Preliminary Results of the Effects of Localized High-Dose Radiotherapy Combined with Total Body Low-Dose Irradiation on Tumor Growth and Stimulating the Immune System in Tumor-Bearing Mice

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ABSTRACT

Background: The immune system plays an extensive role in eliminating tumor cells. On the other hand, low-dose irradiation stimulates the immune system.

Objective: The present study aimed to investigate the therapeutic outcomes of localized high-dose radiotherapy (LH) alone and combined with total body low-dose irradiation (TB).

Material and Methods: In this experimental study, B16F0 tumor cells were injected into the right flank of C57JL/6 mice. The mice were treated with LH alone (13 Gy X-rays to the tumor surface) (LH group) or combined with TB (85 mGy X-rays at the skin) (TB+LH group). Then the tumor volume, the mice's lifespan, the number of lymphocytes extracted from the spleen, and interferon gamma (IFN-γ) production were measured.

Results: Reduced number of lymphocytes, compared to non-irradiated mice (control group), was observed in LH and TB+LH groups. However, the identical number of cultured lymphocytes produced a higher level of IFN-γ in irradiated groups. Comparing the irradiated groups, the number of lymphocytes and their IFN-γ production, tumor growth control, and the mice's lifespan were statistically higher in TB+LH group.

Conclusion: Observing a higher level of IFN-γ in TB+ LH group compared to LH group indicates that low-dose radiation enhanced the stimulating effects of high-dose radiation on the immune system. It caused the mice in TB+ LH group to have a more prolonged lifespan and a lower tumor growth rate. Therefore, it is worth our attention for future studies to investigate whether total body low-dose irradiation can be utilized before radiotherapy to enhance its efficiency.

Keywords

Immune System; Radiotherapy; Whole-Body Irradiation; IFN-γ

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Introduction

Radiation therapy is one of the most essential treatment modalities used to treat tumors. The efficacy of ionizing radiation is mostly ascribed to the direct destruction of tumor cells [1]. However, its impacts on the immune system should also be considered to increase

the efficacy of radiation therapy. The effects of ionizing radiation on the immune system and development of anti-tumor immunity vary according to the factors such as radiation dose, dose rate [2], and the irradiation regimen applied to treat tumors [3].

Based on Kadhim et al.'s classifications of radiation doses, including low (doses of 0.05-0.5 Gy), medium (doses of 0.5-5 Gy), and high (doses of 5-15 Gy), Manda et al. have declared that the effects of each category differ from the others [4]. Radiation with the medium dose used in conformal radiation therapy is immunosuppressive and inhibits the immune response [5], while high-dose radiotherapy, through modulation of the tumor microenvironment, [6] promotes the immune system [7] and consequently develops anti-tumor responses. Meanwhile, some documents indicate that low-dose radiation also activates anti-tumor immune responses [8], even though its biologic effects are fairly different from high-dose radiation effects.

Although both moderate and high doses of radiation have inflammatory effects, only the inflammatory effects of high-dose radiation assist anti-tumor immunity. The underlying cellular mechanisms of this assistance may be attributed to the release of danger associated molecular pattern molecules (DAMPs) from intensively irradiated tumor cells. DAMPs are recognized by dendritic cells through toll-like receptors, which in turn activate integral components of the immune system [8]. Recognition of DAMPs causes dendritic cells to mature and activate effector T-cells. Meantime, high-dose radiation causes an increase in vascular permeability, facilitating T-cell transition through vessel walls and inducing key chemokines that attract effector T-cells to the tumor. These processes prevent tumor evasion from immune system surveillance, occurring through the prevention of tumor antigen presentation and secretion of immunosuppressive cytokines by tumor cells.

Low-dose radiation like high doses results in anti-tumor immune responses [9] and provides the possibility of enhancing the cytotoxic function of natural killer cells against tumors [10, 11]. Experimental and epidemiological findings have pointed out that low-dose irradiation inhibits tumor growth, metastasis, and tumor recurrence [12]. Moreover, as low-dose radiation creates adaptive responses in normal tissues, it may provide a therapeutic strategy for cancer treatment, which encounters the inadequacy of radiation therapy and chemotherapy due to the formation of toxicity in normal tissues.

