MiR-30a and miR-205 are downregulated in hypoxia and modulate radiosensitivity of prostate cancer cells by inhibiting autophagy via TP53INP1

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Introduction

Prostate cancer is a common male malignancy and results in about 300,000 deaths worldwide each year. Currently, radiotherapy has been used for the patients with unresectable prostate tumors and is also an important adjuvant treatment after radical prostatectomy. However, local recurrence after radiotherapy is still common and is a leading cause of treatment failure. Therefore, understanding the mechanisms of radioresistance will help to overcome recurrence after radiotherapy.

Autophagy is a conserved cellular metabolic process involving degradation and recycling of cytoplasmic components for the anabolic process. In prostate cancer, elevated autophagy acts as a protective mechanism to irradiation-induced apoptosis. In fact, this mechanism is also observed in some other types of solid tumors, such as breast cancer and gastric cancer. A large solid tumor is usually characterized as hypoxic tumor microenvironment. Regulation of microRNAs is a feature of an adaptive response to low oxygen in tumors. There are emerging evidence showed that hypoxia responsive microRNAs are involved in regulation of autophagy, as well as autophagy-related radiosensitivity of cancer cells. Our previous study found that miR-205 is significantly downregulated in prostate cancer cells, and its upregulation increases radiosensitivity of the cancer cells via...
targeting tumor protein p53-inducible nuclear protein 1 (TP53INP1) and attenuating autophagy. In fact, TP53INP1 might be an important modulator of autophagy since it directly interacts with some molecular actors of autophagy, such as LC3 and ATG8-family proteins, thereby promoting autophagy. Therefore, it is meaningful to further study how it is dysregulated in prostate cancer.

MiR-30a is also a miRNA usually downregulated in prostate cancer and is significantly associated with pathological stage. It is also a modulator of autophagy via targeting beclin 1. Similar to miR-205, how it is downregulated in prostate cancer is still not clear. In this study, we firstly investigated the association between miR-30a and miR-205 downregulation and hypoxia. Then, we further investigated the regulatory effect of miR-30a on TP53INP1 and autophagy-related radiosensitivity of prostate cancer cells.

Materials and Methods

Cell Culture

Prostate cancer cell lines, including DU145 and LNCaP cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. All cells were cultured in a humidified air containing 5% CO2 at 37°C. Oxygen supply for hypoxic culture was set to 2%.

To detect the change of miR-30a, miR-205 and Dicer expression induced by hypoxia, DU145 and LNCaP cells were subjected to hypoxia up to 72 hours. MiR-30a and miR-205 level at indicating time points were measured using qRT-PCR analysis, while the Dicer expression was detected by using Western blot analysis.

Cell Treatment and Transfection

MiR-30a mimics, miR-205 mimics, Dicer siRNA 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) was 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-30a and miR-205 was synthesized by Genepharm (Shanghai, China).

Cells were transfected with 50 nM miR-30a, 50 nM miR-205, 100 nM Dicer siRNA or 100 nM si-TP53INP1 alone and co-transfected with 50 nM miR-30a or 50 nM miR-205 and pEZ-M02-TP53INP1 plasmid. Transfection was performed using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. DU145 and LNCaP cells without miR-30a or miR-205 overexpression or with TP53INP1 knockdown were treated with 3-MA (5 mM) 1 hour before irradiation, for a duration of 24 hours. DU145 and LNCaP cells with miR-30a or miR-205 overexpression or co-transfected with miR-30a or miR-205 and pEZ-M02-TP53INP1 were also irradiated using indicating dose.

Flow Cytometric Analysis of Cell Apoptosis

Cell apoptosis was detected using the Fluorescein Active Caspase 3 Staining Kit (ab65613, Abcam, Cambridge, MA, USA) and the apoptosis rates were measured by using a flow cytometer (FACSCalibur, BD Biosciences, San Jose, CA, USA).

