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Original Paper

MiR-195/-16 Family Enhances Radiotherapy via T Cell Activation in the Tumor Microenvironment by Blocking the PD-L1 Immune Checkpoint

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Key Words
Mir-195 • MiR-16 • PD-L1 • *Tumor microenvironment* • Immunotherapy • Radiotherapy

Abstract
Background/Aims: Radiotherapy is the standard treatment option for advanced prostate cancer. Unfortunately, despite significant advances in radiation delivery, prostate cancer radioresistance occurs in a large proportion of patients undergoing radiotherapy. As a way to enhance radiotherapy effectiveness, research advances into the mechanisms regulating the immune response have revived interest in combination radiation and immune-based therapies. ***Methods:*** miR-195/-16 family and PD-L1 levels were analyzed in samples from a GSE21032 data set. Kaplan-Meier analysis was used to evaluate the difference in biochemical recurrence-free survival associated with miR-195 and miR-16 expression. qRT-PCR and western blot were used to evaluate the miR-195, miR-16 and PD-L1 expression. Then, we used bioinformatics analysis and luciferase reporter assay to predict and confirm the miR-195 and miR-16 target gene. Finally, we elucidate the miR-195 and miR-16 function on immune evasion in the DU145/T cell co-culture model and syngeneic mouse model treated with radiotion through qRT-PCR, western blot, Flow cytometry and ELISA. ***Results:*** High levels of miR-195 and miR-16 were positively correlated with the biochemical recurrence-free survival of prostate cancer patients. miR-195 and miR-16 were inversely correlated with PD-L1, PD-1, CD80 and CTLA-4 expression. Further mechanistic investigations revealed that miR-195

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and miR-16 inhibited PD-L1 expression. Additionally, restoration of miR-195 and miR-16 expression enhanced radiotherapy via T cell activation in the tumor microenvironment by blocking PD-L1 expression. This synergistic effect of immunotherapy and radiotherapy was associated with the proliferation of functional cytotoxic CD8+ T cells and inhibition of myeloid-derived suppressor cells and regulatory T cells. ***Conclusions****:* Our data revealbiological and functional interactions between immunotherapy and radiotherapy through the miR-195/-16 family regulatory cascade.

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Introduction

Radiotherapy is the standard treatment option for both organ-confined and regionally advanced prostate cancer. Unfortunately, despite significant advances in radiation delivery procedures, prostate cancer radioresistance occurs in a large number of patients undergoing radiotherapy [1]. As a way to enhance radiotherapy effectiveness, research advancesinto the mechanisms regulating the immune response have revived interest in combination radiation and immune-based therapies.

The immune response affects cancer development by acting as an extrinsic tumor suppressor that restrains tumor expansion or destroys tumors [2]. However, tumor cells can escape immune surveillance. T-lymphocyte associated antigen-4 (CTLA-4) and programmed death-1 (PD-1) are the two immunoregulatory proteins expressed on T cell membranes [3-4]. CD80, the CTLA-4 receptor ligand, is expressed in dendritic cells, whereas PD-1 ligand 1 (PD-L1), the PD-1 ligand, is expressed in tumor and/or macrophage cells [5]. The interactions of CD80 with CTLA-4 and of PD-L1 with PD-1 inhibit CD8+ cytotoxic T lymphocyte survival and proliferation and affect the function of tumor-infiltrating T cells, ultimately suppressing the immune response and inducing immune tolerance of cancer [6]. Radiotherapy exerts some potentially anti-therapeutic effects by inducing tumor cell that express PD-L1, secrete TGF-β, and induce regulatory T cells (Tregs) [7]. These findings suggest a potential link between radiotherapy and immunoresistance in prostate cancer.

miR-195 and miR-16 are members of the miR-15/-16/-195/-424/-497/-503 family. miR-195 and miR-16 overexpression increases radiosensitivity of cancer cell [8-9]. miR-195 inhibits human prostate cancer progression by targeting RPS6KB1, and miR-16 acts as a tumor suppressor gene in prostate cancer by controlling cell survival, proliferation and invasion via BCL2, CCND1 and WNT3A [10-11]. However, it is unclear whether the miR-195/-16 family is involved in the regulation of the immune response. In this study, we demonstrated both *in vitro* and *in vivo* that restoration of miR-195 and miR-16 expression enhanced radiotherapy via T cell activation in the tumor microenvironment by blocking the PD-L1 immune checkpoint.

Materials and Methods

Cell culture, Plasmid construction, Reporter genes, Reagents, Expression vectors, and DNA transfection

The prostate cancer cell lines PC-3, DU145 and Trampc1 were purchased from The Cell Bank of Chinese Academy of Science. Cells were maintained in a medium of RPMI 1640 supplemented 10 % FBS and 1 % penicillin/streptomycin and were kept in 5 % CO2 incubator at 37°C. PLe-miR-SCR lentivirus vector and pLe-miR-195 and pLe-miR-16 were purchased from Open Biosystems, AL, USA. Cells infected with pLe-miR-195 or pLe-miR-16. Has-miR-195 and has-miR-16 mimics were purchased from Guangzh Ribobio Co., LTD, China. Hsa-miR-195, hsa-miR-16 mincis and hsa-miR-SC were transfected into cells using Lipofectamine RNAiMAX (Invitrogen, CA, USA) according to the manufacturer's instructions. Protein lysates and total RNAs were collected 48h after the transfection. The expression levels of miRNAs were verified by stem-loop qRT-PCR analysis. All cell lines were tested and free of mycoplasma contamination (MycoAlert Mycoplasma Detection Kit, Lonza).

Blood DCs isolation

Blood DCs were isolated from healthy human donors using a Human Blood Dendritic Cell Isolation Kit II (Miltenyi Biotec, No. 130-091-379) following the manufacturer's instruction.

Tissue samples

A collection of 40 different kinds of fresh-frozen prostate cancer tumor specimens and 20 tumor normal adjacent tissues were from Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology. All tumor samples were collected immediately after the surgical removal and snap-frozen in liquid nitrogen [12].

Biochemical recurrence-free survival analysis in GSE21034 data base.

A normalized mRNA expression dataset for Prostate Adenocarcinoma was downloaded from the cBioPortal for cancer genomics and used to evaluate expression of miR-195/16 family and PD-L1 transcript levels [13]. This dataset includes mRNA and miRNA profiles for 131 primary prostate cancer patient samples. Biochemical recurrence-free survival analysis was calculated for these transcripts for primary tumor samples.

Western Blot Analysis.

