Relationship Between the Invasion of Lymphocytes and Cytokines in the Tumor Microenvironment and the Interval After Single Brachytherapy Hypofractionated Rradiotherapy and Conventional Fractionation Radiotherapy in Non-Small Cell Lung Cancer

Lin Li
affiliated hospital of southwest medical
Hong Cheng Yue
central hospital of bazhong
Yun Wei Han
affilicated hospital of southwest medical university
Wei Liu
people's hospital of dazu
Liang Geng Xiong (755745502@qq.com)
people's hospital of chongqing banan district
Jian Wen Zhang (zhangjianwen66@126.com)
afficated hospital of southwest mnedcical university

Research article

Keywords: cytokines, brachytherapy, hypofractionated radiotherapy, conventional fractionation radiotherapy, non-small cell lung cancer, lymphocytes

Posted Date: May 7th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-23833/v1

Version of Record: A version of this preprint was published on September 17th, 2020. See the published version at https://doi.org/10.1186/s12885-020-07403-1.

Abstract

Background Lymphocytes and cytokines in tumor microenvironment are the key to immunotherapy, The effect of brachytherapy on tumor microenvironment is not clear. The aim of our study was to analyze the relationship between the invasion of lymphocytes and cytokines in the tumor microenvironment and the interval after single brachytherapy hypofractionated radiotherapy (SBHFRT) and conventional fractionation radiotherapy (CFRT) in non-small cell lung cancer (NSCLC).

Methods Lewis tumor-bearing mice were randomly divided into control, CFRT and SBHFRT groups. On the days 7 and 14 after radiation, the expression rates of CD4+, CD8+, Foxp3+, and CD86 + cells and levels of Ki-67 + protein were detected by immunohistochemical analysis, and the tumor necrosis rate was calculated. Following this, interleukin-10 (IL-10), IL-12, and interferon-γ (INF-γ) levels were detected by enzyme-linked immunosorbent assay. The apoptosis rate was evaluated via flow cytometry. The tumor volume and tumor growth inhibition rate (TGIR) were calculated on day 14. Tumor metabolism was assessed via micro 18F-FDG positron emission tomography/computer tomography.

Results The tumor volume in the SBHFRT group reduced by 22.0% and TGIR increased by 92.2% ($P < 0.05$). Further, on days 7 and 14 after radiation, tumor metabolism, Ki-67 + and Foxp3 + expression rates, and IL-10 levels were lower and tumor necrosis and apoptosis rates; CD86+, CD4+, and CD8 + expression rates; and IL-12 and INF-γ levels were higher in SBHFRT group than in the CFRT group, particularly on day 7.

Conclusion SBHFRT could lead to more accumulation of dendritic cells and anti-tumor lymphocytes and cytokines in the tumor tissue, and further reduce the aggregation of immunosuppressive lymphocytes and cytokines in the tumor tissue compared with CFRT, and the difference was the most obvious was day 7 after radiation. Hypofractionated radiotherapy combined with immunotherapy may be better for treating NSCLC, as observed on day 7 after radiation.

Background

Lung cancer, particularly non-small cell lung cancer (NSCLC), is a common malignancy, in which radiotherapy plays an important therapeutic role. The radiotherapy model for malignancies has transformed from conventional fraction radiotherapy (CFRT) to hypofractionated radiotherapy, now considered to be a new and highly effective mode of radiation therapy[1, 2]. Stereotactic body radiotherapy (SBRT) and stereotactic radiosurgery are common procedures in hypofractionated radiotherapy, especially SBRT, which is widely performed for lung cancer, colorectal cancer, liver cancer, and prostate cancer, and shows considerable improvement in the radiotherapeutic outcomes [3, 4, 5, 6, 7, 8]

Immunotherapy has recently emerged as a highly effective and novel therapeutic modality, which is now gaining popularity worldwide in cancer therapy [9]. Immunocheckpoint inhibitors, such as pembrolizumab and ipilimumab, are widely used for melanoma, NSCLC, and pancreatic cancer[10, 11, 12, 13]. The core functionality of immunotherapy relies on the fact that there are enough anti-tumor cells in the tumor tissue. The destruction of immune cells in tumor tissues will lead to the failure of immunotherapy. Accordingly, improving and enhancing immune cells in tumor tissues, especially CD8 + T lymphocytes, is the key to tumor immunotherapy.

The relationship between radiation and immunity is complicated. Radiation was previously thought to have an inhibitory effect on the immune system, particularly because of the sensitivity of lymphocytes to radiation. However, studies have now shown that in addition to inducing lethal DNA damage in tumor and stromal cells, radiation can alter interactions of tumor cells with their microenvironment, and these effects on the tumor microenvironment vary with dose and fractionation schedules [14, 15]. Hypofractionated radiotherapy can stimulate the immune system. NSCLC patients undergoing SBRT have been reported to show increased proportions of CD8 + T cells and decreased levels of inhibitory tregs, with high expression levels of interferon-γ (INF-γ) [16]. In colon cancer, SBRT activates dendritic cells (DCs) and induces immune cell infiltration in tumors and migration of immune cells to tumors [15].

The use of single brachytherapy hypofractionated radiotherapy (SBHFRT) in advanced NSCLC has rarely been reported in the literature, and there is no study on the relationship with immune cells. The results of our previous clinical studies have shown that the effective rate and 2-year overall survival after SBHFRT in patients with NSCLC were 92.3% and 67%, respectively, with a median survival period of 22.5 months [16]. Weather this outcome, in addition to the physical role of radioactive rays, is associated with immune cells and cytokines, especially anti-tumor lymphocytes and cytokines in the tumor microenvironment, is unknown. The main purpose of this study was to investigate the relationship between the invasion of lymphocytes and cytokines in the tumor microenvironment and the

interval after SBHFRT and CFRT for NSCLC through animal experiments, and to provide the basis for radiotherapy combined with immunotherapy of NSCLC.

