in further studies. Third, our study was conducted under limited settings, therefore, the viability of each cell was evaluated by BLI alone. It was not confirmed by other cell viability assays such as MTT assay or Annexin/PI assay. Moreover, this study underscored the diversity of cellular responses to cryoablation protocols at different freezing temperatures. Such findings could be beneficial for future clinical studies aiming to achieve successful cellular death while determining the target temperature for various cancer cells to conduct successful cryoablation treatment. Further studies should concentrate on defining the nadir temperature of each cancer cell line in an *in vivo* setting based on the results of this study. Given the multiple variables that can influence the treatment results in an *in vivo* setting, multiple cycle cryoablation should be performed at comparable temperatures to provide a more accurate and realistic analysis. Our *in vivo* study is ongoing, and it will be included in separate study. Also, we acknowledge that possible recovery after ablation may influence the result in our study. Controlling of time after the treatment could be a good idea to decrease the influence. Unfortunately, this protocol was not part of our initial experimental design. We intend to incorporate this experiment after treatment to better understand the impact of time controlling in further studies.

In conclusion, the average BLI radiance varied among the eight cell lines. The appropriate cryoablation protocols need to be adjusted according to the type of cancer. Cryoablation at 60°C or lower temperatures were sufficient for all cell lines. It should be noted that the melanoma cell line showed specific resistance to cryoablation. Although these cell lines do not

represent all cancers, suitable cryoablation protocols need to be considered depending on the type of cancer.

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Competing interest

The authors have no competing interests to declare that are relevant to the content of this article.

Financial interest

The authors declare they have no financial interests.

Data availability

Data is available upon request

Author contribution

Conceptualization: [Bolortuya Khurelbaatar, A. Adhipatria P. Kartamihardja, Oyunbold LamidOchir, Masaya Miyazaki]; Methodology: [Bolortuya Khurelbaatar, A. Adhipatria P. Kartamihardja]; Formal analysis and investigation: [Bolortuya Khurelbaatar]; Writing - original draft preparation: [Bolortuya Khurelbaatar, A. Adhipatria P. Kartamihardja]; Writing - review and editing: [Xieyi Zhang, Masaya Miyazaki, Yoshito Tsushima]; Funding acquisition: [Masaya Miyazaki]; Supervision: [Masaya Miyazaki, Yoshito Tsushima]

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Tables

Table 1. Cancer cell lines employed in this study.

Name of cell line	Type of cancer	Origin of cancer
Col26-luc	colorectal carcinoma	murine
A549-luc	lung adenocarcinoma	human
NCIH460-luc	lung large cell carcinoma	human
BT549-luc	invasive breast carcinoma	human
B16F10-luc	melanoma	murine
HepG2-luc	hepatocellular carcinoma	human
DU145-luc	prostate adenocarcinoma	human
Caki1-luc	renal clear cell carcinoma	human

Table 1. Eight luciferase-expressed cancer cell lines that were used in this study.

Figure legends

Fig. 1. Steps in experimental procedure before imaging. The steps in the experimental procedure until Bioluminescence imaging are shown.

Fig. 2. The average radiance of BLI was measured according to cell density. A) The colon cancer cell line was seeded at six different densities for determining suitable cell density, followed by bioluminescence imaging. $5*10^6$ were chosen as a suitable density for seeding according to BLI evaluation. B) A positive curvilinear relationship was found between the density of cancer cells and the average radiance of BLI with correlation coefficient of 0.96. A *p* value of less than 0.05 (*) or 0.01 (**) was considered statistically significant.

Fig. 3. BLI: Average radiance of cancer cell lines after single cycle treatment. The results of single cycle cryoablation treatment are shown. The BLI average radiance of -45℃ was significantly effective in all cell lines except melanoma. The average radiance of cell lines at 60℃ and -80℃ showed to be significantly lower than control group. A p value of less than 0.05 (*) or 0.01 (**) was considered statistically significant.