Cells were lysed in RIPA buffer with protein inhibitors. Total protein (20-40g) from each sample was electrophoresised on 8% SDS-PAGE gel and transferred to a nitrocellulose membrane. The membranes were blocked in 5% nonfat milk and probed with primary antibodies overnight at 4 °C. The membranes were washed and probed with secondary antibody conjugated to horseradish peroxidase and the developed with enhanced chemiluminescence (Thermo Scientific).

Quantitative real-time PCR

Total RNA was isolated using Trizol Reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA were synthesized with TaqMan Reverse Transcription Reagents (Life Technologies). The expression levels of miRNAs were analyzed using Taqman MicroRNA Assay Kits (Applied Biosystems, Foster City, CA) specific for hsa-miR-195, has-miR-16 and PD-L1 mRNAs. The fold stimulation was determined using the comparative cycle threshold method (method ($2^{-\Delta\Delta CT}$)). All experiments were performed in triplicate [14].

Luciferase reporter assay

Luciferase reporter assay was done as described previously [15]. PD-L1 3'-UTR regions that contained predicted miR-195 and miR-16 binding sites were amplified by PCR from genomic DNA, and the PCR fragments were inserted into untranslated region (UTR) downstream of the luciferase gene in the pMIR-reporter luciferase vector (Ambion). Luciferase reporter plasmid, β-galactosidase (β-gal) plasmid, and hsa-miR-195, hsa-miR-16 or negative control precursor were cotransfected into cells using Lipofectamine 2000 (Invitrogen). Luciferase activity was measured 48 hours after transfection using β-gal for normalization.

Preparation of DU145 cell lysate

DU145 cell lysate was done as described [16]. Confluent cultures of DU145 cell line was incubated with 0.01% EDTA-solution for 10 min, carefully detached with a cell scraper, washed twice in PBS, and resuspended at a densiry of 5×10^6/ml in serum-free mediun. The cell susupensions were frozen at -80^{0}C and disrupted by four freeze-thaw cycles. For the removal of crude debirs, the lysate was centrifuged for 10 min at 300×g. The supernatant was collected and passed through a 0.2-mm filter.

DU145/T co-culture model

DU145/T cells co-culture model was done as described previously [17]. Peripheral blood mononuclear cells (PBMCs) from healthy human donors were isolated using Lymphoprep density gradient centrifugation (Accurate Chemical). Human PBMCs were plated at a density of 2×10^6 well^{-1} in 6-well plates and stimulated with DU145 cell lysate, anti-CD3e (10 μg mL^{-1}) and anti-CD28 (2 μg mL^{-1}) for 48 h to promote T cell activation. The T cell activation protocol was provided by eBioscience (http://www.ebioscience.com/cell-type/t-cells.htm). DU145 cells that stably expressed miR-195 and miR-16. Stimulated human PBMCs were subsequently

harvested and purified by Lymphoprep density gradient centrifugation, and co-cultured with the DU145 cells at a 10:1 ratio for 16 h. The DU145 cells were then sorted by FCM. The relative expression levels of PD-L1 in the DU145 cells were determined by qRT-PCR assay. Co-culture media were assayed for TNF-α, IFN-γ, IL-2, IL-10, IL-1β and TGF-β using a cytokine ELISA assay.

T cell apoptosis assay

PBMCs were isolated from healthy human donors using Lymphoprep density gradient centrifugation (Accurate Chemical). PBMCs were plated at a density of 2×10^6 well^{-1} in 6-well plates and stimulated with DU145 cell lysate, anti-CD3e (10μg mL mL^{-1}) and anti-CD28 (2μg mL^{-1}) for 48 h to promote T cell activation. The DU145 cells were first exposed to radiation for 48h. Stimulated PBMCs were subsequently harvested and purified by Lymphoprep density gradient centrifugation, and then co-cultured with DU145 cells at a 10:1 ratio for 16 h. Ten micrograms per milliliter anti-PD-L1 were added to the indicated wells to examine PD-1-specific CD8+ T cell apoptosis. The PBMCs were subsequently harvested and stained with PE-conjugated PD-1, Alexa Fluor 488-conjugated annexin V and APC-conjugated CD8 antibodies, respectively. PD-1-dependent CD8+ T cell apoptosis was calculated as the percentage of annexin V+ cells in the gated PD-1+/CD8+ population [17].

Enzyme-linked immunosorbent assays (ELISA)

The production of T cell cytokine was detected by cytokine ELISA using the human or mouse ELISA kit (ExCell Bio, Shanghai, China) as described [17].

Syngeneic orthotopic prostate cancer model

Syngeneic orthotopic prostate cancer model was done as described previously [17]. Trampc1 cells were culture, harvested, and suspended in PBS. For flank injections, a total of 0.5ml containing 5×10^6 cells was injected subcutaneously into the flank of 6- to 8-week-old Tramp mice (Chinese Academy of Sciences at Beijin, China*)*. Tumors were allowed to develop over for 8 days, until tumor diameter was approximately 0.5cm. On day 9, the mice were treated with 20Gy radiation using a PanTak 310 keV X-ray machine at 0.25-mm Cu plus 1-mm Al added filtration at 125 cGy/min. The mice serum was collected at 72h after radiation treatment. Tumor dimensions were measured every 3 days. Analyses included assessment of tumor growth and survival. Tumors tissues were used for enumeration and characterization of MDSCs, Tregs, and CD8+ T cells memory subsets. Mice serum was assayed for IL-10, TNF-α, IFN-γ, and TGF-β by cytokine ELISA assay.

Statistical analysis.

All statistical analyses were performed using EXCEL 2010 (Microsoft, USA). The results reported as mean ± SD. Statistical analyses were conducted using Student's *t*-test and Pearson Correlation Coefficient. $p < 0.05$ were considered statistically significant.