The above findings support the idea of low-dose irradiation alongside the standard or high dose radiation therapy to obtain more favorable anti-cancer effects. Therefore, the present study aimed to investigate the effects of localized high-dose radiotherapy (LH) with and without total body low-dose irradiation (TB) on the tumor growth, lifespan, and immune response of female C57BL/6J mice. The syngergistic B16F0 tumor cells were subcutaneously injected into the right flank of the mice. Some mice received both TB (85 mGy at the skin) and LH (13 Gy to the tumor) with 24 h interval, while others, excluding the control group, were treated with only LH. The number of lymphocytes extracted from the spleen, the amount of interferon gamma (IFN-γ) produced by lymphocytes, tumor growth rate, and lifespan of the mice following irradiation were measured and compared between the groups.

Material and Methods

Mice, cell line, tumor volume measurement, and experimental groups

To perform this experimental study, seven-week old female C57BL/6J mice were acquired from Royan Institute (Karaj, Iran) and kept in appropriate and standard conditions in terms of light, heat, food, and water. The animal experiments were accomplished according to the ethical rules passed by the Ethical Committee of Mashhad University of Medical Science

(ethical code: IR.MUMS.fm.REC.1396.367).

B16F0 cell line was supplied by Pasteur Institute, Tehran, Iran, and cultured in Dulbecco's Modified Eagle (DMEM) medium (Bioidea) supplemented with 10% fetal bovine serum (Gibco), 100 U/ml penicillin, and 100 µg/ml streptomycin (Alfasan, Iran). Subconfluent cells were removed from the flasks and a suspension solution, containing 8×10^5 B16F0 cells and 100 mL phosphate buffered saline, was subcutaneously injected into the right flank of each mouse.

The mice were divided into two groups. The first group received radiation when their tumors reached 100-140 mm^3 in volume (14 days after inoculation). Eight days following irradiation the mice were sacrificed to extract their lymphocytes and measure IFN-γ concentration with ELISA immunoassay. In addition, tumor volume measurement was performed every other day to determine the tumor growth rate over the eight days. Dimensions of the tumors were measured using a digital Vernier caliper, and the tumor volume was calculated based on Equation 1.

1) Tumor volume = length × width × height × 0.52.

The second group was irradiated when the tumors reached 70-80 mm^3 in volume (10 days after inoculation) and allowed to live for a longer period in order to measure their lifespan. For ethical reasons, however, they were euthanized when the size of the tumors exceeded 1,000 mm^3. The tumor growth rate was also determined in the same way as performed for the first group, however, for a longer time (≤ 40 days).

Each of the two groups (first and second groups) was divided into three subgroups: The mice, which received both TB and LH, were named TB+LH group. The mice, which received only LH, were named LH group, and the mice, which received sham irradiation were considered as a control group. For each subgroup, five to six tumor-bearing mice were assigned. Groups and subgroups are shown in Table 1.

Irradiation

Using a superficial X-ray unit (Philips, serial number: 2/625, Amsterdam, The Netherlands), TB+LH subgroups received two subsequent irradiation with a one-day interval. On the first day, they received TB (Dose: 85 mGy at the skin; Energy: 100 kV; Dose rate: 340 mGy/min), and on the second day, only their tumors were locally irradiated (Dose: 13 Gy on the tumor surface; Energy: 100 kV; Dose

Table 1: The type and dose of radiation received by the groups and their subgroups

