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ORIGINAL RESEARCH

MicroRNA-200c Nanoparticles Sensitized Gastric Cancer Cells to Radiotherapy by Regulating PD-L1 Expression and EMT

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Lingyu Qian[1,2,*]
Fangcen Liu[3,*]
Yanhong Chu[1]
Qingqing Zhai[1]
Xiao Wei[3]
Jie Shao[1]
Rutian Li[1]
Qiuping Xu[1]
Lixia Yu[1]
Baorui Liu[1]
Qin Liu[1]

[1]The Comprehensive Cancer Centre of Drum Tower Hospital, Nanjing University Medical School & Clinical Cancer Institute of Nanjing University, Nanjing, People's Republic of China; [2]Department of Oncology, Rudong Peoples' Hospital of Jiangsu Province, Nantong, People's Republic of China; [3]Department of Pathology, Affiliated Nanjing Drum Tower Hospital of Nanjing University Medical School, Nanjing, People's Republic of China

*These authors contributed equally to this work

Introduction: Immuno-checkpoint inhibitors (ICIs) in advanced gastric cancer either as monotherapy or in combining strategies are rapidly evolving but still in early phase. Various efforts have been made to provide insights into regulating immune checkpoint molecule programmed cell death ligand-1 (PD-L1) expression to improve ICIs efficacy. The aim of this study was to investigate the effect and potential mechanism of miR-200c nanoparticles combined with radiotherapy in gastric cancer cells.

Methods: We prepared miR-200c-loaded nanoparticles (miR-200c NPs) to achieve targeted delivery of miR-200c to AGS cells. The roles of miR-200c NPs and radiotherapy in regulating the viability of AGS cells were assessed by CCK-8 toxicity test and Annexin V-FITC/PI apoptosis kit. Flow cytometry was used to analyze expression of PD-L1 and CD44 on the surface of AGS cells treated by miR-200c NPs and/or ionizing radiation. Enzyme-linked immunosorbent assay (ELISA) was used to test the level of transforming growth factor-beta 1 (TGF-β1) secreted by AGS cells. The cooperation mechanism between miR-200c NPs and radiotherapy was also explored in vitro.

Results: Compared with naked miR-200c mimics, miR-200c NPs significantly downregulated PD-L1 expression of gastric cancer cells. The combination of miR-200c NPs and radiotherapy showed significantly synergistic inhibitory effect on gastric cancer cells by inhibiting immune escape mediated by PD-L1, reversing EMT phenotype as well as abrogating cancer stem cells (CSCs)-associated properties of tumor cells.

Conclusion: MiR-200c NPs sensitized gastric cancer cells to radiotherapy by regulating PD-L1 expression and EMT.

Keywords: microRNA-200c, gastric cancer, nanoparticle, PD-L1, radiotherapy

Introduction

Gastric cancer (GC) is the third cause of cancer-related mortality worldwide as described in GLOBOCAN 2018.[1] A large proportion of gastric cancer patients were diagnosed in the late-stage, resulting in poor prognosis. In the few decades, the programmed death receptor-1 (PD-1)/programmed death ligand-1 (PD-L1) axis inhibitors showed improvement in overall survival (OS) and objective response rate (ORR) in advanced gastric or gastroesophageal junction (G/GEJ) cancer treated with ≥2line chemotherapy regimens.[2] However, only 11.9% patients treated with nivolumab showed tumor shrinkage in the Phase 3 ATTRACTION-2 trial.[3] It is imperative to develop new strategies to improve the survival time of gastric cancer.

Correspondence: Qin Liu
The Comprehensive Cancer Centre of Drum Tower Hospital, Nanjing University Medical School & Clinical Cancer Institute of Nanjing University, Nanjing 210008, People's Republic of China
Email liuqinxh@126.com

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The expression of PD-L1 is an important biomarker for immune checkpoint inhibitors (ICIs) in gastric cancer treatment. Objective responses were observed correlatively of PD-L1 tumor expression and across all treatment lines in advanced G/GEJ cancer.[4] As a local intervention, radiotherapy can reshape tumor immune microenvironment[5] and induce PD-L1 expression in tumors.[6,7] Emerging evidence has identified that the combination of radiotherapy and ICIs is promising in clinical outcomes improvement.[8] Furthermore, epithelial-mesenchymal transition (EMT) is closely related to drug and radiotherapy resistance.[9] EMT/Transforming growth factor-β (TGF-β) axis is also critical to PD-L1-based immunosuppression.[10,11] We previously showed that miR-200c could reverse EMT, inhibit tumor metastasis as well as suppress cancer stem cells.[12] Chen et al showed that miR-200c could directly regulate PD-L1 by binding the 3′noncoding region (3-'UTR) of PD-L1.[13] Therefore, miR-200c can sensitize with radiotherapy through reversing EMT, inhibiting cancer stem cells (CSCs) and PD-L1.