Results

miR-195 and miR-16 expression are inversely correlated with PD-L1 levels in prostate cancer

miRNAs act as post-transcriptional modulators of gene expression mainly by binding to the 3' untranslated region (3'-UTR) of their target genes and have been shown to play important roles in regulating the immune response [18]. Through the public database *microRNA.org*, we first analyzed the 3'-UTR sequences of PD-L1, PD-1, CD80 and CTLA-4, finding that PD-L1, which is an immune checkpoint gene, is potentially targeted by miR-15a/-15b/-16/-195/-424/-497/-503 family. To evaluate the physiological interactions between the miR-15a/-15b/-16/-195/-424/-497/-503 family and PD-L1/PD-1/CD80/CTLA-4 expression, we performed *in silico* analysis using a GSE21032 dataset composed of 131 primary prostate cancer patient samples. We also conducted Kaplan-Meier analysis by separating patients into two groups (higher than the 50th percentile and lower than the 50th percentile) according to their miRNA expression levels, to determine whether biochemical recurrence(BCR)-free survival was correlated with the level of miR-15a/-15b/-16/-195/-424/-497/-503 family members in tumors. The results indicated that

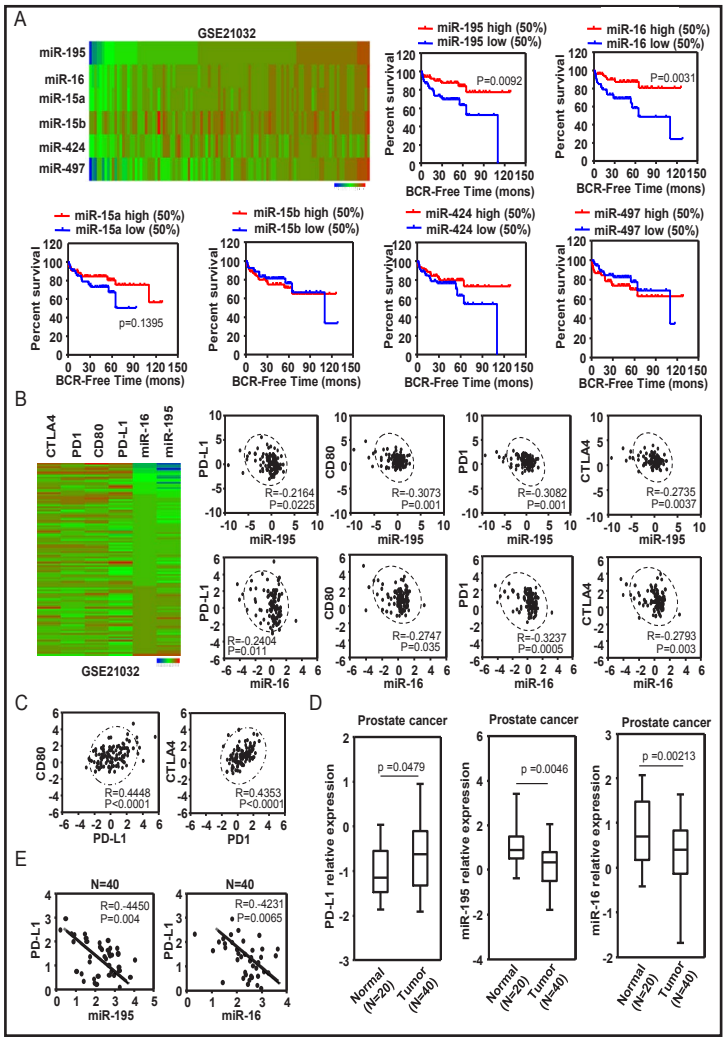

Fig. 1. miR-195 and miR-16 are inversely correlated with PD-L1 levels in prostate cancer patients. (A) Heat map depicting samples from GSE21034 combined human prostate cancer microarray datasets that were assigned to prostate cancer gene expression subtypes (n=150). Kaplan-Meier analysis indicating that patients with samples with highly expressed (> 50%) miR-195 or miR-16 had significantly better BCR-free survival. (B) miR-195 and miR-16 levels are inversely correlated with PD-L1, PD-1, CD80 and CTLA4 expression levels in human prostate cancer. (C) PD-L1 and PD-1 expression levels are positively correlated with CD80 and CTLA-4 expression levels in human prostate cancer. (D) qRT-PCR was performed to determined miR-195, miR-16 and PD-L1 expression in 20 normal prostate tissues and 40 primary prostate tumors. (E) qRT-PCR was performed to determine miR-195, miR-16 and PD-L1 expression in 40 primary prostate tumors. Spearman's rank correlation analysis identified an inverse correlation between miR-195/-16 and PD-L1 levels in the prostate cancer tumor samples.

patients with tumors that expressing high levels of miR-195 and miR-16 had a longer BCR-free survival (Fig. 1A). In addition, miR-195 and miR-16 were negatively associated with PD-L1, PD-1, CD80 and CTLA-4 expression (Fig. 1B), whereas a positive correlation was found among the expressions of PD-L1, PD-1, CD80 and CTLA-4 (Fig. 1C).

To further confirm the interaction between miR-195/-16 and PD-L1, we examined miR-195, miR-16 and PD-L1 levels with quantitative real-time polymerase chain reaction (qRT-PCR) in prostate cancer samples. As shown in Fig. 1D, the miR-195/-16 levels were significantly decreased and the PD-L1 levels were dramatically increased in primary prostate tumors compared with normal tissues. The miR-195 and miR-16 levels were inversely correlated with PD-L1 (Fig. 1E). Taken together, these findings suggested that miR-195 and miR-16 modulated the immune response by interacting with the PD-L1/PD-1 immune checkpoint in prostate cancer.

PD-L1 is regulated by miR-195 and miR-16

To validate the bioinformatics analysis, which indicated that PD-L1 was a potential target of miR-195 and miR-16, we performed luciferase reporter assays with human wild-type PD-L1 3'-UTR and mutants with deletions of the putative miR-195 and miR-16 binding sequences. As shown in Fig. 2A-B, overexpression of miR-195 and miR-16 inhibited wild-type but not mutant luciferase reporter activity in DU145 and PC-3 cells, which suggested that miR-195 and miR-16 targeted PD-L1 through the 3′-UTR binding site. Western blot analyses