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) following manufacturer’s instruction. MiR-30a and miR-205 expression were 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) according to the manufacturer’s instruction. The cDNA library was synthesized using the PrimeScript RT reagent kit (TaKaRa, Dalian, China). The PCR primers for TP53INP1 were: forward, 5'-TCAGCAGAAGAAGAAGAAGAAGAG-3', reverse, 5'-AGCAGGAATCACTTGTATCAGC-3'; for pri-miR-30a were: forward, 5'-ATTGCTGGTTTGAATGAGGCTTCAGTACTTT-3', reverse, 5'-TTCAGCTTTGTAAAAATGTATCAAAGAGAT-3'; for pri-miR-205 were: forward, 5'-TGAAATCTGGTTGGCATAAGACAGACACGAGAG-3', reverse, 5'-GCACTACACGAGGAGGTAGTAGAC-3'. β-actin was used as the internal control. QRT-PCR for miRNA and mRNA was performed using SYBR Premix Ex Taq II (TaKaRa) with an ABI 7500 Sequence Detection System (Perkin-Elmer/Applied Biosystems, Rotkreuz, Switzerland). The results were calculated using the 2-ΔΔCT methods.
Western Blot Analysis

Western blot analysis followed the methods introduced in one previous study. The primary antibodies used include rabbit anti-LC3B (ab51520, 1:3000; Abcam), anti-p62/SQSTM1 (#8025, 1:1000; Cell Signaling, Danvers, MA, USA), anti-Dicer (ab56676, 1:200, H-212; Santa Cruz), anti-TP53INP1 (ab9775, 1:1000; Abcam) and anti-β-actin (ab189073, 1:1000; Abcam). The second horseradish peroxidase-conjugated secondary antibody was purchased from Abcam. The protein band was visualized using the ECL Western blotting substrate (Promega, Madison, WI, USA).

Irradiation and Colonogenic Assay

DU145 or LNCaP cells were firstly transfected with miR-30a or miR-205 mimics, TP53INP1 siRNA, TP53INP1 expression vector alone, or co-transfected with miR-30a or miR-205 mimics and TP53INP1 expression vector. 48 hours after transfection, 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: \( SF = 1-(1-\exp(-x/D0))^N \).

Dual Luciferase Reporter Assay

The 3’UTR fragment of TP53INP1 with or without the putative miR-30a binding site was chemically synthesized and cloned into the downstream of 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-MT respectively. DU145 or LNCaP cells were co-transfected with 200 ng luciferase reporter vector and 50 nM miR-30a mimics or the negative controls. Luciferase activity was examined 48 hours after the transfection using the Dual-Luciferase Assay kit (Promega) and a GloMax 20/20 luminometer (Promega) according to manufacturer’s instruction. Three independent experiments were performed in triplicate.

Statistical Analysis

Data were presented in the form of means ± SD, and analyzed using Student’s t-test. All statistical analyses were performed using SPSS 18.0 software (SPSS, Chicago, IL, USA). \( p \)-value < 0.05 was considered as statistically significant.

Results

MiR-30a and miR-205 are Downregulated Under Hypoxia as a Result of Impaired Dicer Expression in Prostate Cancer Cells

Our previous study observed that miR-205 is downregulated in prostate cancer and can suppress autophagy and enhance radiosensitivity of prostate cancer cells by targeting TP53INP1. In addition, miR-30a is also suppressed in prostate cancer. However, how they are dysregulated in prostate cancer is still not clear. In this study, we firstly detected how the expression of miR-30a and miR-205 changes in hypoxic culture in DU145 and LNCaP cells. The results showed that these two miRNAs were significantly downregulated after hypoxic treatment in both DU145 (Figure 1A-B) and LNCaP cells (Figure 1C-D). One recent study suggests that Dicer expression can be suppressed by hypoxia through an epigenetic mechanism, leading to reduced miRNA processing. Therefore, we decided to investigate whether this mechanism is involved in hypoxia-induced miR-30a and miR-205 downregulation in prostate cancer cells. By performing western blot analysis, we found that Dicer expression is also significantly decreased in both DU145 and LNCaP cells after hypoxic treatment (Figure 1E). Then, we measured to ratio of mature miR-30a and miR-205 to pri-miR-30a and pri-miR-205. QRT-PCR analysis showed that Dicer siRNA (Figure 1F) resulted in significantly decreased ratio of mature miRNA to pri-miRNA, which indicates a defect in miRNA processing (Figure 1G-H).

Both miR-30a and miR-205 Sensitize Prostate Cancer Cells to Irradiation via Attenuating Autophagy

Our previous study showed that autophagy induced by irradiation acts as a protective mechanism in prostate cancer cells. Since prostate cancer is a type of solid tumor usually characterized as hypoxic tumor microenvironment, we, then, further investigate the effects of hypoxia respon-
MiR-30a and miR-205 are downregulated under hypoxia as a result of impaired Dicer expression in prostate cancer cells. A-D, QRT-PCR analysis of miR-30a (A and C) and miR-205 (B and D) expression in normoxic and hypoxic cultured DU145 (A and B) and LNCaP (C and D) cells at indicating time points up to 72 hours. E-F, Western blot analysis of Dicer in normoxic and hypoxic cultured DU145 and LNCaP cells at indicating time points up to 72 hours (E) and in the cells 48 hours after Dicer siRNA transfection (F). G-H, The ratio of mature miRNA to pri-miRNA for miR-30a and miR-205 after hypoxia or si-Dicer in DU145 (G) and LNCaP (H) cells. Mature and pri-miRNA levels were determined by qRT-PCR. *p < 0.05, **p < 0.01.

