

Non-thermal cytocidal effect of infrared irradiation on cultured cancer cells using specialized device

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Abstract

As infrared penetrates the skin, thermal effects of infrared irradiation on cancer cells have been investigated in the field of hyperthermia. We evaluated non-thermal effects of infrared irradiation using specialized device (1100-1800 nm with filtering of wavelengths between 1400 and 1500 nm and contact cooling) on cancer cells. In *in vitro* study, five kinds of cultured cancer cell lines (MCF7 breast cancer, HeLa uterine cervical cancer, NUGC-4 gastric cancer, B16F0 melanoma, and MDA-MB435 melanoma) were irradiated using the infrared device, and then the cell proliferation activity was evaluated by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay. Proliferation of all the cancer cell lines was significantly suppressed by infrared irradiation. Total infrared output appeared to be correlated with cell survival. Increased temperature during infrared irradiation appeared not to play a role in cell survival. The maximum temperature elevation in the wells after each shot in the 20 J/cm² and 40 J/cm² culture was 3.8°C and 6.9°C, respectively. In addition, we have shown that infrared irradiation significantly inhibited the tumor growth of MCF7 breast cancer transplanted in severe combined immunodeficiency mice and MDA-MB435 melanoma transplanted in nude mice *in vivo*. Significant differences between control and irradiated groups were observed in tumor volume and frequencies of TUNEL-positive and Ki67-positive cells. These results indicate that infrared, independent of thermal energy, can induce cell killing of cancer cells. As this infrared irradiation schedule reduces discomfort and side effects, reaches the deep subcutaneous tissues, and facilitates repeated irradiations, it may have the potential application for treating various forms of cancer.

Photodynamic therapy (PDT) is the most common anti-tumor therapy using infrared (IR) light for select forms of cancer.⁽¹⁾ PDT is based on the accumulation of a photosensitizing agent in tumors and uses wavelengths near 800 nm as a photoactivating wavelength to achieve maximum penetration depth.⁽²⁾ This wavelength, however, also has high melanin absorption limiting the ability to deliver light to highly pigmented tumors.⁽³⁾ While wavelengths near 800 nm are the standard activators for PDT, other wavelengths have shown treatment promise. Santana *et al* reported that near-infrared light at 904 nm may have anti-tumor activity and increases cytomorphological changes with apoptosis in neoplastic cells.⁽⁴⁾ Additionally, actively proliferating cells show increased sensitivity to red and near-infrared light.^(5,6) IR irradiation alone appears to induce DNA strand breaks and cell death by apoptosis,⁽⁷⁾ eliciting photodisruptive destruction of tumor tissue.⁽⁸⁾ While IR irradiation appears to damage tumor tissue, it is also shown to reduce cellular protein damage produced by biological oxidants in normal cells.⁽⁹⁾

Many different IR devices and lasers are used in anti-tumor therapies such as PDT and hyperthermia, typically utilizing wavelengths between 750 to 3000 nm. Wavelength selection directly influences target selection and penetration depth. Wavelengths below 1100 nm are absorbed preferentially by melanin in the superficial layers of skin (Fig. 1a). Wavelengths between 1400 to 1500 nm and above 1850 nm are absorbed heavily by water, resulting in heating to the superficial layers of the skin. To deliver IR energy safely to deeper tissues without significant superficial heating requires wavelengths between 1100 nm and 1850 nm excluding the range from 1400-1500 nm.⁽¹⁰⁾

Although many studies elucidate the influences of IR irradiation, no studies have investigated the effects of IR irradiation using a specialized broad spectrum light source emitting light from 1100-1800 nm with a filter to exclude wavelengths between 1400 and 1500 nm) on cancer cells. We hypothesized that this specialized IR irradiation source may kill cancer cells and have beneficial uses for cancer treatment. To investigate the potential effects of this IR irradiation for cancer treatment, we performed both *ex vivo* and *in vivo* testing. 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay investigated the effects on various kinds of cultured cancer cells. *In vivo* studies examined pathology of implanted cancer cells in severe combined immunodeficiency (SCID) mice and nude mice.

Materials and methods

IR device. IR irradiation was performed with a broadband infrared source (Titan; Cutera, Brisbane, CA, USA). The IR device emits a spectrum of IR between 1100 to 1800 nm, with water filtering to remove wavelengths between 1400 and 1500 nm. This delivers IR without the wavelengths that are strongly absorbed by water and hemoglobin (Fig. 1b) allowing for safe delivery of IR energy deeper into tissue. The system delivers energy with a fluence range of from 5 J/cm² to 56 J/cm² using continuous energy single irradiation pulses of 4 to 10 seconds. The IR dosimetry was performed by laser measurement instrumentation L40(150)A-SH-V1 purchased from Ophir, UT, USA. The sapphire contact cooling tip is set to a fixed temperature of 20°C to provide contact cooling. Pre-, parallel-, and post-cooling of the superficial layers accomplished through this temperature-controlled sapphire window further prevents excessive superficial heating.

Cell culture. Testing was conducted on cultures of five cancer cell lines: MCF7 breast cancer cells, HeLa uterine cervical cancer cells, NUGC-4 gastric cancer cells, B16F0 melanoma cells, and MDA-MB435 melanoma cells. MCF7 breast cancer cells, HeLa uterine cervical cancer cells, B16F0 melanoma cells, and MDA-MB435 melanoma cells were obtained from the American Type Culture Collection, and NUGC-4 gastric cancer cells were obtained from Riken BRC Cell Bank. MCF7, HeLa B16F0, and MDA-MB435 cells were maintained in DMEM (Invitrogen, Carlsbad, CA, USA), and NUGC-4 in RPMI1640 (Invitrogen) medium supplemented with 10% fetal bovine serum and antibiotics. Cells were seeded in 96-well microtitre plates at a concentration of 5 x 10³ cells per well in 100 µL of medium. All cells were grown at 37°C in a humidified incubator with 5% CO₂. A total of 726 wells of cancer cell lines were prepared for this study.

Cell culture and IR irradiation. Cancer cells from all five cultures were divided based on the number of individual IR exposures in a single session, and the total irradiation received. The numbers of shots performed per round were 3, 10, and 20 shots, using two separate fluence settings per shot of 20 J/cm² and 40 J/cm², because our preliminary results indicated that 3 shots at 10 J/cm² on cultured cancer cells (NUGC-4 cells and B16F0 cells) were not effective. This resulted in 7 different groups (including control group) within each of the 5 cancer cell lines. The irradiated groups were exposed to a single round of irradiation. The MTS assay was performed after a 3-day incubation period following the exposure session.

Well temperature measurement. Well temperatures were monitored during exposure sessions and sequential pulses were only delivered after well temperature returned to 37°C. This was monitored to prevent accumulated heating from multiple exposures from impacting the data. The temperatures of cultured media in the wells were monitored at 4 mm-depth using a digital thermometer MC3000-000 with

sheathed thermocouple IHKN053 (0.3 mm outer diameter of sheathed temperature sensor) purchased from Chino, Tokyo, Japan. The temperatures were recorded with 2 second sampling, and 5 representative data were averaged to obtain a final score.

Cell proliferation assay. We performed MTS cell proliferation assay to evaluate the effects of IR irradiation. Cell proliferation was analyzed using a CellTiter 96 Aqueous Cell Proliferation Assay kit (Promega, Madison, WI, USA). Aliquots of 20 μ L of MTS reagent were added to the wells and incubated at 37°C in a humidified incubator for 2 hours. Absorbance at 490 nm (OD_{490}) was monitored with a Powerscan HT microplate reader (Dainippon Pharmaceutical, Osaka, Japan).