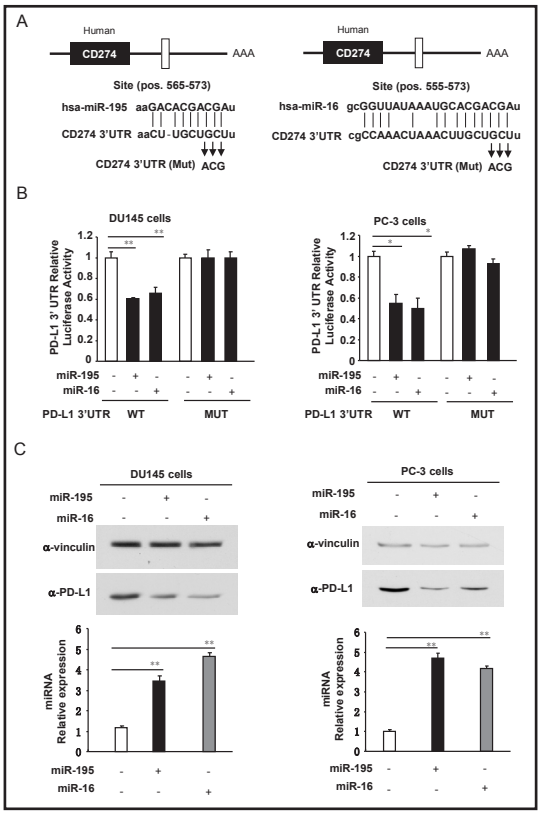

Fig. 2. PD-L1 is directly regulated by miR-195 and miR-16. (A-B) miR-195 and miR-16 targeting sites in the 3-'UTR of human PD-L1 are shown. Luciferase vectors containING the human wild-type (WT) and mutant (MUT) PD-L1 3'- UTR regions were co-transfected into DU145 cells with miR-195 or miR-16. Relative Luciferase/Renila activities were analyzed in the cells 48h after the transfection. (C) Du145 and PC3 cells were transfected with miR-195 or miR-16. miR-195 and miR-16 mRNA levels were determined via qRT-PCR assay. The expression levels of PD-L1 were determined by western blotting. Results represent the mean ± standard error of the mean (SEM) from three independent experiments. * $p \leq 0.05$, ** $p \leq 0.01$.

subsequently demonstrated that miR-195 or miR-16 overexpression in DU145 and PC-3 human cancer cells decreased the protein levels of PD-L1 (Fig. 2C). Taken together, these findings indicated that miR-195 and miR-16 suppressed PD-L1 expression.

miR-195 and miR-16 regulates cytokine secretion in the tumor microenvironment through a PD-L1-dependent pathway

The release of cytokines in the tumor microenvironment is critical in many aspects of T cell function [19-20]. The PD-L1/PD-1 immune checkpoint pathway inhibits the anti-tumor immune response of T cells [6], and PD-L1 preferentially modulates the secretions of regulatory cytokines in the tumor microenvironment [21]. Therefore, we tested whether an increased PD-L1 level through miR-195 and miR-16 signaling in prostate cancer affected the tumor microenvironment. We established a DU145/T cell co-culture model. Co-culture T cells with DU145 cells stably expressing miR-195 and miR-16 decreased IL-1β, IL-10 and TGF-β secretion and increased TNF-α, IFN-γ and IL-2 in the culture media, whereas PD-L1 depletion through a PD-L1-blocking antibody (anti-PD-L1) abolished miR-195- and miR-16-mediated cytokine secretion (Fig. 3A-F). These findings suggested that miR-195 and miR-16 modulated cytokine secretion in the tumor microenvironment by blocking a PD-L1/PD-1-dependent pathway.

miR-195 and miR-16 influences PD-L1-associated T cell apoptosis in the DU145/T cell co-culture model

Previous studies demonstrated that the expression of PD-L1 was directly repressed by miR-195 and miR-16. To investigate the combined effect of miR-195/-16 and radiation in human prostate cancer, we established DU145 cells stably expressing miR-195 and miR-16, and then treated them with radiation or anti-PD-L1. Flow cytometer (FCM) analysis showed that cell surface PD-L1 expression was significantly higher in radiation treated DU145 cells (48h after 20Gy radiation) compared with parental cells (Fig. 4A). Restoration of miR-195 and miR-16 expression in radiation treated DU145 cells decreased cell surface PD-L1 expression, whereas anti-PD-L1 treatment abolished miR-195 and miR-16 overexpression-mediated PD-L1 repression in the radiation treatment group (Fig. 4A).

It has been demonstrated that the PD-L1/PD-1 pathway inhibits the anti-tumor immune response of T cell. To determine whether miR-195 and miR-16 affect the T cell response by regulating PD-L1 expression, we performed DU145/T cell co-culture experiments. Co-culture of T cells with radiated DU145 cells increased PD-1+/CD8+ T cell apoptosis

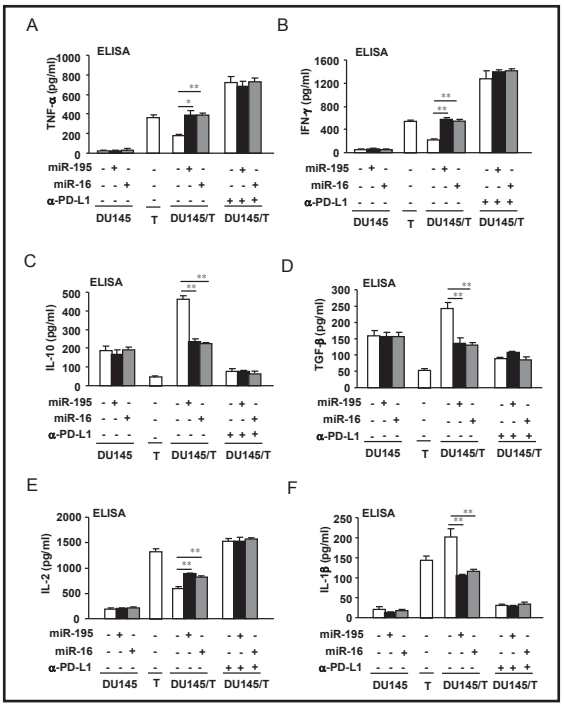

Fig. 3. miR-195 and miR-16 regulate cytokine secretion in the tumor microenvironment through a PD-L1-dependent pathway. (A-F) DU145 cells with stable overexpression of miR-195 or miR-16 treated with anti-PD-L1. After 24 h, T cells were then co-cultured with DU145 cells for 24h. Co-culture medium was assayed for TNF-α, IFN-γ, IL-2, IL-10, IL-1β and TGF-β by cytokine enzyme-linked immunosorbent assay (ELISA). Results represent the mean ± SEM from three independent experiments. ** $p \leq 0.01$.

