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MiR-205 suppresses autophagy and enhances radiosensitivity of prostate cancer cells by targeting TP53INP1

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Abstract. – OBJECTIVE: This study aimed to investigate the role of miR-205 in radiosensitivity and autophagy of prostate cancer cells and to explore its regulative effect on TP53INP1.

MATERIALS AND METHODS: MiR-205 expression was compared in three prostate cancer cell lines (DU145, PC-3 and LNCaP) and one normal human prostate epithelial cell line (RWPE-1). The effect of irradiation-induced autophagy on radiosensitivity of the cancer cells and the effect of miR-205 on irradiation-induced autophagy were explored. The regulative effect of miR-205 on TP53INP1 and the function of this axis was further studied.

RESULTS: Ectopic expression of miR-205 substantially reduced the survival fraction of both DU145 and LNCaP cells to irradiation and inhibited irradiation-induced autophagy. Irradiation-induced autophagy acted as a protective mechanism in prostate cancer cells. TP53INP1 is a direct functional target of miR-205 in irradiation-induced autophagy and radiosensitivity regulation.

CONCLUSIONS: The miR-205/TP53INP1 mediated autophagy pathway might be an important molecular mechanism regulating radiosensitivity of prostate cancer cells and represents a potential therapeutic target for prostate cancer.

Key Words:
Prostate cancer, Autophagy, miR-205, TP53INP1, Radiosensitivity.

Introduction

Prostate cancer is the leading cause of male malignancy related deaths across the world [1]. Radiotherapy is an adjuvant treatment of surgery and is also a therapeutic choice for the patients with regionally unresectable advanced prostate cancers[2]. However, successful radiotherapy largely depends on tumor radiosensitivity and the tolerance of normal tissues. Although technological advances have improved the efficacy of radiation delivery, unfortunately the rate of biochemical/ clinical relapse among the prostate cancer patients undergone radiotherapy remains high[3]. Therefore, it is necessary to further investigate the underlying molecular mechanisms of radioresistance of prostate cancer.

Autophagy is a cellular process that involves degradation of cytoplasmic components and recycling of the destroyed cell organelles for new cell formation. It is a natural pro-survival mechanism of cells under stressful conditions, including tumor cells[4]. Due to the protective effects, some recent studies found autophagy has been implicated in tumor resistance to chemotherapy and radiotherapy[5,6]. MicroRNAs (miRNAs) are one of the key regulators of autophagy[7,8], some studies also found dysregulated miRNAs can regulate chemoresistance and radioresistance through modulating autophagy[9,10].

MiR-205 is usually downregulated in prostate cancer and its low expression is associated with poor survival[11,12]. Importantly, one recent study found miR-205 can impair the autophagic flux and enhance cisplatin cytotoxicity in castration-resistant prostate cancer cells[13], suggesting that miR-205 is implicated with autophagy in prostate cancer. Tumor protein p53-inducible nuclear protein 1 is a protein encoded by the TP53INP1 gene. Its overexpression in prostate cancer correlates with poor prognosis and is predictive of biological cancer relapse[14,15]. In addition, TP53INP1 can directly interact with molecular actors of autophagy, such as LC3 and ATG8-family proteins, thereby promoting autophagy[16,17]. Therefore, it seems these two genes might be connected in some way in autophagy.

This study investigated the role of miR-205 in radiosensitivity and autophagy of prostate cancer cells and also explored its regulative effect on TP53INP1 in irradiation-induced autophagy.

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Materials and Methods

Cell Culture

Prostate cancer cell lines, including DU145, PC-3 and LNCaP cells and normal human prostate epithelial cell line RWPE-1 were obtained from American Type Culture Collection (ATCC). The cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin. All cells were cultured in a humidified air containing 5% CO_2 at 37°C.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total miRNAs were extracted from cell samples using the miRVana miRNA Isolation Kit (Life Technologies, Carlsbad, CA, USA) according to manufacturer's instruction. MiR-205 expression was quantified using TaqMan MicroRNA Assay Kit (Applied Biosystems, Foster City, CA, USA), with U6 snRNA used as the endogenous control.

Total RNA was extracted from the cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. The cDNA library was synthesized using the PrimeScript RT reagent kit (TaKaRa, Dalian, Liaoning, China). The PCR primers for TP53INP1 were: forward, 5'-TCAGCAGAAGAAGAAGAAGAAGAG-3', reverse, 5'-AGCAGGAATCACTTGTATCAGC-3'. β-actin was used as the internal control. QRT-PCR for miRNA and mRNA was performed using SYBR Premix Ex Taq II (TaKaRa, Dalian, Liaoning, China) with an ABI 7500 Sequence Detection System (Perkin Elmer/Applied Biosystems, Rotkreuz, Switzerland). The results were calculated using the $2^{-\Delta\Delta CT}$ methods.

