Original Research Article

Synergistic effect of radiotherapy and dendritic-cell-based immunotherapy via miR-15b-5p regulation in a colon carcinoma mouse model

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Abstract

Purpose: *To determine the combined effect of radiotherapy (RT) and dendritic cell (DC)-based immunotherapy on tumor regression, and the influence of miR-15b-5p on the sensitivity of the tumor to RT/DC.*
Methods: *Colon carcinoma cell line (T-26) was injected subcutaneously into BALB/c mice to generate xenograft tumors. The animals were exposed to radiation therapy (RT) only or DC only, or RT plus DC (RT/DC). Tumor progression was measured and analyzed followed by quantification of miR-15b-5p using qPCR in each group 21 days after tumor inoculation. Overexpressed xenograft tumors were generated by transfecting MiR-15b-5p mimic to CT-26 cell lines and subcutaneously administered to mice. The mice were then subjected to RT/DC treatment and assessed for tumor growth, TUNEL staining, as well as mRNA and protein expressions of B-cell lymphoma-2 (BCL-2), caspase-3 (casp-3), and X-linked inhibitor of apoptosis protein (XIAP).*
Results: *RT/DC combination treatment resulted in tumor regression and increased miR-15b-5p expression, when compared with other groups treated with RT and DC alone (p < 0.001). There was a marked reduction in tumor growth following RT/DC combination therapy in miR-15b-5p overexpressed xenograft tumor (p < 0.001). Moreover, miR-15b-5p/(RT/DC) treatment significantly increased cancer cell apoptosis. These results were substantiated by increased mRNA and protein expressions of casp-3, reduced BCL-2 and XIAP (p < 0.001).*
Conclusion: *These findings demonstrate that RT/DC combination therapy increases miR-15b-5p levels in mice, and that miR-15b-5p upregulation enhances the sensitivity of tumors to RT/DC combination therapy. Therefore, the combination of RT and dendritic cell-based immunotherapy offers significant therapeutic advantage in controlling cancers, including metastatic tumors.*

Keywords: *Radiotherapy, Dendritic cells, Colon carcinoma, Apoptosis, MiR-15b-5p*

INTRODUCTION

Radiotherapy (RT) is one of the mainstream therapeutic options for cancer. Tumor regression by RT is most often associated with disruption of mitotic processes in cancer cells, thereby causing DNA damage [1-3]. Treatment with RT is not directly linked with early cell death/apoptosis, and it has evolved into a promising immunological adjuvant [4-6]. On the other hand,

dendritic cells (DCs) are potent immune system regulators. Indeed, dendritic cell-based vaccines have strong implications for the treatment of cancer [7,8]. Therefore, the combination of RT and dendritic cell-based immunotherapy may offer great advantage in controlling cancers, including metastatic tumors [9-11]. The molecular mechanism of this combination therapy on tumor regression, and strategies to enhance the immunogenicity of DCs still need extensive investigation.

MicroRNAs (miRNAs) are critical regulators of gene expression which influence cellular growth and death [12,13]. It has been postulated that miR-15b-5p acts as a tumor suppressor that negatively regulates post-transcriptional expression of multiple oncogenes [14]. In addition, miR-15b has been studied for its effect on promoting cancer cell apoptosis and inhibiting cellular proliferation, angiogenesis, and metastasis of tumors [14]. Studies have shown that overexpression of miR-15b decreased BCL-2 expression and enhanced the activities of the chief apoptosis mediators, caspase-3 and caspase-7 [15]. Moreover, it has been reported that miR-15b upregulation arrested cell cycle at G0/G1 phase, whereas its downregulation promoted S-phase cell proliferation [16]. A study has shown that irradiation-based DNA damage upregulated miR-15b, thereby modulating cellular stress [17]. Moreover, miRNAs are effective tools for characterizing DC maturation and differentiation, and they are beneficial in the assessing the quality of DCs before their clinical use [18]. The present study was carried out to determine the combined effect of radiotherapy (RT) and dendritic cell (DC)-based immunotherapy on tumor regression, and the influence of miR-15b-5p on the sensitivity of the tumor to RT/DC.

EXPERIMENTAL

Animals

Ten C57BL/6 mice for bone marrow-derived DC culture, and 35 nude/SCID BALB/c mice for development of colon cancer xenograft model were obtained from SLAC Company, Shanghai, China. The experiments on animals were performed in accordance with the International guidelines for study of laboratory animals [19], and were permitted by the local ethical committee (approval no. SCE20160421).