Groups	Subgroups				Endpoints
	Name	Number of irradiation	Type of irradiation	Radiation dose	
First group with 100-140 mm^3 tumor at the time of irradiation	***TB+LH	2	*TB	85 mGy at the skin	Interferon gamma (IFN-γ) concentration, the lymphocyte count and tumor growth rate
			**LH	13 Gy to the tumor	
	LH	1	LH	13 Gy to the tumor	
	Control (1)	0	null	0	
Second group with 70-100 mm^3 tumor at the time of irradiation	TB+LH	2	TB	85 mGy at the skin	Lifespan &Tumor growth rate
			LH	13 Gy to the tumor	
	LH	1	LH	13 Gy to the tumor	
	Control (2)	0	null	0	

* Total body low-dose irradiation, ** Localized high-dose radiotherapy, *** The mice which received both TB and LH

rate: 8.66 Gy/min). Tumors of LH subgroups, only on the second day, received local irradiation (Dose: 13 Gy on the tumor surface; Energy: 100 kV; Dose rate: 8.66 Gy/min). For total body irradiation, a semi-pyramid-shaped applicator with a base of 20×20 cm^2 was attached to the superficial X-ray unit and a source to surface distance (SSD) of 60 cm was adjusted. The mice were kept in a box with a 17×17 cm^2 area in the radiation field and were irradiated when they were not in an anesthesia condition. However, for local irradiation, the mice were anesthetized with an intraperitoneal injection of 2.4 mg ketamine and 0.12 mg xylazine. The anesthetized mice were put on a bed. Afterward, an applicator with 1.5 cm diameter and an SSD of 10 cm, attached to the X-ray tube, was adjusted to surround a margin around the tumor. Finally, the tumor and surrounding margin were irradiated. In order to limit the radiation to the tumor and its margin, a lead shield covered the area around the radiation field (Figure 1).

Spleen removal and lymphocyte extraction

The mice of the first group were sacrificed 8 days following radiation therapy. Their spleens were removed and the lymphocytes were immediately extracted. Then, ammonium chloride was added to the lymphocyte suspension and centrifuged (1200 g at 4 ˚C) for the depletion of red blood cells. The numbers of lymphocytes derived from the spleens were counted using the Trypan blue (Sigma) exclusion test.

Figure 1: Localized irradiation of the tumors. The areas around the radiation field are shielded

Lymphocyte culture

A determined number of lymphocytes (2 × 10^6 per well) were cultured in RPMI-1640 medium, containing 10% FBS in 24-well culture plates (three wells for each sample). Lymphocytes of two wells, among three wells devoted to each sample, were stimulated using highly irradiated (approximately 50 Gy) B16F0 cells, and Phytohemagglutinin Antigen as experimental and positive control groups, respectively. The third well was considered as a control group without stimulation (the negative control).

Analysis of IFN-γ levels by ELISA

Thirty six hours after cultivation of the lymphocytes, the supernatants were collected and immediately frozen at -80 ˚C for further analysis. The concentration of IFN-γ in supernatants was assessed with the use of mouse IFN-γ ELISA kit (MABTECH, Sweden) according to the manufacturer's protocol.

Statistical analyses

The graphs were made using Graph Pad Prism Pro version 7 (Graph Pad Software) and Excel Version 2013 (Microsoft Office). SPSS software, Version 16.0 (SPSS, Inc., Chicago, IL, USA), was used to evaluate the data distribution and to compare the groups. Statistical differences between groups, based on P-values, were evaluated using one way-ANOVA analysis with a post hoc Tukey's test for more than two groups and the independent t-test for two groups. P-values less than 0.05 were regarded as statistically significant.

Results

Lymphocytes counting

Using the trypan blue exclusion test, the numbers of lymphocytes extracted from spleens were assessed following spleen removal. As illustrated in Figure 2, the numbers of lymphocytes were statistically lower in experimental groups (TB+LH and LH) compared to the control group ($P< 0.05$). The lowest number was counted in LH group, which was statistically different from TB+LH group ($P< 0.05$). The decrease in lymphocytes of TB+LH group was expected because lymphocyte apoptosis occurs throughout the body in total body irradiation. However, interestingly, these data indicate that LH decreases the number of lymphocytes more effectively than TB+LH.