MicroRNAs are extremely unstable, so how to deliver them effectively is an important problem to solve. Many studies have reported that nanoparticles (NPs) possess the properties to controlled release, improve drug stability and tumoral targeting delivery strategy.[14] In this study, we prepared miR-200c-loaded nanoparticles (miR-200c NPs) to achieve targeted delivery miR-200c to tumor cells. We also evaluated investigated synergistic inhibition effect and potential mechanism of miR-200c NPs combing with radiotherapy in human gastric cancer cells.

Materials and Methods

Preparation of MiR-200c NPs

MiR-200c mimics and miR-200c-NH_2 were synthesized from Shanghai GenePharm HD company (Shanghai, China). 2-distearoyl-sn-glycero-3-phosphoethanolamine-N-methoxy (polyethylene glycol) N-hydroxylsuccinimide (DSPE-PEG-NHS) was purchased from Seebiotech company (Shanghai, China). The DSPE-PEG-NHS and 12.5 μM miR-200c-NH_2 (1:1, v/v) were mixed in diethyl pyrocarbonate (DEPC) water. The mixture was agitated overnight on a magnetic stirrer at room temperature. Finally, the miR-200c NPs were purified through high speed centrifugation (15000rpm, 10min), washed with DEPC water three times and then stored at −80°C for further use.

The morphology of miR-200c NPs was measured by transmission electron microscope (TEM, JEOL, Japan). The particle size and zeta potential of miR-200c NPs were analyzed using dynamic light scattering (DLS, Malvern Instruments Corporation, UK). The miR-200c in nanoparticles was quantified by microplate spectrophotometer (Tecan Austria GmbH, Grodig, Austria) with the wavelength set to 260nm. The loading efficiency was calculated according to the following equation:

$$\text{Loading efficiency}(\%) = \frac{\text{Weight of the miR} - \text{200c in NPs}}{\text{Weight of the NPs}} \times 100 \quad (1)$$

Cell Viability Assay

The human gastric cancer AGS cells were purchased from the Shanghai Institute of Cell Biology (Shanghai, China) and maintained in Roswell Park Memorial Institute 1640 medium with 10% fetal bovine serum, 50 units/mL penicillin, and 50 units/mL streptomycin in 5% CO_2 at 37°C. The AGS cells were digested and collected, then were inoculated in 96-well plates at a density of 3×10^3 per well. On the second day, the AGS cells were treated with PBS, miR-200c NPs (100nM or 200nM), 10Gy ionizing radiation (IR), miR-200c NPs (100nM or 200nM) + 10Gy IR. After 48 hours, 10μL CCK-8 Solution (Vazyme Biotech Company, Nanjing, China) was added. The absorbance of each well at 450 nm was obtained using a microplate reader (TECAN, Switzerland). We calculated the cell viability according to the following equation:

$$\text{Cell viability}(\%) = \frac{\text{OD of experimental group} - \text{OD of blank group}}{\text{OD of control group} - \text{OD of blank group}} \times 100$$

Radiotherapy was carried out using a 6 MeV electron beam linear Elekta accelerator (Stockholm, Sweden).

Flow Cytometry

The AGS cells were seeded in six-well plates (1×10^5/well). After 24 hours, the AGS cells were treated with various treatments as follows: PBS, 10Gy IR, 20ng/mL TNF-α + 20ng/mL IFN-γ, 20ng/mL TNF-α + 20ng/mL IFN-γ+ 10Gy ionizing radiation. After 48 hours, tumor cells were digested by trypsin and collected, then incubated with anti-human CD274 antibody (Becton, Dickinson and Company, USA) for 30 minutes at room temperature. The expression of PD-L1 on the surface of AGS cells was analyzed by a flow cytometry.

To examine the PD-L1 downregulation effect of miR-200c NPs, the AGS cells were treated with different concentration of miR-200c NPs (0, 100nM, 200nM, 300nM) with or without 10Gy IR after being treated with 20ng/mL TNF-α + 20ng/mL IFN-γ for 48 hours. Then, AGS cells were trypsinized and collected for PD-L1 and CD44 expression as well as apoptosis analysis. AGS gastric cancer cells were co-labeled with Annexin V-Alexa Fluor 488 and propidium iodide for apoptosis analysis according to the manufacturer's protocols. For TGF-β1 examination, the culture supernatant and cells were collected. The level of TGF-β1 in the cell culture supernatant was assessed using an enzyme-linked immunosorbent assay (ELISA) kit (Lianke biotechnology company, Hangzhou, China) in accordance with the manufacturer's protocol.