***In vivo* tumorigenicity and treatment.** *In vivo* testing was conducted on two groups of mice, SCID mice and nude mice. Animals were housed in a temperature-controlled environment under a 12-h light-dark cycle with free access to water and standard mouse chow. Body weight and tumor size were measured every other day. Tumor volumes were defined as $4/3 \times \pi \times (\text{longest diameter})/2 \times (\text{shortest diameter})/2$. This study was approved by Shinshu University Institutional Review Board for Animal Study. National and international principles of laboratory animal care were followed throughout this study. Fourteen female SCID mice (CB17/lcr-Prkdcscid/CrlCrlj) were obtained from Charles River Laboratories (Yokohama, JAPAN). MCF7 cells (2.5×10^6 cell/100 μ L/mouse) were implanted subcutaneously in the right flank of 6-week-old SCID mice. Ten mice were divided into two groups once tumor volume expanded to approximately 60 mm³. This occurred 9 days after cancer transplantation, and is defined as day 1 for testing. Groups consisted of two equal groups ($n = 5$ for each group); one group (control group) was untreated, whereas the other group was treated by IR irradiation. IR irradiation was started on day 1, and performed 7 rounds. These rounds of IR irradiation were performed every other day. One round consists of ten shots of IR irradiation at 20 J/cm². Animals were euthanized at 46 days after cancer transplantation (day 37). In parallel, forty female nude mice (Crlj:CD1-Foxn1nu) were obtained from Charles River Laboratories (Yokohama, JAPAN). MDA-MB435 cells (5.0×10^6 cell/100 μ L/mouse) were implanted subcutaneously in the right flank of 6-week-old SCID mice. Twenty-eight mice were divided into two groups when tumor volume expanded to approximately 100 mm³. This occurred 19 days after cancer transplantation, and is defined as day 1 for testing. Groups consisted of two equal groups ($n = 14$ for each group); one group (control group) was untreated, whereas the other group was treated by IR irradiation. IR irradiation was started on days 1 and 31. Treatments were performed once daily for 13 days. All treatments consisted of 10 exposures of IR irradiation at 40 J/cm². Animals were euthanized at 99 days after cancer transplantation (day 80).

Histological investigation. MCF7 tumors were taken from the SCID mice on days 13 ($n = 2$) and 37 ($n = 8$), and MDA-MB435 tumors were taken from nude mice on days 3 ($n = 4$), 9 ($n = 4$), and 45 ($n = 4$), and 80 day ($n = 16$) for histological examination. Specimens were fixed in 20% neutral buffered formalin and processed for paraffin embedding. MCF7 tumors were then serially sectioned in the horizontal plane and MDA-MB435 tumors were serially sectioned in vertical plane (3–4 μ m thickness). Specimens were evaluated by Hematoxylin and eosin staining, transferase-mediated dUTP nick-end labeling (TUNEL) technique, and immunohistological staining using Ki67. Frequencies of TUNEL-positive cells and Ki67-positive cells were evaluated on digital photographs. Images were scanned and quantified in 5 representative fields per section, then averaged to obtain a final score. The sections were photographed under an Olympus BX51 microscope (Olympus, Tokyo, Japan) equipped with a digital camera system (DP50; Olympus). The digital photographs were processed with Adobe Photoshop (Adobe, San Jose, CA, USA).

Statistical analyses. The differences between groups at each time point were examined for statistical significance with Student *t*-test. $P < 0.05$ was considered to indicate statistical significance.

Results

Cell viability measured by MTS assay. The OD_{490} values of irradiated cell cultures decreased significantly compared with controls in all cultures except at the lowest dose of irradiation at 20 J/cm² three exposures (group 20 J x 3) ($P < 0.001$) (Fig. 2). Group 20 J x 3 showed the smallest decrease with significant reductions in HeLa cells ($P = 0.040$), and MDA-MB435 cells ($P = 0.034$), but only modest reductions were observed in MCF7, NUGC-4, and B16F0 cells. No statistically significant intra-group differences were observed between the 20 and 40 J/cm² group (excluding group 20 J x 3). This was consistently observed for all cancer cell lines.

Well temperatures. In order to exclude the possibility of thermal damage to cancer cells, well temperatures were recorded at the bottom of the culture well (4mm) with 2 second sampling. Temperature rise during each 20 J/cm² shot was $3.76 \pm 0.53^\circ\text{C}$, rising from 37°C to $40.76 \pm 0.53^\circ\text{C}$ during IR irradiation. Temperature returned to baseline (37°C) 43.8 ± 3.58 seconds later. The period over 40°C was 3.6 ± 2.63

seconds during each 20 J/cm² shot. Temperature rise during a 40 J/cm² exposure was 6.85 ± 0.58°C, rising from 37°C to 43.85 ± 0.58°C required 83.4 ± 8.11 seconds to return to baseline temperature. The period over 40°C was 42.1 ± 4.07 seconds during each 40 J/cm² shot (Fig. 3a,b).

The clinical findings during IR irradiation. IR irradiation induced no pain, and mice did not withdraw even though IR irradiation was performed without anesthesia. No side effects, such as epidermal burns, were observed during the study.

The effect of IR irradiation on tumors transplanted to mice. The mean tumor volume of MCF7 in the control group was 62.0 ± 2.4 mm³ on day 1 of the treatment, increased rapidly from through day 7 and ended up at 120.6 ± 15.6 mm³ on day 37. In the irradiated MCF7 group tumor volume was 69.6 ± 4.7 mm³ on day 1 of the treatment and decreased after the 1st round of IR irradiation. Mean tumor volume decreased steadily through day 19 with a minimum volume of 40.2 ± 5.7 mm³. Mean tumor volume began to increase gradually in irradiated group after day 20 ending at 72.9 ± 18.4 mm³ on day 37. Tumor volume differences were statistically significant between control group and IR irradiated group from day 9 when 5th round of IR irradiation was performed through day 37 when the animals were euthanized (Fig. 4a).

The mean tumor volume of MDA-MB435 in control group was 99.0 ± 26.85 mm³ on day 1 of the treatment and increased continuously to 700.5 ± 333.8 mm³ on day 80. The mean tumor volume for the irradiated MDA-MB435 group was 100.0 ± 26.06 mm³ on day 1 of the treatment. Tumor volume decreased after the 1st round of IR irradiation and continued to reduce through day 5 with a minimum volume of 67.0 ± 15.75 mm³. After day 5, the mean tumor volume gradually increase in irradiated group through day 15, resulting in 129.0 ± 44.05 mm³ on day 31 when the second treatment started. Following the second round of irradiation, the mean tumor volume again decreased reducing to 94.0 ± 41.22 mm³ by day 45. The mean tumor volume began to increase gradually, ended up to 357.3 ± 297.2 mm³ (day 80). Significant differences were observed in tumor volume between control group and IR irradiated group from day 5 up to day 77 (Fig. 4b).

Histological analysis of tumor after IR irradiation. The histology of irradiated groups shows tumor shrinkage in MCF7 and MDA-MB435 cells (Figs.5, 6). Injured cells were observed in the center of the tumors especially MCF7 on day 37 and MDA-MB435 on day 45 (Figs.5b, 6b).