IFN-γ concentration

Figure 3 shows the data acquired from the ELISA immunoassay. The results indicate IFN-γ concentration was the highest in TB+LH group (P-value< 0.05 compared to LH and control groups). The next order belonged to LH group, which was statistically different from the control group (P-value< 0.01). According to these data, it can be concluded that LH increases the level of IFN-γ released from lymphocytes, and the increase is greater when LH is combined with low dose total body irradiation.

Mice lifespan

The lifespan was determined from the first day of irradiation (when the whole body of some mice was exposed to a low dose). The results are shown in Figure 4. TB+LH group had the longest lifespan, and three mice out of five mice in this group lived until the end of the experiment (40^{th} day). The differences between this group and others were statistically significant (P-value< 0.001 compared to control, P= 0.005 compared to LH). The next group with a longer lifespan was LH group (by the 30^{th} day, 50% of the mice had survived, and on the 32^{nd} day all of them died). The difference between this group and others was statistically significant (P-value= 0.005 compared to TB+LH group and P-value= 0.006 compared to the control group).

Tumor growth rate

Figure 5a shows the mean tumor volume (in

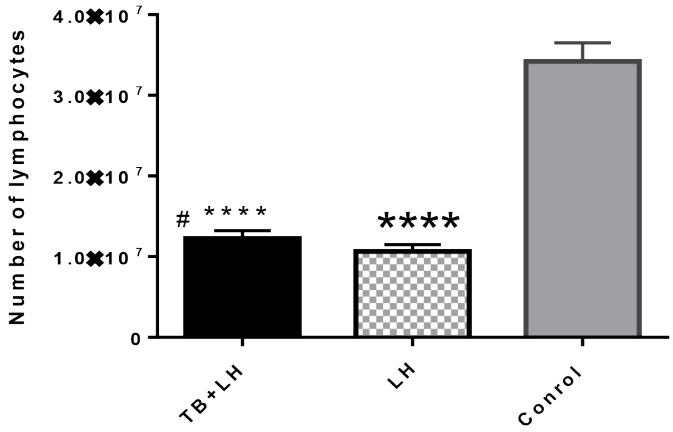

Figure 2: The number of lymphocytes extracted from the tumor-bearing mice. Data are presented as the mean + standard deviation. **** represents significant differences of irradiated groups versus the control group with P-value< 0.0001; # represents a significant difference between the mice which received both TB (Total Body) and LH (Localized High dose) (TB+LH) and Localized high-dose radiotherapy (LH) groups with P-value< 0.05. The mice were divided into three groups based on the radiation dose that they received, including (0.085 + 13) Gy as (TB+LH), 13 Gy as (LH), and 0 Gy as control groups.

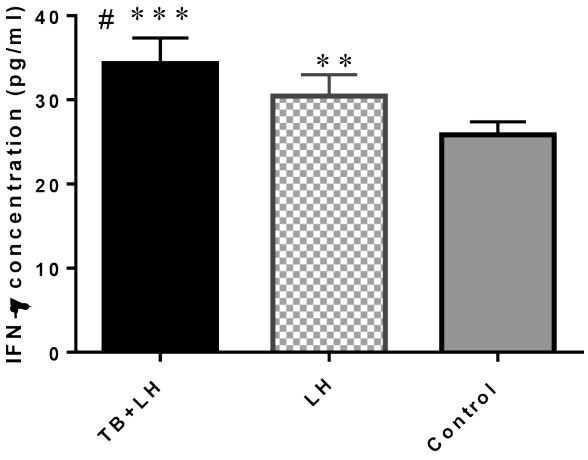

Figure 3: The concentration of interferon gamma (IFN-γ) in the supernatants derived from the lymphocytes' cultures. Data are presented as the mean + standard deviation. *** and ** represent significant differences of irradiated groups versus the control group with P<0.001 and P<0.01 respectively; # represents a significant difference between the mice which received both TB (Total Body) and LH (Localized High dose) (TB+LH) and Localized high-dose radiotherapy (LH) groups with P<0.05. The mice were divided into three groups based on the radiation dose that they received, including (0.085 + 13) Gy as (TB+LH), 13 Gy as (LH), and 0 Gy as control groups.