Methods

Establishment of the tumor-bearing mouse model

Healthy C57BL/16 female mice (aged 4–5 weeks; weighing 16–22 g; Chongqing tianxinhuafu biotechnology company, China; Certificate No: 11401300024918.SPF feeding) were acclimatized for at least a week under standard conditions of 24 ± 2 °C and 50 ± 10% relative humidity before they were enrolled in the study.

The logarithmically grown Lewis cell line (department of oncology, affiliated hospital of Southwest Medical University) was inoculated via a subcutaneous injection into the right hindlimb of healthy C57BL/16 mice. The general state, weight, tumor formation, and tumor volume of C57BL/16 mice were observed and recorded. Experiments were performed when the diameter of the transplanted tumor was approximately 8–10 mm.

All animal experiments were approved by the Institutional Animal Care and Treatment Committee of Southwest Medical University (Luzhou, China).

Main laboratory equipment and reagents

The following equipment and reagents were used in the study: Linear accelerator (Elekta, Sweden), paraffin slicer (Leica instrument company, Germany), new BGZ series II type high precision oven (Shanghai boxun company, China), pipette (Eppende, Germany), inverted phase-contrast microscope (Olympus corporation, Japan), three-dimensional brachytherapy machine and oncentra brachytherapy treatment planning system (brachy TPS. Nucletron company, 4.3.0. 410 version, The Netherlands), high-sugar DMEM (HyClone, USA), fetal bovine serum (Gibco, USA), monoclonal antibodies against mouse CD4, CD8, Foxp3, CD86, Ki-67, and apoptosis detection reagent (Bio-World, USA), detection reagents for interleukin-10 (IL-10), IL-12, and interferon-γ (INF-γ) (andyht (Beijing) company, China)., micro-positron emission tomography/computer tomography (micro-PET/CT) system (Siemens, Germany), nanozoomer digital pathological section scanner (Hamamatsu photonics, Japan), and flow cytometer (BD, USA).

Experimental grouping and specimen collection

Tumor-bearing mice were numerically randomly divided into the control group (12 mice, Dt = 0 Gy) and the experimental groups: (1) CFRT group (12 mice; prescription dose Dt = 20 Gy/10 fraction (F); radioactive source, 6 MV X-ray; source–skin distance, 100 cm) and (2) SBHFRT group (12 mice). The source applicator was implanted (1–2 needles) along the long axis of the tumor, simulated CT scan was performed, gross tumor volume (GTV) at brachy TPS was delineated, and the radiation plan was designed and implemented (prescribed dose Dt = 11.3 Gy/1 F, $D_{95\%} \geq 10$ Gy of GTV). The biologically equivalent dose (BED; tumor $\alpha/\beta = 10$) was calculated according to L-Q model in the CFRT and SBHFRT groups (BED = 24 Gy and 24.07 Gy, respectively; Fig. 1).

On days 7 and 14 after radiation, six mice were euthanized by cervical dislocation in each group, and tumors were completely stripped off, and averagely divided into two parts. One of the two was used for immunohistochemical analysis to detect the expression levels of CD4, CD8, Foxp3, CD86, and Ki-67 in cells and tumor necrosis rate and the other was used for enzyme-linked immunosorbent assay (ELISA) and flow cytometry to detect levels of IL-10, IL-12, and INF-γ, and the rate of apoptosis of tumor cells.

Evaluation of CD4+, CD8+, Foxp3+, and CD86 + cells and Ki-67 expression levels and calculation of tumor necrosis rate

The expression levels of CD4, CD8, Foxp3, CD86, and Ki-67 in tumor tissues were detected by immunohistochemical analysis following the SP method, and the results were independently evaluated by two experienced pathologists. Criteria for the evaluation of CD4+, CD8+, Foxp3+, and CD86 + cells were positive expressions for CD4, CD8, and CD86 staining as shown by brown-yellow or brown cytoplasm or cell membrane, whereas a positive expression of Foxp3 and Ki-67 showed a brown-yellow or brown nucleus. Calculations

of CD4+, CD8+, Foxp3+, and CD86 + cells were performed by selecting five fields for each section. The number of positive cells in each field was counted under a microscope (× 400), and the mean number of positive cells in the five fields was calculated as the number of positive cells in the section.

The positive expression rate for Ki-67 was calculated by counting under a microscope (× 400). Five fields were selected in each section and the number of positive cells in each field was counted along with the total number of cells. The proportion of positive cells among the total cells in the five fields was calculated to determine the positive expression rate of Ki-67.

The stripped tumor tissue was fixed and dehydrated, H□E staining sections were prepared and placed under Nanozoomer digital pathological section scanner to generate a full field of digital image, which was transmitted to the KFBIO, SlideViewer image acquisition system, the tumor boundaries and necrotic areas were delineated, and the total tumor area (S_{tumor}) and necrotic area ($S_{necrotic}$) were calculated. Tumor necrosis rate = $S_{necrotic}/S_{tumor} \times 100\%$.

Levels of IL-10, IL-12, and INF-γ and tumor cell apoptosis

IL-10, IL-12, and INF-γ levels in the tumor tissues were detected by ELISA, and the concentrations of IL-10, IL-12, and INF-γ were calculated according to the standard curve. The apoptosis of tumor cells was analyzed by flow cytometry and the apoptosis rate was calculated.