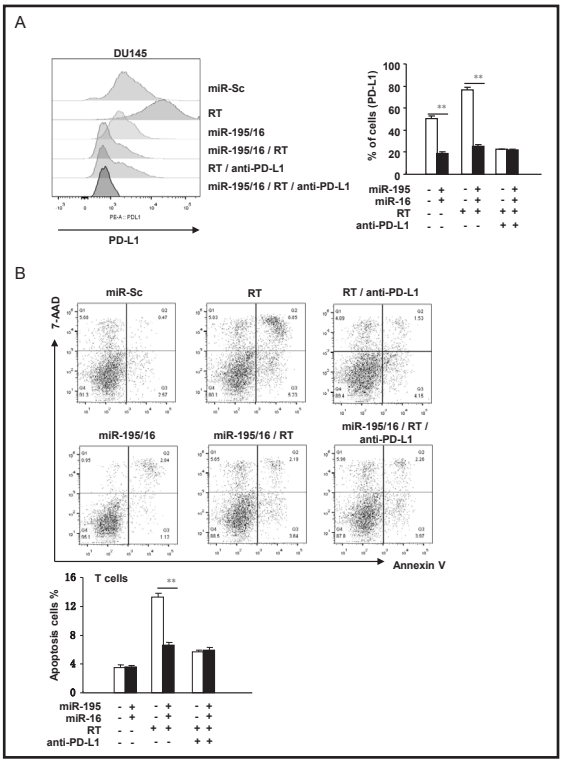

Fig. 4. miR-195 and miR-16 influences PD-L1-associated T cell apoptosis in DU145/T cell co-culture model. (A) DU145 cells with stable overexpression of miR-195 and miR-16 were first exposed to radiation for 48h in the presence or absence of anti-PD-L1. The expression of cell surface PD-L1 was determined by FCM in DU145 cells. Results represent the mean ± SEM from three independent experiments. * $p \leq 0.05$, ** $p \leq 0.01$. (B) T cells were subsequently co-cultured with DU145 cells for 24h. T cells were sorted by FCM, stained with PE anti-PD-1, Alexa Fluor 488 anti-annexin V and APC anti-CD8, and analyzed by flow cytometry for T cell apoptosis in the PD-1+/CD8+ population. ** $p \leq 0.01$. Results represent the mean ± SEM from three independent experiments, and the densitometric level of the apoptosis ratio is shown.

compared with T cells cultured with intact DU145 cells (Fig. 4B). However, in DU145 cells overexpressing miR-195 and miR-16, the apoptosis of PD-1+/CD8+ T cells induced by radiation treatment was completely abolished (Fig. 4B). Similar to anti-PD-L1, miR-195 and miR-16 were also able to mediate apoptosis in PD-1+/CD8+ T cells, which suggested that they decreased radiation-induced PD-1+/CD8+ T cell apoptosis by blocking PD-L1. These results also showed that radiotherapy-induced immune-resistance could be reversed by miR-195 and miR-16 overexpression. These findings indicated that miR-195 and miR-16 improved the therapeutic efficacy of radiation-resistance by suppressing PD-L1.

PD-L1 is required for the efficacy of miR-195 and miR-16 treatment in Trampc1 tumors

Next, we analyzed the mouse PD-L1 3-'UTR through *microRNA.org* and found that mouse PD-L1 was also potentially targeted by mouse miR-195 and miR-16 (Fig. 5A). To confirm this targeting, we generated reporter constructs containing the potential binding site of the PD-L1 gene 3'-UTR regions or their mutants. The luciferase activity of PD-L1 wild-type constructs was inhibited by miR-195 and miR-16 overexpression (Fig. 5B) in Trampc1 cells. Point mutations in the putative binding site abrogated the inhibition by miR-195 and miR-16 (Fig. 5B), which demonstrated that miR-195 and miR-16 specifically targeted the PD-L1 3'-UTR. Western blotting analyses indicated that miR-195 and miR-16 overexpression in Trampc1 cells decreased the protein levels of PD-L1 (Fig. 5C). Furthermore, FCM analysis showed cell surface PD-L1 expression was significantly higher in radiation treated Trampc1 cells (24-72h after 20Gy radiation) compared with untreated cells (Fig. 5D). Restoration of miR-195 and miR-16 expression in Trampc1 cells resulted in decreased cell-surface PD-L1 expression (Fig. 5D).

To investigate the role of miR-195 and miR-16 in regulating Trampc1 tumor growth in vivo, we transplanted Trampc1 cells stably overexpressing miR-195 or miR-16 into syngeneic Tramp mice, followed by anti-PD-L1 treatment. The mouse tumor volumes are shown in Fig

