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Original Article

Radiotherapy suppressed tumor-specific recruitment of regulator T cells via up-regulating microR-545 in Lewis lung carcinoma cells

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Abstract: Objective: Radiotherapy is an important treatment for cancer. The main irradiated action is thought to be the irreversible damage to tumor cell DNA, but recent studies showed that high dose radiotherapy related to the tumor immune response. This study was designed to determine the relationship between Lewis lung tumor radiosensitivity and CD4+CD25+ regulatory T cells (Tregs) infiltration and elucidate the underlying mechanisms in vitro. Methods: With tumor transplantation method to establish mice Lewis lung tumor mice model, to observe the inhibition rate of radiotherapy to tumor growth. Proliferation profiles of CD4+CD25+ Tregs and CD4+ T cells were assessed by flow cytometry. MiR-545 and CCL-22 mRNA were determined by Quantitative Real-Time PCR. CCL-22 protein was determined by western blot assay. Results: Radiotherapy caused a time-dependent inhibition of tumor growth as well as a decrease in the percentage of tumor-infiltrating CD4+CD25+ Tregs of CD4+ T cells compared with no treatment group. And the miR-545 was significantly upregulated and CCL-22 was significantly down-regulated in irradiated tumor and Lewis lung cancer cells. In Lewis lung cancer cell transfection experiments, mimic or inhibitor for miR-545 negatively regulated CCL-22 expression when cells treated or treated without irradiation. Silenced miR-545 promotes CD4+CD25+ Treg proliferation. Additionally, silenced miR-545 reversed radiosensitivity of Lewis lung cancer. Conclusion: Radiotherapy suppressed specific recruitment of regulator CD4+CD25+ Treg cells in Lewis lung carcinoma via up-regulating microR-545.

Keywords: CCL-22, CD4+CD25+ T regulator cells, Lewis lung cancer cells, radiotherapy, miR-545, tumor xenograft

Introduction

Lung cancer is still to be one of the most prevalent malignancies worldwide and in China, more people die from lung cancer than from any other kind of cancer. Therefore, the focus of present study is to explore effective method to improve therapeutic efficacy for lung cancer patients. For conveniently be located, approximately 70% of patients with lung cancer receive external beam radiation treatment as one component of their treatment [1]. Based on this situation, detailed description on mechanism of radiation therapy is extremely essential.

Traditionally, radiation therapy has been viewed as having tumor-suppressive effect due to the result of direct damage to radiosensitive tumor cell DNA, but there is evidence that irradiation mobilizes tumor-specific immunity [2, 3]. It was confirmed by tumor-associated antigens (TAAs) that are targeted by T cells are readily detected in blood, tumors and draining lymph nodes of individuals with cancer [4]. Theoretically, radiotherapy can enhance anti-tumor immunity via increasing the expression of TAAs, inducing immune-mediated targeting of the tumor stroma, and diminishing regulatory T cell (Treg) activity [5]. Tregs, acted as immune suppression for body tissues, have been demonstrated to played an essential role in self-tolerance, transplantation, allergy and tumor/microbial immunity [6]. Indeed, accumulating evidence implicates Tregs as one of the principal cell types suppressing TAA-specific lymphocyte activity and tumor eradication, and thus one of the major obstacles to effective anti-tumor immunotherapy [7-10]. CD25+CD4+ regulatory T cells (Treg), comprised 5-10% of the circulating CD4+ T cell population, suppress tumor immune responses [11]. Indirect evidence suggests that CD4+CD25+ T cells (Tregs) are

important in suppressing TAA-specific immunity [12, 13], and they suppress nonspecific T cell responses in vitro. Thus, it is clear that CD4+CD25+ Treg may be as the obstacle for radiotherapy [14]. However, direct evidence supporting a role for Treg cells in the radiation therapy for lung cancers and the underlying mechanism are lacking.

It is critical that the mechanism that contributes to Treg accumulation in tumors is not fully understood. It has been suggested that Tregs display an enhanced capacity for infiltration of, and accumulation within, the tumor in comparison to effector T cells [7]. In support of this, preferential recruitment has been observed in ovarian carcinoma [4] and human prostate carcinoma cells [15]. It is well known that T cells recruitment is reliant on chemokine-driven mechanisms, and chemokine (C-C motif) ligand 22 (CCL22) has been shown to be produced by both tumor cells and tumor-infiltrating macrophages [16]. Although blockade of CCL22 reduced Treg infiltration into tumors has been demonstrated in a murine xenograft model, there is no evidence focus on its role in lung cancer radiotherapy.

In this study, we established a radiation model of Lewis lung carcinoma in C57BL/6 mice and aimed to identify tumor infiltration of CD4+ CD25+ Treg in irradiating tumor. We also directly assessed whether CCL-22 was involved in this process and aimed to identify the underlying mechanism.

Material and methods

Mice, cell lines and reagents

Animal experiments were conducted in 8-12-week-old female C57BL/6 mice that obtained from the Institute of Laboratory Animal Science (Chinese Academy of Sciences, Shanghai) and were housed under specific pathogen-free conditions.