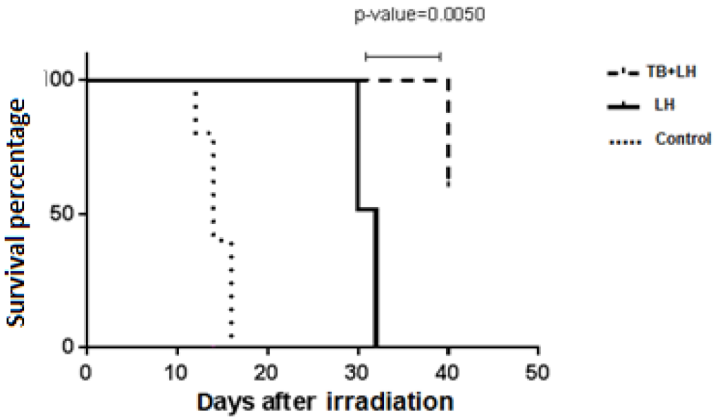

Figure 4: The lifespan of the tumor-bearing mice in different groups. The mice were divided into three groups based on the radiation dose they received, including (0.085 + 13) Gy as the mice which received both TB (Total Body) and LH (Localized High dose)(TB+LH), 13 Gy as (LH), and 0 Gy as control groups.

mm^3) of the three subgroups of the first group on the days after irradiation. The rates of tumor growth in TB+LH and LH groups were lower than that of the control group (P-Value< 0.05), and there was no significant difference between TB+LH and LH groups.

Assessment of the tumor growth rate of the mice belonged to the second group was performed for ≤ 40 days (Figure 5b). Obviously, on each specific day, the mean tumor volumes of TB+LH and LH groups were lower than that of the control group. For instance, as seen in the Figure 5b, on day 12, when all groups had at least one alive mouse, the mean tumor volume of TB+LH and LH groups was lower than that of the control group. Figure 5b also illustrates that before day 18, there was no difference between TB+LH and LH groups; however, since then, the difference was observable (P-value= 0.005) and continued until

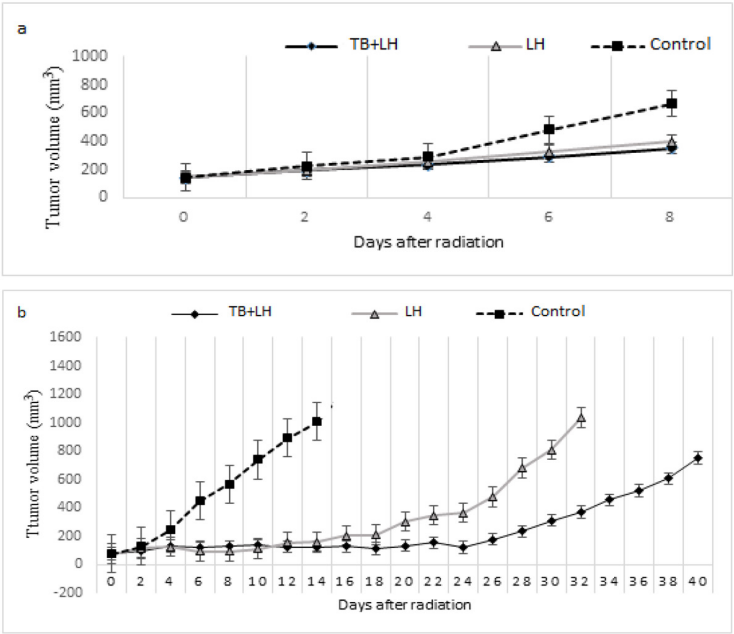

Figure 5: The mean volume of tumors in different groups on days after irradiation: (a) for the mice with larger tumors for 8 days; (b) for the mice with smaller tumors for 40 days. Data are presented as the mean ± standard deviation (5 mice were in each group). The mice were divided into three groups based on the radiation dose they received, including (0.085 + 13) Gy as the mice which received both TB (Total Body) and LH (Localized High dose) (TB+LH), 13 Gy as (LH), and 0 Gy as control groups.