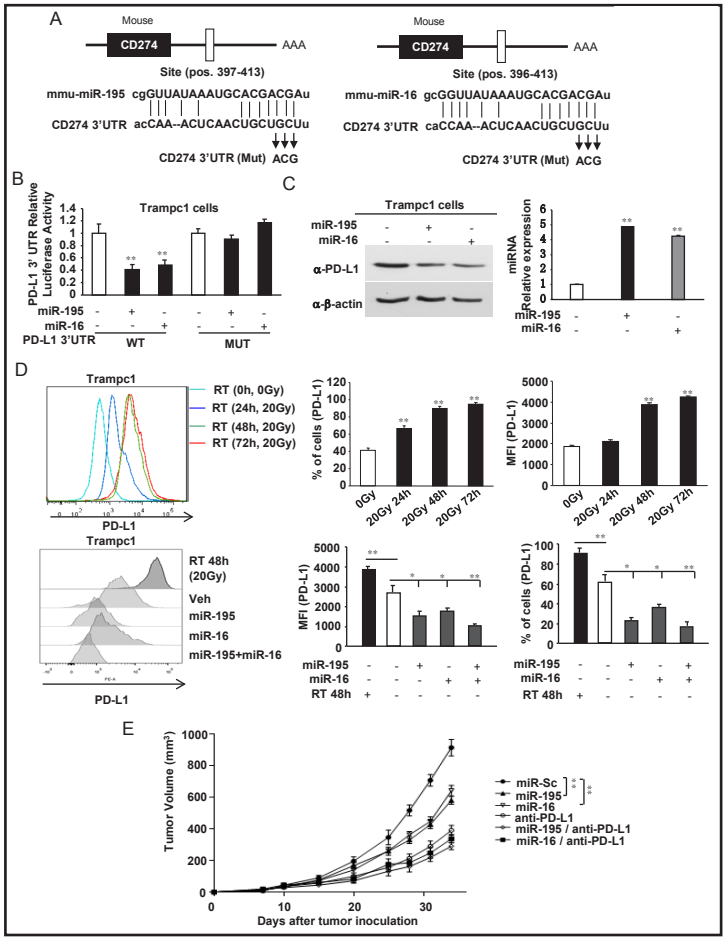

Fig. 5. PD-L1 is required for the efficacy of miR-195 and miR-16 treatment in Trampc1 tumors. (A) The miR-195 and miR-16 targeting sites in 3'-UTR of mouse PD-L1 were shown. (B) The reporter constructs containing the mouse wild-type (WT) and mutant (MUT) PD-L1. 3'- UTR regions were co-transfected into Trampc1 cells with miR-195 or miR-16. The relative Luciferase/Renila activities were analyzed in the cells 48h after the transfection. Results represent the mean ± SEM from three independent experiments. ** $p \leq 0.01$. (C) Trampc1 cells were transfected with miR-195 or miR-16. miR-195 and miR-16 mRNA levels were determined by qRT-PCR assay. Results represent the mean ± SEM from three independent experiments. ** $p \leq 0.01$. Expression levels of PD-L1 were analyzed by western blotting. One representative experiment out of three is shown. (D) Trampc1 cells treated with mir-195/16 mimics or radiation (20Gy) for 24-72h. The expression of cell surface PD-L1 was determined by FCM in Trampc1 cells. The results represent the mean ± SEM from three independent experiments. * $p \leq 0.05$, ** $p \leq 0.01$. (E) Trampc1 cells (5×10^6) with stable overexpression of miR-195 or miR-16 were injected into syngeneic Tramp mice followed by anti-PD-L1 treatment. The mouse tumor volumes of the mice was characterized in different treatment groups. Bar graphs are shown as the mean ± SEM (n = 12 mice/group). ** $p \leq 0.01$.

5E. miR-195 and miR-16 overexpression significantly decreased tumor volumes compared with miR-Sc in untreated Tramp mice, whereas anti-PD-L1 treatment eliminated the efficacy of miR-195 and miR-16 overexpression, suggesting that PD-L1 is required for miR-195 or miR-16 in Trampc1 tumors.

miR-195/-16 enhances the radiotherapy efficacy of Trampc1 tumors by regulating immunocyte production in the tumor microenvironment

To test the effect of combination treatment with miR-195/-16 and radiation in mouse prostate cancer, we implanted Trampc1 cells expressing miR-195/-16 into syngeneic Tramp mice, followed by radiation or anti-PD-L1treatment. Tumor volumes are shown in Fig. 6A. Radiation treatment significantly decreased the tumor volumes in Tramp mice with miR-195 and miR-16 overexpressing tumors, indicating that miR-195 and miR-16 might enhance the efficacy of radiation treatment in suppressing the progression of Trampc1 tumors (Fig 6A). miR-195 and miR-16 overexpression alone exhibited inhibitory effects on tumor growth, which indicated that miR-195 and miR-16 might function as a tumor suppressor (Fig. 6A). To address whether combination therapy resulted in the generation of prolonged protective T cell immunity, 30 days after complete tumor rejection, Tramp mice were re-challenged with a much higher dose of Trampc1 tumor cells on the opposite flank. No palpable tumors were detected in the treated mice after a few weeks, whereas tumors were palpable after 1 week in naive mice (Fig. 6B).

Regulatory T cells (Tregs) and Myeloid-derived suppressor cells (MDSCs) are believed to be the major components of the immune suppressive cells in the tumor microenvironment

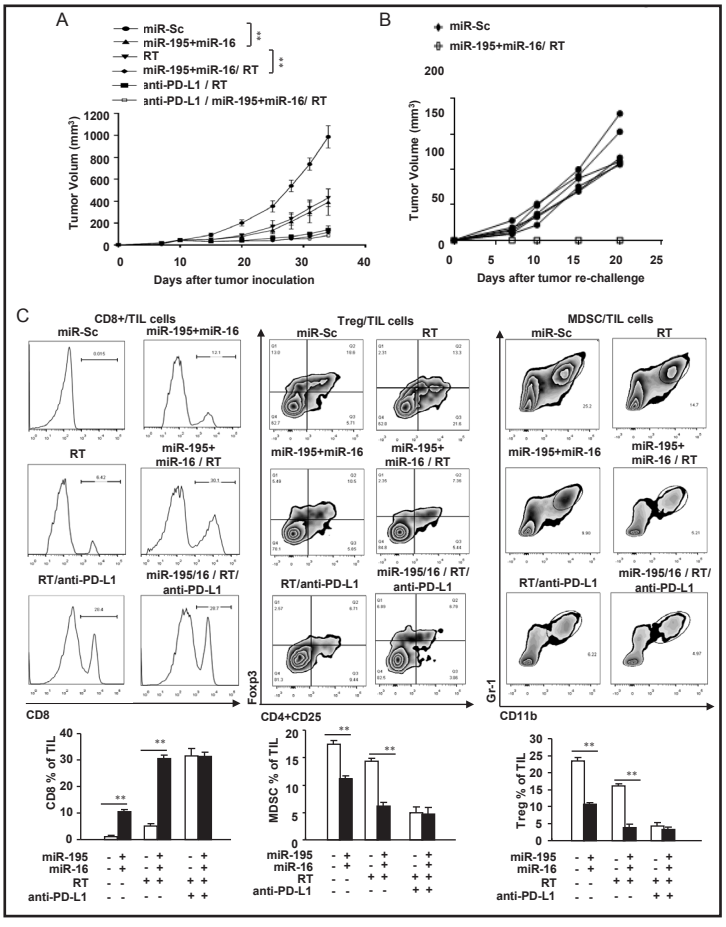

Fig. 6. miR-195/-16 enhanced the radiotherapy efficacy of Trampc1 tumors by regulating immunocyte production in the tumor microenvironment. Trampc1 cells (5×10^6) with stable overexpression of miR-195/-16 were implanted into syngeneic Tramp mice followed by radiation or anti-PD-L1 treatment. (A) Tumor volumes of the mice were characterized in different treatment groups. (B) Tumor-free mice that underwent combination therapy were resistant to the tumor re-challenge. Thirty days after tumor eradication the mice were re-challenge with Trampc1 cells on the opposite flank. (C) CD8+ T cells, Tregs, and MDSCs were isolated from the tumors of Trampc1 tumor-bearing mice and counted by FCM analysis. CD8+ TIL (CD45+) infiltration as a percentage of total leucocytes. Changes in CD4+ CD25+ Foxp3+ Tregs as a percentage of TILs. Representative of MDSCs gated on a CD45+ cell populations are shown. Bar graphs are shown as the mean ± SEM (n = 12 mice/group). * $p \le 0.05$, ** $p \le 0.01$.