In the MCF7 group, frequencies of TUNEL-positive cells in irradiated group were high on days 13 and 37, compared to control group. Frequencies of Ki67-positive cells in irradiated group were low on days 13 and 37, compared to control group (Fig. 7a). Significant differences were observed between control group and IR irradiated group in all the four groups ($P < 0.05$). Frequencies of TUNEL-positive cells in the control group and irradiated group on day 13 were 2.30 ± 0.84%, 19.33 ± 5.05%, respectively. Frequencies of TUNEL-positive cells in control group and irradiated group on day 37 were 3.15 ± 0.71%, 6.63 ± 1.29%, respectively. Frequencies of Ki67-positive cells in control group and irradiated group on day 13 were 72.10 ± 6.40%, 40.1 ± 5.26%, respectively and 57.40 ± 11.73%, 42.12 ± 5.40%, respectively on day 37.

Similarly, in the MDA-MB435 group, frequencies of TUNEL-positive cells in irradiated group were high on days 9 and 45, compared to control group. Frequencies of Ki67-positive cells in irradiated group were low on days 9 and 45, compared to control group (Fig. 7b). Significant differences were observed between control group and IR irradiated group (both in the center and the superficial layer of tumor) in all the four groups ($P < 0.05$). Frequencies of TUNEL-positive cells in control group and irradiated group were 0.84 ± 0.47 %, 15.86 ± 11.18%, respectively on day 9. Frequencies of TUNEL-positive cells on day 45 in the control group, in the superficial layer of the irradiated tumor, and in the center of the irradiated tumor were 1.09 ± 0.44%, 6.68 ± 4.17%, and 78.12 ± 4.93%, respectively. Frequencies of Ki67-positive cells in control group and irradiated group on day 9 were 11.42 ± 6.45%, 0.76 ± 0.12%, respectively. Frequencies of Ki67-positive cells on day 45 in the control group, in the superficial layer of the irradiated tumor, and in the center of the irradiated tumor were 21.06 ± 5.47%, 8.04 ± 3.762%, and 1.21 ± 1.29%, respectively.

Discussion

This study demonstrates that using a specific wavelength of IR (1100-1800 nm with filtering of wavelengths between 1400 and 1500 nm) suppresses tumor growth, independent of thermal energy. Further, due to contact cooling, this light energy can be applied to the body externally without complication.

We previously reported that IR irradiation induces an injury response in the skin and subcutaneous tissue.^(11,12) Thermal damage, including infrared irradiation, denatures the collagen and encourages the generation of new collagen, resulting in tighter skin.⁽¹³⁾ Non-ablative lasers work by thermally stimulating dermal collagen remodeling.⁽¹⁴⁾ To protect the subcutaneous tissues against IR, hemoglobin and fluid absorb IR through blood vessel dilation and the skin turning red or foam formation.⁽¹²⁾ This is because IR is strongly absorbed by hemoglobin and fluids.⁽¹²⁾ Thus, the dermis tends to increase the amount of fluid by inducing increases in collagen, elastin and water-binding protein to protect the subcutaneous tissues against IR.^(11,12) These increases in the amount of fluid may indicate an attempt by the body to further reduce the level of future IR penetration to deeper tissue structures.^(11,12)

IR irradiation can penetrate deep into subcutaneous human tissue (up to 12 mm), where it can cause photochemical changes.⁽¹⁵⁻¹⁷⁾ The penetrating 600-1300 nm wavelength region causes photochemical changes and affects a large volume and depth of tissue.⁽¹⁸⁾ In a study using near-infrared irradiation at 904 nm, irradiation was shown to increase cytomorphologic changes with programmed cellular death in neoplastic cells, however no apparent changes were observed in non-neoplastic cells.⁽⁴⁾ Unlike wavelengths beyond 1100 nm where melanin absorption is negligible,⁽¹⁸⁾ absorption at 904 nm is significant. This may limit possible uses of the 904 nm wavelength for certain body areas in races with skin rich in melanin.

To deliver light energy or IR safely to the deeper tissues, wavelengths below 1100 nm, from 1400 to 1500 nm and above 1850 nm need to be filtered out of the delivered spectrum. Without filtering, too much energy is absorbed in the superficial layers of skin, limiting safe energy delivery to deeper tissue. In this study, we used an infrared device that emits a spectrum of IR from 1100 to 1800 nm, with filtering of wavelengths between 1400 and 1500 nm that are strongly absorbed by fluid and hemoglobin (Fig. 1b). Internal to the device, a water filter absorbs high water absorption IR wavelengths preventing emission of wavelengths that would lead to superficial heating, which can lead to painful sensations and burns.⁽¹⁷⁾ This IR device cools the superficial layers through a temperature-controlled sapphire window to prevent the surface temperature from increasing severely during irradiation.⁽¹³⁾ This specific wavelength and cooling system enable IR to penetrate to deeper tissues without any pain or epidermal burns. This was evidenced by the ability to treat mice without anesthesia and without contact burns or other adverse events.

Irradiated cell cultures showed significant decreases in cell counts in all cultures except at the lowest dose of irradiation at group 20 J x 3. Correlation to efficacy seemed to be highest with total delivered energy, not per pulse fluence, as lower output, multiple irradiations appeared equally effective to higher fluence irradiations. Ten shots at 20 J/cm² achieved a comparable significant reduction in cell count as that of 3 shots at 40 J/cm². Three shots at 20 J/cm² appeared close to a threshold energy dosage. Further studies are required to determine the accurate correlations between irradiation dose and cell survival, and the different effects in each cancer cell lines.

We have shown that infrared irradiation significantly inhibited the tumor growth of MCF7 in SCID mice and MDA-MB435 in nude mice *in vivo* level. Significant differences were observed in tumor volume, frequency of TUNEL-positive and Ki67-positive cells between control group and IR irradiated groups. The histology of irradiated groups shows tumor shrinkage and injured cells in the center of tumor. Contact cooling (20°C) by this infrared device allowed IR to penetrate deeper tissues, thus inducing central tumor injury. Frequency of TUNEL-positive cells in irradiated group were significantly higher than control group in both MCF7 and MDA-MB435, which supports that IR irradiation induces apoptosis of cancer cells. Frequency of Ki67-positive cells in irradiated group were significantly lower than control group in both MCF7 and MDA-MB435, which suggests that IR irradiation could suppress proliferation of cancer cells.

IR typically induces molecular vibrations^(16,19) and rotations causing an increase in temperature. However, in *in vivo* studies, IR penetrates the skin and reaches the subcutaneous tissue without a significant increase in skin temperature.⁽¹⁶⁾ The effects of IR are independent of generated heat.⁽²⁰⁾ In the present study, increased temperature appeared not to play a role in cell culture survival rate. Results in the 20 J/cm² group (excluding group 20 J x 3) were statistically equivalent to the 40 J/cm² group and the maximum temperature rise during exposure in the 20 J/cm² group was only 3.76°C (temperature = 40.76°C). This is roughly equivalent to a bad illness induced fever. Further, elevated temperatures only remained above 40°C for 3.6 seconds, far short of damages observed during prolonged high grade fevers. The rationale of hyperthermia is based on a direct cell-killing effect at temperatures above 41-42°C.⁽²¹⁾ With whole-body hyperthermia, tumor growth suppression requires temperatures of about 42°C with exposure of at least 60 minutes.⁽²²⁾ Even though the extent and the duration of temperature elevation were not significant in relation to the cases of hyperthermia, irradiated cell cultures showed significant decreases in cell counts in the present study. This level of temperature rise is not associated with tumor growth suppression indicating a factor other than hyperthermia is responsible for growth suppression of the cancer cell lines.