Lewis lung carcinoma cell line was purchased from ATCC (USA) and cultured in RPMI 1640 medium containing 10% fetal bovine serum. After grew against the wall of flask, formed a monolayer, the cells were digested by 0.25% trypsin to prepare cell suspensions at 1×10^7/ml. T Cells were obtained from blood and tumors as described [17-19]. CD4+ T cells were purified with Untouched kits (Miltenyi). CD4+ CD25+ T cells were purified with paramagnetic beads (Miltenyi) and sorted with phycoerythrin (PE)-conjugated antibody to CD25 (PharMingen). Cell populations were assessed to be more than 90% pure by flow cytometry as below.

RPMI 1640 medium and fetal bovine serum were purchased from Gibco (Invitrogen Company, USA). Lipofectamine 2000 transfection reagent was obtained from Invitrogen Life Technologies (Grand Island, NY, USA). Anti-CCL-22 antibody was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA).

Tumor xenograft and irradiation therapy

Tumors were generated by subcutaneous injection into the abdomen of C57BL/6 mice with 1×10^7 cells/ml of Lewis lung carcinoma cells with or without transfection with miR-545 inhibitor that suspended in 100 μL PBS and mixed with 20% matrigel. Seven days after the cell inoculated, tumors were irradiated with a single fraction of 12 Gy/1f/1 d for 16 days. The tumor volume was regularly measured using calipers as *length* × *width* × *depth* per two days. After inoculation, animals were euthanized and tumors were removed for T cells count, Quantitative Real-Time PCR or protein expression assay.

Lewis lung carcinoma cells treated with or without miR-545 inhibitor were exposed to radiation single in a fraction of 5 Gy. After 24 hours irradiation, the cells used for analysis on mRNA or CCL-22 protein expression.

Flow cytometry

Flow cytometric analysis CD4+CD25+ proliferation was performed using primary labeled antibodies matched with the appropriate isotype controls and the experiment was conducted 48 hours after co-cultured Lewis lung cancer cells with T cells. The cells were first washed with phosphate-buffered saline and stained with anti-CD4 antibody (BD Bioscience, San Jose, CA, USA). The antibody-bound splenocytes were then washed and resuspended in fluorescence-activated cell-sorting (FACS) buffer. Finally, the antibody-bound cells were analyzed by FACS. To analyze the CD4+CD25+ Treg cell population, cells were first stained with anti-CD4 and CD25 antibodies, then fixed and permeabilized before they were stained with PE-Cy5-labeled anti-FoxP3 antibody (eBiosci-

ence Co.). After washing, cells were detected by FACS Calibor and analyzed with Cellquest software (BD Bioscience).

Cell migration assay

After 24 hours stimulated by medium supernatant, CD4+CD25+ Treg cells were washed with phosphate-buffered saline and resuspended in RPMI 1640 medium. 0.1 mL cells (1×10^4)/mL containing 0.15% BSA was placed in the upper chamber of the Transwell culture system (Falcon, Franklin Lakes, NJ). And DMEM (0.6 mL) containing 0.5% FCS or 100 ng/mL chemokine (interleukin-8, lymphotactin, or monocyte chemoattractant protein-2) was added to the lower chamber. Background (control) levels of migration were determined by placing DMEM (0.6 mL) containing 0.5% BSA in the lower chamber. The Transwell culture system was incubated at 37°C with 5% CO_2 for 6 h. Cells on the upper membrane surface were gently removed with a cotton bud. The microporous membrane was fixed in methanol for 20 min at room temperature and stained using 5% crystal violet for 15 min. The cells on the downside surface of the membrane were counted. The experiment was repeated at least three times, each in triplicate.

Cell treatment with miRNA inhibitor or mimics

Lewis lung carcinoma cells were treated with miR-545 mimic or miR-545 inhibitor (Ambion Pre-miR miRNA Precursors, Life Technologies) using Oligofectamine (Life Technologies) according to the manufacturer's instructions. miRNA mimics negative control (mimic-NC) and miRNA inhibitor negative control (inhibitor-NC) was severed as negative controls in the experiments respectively. Further analysis of the samples (infection or RNA isolation) was performed at 24 h post-transfection unless specific indication. While the Lewis lung cancer cells were irradiated for 6 h before transfection. The sequence for this experiment as follows: Hsa-miRNA-545 mimic: Sense strand: 5'-UCAGCAAACAUUUAUUGUGUGC-3'; Anti sense strand: 5'-GCACACAATAAATGTTTGCTGA-3'. Hsa-miR-545 inhibitor: 5'-mGmCmAmCmAmCmAmAmUmAmAmAmUmGmUmUmGmCmUmGmA-3'.

MTT assay

After locally irradiation, Lewis lung carcinoma tumors were isolated and were made into cell suspension. The cells were seeded at a density of 1400 cells/well. On the next day, the wells were added with 50 mL of 5 mg/mL 3-(4,5-Dimethylthiazol-2-yl) 22,5-diphenyltetrazolium bromide (MTT) and incubated for 4 h with 5% CO_2, and then the reaction was stopped by media replaced with DMSO. Optical density was analyzed by using an mQuant Microplate Spectrophotometer (BioTek, UK) at a wavelength of 540 nm.