Fig. 4. BLI: Average radiance of cancer cell lines after double cycle treatment. The results of double cycle cryoablation treatment are shown. The average radiance of BLI at -45℃, -60℃ and -80℃ showed to be significantly effective in all cancer cell lines. A p value of less than 0.05 (*) or 0.01 (**) was considered statistically significant.

Fig. 5. BLI: Average radiance of cancer cell lines after triple cycle treatment. The results of triple cycle cryoablation treatment are shown. -45℃, -60℃ and -80℃ showed to be significantly effective in all cancer cell lines. A p value of less than 0.05 (*) or 0.01 (**) was considered statistically significant.

Fig. 6. BLI: Average radiance of cancer cell lines at -45℃. The average radiance of BLI at 45℃ cryoablation treatment was significantly lower than untreated group even after single cycle treatment in all cell lines, but the average radiance of melanoma was significantly higher than control group. A p value of less than 0.05 (*) or 0.01 (**) was considered statistically significant.

Fig. 7. BLI: Average radiance of cancer cell lines at -60℃. The average radiances of BLI at 60℃ cryoablation treatment are shown. Not regarding to the number of cycles, all cell lines showed significantly lower average radiance compared to control group. A p value of less than 0.05 (*) or 0.01 (**) was considered statistically significant.

Fig. 8. BLI: Average radiance of cancer cell lines at -80℃. The average radiance of BLI at 80℃ cryoablation treatment was significantly lower than untreated group in all treatment cycles in all cancer cell lines. A p value of less than 0.05 (*) or 0.01 (**) was considered statistically significant.