Tumor volume and tumor growth inhibition rate

The maximum (d_{max}) and minimum diameters (d_{min}) of transplanted tumors in each group were measured every 2 days from the 12th day after inoculation until the 14th day after radiation: tumor volume (V_{tumor}) = $d_{max} \times d_{min}^2/2$. Tumor growth inhibition rate (TGIR) was calculated on the 14th day after radiation: TGIR = (control group V_{tumor} – experimental group V_{tumor})/control group $V_{tumor} \times 100\%$.

Micro-PET/CT

On days 7 and 14 after radiation, six mice from each group underwent 18 F-FDG micro-PET/CT (parameters 80 Kv, 500 uA; Space 1.5 mm). The images of micro-PET/CT were analyzed by two experienced physicians in the Nuclear Medicine Department, and the maximum standardized uptake value (SUV_{max}) of tumor tissues was calculated.

Statistical analysis

SPSS 17.0 software was used for statistical analysis, and the measurement data are expressed as ± S. One-way ANOVA and LSD-t test were used for comparisons between groups. TGIR was compared by t test, and $P < 0.05$ indicated that the difference was statistically significant.

Results

Tumor volume, TGIR, and SUV_{max}

The tumor volume in the SBHFRT group reduced by 22.0% and 37.0% compared with that in the CFRT and control groups, respectively ($P < 0.05$), whereas the volume in the CFRT group reduced by 19.2% compared with that in the control group ($P < 0.05$). TGIR in the SBHFRT group increased by 92.2% compared with that in the CFRT group ($t = 10.70$, $P < 0.05$) (Fig. 2-A; Table 1).

Table 1
Comparisons of tumor volume, SUV_{max}, TGIR, tumor necrosis rate, apoptosis rate, and ki-67 positive expression rate (± S) among groups

Groups	n	Tumor necrosis rate		Apoptosis rate		Rate of Ki-67+		V_{tumor}	SUV_{max} value		TGIR§
		Day 7 (ratio, %)	Day 14 (ratio, %)	Day 7 (ratio, %)	Day 14 (ratio, %)	Day 7 (ratio, %)	Day 14 (ratio, %)	(mm^3)	Day 7 (ratio, %)	Day 14 (ratio, %)	(%)
Control	6	18.3 ± 3.6	16.0 ± 3.8	3.4 ± 0.3	2.8 ± 0.4	79.0 ± 5.6	89.5 ± 4.5	4169.8 ± 123.3	2.4 ± 0.2	2.9 ± 0.3	
CFRT[]	6	23.1 ± 4.6 (26.2△)	18.9 ± 4.0 (18.1△)	3.9 ± 0.4 (14.7△)	3.2 ± 0.5 (14.3△)	72.5 ± 9.1 (-8.2△)	81.0 ± 5.8# (-9.5△)	3368.7 ± 134.9#	2.2 ± 0.2# (-8.3△)	2.6 ± 0.2# (-10.3△)	19.2 ± 3.2
SBHFRT[]	6	51.8 ± 7.1#※ (183.1△ /124.2▼)	47.4 ± 6.8#※ (196.3△ /150.8▼)	7.3 ± 0.7#※ (114.7△ /87.2▼)	5.7 ± 0.7#※ (103.6△ /78.1▼)	41.8 ± 4.6#※ (-47.1△ /-42.3▼)	44.8 ± 7.2#※ (-49.9△ /-44.7▼)	2627.3 ± 103.4#※	1.5 ± 0.1#※ (-37.5△ /-31.8▼)	1.8 ± 0.2#※ (-37.9△ /-30.8▼)	37.0 ± 2.5※
F		70.22	71.28	114.06	52.31	52.12	95.83	242.94	42.60	43.03	10.70(t)
P		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

[]Conventional fractionation radiotherapy.

[]Single brachytherapy hypofractionated radiotherapy.

§Tumor growth inhibition rate

#Compared with the control group, $P < 0.05$

※Compared with the CFRT group, $P < 0.05$.

△Ratio compared with the control group

▼Ratio compared with the CFRT group

SUV_{max} in the SBHFRT group was lower than that in the CFRT and control groups ($P < 0.05$) on days 7 and 14 after radiation, whereas SUV_{max} in the CFRT group was only lower by 8.3% and 10.3% compared with that in the control group, respectively ($P < 0.05$). Further more, the difference was most obvious on day 7 after radiation($t = 3.57, 3.29$; $P < 0.05$; Fig. 2-B; Table 1).

Tumor cell necrosis and apoptosis rates and Ki-67 + expression rate

Tumor cell necrosis and apoptosis rates in the SBHFRT group were higher than those in the CFRT and control groups on days 7 and 14 after radiation ($p < 0.05$), whereas there were no significant differences in the rates in the CFRT group compared with those in the control group ($P > 0.05$). The most obvious difference in apoptosis rates was observed on day 7 after radiation($t = 3.91, 2.93$; $P < 0.05$) (Fig. 2-C,D,E; Table 1).

The expression rates of Ki-67 + in the SBHFRT group were lower than those in the CFRTand control groups on days 7 and 14 after radiation($p < 0.05$). However, there were no differences in the expression rates between the CFRT group and the control group on day 7 than on day 14($P > 0.05$). There were no differences on days 7 and 14($t = 0.86,1.92$; $P > 0.05$; Fig. 2-F; Table 1).

Expression rates of CD86+, CD4+, CD8+, and Foxp3+ cells

The rates of CD86+, CD4 + and CD8 + cells in the SBHFRT group were higher than those in the CFRT and control groups on days 7 and 14 after radiation($p < 0.05$). The most obvious differences were observed on day 7 after radiation($p < 0.05$). There were higher differences in the rates of CD4 + and CD8 + cells on day 7 than on day 14 in the SBHFRT group ($t = 3.04, 2.85$; $P < 0.05$; Fig. 3-A,B,C;Table 2).