Bone marrow-derived DC culture

Bone-marrow cells were acquired from C57BL/6 mice (n = 10) as previously described [20]. The

BM cells were suspended in Tris-NH$_4$Cl potassium lysis buffer for 2 min at room temperature to lyse the red blood cells, followed by centrifugation. The cells were washed in PBS and cultured in RPMI-1640 medium with recombinant mouse interleukin (IL)-4 (10 ng/mL, Thermo Fisher) and 20 ng/mL recombinant mouse granulocyte-macrophage-colony-stimulating factor (Thermo Fisher) at a density of 2×10^6 cells/mL. After 6 days of culture, non-adherent cells were harvested and observed using FC500.

Colon carcinoma model and treatment

A colon carcinoma metastatic model was generated using BALB/c mice (n = 35). Xenograft tumors were produced by injecting 2×10^6 CT-26 colon carcinoma cells subcutaneously into mice. After seven days, tumor growth, measured as tumour volume (V), was calculated as shown in Eq 1.

$$V = LS^2/2 \dots\dots\dots\dots\dots (1)$$

where L (mm) and S (mm^2) are the long and short axes, respectively.

When the tumor volume reached 50 - 100 mm^3 after seven days of inoculation, the mice were divided into four groups: vehicle, radiation alone (RT), intratumoral injection of DCs only (DC), and radiation with DCs (RT/DC). Each group was given treatment three times at an interval of 7 days. The optimized dose of radiation i.e. 15 Gy, and 48 h between radiation and DC administration were followed. After 21 days, the mice were sacrificed, and the tumor tissues were excised and processed for histological examination.

MiRNA RT-qPCR

Isolation of total RNA was done using miRVana miRNA isolation kit (Thermo Fisher) based on manufacturer's guidelines. The RNA was reverse-transcribed to cDNA with TaqMan® MicroRNA Reverse Transcription Kit, and the cDNA was subjected to qPCR using miRVana quantitative RT-PCR primer set for miR-15b-5p (Thermo Fisher). The qPCR was monitored using ABI PRISM 7000, with U6 RNA as the endogenous control for normalization of mean Ct values using the $2^{-\Delta\Delta Ct}$ method.

MiR-15b-5p mimic transfection

The MiR-15b-5p mimics and negative control were transiently transfected into CT-26 cells using Lipofectamine RNAiMax (Thermo Fisher,

USA.), and were harvested at 48 h following transfection. The stable transfected cells were identified using puromycin (0.85 µg/mL), which was added to the culture medium two days after transfection. The selected puromycin-resistant colonies were expanded for two weeks. Thereafter, xenograft tumors were produced by subcutaneous administration of 2×10^6 CT-26/vector or CT-26/15b mimic cells. All groups then received RT/DC treatment as described previously.

Tumor growth measurement

Tumor volume was assessed following tumor cell injection every 3 days. Each treatment was started when the measured tumor volumes were 50 - 100 mm^3. Following the first treatment, tumor volumes were continuously measured, and analysed to determine differences between treatment groups. Following 21 days of treatment, the mice were sacrificed, and the tumors were excised and subjected to histological examinations.

TUNEL staining

The TUNEL staining was executed using ApopTag plus peroxidase *in situ* apoptosis detection kit (Sigma Aldrich) in line with the protocol described in the kit manual. The tumor samples were fixed in 4 % paraformaldehyde overnight and paraffin-embedded before TUNEL staining.

Determination of mRNA expressions

Homogenised samples were used to isolate RNA using PureLink RNA mini kit as described in the manual (Thermo Fisher). The concentration of RNA extract was measured using NanoDrop 1000 system (Thermo Fisher). The RNA was converted to cDNA using SuperScript™ IV VILO™ Master Mix (Thermo Fisher). Then, mRNA expressions of B-cell lymphoma-2 (BCL-2), caspase-3 (casp-3), and X-linked inhibitor of apoptosis protein (XIAP) genes were determined with SYBR™ Green PCR Master Mix (Applied Biosystems). Primers were acquired commercially (Thermo Fisher) and GAPDH served as the endogenous control for normalizing the mean Ct values using the $2^{-\Delta\Delta Ct}$ method.