Fig. 1. Steps in experimental procedure before imaging

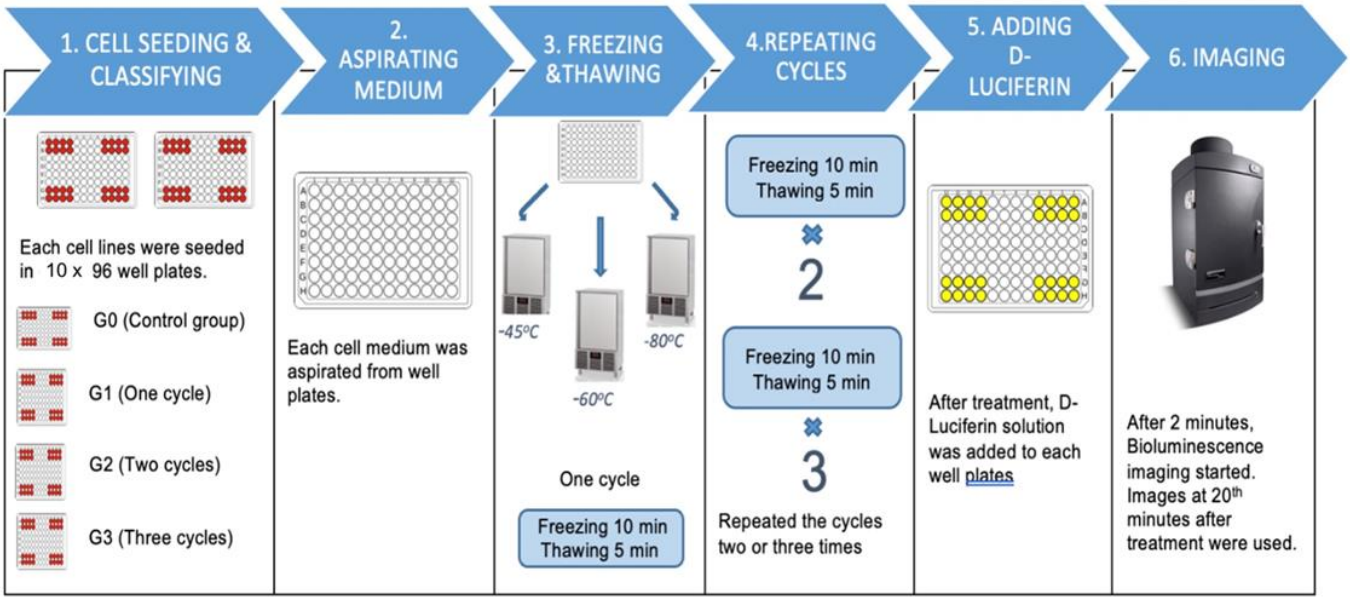

Fig. 2. A) The average radiance of BLI in Colon 26-luc cell line among six different cell density. $5*10^6$ were chosen as a suitable density for seeding according to BLI evaluation. B) BLI average radiance correlation according to different cell density. A positive curvilinear relationship was found between the density of cancer cells and the average radiance of BLI.