Table 2
Comparisons of the expression rates of CD86+, CD4+, CD8+, and Foxp3 + cells in tumor tissue (rate/field;±S).

Groups	n	Rate of CD86 + cells		Rate of CD4 + cells		Rate of CD8 + cells		Rate of Foxp3 + cells	
		Day 7 (ratio,%)	Day 14 (ratio,%)	Day 7 (ratio,%)	Day 14 (ratio,%)	Day 7 (ratio,%)	Day 14 (ratio,%)	Day 7 (ratio,%)	Day 14 (ratio,%)
Control	6	2.7 ± 1.4	1.9 ± 1.0	36.8 ± 4.6	34.3 ± 3.3	27.5 ± 4.5	24.2 ± 4.0	16.2 ± 3.5	17.5 ± 3.6
CFRT[]	6	3.2 ± 1.7 (18.5△)	3.0 ± 1.5 (57.9△)	42.3 ± 6.8 (14.9△)	37.3 ± 6.8 (8.7△)	50.7 ± 7.6# (84.4△)	39.8 ± 6.6# (64.5△)	12.5 ± 2.7# (-22.8△)	15.2 ± 3.3 (-13.1△)
SBHFRT[]	6	6.7 ± 2.6#※ (148.1△/ 109.4▼)	5.2 ± 2.3#※ (173.7△/ 73.3▼)	62.7 ± 9.1#※ (70.4△/ 48.2▼)	49.2 ± 6.0#※ (43.4△/ 31.9▼)	86.0 ± 9.6#※ (212.7△/ 69.6▼)	71.3 ± 8.2#※ (194.6△/ 79.1▼)	7.3 ± 1.9#※ (-54.9△/ -41.6▼)	9.2 ± 1.9#※ (-47.4△/ -39.5▼)
F		7.44	5.63	22.24	11.89	92.24	82.05	15.6	12.13
P		0.01	0.02	0.00	0.00	0.00	0.00	0.00	0.00

[]Conventional fractionation radiotherapy

[]Single brachytherapy hypofractionated radiotherapy

#Compared with the control group, $P < 0.05$

※Compared with the CFRT group, $P < 0.05$

△Ratio compared with the control group

▼Ratio compared with the CFRT group

The rates of Foxp3 + cells in the SBHFRT group were lower than those in the CFRT and control groups on days 7 and 14 after radiation($P < 0.05$). The rate of Foxp3 + cells in the CFRT group were too lower than those in the control group($P < 0.05$). The most obvious difference was on day 7. There were no differences between day 7 and day 14 in the SBHFRT and CFRT groups ($t = 1.68, 1.56$;$P > 0.05$; Fig. 3-D, Table 2).

Levels of IL-10, IL-12, and INF-γ in tumor tissues

IL-10 levels in the SBHFRT group were lower than the levels in the CFRT and control groups on days 7 and 14 after radiation ($p < 0.05$). The most obvious difference was on day 7. The IL-10 levels were not different between the CFRT and control groups ($p > 0.05$). However, there were differences observed between days 7 and 14 in the SBHFRT and CFRT groups ($t = 2.55, 2.49$; $p < 0.05$;Table 3).

Table 3
Comparisons of IL-10, IL-12, INF-γ levels in tumor tissues (pg/ml, ±S).

Groups	n	IL-10 levels		IL-12 levels		INF-γ levels	
		Day 7 (ratio,%)	Day 14 (ratio,%)	Day 7 (ratio,%)	Day 14 (ratio,%)	Day 7 (ratio,%)	Day 14 (ratio,%)
Control	6	190.9 ± 53.0	257.4 ± 65.4	18.1 ± 4.3	17.4 ± 2.0	201.9 ± 39.7	184.9 ± 31.6
CFRT[]	6	155.3 ± 48.8 (-18.6△)	219.9 ± 54.1 (-14.6△)	21.7 ± 2.5# (19.9△)	19.1 ± 1.9 (9.8△)	222.2 ± 38.8 (10.1△)	210.8 ± 36.1 (14.0△)
SBHFRT[]	6	91.6 ± 31.5#※ (-52.0△ /-41.0▼)	135.8 ± 47.6#※ (-47.2△ /-38.2▼)	26.8 ± 2.8#※ (48.1△ /23.5▼)	22.5 ± 2.5#※ (29.3△ /17.8▼)	348.4 ± 53.9#※ (72.6△ /56.8▼)	270.7 ± 44.6#※ (46.4△ /28.4▼)
F		7.38	7.38	19.68	8.70	18.94	8.11
P		0.01	0.01	0.00	0.00	0.00	0.00

[]Conventional fractionation radiotherapy

[]Single brachytherapy hypofractionated radiotherapy

#Compared with the control group, $P < 0.05$

※Compared with the CFRT group, $P < 0.05$

△Ratio compared with the control group

▼Ratio compared with the CFRT group

IL-12 and INF-γ levels in the SBHFRT group were higher than those in the CFRT and control groups on days 7 and 14 after radiation ($p < 0.05$). The most obvious differences were on day 7. IL-12 and INF-γ levels in the CFRT group were considerably higher than those in the control group ($p < 0.05$). Differences in levels between days 7 and 14 were only observed in the SBHFRT group ($t = 2.79, 2.72$; $p < 0.05$;Table 3).