the end of the experiment. In order to analyze the tumor growth more precisely, gradients of the lines, which fitted the data in Figure 5b, were determined. Table 2 shows the equations of the fitted lines and corresponding gradients. The gradient of TB+LH line was lower than that of LH line, indicating less tumor growth in TB+LH group. The same analysis indicates that the tumor growth of TB+LH and LH groups was less than that of the control group.

Correlation between IFN-γ and tumor growth

Statistical analysis performed by prism7 software showed that the tumor volumes of the mice sacrificed on the 8th day and the amount of IFN-γ, which their lymphocytes produced, were inversely correlated (P-Value< 0.05, Pierson coefficient< 0). The inverse correlation was higher in TB+LH group compared to LH and control groups (Table 3).

Discussion

The results of the current study revealed that LH had positive impacts on the level of IFN-γ, tumor growth reduction, and lifespan of the tumor-bearing mice. As IFN-γ production is the result of immune system stimulation, it may be concluded that LH, apart from direct effects (eradication of the tumor cells), stimulates the

Table 2: Equations of the fitted lines to the curves of each mean tumor volume of the groups

Groups	Control	LH	TB+LH
The fitted line equation	y = 147.4x - 143.93	y = 48.822x - 123.82	y = 25.723x - 44.198

TB: Total body low-dose irradiation, LH: Localized high-dose radiotherapy, TB+LH: The mice which received both TB and LH

Table 3: The results of statistical analysis which show an inverse correlation between interferon gamma (IFN-γ) concentration and tumor volume

Group Name	P-Value	Pierson Correlation
TB+LH	0.045	-0.801
LH	0.05	-0.54
Control	0.023	-0.573

TB: Total body low-dose irradiation, LH: Localized high-dose radiotherapy, TB+LH: The mice which received both TB and LH

immune system. The combination of TB with LH increased the aforementioned beneficial effects, which may indicate that the immune-stimulatory effects of TB have been added to the stimulatory effects of LH.

IFN-γ has anti-tumor immunity effects, activates macrophage and natural killer cells against tumor cells, upregulates anti-angiogenic chemokines within tumor cells [13], promotes the surface expression of MHCI (Major Histocompatibility Complex class 1) receptors, and enhances antigen presentation on tumor cells [14]. On the other hand, radiation has been shown to have inflammatory effects on the tumor environment, and undoubtedly the release of IFN-γ cytokines has been implicated in the development of radiation-induced inflammation. Besides, Persson et al. inoculated IFN-γ secreting cells into tumor-bearing rats and irradiated the tumors [14]. They observed that IFN-γ secreting cells increased the effect of radiation and reduced the tumor growth rate of the animals treated with IFN-γ secreting cells. Based on these facts, the immune-stimulating effects of radiation can be evaluated by measuring the amount of IFN-γ produced by lymphocytes, as performed in the present study. Our results revealed that the level of IFN-γ produced by lymphocytes was enhanced in LH group, predicating the positive impact of high dose irradiation on the immune system. This observation supports the findings obtained by Schaue et al. [3]. They irradiated the mice with 15 Gy or two fractions of 7.5 Gy and observed IFN-γ production. IFN-γ production by lymphocytes, as a result of high dose irradiation (13 Gy) in this study, confirms the statement suggested by Persson that very high dose radiation is necessary to stimulate immune response [14]. On the other hand, many researchers have proved that applying fractionated radiotherapy with a high dose per fraction regimen is more effective than single-dose irradiation [3, 15].

Other quantities measured in this study, in harmony with IFN-γ production, indicate that LH resulted in improved outcomes compared to the control group.