Quantitative real-time PCR

Total RNA was extracted from the Lewis lung carcinoma cells with the Trizol Reagent (Invitrogen, Carlsbad, CA, USA). The mature miR-545 and CCL-22 mRNA were quantified by using Quantitative Real-Time PCR (Q-RT-PCR) assays with fluorescent nucleic acid dye. Each sample (1 μg) was reverse-transcribed into cDNA by using the RealMasterMix First Strand cDNA Synthesis Kit (Tiangen). Real-time PCR was conducted by using SYBR Premix ExTagTM (Takara) according to the manufacturer's protocols and performed in the Applied Biosystems 7500 Real-time PCR system. The threshold cycle (CT) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. The miRNA expression levels were normalized to U6 RNA and the CCL-22 mRNA levels were normalized to actin mRNA. All reactions were run in triplicate.

Western blot

Proteins in Cells were extracted by inM-PER mammalian protein extraction reagent (Pierce Biotechnology) followed by centrifugation at 15 000 g for 10 min. Protein concentration of cell lysates was measured by using DC protein assay kit (Bio-Rad). Proteins (10-20 mg) were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to PVDF membrane from Bio-Rad (Hercules, CA, USA). Two hours after blocking in 5% nonfat milk, the protein blots were then incubated with primary antibodies (CCL-22, 1:200; β-actin, 1:1000) in 3% bovine serum albumin at 4°C overnight, followed by incubation with secondary antibodies (1:1000) at room temperature for 2 h. The protein signals were detected by ECL method.

Statistical analysis

All statistical analyses were performed using SPSS 16.0 software (Chicago, IL). Student's *t*

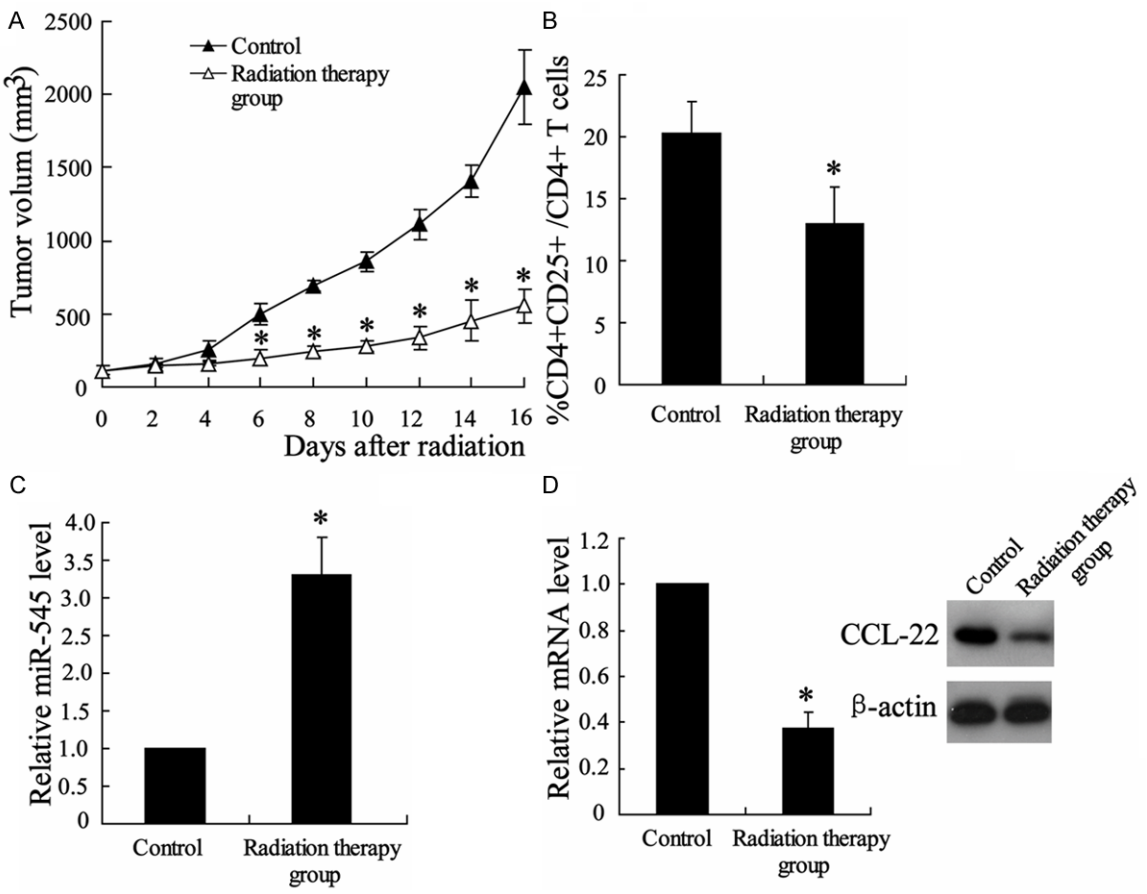

Figure 1. Radiotherapy in mice bearing Lewis lung carcinoma tumor. Seven days after C57BL/6 mice injected with Lewis lung cancer cell lines, transplanted tumors were irradiated with a single dose of 12 Gy/1f/1 d for 16 days, whereas control mice received no radiotherapy. A. Tumor growth in the period of irradiation therapy. Sixteen days after irradiation therapy, mice were euthanized, and tumor infiltrate was moved to analyzed; B. Percentage of CD4+CD25+/CD4+T cells by using flow cytometry, C. miR-545 level by using Quantitative Real-Time PCR, D. mRNA and protein expression level of CCL-22 by using Quantitative Real-Time PCR and western blot, respectively. Experimental groups consisted of six mice. The data are represented as mean ± SD. $^{*}P < 0.05$ compared with corresponding control.