Western Blot

For Western blot analysis, AGS cells treated by different groups were lysed in RIPA Lysis Buffer supplemented with a protease inhibitor. The protein lysates were separated by SDS-PAGE and transferred to nitrocellulose (NC) membranes. After blocking with nonfat milk, the membranes were sequentially incubated with primary antibodies and secondary antibodies. The immunoreactive protein images were captured using a G: BOX chemiXR5 system and GAPDH was used as control.

The Statistical Analysis

Each experiment was performed independently at least three times. The values were shown as means ± standard deviation (SD) in the manuscript were means. Comparisons between groups were evaluated by the Student's *t*-test. $P < 0.05$ were considered to be statistically significant values.

Results

Characterization of MiR-200c-Loaded Nanoparticles

MiR-200c NPs are self-assembled micelles from amphiphiles composed of miR-200c sequence and a diacyl lipid tail. In aqueous solutions, these amphiphiles self-assembled into three-dimensional spherical micelles. As shown in Figure 1, miR-200c NPs showed a miR-200c corona and a lipid cores with a diameter of around 20 nm. The diameter size obtained by DLS analysis was 17.47nm. The miR-200c NPs exhibited a negative surface charge with -22.57 ± 0.42mV zeta potential. The loading efficiency of miR-200c in nanoparticles was 6.22%.

Cell Proliferation Inhibition of MiR-200c NPs Combined with Radiotherapy

We next evaluated whether miR-200c NPs could enhance the antitumor effects of radiotherapy. As shown in Figure 2A, the cell suppression rates of miR-200c NPs (100nM), miR-200c NPs (200nM) and radiation against AGS cells were 18.86%, 49.18% and 20.18%, respectively. Compared with radiation alone, miR-200c NPs + IR showed a higher cytotoxic effect ($p < 0.01$). When the concentration of miR-200c increased to 200nM, the cell growth inhibition was 71.87% ($p < 0.001$). These results indicated that miR-200c NPs could act as a sensitizer of radiation.

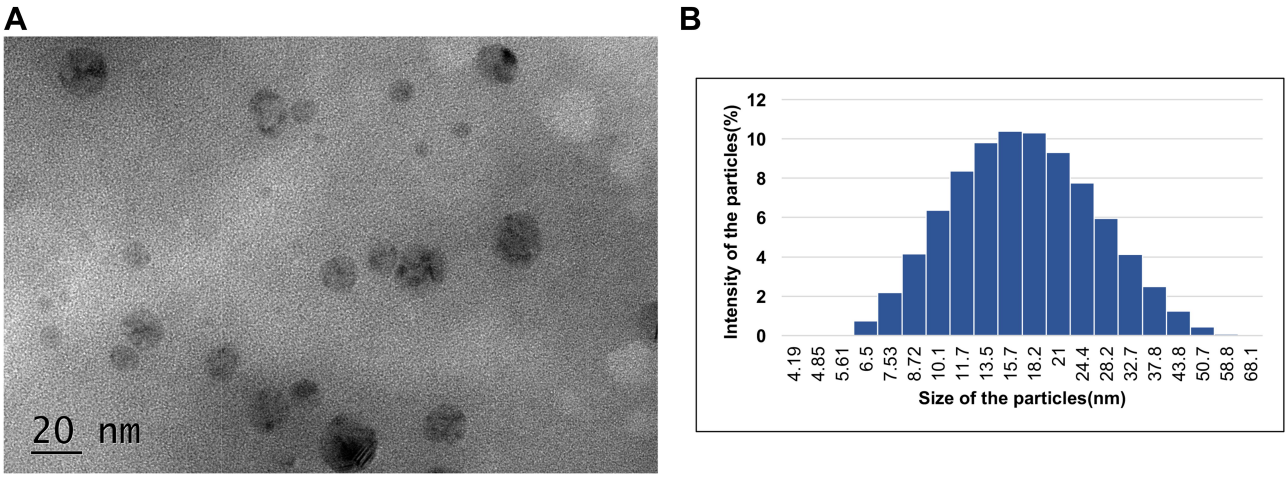

Figure 1 Preparation and characterization of miR-200c NPs. (**A**) The TEM images of miR-200c NPs; (**B**) the particle size distribution of miR-200c NPs by DLS.