Discussion

Radiation induces tumor cell DNA damage and death, and leads to in-situ vaccination, promoting DCs, antitumor lymphocytes, and cytokines accumulated in tumor tissues [18]. The quantity of in-situ vaccine produced by radiation is closely associated with tumor cell death. The result of our study indicated that under the same BED, the tumor showed a larger decrease in volume after SBHFRT than after CFRT, and TGIR after SBHFRT was nearly twice that after CFRT. On days 7 and 14 after radiation, SUV_{max} and Ki-67 + expression rates showed a larger decrease after SBHFRT than after CFRT. Further, tumor necrosis and apoptosis rates were considerably higher after SBHFRT than after CFRT on days 7 and 14. This indicated that SBHFRT was more effective than CFRT in terms of the physical role of radiation, and could result in a higher rate of tumor cell death, and lead to a level of in-situ vaccination.

The relationship between radiation and immunity is complex. Immunologic effects induced by radiation are not only sensitive to variations in dose and fractionation but are also time-dependent [18]. The results of a comparative study with a 48 Gy/8 F or 48 Gy/6 F regimen for treatment by SBRT in NSCLC patients indicated that the levels of CD3 + T lymphocytes were slightly higher after SBRT, and they significantly increased at 3 weeks. The ratio of CD8 + T cells/CD3 + T cells increased significantly 3 weeks after SBRT [16]. In our study, we selected days 7 and 14 as the observation time windows, and it was possible to observe the high expression of tumor

invasion lymphocytes and cytokines and the relationship between hypofractionated radiotherapy and tumor immune microenvironment.

Radiation can enhance the efficacy of immune checkpoint blocker and promote immune reponse, but the dose and fraction mode of radiation that induces this immune effect is not clear, and the recruitment of DCs is closely related to radiation dose and fraction mode [19]. The co-stimulatory factor CD86 is a surface marker of mature DCs [20]. Colon cancer studies have shown that 48 h after 10 Gy/F radiation, mature CD86 + DCs in tumor tissues significantly increased [21]. In our study, on days 7 and 14 after radiation, the number of CD86 + cells in the SBHFRT group showed a higher increase than that in the CFRT group. On comparing the results on days 7 and 14, the number of CD86 + cells in the SBHFRT group was higher on day 7 than on day 14; the number of CD86 + cells was higher on day 7 in the CFRT group too, but only by 6.7%. The reason may be the increased release of radiation-associated antigenic proteins induced by SBHFRT. SBHFRT can lead to a higher accumulation of mature DCs than CFRT, especially on day 7, with radiation-induced dead tumor cells acting as antigens to play a more effective role in antigen presentation.

Lymphocytes and cytokines in the tumor immune microenvironment play an important role in anti-tumor immunity [14, 22, 23]. T cells are the main cell type in the tumor microenvironment. Without intervention, CD4 + T cells and Tregs are the main cell types, and the level of CD8 + T cells is usually very low [24]. An increase in the number of anti-tumor lymphocytes and cytokines in the tumor microenvironment can enhance the anti-tumor immune response [14, 15]. Studies have shown that high expression of CD4 + and CD8 + T lymphocytes in NSCLC led to higher the median survival and 3-year survival rates than low expression and no expression, whereas low expression of Foxp3 + T lymphocytes led to higher median survival and 3-year survival rates than high expression [25]. In our study, on days 7 and 14 after radiation, the expressions of CD4 + and CD8 + cells in the SBHFRT group were higher than those in the CFRT group; further, the expression of Foxp3 + T lymphocytes was lower in the SBHFRT group than in the CFRT group. In the SBHFRT group, the rate of increase of CD4 + and CD8 + cells on day 7 was higher than that on day 14, and the rate of decrease of Foxp3 + cells was higher than that on day 14. The reason for this may be that SBHFRT rather than CFRT can induce higher expression levels of T helper lymphocytes and cytotoxic T lymphocytes, especially 7 days after radiation; promote the accumulation of the anti-tumor cytokine IFN-γ [26]; regulate T lymphocyte decrease, which is beneficial to reduce tumor cell immunosuppression; and recruit more DCs and cytotoxic T lymphocytes, enhancing tumor cell antigen recognition, and in turn having a stronger antitumor effect [24, 27].

Cytokines also play an important role in the anti-tumor immune response. IL-12 can induce the production of IFN-γ, stimulate the proliferation and activation of CD8 + T lymphocytes, and play a role in promoting the anti-tumor immune response [28, 29]. As an immunosuppressive factor, IL-10 can not only inhibit the apoptosis of tumor cells but also inhibit the role of IFN-γ and the anti-tumor immune response [28, 30]. In our study, on days 7 and 14 after radiation, IL-12 and INF-γ levels in the SBHFRT group were obviously higher than those in the CFRT group, and IL-10 level was obviously lower than that in the CFRT group. On day 7, the rate of increase of IL-12 and INF-γ levels, and the rate of decrease on IL-10 level in the SBHFRT group were significantly higher than those on day 14. The reason for this may be because SBHFRT resulted in a high rate of tumor cell necrosis in a short term; released a large number of tumor-associated antigens; led to a higher accumulation of DCs to secrete higher levels of IL-12; activated cytotoxic T lymphocytes and CD4 + T cells; produced more IFN-γ, promoting cytotoxic T cell proliferation and activation; and played a role in the positive feedback to adjust the secretion of IL-12 by DCs, while reducing the secretion of IL-10, which is beneficial for the generation of IL-12 and IFN-γ [27, 29, 31].

Conclusion

SBHFRT could lead to a higher accumulation of DCs, anti-tumor lymphocytes, and cytokines in the tumor tissue, and reduce the aggregation of immunosuppressive lymphocytes and cytokines in the tumor tissue compared with CFRT; this difference was the most obvious on day 7 after radiation. Thus, hypo-fractionated radiotherapy combined with immunotherapy may be better for treating NSCLC, as observed on day 7 after radiation.