In the present study, even very low output and fewer shots of IR (Ten IR shots at 20 and 40 J/cm²) inhibited the tumor growth. This output is so low that in human skin even the sensation of heat will not be felt due to contact cooling. This IR irradiation induced no pain, and mice did not withdraw even though IR treatment was performed without anesthesia. Further, side effects such as epidermal burns were not observed, and mice looked healthy throughout of the study. Further studies are necessary to determine if more output, increased frequency of treatments and/or longer periods of irradiation may be even more effective in suppressing tumor growth.

The advantages of this IR irradiation schedule are reducing discomfort and side effects, and low cost. Taken together, these characteristics facilitate repeated irradiations, which if proven beneficial for cancer cell reduction in human may provide an alternative or adjunct treatment for patients, offering improved results and / or patient quality-of-life. This IR irradiation is frequently performed for other indications at a level of 40 J/cm², with a very high safety record and no significant complications.⁽¹³⁾

It should be noted that this was a preliminary study based on experiments in a limited variety of cancer

cell lines. Further studies are warranted in larger numbers and various types of cancer cells and with longer post-treatment periods to evaluate variations in treatment parameters and correlations with other anti-tumor therapies. These studies promise to contribute to the design of more efficacious cancer treatments.

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Disclosure

None of the authors of this study have a conflict of interest.

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Figure legends

Fig. 1. (a) The absorption coefficients of melanin (brown), hemoglobin (red) and water (blue). (b) IR device used in the present study emits a spectrum of IR from 1100 to 1800 nm (bold red), with filtering of wavelengths between 1400 and 1500 nm (blue belt), which are strongly absorbed by water and hemoglobin. This specialized spectrum with water filter and the contact cooling system prevent pain and epidermal burns.

Fig. 2. The values of OD₄₉₀ in MCF7 breast cancer cells (a), HeLa uterine cervical cancer cells (b), NUGC-4 gastric cancer cells (c), B16F0 melanoma cells (d), and MDA-MB435 melanoma cells (e). The OD₄₉₀ values of irradiated cell cultures excluding the lowest irradiation group of 3 shots at 20 J/cm² decreased significantly ($P < 0.001$, compared with controls) in all cultures.

Fig. 3. The well temperatures irradiated three (a), and twenty (b) shots at 20 J/cm² (blue) and 40 J/cm² (red). The temperatures of cultured media were monitored at 4 mm-depth of the wells during exposure sessions and sequential pulses were only delivered after well temperature returned to 37°C.

Fig. 4. Relative tumor volume of MCF7 (a) and MDA-MB435 (b). Significant differences were observed in MCF7 tumor volume between control and IR irradiated group from day 9 through 37 when the animals were euthanized. Significant differences were observed in MDA-MB435 tumor volume between control group and IR irradiated group from day 5 up to day 77. Significant differences are indicated (*: $P < 0.05$, **: $P < 0.01$). Cross sign indicated when two animals of each group were euthanized for histological investigation (day 45).

Fig. 5. The horizontal sectional histology of control and IR irradiated MCF7 breast cancer on day 13 (a) and 37 (b). The left column shows control tissues, and the right column shows IR irradiated tissues. Images from top to bottom show hematoxylin and eosin staining, TUNEL technique, and immunohistological staining using Ki67. Scale bars = 400 μm ($\times 40$ magnification); insets: Scale bars = 40 μm ($\times 400$ magnification).

Fig. 6. The vertical sectional histology of control and IR irradiated MDA-MB435 melanoma on day 9 (a) and 45 (b). The left column shows control tissues, and the right column shows IR irradiated tissues. Images from top to bottom show hematoxylin and eosin staining, TUNEL technique, and immunohistological staining using Ki67. Scale bars = 400 μm ($\times 40$ magnification); insets: Scale bars = 40 μm ($\times 400$ magnification).

Fig. 7. Mean scores of frequencies of TUNEL-positive cells and Ki67-positive cells. MCF7 (a) and MDA-MB435 (b). Significant differences were observed between the control and IR irradiated group in all the four groups ($P < 0.05$)

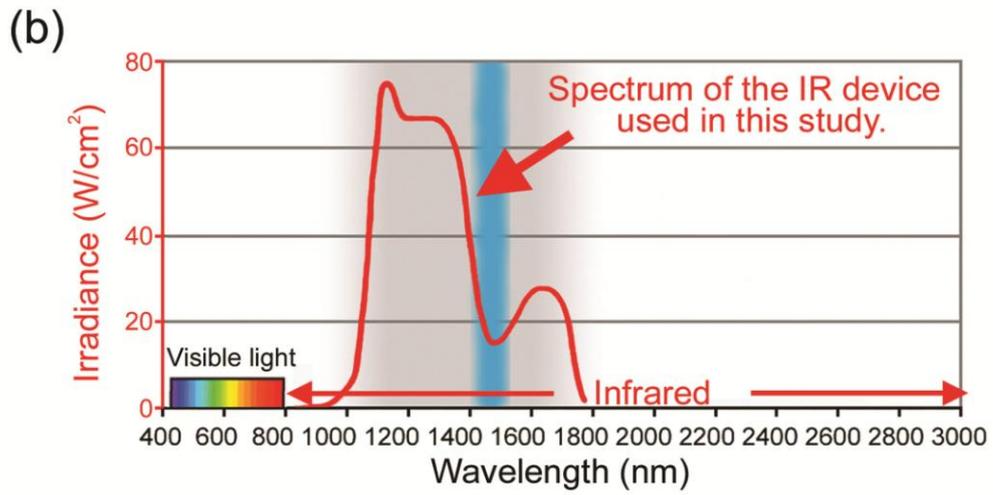
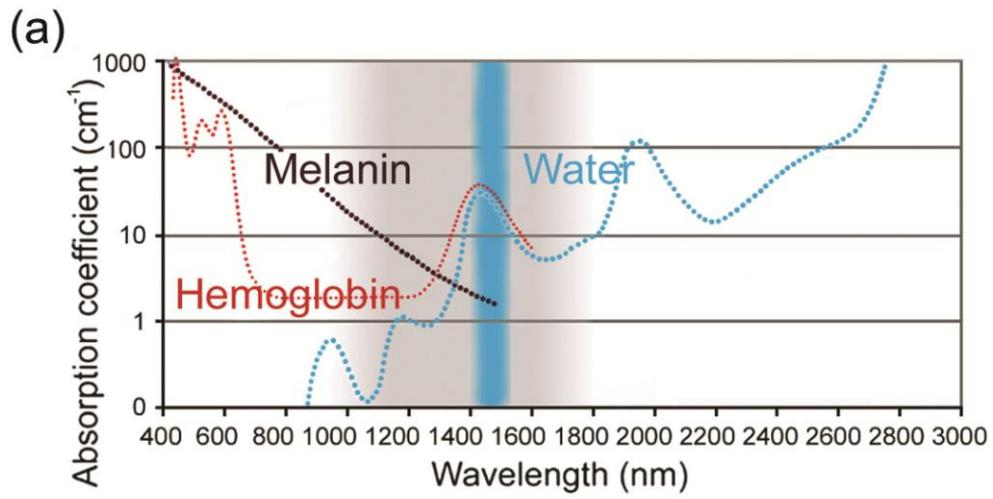


Fig. 1.