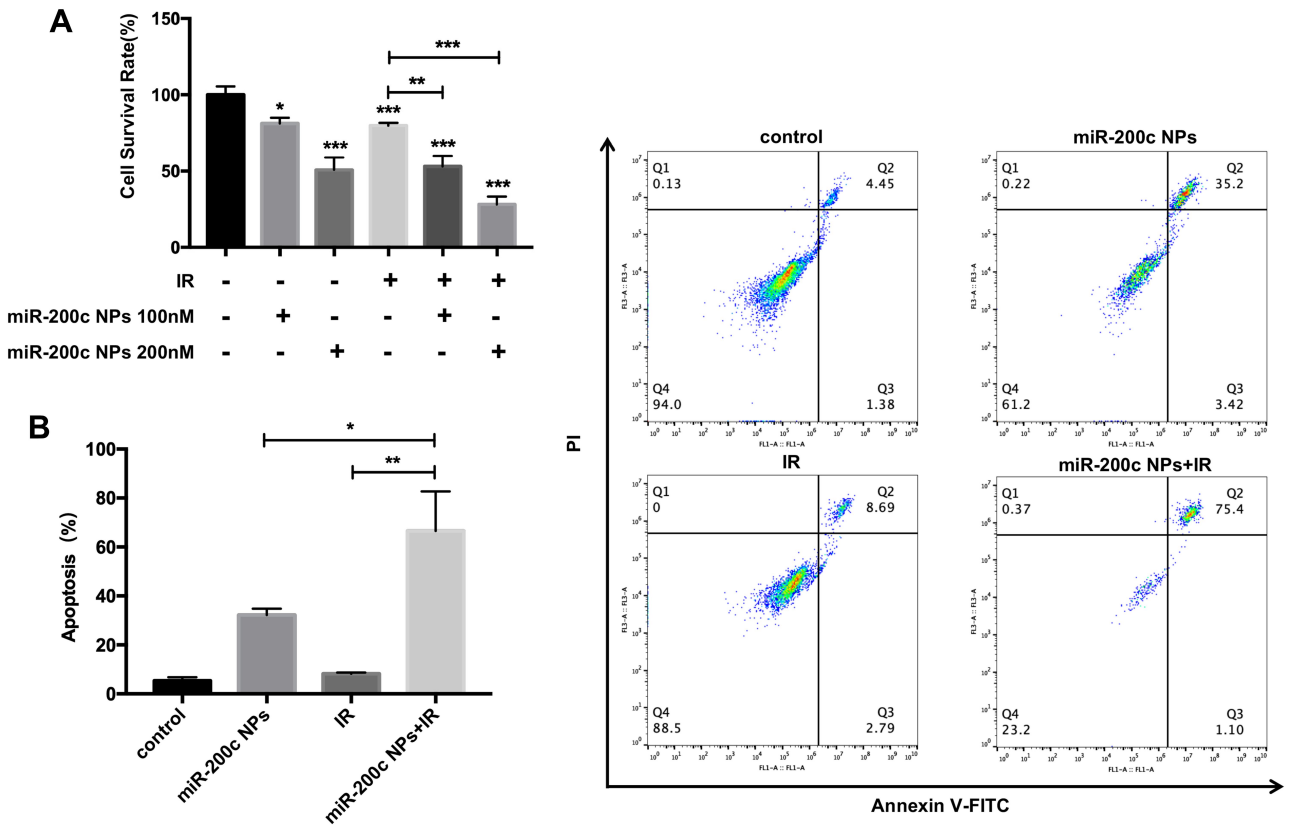

Figure 2 (**A**) The cell survival rate of miR-200c NPs combined with radiotherapy on AGS cells. (**B**) The apoptosis percentage of AGS cells treated with IR and/or miR-200c NPs (*P < 0.1, **P < 0.01, ***P < 0.001).

MiR-200c NPs Combined with Radiotherapy Induced the Apoptosis of the AGS Cells

Apoptosis resistance is an important characteristic of mesenchymal cells or tumor stem cells, and it is also one of the key reasons for radiotherapy failure. As shown in Figure 2B, the apoptosis rate induced by miR-200c NPs and IR alone were 35.20% and 8.69%, respectively. Compared with IR or miR-200c NPs alone, the percentage of apoptotic cells was significantly increased after treatment with miR-200c NPs combined with IR ($P < 0.01$, $P < 0.1$).