and have been associated with radioresistance [22]. These two cell types have been shown to be expanded in preclinical tumor models and to promote T cell dysfunction that favors tumor progression [22]. Our present work demonstrated that PD-L1 is required for miR-195 and miR-16 to inhibit Trampc1 tumor growth. We examined the abundance of CD8+ T cells, MDSCs and Tregs in Trampc1 tumors using a FACScan flow cytometer and the same animal model. As shown in Fig. 6C, radiation treatment significantly increased the number of CD8+ T cells and decreasedMDSC and Treg levels in the Tramp mice with miR-195 and miR-16 overexpressing tumors, whereas anti-PD-L1 treatment blocked the efficacy of the combination treatment. These results indicate that miR-195 and miR-16 might activate the T cell immune response and repress T cell dysfunction during radiotherapy through a PD-L1-dependent pathway. Taken together, these findings further demonstrated that miR-195 and miR-16 enhanced the efficiency of radiotherapy by regulating CD8+ T cell, MDSC and Treg abundance in the tumor microenvironment through a PD-L1-dependent pathway.

miR-195/-16 enhances the radiotherapy efficacy of Trampc1 tumors by activating cytotoxic T cells and reducing regulatory cytokine secretions in the tumor microenvironment

Antigen-specific cytotoxic T cells (CTLs) play indispensable role in the immune response to infectious pathogens [23]. IFN-γ expression is a surrogate marker of CTL levels [23]. As shown in *Fig. 7A*, radiation therapy significantly increased the number of IFN-γ+/CD8+ T cells in Tramp mice with miR-195 and miR-16 overexpressing tumors, whereas anti-PD-L1 treatment eliminated the efficacy of the combination treatment, which suggested that miR-195 and miR-16 also enhanced the efficacy of radiotherapy by CTL activation through a PD-L1-dependent pathway.

We have shown that miR-195/-16 directly regulates PD-L1, and also that miR-195/-16 blocks the expression of PD-L1 to regulate T cell cytokine secretions in a DU145/T cell co-culture model. Subsequently, we measured the cell surface PD-L1 expression in Trampc1 tumor cells. FCM analysis showed that cell surface PD-L1 expression was significantly increased following treatment with radiation alone (Fig. 7B). Restoration of miR-195 and miR-16 expression in radiation-treated Trampc1 tumors resulted in decreased cell surface PD-L1 expression (Fig. 7B). Furthermore, we measured the mRNA levels of PD-L1, CD80, CD8, TNF-α and IFN-γ via qRT-PCR in Trampc1 tumors. A similar result was observed. As shown in Fig. 7C, the mRNA levels of PD-L1 and CD80 significantly increased in Trampc1 tumors following radiation therapy alone. Restoration of miR-195 and miR-16 expression in radiation-treated Trampc1 tumors resulted in decreased PD-L1 and CD80 expression. Furthermore, the mRNA levels of CD8, TNF-α and IFN-γ were increased in Trampc1 tumors following treatment with radiation alone. The effects of radiation on CD8, TNF-α and IFN-γ were enhanced when miR-195 and miR-16 were overexpressed (Fig. 7C). We subsequently measured T cell cytokine secretions in the serum of Tramp mice. As shown in Fig. 7D, radiation treatment did not inhibit the secretion of IL-10 and TGF-β in the Tramp mice carrying miR-Sc-overexpressing tumorsbut clearly inhibited the secretion of IL-10 and TGF-β in the Tramp mice with miR-195 and miR-16 overexpressing tumors. Moreover, radiation treatment increased the secretion of TNF-α and IFN-γ in the Tramp mice carrying miR-Sc overexpressing tumors. However, radiation treatment significantly induced the secretion of TNF-α and IFN-γ in the Tramp mice with miR-195 and miR-16 overexpressing tumors (Fig. 7D), which suggested that miR-195/-16 enhanced the efficacy of the radiotherapy by regulating the secretions of regulatory cytokines. Taken together, these findings demonstrated that miR-195/-16 might improve the immune evasion of radiotherapy-induced PD-L1-associated T cells through PD-L1 signaling (Fig. 7E).

Discussion

Unfortunately, many more patients will experience increasing levels of prostate-specific antigen (PSA) following radiotherapy, a condition BCR [24]. Physicians treating patients with BCR are faced with a difficult series of decisions, attempting to delay the occurrence of