test was used to analyze the significance between two groups. One-way Analysis of Variance (ANOVA) was used to test the significance of the tumor growth. Error bars represent SD. *P*-values < 0.05 were considered statistically significant. Data are representative of four independent experiments.

Results

Local high-dose radiotherapy suppressed tumor growth in mice bearing Lewis lung carcinoma tumor and reduced CD4+CD25+ Treg infiltration

C57BL/6 mice bearing Lewis lung carcinoma tumors were locally irradiated with a single dose of 12 Gy/1f/1 d for 16 days, which resulted in a continuously significant reduction in tumor growth (**Figure 1A**). Upon flow cytometric analysis of digested tumors 7 days after radiation, we found a striking decrease in the percentage of tumor-infiltrating CD4+CD25+ T cells of CD4+ T cells (**Figure 1B**). To identify the possible mechanism underlying radiotherapy, we determined signaling molecular expression in transplanted tumor. As shown in **Figure 1C** and **1D**, compared to tumor without irradiation treatment, radiotherapy promoted miR-545 expression and sharply decreased CCL-22 expression in levels of both mRNA and protein.

miR-545 is crucial for CCL-22 expression in Lewis lung cancer cells

To examine whether exist the possible regulation effect of miR-545 on CCL-22 expression in

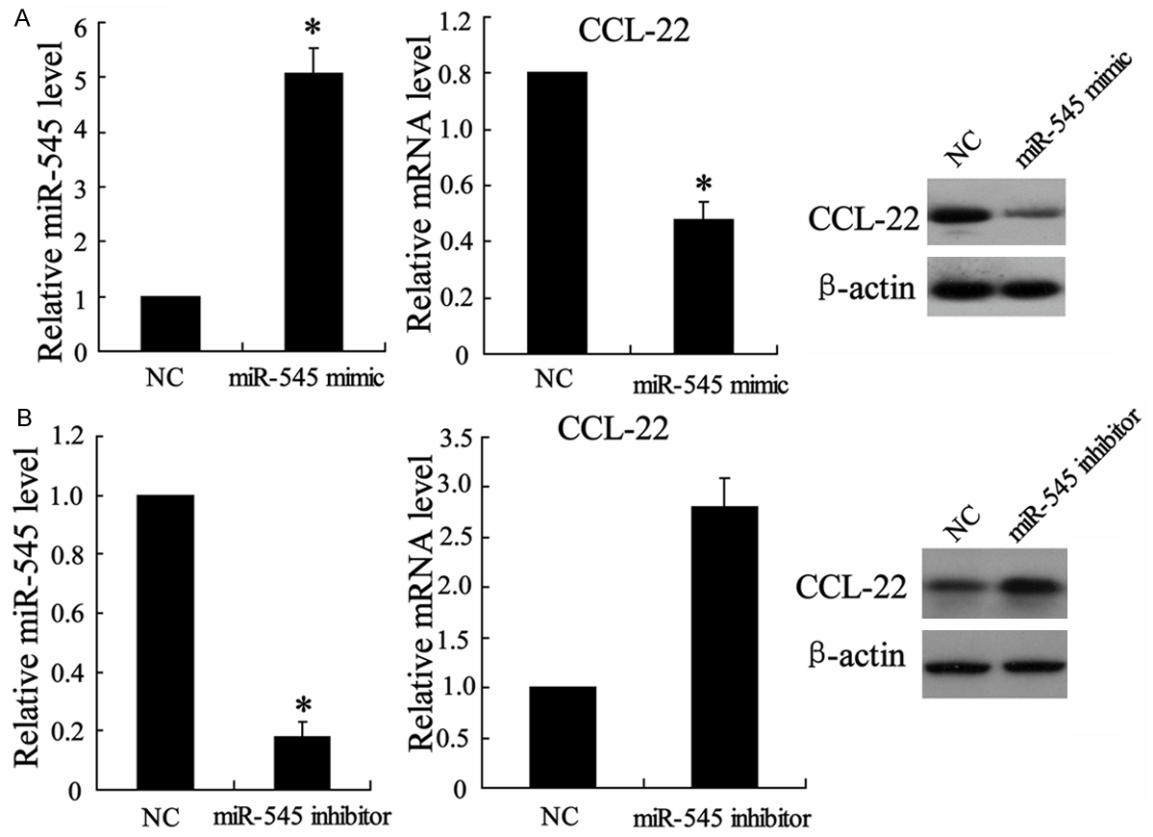

Figure 2. Negative regulation of CCL-22 expression by miR-545 in Lewis lung cancer cells. A. Forty-eight hours after Lewis lung cancer cells transfected with miR-545 mimic or negative control, the cells were lyzed to determine levels of miR-545 and CCL-22 mRNA expression by using Quantitative Real-Time PCR and CCL-22 protein expression by western blot. B. Lewis lung cancer cells were transfected with miR-545 inhibitor and expression of miR-545 and CCL-22 was determined 48 hours post treatment. The data are represented as mean ± SD. *$P < 0.05$ compared with negative control.