List Of Abbreviations

NSCLC: non-small cell lung cancer; CFRT: conventional fraction radiotherapy; SBRT: Stereotactic body radiotherapy; INF-γ: interferon-γ; DCs: dendritic cells; SBHFRT: single brachytherapy hypofractionated radiotherapy; IL-10: interleukin-10; micro-PET/CT: micro-positron emission tomography/computer tomography; F: fraction; GTV: gross tumor volume; BED: biologically equivalent dose; ELISA: enzyme-linked immunosorbent assay; d: diameter; V_{tumor}: tumor volume; TGIR: Tumor growth inhibition rate; SUV: standardized uptake value.

Declarations

Ethics approval and consent to participate

The Committee for Research Ethics and for Animal Care and Use in Research, Southwest Medical University, Luzhou (China) approved the present study. We handled animals in compliance with the revised Animals (Scientific Procedures) Act 1986.

Consent to publish

All authors agree to publish.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing Interests

The authors declare that they have no competing interests.

Funding

This work was supported by the Chongqing natural science foundation (NO: cstc2018jcyjA0794).

Authors Contributions

LL performed the study design, animal studies, statistical analysis and drafted the manuscript YHC and HYW performed the statistical analysis and the animal studies. LW performed software analysis and Visualization. XLG performed conceptualization, methodology and supervision.ZJW performed methodology, project administration, editing acquisition and funding acquisition.

Acknowledgements

We thank the Oncology Department, central laboratory of the affiliated hospital of Southwest Medical University, for providing assistance, and thank the Chongqing natural science foundation.

References

1. Deloch L, Derer A, Hartmann J, et al. Modern Radiotherapy Concepts and the Impact of Radiation on Immune Activation. Front Oncol. 2016,6:141.
2. Zhang H, Wan C, Huang J, et al.In Vitro Radiobiological Advantages of Hypofractionation Compared with Conventional Fractionation: Early-Passage NSCLC Cells are Less Aggressive after Hypofractionation. Radiat Res. 2018,190(6):584-595.
3. Timmerman RD, Paulus R, Pass HI, et al.Stereotactic Body Radiation Therapy for Operable Early-Stage Lung Cancer: Findings From the NRG Oncology RTOG 0618 Trial. JAMA Oncol. 2018,4(9):1263-1266.
4. Ouyang W, Yu J, Nuerjiang S, et al.Stereotactic body radiotherapy improves the survival of patients with oligometastatic non-small cell lung cancer. Cancer Med. 2019,8(10):4605-4614.
5. Thompson R, Cheung P, Chu W, et al.Outcomes of extra-cranial stereotactic body radiotherapy for metastatic colorectal cancer: Dose and site of metastases matter. Radiother Oncol.2020,142:236-245.
6. Li S, Dong D, Geng J, et al.Prognostic Factors and Optimal Response Interval for Stereotactic Body Radiotherapy in Patients With Lung Oligo metastases or Oligoprogression From Colorectal Cancer. Front Oncol. 2019,9:1080.