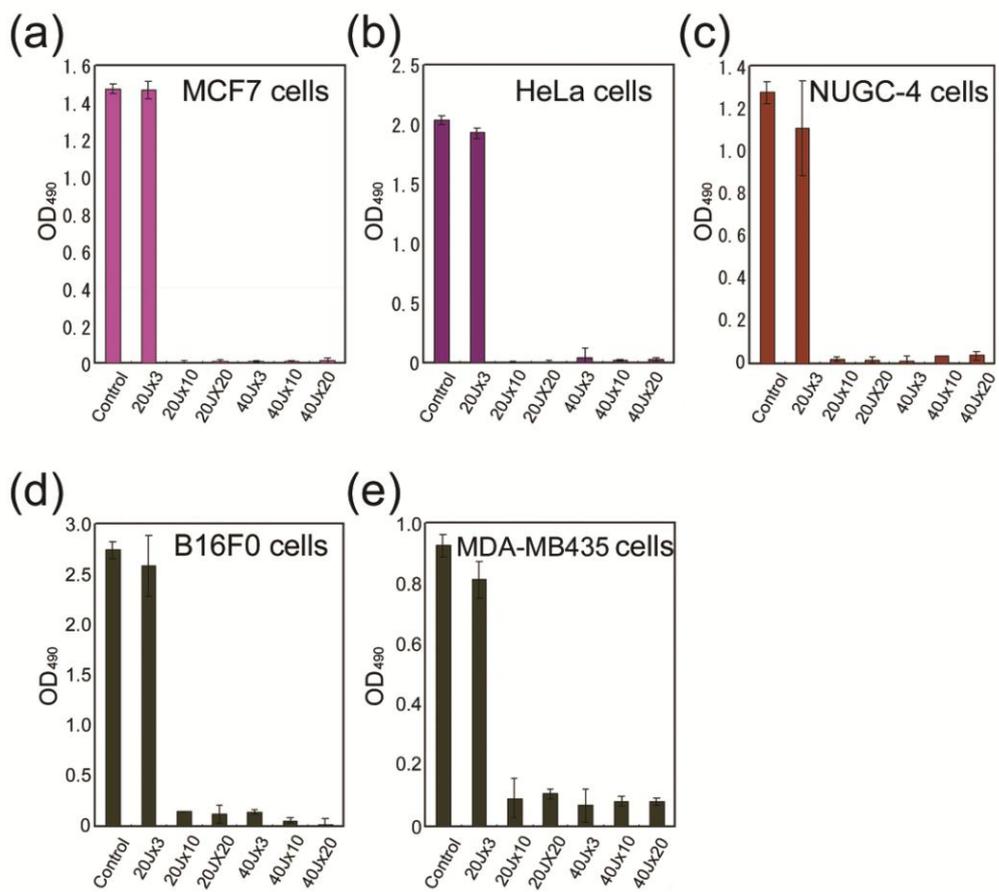


Fig. 2.

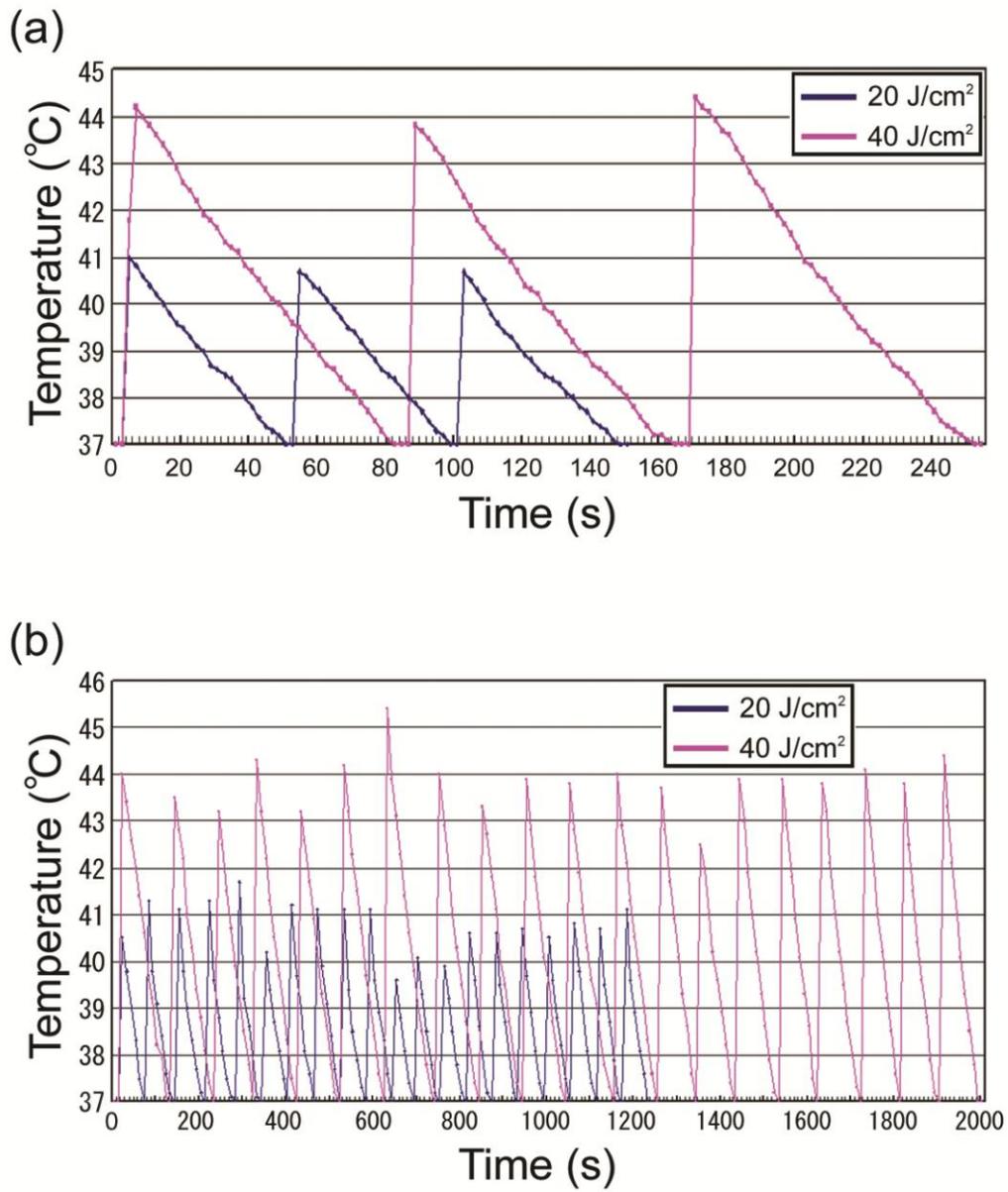


Fig. 3.