Lewis lung cancer cells, we treated cells with mimic or inhibitor for miR-545 and then evaluated mRNA and protein level of CCL-22 in vitro. miR-545 mimic transfection largely enhanced miR-545 expression, whereas the over-expressed miR-545 had a potential inhibitor effect on CCL-22 expression in both mRNA and protein level (**Figure 2A**). To further ascertain the negative regulating role of miR-545, the miR-545 was silenced by miR-545 inhibitor treatment and CCL-22 expression was determined. As shown in **Figure 2B**, both miRNA and protein level of CCL-22 expression was upregulated when miR-545 was silenced.

Involvement of up-regulated CCL-22 by miR-545 inhibition in Lewis lung cancer cell radiotherapy

In vivo experiment has showed that miR-545 and CCL-22 are involved in Lewis lung cancer tumor radiotherapy. We next irradiated Lewis lung cancer cells with fraction of 5 Gy/1f/1 d in vitro. Consistent with result from irradiated tumor, we identified an up-regulation of miR-545 expression (**Figure 3A**) and decreases in levels of CCL-22 mRNA and protein (**Figure 3B**). Notably, the above results indicating the negative regulation of CCL-22 expression by miR-545. To testify the process is involved in Lewis lung cancer cell radiotherapy, cells were treated with miR-545 inhibitor before exposed to irradiation. The result showed that silenced miR-545 abrogated radiotherapy-inhibited down-regulated CCL-22 expression in levels of mRNA and protein (**Figure 3C**).

Silenced miR-545 promoted migration and infiltration of CD4+CD25+ Treg

CD4+CD25+ Treg is essential for caner cells survival via suppressing immune responses. As described above, we identified a reduction in tumor infiltration of CD4+CD25+ Treg in trans-