Irradiation and Clonogenic Assay

48 hrs after treatments, cells were seeded in six-well plates and exposed to radiation using 6 MV X-ray generated by a linear accelerator (Varian 2300EX, Varian, Palo Alto, CA, USA) at a dose rate of 5 Gy/min. Then, the plates were further incubated for 10 to 14 days and then the cells were fixed with 10% methanol and stained with 1% crystal violet in 70% ethanol. Colonies with more than 50 cells were counted under a light microscope. Survival fraction was defined as the number of colonies/ (cells inoculated × plating efficiency). Radiation survival curve was derived from multi-target single-hit model: SF = 1-(1-exp(-x/D0))^N.

Western Blot Analysis

Whole-cell lysate was prepared using a lysis buffer (Beyotime, Shanghai, China) containing proteinase and phosphatase inhibitor cocktails (Sigma-Aldrich, St. Louis, MO, USA). The protein concentration was determined using a BCA protein assay kit (Thermo Scientific, Rockford, IL, USA). The proteins were separated on 10% SDS PAGE gel and transferred onto polyvinylidene difluoride membranes. The membranes were blocked with 5% nonfat milk for 1 hr and then incubated with primary antibodies overnight at 4□. The primary antibodies used include rabbit anti-LC3B (ab51520, 1:3000; Abcam, Cambridge, MA, USA), anti-p62/SQSTM1 (#8025, 1:1000; Cell Signaling, Danvers, MA, USA), anti-cleaved PARP (#9546, 1:2000: Cell Signaling, Danvers, MA, USA), anti-TP53INP1 (ab9775, 1:1000; Abcam, Cambridge, MA, USA) and anti-β-actin (ab189073, 1:1000; Abcam, Cambridge, MA, USA). After three times washing, the membranes were incubated in horseradish peroxidase-conjugated secondary antibody for 1 hr at room temperature. Then, the protein band was visualized using the ECL Western blotting substrate (Promega, Madison, WI, USA).

Preparation of Stable GFP-LC3-Expressing LNCaP Cells

For stable infection, a recombinant lentivirus containing GFP-LC3 was obtained from Genepharma (Shanghai, China). LNCaP cells were infected with the recombinant lentivirus particles and the cell expressing GFP-LC3 were isolated using fluorescence-activated cell sorting. The cells with stable GFP-LC3-expressing were selected and cultured in 400 μg/ml geneticin (Gibco, Grand Island, NY, USA).

Cell Reagent and Transfection

MiR-205 mimics and the negative controls were purchased from RiBoBio (Shanghai, China). TP53INP1 siRNA was purchased from Santa Cruz Biotech (Santa Cruz, CA, USA). 3-methyladenine (3-MA) and bafilomycin A1 (Baf. A1) were purchased from Sigma-Aldrich (St. Louis, MO, USA). A pEZ-M02-TP53INP1 plasmid in which the TP53INP1 cDNA does not include the 3'UTR targeted by miR-205 was synthesized by Genepharma (Shanghai, China). Cells were transfected with miR-205 or si-TP53INP1 alone and co-transfected with miR-205 mimics with pEZ-M02-TP53INP1 plasmid. Transfection was performed using Lipofectamine

2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Clonogenic assay was carried out at 48 hr after the transfection. In some groups, cells were treated with 3-MA or Baf. A1 1 hr before radiation, for a duration of 24 hr.

Dual Luciferase Reporter Assay

The 3'UTR of TP53INP1 with or without the putative miR-205-binding site were chemically synthesized and cloned into the Renilla luciferase gene (pLUC-REPORT vector; Promega, Madison, WI, USA). The recombinant plasmids carrying wild-type or mutant sequences are named as pLUC-TP53INP1-WT and pLUC-TP53INP1-MUT respectively. DU145 or LNCaP cells were co-transfected with 200ng luciferase reporter vector and 100 nM miR-205 mimics or the negative controls. Luciferase activity was examined 48 hrs after the transfection using the Dual-Luciferase Assay kit (Promega, Madison, WI, USA) according to manufacturer's instruction. Three independent experiments were performed in triplicate.

Statistical Analysis

Data were presented in the form of means ± standard deviation, and analyzed using Student's *t*-test. All statistical analyses were performed using SPSS 18.0 software (SPSS, Inc., Chicago, IL, USA). p value of < 0.05 was considered as statistically significant. Data was representative of more than three independent experiments, with each performed in triplicate.