MiR-200c NPs Inhibited PD-L1 Induced by Radiotherapy

TNF-α + IFN-γ was reported to stimulate PD-L1 expression. As shown in Figure 3A, IR could also up-regulate PD-L1 expression of gastric cancer cells. The combination of TNF-α + IFN-γ and radiation showed the most significant PD-L1 regulation ($P < 0.001$). We also examined the effects of different miR-200c formulations on the PD-L1 expression of AGS cells. Compared with naked miR-200c, miR-200c NPs significantly inhibited PD-L1 levels (Figure 3B). The PD-L1 inhibition ratio of miR-200c NPs increased as the concentration of miR200c increased (Figure 3C). More importantly, miR-200c NPs could inhibit the PD-L1 expression induced by radiotherapy on gastric cancer cells (Figure 3D). As the concentration of miR-200c increased, the inhibitory effect on the PD-L1 expression was strengthened.

MiR-200c NPs Combined with Radiotherapy Regulated EMT-Related Pathways

We also investigated the role of miR-200c NPs on regulating EMT signal ways. TGF-β acts as a critical immune suppressor in EMT-related tumor microenvironment.[15–17] We found miR-200c NPs or IR could inhibit TGF-β1 expression secreted by cancer cells. As miR-200c concentration increasing, the inhibition rate of TGF-β1 increased (Figure 4A). MiR-200c NPs combined with IR significantly inhibited the secretion of TGF-β1 in gastric cancer