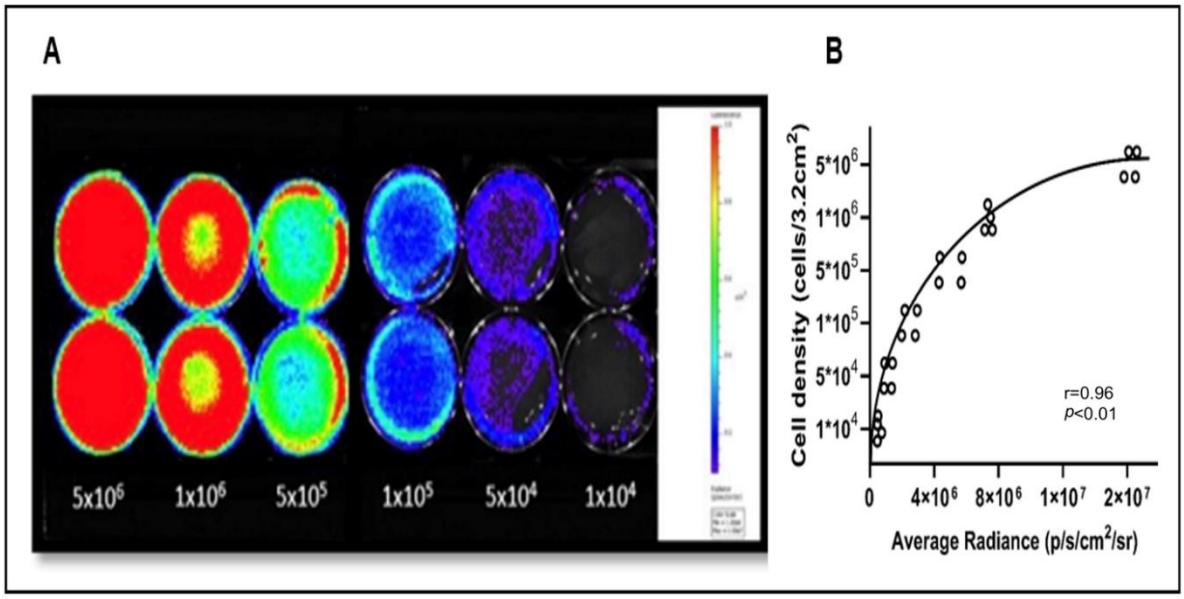

Fig. 3. Average radiance of cancer cell lines after single cycle treatment