Results

MiR-205 Sensitized Prostate Cancer Cells to irradiation

MiR-205 has been reported as a tumor suppressor and was usually downregulated in prostate cancer[11,12]. We firstly investigated miR-205 expression in three prostate cancer cell lines and one normal prostate epithelial cell line RWPE-1. In consistent with previous studies, we also confirmed that miR-205 expression was significantly lower in PC-3, DU145 and LNCaP cells than in the normal prostate epithelial cell line (Figure 1A). Then, we explored the effect of miR-205 on the radiosensitivity of DU145 and LNCaP cells. MiR-205 overexpression (Figure 1B) substantially reduced the survival fraction of the both DU145 and LNCaP cells to irradiation (Figure 1C-D).

Irradiation Induced Autophagy Acted as a Protective Mechanism in Prostate Cancer Cells

Autophagy may abrogate or enhance the cytotoxic effects of irradiation, depending on the type of cancer cells and the environmental stress[18-20]. In prostate cancer cells, the effects of autophagy on radiosensitivity are not quite clear. In this study, we observed that irradiation activated autophagy in DU145 and LNCaP cell lines, which were reflected by enhanced expression of LC3-II and reduced level of p62 (Figure 2A). Treatment with 3-MA, an autophagy inhibitor that blocks autophagosome formation, remarkably reduced the expression of LC3-II and inhibited degradation of p62 after irradiation (Figure 2B). In addition, 3-MA also enhanced the radiosensitivity of DU145 and LNCaP cells (Figure 2C-D). These results showed that autophagy is activated as an adaptive response to irradiation and inhibition of autophagy reduced survival of prostate cancer cells after irradiation.

MiR-205 Inhibited Irradiation-Induced Autophagy

In LNCaP cells with stable GFP-LC3 expression, miR-205 overexpression significantly decreased the lipidation of LC3 (Figure 3A) and inhibited degradation of p62 protein induced by irradiation (Figure 3B). To further explore the exact stage of autophagy in which miR-205 was involved in, we used bafilomycin A1, an inhibitor of the late phase of autophagy by preventing maturation of autophagic vacuoles via inhibiting fusion between autophagosomes and lysosomes[21]. 24 hrs after irradiation, the bafilomycin A1-treated negative control group showed significantly accumulation of LC3-II. In comparison, enforced miR-205 expression attenuated the accumulation (Figure 3C). These results suggest that miR-205 might inhibit autophagy since the early autophagosome formation, rather than from autophagosome degradation.

Then, we tried to investigate whether miR-205 induced sensitization to irradiation was dependent on inhibition of autophagy. In LNCaP cells without irradiation exposure, neither 3-MA nor miR-205 mimic alone affected the apoptosis rate cells (Figure 3D-E). However, combination of miR-205 transfection and 3-MA treatment resulted in increased apoptosis rate (Figure 3D-E). 24 hrs after 6 Gy exposure, miR-205 overexpression and 3-MA treatment significantly increased apoptosis (Figure 3 D-E). Combined miR-205