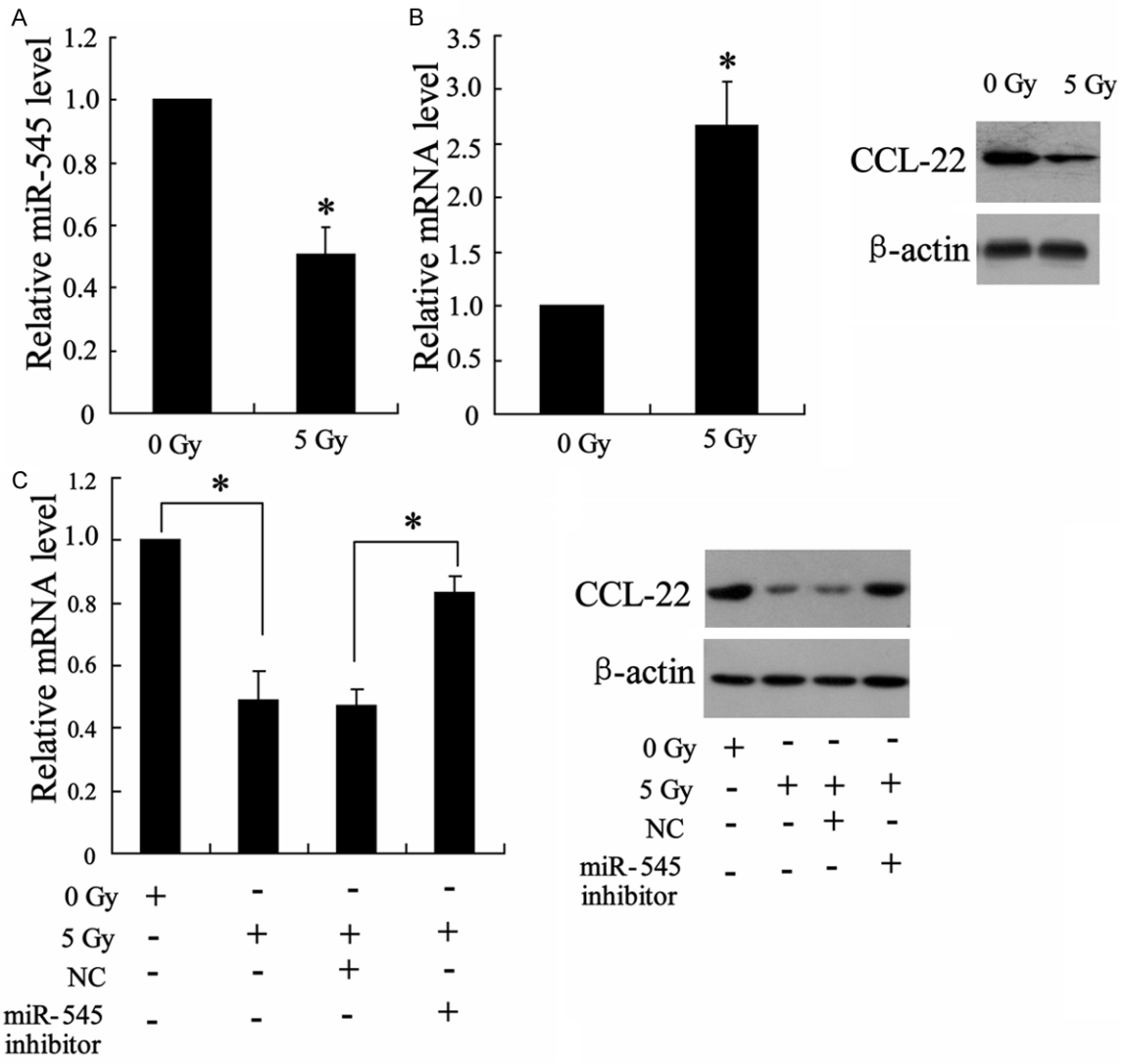

Figure 3. Radiation treatment upregalated CCL-22 expression through inactivating miR-545 in Lewis lung cancer cells. Cells were irradiated with a signal dose of 5 Gy/1f/1 d, whereas the control cells did not expose to radiation. A. miR-545 level was determined by using Quantitative Real-Time PCR. B. Levels of CCL-22 mRNA and protein were assessed by using Quantitative Real-Time PCR and western blot, respectively. The cells were incubated with miR-545 inhibitor for 48 hours before single dose of 5 Gy/1f/1 d radiotherapy. C. Levels of CCL-22 mRNA and protein were assessed as described above. The data are represented as mean ± SD. $^{*}P < 0.05$ compared with corresponding control.

planted tumor, suggesting the possible therapeutic target for radiotherapy. It has been reported that the infiltration of Treg relied on chemokine-driven mechanisms. To evaluate whether induced CCL-22-driven infiltration, we next obtained culture supernatant from cultured Lewis lung cancer cells that has been transfected with miR-545 inhibitor and testified its impact on migration of CD4+CD25+ Treg. As shown in **Figure 4A**, silenced miR-545 promoted CD4+CD25+ Treg migration, whereas the migration was abolished by cells treated with CCL-22 antibody. To further determine the impact of CCL-22 on CD4+CD25+ Treg proliferation, rats T cells were co-incubated with Lewis lung cancer cells transfected with miR-545 inhibitor and proliferation of the CD4+CD25+ Treg was evaluated by flow cytometry. The result showed that percentage of CD4+CD25+ Treg was significantly increased, which can attenuated by CCL-22 antibody treatment (**Figure 4B**).

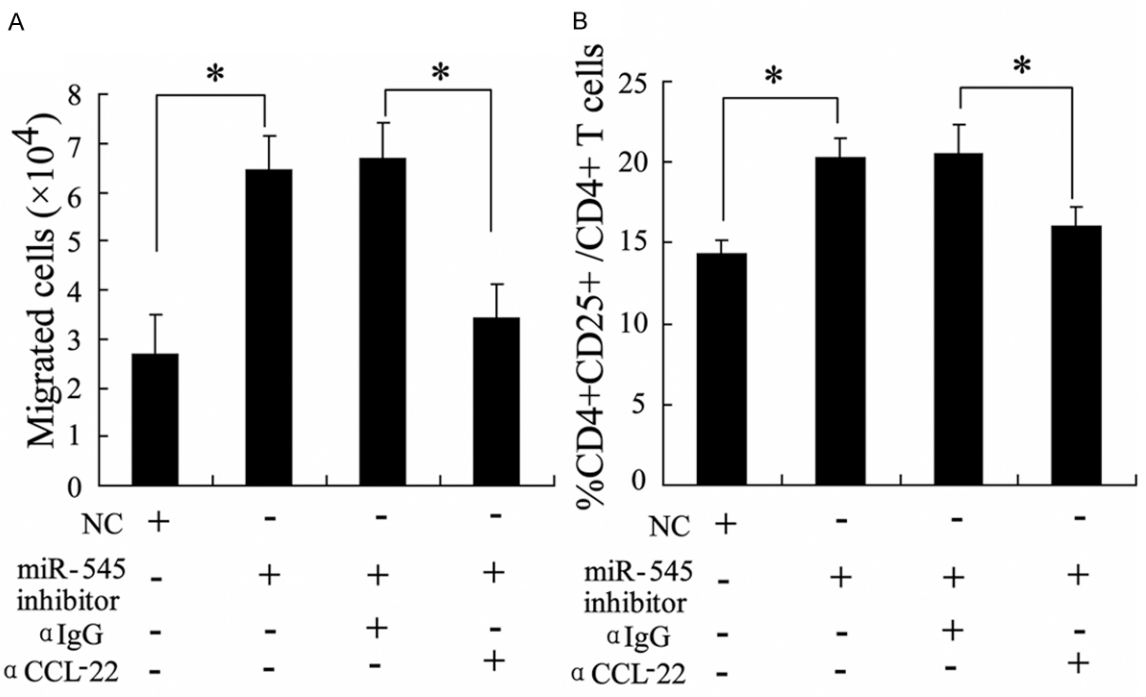

Figure 4. Silenced miR-545 promote CD4+CD25+ Treg proliferation. A. Forty-eight hours after miR-545 transfection, supernatant of cultured Lewis lung cancer cells was separated and was used to culture CD4+CD25+ Treg cells. After 24 hours incubation, followed with 4 hours cell migration experiment. B. CD4+ Treg cells were co-cultured with Lewis lung cancer cells which has been incubated with miR-545 inhibitor for 24 hours. After 48 hours culture, CD4+CD25+ Treg cells analyzed by flow cytometry. The data are represented as mean ± SD. $^{*}P < 0.05$ compared with corresponding control.