7. Mishal Mendiratta-Lala, William Masch, Prasad R. Shankar, et al. MR Imaging Evaluation of Hepatocellular Carcinoma Treated with Stereotactic Body Radiation Therapy (SBRT): Long Term Imaging Follow-Up. Int J Radiat Oncol Biol Phys. 2019,103(1): 169–179.
8. Mark E. Hwang, Mark Mayeda, et al.Stereotactic body radiotherapy with periprostatic hydrogel spacer for localized prostate cancer: toxicity profile and early oncologic outcomes. Radiat Oncol. 2019,14: 136.
9. Steven A, Fisher SA, Robinson BW. Immunotherapy for lung cancer. Respirology. 2016,21 (5):821 - 33.
10. Long GV, Atkinson V, Cebon JS, et al.Standard-dose pembrolizumab in combination with reduced-dose ipilimumab for patients with advanced melanoma (KEYNOTE-029): an open-label, phase 1b trial. Lancet Oncol. 2017,18(9): 1202 - 1210.
11. Giaj Levra M1, Cotté FE2, Corre R, et al.Immunotherapy rechallenge after nivolumab treatment in advanced non-small cell lung cancer in the real-world setting: A national data base analysis. Lung Cancer. 2020,140:99-106.
12. Crinò L, Bidoli P, Delmonte A, et al.Italian Cohort of Nivolumab Expanded Access Program in Squamous Non-Small Cell Lung Cancer: Results from a Real-World Population. Oncologist. 2019,24(11):e1165-e1171.
13. Banerjee K, Kumar S, Ross KA,et al.Emerging trends in the immunotherapy of pancreatic cancer. Cancer Lett. 2018,417:35-46.
14. Arnold KM, Flynn NJ, Raben A, et al. The Impact of Radiation on the Tumor Microenvironment: Effect of Dose and Fractionation Schedules. Cancer Growth Metastasis. 2018,11: 1179064418761639.
15. Frey B, Rückert M, Weber J,et al.Hypofractionated Irradiation Has Immune Stimulatory Potential and Induces a Timely Restricted Infiltration of Immune Cells in Colon Cancer Tumors. Front Immunol. 2017,8: 231.
16. Zhang T, Yu H, Ni C,et al.Hypofractionated stereotactic radiation therapy activates the peripheral immune response in operable stage I non-small-cell lung cancer. Sci Rep. 2017,7(1):4866.
17. Xiang L, Zhang JW, Lin S, et al. Computed Tomography-Guided Interstitial High-Dose-Rate Brachytherapy in Combination With Regional Positive Lymph Node Intensity-Modulated Radiation Therapy in Locally Advanced Peripheral Non-Small Cell Lung Cancer: A Phase 1 Clinical Trial. Int J Radiat Oncol Biol Phys. 2015,92(5): 1027-1034.
18. Mathieu Spaas, Yolande Lievens.Is the Combination of Immunotherapy and Radiotherapy in Non-small Cell Lung Cancer a Feasible and Effective Approach? Front Med (Lausanne) . 2019,6: 244.
19. Vanpouille-Box C, Formenti SC, Demaria S. Toward Precision Radiotherapy for Use with Immune Checkpoint Blockers. Clin Cancer Res. 2018, 24(2):259-265.
20. Dong P, Ma L, Liu L, et al.CD86/CD206, Diametrically Polarized Tumor-Associated Macrophages, Predict Hepatocellular Carcinoma Patient Prognosis. Int J Mol Sci. 2016,17(3):320.
21. GuPta A, Probst H C,Vuong V, et al. Radiotherapy Promotes Tumor-SPecific Effector CD8+ T Cells via Dendritic Cell Activation. J Immunol. 2012,189(2):558-566.
22. Koff JL, Ramachandiran S, Bernal-Mizrachi L. A time to kill: targeting apoptosis in cancer. Int J Mol Sci. 2015,16(2):2942-55.
23. Sakai C, Nishikawa H. Immunosuppressive Environment in Tumors. Gan To Kagaku Ryoho. 2018,45(2): 222-226.
24. Donatella Aldinucci, Cinzia Borghese, Naike Casagrande. Formation of the Immunosuppressive Microenvironment of Classic Hodgkin Lymphoma and Therapeutic Approaches to Counter It. Int J Mol Sci. 2019,20(10): 2416.
25. Yamei Zhao, Xiaoxu Ge, Jiawei He, et al.The prognostic value of tumor-infiltrating lymphocytes in colorectal cancer differs by anatomical subsite: a systematic review and meta-analysis. World J Surg Oncol. 2019,17: 85.
26. RaPhael I, Nalawade S, Eagar TN, et al T cell subsets and their signature cytokines in autoimmune and inflammatory diseases Cytokine. 2015,74(1): 5-17.
27. Aleksandr Barinov, Alessia Galgano, Gerald Krenn, et al. CD4/CD8/Dendritic Cell Complexes in the Spleen: CD8+ T Cells Can Directly Bind CD4+ T Cells and Modulate Their Response. PLoS One. 2017,12 (7): e0180644.
28. Galdiero MR, Marone G, Mantovani A. Cancer Inflammation and Cytokines. Cold Spring Harb Perspect Biol. 2018,10(8). pii: a028662.
29. Wu SY, Chen CL, Tseng PC, et al.Fractionated ionizing radiation facilitates interferon-γ signaling and anticancer activity in lungadenocarcinoma cells. J Cell Physiol. 2019 Feb 14. [Epub ahead of print]
30. Vahl JM, Friedrich J, Mittler S, et al. Interleukin-10-regulated tumour tolerance in non-small cell lung cancer. Br J Cancer. 2017,117(11):1644-1655.
31. Lu X.Impact of IL-12 in Cancer. Curr Cancer Drug Targets. 2017, 17(8): 682-697.