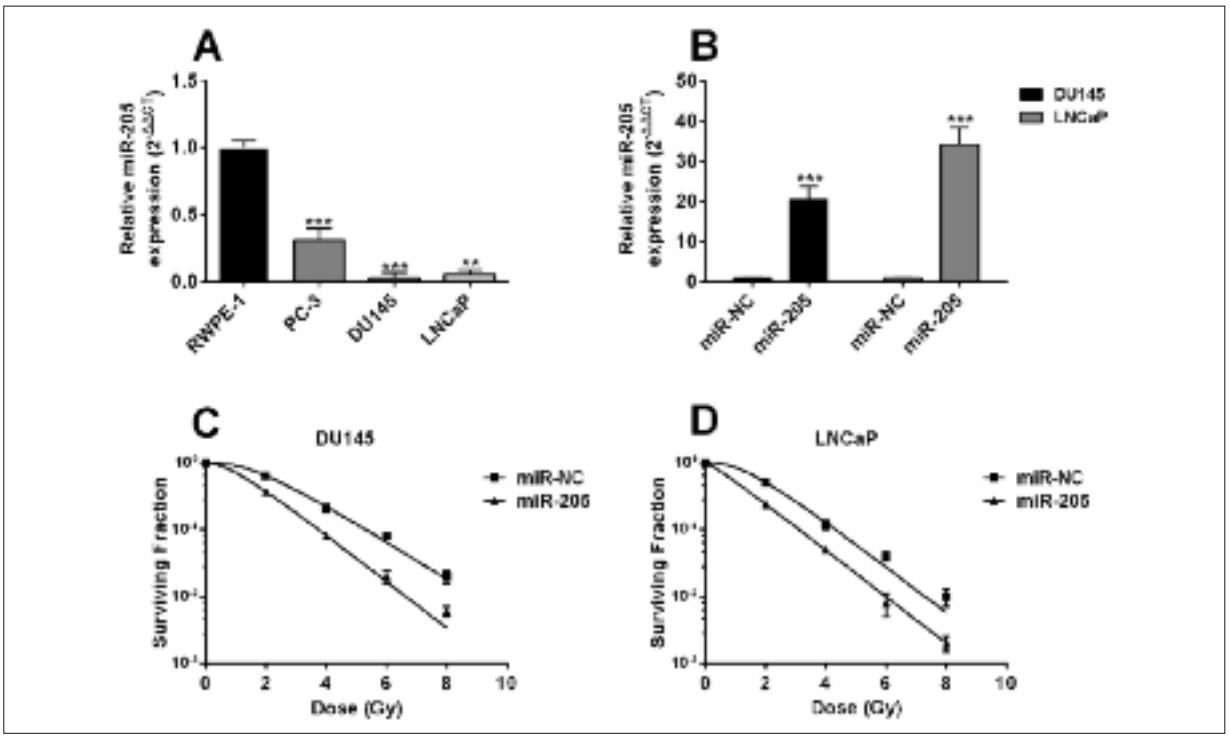

Figure 1. MiR-205 sensitized prostate cancer cells to irradiation. ***A,*** QRT-PCR analysis of the relative miR-205 expression in normal human prostate epithelial cell line RWPE-1 and in prostate cancer cell lines. ***B,*** QRT-PCR analysis of the relative miR-205 expression in DU145 and LNCaP cells transfected with miR-205 mimic or the scrambled negative control. ***C,*** and ***D,*** The survival fraction of DU145 ***(C)*** and LNCaP ***(D)*** cells with or without miR-205 overexpression after exposure to indicate dose. Colonies containing > 50 cells were counted via microscopic inspection. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

overexpression and 3-MA treatment had a stronger effect on promoting apoptosis (Figure 3D-E). Western blot analysis also confirmed that miR-205 overexpression or 3-MA treatment increased the irradiation-induced activation of caspase-3 and PARP (Figure 3F). Clonogenic assay showed that miR-205 or 3-MA significantly enhanced radiosensitivity of LNCaP cells to irradiation (Figure 3G).

MiR-205 Inhibited Irradiation-Induced Autophagy by Targeting TP53INP1

To further explore the underlying mechanisms of miR-205 in irradiation-induced autophagy, the putative targeting genes were searched using online databases (TargetScan 6.2). Further analysis using Gene Ontology Analysis and manual search of previous studies, we identified three possible genes related to autophagy, including ACSL1, TP53BP2 and TP53INP1. Due to previously reported role in autophagy[16,17,22], this study focused on TP53INP1.The predicted putative binding site between miR-205 and TP53INP1 and the designed mutant sequence were showed in Figure 4A. Ectopic expression of miR-205 did not affect the TP53INP1 mRNA level (Figure 4B), but significantly suppressed TP53INP1 protein expression in both DU145 and LNCaP cells (Figure 4C). Dual luciferase assay showed that miR-205 inhibited the relative luciferase activity of the reporter with wild-type of TP53INP1 sequence, but not the mutant gene in the two cancer cell lines (Figure 4D-E).

Then, we examined the effect of TP53INP1 knockdown on radiosensitivity. Both DU145 and LNCaP cells with TP53INP1 knockdown had reduced survival fraction after irradiation (Figure 4F-G). Besides, TP53INP1 knockdown also suppressed irradiation-induced autophagy (Figure 4H). These results suggest that miR-205 suppressed irradiation-induced autophagy by directly targeting TP53INP1. To further confirm the functional role of miR-205 and TP53INP1 in rediosensitivity, we constructed a TP53INP1 expression plasmid in which the 3'UTR sequence targeted by miR-205 was deleted (Figure 4I). Co-transfection of TP53INP1 and miR-205 significantly reversed the enhanced radiosensitivity of