Silenced miR-545 attenuated radiosensitivity of Lewis lung cancer

The above-mentioned data suggest that irradiation negatively related with tumor specific effector CD4+CD25+ Terg, which mediated by miR-545 up-regulation. To address the question whether the presence of miR-545 is sufficient to control the tumor or CD4+CD25+ Terg proliferation, we treated Lewis lung cancer cells with miR-545 inhibitor before tumor transplantation. C57BL/6 mice bearing Lewis lung carcinoma tumors were locally irradiated with a single dose of 12 Gy/1f/1 d for 16 days. The result showed that compared with its negative control, miR-545 inhibitor treatment leaded to Lewis lung cancer tumor radioactive insensitivity that caused an increase in tumor growth under irradiation treatment (**Figure 5A**). We next identified high percentage of infiltrated CD4+CD25+ Terg of CD4+ effector T cells in transplanted tumor when miR-545 was silenced (**Figure 5B**). Whereas, miR-545 inhibitor has no effect on growth of CD4+CD25+ Terg (**Figure 5C**).

Discussion

In this study, Lewis Lung Carcinoma bearing mice model was established and locally irradiated with high dose of X-Irradiation. The data showed that radiation effectively suppressed tumor growth. Notably, the transplanted Lewis lung tumor contained a decreased percentage of infiltrated CD4+CD25+ T regulator cells/CD4+ effector T cells, suggesting that radiotherapy attenuated tumor immune suppression. We next confirmed that reduction in tumor CD4+CD25+ Treg infiltration was mediated by down-regulated CCL-22. Moreover, our data also showed that miR-545 was the key regulator for CCL-22 expression in both vivo and vitro. Thus, these results suggested that radiotherapy suppressed tumor growth via up-regulating miR-545 expression, which effectively inhibited CCL-22 recruiting CD4+CD25+ Treg.

It is well accepted that miRNAs were the key regulatorforvariouscellularfunctions[20]2541q. Accumulated evidence pointed out that miRNAs are also involved in many signaling path-