Western blot analysis

Protein was isolated from homogenized tumor samples, and the extracted protein concentration was assayed using BCA kit (Sigma-Aldrich). The protein was subjected to SDS PAGE (10%), and the separated proteins were transferred to a nitrocellulose membrane using the electroblotting technique. After electro-blotting, the membrane was blocked with 5 % non-fat milk, and incubated overnight at 4 °C with primary antibodies i.e. rabbit anti-BCL-2 (diluted 1:1000), anti-casp-3 (diluted 1:1000), and anti-XIAP (diluted 1:1000) (Santa Cruz Biotechnology). The membrane was then incubated with goat secondary antibody labeled with horseradish peroxidase (diluted 1:1000) in blocking buffer. The proteins were detected using Chemiluminescence kit (Thermo Fisher Scientific).

Statistical analysis

All numerical data are represented as the mean ± standard error of mean (SEM). One-way analysis of variance (ANOVA) was employed to compare the variables followed by Bonferroni post-test and Student's t-test to analyse two independent groups using GraphPad Prism 5.0 for Windows software. The differences were considered statistically significant at $p < 0.05$.

RESULTS

RT/DC combination therapy reduced tumor volume
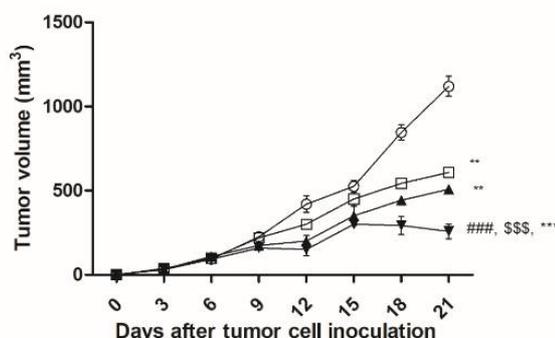
Figure 1: Effect of RT/DC on tumor volume 0 - 21 days after tumor cell inoculation. RT/DC treated tumors showed a remarkably significant tumor regression, when compared to other groups. Data are represented as mean ± SEM; [**]$p < 0.01$, compared with vehicle; [***]$p < 0.001$, compared to vehicle; [###]$p < 0.001$, compared with RT; [$$$]$p < 0.001$, compared with DC. The groups are represented by symbols: ○ = vehicle, □ = RT, ▲ = DC, and ▼ = RT/DC

Tumor volume was recorded every 3 days from the day of tumor cell inoculation. Compared to the vehicle-treated group, the other groups showed statistically significant changes in tumor regression over the course of 21 days (Figure 1). The groups subjected to RT or DC treatment only exhibited slight tumor regression, when compared to the vehicle-treated group ($p < 0.01$).

However, the RT/DC group which received the radiation and intratumoral DC combination therapy showed remarkable decreases in tumor volume, when compared with vehicle group ($p < 0.001$), RT-only group ($p < 0.001$) and DC group ($p < 0.001$).

RT/DC therapy increased miR-15b-5p levels

To assess the potential function of miR-15b-5p in the RT/DC combination therapy, the miR-15b-5p level was quantified in samples from the three groups: vehicle, RT, DC and RT/DC. The group subjected to RT showed 0.912-fold increase, while the DC group showed 0.81-fold increase in miR-15b-5p expression, when compared to the vehicle-treated group ($p < 0.01$). The RT/DC group presented a remarkable 2.445-fold increase in miR-15b-5p expression, relative to the vehicle-treated group (($p < 0.001$; Figure 2). The change in miR-15b-5p level in the RT/DC group was also significant relative to the RT-only and DC-only groups ($p < 0.001$).

Figure 2: Effect of RT/DC combination on miR-15b-5p expression. Values are presented as mean ± SEM of fold-change; [**]$p < 0.01$, relative to vehicle; [***]$p < 0.001$, relative to vehicle; [###]$p < 0.001$, relative to RT; [$$$]$p < 0.001$, compared to DC
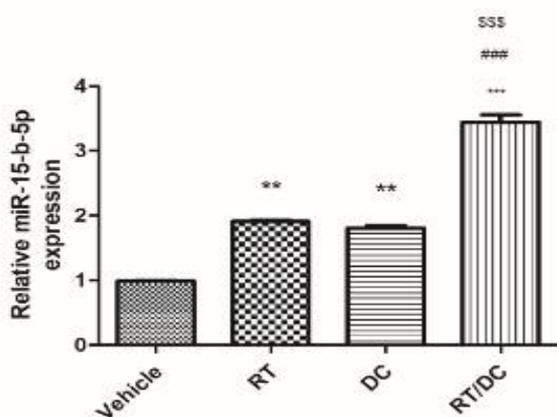
MiR-15b-5p promoted the sensitivity of the tumor to RT/DC treatment