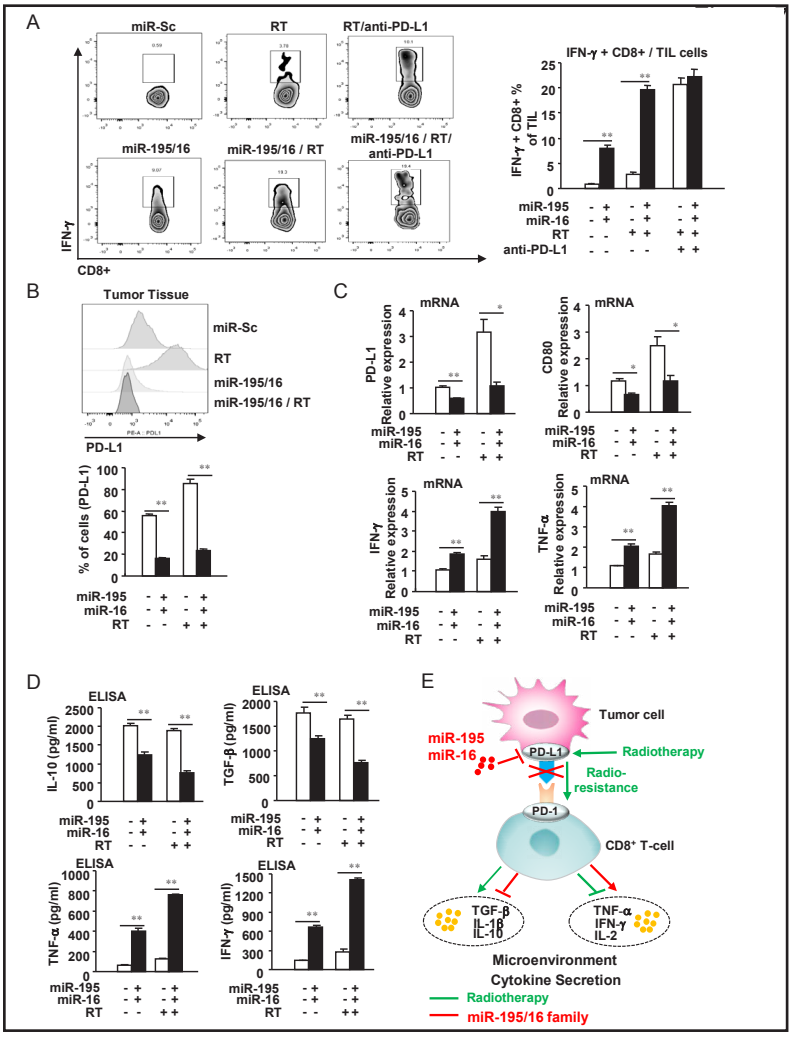

Fig. 7. miR-195/-16 enhance the radiotherapy efficacy of Trampc1 tumors by reducing regulatory cytokine secretions in the tumor microenvironment. Trampc1 cells (5×10^6) with stable overexpression of miR-195/-16 were implanted into syngeneic Tramp mice, followed by radiation or anti-PD-L1 treatment. T cells were harvested from regressing tumors and stained with various markers. Mouse circulating serum was collected 72h after radiation treatment. (A) IFN-γ+/CD8+ T cells were isolated from tumors of Trampc1 tumor-bearing mice and counted. * $p \leq 0.05$, ** $p \leq 0.01$. Bar graphs are shown as mean ± SEM (n = 12 mice/group). (B) Expression of cell surface PD-L1 was determined by FCM in Trampc1 tumors. * $p \leq 0.05$, ** $p \leq 0.01$. Bar graphs are shown as the mean ± SEM (n = 12 mice/group) (C) Relative expression levels of PD-1, PD-L1, CD80, TNF-α and IFN-γ from tumors of Trampc1 tumor-bearing mice were determined via qRT-PCR assay. * $p \leq 0.05$, ** $p \leq 0.01$. Bar graphs are shown as mean ± SEM (n = 12 mice/group). (D) Circulating serum from Tramp mice was assayed for IL-10, TNF-α, IFN-γ, and TGF-β by cytokine ELISA. ** $p \leq 0.01$. Bar graphs are shown as the mean ± SEM (n = 12 mice/group). (E) Schematic representation of the biological and functional interaction between PD-L1 and radiotherapy through the miR-195/-16 family regulatory cascade.

metastatic disease and death while keeping patients whose disease may never affect their overall survival or quality of life from being over-treated [24]. Radiotherapy is a standard treatment option for both organ-confined and regionally advanced prostate cancer [25]. Radiotherapy enhances tumor immunogenicity and antigen presentation, increases cytokine production, and alters the microenvironment of tumor cells. Radiation-induced adaptive immunity has the potential to improve outcomes in aggressive prostate cancer because it involves high levels of PD-L1 expression [26]. Additionally, fractionated radiotherapy induces the up-regulation of PD-L1 through CD8+ T cell production of IFN-γ. Therefore, further investigation into the mechanisms regulating the immune response have revived interest into the use of combination radiation and immune-based therapies to improve local control and prolong BCR-free survival in prostate cancer. The evidence shows that a growing number of miRNAs have been identified to be important regulators of radiosensitivity in kinds of tumors [27-29], however, the mechanism remains unclear.Here, we found that high expression levels of miR-195 and miR-16 were positively correlated with the BCR-free

survival time of prostate cancer patients but inversely correlated with PD-L1, PD-1, CD80 and CTLA-4 expression in this dataset. Functional investigations revealed that miR-195/-16 induced an immune response to radiotherapy via activation of the T cell response by blocking the PD-L1 immune checkpoint. These results indicated that endogenous miR-195/-16 plays an important role in the radioresistance of prostate cancer. Suppression of PD-L1 may be possible by promoting the expression of endogenous miR-195 and miR-16, thereby improving the therapeutic index of the radioresistance-based increase in PD-L1 expression.

A MRX34 clinical trial is currently underway at MD Anderson. MRX34, an investigational drug with the ability to mimic the tumor-suppressing capacity of miR-34, increases CD8+ T cells of the immune system when combined with radiotherapy [30]. Gene therapy based on miRNA provides an attractive antitumor approach to comprehensive cancer therapy. In our previous work, we found that miR-424(322) could reverse chemoresistance via T-cell activation by blocking PD-L1 expression in ovarian cancer [17]. In the current study, mechanistic investigations indicated that the miR-195 and miR-16 family also induced an immune response by blocking the PD-L1 immune checkpoint of radiotherapy in prostate cancer. Our research revealed a novel mechanism by which this miRNA family influenced the PD-L1/PD-1 signaling pathway. Further studies are needed to confirm these findings in other tumors.

By creating a tumor immune environment that allows the lesion to lude immune system attack, cancer cells that grow to form invasive tumors may escape the immune system [31]. Modification of the tumor microenvironment can involve the down regulation of major histocompatibility complex molecules or the induction of immune inhibitory cytokines and chemokines, which leads directly to activated T cells and the proliferation and activation of immunosuppressive cell populations such as Tregs and MDSCs [32]. In this study, the tumor immune environment could be actively manipulated by the miR-195/-16 family to revert the tumor environment, allowing the activation of CD8+ T cells, inhibition of MDSCs and Tregs, and induction of cytokines such as IFN-γ, TNF-α, and IL-2 that are associated with cancer eradication.

A recent study demonstrated that blockage of PD-L1 restored immune suppression and resulted in the expansion of tumor-infiltrating lymphocytes (TILs) [33]. An increased number of CD4 and CD8+ cells is related to higher CD8/Treg and CD4/Treg ratios [34]. This is related to a moderate clinical response, together with molecular evidence of functional activation of TILs. A parallel increase in MDSCs reveals the archetypal tumor immunosuppressive environment [35]. In this model, miR-195/-16 in combination with radiotherapy was shown to be highly effective. This effect is likely due to the downregulation of high expression levels of PD-L1 by miR-195/-16 in Trampc1 tumor cells as well as tumor-derived myeloid cells. The current research results show that tumors lacking TILs may have PD-L1 mediated suppression, leading to TIL depletion. Our findings suggest that high frequency of Tregs and MDSCs in tumors lacking TILs is associated with poorly performing Tregs in human prostate cancer. Our work has shown, using the combination of miR-195/-16 and radiotherapy that miR-195/16 regulates the production of CD8+ T cells, MDSCs and Tregs, which activate CTLs and reduce the secretions of regulatory cytokines and ultimately, enhance radiotherapy efficacy.

Conclusion

In summary, we identified a novel mechanism by which miR-195 and miR-16 influence PD-L1/PD-1 immune checkpoint signaling pathway. Restoration of the expression of miR-195/-16 could enhance radiotherapy via activation of the T cell response in the tumor microenvironment by blocking the PD-L1 immune checkpoint. This synergy between radiotherapy and immunotherapy was accompanied by the proliferation of functional cytotoxic CD8+ T cells and inhibition of MDSCs and Tregs. This work indicates a biological and functional interaction between immunotherapy and radiotherapy through the miR-195/-16 family regulatory cascade.

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Disclosure Statement

The authors declare to have no competing interests.

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