Although LH group showed superiority over the control group, TB+LH group displayed better outcomes compared to LH group. Figure 3 illustrates that the yield of IFN-γ produced by lymphocytes derived from TB+LH group was more than the corresponding value for LH group (P-value= 0.036). The number of lymphocytes and the lifespan of TB+LH group were also more than those of LH group (Figures 2 and 4). In addition, it was observed that, after 18 days, the mean tumor volume of TB+LH group was less than that of LH group (Figure 5b). Besides, statistical analyses showed that there was a stronger inverse correlation between tumor volume and IFN-γ concentration in TB+LH group compared to LH group. It may imply that TB+LH enhanced the immune system more efficiently and consequently resulted in a higher tumor volume reduction and increased lifespan.

Feng et al. [16] irradiated the mice with 75 mGy priming electron rays, and 24 h later irradiated the tumors previously injected into the flank of the mice with 2 Gy X-rays. They also irradiated another group of mice with only 2 Gy localized irradiation. Measuring tumor cell apoptosis, tumor volume, and IFN γ production, their results indicated that the combination of priming radiation and localized irradiation was more advantageous than localized irradiation alone. These findings are consistent with our results; however, unlike us, they ob-

served that the response of the immune system to localized irradiation (the level of cytokine production) was similar to that of non-irradiated mice. This difference can be attributed to the moderate dose (2 Gy) that they applied instead of the high dose (13 Gy), utilized in the current study. The discrepancy between the two studies confirms the view that the radiation dose must be very high (more than 2 Gy) to stimulate the immune system, as demonstrated via stereotactic ablative radiotherapy [8, 17]. Moreover, despite the lack of cytokine production due to 2 Gy irradiation alone, Feng et al. observed that the level of cytokines (IL 1β, TNF α, and IFN γ) in the peripheral blood of the mice, which received both priming and 2Gy irradiation, was higher than that of non-irradiated mice. This observation is consistent with the result of the present study regarding the higher level of IFN γ production in TB+LH group compared to LH group.

Beyond the beneficial effects of TB+LH on the immune system, the superiority of TB+LH towards LH alone may be attributed to the favorable impact of low-dose irradiation on the cancer stem cells. Kaushik et al. irradiated breast cancer cell lines with a low dose (0.1 Gy) and noticed that the number of cancer stem cells decreased and JAK1/STAT3 signaling was suppressed in the irradiated cells [18].

In addition to the benefits of TB observed in this study, many studies have proved that low dose irradiation of total body protects normal tissues from damage induced by challenging dose [19-21]. Therefore, it may be suggested that applying TB before standard radiotherapy (2 Gy per fraction) and hypofractionated radiotherapy (SABR (Stereotactic ablative radiotherapy), SBRT (Stereotactic body radiation therapy)) provides a therapeutic strategy for cancer treatment through stimulating the immune system or protecting normal tissues.

Conclusion

This paper represents a preliminary study that investigated whether the combination of TB with LH could be advantageous. The results indicated that the therapeutic outcomes of the TB plus LH regimen and its stimulatory effects on the immune system were more than LH alone. However, more studies should be performed to challenge these results.

We did not evaluate the effects of TB combined with moderate-dose irradiation in the current study. Therefore, it may be beneficial to plan a new project to investigate the effects of TB plus moderate-dose irradiation on the immune system. If its favorable effects are confirmed through future studies, TB might be utilized in clinical trials. It is not unlikely that TB provides a therapeutic strategy for cancer treatment and compensates for the immune suppressive effects of the moderate radiation dose implemented in the standard radiotherapy. Moreover, TB, through radiation-induced adaptation, may decrease normal tissue damage induced by standard or high-dose radiotherapy. On the other hand, there might be an adaptive response in tumor cells, which might protect them against moderate or high dose radiation. Hence, investigating the probable adaptive response of tumor cells as well as of normal cells will be helpful.

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Conflict of Interest

None

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