The upregulation of miR-15b-5p enhanced the sensitivity of the tumor to RT/DC treatment, resulting in a remarkable decrease in tumor volume from day 9 of tumor cell inoculation, up to the 21st day, relative the vector/untreated group ($p < 0.001$). The vector/(RT/DC) group and miR-15b-5p/untreated group showed decreases in tumor volume, when compared with the vector/untreated group. Thus, miR-15b-5p upregulation directly enhanced the sensitivity of tumors to RT/DC.
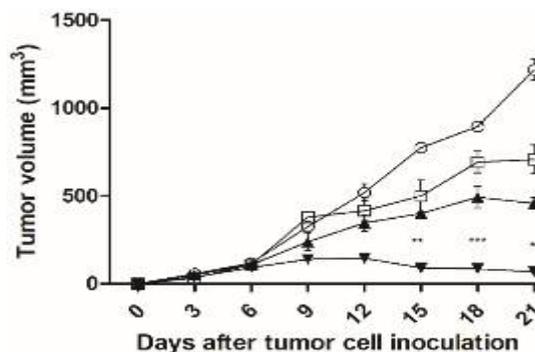
Figure 3: Effect of RT/DC on tumors with miR-15b-5p overexpression. Treatment with miR-15b-5p/(RT/DC) resulted in a significant tumor regression starting from day 9, indicating the relevance of miR-15b-5p upregulation in enhancing the sensitivity of tumors to RT/DC treatment. Values are presented as mean ± SEM; ***$p < 0.001$, **$p < 0.01$, compared with vector/untreated. The groups are represented by symbols: ○ = vector/untreated, □ = miR-15b-5p/untreated, ▲ = vector/(RT/DC), and ▼ = miR-15b-5p/(RT/DC)

MiR-15b-5p upregulation promoted cell death in RT/DC-treated tumor
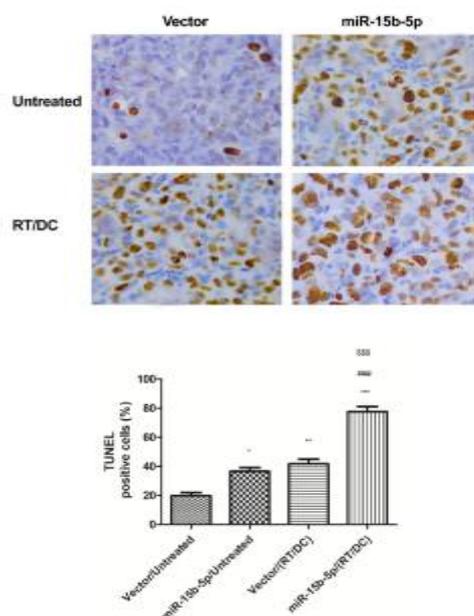
Figure 4: Effects of RT/DC and MiR-15b-5p overexpression on apoptosis. (a) Representative images of TUNEL-stained cells in vector/untreated, vector/(RT/DC), miR-15b-5p/untreated, and miR-15b-5p/(RT/DC) cells (x40). (b) Percentage of TUNEL-positive cells (%). [*]$p < 0.05$, [**]$p < 0.01$, [***]$p < 0.001$, compared with vehicle/untreated; [###]$p < 0.001$ compared to vector/(RT/DC), and [$$$]$p < 0.001$, compared to miR-15b- 5p/untreated

TUNEL staining demonstrated that miR-15b-5p upregulation significantly increased cell death in tumor samples treated with RT/DC (Figure 3a, &

3b). The average cell death increased by 57.8% in miR-15b-5p/(RT/DC) group, when compared to other groups ($p < 0.001$). Vector/(RT/DC) and miR-15b-5p/untreated groups also exhibited significant increases in cell death (20.9 %, $p < 0.01$ *vs* vector/untreated; and 16.76 %, $p < 0.05$ *vs* vector/untreated, respectively). These results imply that RT/DC and miR-15b-5p also had individual effects on cell death.