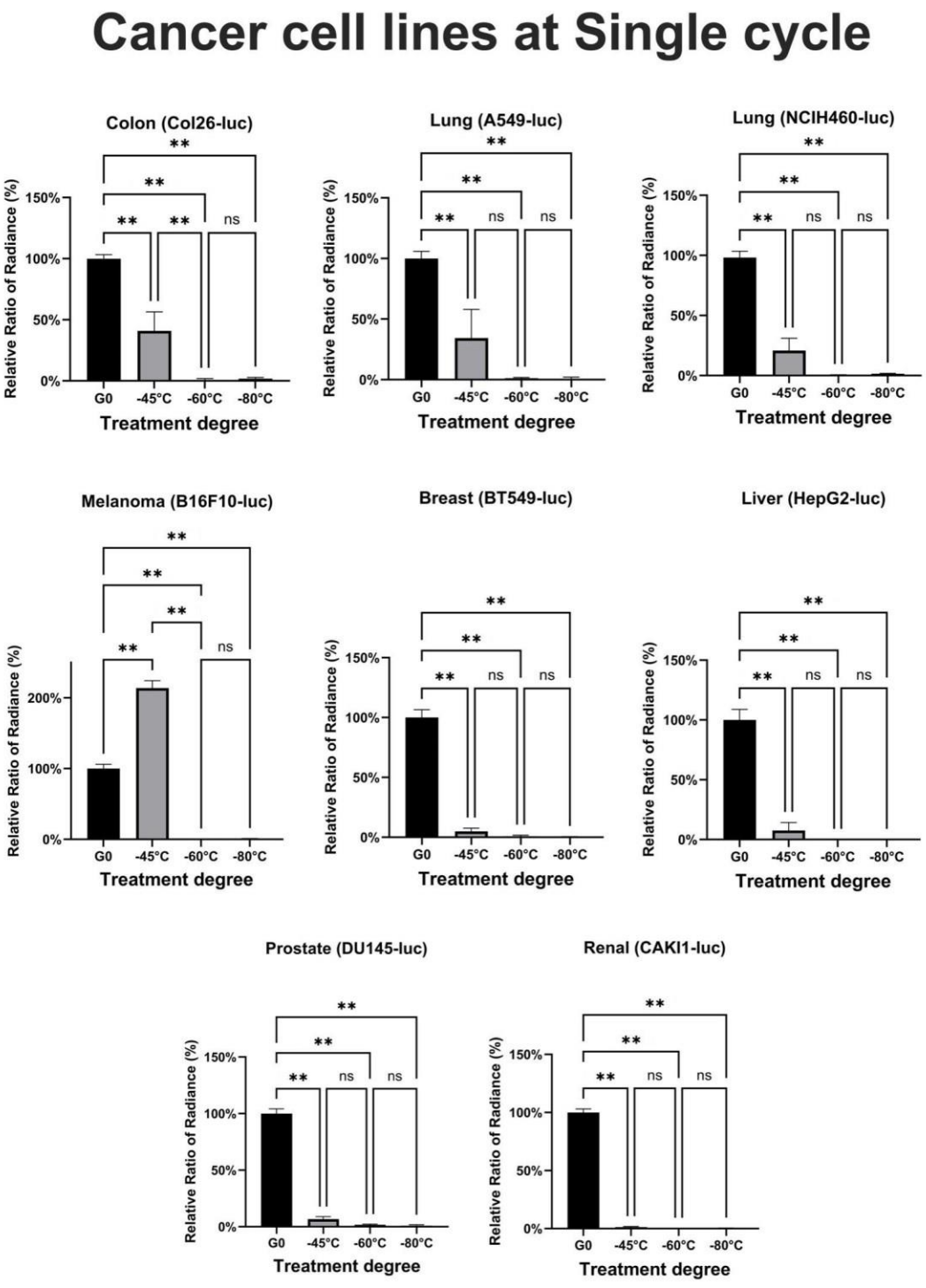

Fig. 4. Average radiance of cancer cell lines after double cycle treatment

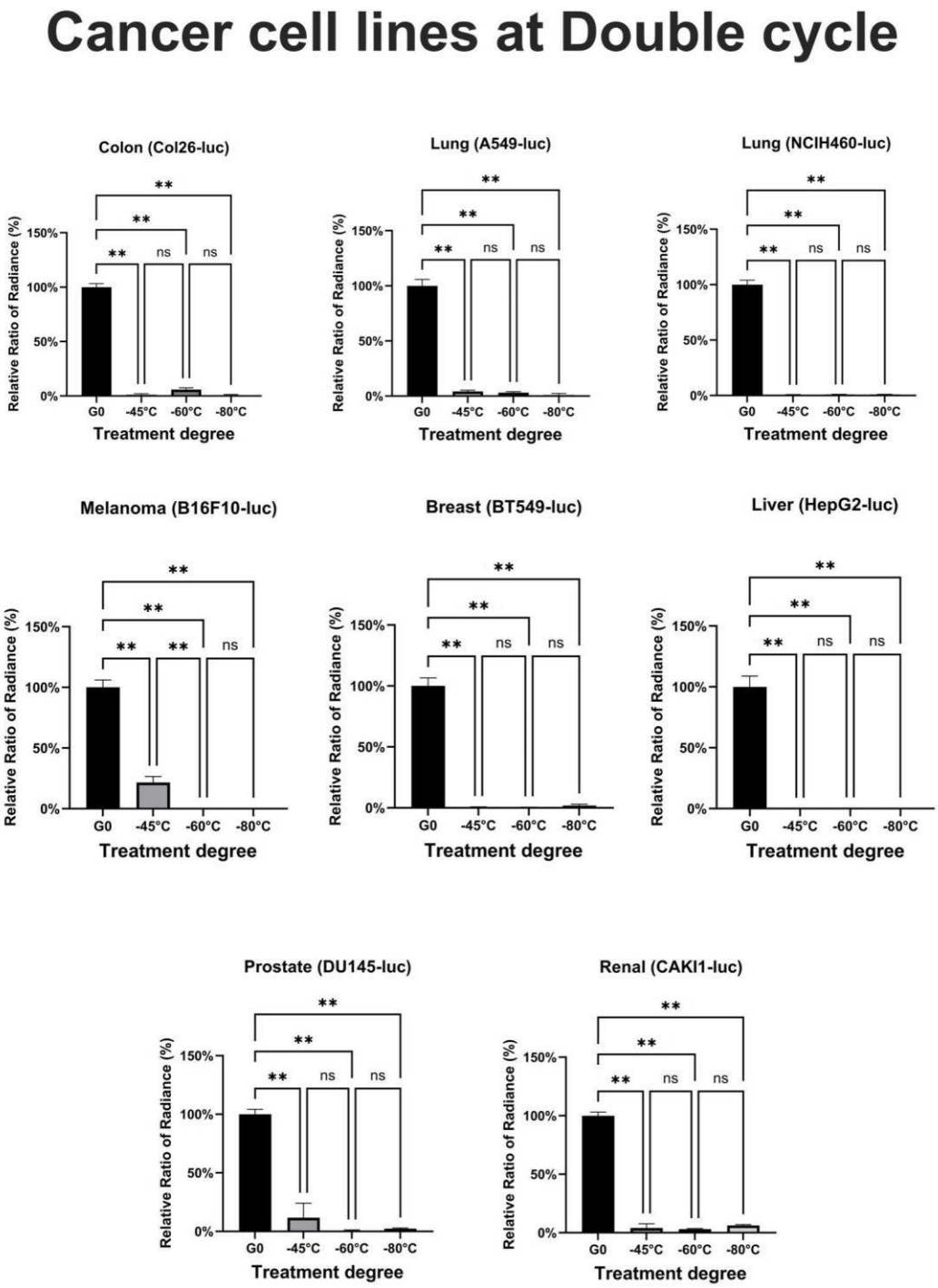