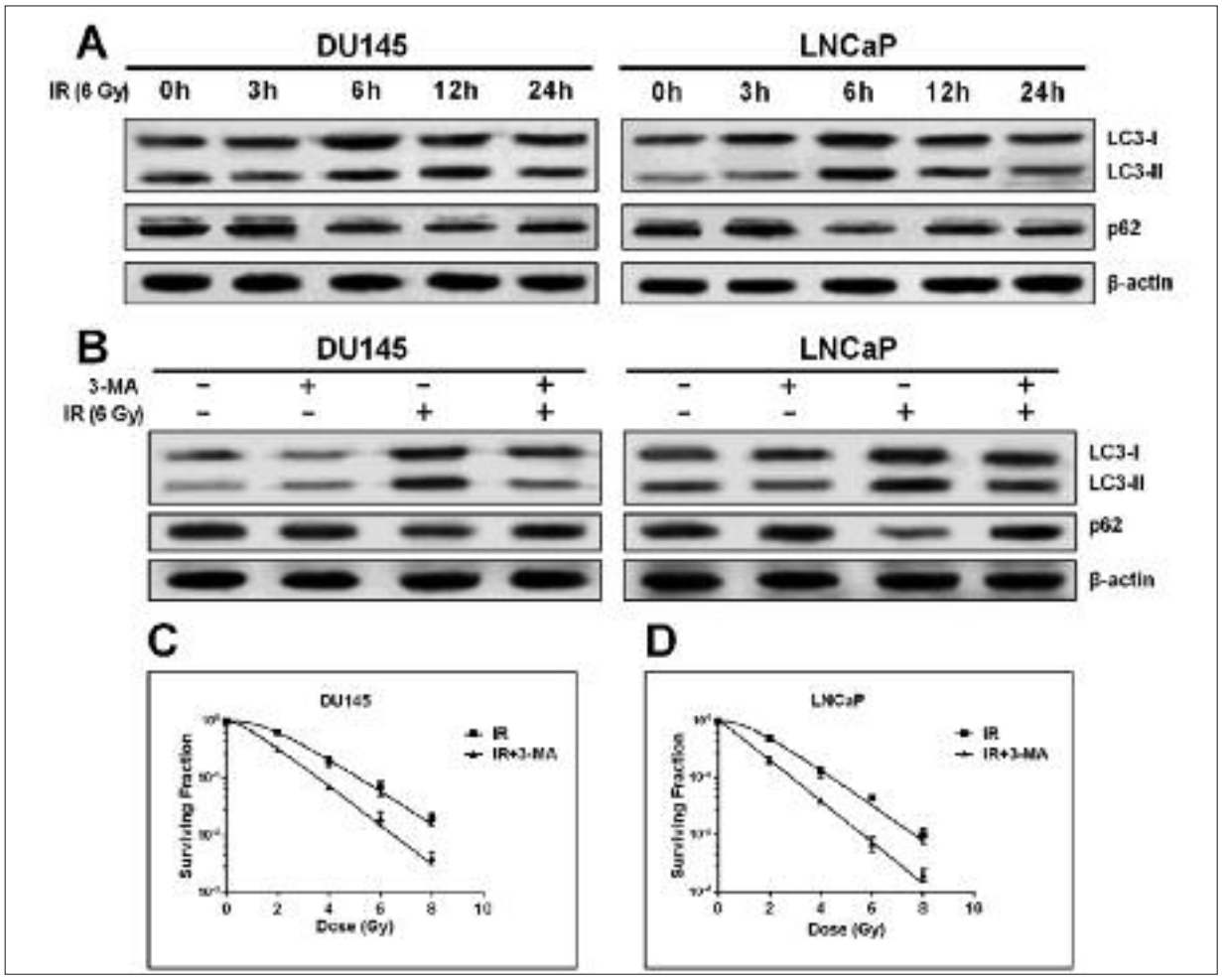

Figure 2. Irradiation induced autophagy, which acted as a protective mechanism in prostate cancer cells. ***A,*** DU145 and LNCaP cells were exposed to 6 Gy radiations. Autophagosome formation over time was detected via Western blot using antibodies against LC3 and p62. ***B,*** Western blot analysis of autophagy in DU145 and LNCaP cells with or without treatment with 3-MA (5 mM) after irradiation (6 Gy) for 24 hr. ***C,*** The survival fraction of DU145 and LNCaP cells with 6 Gy exposure alone or with combined irradiation and 3-MA (5 mM) treatment.

DU145 and LNCaP cells induced by miR-205 overexpression (Figure 4J-K). In addition, transfection with this TP53INP1 vector also restored the expression of LC3-II and degradation of p62 induced by irradiation (Figure 4 L), suggesting upregulated autophagy activity. Taken together, these results showed that TP53INP1 is a functional target of miR-205.