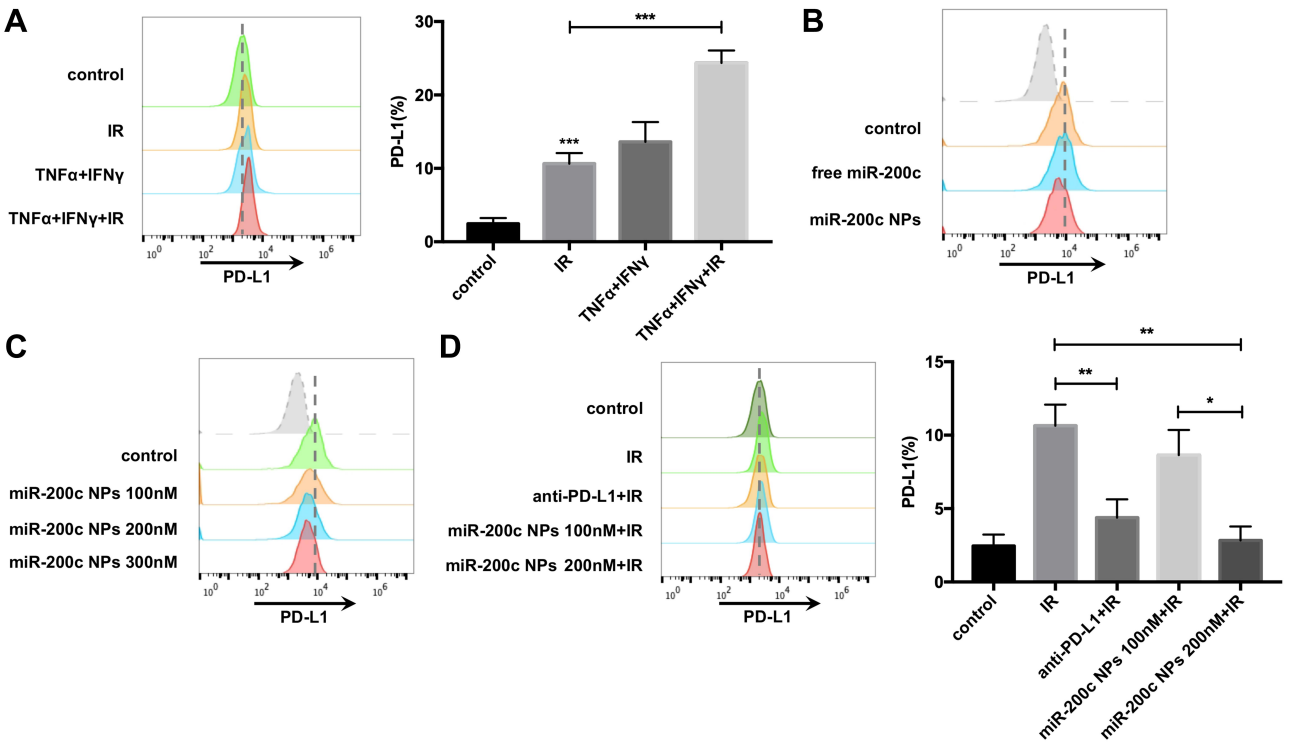

Figure 3 MiR-200c inhibited the expression of PD-L1. (**A**) Radiotherapy induced PD-L1 expression of AGS cells. (**B**) MiR-200c NPs significantly inhibited the expression of PD-L1 on gastric cancer cells. (**C**) The inhibitory effect on the PD-L1 expression was gradually strengthened as the concentration of miR-200c NPs increased. (**D**) Both miR-200c NPs and anti-PD-L1 could inhibit PD-L1 expression caused by radiotherapy (*P < 0.1, **P < 0.01, ***P < 0.001).

cells compared with IR alone (Figure 4B). EMT was also regulated by CD44, a marker of gastric cancer stem cells (CSC).[18] We investigated the effect of miR-200c NPs combined with IR on the proportion of CD44 positive AGS cells by flow cytometry. As shown in miR-200c NPs Figure 4C, 10Gy IR increased the proportion of CD44 positive AGS cells, while miR-200c NPs + IR significantly reduced CD44-positive AGS cells. The AGS cells treated by miR-200c NPs + IR showed increased E-cadherin expression, decreased β-catenin protein level (Figure 4D). Therefore, the miR-200c NPs can reverse EMT by inhibiting TGF-β1 and regulating E-cadherin/β-catenin pathway of gastric cancer.

Discussion

Immunotherapy has emerged as a major therapeutic modality in gastric cancer treatment. However, most patients with metastatic gastric cancer are unable to respond to PD-1/PD-L1 blockade therapy. Integrating radiotherapy into immunotherapy has gained substantial interest and improve therapeutic response.

PD-L1 inhibits T cell activation and causes T cell exhaustion by binding with T cell checkpoint PD-1. Abnormally high PD-L1 expression on tumor cells and antigen-presenting cells (APCs) in the tumor microenvironment mediates tumor immune escape and is related to the poor prognosis.[19] Tumor microenvironment (TME) plays an important role in immunotherapy. Radiotherapy is an important topical treatment strategy for gastric cancer. Radiotherapy can improve anti-tumor responses through attracting cytotoxic T lymphocytes, APCs to irradiated TME, increasing antigens release as well as MHC class I expression. By contrast, radiation therapy also showed inhibitory effects on immunomodulatory.[20] In preclinical models, radiotherapy could attract regulatory T cells (Tregs) infiltrating to tumor sites, induce immunosuppressive molecules, such as TGF-β and PD-L1 expression on macrophages, tumor cells as well as dendritic cells. On the other hand, due to the influence of tumor microenvironment and the limitation of surrounding normal organs, the curative effect of radiotherapy for gastric cancer is limited. Radiation-induced tumor regression needs a balance between adverse effects. In contrast to high-dose single-fraction radiation regimens, low-dose and repeated radiation might cause inappropriate IFN signaling way activation and tumor cell radioresistance. Therefore, combing treatments with