Fig. 5. Average radiance of cancer cell lines after triple cycle treatment

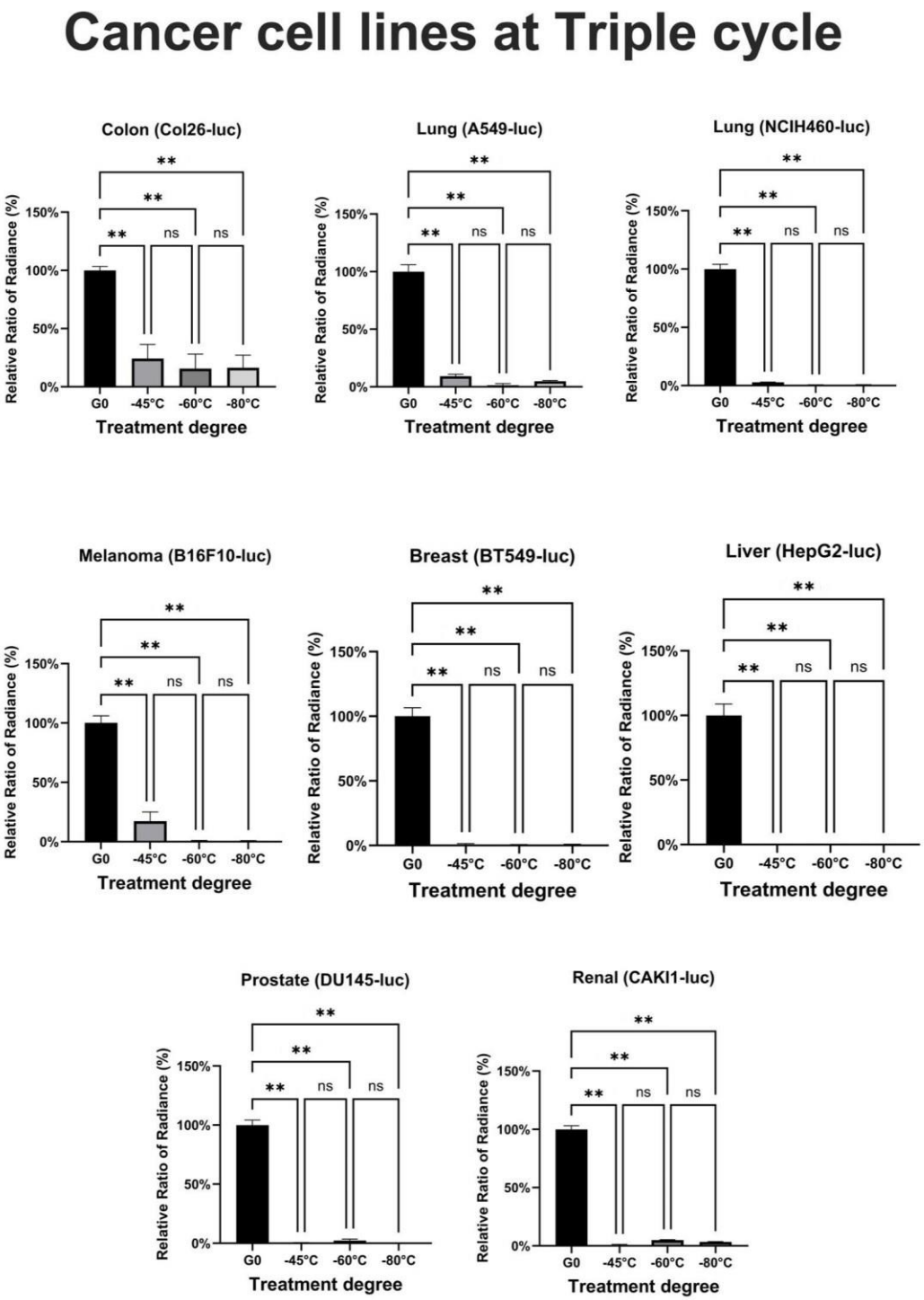