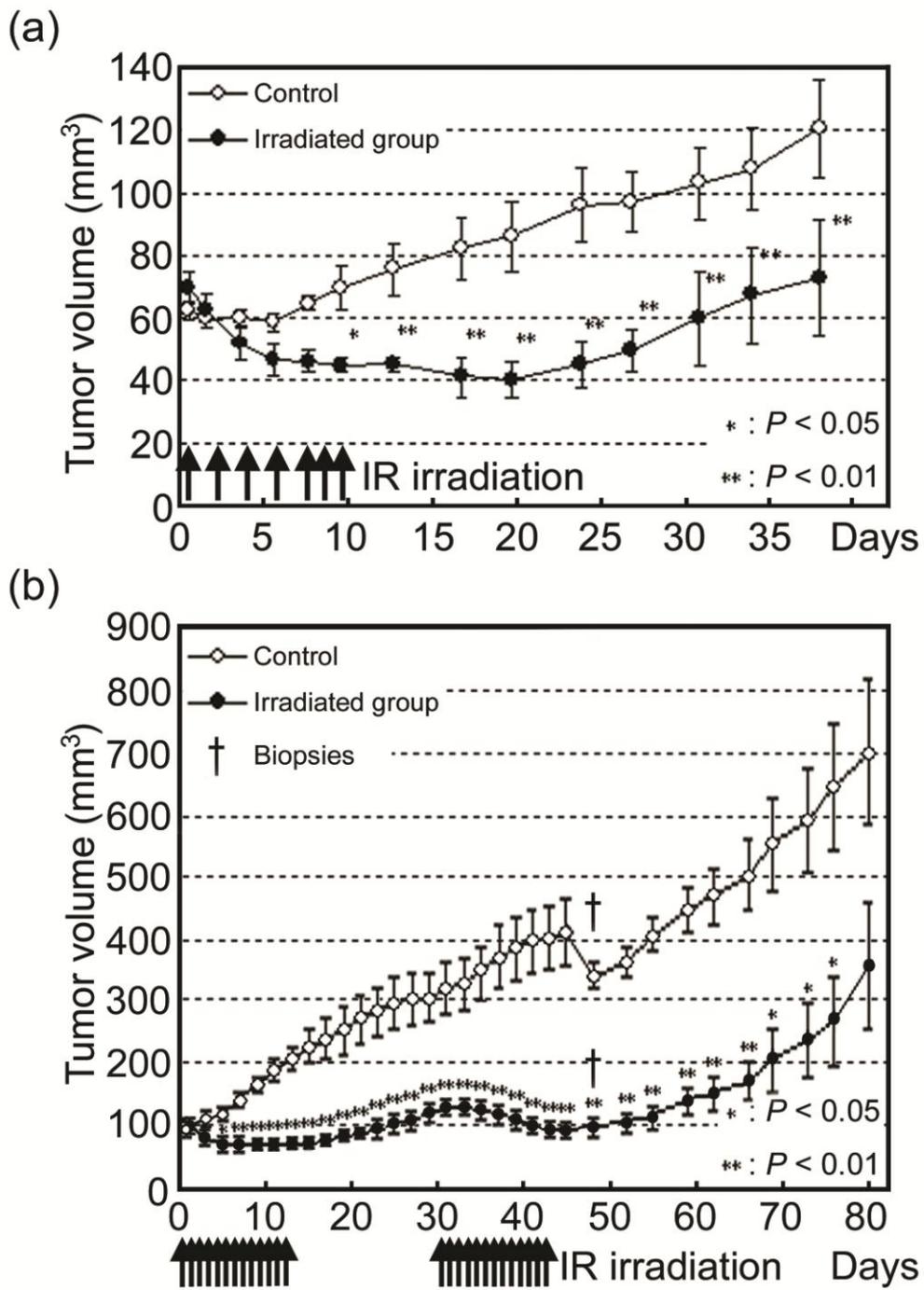


Fig. 4.

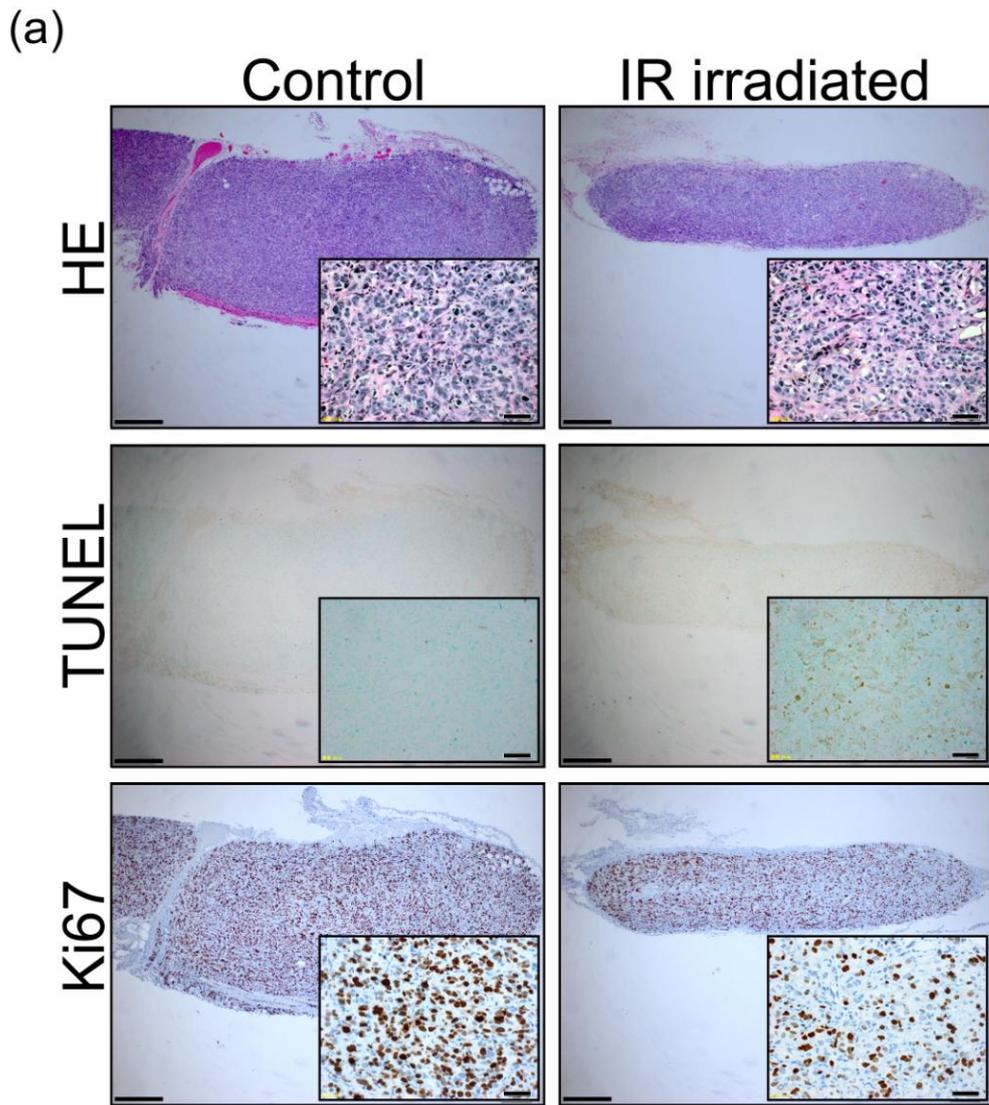


Fig. 5.