MiR-15b-5p promoted the expression of cell death-related genes in RT/DC-treated tumor sample

Figure 5: Effect of RT/DC on relative mRNA expression of apoptosis-linked genes. (a) Casp-3, (b) BCL-2, and (c) XIAP. Values are presented as mean fold-change ± SEM, relative to endogenous control GAPDH. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, compared to vehicle/untreated; $^{###}p < 0.001$, compared to vector/(RT/DC), and $^{$$$}p < 0.001$, $^{$$}p < 0.01$, compared to miR-15b-5p/untreated
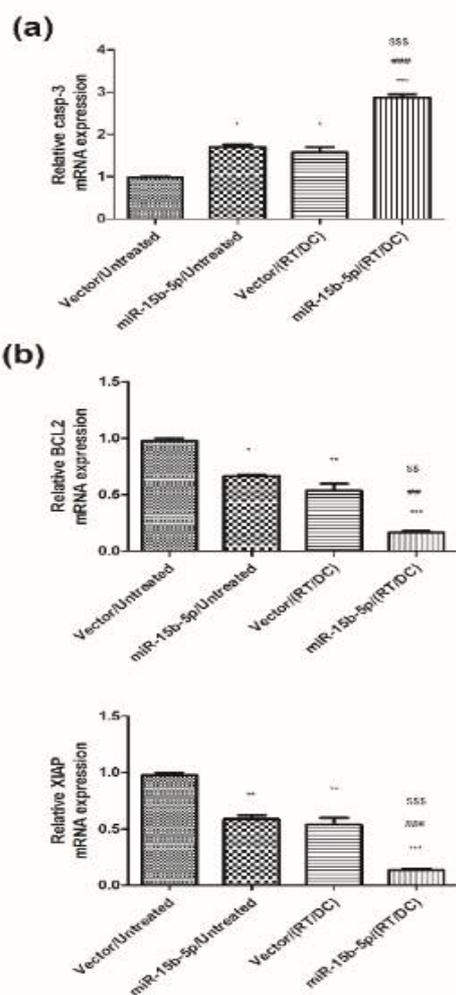
To further investigate the mechanism involved in the enhancement of the sensitivity of tumors to RT/DC combination treatment by miR-15b-5p upregulation, mRNA expression of the apoptosis-associated genes i.e. casp-3, BCL-2 and XIAP were determined. Treatment with miR-15b-5p/(RT/DC) resulted in a significant 1.86-fold rise in casp-3, 0.834-fold decrease in BCL-2, and 0.864-fold decrease in XIAP mRNA expressions, when compared to vector/untreated group ($p < 0.001$; Figures 5a, 5b and 5c). However, significant fold-changes were seen in casp-3, BCL-2 and XIAP expressions, relative to the miR-15b-5p/untreated and vector/(RT/DC) groups ($p < 0.001$, compared to vector/(RT/DC); $p < 0.001$, compared to miR-15b-5p/untreated) (Figures 5a, 5b and 5c). The corresponding changes in miR-15b-5p/untreated and vector/(RT/DC) groups were marginal, when compared to miR-15b-5p/(RT/DC), indicating the potential apoptosis-modulating effect of miR-15b-5p/(RT/DC).

Verified levels of apoptosis-linked proteins casp-3, BCL-2, and XIAP

Western blot confirmed the qPCR mRNA expressions of casp-3, BCL-2 and XIAP in miR-15b-5p-upregulated, RT/DC-treated tumors. There were decreases in BCL-2 and XIAP mRNA, whereas casp-3 increased in miR-15b-5-/(RT/DC) group. Altered expressions of these proteins in miR-15b-5p/untreated and vehicle/(RT/DC) groups were observed. However, the effect was maximum in the miR-15b-5p/(RT/DC) group. These results indicate that miR-15b-5p upregulation enhanced the sensitivity of the tumor to RT/DC treatment.
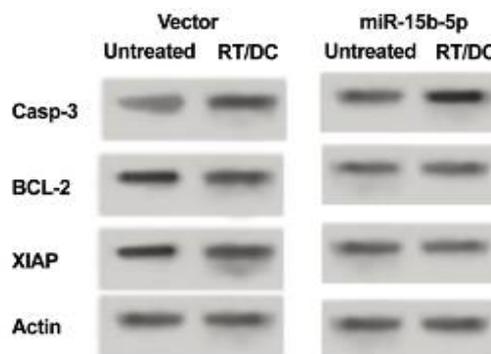
Figure 6: Western blot results for apoptosis-linked proteins casp-3, BCL-2, and XIAP. The protein expression analysis for vector/untreated, vector/(RT/DC), miR-15b-5p/untreated and miR-15b-5p/(RT/DC) is shown. Casp-3 expression increased, whereas BCL-2 and XIAP protein expressions decreased markedly in miR-15b-5p/(RT/DC) group, relative to the other groups