Fig. 6. Average radiance of cancer cell lines at -45°C

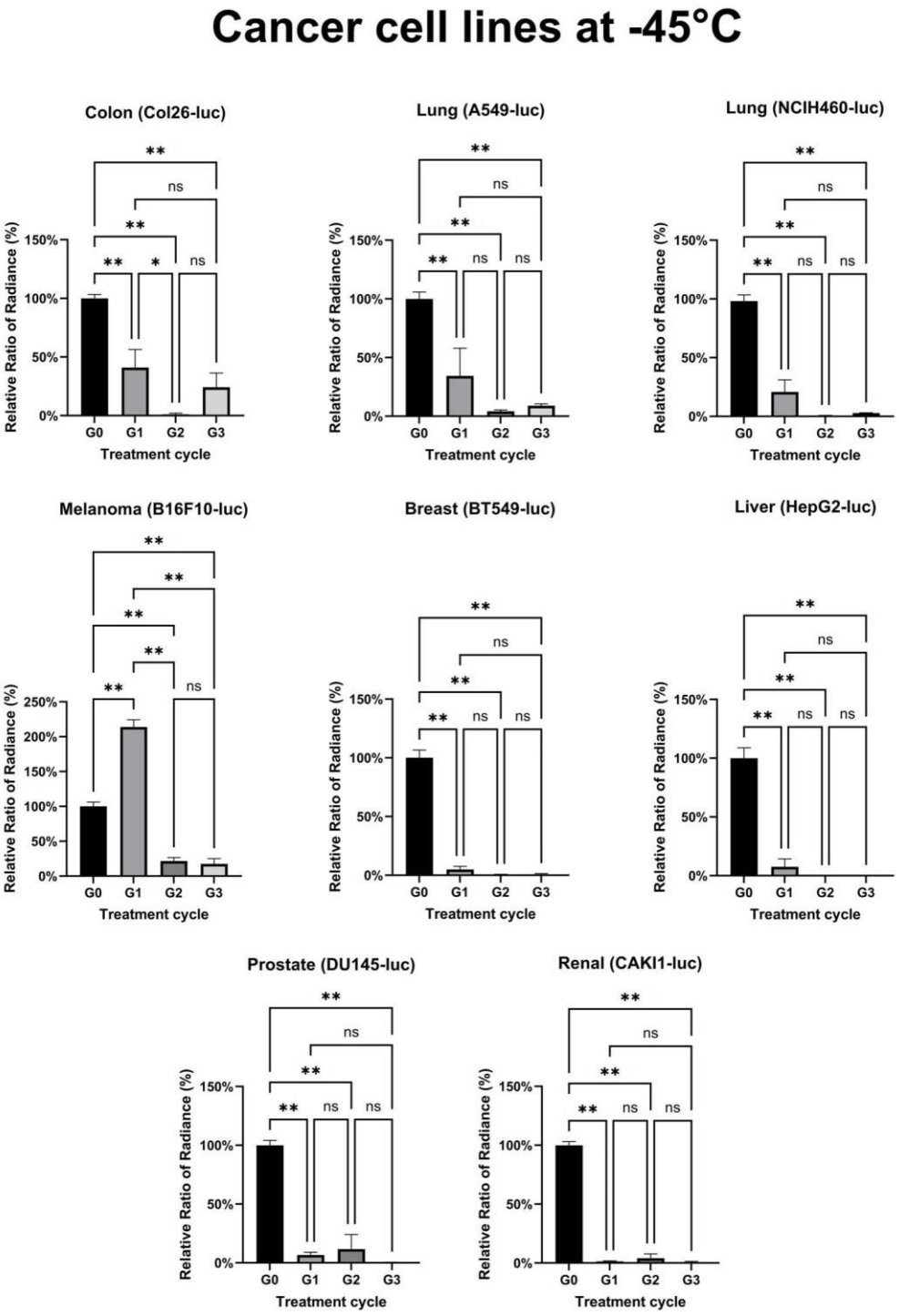

Fig. 7. Average radiance