(b)

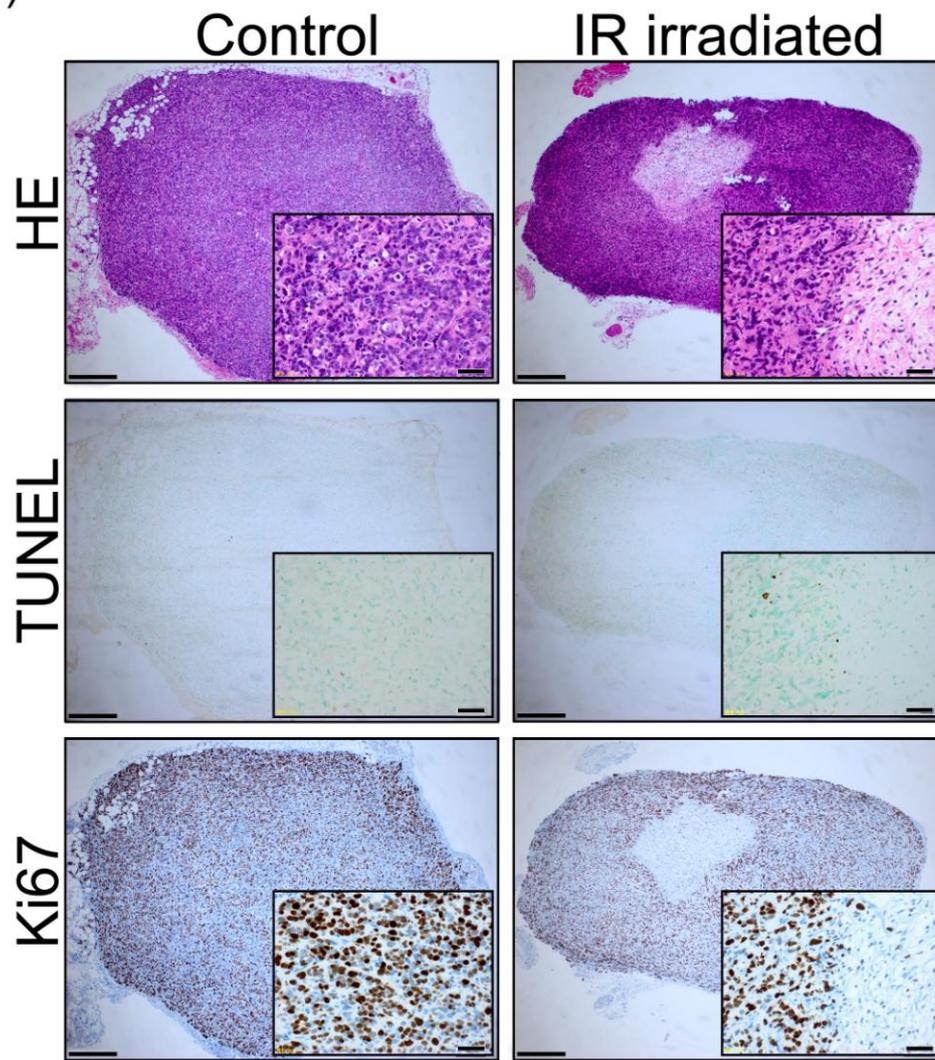


Fig. 5.

(b)

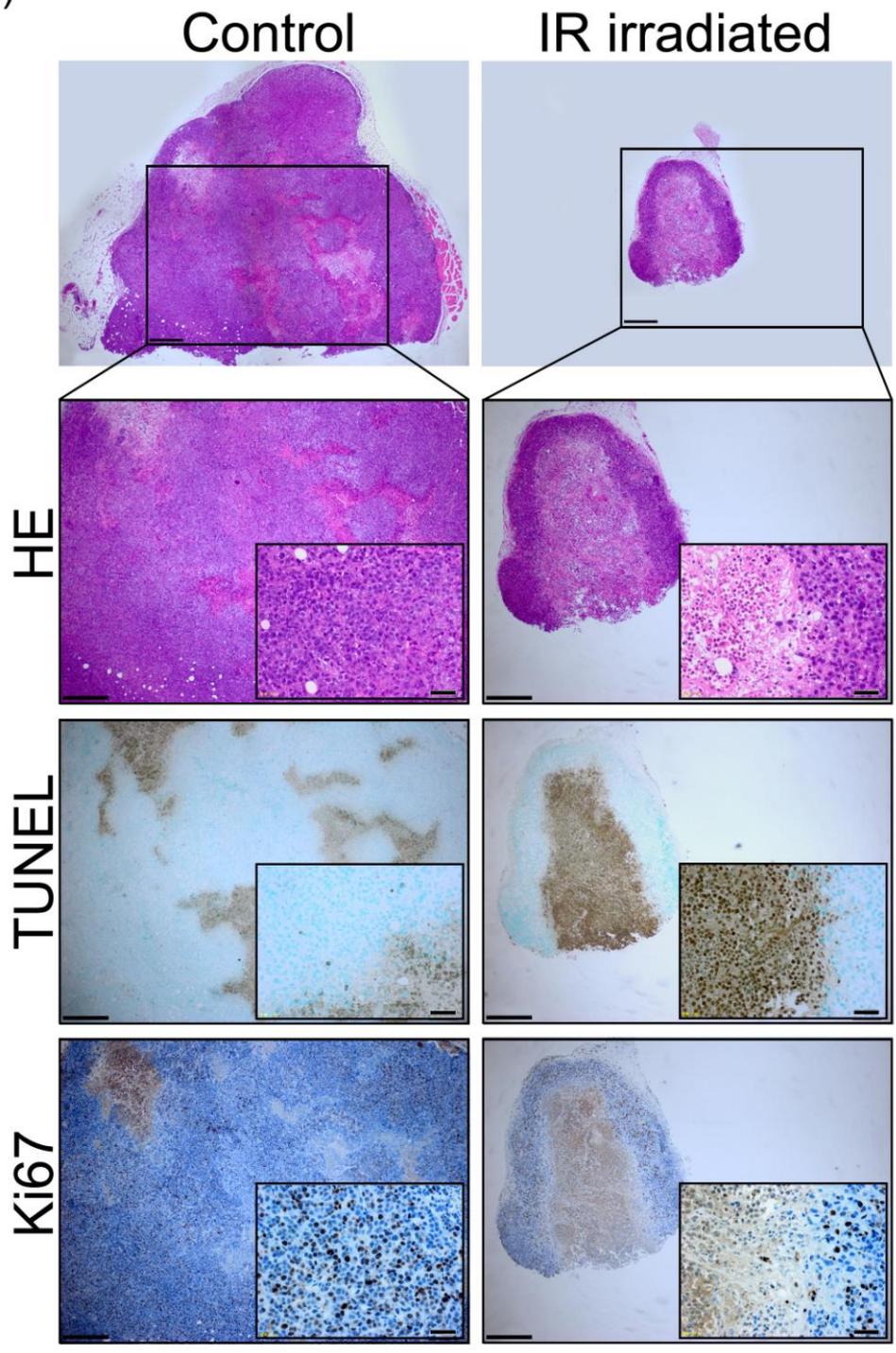


Fig. 6.