DISCUSSION

The present study demonstrates the synergistic effect of RT and DC-based immunotherapy on

tumor regression with mechanistic insight on the function of miR-15b-5p in enhancing the sensitivity of tumor to RT/DC combination treatment. The understanding of immune mechanisms and the implications of RT on tumor microenvironment has resulted in the development of innovative treatment approaches for cancer. Many reports support the use of combination of RT and immunotherapy to achieve a better local/systemic control of tumors [9-11]. The irradiation of tumor predominantly leads to DNA damage, resulting mitotic catastrophe which is not directly linked to pre-mitotic cell death or apoptosis [4,5]. The RT-induced DNA damage activates the immune regulatory mechanism. Accumulating evidence show that the immune system of the host generates cytotoxic adaptive response which enables immunogenic cell death and tumor regression [21]. The efficiency of immunogenic cell death also depends on tumor type and immunogenic capacity, while RT enhances this fine-tuned process *via* the activation of critical inflammatory processes [21].

The preparation of DCs with tumor-associated antigens (TAAs) is crucial for generating DC vaccines for the induction of immunotherapy in tumors, and it can be attained by culturing DCs with irradiated cancer cells *ex vivo* or *in vivo* [22]. The irradiation of cancer cells promotes the generation of TAAs and increases the stimulation of DCs. A study has reported thatγ-irradiation of DCs resulted in enhanced anti-cancer activity by increasing the cytotoxicity and activation of T cells [23]. In view of extant studies on the role of DCs against cancer and the influence of RT as an immunological adjuvant, the effect of RT/DC combination therapy compared with sole treatment with RT or DC, was investigated in the present study in a colon carcinoma metastatic mouse model. The findings showed that RT/DC combination treatment resulted in remarkably significant tumor regression, when compared with other groups. However, the application of RT/DC therapy in cancer is still in its infancy. Extensive investigations are required on ways to enhance the immunogenicity of DCs and sensitization of tumor to the treatment.

Furthermore, the regulatory function of miR-15b-5p following RT/DC combination therapy was assessed. It is known that miR-15b which is aberrantly expressed in malignant tumors, is involved in cancer progression [14]. The upregulation of miR-15b induces cell death, reduces cancer cell proliferation, and decreases angiogenesis and metastasis of tumors [14]. In the present study, miR-15b-5p was significantly downregulated in the untreated group, while the

RT/DC treatment significantly increased miR-15b-5p level, relative to the other groups. This observation led to investigations on the upregulation of miR-15b-5p in colon carcinoma model *in-vivo,* and its effect on the sensitivity of tumor to RT/DC treatment.

Another objective of this study was to investigate the impact of miR-15b-5p upregulation on tumor regression following RT/DC treatment. The results showed that miR-15b-5p mimic and RT/DC treatment had individual influences on tumor regression and cellular apoptosis. However, the effect was remarkably enhanced in the group treated with both miR-15b-5p mimic and RT/DC combination. A recent study reported that miR-15b-5p upregulation enhanced 5-fluorouracil-induced cell death in a colorectal cancer model. MiR-15b-5p mimic transfection also reversed the chemo-resistance of cancer cells to 5-fluorouracil *via* NFkB1 signaling pathway [24].

Therefore, in this study, the marked reduction in tumor growth and enhanced apoptosis of cancer cells imply that miR-15b-5p upregulation indeed promotes the sensitivity of tumors to RT/DC treatment. It has been reported that miR-15b-5p directly targeted BCL-2, and that increases in miR-15b-5p reduced BCL-2 while increasing casp-3 and casp-7 protein levels [15]. A previous study showed that inhibition of miR-15b attenuated TRAIL-mediated apoptosis, whereas miR-15b precursor increased cell death [25]. Another study demonstrated that administration of dihydroartemisinin for the treatment of gastric cancer resulted in an increased miR-15b expression concomitant with reduced expression of BCL-2 [26]. Moreover, increased XIAP level has been associated with progression of cancer, and miR-15b-5p mimic was reduced with XIAP expression, thereby promoting apoptosis of cancer cells [24]. The results obtained in the present study, wherein miR-15b-5p upregulation resulted in a significant increase in casp-3 and marked reductions in BCL-2 and XIAP levels in RT/DC treated group, are consistent with these findings.

CONCLUSION

The findings of this study show, for the first time, that RT/DC combination therapy increases miR-15b-5p expression. Furthermore, miR-15b-5p upregulation enhances the sensitivity of tumors to RT/DC combination therapy *via* the regulation of genes and proteins associated with cancer cell apoptosis. Thus, this study opens a new avenue for optimization of DC preparation and RT, and enhancing the immunogenicity of the DCs.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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