Figures

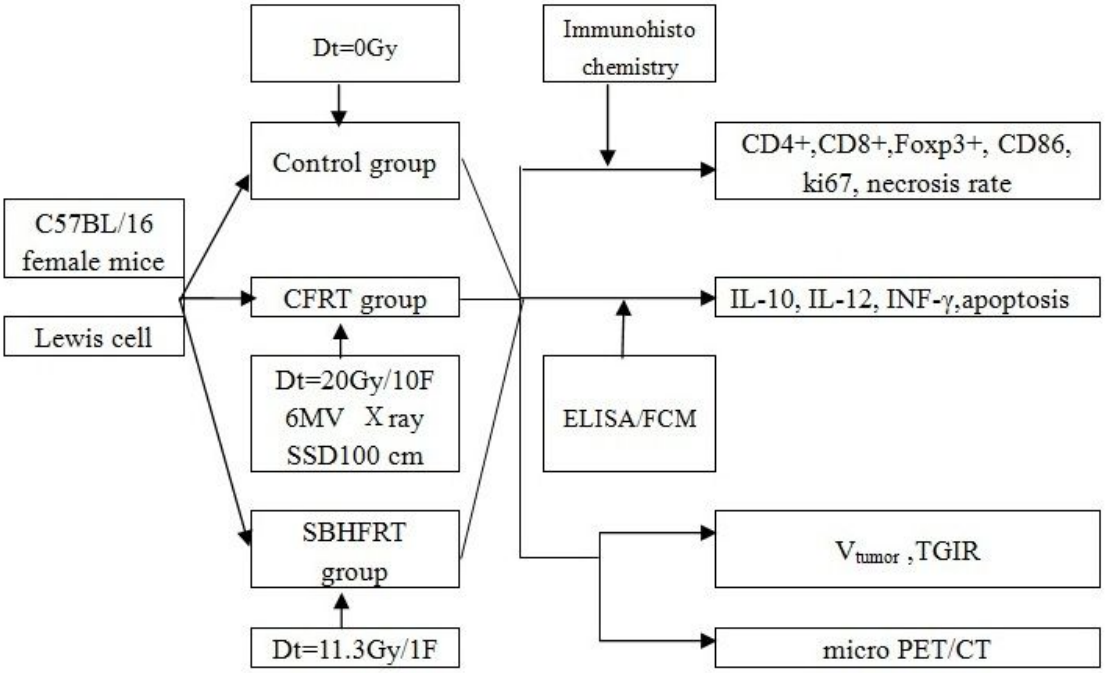

Figure 1

Research and design

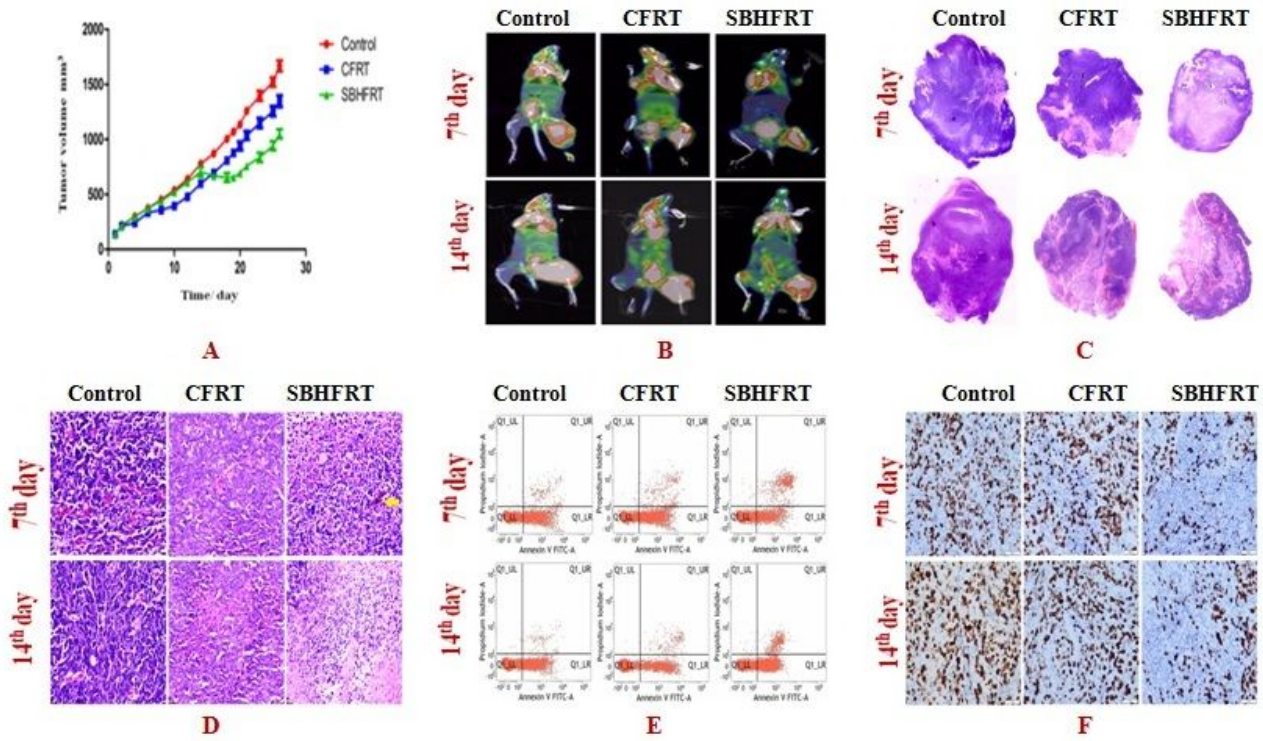

Figure 2

Tumor growth curve (A), 18 F-FDG micro-PET/CT for evaluating tumor metabolism (B), tumor tissue necrosis [(C): KFBIO, SlideViewer, (D):H&E staining], apoptosis (E), and ki-67 positive expression (F).

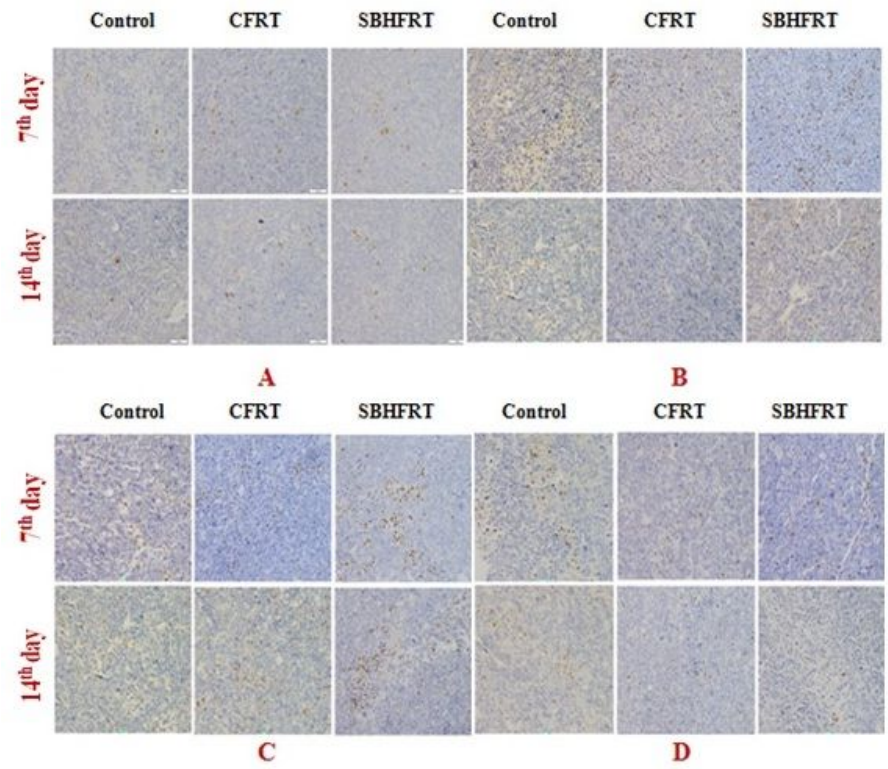

Figure 3

Expressions of CD86+ (A), CD4+ (B), CD8+ (C), Foxp3+ T lymphocytes (D) as shown by yellow particles (×400).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- checklist.doc