(b)

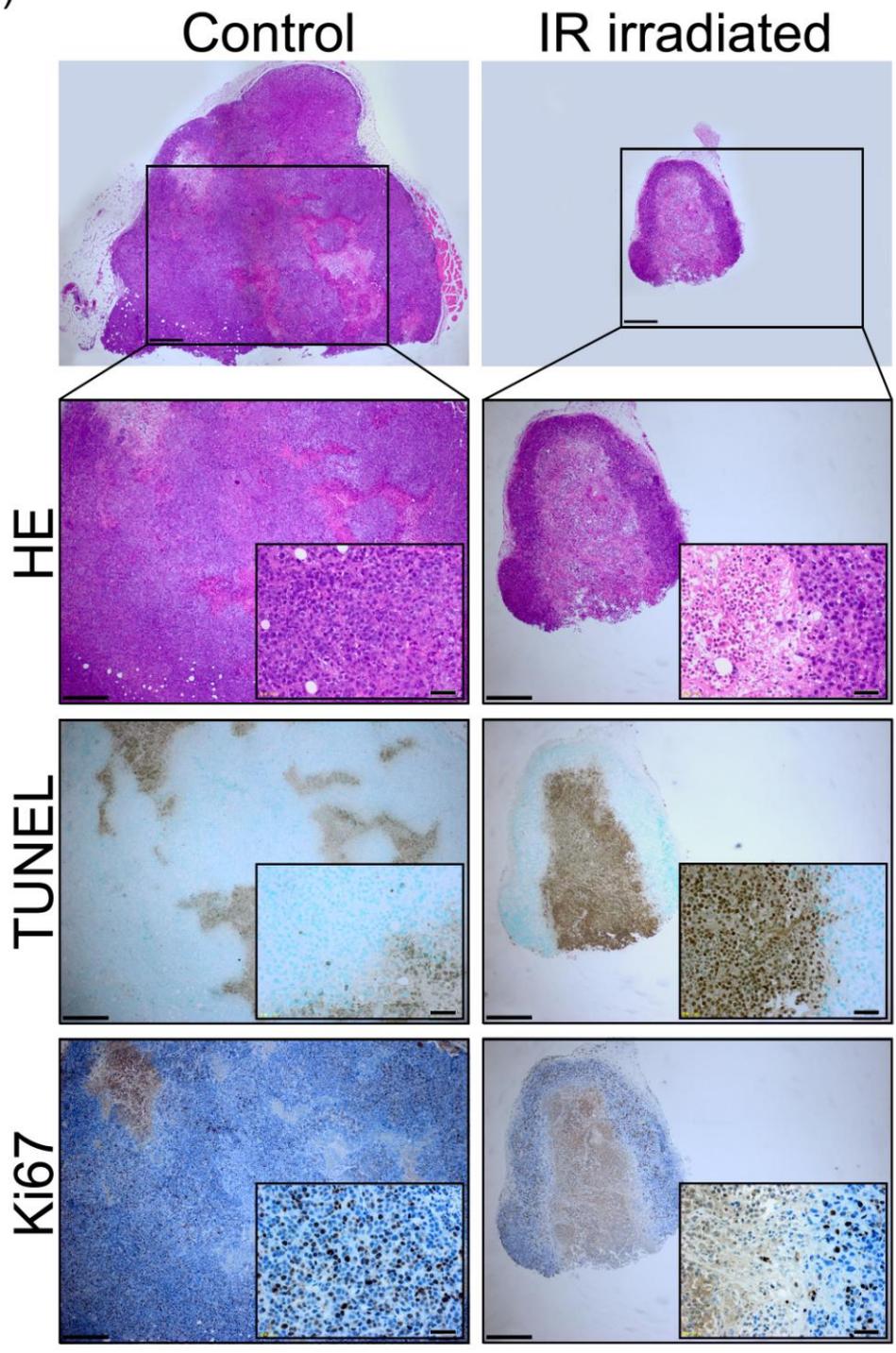


Fig. 6.

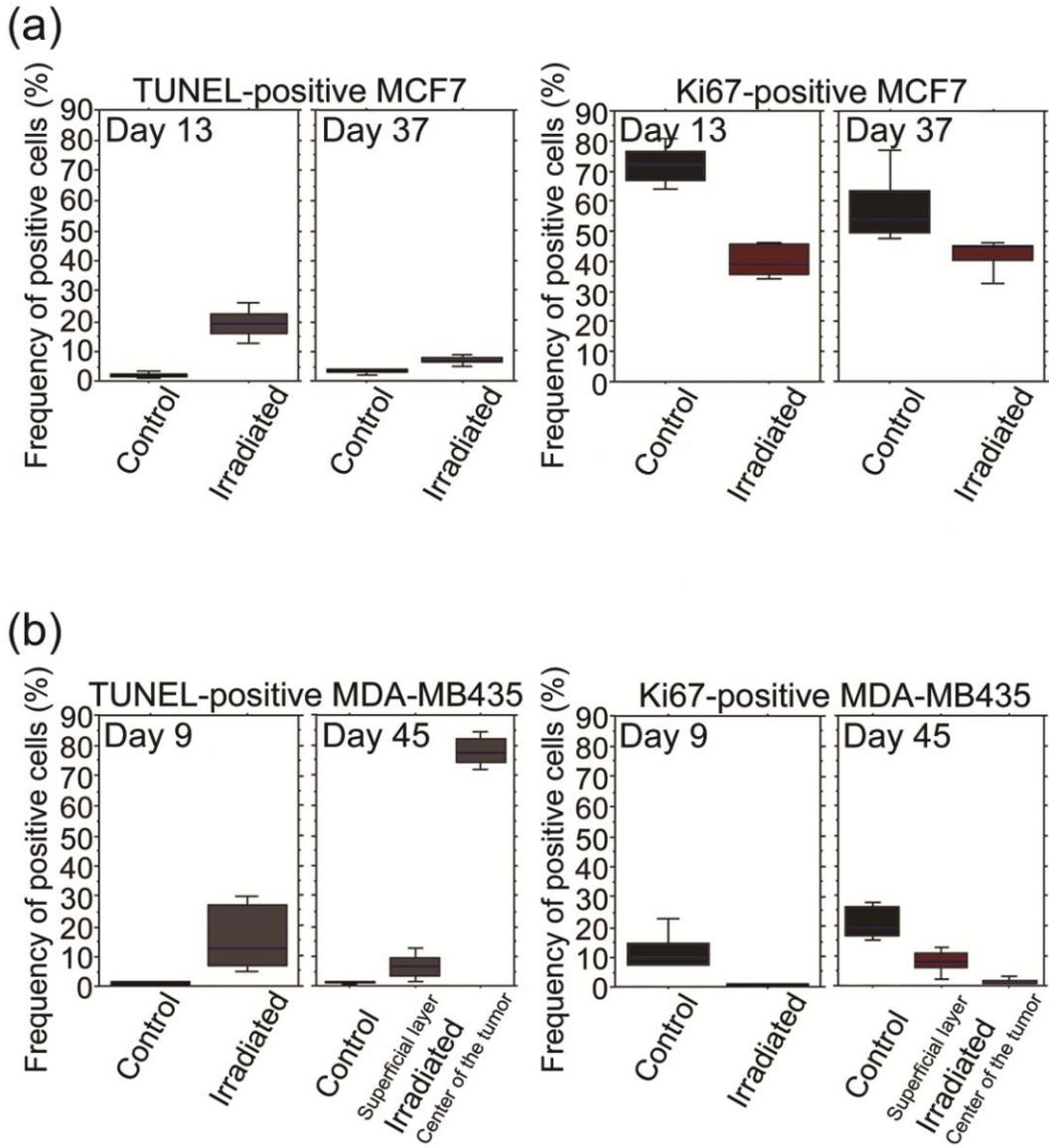


Fig. 7.