Discussion

MiR-205 is generally viewed as a tumor suppressor in prostate cancer by targeting several important genes regulating epithelial-to-mesenchymal transition and cell migration and invasion[23,24]. Low miR-205 expression also confers chemoresistance of prostate cancer cells[13,25]. Recently, miR-205 has been reported as a tumor radiosensitizer, by targeting multiple genes, such as PTEN[26], ZEB1 and Ubc13[27]. In this study, we confirmed that miR-205 overexpression could sensitize DU145 and LNCaP cells to irradiation. Therefore, we decided to further investigate the underlying mechanisms.

Autophagy is an evolutionarily conserved catabolic process that involves the degradation of cytoplasmic materials through lysosomal degradation pathway[28]. It is critical for maintaining cellular integrity. Activation of autophagy may suppress tumorigenesis through degrading defective organelles and other cellular components[28]. How-

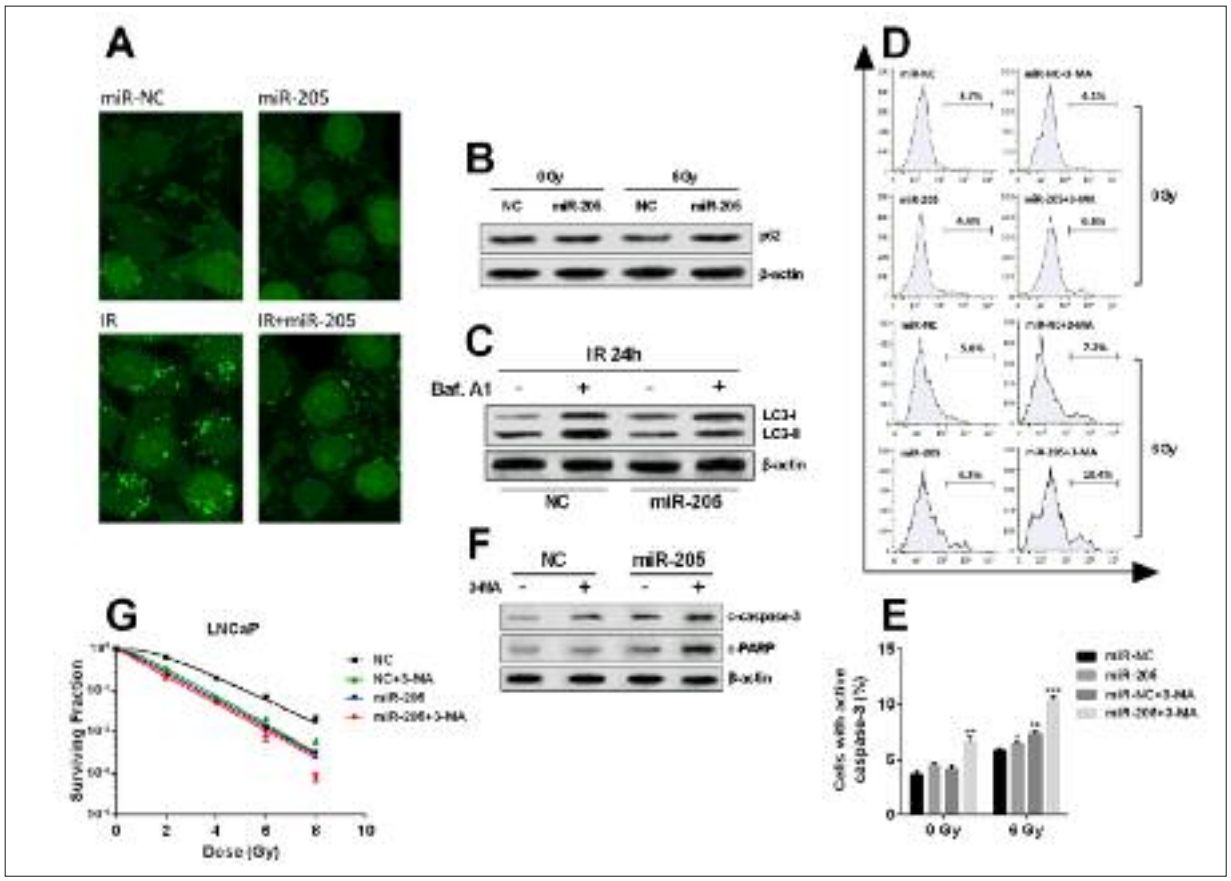

Figure 3. MiR-205 inhibited irradiation-induced autophagy. ***A,*** LNCaP cell line stably expressing GFP-LC3 was established. The cells were then transfected with a miR-205 mimic or a scrambled negative control. 48hrs after transfection, the cells were exposed to 6 Gy radiation and incubated for another 24 hrs. Then, the accumulation of GFP-LC3 puncta was captured using a fluorescence microscope. ***B,*** Western blot analysis of autophagy 48 hrs after transfection in LNCaP cells using an antibody against p62. ***C,*** 48 hrs after transfection, LNCaP cells were treated with bafilomycin A1 (100 nM) for 1 hr and, then, exposed to 6 Gy radiations. The expression of LC3-I/-II was analyzed 24 hrs postirradiation. ***D,*** LNCaP cells with or without miR-205 overexpression were treated with 3-MA (5 mM) for 1 h, followed by exposure to 6 Gy radiations. 