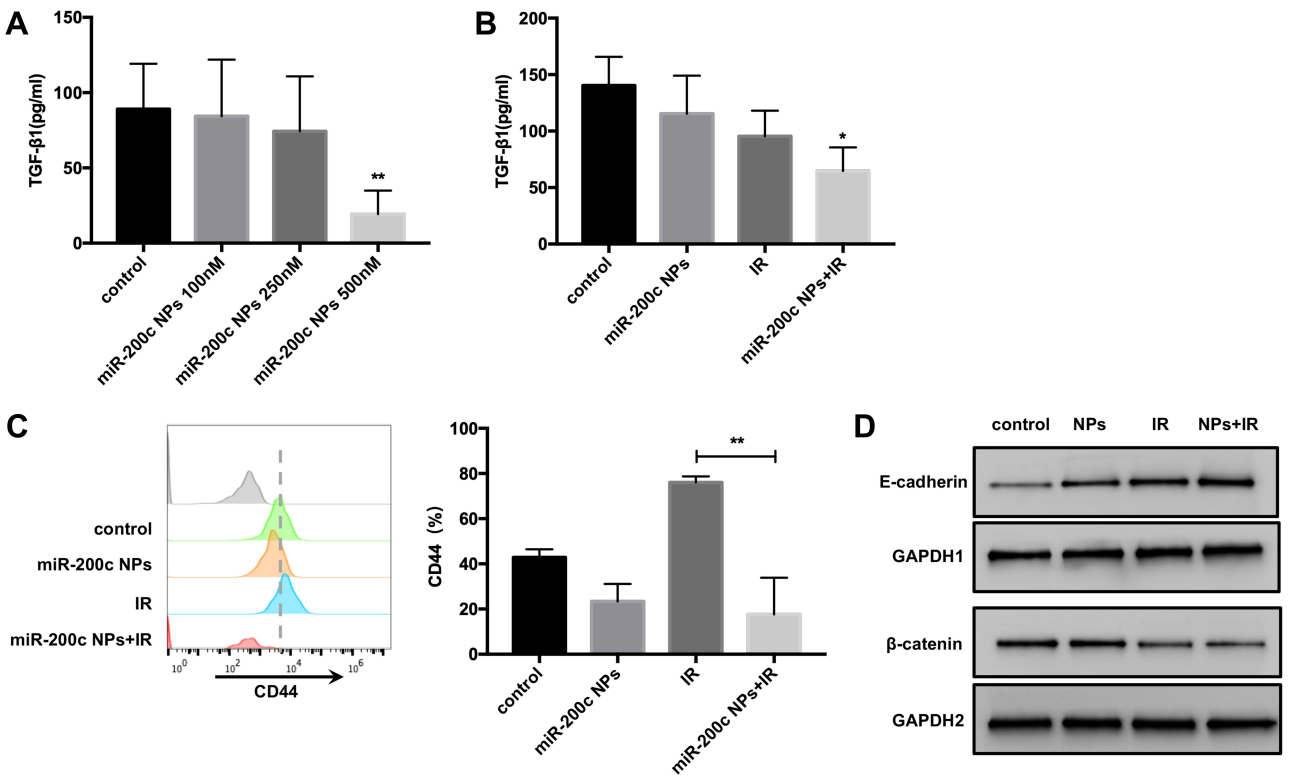

Figure 4 MiR-200c NPs and radiotherapy regulated EMT-related pathways. (**A** and **B**) The level of TGF-β1 in the cell culture supernatant was assessed after different treatments. (**C**) The CD44 expression was detected by flow cytometry. (**D**) The β-catenin and E-cadherin levels of AGS cells after different treatments (control, miR-200c NPs 200nM, 10Gy IR, miR-200c NPs 200nM + 10Gy IR) were analyzed by Western blot. (*$P < 0.1$, **$P < 0.01$).

radiotherapy and immune checkpoint inhibitors can reverse T-cell exhaustion and enhance radiosensitivity.[21,22] MiR-200c is a kind of endogenous noncoding small molecular RNA, which induces the degradation of mRNA or the inhibition of translation via binding to target mRNA 3′UTR.[23] Some studies have shown that PD-L1 is the downstream target of miR-200c, and miR-200c can directly target the 3′UTR of PD-L1 and inhibit the expression of PD-L1.[13] PD-L1 has been confirmed to negatively regulate tumor infiltrating lymphocytes (TILs), remodeling tumor immunosuppressive environment and increase the immune resistance of tumors. PD-L1 deficiency in tumor cells suppressed tumor growth and metastasis, high miR-200c and low PD-L1 expression manifested a significantly prolonged survival time.[24] In order to improve stability and decrease degradation, we prepared miR-200c NPs by FDA approved material DSPE-PEG. When given one-dose 10Gy irradiation, the PD-L1 expression on the surface of human gastric cancer cell AGS was greatly regulated. In the in vitro study, miR-200c NPs + IR showed a greater cytotoxic effect on the AGS cells than miR-200c NPs or IR alone. Apoptosis resistance is an important characteristic of mesenchymal cells or tumor stem cells, and it is also one of the key reasons for the failure of radiotherapy.[25] Then, we investigated the effect of miR-200c NPs + IR on the apoptosis of the AGS cells. It was found that the apoptosis of the AGS cells pretreated with miR-200c NPs was significantly higher than that only treated with radiotherapy. Based on the above results, miR-200c NPs and radiotherapy exhibited synergistic inhibitory effects on the gastric cancer cell proliferation and apoptosis.