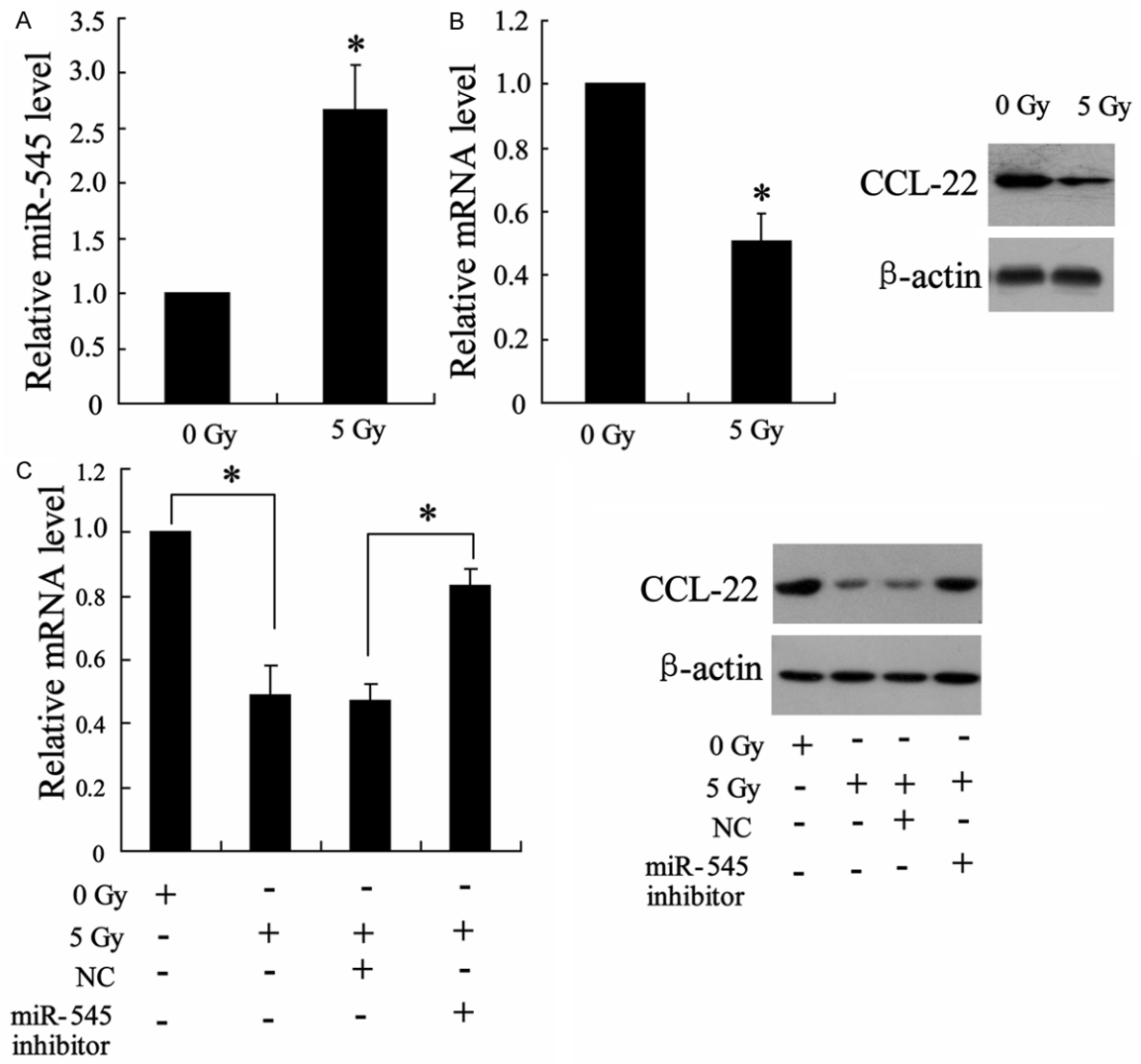

Figure 5. Silenced miR-545 reversed radiosensitivity of Lewis lung cancer. C57BL/6 mice were injected with Lewis lung cancer cells which has been transfected with miR-545 inhibitor. Seven days after transplantation, tumors were irradiated with a single dose of 12 Gy/1f/1 d for 16 days. A. Tumor growth was measured every two days. Sixteen days after irradiation therapy, mice were euthanized, and tumor infiltrate was moved to analyze. B. percentage of CD4+CD25+/CD4+T cells by using flow cytometry. C. Cells were transfected with miR-545 inhibitor and its viability was determined by using MTT assay. The data are represented as mean ± SD. $^{*}P < 0.05$ compared with corresponding control.

ways and DNA damage repair processes, which is essential for cancer radiotherapy [21]. Previously, miR-545 has been reported to suppress cell proliferation by targeting cyclin D1 and CDK4 in lung cancer cells [22]. Thus, we hypothesized that miR-545 may be involved in the process of radiotherapy in lung cancer, which was supported by up-regulation of miR-545 during irradiation of xenograft tumor and Lewis lung cancer cells. Based to its inhibition effect on proliferation of lung cancer cell [22], overexpression of miR-545 was assumed to exert resistance impact on tumor growth during radiotherapy, which was supported by tumor growth inhibition in vivo and Lewis cell apoptosis in vitro in our research.

Strategies for tumor eradication have focused mainly on either enhancing tumor immunogenicity or promoting anti-tumor effector responses, although both have been largely unsuccessful [23]. This is thought be due primarily to the highly immunosuppressive environment found within the tumor. Regulatory T cells (Tregs) are

crucial in mediating immune homeostasis and promoting the establishment and maintenance of peripheral tolerance. In this study, high dose of local 12 Gy/1f/1 d irradiation largely suppressed mice xenograft tumor growth as well as blocked CD4+CD25+ Treg infiltration. As cancer cells express both self- and tumor-associated antigens, Tregs are key to dampening effector cell responses, and therefore represent one of the main obstacles to effective antitumor responses. Thus, fully elaborate relationship between immune suppression and radiotherapy is conducive to cancer responds to treatment.

It is well known that chemokine-driven mechanisms are essential for CD4+CD25+ Treg infiltration. Among these, CCL-22 has been shown to be produced by both tumor cells and tumor-infiltrating macrophages. Although, previously, blockade of CCL22 has been demonstrated to reduce Treg infiltration into ovarian tumors and induce tumor rejection in a murine xenograft model [16], few research focus on its role in radiotherapy for lung cancer. The data in our study showed that radiation therapy induced reduction of CCL-22 expression in both transplanted tumor and Lewis lung cancer cells. Notably, this process was mediated by up-regulated miR-545. Given to this, we culture miR-545-silenced cancer cells and applied culture supernatant to stimulate CD4+CD25+ Treg. The results showed facilitation of CD4+CD25+ Treg migration and proliferation. Inhibitor for miR-545 treatment reduced radio-sensitivity of mice xenograft tumor. These data indicated the key role of CCL-22 in lung cancer radiotherapy.

In conclusion, we established Lewis Lung Carcinoma bearing mice in C57BL/6 mice. The findings suggest that CD4+CD25+ Treg plays an important role in the mechanism underlying the radioresistance of Lewis Lung Carcinoma in vivo and in vitro. Further studies to elucidate the molecular mechanism underlying the radioresistance of the Lewis Lung Carcinoma cell lines indicated that over-expressed miR-545 inhibited CCL-22 activity. These founding will contribute to a better understanding of not only the molecular mechanisms of radiotherapy in lung cells, but also the development of combination between new gene therapies and radiotherapies for types of malignancies.

Disclosure of conflict of interest

None.

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