24 hrs postirradiation, the cells with active caspase-3 were detected using a flow cytometry. ***E,*** Quantification of the proportion of cells with active caspase-3 in Figure ***(D)***. ***F,*** Cleaved caspase-3 (c-caspase-3) and cleaved PARP (c-PARP) protein expression were further detected using Western blot. ***G,*** The survival fractions of cells with the indicated treatment. The data are presented as means ± standard deviation from three independent experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

ever, this pathway may also be utilized by cancer cells to generate nutrients and energy in stressful conditions, such as hypoxia, starvation, and stress caused by chemotherapy and radiotherapy[28]. Some recent studies showed that several miRNAs can regulate autophagy, thereby affecting radiosensitivity of cancer cells. For example, miR-200c can inhibits autophagy and enhances radiosensitivity in breast cancer cells by targeting UBQLN1[29]. Inhibition of miR-191 can promote autophagy in lung cancer cells, leading to a higher level of radioresistance[30]. In consistent with this notion, this study showed that autophagy induced by irradiation functions as a survival mechanism of prostate cancer cells. In addition, the results also suggest that miR-205 may enhance radiosensitivity through suppressing irradiation-induced autophagy and promoting apoptosis. Besides, combined 3-MA treatment and miR-205 overexpression showed some level of additive effects on promoting apoptosis. This phenomenon is consistent with the distinct and/or parallel mechanisms of their actions. 3-MA can inhibit growth via blocking the PI3K-AKT-mTOR pathway[31,32], while miR-205 can induce apoptosis through targeting several components of the mitogen-activated protein kinase (MAPK) and androgen receptor (AR) signaling pathways[33].