Figure 1. MiR-30a and miR-205 are downregulated under hypoxia as a result of impaired Dicer expression in prostate cancer cells. A-D, QRT-PCR analysis of miR-30a (A and C) and miR-205 (B and D) expression in normoxic and hypoxic cultured DU145 (A and B) and LNCaP (C and D) cells at indicating time points up to 72 hours. E-F, Western blot analysis of Dicer in normoxic and hypoxic cultured DU145 and LNCaP cells at indicating time points up to 72 hours (E) and in the cells 48 hours after Dicer siRNA transfection (F). G-H, The ratio of mature miRNA to pri-miRNA for miR-30a and miR-205 after hypoxia or si-Dicer in DU145 (G) and LNCaP (H) cells. Mature and pri-miRNA levels were determined by qRT-PCR. *p < 0.05, **p < 0.01.

miR-30a and miR-205 on radiosensitivity of prostate cancer. DU145 and LNCaP cells were firstly transfected for miR-30a and miR-205 overexpression (Figure 2A-B). The results of colonogenic assay showed that miR-30a and miR-205 overexpression both enhanced radiosensitivity of prostate cancer cells (Figure 2C-D). MiR-30a and miR-205 overexpression significantly reduced autophagy induced by irradiation in both DU145 and LNCaP cells (Figure 2E). Then, we investigate how these two miRNAs influence apoptosis of these two cell lines after irradiation. Flow cytometry analysis showed that miR-30a and miR-205 increased the ratio of apoptotic cells after irradiation (Figure 2F-G). In addition, they both showed synergetic effects when combined with 3-MA (Figure 2F-G).

MiR-30a Directly Targets 3’UTR of TP53INP1 and Decreases its Expression

We previously demonstrated that miR-205 directly targets 3’UTR of TP53INP1 and decreases its expression. In fact, our preliminary bioinformatics study also suggests that miR-30a has a putative binding site with 3’UTR of TP53INP1 (Figure 3A). In both DU145 and LNCaP cells, miR-30a overexpression could not reduce TP53INP1 mRNA (Figure 3B-C), but substantially suppressed TP53INP1 protein expression (Figure 3D). Then, we performed dual luciferase assay to verify the binding between miR-30a and 3’UTR of TP53INP1. The results showed that miR-30a suppressed luciferase expression of the reporter carrying the wild-type of TP53INP1 sequence, but not the reporter with mutant sequence in DU145 cells (Figure 3E). These results suggest that miR-30a directly targets 3’UTR of TP53INP1 and decreases its expression.

Both miR-30a and miR-205 Modulate Radiosensitivity of Prostate Cancer Cells at Least via TP53INP1

Firstly, DU145 and LNCaP cells were firstly transfected with TP53INP1 siRNA (Figure 4A). TP53INP1 inhibition significantly reduced autophagy (Figure 4A). Inhibition of endogenous
TP53INP1 resulted in substantially decreased cell survival after irradiation (Figure 4B-C). Then, DU145 and LNCaP cells were transfected with TP53INP1 expression vector without miR-30a or miR-205 mimics (Figure 4D). TP53INP1 overexpression substantially enhanced autophagy level in both DU145 and LNCaP cells (Figure 4E). In addition, miR-30a and miR-205 could not suppress the increased autophagy (Figure 4E). TP53INP1 overexpression increased cell survival (Figure 4F-G) and decreased the ratio of apoptotic cells (Figure 4H-I) after irradiation, which could not be reversed by miR-30a or miR-205 overexpression (Figure 4F-I).

**Discussion**

MiR-30a downregulation was observed in prostate cancer and was significantly associated with pathological stage. However, how it is
Figure 3. MiR-30a directly targets 3’UTR of TP53INP1 and decreases its expression. A, Predicted binding sites between miR-30a and 3’UTR of TP53INP1. The designed mutant sequence was also given. B-D, QRT-PCR analysis of TP53INP1 mRNA (B-C) and Western blot analysis of TP53INP1 protein (D) in DU145 and LNCaP cells transfected with miR-30a, miR-205 or TP53INP1 siRNA. E, The relative luciferase activity in DU145 cells after cotransfection with wild-type or mutant TP53INP1 3’UTR reporter gene and miR-30a mimic or the negative control. *p < 0.05, **p < 0.01.