of cancer cell lines at -60°C

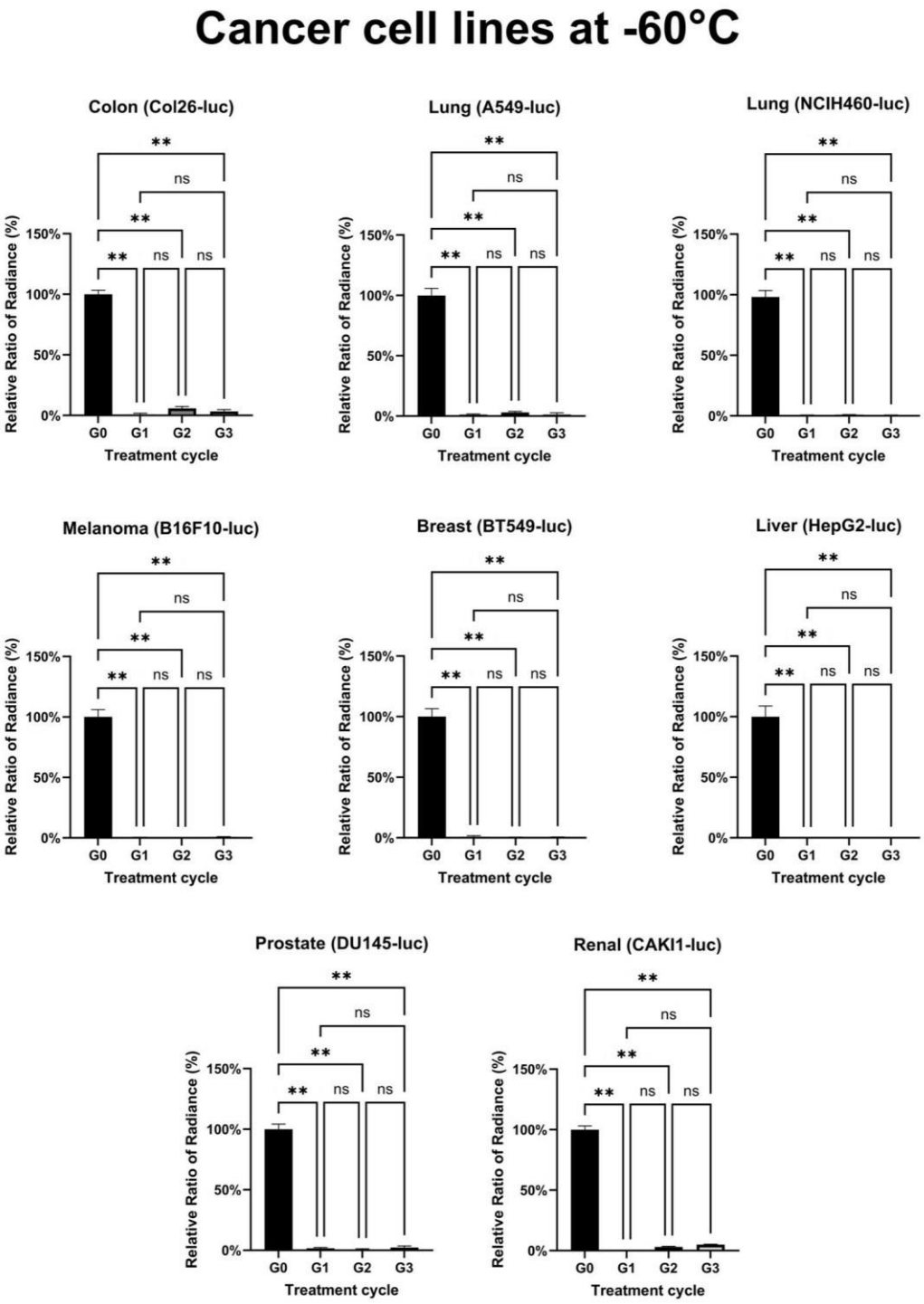

Fig. 8. Average radiance of cancer cell lines at -80°C