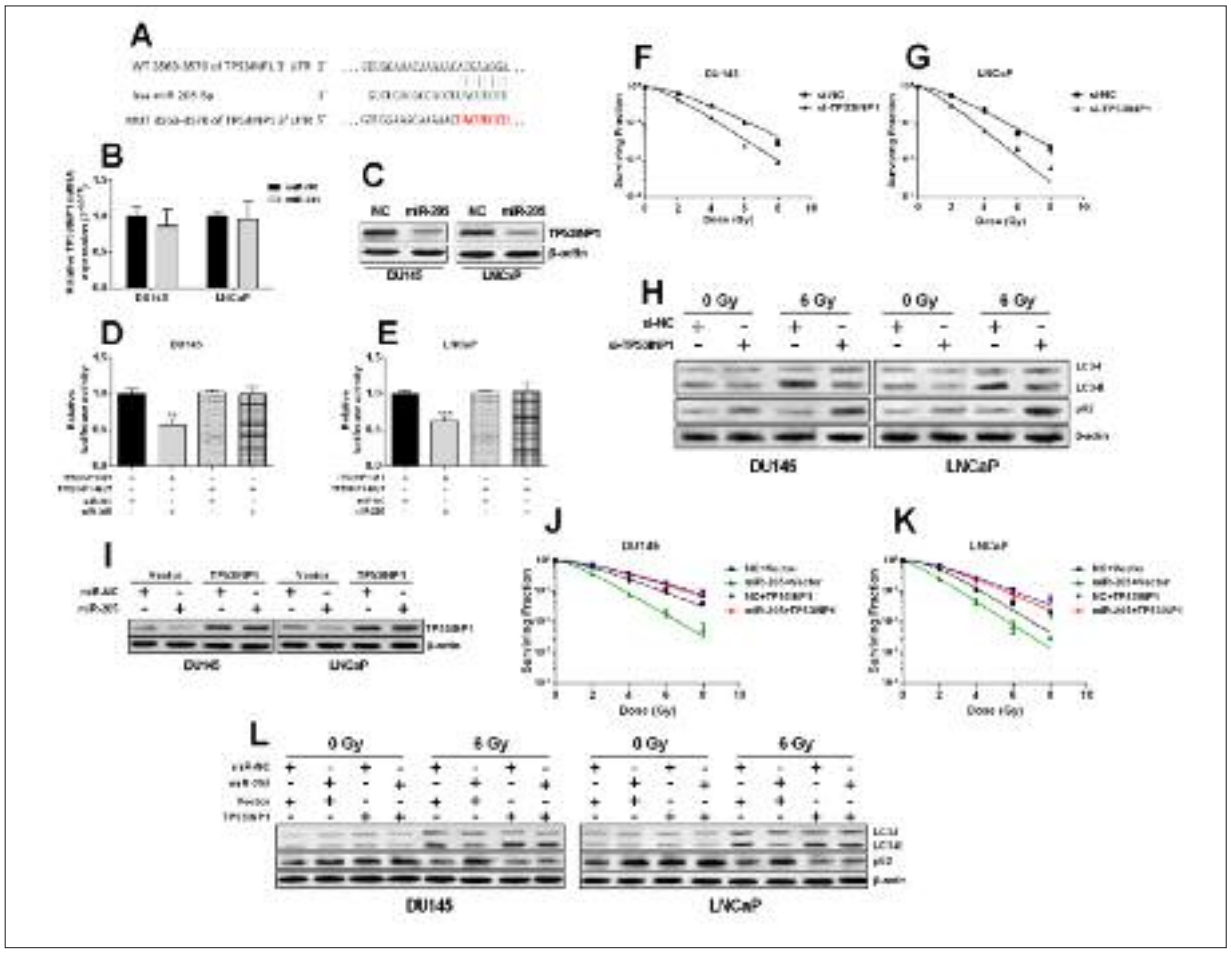

Figure 4. MiR-205 inhibited irradiation-induced autophagy by targeting TP53INP1. ***A,*** Predicted binding sites between miR-205 and 3'-UTR of TP53INP1. The designed mutant sequence was also given. ***B,*** and ***C,*** TP53INP1 mRNA ***(B)*** and protein ***(C)*** in DU145 and LNCaP cells with or without miR-205 overexpression. ***D,*** and ***E,*** The relative luciferase activity of DU145 ***(D)*** and LNCaP ***(E)*** cells after cotransfection with a wild-type or mutant TP53INP1 3'UTR reporter gene and miR-200c mimic or the negative control. ***F,*** and ***G,*** Survival fraction of DU145 ***(F)*** and LNCaP ***(G)*** cells with or without knockdown of TP53INP1. ***H,*** The activity of irradiation-induced autophagy in DU145 and LNCaP cells (with or without knockdown of TP53INP1) 24 hrs after irradiation via using antibodies against LC3 and p62. ***I,*** Western blot analysis of TP53INP1 expression in DU145 and LNCaP cells co-transfected with pEZ-M02-TP53INP1 and miR-205 or the negative control. ***J,*** and ***K,*** Survival fraction of DU145 ***(J)*** and LNCaP ***(K)*** cells with treatment indicated in Figure ***(I)***. ***L,*** The activity of irradiation-induced autophagy in DU145 and LNCaP cells with treatment indicated in Figure ***(I)***. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Dual luciferase assay in this study revealed that TP53INP1 is a direct target of miR-205. Enforced miR-205 expression significantly suppressed TP53INP1 expression at the protein level. In some types of cancers, TP53INP1 functions as a tumor suppressor[16,34]. However, in prostate cancer, it acts as an oncogene and its overexpression is associated with castration-resistant prostate cancer[15]. MiR-205 overexpression has been considered as a worse prognostic factor of prostate cancer[14]. As a cell stress response protein, the increased TP53INP1 expression in castration resistant prostate cancer after hormone therapy could be an adaptive response induced by castration to enhance cell survival[15]. Therefore, TP53INP1 might be a critical gene modulating stress responses of prostate cancer cells. In this study, we observed that TP53INP1 knockdown could substantially enhance radiosensitivity and suppress irradiation-induced autophagy in prostate cancer cells. However, restoring TP53INP1 reversed the enhanced radiosensitivity and autophagy suppression induced by miR-205 overexpression. These findings are consistent with a previous study which reported that miR-205 can impair the autophagic

flux in prostate cancer cells[13]. Put together, this study revealed that miR-205 can suppress autophagy and enhance radiosensitivity of prostate cancer cells by targeting TP53INP1.

Conclusions

The miR-205/TP53INP1 mediated autophagy pathway might be an important molecular mechanism regulating radiosensitivity of prostate cancer cells and represents a potential therapeutic target for the treatment of prostate cancer.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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