Epithelial mesenchymal transition (EMT) is closely associated with disease progression and poor prognosis of various malignancies. TGF-β induced EMT of gastric cancer cells in preclinical model.[26] The levels of ZEB1 and PD-L1 were significantly upregulated in lung cancer cells treated with TGF-β. Bifunctional Fusion Protein Targeting TGF-β and PD-L1 demonstrated a manageable safety profile and clinical activity in heavily pretreated advanced GC/GEJC.[27] MiR-200c plays an important role on cancer invasion and metastasis as well as reversion of EMT.[28,29] In this study, we verified that the TGF-β and PD-L1 expression was significantly inhibited as miR-200c concentration increasing. Cancer stem cells (CSCs) play an important role in the development of cancer for their ability of self-renewal and

differentiation.[30] Moreover, EMT is involved in the development of cancer caused by CSCs, which is related to the acquisition of stem cells in epithelial tumor cells.[31,32] Our previous study showed that miR-200c is a highly selective and effective radiosensitizer in gastric cancer by inhibiting cancer stem cells (CSC)-like properties and the EMT process.[12] Stem cell marker CD44 is not an important regulator of mesenchymal phenotype.[33–35] The TGF-β1/CD44 dual inhibition showed stronger antitumor activity than the single inhibition.[18] In this study, we found that miR-200c NPs could not only decrease the level of TGF-β1 secreted by the AGS cells, but also reduce the percentage of CD44 positive cells in the AGS cells. Therefore, miR-200c NPs may effectively reverse EMT and improve the sensitivity of radiotherapy by means of multiple-suppressing for TGF-β1, PD-L1 and CD44.

Besides tumor cells, PD-L1 is also expressed on numerous host cells, such as dendritic cells and tumor-associated macrophages, promoting immune evasion. PD-L1 ligand on tumor cells also induce the expression of PD-1 on lymphocytes and activating PD-1/PD-L1 signal pathway caused T cell apoptosis or exhaustion. MiR-200/ZEB1 axis in tumor cells was reported to regulate $CD8^+$ TIL phenotype. MiR-200c helps efficiently exert antitumor effect by maintaining T cell activation and hindering T cell inhibition from tumor microenvironment. Over-expression of miR-200c could inhibit PD-L1 expression, reverse CD8+ T cell exhaustion and subsequent CD8+ T cell activation.[13] Tang et al reported that PD-L1 expressed in APCs negatively regulated and inhibited T cell activation.[36] Sufficient tumor regression needs blocking PD-L1 both in tumor cells and host myeloid cells. In the further in vivo experiments, we will explore the antitumor advantages of miR-200c and radiotherapy combination.

In summary, miR-200c NPs combined with radiotherapy have synergistic inhibitory effect on gastric cancer cells. The possible mechanisms include (Figure 5): (1) stable and effective delivery of miR-200c into the AGS cells by nanoliposomes; (2) inhibit the immunosuppressive action of PD-L1 on the surface of the AGS cells; (3) reverse EMT by co-suppressing TGF-β1 and CD44. MiR-200c NPs combined with radiotherapy may be a potential strategy for the treatment of gastric cancer. Further work should focus on in vivo research, especially the relevant specific mechanism on tumors and T cells in the tumor microenvironment.

Conclusions

Our study shows that miR-200c NPs combined with radiotherapy have a significant synergistic inhibitory effect on gastric cancer cells through reversing EMT and inhibiting immune escape mediated by PD-L1. In general, miR-200c NPs combined with radiotherapy provide a new idea for the treatment of gastric cancer, and provide experimental basis for the follow-up in vivo preclinical and clinical research.

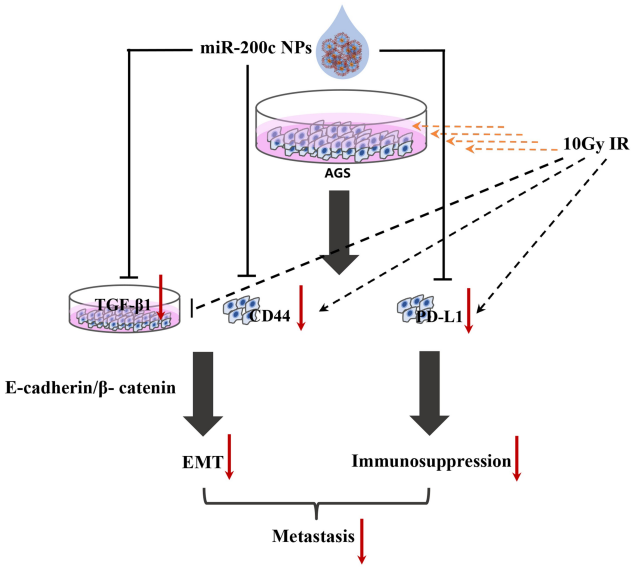

Figure 5 The mechanism of miR-200c NPs combined with radiotherapy inhibiting the proliferation of gastric cancer cells (indicated by the red arrow).

Acknowledgments

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Disclosure

The authors declare no competing interests.

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