Figure 4. Both miR-30a and miR-205 modulate radiosensitivity of prostate cancer cells at least via TP53INP1. A, Western blot analysis of autophagy in DU145 and LNCaP cells transfected with TP53INP1 siRNA. B-C, The survival fraction of DU145 (B) and LNCaP (C) cells with or without TP53INP1 knockdown. D-E, Western blot analysis TP53INP1 expression (D) and autophagy (E) in DU145 and LNCaP cells after transfection with TP53INP1 expression vector alone or in combination with miR-30a or miR-205 mimics. F-G, The survival fraction of DU145 (F) and LNCaP (G) cells with TP53INP1 overexpression alone or in combination with miR-30a or miR-205 mimics. H-I, Quantification of flow cytometry analysis of irradiation induced apoptotic DU145 (H) and LNCaP (I) cells with transfection described in Figure F. *p < 0.05, **p < 0.01.
downregulated and its downstream regulation in the cancer is still not well demonstrated. Cancer cells usually make adaptive responses in the hypoxic tumor microenvironment. Regulation of microRNAs is one of the feature of adaptive responses to low oxygen. One previous study suggests that hypoxia induces epigenetic inhibition of oxygen-dependent H3K27me3 demethylases KDM6A/B, which results in silencing of the Dicer promoter and subsequently suppressed Dicer expression. This silencing findings in depression of miR-200. Impaired Dicer expression is also observed in decreased miR-143 and miR-155 expression in lung cancer cells in hypoxia. One previous work observed miR-205 is also significantly downregulated in prostate cancer. Therefore, in this study, we firstly investigated whether miR-30a and miR-205 downregulation are a result of impaired Dicer expression in prostate cancer. Our results showed that Dicer expression was significantly decreased after hypoxic treatment. In addition, Dicer inhibition directly resulted in decreased ratio of mature miRNA to pri-miRNA, which indicates a defect in miRNA processing.

Previous investigations reported that miR-30a is involved in regulating of autophagy. It directly targets 3’UTR of Beclin-1, thereby decreasing autophagic activity. MiR-30a downregulation and associated elevated autophagy is considered as an important mechanism of acquired chemoresistance in some types of cancer. In this research, our preliminary bioinformatics study suggests that miR-30a also has a putative binding site with 3’UTR of TP53INP1. Therefore, we decided to further investigate whether it has a similar effect as miR-205 in modulating autophagy and related radiosensitivity. By performing dual luciferase assay, we confirmed that miR-30a directly interacts with the predicted sequence and suppresses TP53INP1 expression at protein level. Therefore, miR-30a and miR-205 can simultaneously target TP53INP1 and suppress its expression.

Autophagy is an evolutionarily conserved catabolic process that involves degradation and recycling of cytoplasmic components for anabolic process. In prostate cancer cells, autophagy functions as a protective mechanism of cells to stressful conditions, including irradiation-induced cell apoptosis. For example, hypoxia-induced MiR-301a/b upregulation, which resulted in decreased NDRG2 expression, increased autophagy and cell viability and reduced cell apoptosis. Some hypoxia responsive miRNAs are involved in regulating autophagy and radiosensitivity of the cancer cells. For example, in colon cancer cells, HIF-1α induced in hypoxia enhances miRNA-210 expression, which in turn enhances autophagy and reduces radiosensitivity by downregulating Bcl-2 expression. In prostate cancer, TP53INP1 acts as an oncogene and its overexpression leads to enhanced castration-resistance. TP53INP1 is a cell stress response protein, its overexpression acts as an adaptive response to enhance cell survival in stressful conditions. By using TP53INP1 expression vector without miR-30a and miR-205 binding sites, we found that TP53INP1 overexpression significantly increased autophagy and radioresistance of the cancer cells, the effect of which could not be abrogated by miR-30a and miR-205 overexpression. These results further confirmed that TP53INP1 plays an important role in modulating autophagy and is a survival related protein of prostate cancer cells after irradiation.

Conclusions

We observed that miR-30a and miR-205 are two hypoxia responsive miRNAs, which simultaneously target TP53INP1 and suppress its expression. The miR-30a/miR-205/TP53INP1 axis is involved in autophagy and radiosensitivity regulation, which represents a potential therapeutic target for the treatment of prostate cancer.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

References


